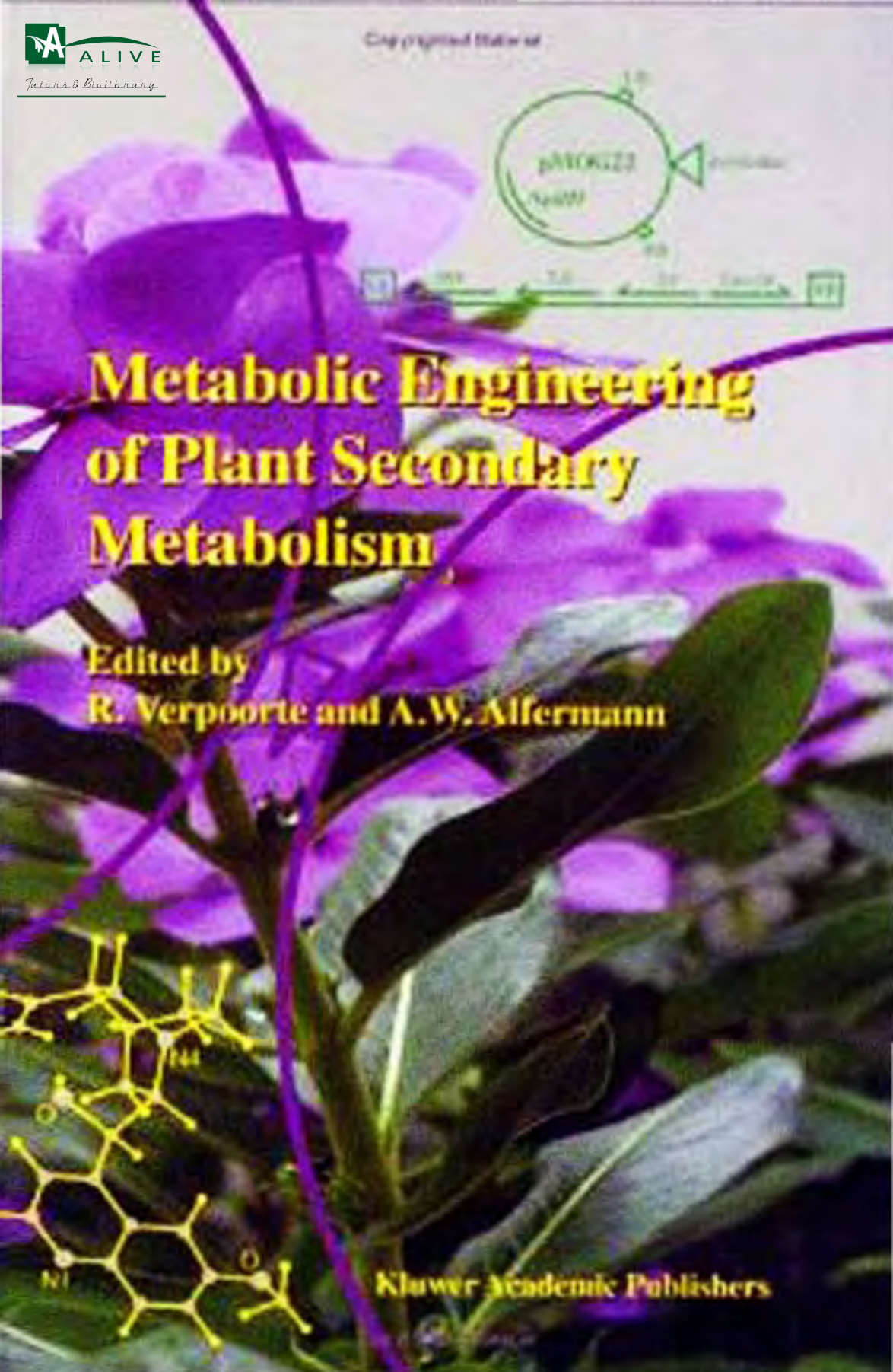

Metabolic Engineering of Plant Secondary Metabolism

Edited by
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PREFACE

In this book we aim at giving a general overview on metabolic engineering of plant secondary metabolism, and show by a series of reviews the progress made in applying molecular biology to alter the production of certain compounds. Several approaches are presently of interest

I - improve the production of secondary metabolites used as specialty chemicals, such as drugs, insecticides, dyes, flavours and fragrances. This includes improving the production in plants or plant cells, introducing the production of a compound of interest in another plant species, e.g. more suitable for cultivation, or even the production of complete new compounds.

II - altering the quality of a plant, e.g. used as food or an ornamental plant. This includes altering flower colours, changing taste, smell or colour of food, reducing level of toxic or unwanted compounds in food or fodder plants.

III - increase resistance against pest and diseases.

These different aspects will be the basis of the book. In the two introductory chapters we will first discuss the general background of secondary metabolism and the possibilities to alter secondary metabolite pathways. The next two chapters deal with the state-of-the-art of the transformation technologies: the *Agrobacterium* system and the particle gun. The next chapter will deal with the possibilities of producing antibodies in plants, this is potentially also applicable for altering secondary metabolite pathways. As secondary metabolite pathways might be under the control of one or just a few genes, Chapter 6 deals with work on transcriptional regulators as possible targets for genetic engineering. The subsequent chapters deal with agricultural applications of metabolic engineering, aiming at improving the quality of plants. The last chapters concern the possibility of altering the production of pharmaceutically interesting compounds in plants or plant cell cultures.

Certainly there would have been further examples of the application of metabolic engineering. However, being complete in such a fast moving field would be impossible, rather we preferred to give an overview for some important fields. In principle the strategies can be used for any type of secondary metabolite, taking into account that a number of constraints exist. Anyway we hope that this book will help the reader to have an overview on the posibilities to overproduce compounds, to block the production of unwanted compounds, or produce new compounds in plants or plant cells.

Rob Verpoorte and Willi Alfermann

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CHAPTER 1

SECONDARY METABOLISM

R. VERPOORTE

Introduction

Definition

Many authors have discussed the problem of a proper definition of secondary metabolites.[1,2,3] Bennett and Bentley[1] extensively discussed the history of the term secondary metabolites, with special reference to microbial metabolites. They gave the following definition:

> "General metabolites (hence general metabolism): A metabolic intermediate or product, found in most living systems, essential to growth and life, and biosynthesized by a limited number of biochemical pathways. Secondary metabolites (hence secondary metabolism): a metabolic intermediate or product, found as a differentiation product in restricted taxonomic groups, not essential to growth and life of the producing organism, and biosynthesized from one or more general metabolites by a wider variety of pathways than is available in general metabolism."

The secondary metabolites are characterized by an enormous chemical diversity, every organism has its own characteristic set of secondary metabolites, some of which they may share with other related or totally unrelated organisms. For many years secondary metabolites have been considered as more or less waste products, with no apparent use for the plant. Still our knowledge about the role of the secondary metabolites is limited, but now it is generally accepted that secondary metabolism is involved in the relationship of the organism with its environment, *e.g.* in resistance against pests and diseases, as attractant of pollinators, or as signal compound. As the definition given by Bennett and Bentley does not say anything about the role of secondary metabolites we are not fully satisfied with this proposal. But the chemical diversity and the limited knowledge on their role, hamper efforts to more sharply define the group. Our best effort is the following:

> "Secondary metabolites are compounds with a restricted occurrence in taxonomic groups, that are not necessary for a cell (organism) to live, but play a role in the interaction of the cell (organism) with its environment, ensuring the survival of the organism in its ecosystem."

In this view secondary metabolites are essential for an organism to survive as a species in its ecosystem. We thus fully agree with the final conclusion of Bennett and Bentley that "There is little that is 'secondary' about secondary metabolism".

R. Verpoorte and A.W. Alfermann (eds), Metabolic engineering of plant secondary metabolism, 1-29.

Number of compounds

Plants produce a broad spectrum of so called secondary metabolites. In 1988 the NAPRALERT database contained more than 88,000 compounds (N.R. Farnsworth, personal communication), most of which were derived from plants. Every year about 4000 new are reported. Similar numbers can be learned from the Dictionary of Natural Products (Table 1)[4], which in 1998 had about 85,000 entries for secondary metabolites. The largest groups are the terpenoids and the alkaloids, in 1988 NAPRALERT contained about 33,000 terpenoids and 16,000 alkaloids, numbers similar to those in Table 1.

Table 1. Number of secondary metabolites as present in the Dictionary of Natural Products (Chapman and Hall, 1998)[4]

Total number of entries:	85,058		
among others of which:			
aliphatics	5200		
polyketides	2442		
carbohydrates	3210		
oxygen heterocycles	1348		
simple aromatics	4527		
benzofuranoids	387		
benzopyranoids	2694		
flavonoids	8128		
tannins	750		
lignans	1565		
polycyclic aromatics	2448		
terpenoids	27,463	hemi-	56
		mono-	1946
		sesqui-	8650
		di-	7834
		tri-	5582
		tetra-	352
		poly-	51
		steroids	4600
amino acids, peptides	3921		
alkaloids	15,765	indole	3693
		isoquinoline	3498
		steroidal	873

Economic importance

Plant secondary metabolites represent an enormous value from economical point of view. First of all quite a few are used as specialty chemicals, such as drugs, flavours, fragrances, insecticides and dyes. Of all drugs used in western medicine about 25% is

derived from plants, either as a pure compound or as derived from a natural synthon.[5] Examples of the former are morphine, codeine, paclitaxel, vinblastine, vincristine, scopolamine, atropine, pilocarpine, physostigmine and digoxin. An inventory in the eighties identified about 121 plant compounds from about 95 different plant species which were used in western pharmacotherapy.[6,7] Best example of the synthon is the steroid skeleton, which is the basis of a large series of drugs, such as contraconceptives and corticosteroids.

Besides this actual real economical value, there is also an enormous potential value for new drug development. Nature's biodiversity is an important source for new leads for drug development. Cragg *et al.*[8] showed that from all new approved drugs in the years 1983-1994, about 6% are natural products, 24% natural products derivatives and 9% based on natural product leads. In the field of antibiotics the percentage natural products and natural products derivatives is even much higher, 78%. For anticancer drugs this is 48%, with another 13% for compounds derived from natural product leads.

Nature has developed an enormous diversity during several billion years of evolution. Presently it is estimated that there exist at least 250,000 different plant species, up to 30 million species of insects, 1.5 million species of fungi and similar numbers of algae and prokaryotes.[9] All these species live together in ecosystems, where they adapt to the physical conditions and interact with each other, including interactions such as defence, symbiosis, pollination, etc. All examples in which chemistry plays a major role. Considering the number of organisms, the number of interactions is almost infinite, and thus an enormous wide variety of secondary metabolites has been developed in the course of evolution. Nature thus is a very important source for new leads for drug development. Mostly plants and microorganisms have so far been screened for this purpose. However, as it can be seen from Table 2, the number of plants studied so far for new leads is limited. Less than 5% has been studied for one or sometimes more biological activities, about 15% has been subject for some phytochemical study (N.R. Farnsworth, personal communication, 1996). Obviously there is an enormous potential in plants for drug development.

Table 2. Number of plant species, which have been studied phytochemically or for at least one type of biological activity, as on December 1, 1995, in the database NAPRALERT (N.R. Farnsworth, personal communication):

	Species studied for biological activity	Species studied phyto-chemically
Monocots	1,283	3,721
Dicots	11,924	31,126
Gymnosperms	239	638
Pteridophytes	349	961
Bryophytes	39	457
Lichens	118	625

With a total world market for medicines of about 250 billion US $ per year, it is obvious that natural products from plants are a valuable commodity. A much more difficult group to assess in terms of economical terms, at least in value of money, is that of the medicinal plants.[10] It is estimated that about 80%[6,7,11] of the world population depends on traditional medicinal plants for their primary health care. Though in most cases no scientific studies have been made to confirm their activity, of those studied quite a few showed activities related to their use. Traditional medicine has served as lead for many important drugs, such as morphine, digoxin, quinine, hyoscyamine, salicylic acid and artemisinin.[5] Probably the activity is in many cases due to a combination of secondary metabolites present in these plants. Traditional medicines are very important in primary health care, where they can be used instead of expensive western medicines. Their potential value is in the possibility that they may contain new biologically active compounds, which can be further developed into drugs for the international market.

The other major group of economically important natural products is that of flavours and fragrances. This group comprises both pure chemical entities and mixtures of compounds (*e.g.* various essential oils). These compounds are on the market as such, but of course they are also of great importance for the quality of our food and spices. For example the bitter taste of beer is dependent on the bitter acids from hops. The whole world market of beer, with about the same value in terms of money as the total drug market, is dependent on the three bitter acids from hops: humulone, co-humole and ad-humulone.[12] Taste and colour of our food and colour of flowers is determined by secondary metabolites. Moreover, food plants also contain all kinds of other compounds which are very much quality determining, such as caffeine. Presently there is much interest in health promoting effects of secondary metabolites in food. Anthocyanins, flavonoids and carotenoids are now well known examples, but certainly one may expect others that will be discovered in the coming years.

Plants do not only contain compounds, which are favorable for our health, but also toxic compounds. This can be acute toxicity but also chronic toxicity, for example toxic for the liver (hepatotoxicity). Pyrrolizidine alkaloids are well-known hepatotoxic compounds among others found in some fodder plants.[13,14,15] The *Solanum* glyco-alkaloids in potatoes are an example of toxic compounds, which may occur in our food.[13]

Some plants are used because of their effect on the central nervous system, *e.g.* hallucinogenic effects, and may cause addiction. Morphine, cocaine, mescaline, psilocybine, and tetrahydrocannabinol are well known examples of compounds with such activities.[16,17]

Obviously, because of the quality traits of plants connected with secondary metabolites, they are important from an agricultural point of view.[18,19] An even more complex, but at least as important, aspect of secondary metabolism in plants is the role in the plant against pests and diseases.[21,22,23,24] A role which one gradually starts to unravel and which results in the identification of further important targets for metabolic engineering of secondary metabolism.[19] For example our increasing knowledge on the biosynthesis phytoalexins and antifeedants opens the way for genetic engineering of such pathways aiming at increased resistance against infections[25] or insects.[19]

Classification of secondary metabolites

Secondary metabolites can be classified in different ways: based on chemical characteristics, plant origin or biosynthetic origin. From a chemical point of view, the compounds can be divided in a number of groups based on a typical characteristic, such as alkaloids, characterized by a basic nitrogen function, or phenolics, which are characterized by aromatic ring systems having a phenolic hydroxyl group. Other groups, or subgroups are based on the presence of a certain type of basic skeleton, *e.g.* anthracene, coumarine, quinone, indole, isoquinoline, etc. Examples of classification based on plant origin are the opium alkaloids, *Strychnos* alkaloids, and *Digitalis* cardenolides. Often these groups of compounds are connected with pharmaceutical applications.

The classification based on biosynthetic origin has as major examples the terpenoids, phenylpropanoids and polyketides. The largest group in fact is that of the terpenoids (Table 1). These compounds have in common that they are all derived from the isoprenoid biosynthetic pathway which uses a C_5 building block to build up C_{10} (monoterpenes), C_{15} (sesquiterpenes), C_{20} (diterpenes), C_{30} (steroids and triterpenes) and C_{40} (carotenoids) compounds. Also in the other two groups mentioned a few basic building blocks are used to assemble a basic skeleton. These building blocks are phenylalanine/tyrosine (C_9) and acetate (C_2), from which respectively the phenylpropanoids and polyketides are derived. In Figure 1 some major group of secondary metabolites derived from the terpenoid and phenylpropanoid pathways in plants are summarized. These two pathways are the most important for secondary metabolite formation in plants, the polyketide pathway is particularly well-developed in microorganisms.

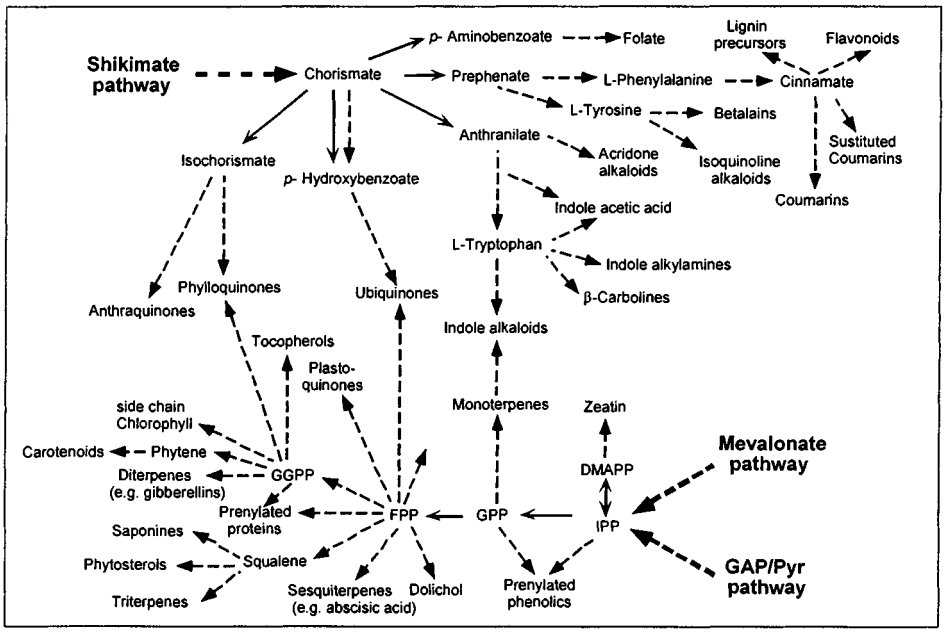


Figure 1. Terpenoid and shikimate pathways, two major routes leading to various secondary metabolites.

Smaller series of compounds are derived from some amino acids, such as various types of alkaloids derived from tryptophan (*e.g.* indole alkaloids), lysine (*e.g.* quinolizidine alkaloids), ornithine (*e.g.* pyrrolizidine alkaloids, tropane alkaloids).[26,27,28]

Most plants make similar basic skeletons of compounds, *e.g.* terpenoids, but differ in the "decoration" of the basic structure with various functional groups. Combinatorial chemistry is thus as old as evolution. Nature is continuously making new molecules, evolution selects the ones which gives the producer an advantage.[29,30]

In the following paragraphs some more details will be given about the general parts of the major secondary biosynthetic pathways mentioned. These pathways are found in most, if not all, plants.

Major secondary metabolite pathways

In plants particularly three pathways are the source of most secondary metabolites: the shikimate pathway, the isoprenoid pathway and the polyketide pathway. After the formation of the major basic skeletons, further modifications result in plant species specific compounds. The "decorations" concern for example hydroxy, methoxy, aldehyde, carboxyl groups and substituents adding further carbon atoms to the molecule, such as prenyl-, malonyl-, and glucosyl-moieties. Moreover, various oxidative reactions may result in loss of certain fragments of the molecule or rearrangements leading to new skeletons.

Shikimate pathway

The shikimate pathway is the major source of aromatic compounds.[31,32,33,34] It is found in microorganisms and plants, but not in mammals, making it an interesting target for herbicides and antibiotics, as these compounds are expected not to have any effect on the mammalian system. Glyphosate is a well known example. The pathway starts with the condensation of D-erythrose 4-phosphate and phosphoenolpyruvate (Fig. 2). In a series of reactions a cyclic compound, 3-dehydroquinate is obtained. In two further steps this yields shikimate, which after phosphorylation, is coupled by the enzyme EPSP synthase with phosphoenolpyruvate to give 5-enolpyruvylshikimate-3-phosphate (EPSP). This enzyme is the target for glyphosate, the herbicide mentioned above. Dephosporylation of EPSP eventually results in chorismate, from where the pathway diverges into two major branches, leading to respectively phenylalanine/tyrosine and tryptophan. In terms of carbon fluxes some minor branches lead to isochorismate, 4-hydroxybenzoic acid and 4-aminobenzoic acid, from which series of different secondary metabolites are derived (Fig. 1). All branches lead to products necessary for primary metabolism and primary functions in cells, but also secondary metabolite pathways are derived from these branches. From an early intermediate of the shikimate pathway (3-dehydroshikimate) gallic acid derivatives are formed (Fig. 2).

The enzymes channeling chorismate into the aromatic amino acids pathways are chorismate mutase and anthranilate synthase (Fig. 2). Although it has been hypothesized that some plants have dual shikimate pathways for the aromatic amino acids[35], one plastidic leading to primary and one cytosolic to secondary metabolites, so far no

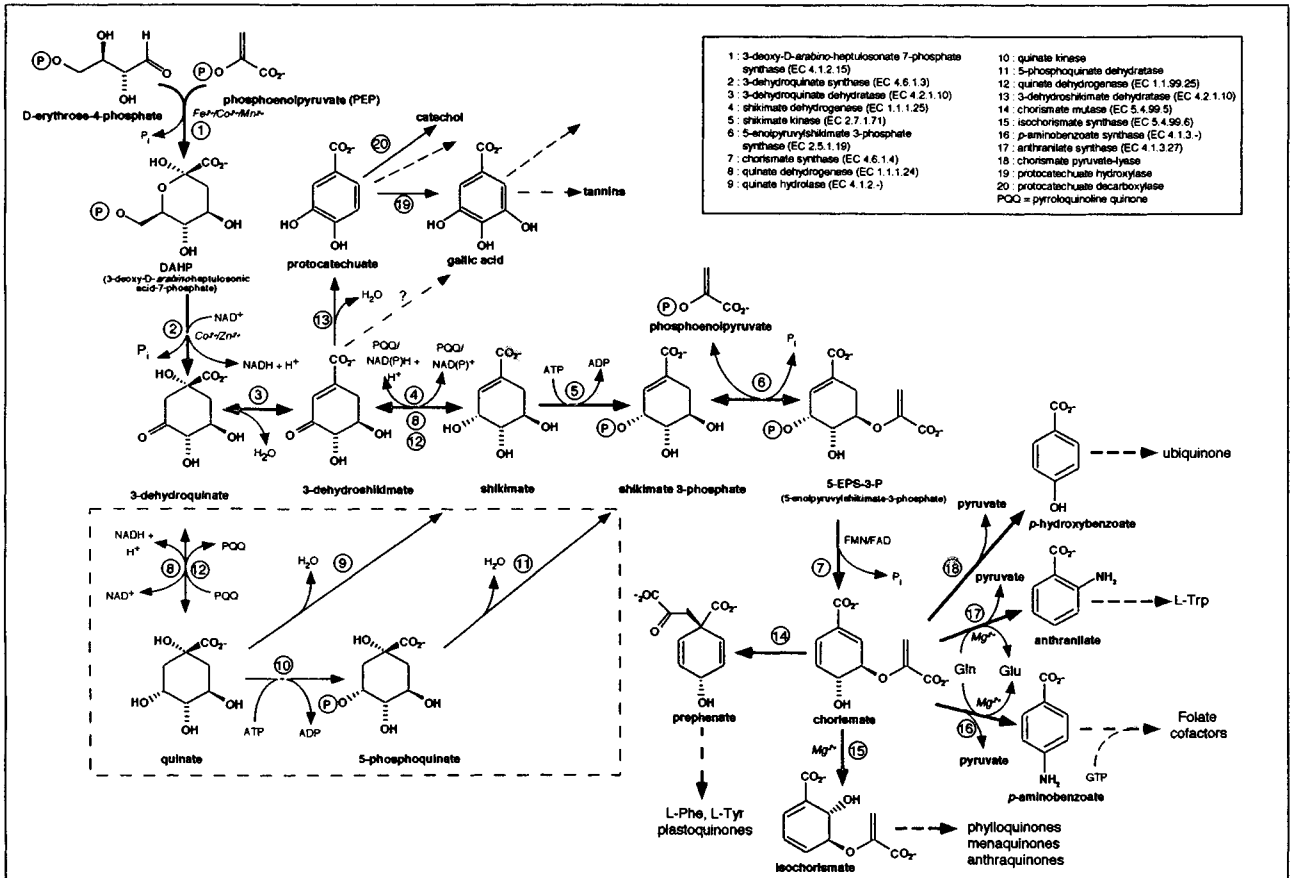


Figure 2. Shikimate pathway.

convincing evidence has been found for this theory. Although, in several plant species for both chorismate mutase and anthranilate synthase more than one gene has been cloned, only in case of chorismate mutase a plastidial and a cytosolic enzyme have been found.[34,36,37]

The phenylpropanoid pathway is one of the most important metabolic pathways in plants in terms of carbon flux.[31,32,38] In a cell more than 20% of the total metabolism can go through this pathway, the enzyme chorismate mutase is an important regulatory point. The importance of this pathway is due to the fact that it leads to among others lignin, lignans, flavonoids, and anthocyanins (Chapters 7 and 8) (Fig. 3). Key to these products is the enzyme phenylalanine ammonia lyase (PAL), which converts phenylalanine into *trans*-cinnamic acid by a non-oxidative deamination. This enzyme can be found in all plants, in some plants a single enzyme is found, whereas others may have several iso-enzymes. These enzymes may be under different regulation, *e.g.* inducible after wounding or by UV-light. In alfalfa as many as 6 PAL genes have been found.[38] PAL plays an important role in controlling the flux into the phenylpropanoid pathway as was shown among others by overexpression of a gene encoding PAL in tobacco.[39]

The product of the deamination by PAL, cinnamate, can be further hydroxylated and methylated, leading to compounds such as coumaric acid, caffeic acid, ferulic acid, 5-hydroxyferulic acid, and sinapic acid. The introduction of the various hydroxy groups is catalyzed by monooxygenases, which are membrane bound cytochrome P-450 enzymes, or soluble phenolases. The hydroxycinnamates are activated by ligases that produce the CoA-esters of the compounds. The CoA-esters are the starter molecules for various biosynthetic routes. The most important being that leading to the flavonoids, in which coumaryl-CoA is coupled with three C_2-units coming from malonyl-CoA. This reaction is catalyzed by the enzyme chalcone synthase (CHS), the product is the chalcone naringenin (see Chapters 7 and 8). A further ring closure leads to the basic skeleton of the flavonoids. Until this point, this pathway is probably the same in all plants, from thereon a variety of reactions lead to the various flavonoids, anthocyanidins, isoflavonoids, etc. These reactions include the introduction of further hydroxy or methoxy groups, conjugations with various sugars or acyl groups (*e.g.* hydroxycinnamyl, malonyl). The enzyme CHS has been extensively studied and a number of genes have been cloned from various plants.[40] A closely related enzyme is stilbene synthase, which has a high degree of homology with CHS. This enzyme leads to a different type of ring closure in the condensation product of coumaryl-CoA and the three malonyl-CoA molecules. The compound formed is the stilbene resveratrol, which has only two carbons in between the two aromatic moieties instead of three as in the chalcones (Fig. 3) (Chapter 11).[41] Again this compound is the basis of a series of secondary metabolites.

Cinnamate and its hydroxy-derivatives are also the precursor for a broad variety of other phenolics such as coumarines, formed by lactonization after introduction of an ortho hydroxy group in cinnamate, and benzoic acid derivatives such as salicylic acid by cleavage at the double bond in the side-chain of cinnamate. Conversion of the carboxylic group in the (hydroxy) cinnamates to an alcohol, yields the building blocks for lignin and the lignans[19]. Lignin is an important constituent of secondary cell walls.

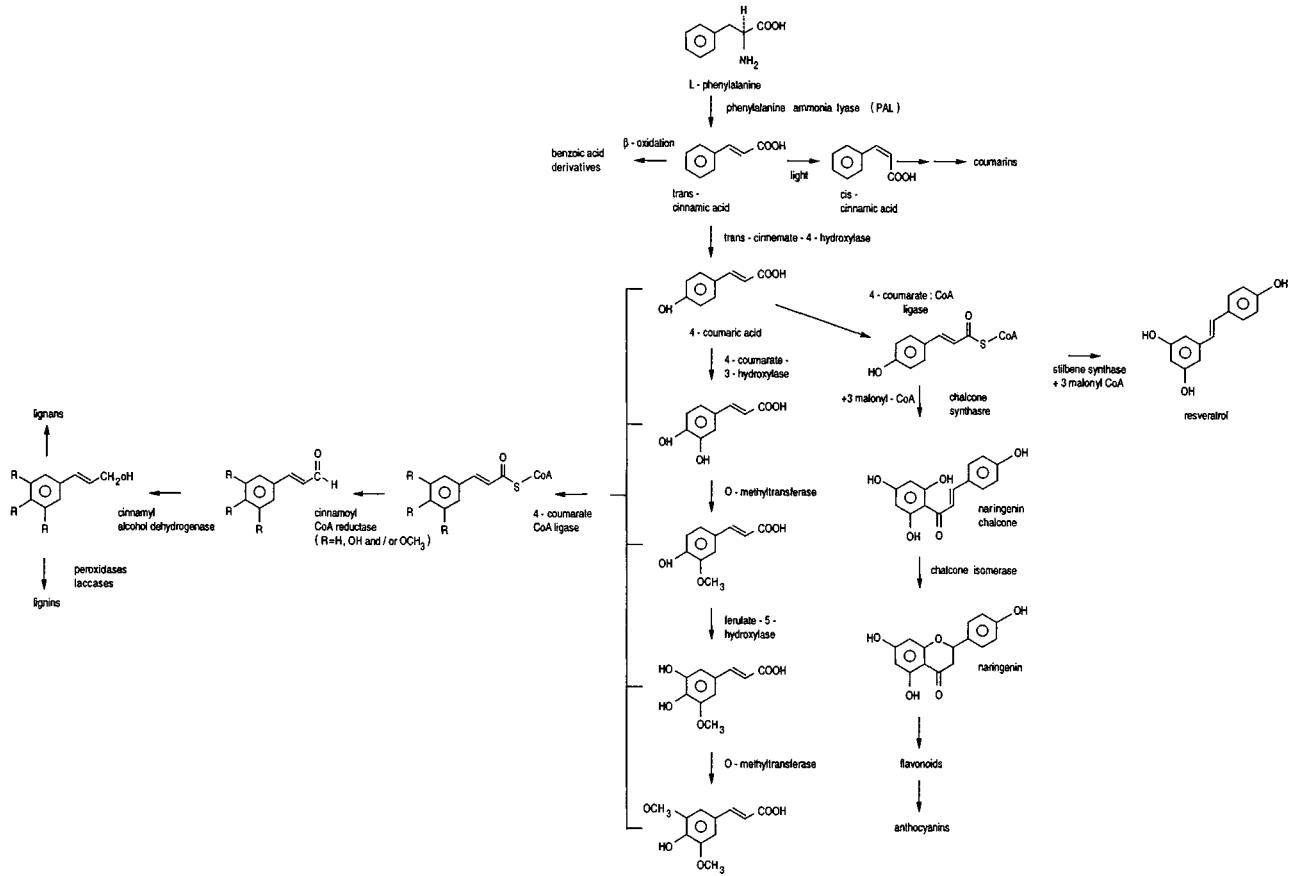


Figure 3. Phenylpropanoid pathways.

It is formed through the condensation of the hydroxycinnamyl alcohols into polymeric structures, which are attached to the polysaccharides in the cell wall. The coupling is an oxidative process catalyzed by peroxidases. Lignans are dimers of hydroxycinnamic alcohols, such as the antitumor drug podophyllotoxin.

The two major classes of alkaloids, the isoquinoline and the indole alkaloids, are derived from the aromatic amino acids. Decarboxylation of these amino acids is an important step in the biosynthesic pathway of these alkalods. The isoquinoline alkaloids are formed from dopamine which is condensed with 4-hydroxyphenyl acetaldehyde (both formed from tyrosine), yielding the benzylisoquinoline norcoclaurine. This compound is in a series of steps (O-methylation, N-methylation, hydroxylation, O-methylation) converted into reticuline, the precursor for a large number of isoquinoline alkaloids such as morphine, sanguinarine and berberine.[42,43] The diversity of structures in the isoquinoline group of alkaloids is first of all based on oxidative phenol couplings, giving rise to new skeletons, *e.g.* apomorphine-, morphine- and bisbenzylisoquinoline-type (Fig. 4).

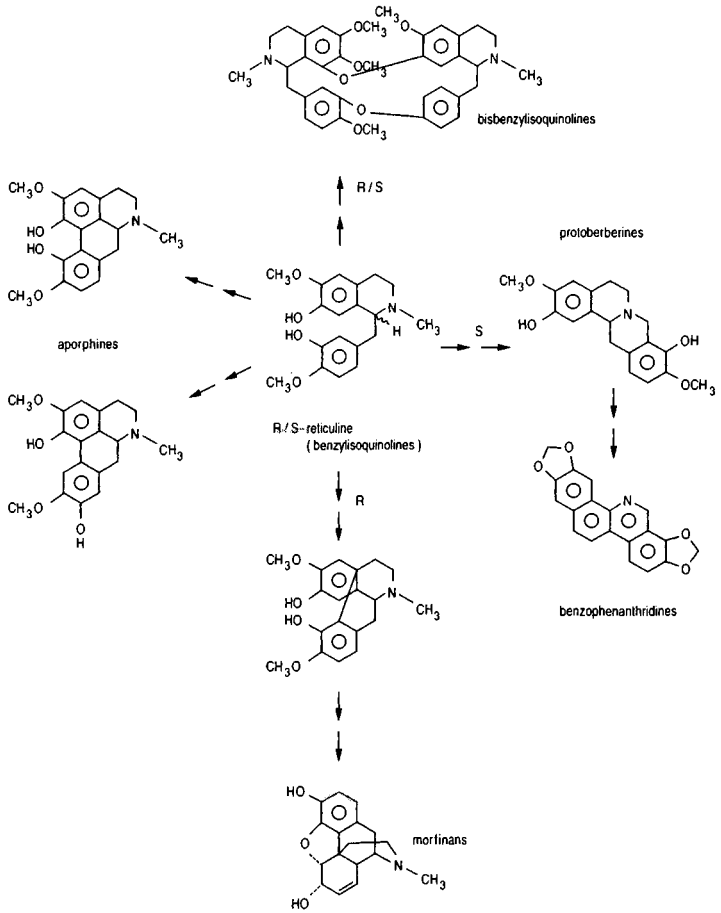


Figure 4. Some basic skeletons of isoquinoline alkaloids formed through oxidative phenol coupling.

The terpenoid indole alkaloids are formed by a Pictet-Spengler-type condensation reaction of the aldehyde secologanin and tryptamine, yielding strictosidine (see Chapters 4, 6, 9 and 10). Strictosidine is the precursor for a large number of terpenoid indole alkaloids, such as strychnine, vinblastine, reserpine, ajmaline, ajmalicine, and some related quinoline alkaloids, such as quinine.[42,43,44] In the formation of the various skeletons of indole alkaloids, the condensation of an aldehyde function with an amine plays a major role (Fig. 5), as after hydrolysis of strictosidine a molecule is formed which has two aldehyde and two amine functions.

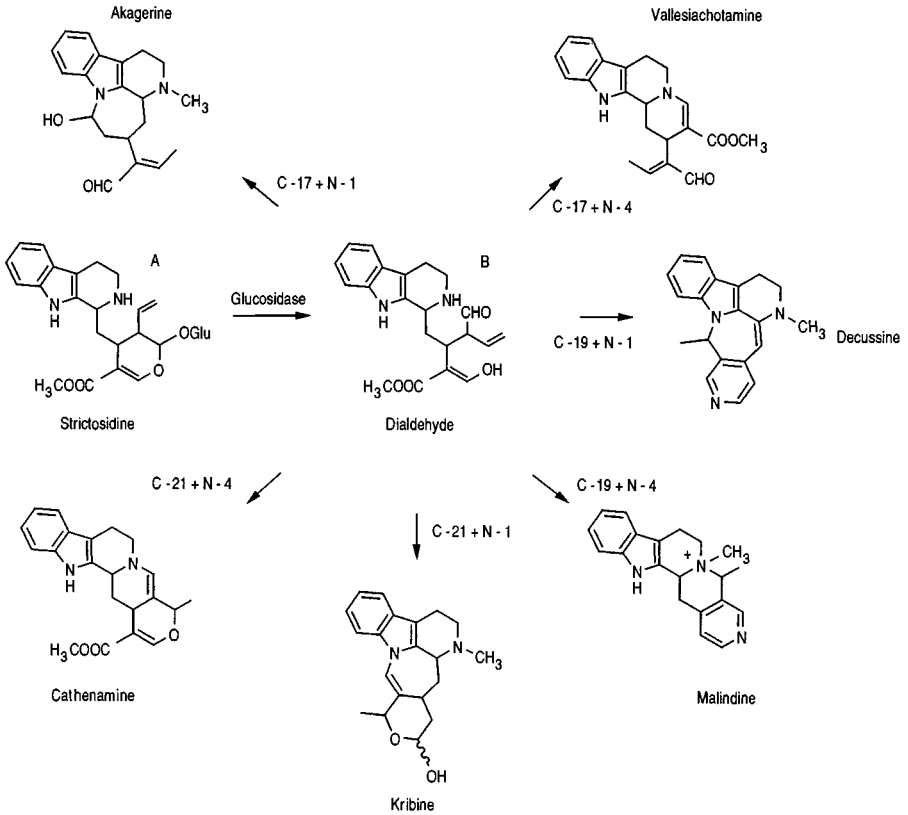


Figure 5. Various skeletons of terpenoid indole alkaloids formed through the intramolecular reaction of an aldehyde group and an amine function.

Some other types of phenolic compounds are derived from other branches of the chorismate pathway (Fig. 1). For example, the isochorismate branch leads to anthraquinones (*e.g.* in some Rubiaceae plants). Naphtoquinones are derived from 4-hydroxybenzoic acid. However, anthraquinones and naphtoquinones can also be derived from the polyketide pathway, *e.g.* anthraquinones in the Rhamnaceae family and naphtoquinones in the Plumbaginaceae. Also for benzoic acid derivatives, such as salicylic acid, 2,3-dihydroxy-benzoic acid and 4-hydroxybenzoic acid, two possible

pathways exist. One pathway *via* cinnamic acid (commonly found in plants) and another directly from chorismate *via* isochorismate (commonly found in microorganisms).[45]

Terpenoid pathway

The other important pathway in plants is that of the terpenoids, also known as isoprenoid pathway.[28,46,47,48,49,50,51] Terpenoids include more than one third of all known secondary metabolites (Fig. 1). Moreover, the C_5-building block is also incorporated in many other skeletons, *e.g.* in anthraquinones, naphtoquinones, cannabinoids, furanocoumarines, and terpenoid indole alkaloids. In the "decoration" type of reactions in various types of secondary metabolites C_5-units are attached to the basic skeleton, *e.g.* hop bitter acids, flavonoids and isoflavonoids.[52,53]

The C_5-buiding block is isopentenyl diphosphate (IPP), which is isomerized into dimethylallyl diphosphate (DMAPP) by the enzyme IPP isomerase. The highly reactive DMAPP is the starter molecule of the terpenoid biosynthesis.[28,54,55] (Fig. 6). The allylic phosphate group is an excellent leaving group, yielding a carbonium ion, stabilized by the allyl function. This carbonium ion is a reactive alkylating agent, which readily reacts with IPP, giving geranyl diphosphate (GPP). This molecule again has the active allylic phosphate group, and can thus further react with a molecule of IPP to give farnesyl diphosphate (FPP). A further reaction yields geranylgeranyl diphosphate (GGPP). These reactions catalyzed by prenyltransferases yield respectively monoterpenes (C_{10}), sesquiterpenes (C_{15}) and diterpenes (C_{20}).

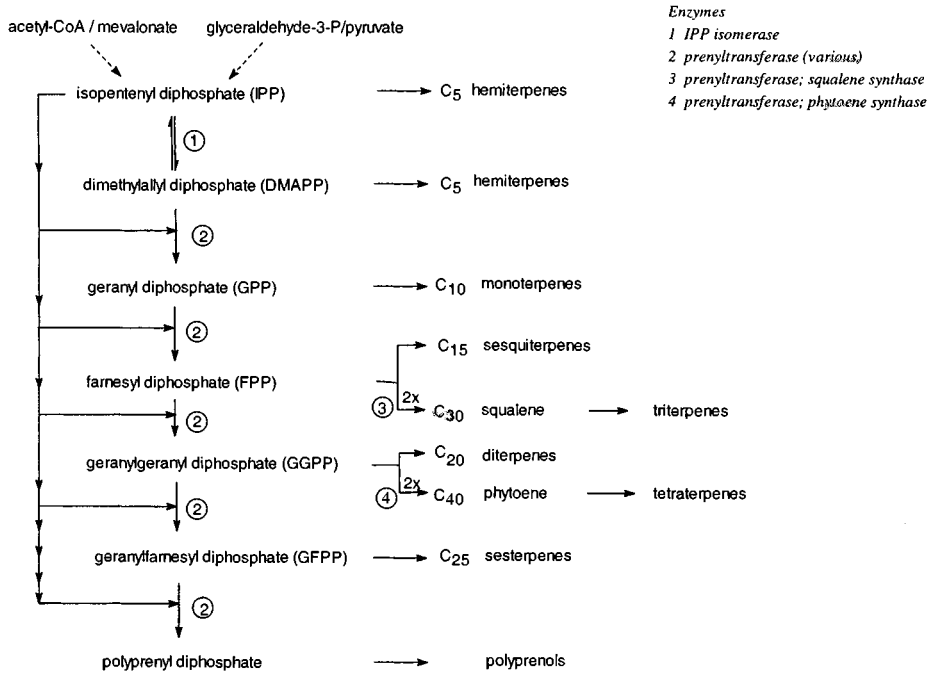


Figure 6. Biosynthesis of the various groups of terpenoids.

The tail-to-tail coupling of two all-trans molecules of FPP results in the formation of squalene, the precursor for the steroids and triterpenoids. These two important groups of terpenoids are both formed from squalene oxide, but from two different conformations of this precursor. Tail-to-tail coupling of two GGPP molecules results in the formation of phytoene (C_{40}), the precursor of the carotenoids.

In each of the groups of terpenoids different skeletons are found. The enormous diversity in mono-, sesqui-, and diterpene skeletons is first of all due to selective terpenoid synthases and cyclases, a large family of enzymes which catalyze the cyclization of differently folded GPP-, FPP-, or GGPP-molecules, which also may have undergone cis-trans -isomerizations.[51] From the various basic skeletons further skeletal diversity can be introduced in subsequent biosynthetic steps in which among others various cytochrome P-450 enzymes play a major role.[51,56]

Compartmentation is an important aspect of the terpenoid biosynthesis. Although several theories exist, mainly differing in the source of the IPP/DMAPP precursor, it is now generally accepted that the C_{10}, C_{20} and C_{40} compounds are formed in plastids, whereas the C_{15} and C_{30} compounds are formed in the cytosol.[47,54,55] This compartmentation coincides with the crucial role of some C_{20} and C_{40} type compounds in photosynthesis, and of some C_{30} type of compounds as constituents of plant membranes.

From all the mentioned groups of terpenoids, a large number of secondary metabolites is derived, all having different roles for the plant, *e.g.* essential oils (monoterpenoids) and flower colours (carotenoids) as insect attractants, phytoalexins (sesqui-, di- and triterpenes)[59] as antimicrobial agents, and antifeedants (mono- and sesquiterpenes) as defence against various predators.[60,61] Also among plant hormones terpenoids are found, *e.g.* abscisic acid, gibberellins, and brassinolides (respectively sesqui-, di- and triterpenes).[62,63,64,65]

For many years, it was thought that all terpenoids derive from mevalonate. Radioactive ^{14}C-mevalonate was shown to be incorporated in all kinds of terpenoids, however, in many cases the precise site of incorporation was not determined. Recently by using ^{13}C-labeled intermediates, it was found that in microorganisms certain terpenoids were not formed from mevalonate but from a very different pathway, also yielding IPP/DMAPP as final product.[55,58,66,67] This pathway is now also shown to be involved in the biosynthesis of terpenoids in plants. Particularly the plastidial terpenoids seem to be derived from the new pathway, *i.e.* the monoterpenes, diterpenes and carotenoids.[55,58] Whereas the cytosolic terpenoid pathway seems to use IPP and DMAPP derived from the mevalonate pathway, *i.e.* the sesquiterpenes, steroids and triterpenes. The alternative pathway starts with glyceraldehyde-3-phosphate and pyruvate as precursors, which in several steps yield D-1-deoxyxylulose-5-phosphate (Fig. 7). A rearrangement of this molecule results in the branched skeleton of IPP.[66,67] In plants this pathway is operative in plastids.[55,58]

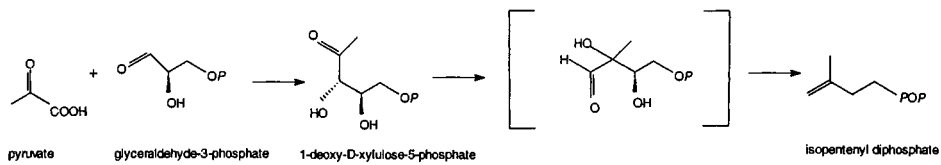


Figure 7. Deoxy-xylulose pathway leading to IPP/DMAPP, precursors for the terpenoids.

The mevalonate pathway starts with the coupling of three molecules of acetyl-CoA (Fig. 8). [28,68,69,70,71] The hydroxymethylglutaryl-CoA (HMG-CoA) formed is subsequently reduced by the enzyme HMG-CoA reductase to yield mevalonic acid. In two steps mevalonate kinase and mevalonate-5-phosphate kinase form mevalonate diphosphate, which is subsequently decarboxylated to yield IPP. Particularly the enzyme HMG-CoA reductase has obtained a lot of attention as it has an important regulatory role in the biosynthesis of the terpenoids including cholesterol. It is the target of the statins, drugs lowering the blood cholesterol levels. Also in plants HMG-CoA reductase has extensively been studied. Several isoenzymes seem to occur, each encoded by differently regulated genes.[72]

Because of these new findings, most major terpenoid biosynthetic pathways need reinvestigation concerning the source of the C_5-units used. With feeding early, ^{13}C-labeled, precursors and determining the site of incorporation in a molecule by means of $^{13}CNMR$, the origin of IPP/DMAPP from either pathway (or both) can be established.[55,58,73]

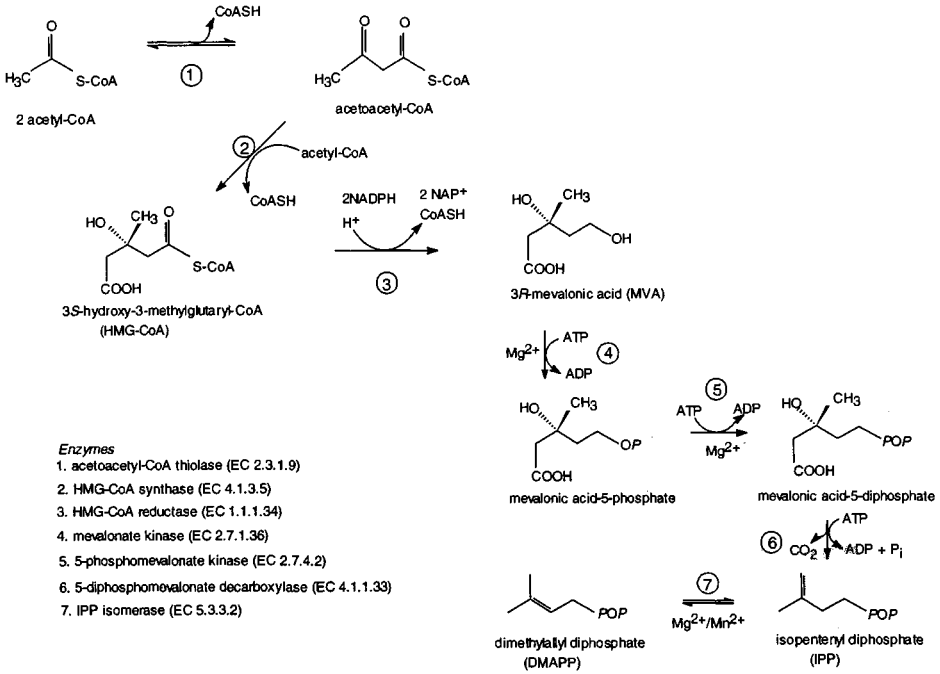


Figure 8. Mevalonate pathway leading to IPP/DMAPP, precursors for the terpenoids.

Polyketide pathway

The poyketide pathway plays an important role in primary metabolism in the biosynthesis of fatty acids. The fatty acids are basis for various secondary metabolites, but the polyketide pathway is also directly leading to secondary metabolites, particularly

in micro-organisms, but also in plants.[27,28,74] The C_2 polyketide building block acetyl-CoA ester is first converted into the more reactive malonyl-CoA ester. This compound is then used in a variety of reactions, among others, also in the start of the mevalonate pathway (Fig. 8). In the fatty acid biosynthesis, acetyl-CoA is the starter molecule, bound to a thiol group in the fatty acid synthetase enzyme complex, the malonyl-CoA is bound to another vicinal thiol group in the acyl carrier protein (ACP) and is subsequently condensed with the acetyl group. The acetoacetyl-ACP is reduced to give the butyryl-ACP, which reacts with a further malonyl-CoA, thus in a series of reactions the fatty acids are built up. From the fatty acid pathway various secondary metabolites are formed, such as alkanes, acetogenins and jasmonates.

The malonyl-CoA is also part of the flavonoid biosynthesis, coumaryl-CoA is condensed with 3 molecules of malonyl-CoA, after which ring closure yields naringenin (see above). The enzyme stilbene synthase results in the formation of stilbenes such as resveratrol using the same substrates (Chapter 11). The condensation of coumaryl-CoA with one malonyl-CoA leads to benzalacetones.[74] Other examples of plant secondary metabolites derived from the polyketide pathway are 6-methylsalicylic acid and coniine (4 C_2 units), plumbagin (6 C_2 units) and anthraquinones (8 C_2 units) (Fig. 9). However, the anthraquinones are in some plant families derived from the chorismate pathway.

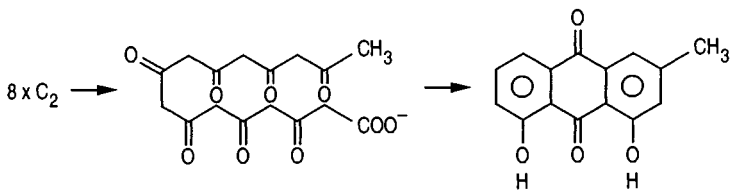


Figure 9. Polyketide biosynthetic pathway leading to anthraquinones.

Chemodiversity and pathway architecture

Chemodiversity

The basic skeletons resulting from the above mentioned general secondary metabolite pathways are in each plant species further modified in a specific way. The modifications concern among others the introduction of further substituents, new functional groups, isomerizations, ring opening followed by new ring closures, and coupling of different secondary metabolites. Examples of the introduction of further substituents are best illustrated by prenylgroups as for example found in flavonoids[53] and glycosides in which different sugars are coupled usually to hydroxygroups, but sometimes even directly to carbons or other functional groups of a molecule. In a number of cases, it has been shown that the glycosides are a storage form of toxic compounds which can be liberated by glucosidases in case of cell damage. The cyanogenic glycosides are a well-known example.[75,76] Also in the terpenoid indole alkaloid biosynthesis a similar activation is observed with strictosidine.[77]

The introduction of various functional groups is particularly due to oxidative reactions. It includes the introduction of hydroxy, methoxy, epoxy, aldehyde and carboxyl

groups. The enzymes involved are often cytochrome P450 enzymes, dioxygenases and peroxidases. In plants these types of enzymes occur as a number of closely related isoenzymes, all encoded by separate genes. For example in *Catharanthus roseus* one can detect 16 closely related cDNAs by means of PCR using conserved areas of P450 enzymes as primers.[78] The P450 monooxygenases consist of a haemgroup containing protein which determines the substrate selectivity and a flavoprotein, the NADPH-cytochrome P450 reductase.[79,80,81] In contrast to mammalian P450 enzymes, most plant P450 enzymes are highly substrate specific and have well defined roles in (secondary) metabolic pathways; however, also P450 enzymes with broad specificity have been reported which play a role in detoxification of xenobiotics. Generally, the P450 enzymes are bound to the endoplasmic reticulum membranes. They are among others involved in the oxidative phenol coupling, which plays an important role in the isoquinoline alkaloid biosynthesis[42], aromatic hydroxylations in various phenylpropanoid pathways (*e.g.* the ubiquitous cinnamic acid 4-hydroxylase) and hydroxylations in terpenoids.[81]

Dioxygenases, iron containing enzymes without a haem-group, occur also widely in plants and are encoded by multigene families. They include among others lipoxygenases in lipid metabolism, several enzymes in the flavonoid biosynthetic network and some gibberellin hydroxylases and oxidases.[82] In Chapter 13 hyoscyamine 6β-hydroxylase is discussed, this enzyme is also a dioxygenase.

Peroxidases are a further group of haem-containing enzymes found in all plants, which play a role in oxidative conversions of secondary metabolites.[83] These enzymes require H_2O_2 as co-factor, and in general have broad substrate specificity.

Pathway architecture

Usually in plants a number of closely related compounds is found. From an evolutionary point of view, this makes sense as it may result in a broader spectrum of activity, *e.g.* broadspectrum antimicrobial activity. Moreover, development of resistance against such mixtures of compounds might be more difficult than in case of a single compound, again antibiotics may serve as an example. The formation of a number of related compounds is due to different mechanisms, in part this can be the chemistry of intermediates, in part the substrate specificity of the enzymes of a pathway.

Examples of the role of chemistry are isomerizations or intramolecular rearrangements. Chemical reactions may occur in a biosynthetic pathway, which are not controlled by an enzyme, and which may result in, for example, different stereoisomers (chiral compounds). Isomerization can be illustrated with the biosynthesis of the *Cinchona* alkaloids, in which cinchoninone and quinidinone are intermediates that are in equilibrium with respectively cinchonidinone and quininone (Fig. 10). The equilibrium is dependent on the environment, *e.g.* the pH. In the plant two NADPH-oxidoreductases are found, one of which can only reduce the non-methoxylated compounds into cinchonine and cinchonidine, another which can also reduce the methoxylated compounds, thus yielding the already mentioned alkaloids as well as quinine and quinidine. The ratio between the two diastereisomers is thus determined by the chemical equilibrium (for a review see [84]).

The role of substrate specificity of the enzymes in a pathway is a crucial one. Assuming that each enzyme in a pathway is strictly selective for the substrate, one obtains a linear

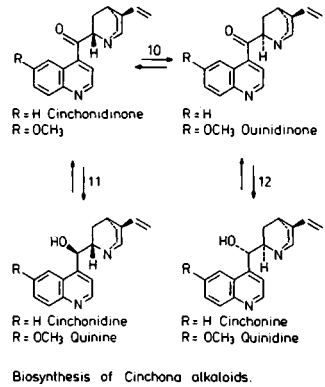


Figure 10. Isomerization of intermediates in the quinoline alkaloid biosynthesis results in the biosynthesis of two stereoisomers.

pathway, and a mixture of different compounds is only due to the fact that a pool of different intermediates exists. However, if enzymes are also capable of doing more than one reaction or accept different substrates, a completely different picture evolves. One obtains metabolic grids or networks in which different compounds can be formed and accumulated. In Fig. 11a theoretical example is given, it assumes a basic skeleton S in which three functional groups can be introduced. If all steps would be catalyzed by highly specific enzymes, 12 enzymes could be involved in the formation of three different products with one functional group, three different products with two functional groups, one final product with all three functional groups). The other extreme would be only one enzyme, capable of doing all functionalizations, which in case of oxidation might be possible, but more likely is three enzymes, each for one functional group, but with a broad substrate specificity. By a partial substrate specificity, *e.g.* the enzyme responsible for the introduction of group 3 is unable to utilize $S_{1,2}$, this compound will accumulate. Affinities and activities of the enzymes for the different substrates will thus regulate the relative amounts of all compounds. Examples of such pathways are many, terpenoids which carry numerous functional group, introduced on the basic skeleton formed in the early stages of the biosynthesis (mono-, sesqui-, di-, triterpenoids and steroids), pyrrolizidine alkaloids, and flavonoids. Clear examples of high substrate specificity are found in the extensively studied biosynthesis of isoquinoline alkaloids.[42,85] The substrate specificity of the berberine bridge enzyme plays a role in the utilization of R/S-reticuline (Fig. 4). This enzyme can only utilize S-reticuline as substrate, and is the start of the pathway leading *via* scoulerine to the various protoberberine and benzophenanthridine alkaloids. R-reticuline is the precursor for the pathway leading to morphinan alkaloids, the first step being an intramolecular oxidative phenol coupling yielding salutaridine. This step is catalyzed by a highly selective cytochrome P450 enzyme, unable to utilize the S-isomer. Another interesting example is the biosynthesis of berberine in *Berberis stolonifera* and *Coptis japonica*. In both systems tetrahydrocolumbamine is the precursor, but in *Berberis* first the third aromatic ring is formed and then the methylenedioxy-function, in *Coptis* the sequence is the opposite (Fig. 12).[86] Apparently one and the same compound can be formed through different pathways, in this case in *Coptis* a different

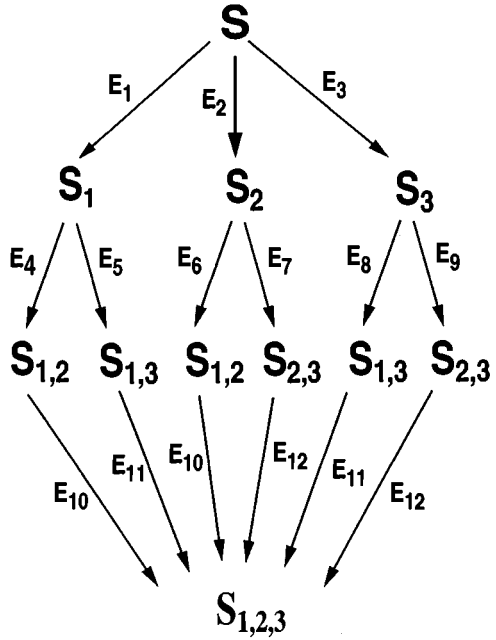
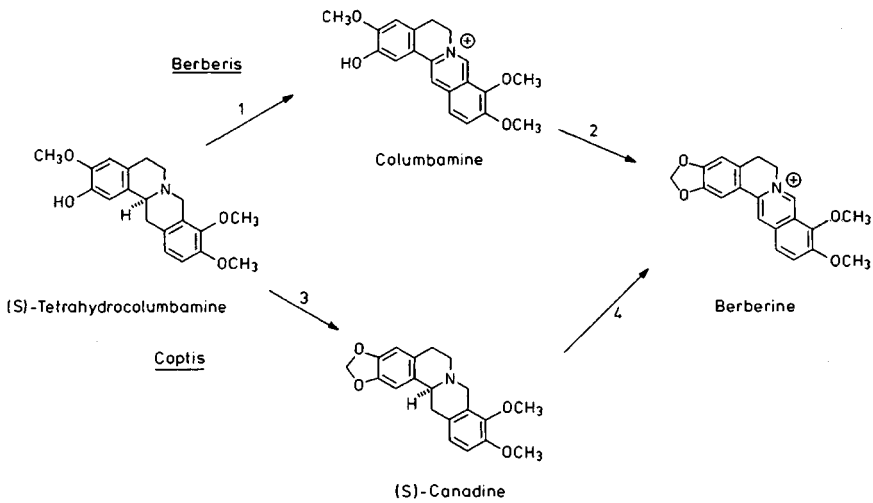


Figure 11. Schematic biosynthetic network, S is a basic skeleton to which the functional groups 1, 2 and 3 are added. $E_{1\text{-}12}$ are enzyme catalyzed steps. With high enzyme substrate specificity 12 different enzymes are necessary to produce $S_{1,2,3}$, with very low substrate specificity 3 enzymes would be sufficient.



1 (S)-Tetrahydroprotoberberine oxidase (STOX)
2 Berberine synthase
3 (S)-Canadine synthase
4 (S)-Canadine oxydase

Figure 12. Two different pathways may lead to the same product in two different plant species.

type of oxidase is involved in the formation of the aromatic ring. Also the stereospecificity of the reaction catalyzed by an enzyme can be reason for further chemodiversity, this is best illustrated by the reduction of tropinone. Two different reductases have been found in the plant *Hyoscyamus niger* which each give only one stereoisomer of tropine.[87] Tropine is further channelized into the tropane alkaloids, pseudotropine probably into the calystegines (see Chapter 13).

Regulation

Genes

Each plant produces a broad spectrum of secondary metabolites, all serving different purposes, such as antifeedant, antimicrobial, attractant and signal compound. Based on the different functions, the expression of the pathways involved will be different. Some pathways will be expressed constitutively in a certain tissue. This can be in young tissues to be protected against insects, or in flowers to attract pollinators by colour or volatile attractants. Other pathways are induced on gene level after wounding or infection. Induction can also occur on the level of compounds. Certain constitutively expressed compounds are enzymatically converted into biologically active compounds after wounding, *e.g.* compounds liberated through the effect of a glucosidase such as HCN from cyanogenic glycosides.[75,76] Similar systems of an inactive glycoside activated by a specific glucosidase are also found in other plants, *e.g.* strictosidine and strictosidine glucosidase in *Catharanthus roseus.*[77]

The different functions of secondary metabolites mean that the regulation of certain pathways on the gene level is part of the developmental process of the plant cells, *i.e.* the process of differentiation. On the other hand, other pathways are inducible on gene level by external signals, such as elicitors or endogenous signal molecules such as salicylic acid[88] or jasmonate.[89] Such signals can thus induce complete pathways.

As discussed above, there are some basic pathways, which can be found in most plants. But apparently such pathways can be regulated quite differently in plants. For example, the biosynthesis of certain sesquiterpenes in some Solanaceae plants is inducible by microbial infection[90], whereas in other plants the sesquiterpenoid biosynthesis is expressed constitutively. Another example is the biosynthesis of anthraquinones in Rubiaceae plant species. In *Rubia tinctorum* these compounds are constitutively produced in the roots, whereas in *Cinchona* species these compounds are only produced after induction through infection with plant pathogenic microorganisms. In *Morinda citrifolia* anthraquinones are found constitutively in all parts of the plant.[91] Apparently similar genes encoding enzymes of certain secondary metabolite pathways are differently regulated, by having different promoters. The homology between genes encoding certain enzymes of secondary metabolism which have so far been cloned support this view. Chalcone synthase is such an example[40], this enzyme occurs in most plants, if not in all, as it plays a major role in flavonoid biosynthesis. However, this pathway is quite differently regulated in different plant species, and even in different tissues from one plant. Best studied in this respect is the flavonoid biosynthetic pathway.[92,93,94] Flavonoids play a crucial role in plants such as in protection against UV-light damage, in the sexual reproduction process and in

the interaction with other organisms in the environment. Through thorough analysis of gene expression patterns and taxonomy one has been able to get insight in the evolutionary development of the various branches of the flavonoid-anthocyanin pathway.[92] Structural genes of the pathway are probably derived from genes from primary metabolism, as can be concluded from homology in their respective sequences, such as between chalcone synthase and genes from the fatty acid biosynthesis (polyketide formation). Through gene duplication and combination with other regulatory elements in the promotors new specialized structural genes have developed. In the course of time, these genes might have gradually changed, resulting in enzymes with an altered substrate specificity. Homology found between genes from different plants and different steps in the secondary metabolic pathways, support this. Eventually this has resulted in the enormous diversity in structures and roles of the flavonoids/anthocyanins in plants.[92] Consequently within one plant differently regulated isoenzymes may occur. Chalcone synthase[40] and phenylalanine ammonia lyase[38] are examples from the flavonoid pathway. HMG-CoA reductase[72,95,96] is an example from the terpenoid pathway. Tyrosine decarboxylase in poppy is an example from the isoquinoline alkaloid pathway.[97,98,99,100]

The role of regulatory genes in the biosynthesis of secondary metabolites is discussed in Chapter 2 and 6. These genes are interesting targets for turning on or off complete secondary metabolite pathways.[94,101]

Enzymes

Regulation of a pathway also occurs at the level of enzymes. Posttranslational modifications, enzyme turnover, feedback inhibition or activation, allosteric interactions, co-factor availability, and phosporylation, are just a few examples of possible regulatory mechanisms at this level. Catabolism can be illustrated with tryptophan decarboxylase (TDC) in *C. roseus*. Fernandez *et al.*[102] reported that TDC is stabilized by Mn^{2+} or Mg^{2+}, whereas ATP increased the rate of inactivation of TDC. The monomer of which TDC is built up was found to be ubiquinated, and thus inactivated.[103] Increasing the stability of an enzyme might thus also be an approach for enhancing the activity.

The regulation of the biosynthesis of the aromatic amino acids around chorismate may serve as an illustration of feedback inhibition and activation (Fig. 13). Anthranilate synthase, the first enzyme in the tryptophan branch is strongly inhibited by its end product, tryptophan. In the branch leading to phenylalanine, the first enzyme chorismate mutase is induced by tryptophan and inhibited by phenylalanine and tyrosine.[34,36]

Even pH might be a regulatory factor, as was described for the hydroxylase converting coumaryl-CoA into caffeoyl-CoA in parsley. This cytosolic enzyme is only active in a very narrow pH-range, having it maximum at pH 6.5, whereas at pH 7.5 and 4.5 it is almost inactive (the activity being restored by adjusting the pH to 6.5). Only after elicitation, when the intracellular pH drops, the enzyme becomes active.[104]

Compartmentation

Also compartmentation is an important aspect of regulation, both at cellular and subcellular level. Above we mentioned already the subcellular compartmentation of the aromatic amino acid biosynthesis and the terpenoid pathways. At cellular level many examples exist of compounds biosynthesized in roots and subsequently transported to

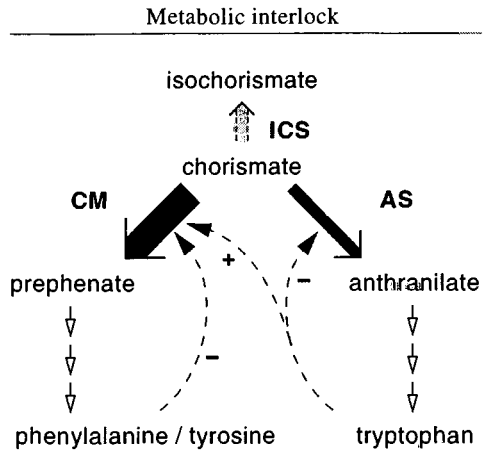


Figure 13. Regulation of the enzymes channeling chorismate into the pathways leading to the aromatic amino acids.

other plant organs, where they are stored. Well known is the production of hyoscyamine in roots of *Hyoscyamus niger*, from where it is transported to the leaves, in the cells of the pericycle the alkaloid is converted to scopolamine by an epoxidation (Chapter 13).[105]

Subcellular compartmentation is well documented for both indole and isoquinoline alkaloid biosynthesis. The biosynthesis of indole alkaloids in *Catharanthus roseus* may thus serve as an example (Fig. 14).[106] The two primary metabolism pathways that yield the precursors for the indole alkaloid biosynthesis in *C. roseus*, the terpenoid pathway leading to geraniol and the tryptophan pathway are thought to occur in plastids (for reviews see [47,54,55,58,68,107]). The site of the secologanin pathway remains unknown. Tryptophan decarboxylase is known to be cytosolic[77,108,109,110,111,112] whereas strictosidine synthase is a vacuolar enzyme.[77,108,109,110,113,114,115] The next enzyme in the indole alkaloid biosynthesis is a specific glucosidase, which is located outside the vacuole in the endo-plasmic reticulum.[77] The step from ajmalicine to serpentine is catalyzed by vacuolar peroxidases.[116] The biosynthesis of catharanthine occurs in the cytosol, whereas one of the enzymes in the vindoline pathway is localized in the thylakoids.[111]

Subcellular compartmentation also plays an important role in the biosynthesis of the isoquinoline alkaloid berberine (for a review see [43,117]). Several of the enzymes involved in the biosynthesis of this alkaloid and related compounds in *Berberis wilsoniae* are found in distinct vesicles, such as the berberine bridge enzyme responsible for the oxidation of the N-methylgroup in (S) -reticuline, and (S) -tetrahydroprotoberberine oxidase converting the tetrahydroderivative into the quaternary protoberberine compound.[118]

Compartmentation means that transport is included in a biosynthetic pathway. Such transport can be active or passive (diffusion). Energy requiring carriers are responsible for the active transport of a molecule through subcellular membranes. Selective and

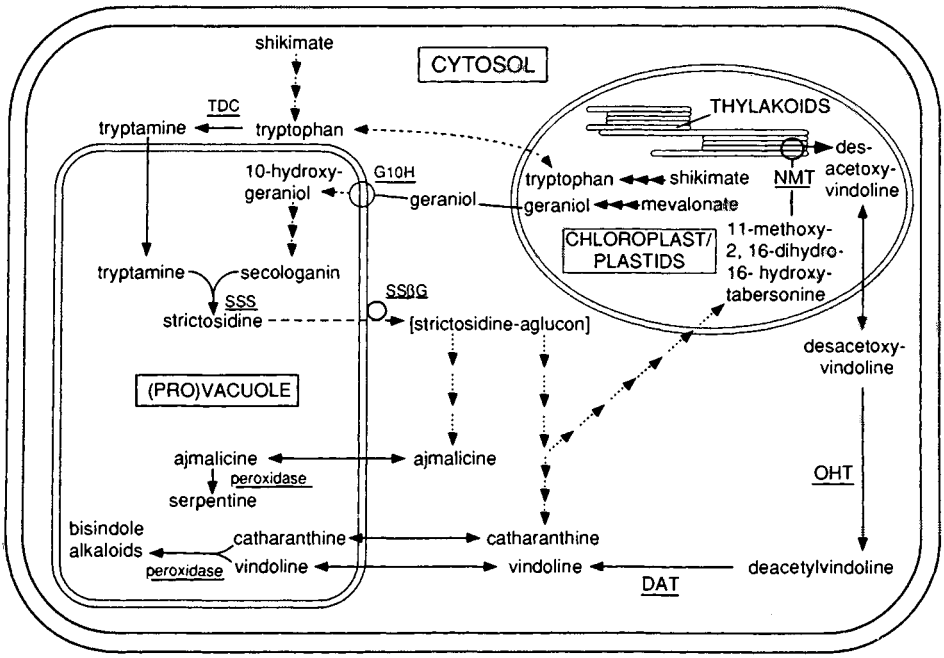


Figure 14. Summary of the knowledge on the compartmentation of the various parts of the biosynthesis of terpenoid indole alkaloids in *Catharanthus roseus* cells. Not all pathways will occur in one single cell.

active transport of the indole alkaloids ajmalicine and vindoline into vacuoles of *C. roseus* cells was postulated by Deus-Neumann and Zenk[119,120] They showed that the transport is ATP-ase dependent. Other alkaloids, such as morphine, codeine, scopolamine, and nicotine were not taken up by these vacuoles. On the other hand, ample evidence for the involvement of an ion-trap mechanism has been reported for the accumulation of ajmalicine[116,121] in *C. roseus* vacuoles and for the quinoline alkaloids in *Cinchona* cells.[122] In the ion-trap model, transport is driven by the difference in pH between cytosol and vacuole. The lower the pH in the vacuole, the stronger the accumulation of the alkaloids. Consequently, accumulation is controlled by ATP-dependent proton pumps, which are responsible for acidifying vacuoles. Tryptamine is produced in *C. roseus* cells by decarboxylation of tryptophan in the cytosol. The enzyme catalyzing the next step of the biosynthesis of terpenoid indole alkaloids is located in the vacuole. The transport of the strongly basic tryptamine into the vacuoles is probably also driven by the pH gradient. The example of the indole alkaloid biosynthesis clearly shows that selective transport proteins and/or pH-controlling genes may play an important role in the regulation of secondary metabolism.

On top of subcellular compartmentation, even molecular compartmentation may occur. Enzymes from a metabolic pathway might be arranged in a sequential manner in metabolic channels or metabolons as postulated by several authors (*e.g.* [57,123,124,125]). For example, separate metabolic channels are thought to occur in the endoplasmic

reticulum for the biosynthesis of sesquiterpenes and sterols.[123,125] Also the pathway from phenylalanine to flavonoids is thought to occur in the endoplasmic reticulum involving a multienzyme complex, with the first (PAL) and the last step (a flavonoid glucosyltransferase) located in the lumen of the membranes.[124]

Conclusions

This chapter gives just a very brief summary of secondary metabolite pathways. Nevertheless, here I should like to make some general conclusions, which are of importance in connection with developing strategies for engineering secondary metabolite pathways.

The basic skeletons of a number of secondary metabolites are formed in most plants, but the regulation of these early steps might be quite different. The diversity in secondary metabolites found in plants is due to specific enzymes, which add new structural elements to the basic skeletons. However, one and the same compound may derive from completely different pathways in different plants.

Pathways are partly controlled by the cellular development, partly induced by exogenous or endogenous signals. Regulation of pathways is not only at the level of genes, but also enzymes are regulated, moreover compartmentation and thus transport plays a major role. Not all steps in biosynthetic pathways are catalyzed by enzymes, some are just spontaneous chemical reactions.

An important point to consider is that metabolism is a dynamic process, at any point in time, compounds are formed and often further metabolized. One can thus distinguish, developmental, seasonal as well as diurnal variations in secondary metabolite profiles in plants.[126] Unraveling secondary metabolism is thus like working on a 4-dimensional jig-saw puzzle, in which time and space are the dimensions.

Before a strategy for the modification of a pathway can be developed, it is thus of utmost importance to thoroughly investigate biosynthetic pathways at the level of intermediates, enzymes and genes. This pathway mapping should be followed or combined with cell biological studies. In the case of overproduction of specialty chemicals or blocking of the production of unwanted compounds, knowledge on the possible role of the secondary metabolites involved is also of interest, as it will allow the evaluation of the viability of a plant in which the secondary metabolite pathway is altered.

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CHAPTER 2

GENERAL STRATEGIES

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Introduction

In the past years different methods have been developed for the genetic transformation of plants and plant cells. The two most successful ones are direct introduction of genes using the particle gun and *via* a (binary) vector transferred by *Agrobacterium*. In the Chapters 3 and 4 these methods and some applications are described in more detail. *Agrobacterium tumefaciens* is widely used for transformation of plants. A major restriction is the limited host range, which is mostly dicotyledonous plants, and even in this group not all plants. The particle gun is, in principle, applicable to any plant species. Though transformation is feasible, the regeneration of a plant from the transformed cells is often a difficult task.

For the production by *in vitro* cultured cells, transformation with the *Agrobacterium rhizogenes* is also used. Infection with this bacterium results in the tumorous growth of roots. These so-called "hairy roots" can be cultured *in vitro* and produce secondary metabolite profiles similar to the roots of the plants. They are interesting for studying secondary metabolism. For the production of rare compounds such hairy roots systems are of interest[1], though their growth on large scale is quite expensive.[2] With *A. rhizogenes* also new genes can be introduced into a plant (cell).

The transformation of plants and plant cells being feasible, the question is what applications are possible in connection with secondary metabolism. One can distinguish three major areas:

- production of specialty chemicals;
- altering quality traits of food, fodder or horticultural plants;
- increasing the resistance of plants against pest and diseases.

In the first category the aim is to produce economically interesting levels of specialty chemicals, such as drugs, dyes, flavours, fragrances, and agrochemicals. Production of known compounds can be envisaged by engineering the original plant itself as well as by introduction of the pathway in another plant species. Also the production of novel compounds might be of interest for screening for new lead compounds in the development of drugs or agrochemicals.

For the second category several applications can be thought of. The aim can be to increase the level of a certain compound (or compounds), which is of interest for the taste or flavour of food, or has health promoting effects.[3] Also the introduction of genes which open new pathways for the plant could be of interest. For example, with the aim to improve the taste of flavour, to alter colour of food, fruits or flowers,

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or to introduce a pathway leading to health promoting compounds. Both colour and health promoting effects (*e.g.* antioxidants) are strongly linked with the carotenoid and flavonoid/anthocyanin pathways (see Chapter 7).

A completely different approach in this category of applications is decreasing the levels of unwanted compounds. These could be compounds with less desired properties, such as strong (bitter) taste, or central nervous effects, such as caffeine. Also blocking the biosynthesis of toxic compounds could be an interesting goal, *e.g.* the glycoalkaloids in potatoes, or pyrrolizidine alkaloids or glucosinolates (Chapter 9) in some fodder plants. Blocking pathways can also be used to change colours of *e.g.* fruits or flowers.

The third category requires insight in the role of secondary metabolites in the defense systems of plants. Secondary metabolites play a role as antifeedant against various predators such as mammals and insects. Also in attracting pollinators, or insects preying on the predators, volatile secondary metabolites do play an important role.[4,5,6,7] In the resistance against microorganisms both phytoanticipins and phytoalexins play an important role in combination with various other systems, such as microbial cell wall digesting enzymes and proteases. Phytoanticipins are antimicrobial active compounds stored in a plant, which are liberated, and sometimes even activated, after wounding.[8] The biosynthesis of phytoanticipins is constitutively expressed. Phytoalexins are antimicrobially active compounds, which are *de-novo* produced after wounding and infection. Their biosynthesis is induced at the gene level.[9] Through metabolic engineering one can envisage an increase of the levels of the active compounds, or to introduce new compounds in a plant, known to be active from others. Depending on their role this can be compounds, which are constitutively expressed in certain tissues, or compounds of which the biosynthesis is induced after wounding. The introduction of stilbene synthase in plants resulting in the production of the phytoalexin resveratrol is such an example (Chapter 11). Another approach is to overexpress genes involved with the signal compounds of the inducible defence systems in plants, such as salicylic acid. The constitutive production of this signal compound in the systemic acquired resistance in plants, can be achieved by transformation with some bacterial genes encoding the biosynthesis of salicylic acid in two steps from chorismate. This pathway is different from the endogenous, which is thought to include phenylalanine and cinnamic acid.[10,11]

In altering plant secondary metabolism one has to keep in mind that blocking pathways may result in plants which are more vulnerable for certain pests of diseases, depending on the role of the pathway involved. In case of increasing levels of secondary metabolites or the introduction of new pathways in food plants, one has to consider the possibility of increased toxicity. Blocking pathways may also result in the accumulation of toxic intermediates.

Biosynthetic pathway mapping and gene cloning

BIOSYNTHETIC PATHWAY MAPPING

For each application of metabolic engineering a thorough knowledge of the biosynthetic pathway involved is needed, in order to develop a strategy to increase or decrease levels of a compound. Ideally this means that all steps at the level of intermediates and enzymes should be known. Based on this information certain steps can be selected as subject for metabolic engineering, and if necessary the gene(s) involved can be cloned. However, in many cases the secondary metabolite pathways are not well known (see Chapter 1). At the level of enzymes only a few pathways have been extensively studied.[12] But even known pathways could need confirmation by using ^{13}C-labeled precursors. The recent discovery of a new terpenoid pathway in plants (see Chapter 1), clearly demonstrates this need. Even between plant species differences may occur in seemingly similar pathways. The reversal of the last two steps in the biosynthesis of berberine is such an example (see Chapter 1).[13] The availability of the various, sometimes unstable, intermediates are a major constraint in such studies. Because of the complex structures, sometimes combined with instability, synthesis of the necessary labelled intermediates is not always possible or very elaborate. Moreover, feeding of intermediates to plants usually results in too low yields of the products to determine by spectrometric analysis the site of incorporation of the labels in the final products. Nowadays, plant cell suspension cultures are an important tool for such studies. The major advantage is the ease of feeding compounds to cultured cells and the inherent high incorporation levels. Even very early precursors such as amino acids, acetate, pyruvate or sugars can be easily fed in such systems. The limitation is that not all secondary metabolite pathways are expressed in cell cultures. The use of root or shoot cultures can usually solve this problem.

Another approach is the isolation of crude protein extracts from plants and incubation of these with intermediates of the pathway and detection of the formation of the products. Besides the need of the availability of the intermediates, this approach is sometimes hampered by the fact that enzymes are not stable and cannot be isolated in active form. An advantage of this approach is that it can be linked with efforts to identify possible limiting steps in pathways and to purify and characterize the enzymes as basis for future cloning of the genes (see below).

Mapping of the biosynthetic pathways is of great importance and is the basis for metabolic engineering. First the pathways need to be established at the level of the intermediates and then at the level of the enzymes. The most important constraint is that many of the target enzymes of secondary metabolism occur at low levels, requiring extensive purification. In combination with the instability of proteins, this often results in low yields of the protein.

CLONING GENES INVOLVED IN SECONDARY METABOLISM

Engineering secondary metabolite pathways requires the availability of genes encoding enzymes, which are able to catalyze steps from the pathway involved. Unfortunately the

number of genes known from secondary metabolite pathways in plants is rather limited. As many pathways are not known in detail, cloning of the genes involved is time consuming, as it requires good insight in the pathway at all levels, *i.e.* intermediates, enzymes and genes. Before one can start efforts to engineer a pathway, a suitable gene needs to be obtained from some source, thus necessitating extensive studies of the biosynthetic pathways.

In microorganisms secondary metabolite pathways have, in many cases, been elucidated with the aid of mutants. However, in plants this approach is hampered by the lack of extensive series of mutants. Obtaining the necessary mutants requires that the pathway is known, and intermediates can be detected. Which is quite an elaborate procedure, not even considering the difficulties in generating a large series of mutants of a plant and analyzing these for the desired mutation. In recent years molecular biology has developed relatively rapid methods to clone a certain gene, such as complementation of mutant microorganisms, transposon tagging, and differential screening. However, secondary metabolite pathways usually consist of a large number of specific steps and thus genes, consequently these approaches have so far not been very successful in unravelling complete pathways. Complementation of microbial pathways requires that the step involved is present in a suitable microorganism. In case of secondary metabolism it is almost by definition very difficult to find another organism having a similar pathway, which can be used to select for a successful complementation. With transposon tagging, selection of successful events is difficult just like it is in mutants obtained *via* other methods. Except in case of a visible trait, one has to analyze for the occurrence of the target compound which is accumulating as a result of the mutation, as well as to analyze for the other intermediates of the pathway involved.

For differential screening one needs to have a cell line or plant tissue in which under well-defined conditions the production of a certain compound is induced, so a high level of the mRNA involved is expected. This method has for example been applied with *Catharanthus roseus* cell cultures in which on an induction medium alkaloid production is increased.[14,15] This study aimed at cloning the gene encoding geraniol-10-hydroxylase (G10H), a cytochrome P450 dependent enzyme, which catalyzes the first step in the biosynthesis of secologanin, one of the precursors of strictosidine. Strictosidine is an important intermediate for the production of indole alkaloids (Fig. 1). A plant contains a large number of cytochrome P450 enzymes, and thus a correspondingly large number of P450 genes. Schroeder and co-workers[14,15] compared a cell culture induced for alkaloid production and a non-induced cell culture of *C. roseus*, in this way they found a cDNA encoding a cytochrome P450 enzyme. But no G10H activity could be confirmed for the protein expressed by this cDNA in various organisms.[15] Such more general approaches to clone a gene require solid proof for the activity of the enzyme after expression of the cloned gene in a suitable organism.

Also more specific approaches of cloning genes might offer problems. For example, the cloning of a gene through screening of a cDNA expression library may be difficult because the antibodies raised against the plant enzyme may lack specificity due to glycosylation or other posttranslational modifications of the protein. The presence of closely related proteins constitutes a further problem in gene cloning strategies based on

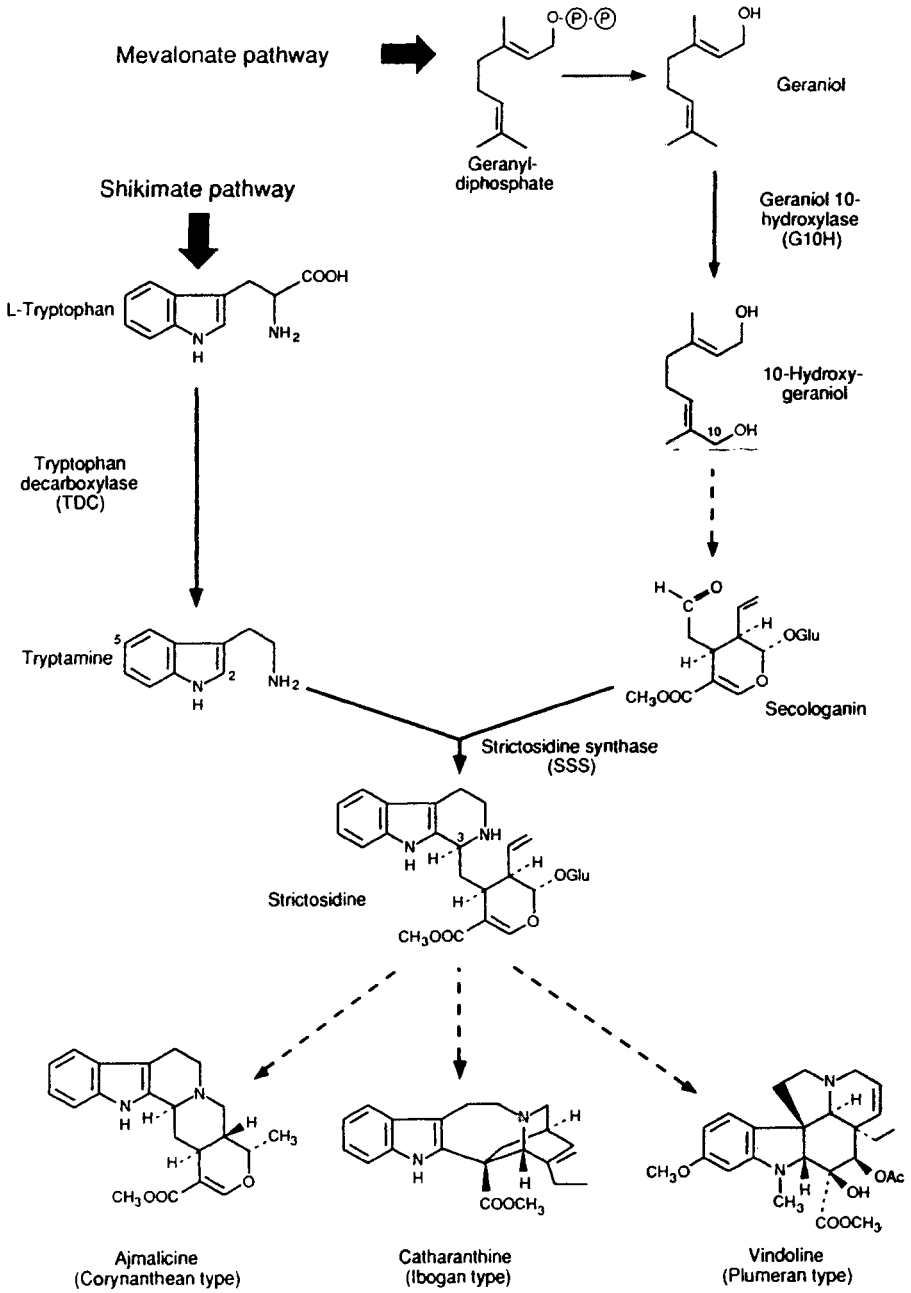

Figure 1. Biosynthesis terpenoid indole alkaloids.

the use of antibodies. The latter we experienced for example in our efforts to clone the gene for G10H. Polyclonal antibodies raised against purified G10H did not lead to the

isolation of a clone encoding G10H, due to the lack of specificity and the occurrence of a large number of P450 enzymes.

Homology of conserved areas can be used to clone an already known gene from another plant species. This approach has also been used for cytochrome P450 genes in order to clone the gene encoding G10H. However, by means of PCR with degenerate primers based on conserved areas of cytochrome P450 genes, 16 highly similar genes were detected. Only by expression of all these genes, one can identify the one encoding G10H-activity.[16] By using internal amino acid sequences of the enzyme, chances for cloning of the encoding gene might be better in case of a large gene family such as for cytochrome P450 enzymes.[17,18,19]

Because of these difficulties only few genes from secondary metabolism are knownso far. Most of these were cloned by using the information obtained from the amino acid sequence of the encoded enzyme or by screening a cDNA expression library with antibodies against the enzyme. Very few secondary metabolite pathways are known at the level of (some of) the enzymes involved. Some examples of pathways, which have been studied more extensively are the pathways leading to flavonoids/anthocyanidins (see Chapter 1 and 7), tropane alkaloids[20] (see Chapter 13), indole alkaloids from *C. roseus*[21,22,23] and *Rauvolfia*[24], and isoquinoline alkaloids.[21,25,26,27,28,29,30] The pathway leading to flavonoids/anthocyanidins is best known, even down to the level of the genes. This pathway has been subject to metabolic engineering, particularly with the aim to modify flower colours[31,32,33], or to study the response to various forms of stress in plants.[34] Besides the structural genes from this pathway, also regulatory genes are known, which have been used to turn on this pathway in different plant species (see also Chapter 2, 6 and 7).[35,36,37]

Finally it has to be noted that not all steps of secondary metabolite pathways are due to enzyme catalyzed reactions. Some can proceed chemically, *e.g.* the conversion of neopine to codeinone in the morphinan biosynthetic pathway.[38] Moreover, transport can play an important role in the regulation of biosynthetic pathways (see Chapter 1).

Strategies to increase the level of secondary metabolite production

As discussed above, increasing levels of secondary metabolites can serve different goals, such as to make more of a specialty chemical, to improve taste or flavour, or to increase levels of defense compounds. In general the following strategies can be envisaged to increase secondary metabolite production using molecular genetic methods:

- increase the flux through the pathway to the desired product;
- decrease catabolism of the desired product;
- increase the percentage of producing cells;

These approaches will be discussed in more detail.

INCREASE FLUX THROUGH PATHWAY

For increasing the level of a desired compound the first thought is to try to increase the total flow in the pathway towards the desired product. The flux might be controlled by various factors (Fig. 2):

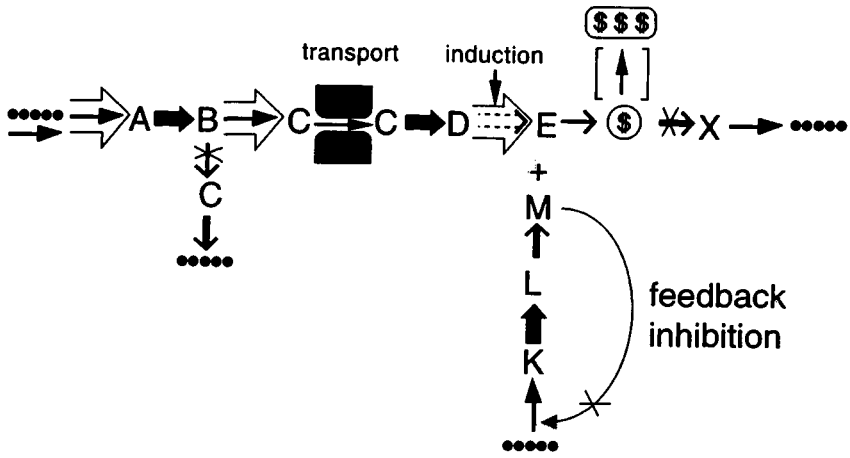

Figure 2. Various factors which might influence the flux through a pathway. Closed arrows: wild-type situation. Open arrows: improved flux by genetic engineering. Crosses: processes to be blocked by genetic engineering. [ ]: step added to pathway.

- relatively low activity of an enzyme(s) in the pathway including the occurrence of inducible enzyme(s);
- feedback inhibition of certain enzyme(s) in the pathway by an intermediate or the end product;
- competition with other pathways for certain intermediates;
- availability of co-factor(s);
- different compartmentation of enzymes and substrates.

In this approach the identification of the targets for genetic engineering thus requires detailed knowledge of the intermediates and enzymes of the pathway.

Rate limiting enzyme

The flux can be controlled by regulation of the level of enzyme activity. This can be a constant low level or even an enzyme, which is induced under certain conditions only. For example in phytoalexin biosynthesis induction at gene level is necessary for the production. To increase the amount of enzyme, one can use the gene from the plant itself or a gene from another plant or organism encoding an enzyme with a similar function, in combination with a strong (constitutive) promoter. Several examples of this approach have been reported in the past years as can be learned from the subsequent chapters in this book. In cases where low activity of an enzyme is due to rapid turnover, protein engineering might be an approach to increase stability of the enzyme. Protein engineering may also lead to more active enzymes.

Feedback inhibition

Inhibition by an intermediate or an end product of the pathway is quite common. The regulation of fluxes from chorismate into the aromatic amino acid pathways is such

an example (see Chapter 1, Fig. 13). Overexpression of the enzyme involved may have some effect, but the introduction of a gene encoding an enzyme, which is not sensitive to feedback-inhibition will be more efficient. Such an enzyme could be obtained from another organism or alternatively be engineered based on the knowledge of the site involved in the interaction with the inhibitor.

In case of anthranilate synthase, which is inhibited by tryptophan (for reviews see [39,40,41]), tryptophan resistant isoenzymes have been obtained from, among others, *Nicotiana tabacum*[42] and *Solanum tuberosum* cell cultures after selection for resistance to 5-methyltryptophan.[43] Also mutant *Arabidopsis* plants have been reported, that contain tryptophan-feedback resistant anthranilate synthase.[41,44]

Competitive pathways

In biosynthetic pathways many branching points are found where enzymes compete for a common precursor. Obviously, blocking the competitive pathway is a way to increase the flux into the desired pathway. This can be achieved by using antisense genes. Blocking of competitive pathways can also be achieved by expression of genes encoding ribozymes[45] or antibodies against the target enzyme.[46,47] In principle the approaches used for blocking competitive pathways are the same as described below to reduce the flux towards an undesired compound.

Blocking of pathways is not always possible, as the pathway might be important for the plant (cell), particularly at branching points from primary metabolism. Again the chorismate branching point may serve as an example: cutting off the phenylalanine pathway is not feasible. Instead of blocking the competitive pathway, opening up the pathway of interest at the branching point should be considered. The mechanism of controlling the flux into the competitive pathways can be many. It includes activity levels of the competing enzymes, relative affinities of the enzymes for the branch point substrate, feedback mechanisms (see chorismate branchpoint, Chapter 1, Fig. 13), co-factor availability, and compartmentation.

If the fluxes are controlled through the relative activities of the competing enzymes, overexpression of the rate-limiting enzyme can be a solution. If the relative affinities of the enzymes for the substrate are the controlling factors, the introduction of an enzyme from another plant or organism with a higher affinity should be the aim. A new approach in this field could be the recently reported catalytic antibodies (abzymes). These antibodies bind unstable intermediates and thus catalyse steps in a biosynthetic route.[48] This approach has been used for the first intermediate in the conversion of chorismate into prephenate. The gene for the antibody raised against this intermediate was shown to be able to complement a yeast mutant blocked in chorismate mutase activity.

For overcoming the feedback mechanisms, the protein engineering approach holds promise.

Availability co-factors

Co-factors might be the limiting factor for a certain step. Limitation can be due to competition with other pathways or compartmentation. Increasing of the levels of co-factors thus requires good insight in the regulation of the enzymatic step involved. Based on that strategies can be developed to improve co-factor availability. Such strategies can thus include blocking of competitive pathways or increasing levels of enzymes that

regulate the level of the co-factor. This means a major intervention in the primary metabolism of the cells as well and thus might be a difficult target.

Compartmentation

Compartmentation as a regulatory factor means that transport is involved. This can, for example, be active transport through transporter proteins, or driven by pH-gradients. Genetic engineering of transport, however, is not a straightforward approach. For example, a change in pH through genetic engineering might influence many other processes as well. In case of specific transporter proteins genetic engineering should be more straightforward, though for secondary metabolism no examples of such an approach have been reported to our knowledge. Targeting of the enzyme to the compartment of the substrate is feasible, as specific peptide signals are known for all cell compartments. However, such an approach may cause problems of toxicity of the products formed in another compartment than usual.

DECREASE CATABOLISM

Secondary metabolites found in a plant (cell) are part of a dynamic process, which has seasonal and diurnal variations and is influenced by external conditions as well. In fact there is a continuous turnover of secondary metabolites (see Chapter 1). For example, during the end of the growth phase of *Catharanthus roseus* and *Tabernaemontana* cell cultures the *de-novo* production of terpenoid indole alkaloids is almost equalled by the rate of catabolism.[49,50,51,52,53] In case enzymes are responsible for the catabolism, they can be blocked in the same way as described above for competitive pathways. If catabolism is due to chemical instability, metabolic engineering does not offer a solution.

INCREASE OF PERCENTAGE OF PRODUCING CELLS

Many secondary metabolites have a restricted occurrence in plants, *e.g.* only in some specialized cells or tissues. Flowers and glandular hairs are very obvious examples. Even in cell cultures not all cells necessarily produce the desired product. For example, alkaloids were found to accumulate in only 50% of the cells in *C. roseus* cell cultures.[54] Apparently there is a physiologically determined maximum level of secondary metabolites in cells, as can be concluded from the production of anthocyanidins in *C. roseus* cell cultures in which the level is determined by the percentage of anthocyanidin containing cells. These cells all accumulate similar levels of these compounds.[55,56] Also berberine levels in *Coptis japonica* cell cultures were correlated with the number of accumulating cells, the berberine content in an accumulating cell being similar in low and high producing strains.[57,58,59] Increasing the number of producing cells by means of genetic engineering is thus of interest. However, that requires understanding the process of cellular differentiation. It requires the identification of regulatory genes. The feasibility of this approach has been shown by the activation of the production of anthocyanins in *Arabidopsis* and tobacco by the maize regulators R and C1.[35] However, at the present for most secondary metabolite pathways we still do not know anything about regulatory genes. Though recent work on alkaloid biosynthesis

in *Catharanthus roseus* resulted in the isolation of some regulatory genes (Memelink *et al.*, unpublished results), showing that with a pathway in which some genes have been cloned it is feasible to pick up regulatory genes, using different molecular approaches. A complicating factor might be the possibility that more than one cell type is involved in biosynthesis (see Chapter 1).

Strategies to produce new compounds

The production of new compounds can be interpreted in two ways, a new compound for the plant, but known from other sources, or a completely new compound, not known before. The first is of interest for most applications of metabolic engineering as mentioned above, the second possibility is of interest for producing new leads for development of drugs or agrochemicals (recombinatorial biochemistry) or to create more resistant plants.

To produce a known compound in another plant species can be achieved by transformation with genes of the pathway from the original producing plant. However, this requires that the necessary intermediates are present in the host plant. For short pathways this is feasible. It requires the expression of the genes concerned, in the cells (tissue) and cellular compartment where the precursor is found (*e.g.* see Chapters 11 and 12). For more complex compounds this is not possible, *e.g.* paclitaxel production in other plants than *Taxus* species will be difficult to achieve, as the basic taxane skeleton is not found in other plants.

For the recombinatorial biochemistry different approaches can be envisaged. In the polyketide antibiotics pathway in microorganisms, different genes encoding different steps of the biosynthesis (*e.g.* controlling chain length, reductases, cyclases) were combined to produce new compounds.[60,61] This is based upon the fact that in microorganisms the encoding genes are organized in an operon. Reshuffling of such operons from different organisms results in the production of new compounds. In plants such operons are not found. The genes encoding the various steps in a pathway are scattered throughout the genome.

For plants other approaches can be envisaged such as the introduction of enzymes with a broad substrate specificity, *e.g.* cytochrome P450 enzymes. Such oxidizing enzymes might further modify already present compounds by the introduction of new functional groups such as hydroxy or epoxy functions. Another approach is the introduction of an enzyme that opens up a new pathway from a readily available primary metabolite. Such an enzyme should be selective. Proof of this concept can be found in Chapters 11 and 12. Similarly the microbial salicylate biosynthetic pathway in tobacco, starting with the enzyme isochorismate synthase, which uses the readily available intermediate chorismate, has been introduced in plants.[11] Other examples are the introduction of terpene cyclases in plants, giving rise to new terpenoid skeletons for the plants, which might be further modified by various enzyme systems already present in the plant.[62]

Strategies to reduce levels of a certain compound

The level of a certain compound can be reduced by:

- decreasing the flux towards that product by reducing the level of an enzyme in the pathway;
- increase catabolism;
- increase flux into competitive pathways.

The first approach requires reduction of the expression of the gene concerned. A variety of methods can be used to achieve this goal. Cosuppression[63] or antisense technology[64] are very successful methods to suppress gene expression in plants. The reduction of the lignin content in plants, for example, has been obtained by expression of an antisense caffeic acid O-methyltransferase gene.[65] Homologous recombination would be an interesting option, as it has shown to be a successful method to knock out the expression of a gene in yeasts and animals. It has been shown that homologous recombination between introduced DNA and an endogenous plant gene is possible[66], however, it needs considerable further improvement to become a standard technique in plants.[67] Genes can also be knocked out by mutagenesis, either chemically or using T-DNA or transposon insertions. It requires an appropriate selection procedure for the plants with the desired trait. Also after transcription a biosynthetic step can be blocked. This can be achieved by degradation of specific mRNAs using ribozymes[45], or by expressing a gene encoding enzyme-specific antibodies.[46,47] A completely different approach is to sequester the undesired metabolites by expressing antibodies, as described for abscisic acid. This approach will influence the effect of the compound in the plant, but not lower the amount present.[48] The two other possibilities, increasing catabolism or the flux into comptetitive pathways, require similar approaches as discussed above for increasing flux through a pathway. *I.e.*, transform the plant with sense genes encoding selective enzymes. The reduction of indole glucosinolate levels in canola by introducing tryptophan decarboxylase into the plants, is an interesting application of introducing a competitive pathway to reduce levels of an undesired compound.[68]

Further considerations

Above the different goals in metabolic engineering of secondary metabolism were discussed and the strategies which one could think of to achieve these goals. In the subsequent chapters a number of examples will be given in more detail. Based on these results, and others encountered in our own research or learned from literature, there are some issues which needs attention in planning approaches to engineering of secondary metabolite pathways. It concerns problems which might hamper a successful application of this new tool molecular biology is offering for plant breeding.

GENE EXPRESSION

Techniques to introduce new genes into plants do not allow a prediction about the site of integration and the level of gene expression levels, even when a strong promoter

is used. To obtain a transgenic plant (cell) line with a high expression level, a large number of transformants needs to be screened.

From the various reports in literature it is clear that the efficiency of expressing a certain gene in different plant species may vary considerably. For example, expression of the *Tdc*-gene in tobacco, potato and canola resulted in quite different levels of enzyme activity.[69] The commonly applied *35S* promoter is a viral promoter, which uses the transcription factors of the host for its activity. The efficiency of this promoter differs for plant species. Even within a single plant the level of the resulting enzyme activity may vary in different plant parts, despite the use of a constitutive promoter.[70] This can be illustrated with the heterologous expression of a stilbene synthase genomic clone from peanut (including its own promoter) in tobacco (see also Chapter 11). This gene is inducible by elicitors, and showed a similar behaviour in aerial parts of transgenic tobacco, but in roots it was constitutively expressed.[71] The levels of the product of this enzyme, resveratrol, are about 100 fold higher in peanut callus after elicitation than in tobacco. With a grapevine genomic stilbene synthase gene much higher levels could be reached in the leaves of tobacco.[72] The different levels of expression can, among others, be due to differences in the promoter activity, or mRNA- and enzyme stability. Also positional effects might play a role in the efficiency of the expression of a transgene.

The expression of a gene not always results in the accumulation of a functional protein, as was observed for example after expressing a bacterial gene encoding lysine decarboxylase in tobacco (see also Chapter 10).[70,73] Even a different type of activity was observed in one occasion after the introduction of the *C. roseus* gene encoding tryptophan decarboxylase into tobacco. This resulted not only in the accumulation of tryptamine, but also of tyramine in some of the transgenic plants. *In vitro* the enzyme is only able to decarboxylate tryptophan.[74] The results observed in transgenic plants often concern a single individual plant, therefore one should be very careful in making general conclusions, since it may involve a mutation not connected with the introduction of the transgene. General conclusions on the effect of genetic engineering can only be drawn when the observed phenotype is reproducible and is correlated with the expression of the introduced construct.

STABILITY

The stability of a transgenic trait is an important aspect of genetic transformation. The gradual silencing of the transgenes has been observed in subsequent generations of transgenic plants.[75] The mechanisms of gene silencing are not well understood. Often silencing is associated with DNA methylation.[76] Reliable methods to prevent transgene inactivation are not available at the moment, although some general measures can be taken to minimize the problem.[76]

In *Drosophila* transgenes can be inactivated by mechanisms resembling position effect variegation. A possible solution for this problem is to introduce matrix-attachment regions and/or insulator elements on the construct.[77] Alternatively one could try to target the construct to a chromosomal region that provides a favourable environment for stable transgene expression. This can be achieved by homologous recombination to an

endogenous site or to a previously introduced recombination site for a sequence-specific recombination system, such as the Cre-loxP system. Transgenes can also be inactivated by cosuppression.[63,78] Cosuppression seems to be associated with multiple copies of rearranged transgenes, consequently a solution might be the selection of transformants with only a single copy of the transgene.

In plant cell cultures, a continuous selection for rapid cell division and growth occurs. The transgenic trait might be a negative selection criterium, and thus gradually be lost. Studies on the stability of transgenic cells will be necessary to assess the feasibility of using transgenic cell cultures for secondary metabolite production. Some metabolites may be toxic, and their production may not be compatible with cell division. By placing transgenes under the control of inducible promoters and inducing the pathway after the growth phase of the cells, this problem can be solved. For plants, a number of inducible promoter systems have been reported, *e.g.* based on regulation by tetracyclin[79], steroid hormones[80], or copper.[81] A restriction is that uptake of the inducer by plants or plant cells is required. Toxic metabolites or their biosynthetic enzymes could also be targeted to cellular compartments that are insensitive to the toxic effects. A specific problem in plant cell suspensions is the instability of the ploidy level of the genome[82], which causes an inherent genetic variability, and also interferes with the ability to regenerate plants.

PATHWAY ARCHITECTURE AND LIMITING STEPS

The pathway architecture (see Chapter 1) is important to decide about the approach to follow to increase the flux to a certain compound. In case of a linear pathway limiting steps need to be identified. Any enzyme, which is a limiting factor in a pathway, either because of low activity or as it is only induced under certain conditions, is an interesting target for genetic engineering. The already mentioned hyoscyamine/scopolamine conversion is a clear example of such an approach being successful (see also Chapter 13). However, there are also examples where this approach did not give the expected results. The introduction of the gene encoding tryptophan decarboxylase into *Catharanthus roseus* cells is such an example. Though this gene is strongly regulated in the cell cultures,[22] overexpression only results in an increase of tryptamine, but not in an increase of terpenoid indole alkaloids production. Apparently, the other precursor needed for this pathway, secologanin, is also a limiting factor. Surprisingly, overexpression of strictosidine synthase, the enzyme that couples tryptamine and secologanin, does result in increased alkaloid production.[83,84] Also the work of Berlin and co-workers on the introduction of the *tdc*-gene into *Peganum harmala* cells[85], with the aim to increase the production of harmane-type of alkaloids, shows similar results (see also Chapter 10). A higher level of tryptamine, but no increase of harmane alkaloids was observed. In fact both examples clearly show a very basic rule, once you have overcome a limitation, you will directly meet the next one. A biosynthetic pathway may thus contain many steps, which need to be engineered. This is in fact an important argument for looking for the regulatory genes, which might turn on the whole pathway. This also requires the cloning of some of the individual genes of a pathway, if the strategy is to find regulatory genes *via* promoter analysis.

In case of a network as discussed in Chapter 1 (Fig. 11), it is important to know the selectivity of the enzymes involved. If, for example, $S_{2,3}$ is the desired compound, in case of high selectivity of the enzymes in the network, the genes encoding the enzymes E_2, E_3, E_7 and E_9 could be targets for overexpression, and the genes encoding the enzymes E_1, E_6, E_8 and E_{12} for blocking by antisense genes. In case of low selectivity, *i.e.* only three enzymes are involved in the introduction of the three substituents, one should block E_1 and/or increase E_2 and E_3 in activity. Clearly different strategies are thus required, which have to be based on a thorough knowledge of the pathway.

REGULATION

In Chapter 1 we briefly discussed some aspects of regulation of secondary metabolism, both on the level of genes and enzymes. Feedback effects and compartmentation are particularly important aspects to take into account for metabolic engineering.

Feedback effects

Stephanopoulos and Vallino[86] extensively discussed the problems of metabolic engineering in primary metabolism. They postulated that because of the rigidity of the metabolic networks, it is difficult to redirect a larger part of the carbon flux towards certain desired products. Redirection means that at certain branching points the carbon flux has to be increased into a certain pathway. Three types of such branching points (nodes) can be distinguished. Nodes in which the enzymes at the branching point are not regulated. Nodes where one branch is dominating, because one enzyme has higher level of activity or higher affinity and is not regulated by feedback inhibition, as the minor branch might be. In the third type of node the split ratio is tightly regulated, usually by feedback from the products of each of the branches. In such nodes sometimes trans-activation between the branches by their respective products occurs.

In the first case, there will be no difficulty in redirecting the flux. The other two types are more difficult to affect. Overproduction in one branch will cause feedback inhibition of that branch and possibly induction of the other. The regulation of the enzymes at the branching point of the biosynthetic pathways leading to the aromatic amino acids, anthranilate synthase and chorismate mutase, is an example of a rigidly controlled node (Chapter 1, Fig. 13). Anthranilate synthase is feedback inhibited by tryptophan, the final product of this pathway. Moreover, tryptophan activates the plastidial chorismate mutase isoenzyme, the first enzyme of the pathway leading to phenylalanine and tyrosine. These compounds inhibit the first enzyme in their biosynthetic route, chorismate mutase. This inhibition is counteracted by tryptophan.[39,40,41,87] This interlock might cause the lower levels of phenylalanine, lignin and phenolic compounds in transgenic potato tubers with low tryptophan levels through the introduction of the *Tdc*-gene.[88]

Particularly in networks as common in primary metabolism, such regulation mechanisms make it difficult to engineer major changes in fluxes. Fortunately, most secondary metabolite pathways are not networks, but directing more carbon from primary metabolism into secondary metabolism might be hampered by the above mentioned type of regulation. Not only increasing levels of enzyme activity but

also overcoming the feedback inhibitions and transactivations will be important in increasing the flux of carbon into secondary metabolite pathways. In developing a strategy for redirecting fluxes by metabolic engineering one has thus to take such considerations into account.

Cellular compartmentation

As discussed above, in plants and plant cell cultures not all cells are producing secondary metabolites. An important question to be solved is whether the whole pathway is downregulated in non-producing cells, or only one or a few key steps? In the latter case the above mentioned approach of increasing constitutively the activity of such enzyme(s) could be followed. In the case of downregulation of the complete pathway, one needs to identify the regulatory mechanism in order to be able to switch on the pathway (see above). Another point to be considered is that (part of) biosynthesis might occur in other cells than where storage is taking place. For example, Hashimoto and co-workers reported that the final step in the biosynthesis of scopolamine, the epoxidation of l-hyoscyamine occurs in pericycle cells of young roots, subsequently the alkaloid is transported to the aerial parts where storage occurs.[89,90] Constitutive expression of the gene encoding hyoscyamine-6-hydroxylase[91,92], however, results in the production of scopolamine. In this case, metabolic engineering thus is not hampered by the compartmentation (see also Chapter 13).

Subcellular compartmentation

Secondary metabolite pathways are not just a series of consecutive reactions, but steps may occur in different subcellular compartments.

Compartmentation of biosynthetic pathways have been studied quite extensively for both terpenoid indole (for a review see [22], see also Chapter 1, Fig. 14) and isoquinoline alkaloids (for a review see [20,21]).

The subcellular compartmentation of biosynthetic pathways, requires targeting of proteins to the appropriate compartment, otherwise the substrate might not be available for the enzyme. This was for example clearly shown for lysine decarboxylase[70] (see Chapter 10) and some chorismate utilizing enzymes leading to 4-hydroxybenzoic acid[93] (see Chapter 12) and salicylic acid.[11]

In Chapter 1 we discussed the possible occurrence of metabolons, or metabolic channels in which a series of enzymes is organized in aggregates, which for example can be part of a cellular membrane.[94,95,96] Genetic engineering of enzymes in such metabolons requires specific targeting information for the proteins in such a molecular compartment. For metabolic engineering the occurrence of such metabolons would imply that the possibilities of increasing a single step in such a metabolon are limited. The regulation of the expression of the genes encoding the enzymes in a metabolon is expected to be coordinated. The regulatory genes should thus be interesting targets to increase productivity in case of such metabolons.

Conclusions

Though genetic engineering of plants and plant cells is feasible, still quite some bottlenecks exist, *e.g.* difficulties in transformation and regeneration of plant species. Any project in this field should first start with developing the necessary protocols for this purpose. Assuming that transformation is feasible, the next constraint will be the lack of knowledge of many secondary metabolite pathways. For most pathways no detailed information is available for the intermediates, enzymes and genes. That means that this biochemistry needs first to be solved. For those pathways for which this has been done, interesting results have been obtained with metabolic engineering as can be learned from the various chapters in this book. The genetic engineering also learns us more about the regulation of pathways, in fact it might be a very useful tool in better understanding regulation of secondary metabolism. In terms of results some interesting applications have been reported, for the production of specialty chemicals, for changing quality traits of plants and in producing new compounds for a plant. The perspectives for engineering plant secondary metabolism are thus very bright, but it requires a close collaboration between phytochemists, biochemists, plant physiologists and molecular biologists.

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Presently, we have to conclude that transgenic plants and root cultures have not yet provided clear evidence that secondary products such as HCPs and nicotine levels can be improved by overexpression of heterologous ADC or ODC activity.

Cadaverine is a polyamine which has attracted less attention because it does not seem to have a role as modulator of growth related responses. Cadaverine levels in higher plants are often low and hardly to detect. It may thus be regarded as secondary metabolite formed by the decarboxylation of lysine (Fig. 6). Cadaverine is precursor of several groups of alkaloids - *e.g.* quinolizidine alkaloids in lupins. Indeed, the interest in lysine decarboxylase (LDC) resulted from the idea to endow lupin cells with high LDC activity and to increase by this quinolizidine alkaloids. However, we rejected this idea when we had a closer look to this seemingly simple 2-step pathway.[35] Our unpublished studies told us that cadaverine is not the main limiting factor in the biosynthesis. The pathway is well expressed in leaves, but poorly in cultured cells. Transformation of lupins with genetically constructed strains of *Agrobacterium* sp. and regeneration of intact plants are not trivial. Therefore we looked for an an attractive alternative to use the *ldc* gene we had cloned from the enterobacterium *Hafnia alvei,* in metabolic engineering experiments.

Anabasine is a structural analog of nicotine in which the putrescine-derived N-methylpyrrolinium ring is replaced by a cadaverine-derived Δ^1- piperidinium ring (Fig. 6). In most tobacco varieties anabasine is a minor alkaloid, often hardly detectable. The reason for this is evidently the lack of cadaverine. Feeding of cadaverine to hairy root cultures of various tobacco ssp. greatly increased the levels of anabasine.[36] Despite the fact that the way is not fully elucidated how cadaverine is incorporated into anabasine, it is clear that cadaverine is limiting in anabasine biosynthesis. Consequently, overexpression of LDC in *ldc*-transgenic tobacco should result in the overproduction of anabasine in tissues where nicotine formation occurs. In addition, one could also expect that novel hydroxycinnamoylcadaverines (HCCs) might be formed in cadaverine-overproducing cultures, as feeding of cadaverine led to the formation of these compounds.[37] The enzyme hydroxycinnamoyl-CoA:putrescine hydroxycinnamoyltransferase accepts also cadaverine as substrate.[38] Thus, if it works as anticipated, one would have a system where a) two pathways are affected by the overexpression of LDC and b) due to the almost complete absence of the target compounds in controls, product increases are easily recognized. In the following we report only about those *ldc*-transgenic cultures which led to significantly enhanced product levels of anabasine and HCCs. We can conclude from all our experiences that high expression of the *ldc* gene requires a) a leader sequence and b) a targeting signal for import into plastids. The LDC must in addition be present in tissues where the pathways of the target compounds are active.

For fulfilling the above demands, an expression cassette was constructed in which the *ldc* gene fused to the coding region of a *rbcS* (small subunit of ribulose biphosphate carboxylase) transit peptide (*tp*) was under the control of the *CaMV35S*-promoter. This was cloned into a binary vector and introduced by triparental mating into an *A. tumefaciens* strain with an Ri-plasmid. Tobacco seedlings were transformed with this *Agrobacterium* strain and many *ldc*-transgenic root cultures were obtained. These lines were screened for those having high LDC activity. We had to screen a larger number

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CHAPTER 3

AGROBACTERIUM, A NATURAL METABOLIC ENGINEER OF PLANTS

P.J.J. HOOYKAAS

Introduction

The soil bacteria *Agrobacterium tumefaciens* and *A. rhizogenes* are the etiological agents of the plant diseases crown gall and hairy root, respectively. They belong to the family of Rhizobiaceae, and thus are related to the nitrogen fixing rhizobia. Whereas crown gall is characterized by the presence of tumors on plants, the hairy root disease is so called because of a conspicuous proliferation of roots from infection sites (Fig. 1). Plant cells in crown galls and hairy roots have two features with which they are distinguished from normal plant cells: 1) they are tumorous *i.e.* they proliferate in the absence of added growth factors in *in vitro* culture, and 2) they produce and secrete specific compounds which have been given the generic name of opines. It is now known that these novel properties of crown gall and hairy root cells are a consequence of the presence of a segment of bacterial DNA, the T(ransferred)-DNA within these cells. This bacterial DNA forms part of a large (about 200 kbp) bacterial plasmid which is present in virulent strains of these *Agrobacteria*, and is known as Ti (tumor inducing) plasmid in the case of *A. tumefaciens* and Ri (root inducing) plasmid in the case of *A. rhizogenes*. The T-DNA of the Ti plasmid contains a number of genes which are expressed in the transformed plant cells. Some of these are *onc*-genes which encode enzymes involved in the production of plant growth regulators, *viz.* the phytohormones indole acetic acid (an auxin) and isopentenyl-AMP (a cytokinin). Other determine enzymes which catalyze the production of the opines. The presence of these genes in the T-DNA explains the novel properties of crown gall cells. Similarly, the T-DNA of Ri plasmids contains genes called *rol* genes which confer the capacity to differentiate into roots on transformed cells as well as opine synthase genes. There are several different classes of opines which may be present in the T-DNA transformed plant cells (Fig. 2). This depends entirely on the types of opine synthase genes present on the T-DNA. Opine producing overgrowths form a specific ecological niche for the infecting agrobacteria, since only these can utilize the opines, which are secreted from the tumor cells, for growth. Apparently, agrobacteria are metabolic engineers which apply genetic transformation to force plant cells into producing the required metabolites. For further detailed information on *Agrobacterium* the reader may consult a number of recent reviews[1-4].

R. Verpoorte and A.W. Alfermann (eds), Metabolic engineering of plant secondary metabolism, 51-67.

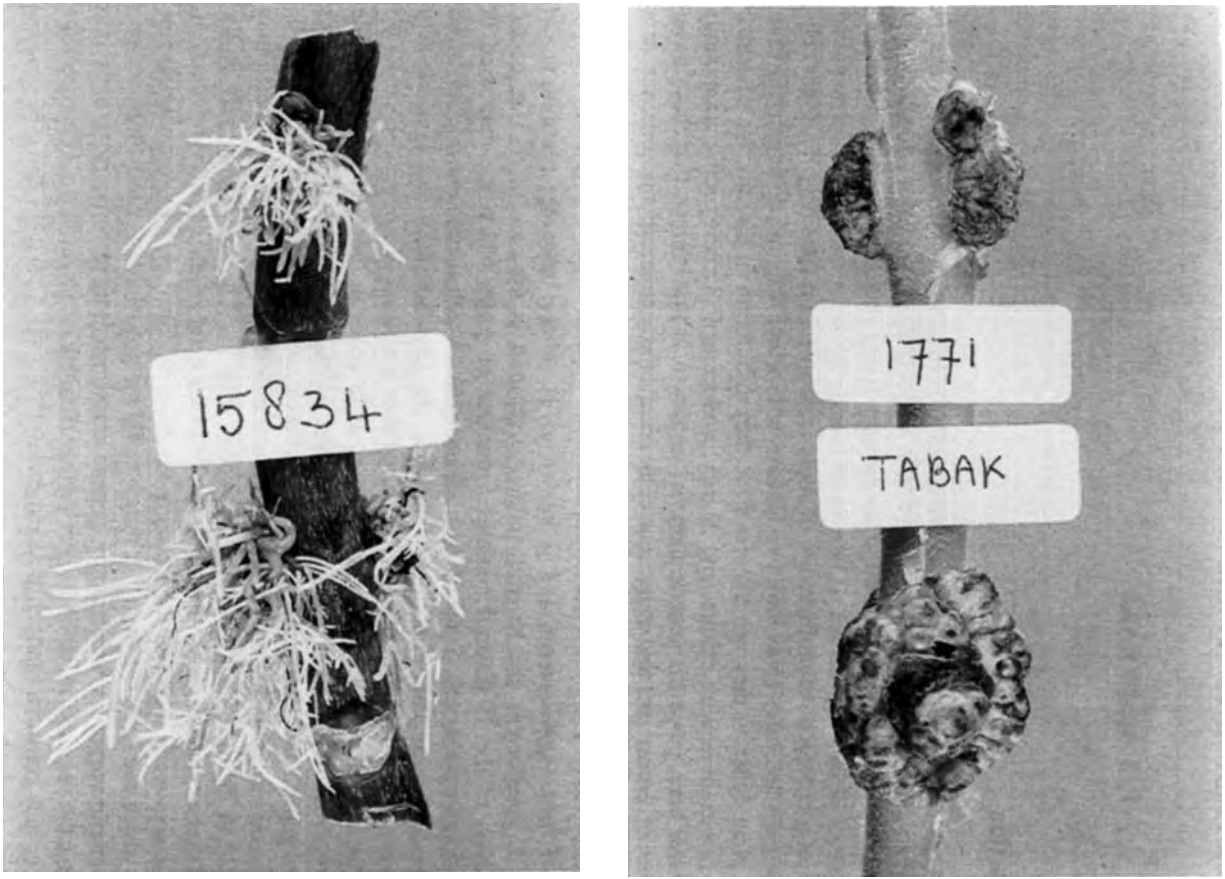

Figure 1. Crown gall tumors and hairy roots induced by Agrobacterium tumefaciens and A. rhizogenes, respectively.

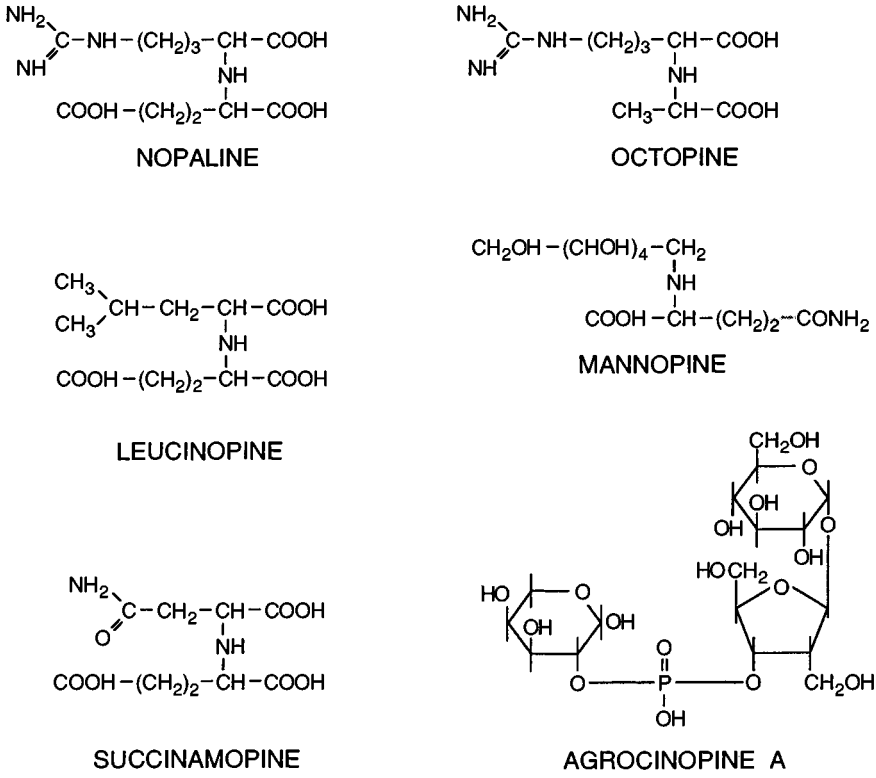

Figure 2. Structural formulae of opines.

Virulence genes involved in DNA transfer

Transfer of the T-region of the Ti plasmid to plant cells is mediated by the products of virulence (*vir*-) genes[2,3], which are located in the Virulence region adjacent to the T-region in the Ti plasmid (Fig. 3). The *vir*-genes are silent, until they are induced by the presence of specific phenolic compounds such as acetosyringone[5] and other lignin precursors and degradation products[6] (Fig. 4). These inducers become abundant in plant tissue after wounding[5] which is long known to be a prerequisite for efficient tumorigenesis. Induction of the *vir*-genes also requires a low pH (5-6) and a temperature below 29°C. The low pH matches of course the conditions in plant (wound) sap. Regulation of the *vir*-genes is mediated by the proteins of the *virA* and *virG* genes, which together encode a two component regulatory system with the VirA protein acting as the chemoreceptor (of the phenolic compounds) and VirG functioning as a transcriptional activator of the *vir*-genes after having been forforylated by VirA on a critical aspartate residue. Regulation of the *vir*-genes is discussed in more detail in a review by Winans[7].

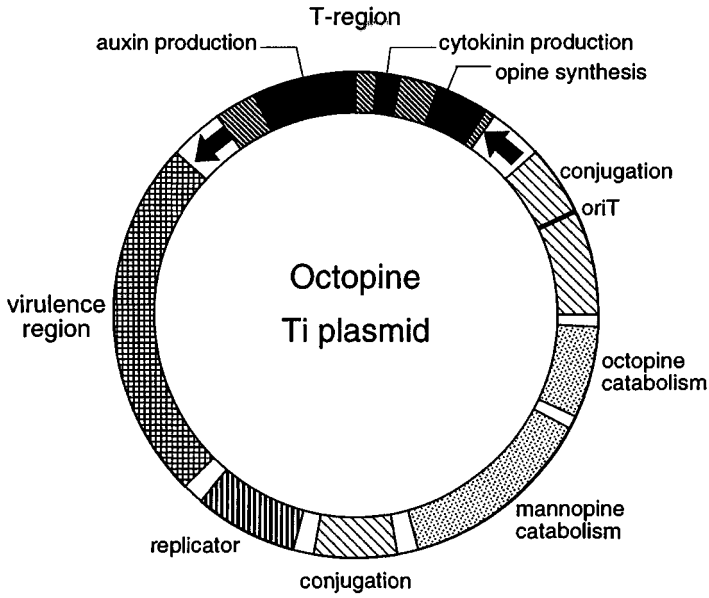

Figure 3. Genetic map of an (octopine) Ti plasmid. Arrows denote the border repeats.

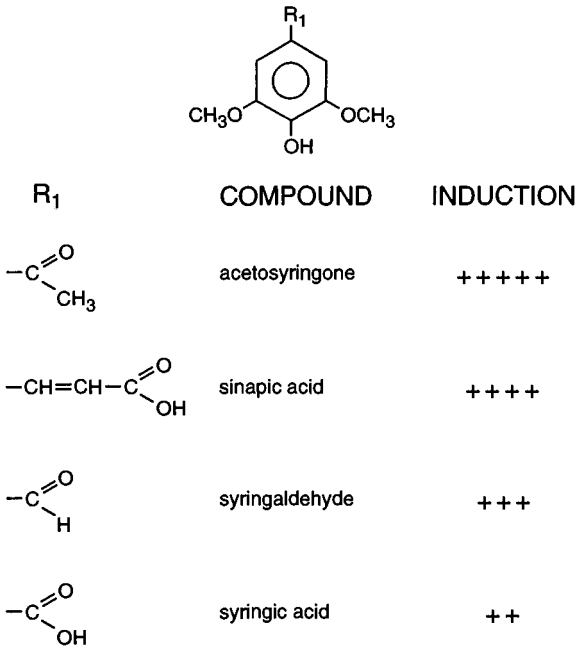

Figure 4. Inducers of the virulence genes.

Besides *virA* and *virG* the *vir*-regulon consists of about 20 genes which are divided over several operons[2,3]. Two of these operons, the *virB* and *virD* operons, determine a transfer system which resembles closely the conjugative transfer systems of the wide host range *incN*, *incP* and *incW* plasmids[8]. This transfer system mediates the transfer of single stranded copies of the T-region (T-strands) to plants. In fact it is a nucleoprotein complex which is introduced into recipient plant cells. The T-strand is covalently bound to the VirD2 protein at its 5' end and can also become coated by the single stranded DNA binding protein VirE2[3,9]. This latter protein protects the T-strand against nucleases during its journey to the plant cell nucleus[10], while VirD2 has a nuclear localization sequence in its C-terminal part which is essential to bring the T-strand into the plant cell nucleus[11]. The transfer system not only mediates transfer of the nucleoprotein T-complex to the recipient plant cells, but also of certain proteins, such as the VirF protein, which travel independently to the plant cells in order to increase the efficiency of transformation[12].

Since agrobacteria induce tumors only on dicotyledonous plants, it was long thought that the host range of T-DNA transfer was restricted to this class of plants. However, later it was found that transfer is possible to monocotyledonous plants as well, but that transfer does not lead to tumorigenesis here[13]. More recently it was found that the virulence system of *Agrobacterium* can mediate DNA-transfer even to other bacteria[14] and to yeast[15].

Plant vectors

The natural vector system of *Agrobacterium* is now generally used for the genetic modification of plants. This was possible, because it was found that a) the Ti plasmid could be disarmed *i.e.* rendered non-oncogenic by deletion of the *onc*-genes without a loss of its transfer abilities, b) genes added to the T-region were co-transferred to the recipient plant cells. Now it is known that the Vir-system of *Agrobacterium* can mediate transfer of any genes to plant cells, provided that they are surrounded by the 24 bp border repeats which form the ends of the natural T-region in the Ti plasmid[16]. These repeats are recognized by proteins encoded by the Vir-region which use these as start and end points of transfer[8]. Since the Ti and Ri plasmids are large (about 200 kbp) it is impossible to manipulate them efficiently *in vitro*. Two strategies have been devised to circumvent this problem. The first is to use so called intermediate vectors, into which genes of interest can be cloned in *E. coli* and which subsequently are introduced into *Agrobacterium* and integrate between the border repeats of a (disarmed) Ti plasmid by homologous recombination[17]. This cointegrate system is now only rarely used. Instead the binary vector system is applied for the genetic modification of plants almost invariably[18]. In this system the genes of interest are cloned directly between the border repeats which are present on a shuttle plasmid that replicates both in *E. coli* and *Agrobacterium*. After manipulation the loaded binary vector is introduced into a disarmed *Agrobacterium* strain, which has a Ti plasmid from which the entire T-region has been deleted, but still has an intact Vir-region (Fig. 5). This *Agrobacterium* host then transfers the T-region of the binary vector to the plant cells. In order to be able

to detect plant cells transformed by *Agrobacterium* markers are added to the T-region which confer a selective phenotype on the transformed plant cells. Often bacterial antibiotic resistance genes serve this purpose, since plant cells - due to the presence of 'prokaryotic' chloroplasts and mitochondria - are as sensitive to certain classes of antibiotics as bacteria. The antibiotic resistance genes are modified so that they are expressed in plant cells[19]. Genes conferring resistance to the antibiotics kanamycin, hygromycin and gentamycin have been used in this way. Similarly, genes conferring tolerance to herbicides such as sulfonylureas and bialaphos have been employed. Transformation can be directly visualized by linking a reporter gene to the T-region. For plants the *E. coli* gene coding for β-glucuronidase (GUS) has become very popular, since the presence of this gene can be detected histochemically by adding X-Gluc (5-bromo-4-chloro-3-indolyl-β-d-glucuronide) to the plant tissue[20]. Presence of the gene in plant cells becomes visible by blue staining. In order to avoid potential expression (and blue staining) due to the presence of remaining agrobacteria, the gene was tailored by insertion of an intron to be expressed only by the transformed plant cells and not by the bacteria[21]. Recently, the gene coding for the *Aequoria victoria* green fluorescent protein (GFP) was modified to serve as a reporter in plant cells[22]. An advantage of this gene is that for the detection of its expression no substrate has to be added; the fluorescence of GFP can be seen after illumination of the transformed tissue with UV- or blue light. This allows the tissue to be maintained and propagated in contrast to tissue stained for the presence of β-glucuronidase.

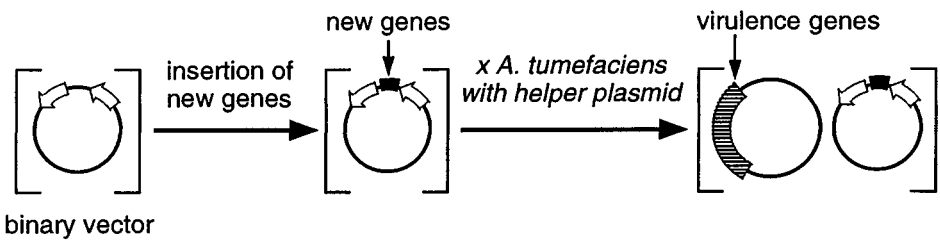

Figure 5. The binary vector system.

T-DNA integration

The T-DNA integrates into the plant genome by a process of illegitimate recombination, whereby the ends are most often well preserved[23,24]. Integration is accompanied by the formation of small deletions at the target locus. The process of T-DNA integration is effectuated in a similar way in different recipients including dicotyledonous and monocotyledonous plants[25] and yeast[26]. Transgenic lines often contain only one copy of the T-DNA, but lines with multiple T-DNA copies at one or more loci in the genome are also prevalent[27]. Sometimes partial, truncated T-DNA copies may be present, or T-DNAs which are accompanied by segments of the original binary vector[28]. Integration of the T-DNA takes place at random positions in the genome, although there

seems to be a clear preference to land into transcriptionally active regions[29]. In model plants such as tobacco and arabidopsis, the T-DNA has therefore been used for gene tagging[29,30]. Mutants obtained from T-DNA transfer experiments which have interesting phenotypes are propagated. The mutant phenotype may however not be linked to the T-DNA insertion, since plant cells receiving T-DNA seem to accumulate spontaneous mutations. However, if linkage between the mutant phenotype and the presence of the T-DNA is established in genetic experiments, the gene affected can be cloned by using the T-DNA tag as a physical marker. In this way genes involved in plant development, phytohormone signalling and brassinosteroid biosynthesis have been cloned[31,32]. Tagging experiments are often done not by using ordinary binary vectors, but by employing special purpose vectors such as promoter trap, enhancer trap or activation vectors (Fig. 6). Promoter and enhancer trap vectors have a reporter gene (usually the β-glucuronidase gene) located close to one of the border repeats of the T-region[29]. The GUS genes in these vectors are not expressed in plant cells, unless the T-DNA inserts close to a plant promoter (promoter trap) or a plant enhancer (enhancer trap). Usually a collection of transgenic plants with these entrapment vectors is isolated which is then screened for individuals showing the desired GUS-expression pattern[33]. In this way developmentally regulated, organ or cell-type specific and conditional (stress or signal compound inducible) GUS gene fusions have been obtained. The promoter/enhancer elements entrapped can then be cloned and used to drive transgenes in transgenic plants in the desired fashion.

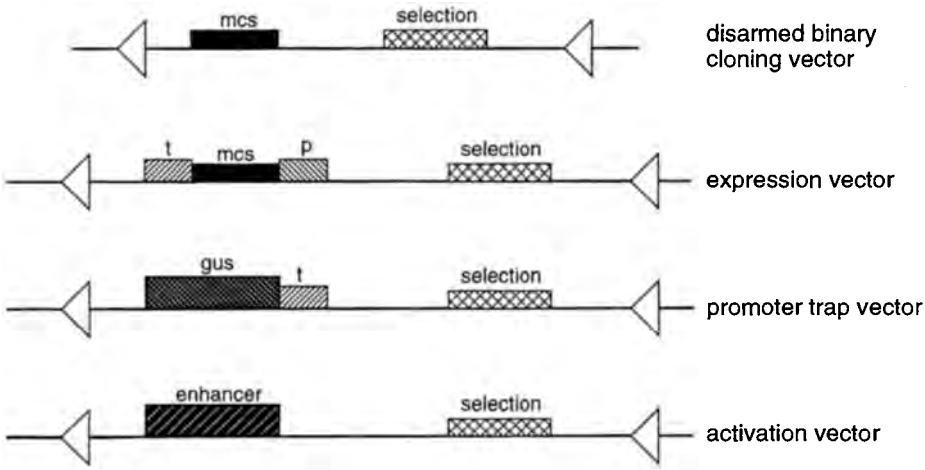

Figure 6. Plant vectors.

Activation vectors are used to make dominant mutations in plants by inducing alterations in gene expression at and around the locus of insertion[34,35]. These vectors have an enhancer or outward-reading promoter located close to one of the border repeats. These may induce overexpression (activation) of genes located in the vicinity of the landing site. In case an outward-reading promoter is present, it is also possible

that transcription in the antisense direction may lead to silencing of a gene at the site of integration. Activation vectors have been used to isolate, arabidopsis calli which regenerate shoots in the absence of cytokinin[36], a dehydration tolerant callus of *Catherostigma plantagineum* [37], and an arabidopsis mutant with large lobed leaves (our unpublished results).

Recently it was found that extremely large stretches of DNA can be transferred to and stably integrated into the plant genome by the T-DNA vector system. The Binary BAC (BIBAC)-vectors used for this purpose are based on the bacterial artificial chromosome (BAC)-vectors which can contain up to 300 kbp of cloned DNA. In the example described in literature a fragment of 150 kbp of DNA cloned from the human genome was inserted in the tobacco genome without deletion or permuation[38].

Applications

Tissues transformed by the *Agrobacterium* vectors described above can be regenerated into complete, fertile plants using tissue culture technology which is used for the regeneration of normal, untransformed tissue. In the fifteen years since the first transgenic (tobacco) plants were isolated many different plant species (including crops such as potato[39], tomato[40], cassave[41], rice[42], maize[43] and wheat[44]) have been transformed with genes conferring a variety of traits. Initially these were mainly antibiotic resistance genes and reporter genes used for selection and detection of transformants. Later plants were made tolerant for various herbicides by the insertion of herbicide-insensitive target genes or of herbicide detoxifying enzymes[45]. Virus resistant plants were obtained by expressing the genes for viral coat proteins in them[46]. Crop plants were made male-sterile, an important trait in breeding, by the expression of toxic genes such as those coding for an RNAse or for the catalytic subunit of the diphtheria toxin, in the tapetum cells, where the gametophytes are generated, using a tapetum-cell specific promoter[47]. By cloning a cDNA in the reverse orientation between a promoter and a terminator, a socalled antisense construct can be obtained. Introduction of these in plants leads to the silencing of the homologous endogenous genes[48]. This possibility has been used to inactivate many genes in plants. Thus petunia plants with altered flower colour[48], tomato producing fruits with delayed ripening[40] and potato producing tubers with optimized starch content[39], became available. Plants less suffering from insect damage were produced by the introduction of genes encoding *Bacillus thuringiensis* endotoxins[49]. Many more applications have gone into field trials in the meantime, and it is expected that products will find their way to the market more and more. The application of the transformation technology is limited now predominantly by the unavailability of genes which can confer the desired traits to plants. Other limiting factors concern the recalcitrance of certain plant species for transformation and variation and instability in transgene expression. Below I shall separately deal with the perspectives to solve each of these three limitations in the future.

Identifying useful genes

One of the factors limiting the possibilities for metabolic engineering of secondary metabolism is the scarcity of cloned genes coding for enzymes participating in secondary metabolism. The focus in plant genetics is on crop species, which are important in agriculture, and on some model species such as *Arabidopsis thaliana.* This has made available genes involved in metabolic pathways operative in these species, some of which are present in many other plant species as well such as those for anthocyanin pigment and lignin biosynthesis. The cloned genes can now be used to find homologous genes in other species. To isolate genes for which no homologs are available requires a considerable effort. This can and has been done by purifying the enzymes first and then to use either antibodies against these or oligonucleotides synthesized on the basis of partial protein sequences to screen cDNA (expression) libraries. In this way genes involved in indole alkaloid biosynthesis in *Catharanthus roseus* were cloned[50,51]. Novel developments in map-based cloning such as the use of AFLP-markers allow to clone genes for interesting traits (which give a phenotype) now also in this way. Thus a fungal resistance gene was recently cloned from barley[52]. Activation tagging may be a rapid alternative to clone genes of which upregulation leads to an immediately visible phenotype (*e.g.* pigment formation, or tolerance of a metabolic inhibitor). In this way tagged tobacco lines were selected which could grow in the presence of an inhibitor of polyamine biosynthesis[34].

Recalcitrance to transformation

A second limiting factor concerns the recalcitrance of certain plant species to transformation by *Agrobacterium.* This can relate to a total lack of T-DNA transfer, lack of T-DNA expression or integration, or an impossibility to regenerate the transformed cells into fertile plants. For cereals this was solved by using embryogenic callus derived from the scutellum layer of embryos as a target for transformation by *Agrobacterium*[42]. Selection of the proper target tissue for transformation was an important factor by which recalcitrance was overcome in a number of species. A number of other factors have also contributed to this. Firstly, the availability of a number of different selection markers. Some of these cannot inhibit growth in some plants or are selective, but inhibit regeneration in certain species. In such cases the availability of an alternative selection gene is essential. Secondly, the increase in knowledge about the *Agrobacterium* vector system itself. This taught that different *Agrobacterium* strains have slightly different host ranges[12] and thus that it is best to compare a set of strains for effectiveness on a particular host. Also it became apparent that the *Agrobacterium vir*-genes need to be induced by factors present in plant wound sap[5,6] and that some plants do not secrete enough of these factors for optimal induction. For that reason in many transformation protocols the addition of acetosyringone to the cocultivation medium is recommended. This same compound may also help by advancing competent cells into the S-phase of the cell cycle which is necessary for transformation[53,54]. Superbinary vectors on which extra copies of some of the *vir*-genes are present, turned out to be more effective in

gene delivery in a number of species[42]. Presence of *Agrobacterium* also may induce tissue necrosis and cell death, for instance in *Vitis vinifera*[55]. This is due to an oxygen-dependent defense response of the plant cells, which can be inhibited by the addition of antioxidants to the cocultivation medium.

Development of an *Agrobacterium* transformation protocol for a new species may not be straightforward, but many of the tricks that were learned over the years in the many different systems will be very helpful to proceed. Alternatively, the use of *Agrobacterium rhizogenes* may be considered. This bacterium naturally provokes the formation of hairy roots in many plant species. Hairy roots can be propagated easily *in vitro*[56], but often can be regenerated into complete plants. Such plants usually have phenotypes by which they are distinguished from normal plants. The most conspicuous differences are that they are shorter, bushier and have leaves which are wrinkled[57]. The T-DNA of the Ri plasmid can be used for the delivery of novel genes into plants, after these have been inserted in the T-region by an intermediate vector (cointegrate system). *Agrobacterium rhizogenes* can also deliver the T-region from a binary vector (with border repeats from a Ti plasmid) efficiently into plants[58]. Co-transformation of the Ri plasmid T-DNA and the binary vector T-DNA is high, so that it suffices to screen amongst hairy roots for the presence of this latter T-DNA. If the two T-DNAs integrate at different positions in the genome (often but not always the case) they can be separated from each other in the offspring. Amongst the offspring thus seedlings are present which lack the Ri T-DNA and have a normal phenotype, but still have the binary vector T-DNA. When it is not necessary to obtain complete transgenic plants, the use of transgenic cell or tissue cultures can be considered. By transformation with Ri T-DNA fast growing hairy root cultures can be obtained, which may produce secondary metabolites as efficiently as normal roots; in case the interesting metabolites are produced in the shoot, shoot cultures may be useful, which can be produced by transformation with a "shooty" *Agrobacterium* strain[59], in the possession of an active *ipt* gene.

T-DNA expression

The genes in the natural T-DNAs of *Agrobacterium* are expressed in plant cells due to the presence of regulatory signals (promoters, terminators) surrounding the coding regions of these genes which are recognized by the transcription machinery of the plant. These same regulatory signals have been used extensively also to drive other genes for expression in plants, besides other derived from plant genes or from plant virusses such as the 35S promoter of cauliflower mosaic virus. For a detailed discussion see the chapter by Memelink. A problem which is often encountered in transgenic plants relates to variation and instability of the expression of the introduced transgenes, and aberrant segregation of the transgene in the offspring. The T-DNA itself is stable and behaves as a normal plant gene in the sense that mutation of it is extremely rare (frequency less than 10^{-4})[60]. Instability of T-DNAs may be more apparent if there are (direct) repeats present within the T-DNA due to intrachromosomal recombination[61]. Besides due to problems with expression (see below) aberrant segregation ratios may sometimes be caused by the fact that the transgenic plant under study is a periclinal chimera, consisting of both transgenic

cells and normal cells[62]. Also the occurrence of polyploidy or aneuploidy during transformation may lead to aberrant segregation of the transgene. It is therefore advisable to check the genome composition of the transgenic plants and select those which have remained normal diploids. However, the most important causes of variation and instability of the expression of transgenes in different transgenic plants are differences in copy number or organization of the transgenes and position effects, the influence of the surrounding chromosomal DNA on the transgenes. It can be imagined that after insertion within a transcriptional unit of the plant chromosome transcription of the transgenes may be inhibited (due to generation of antisense transcripts from the neighbouring plant promoter and collision of the two transcriptional apparatuses transcribing the chromosomal plant gene and the transgene near the border repeat, respectively) or stimulated due to the presence of plant enhancers or plant promoters effective in the same direction as the transgene[63]. Besides the neighbouring chromosomal DNA may have sequences that direct DNA-methylation and thus silencing of the area[64]. Evidence suggests that the chromosomes are attached at many sites to a protein core, the matrix or scaffold, by specific sequences, the matrix or scaffold attachment sequences (MARs or SARs)[65]. The chromosomal DNA located between two MARs is insulated against influences from sequences located in other neighbouring loops. Surrounding transgenes by cloned MAR sequences may prevent position effects to a large extent, probably by insulating the transgenes against the influence of neighbouring genes of the host plant[65,66].

Most observations suggest that there is no linear relationship between the copy number of the transgene and the level of expression. This is not only due to the important influence of neighbouring sequences on expression as described above, but also due to interactions between the transgenes themselves which may lead to silencing. This penomenon is related to co-suppression, the silencing of an endogenous plant gene due to the introduction of a homologous transgene[67,68]. The molecular mechanism underlying co-suppression is not known, but it is being used to inactivate plant genes just like antisense suppression. Co-suppression and antisense expression can lead to the inactivation of transgenes, but inactivation is often not complete[67]. This can be seen for instance in petunia, where such constructs were introduced to inhibit pigment formation[48]. However, not only plants with white flowers were obtained, but also plants with variegated flowers which still had a faint coloration overall or which were still coloured in certain parts of the flowers. The frequent occurrence of co-suppression phenomena in plants has made transgenic lines with simple, single copy inserts the preferred choice in research and application. This is a major reason that *Agrobacterium* transformation by which such single copy lines can be readily obtained is now replacing methods of direct gene transfer (electroporation, particle gun) which almost invariably give complex integration patterns of transgenes.

Novel tools

To increase the flux through a pathway for secondary metabolite production it may be desirable to block a competitive pathway. As described above this may be accomplished by the use of antisense or sense (co-suppression) constructs. However, this usually does

not lead to complete inactivation. A gene disruption would however lead to a complete loss of gene activity. Transposons form a useful tool to accomplish this[69]. If these are not present naturally in the host plant of interest, they can be introduced from a heterologous source. The Ac-Ds and En-I transposon systems from maize have been introduced into several other plant species to induce transposon-tagged mutations[69,70]. Mutants can be identified by phenotype. Reversely, one can screen in libraries of transposon-tagged mutants, which are available for arabidopsis and petunia, for insertions in the gene of interest by PCR, and thus identify individuals in which there is a mutation in this gene[71]. A similar strategy has been used to identify mutants with a T-DNA insertion in the gene of interest in a large collection of T-DNA insertion mutants of *Arabidopsis thaliana* [72,73]. However, it will be feasible only for few plant species to assemble such a large collection of insertion mutants. Gene targeting might become an alternative especially for plants for which such collections are not readily available. Gene targeting is the directed integration of transgenes at a preferred position in the genome by homologous recombination. This has been used to disrupt or modify genes not only in prokaryotes and unicellular eukaryotes such as yeast, but also in mammals such as the mouse. Knock-out mice in which a specific gene is disrupted by an introduced transgene now play an important role in medical research. Unfortunately, using similar technology as in mouse did not lead to an efficient system for routine gene targeting in plants yet[74], but there is optimism that for instance the introduction of enzymes from other organisms[75] may help to overcome this barrier soon. The recent finding of efficient gene targeting in the moss *Physcomitrella patens* suggests that there may be significant interspecies differences for targeting, or that targeting may be achieved much more readily in haploid or gametophytic cells[76].

Transgenic plants often contain T-DNA constructs in which not only the transgene of interest is present, but also certain selection markers for instance. This may be an undesired situation, since it prevents the use of the same selection marker in renewed transformation of the already transgenic line or to perform crosses with other transgenic lines carrying the same selection gene. Also for public acceptance in the market it may be beter if these selection genes are absent from transgenic crops. Recently, a method was developed by which such markers can be deleted from the genome[77]. The method uses site specific recombination systems from bacteria (Cre, lox system) or yeasts (FLP, frt system). The genes to be deleted from the plant genome are surrounded by the recognition sequences (30-50 bp sequences) of such a site specific recombinase. If these sequences are in a direct repeat orientation the sequences in between can be deleted by the recombinase expressed from a plant promoter. The recombinase gene can be introduced or expressed transiently in the transgenic plant from which the selection genes have to be deleted, or can be introduced by crossing with a plant in which this gene is stably present already. Another possibility to obtain marker-free plants was recently suggested. Here the ipt gene, which is naturally present in the T-DNA and which determines cytokinin production, is present within the Ac transposon located in the binary vector T-DNA[78]. Presence of *ipt* induces shoot formation. Selection now can be on shoots which are formed in the absence of cytokinin in the culture medium. These abnormal (due to the presence of the *ipt* gene) shoot lines regenerate normal shoots spontaneously, which lack the *ipt* gene due to the loss of the Ac element.

Although much has been achieved during the last fifteen years in the refinement of the *Agrobacterium* system as a tool for plant transformation it will be clear that further modifications and assets are needed to comply with specific demands. It is in my mind without doubt that future developments will turn this vector system even more sophisticated and versatile than it is already today.

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CHAPTER 4

PARTICLE GUN METHODOLOGY AS A TOOL IN METABOLIC ENGINEERING

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Summary

Gene transfer into plants is an essential component of any genetic engineering program. Over the past fifteen years major advances in the genetic engineering of plants focused on major crop species such as cereals, legumes and woody species and model systems such as tobacco, petunia and *Arabidopsis*. This was a reflection of the focus on fundamental studies in gene expression and regulation using single genes or applied targets using genes of agronomic interest such a herbicide, pest and disease resistance. One of the limitations of available methodology has been the difficulty to introgress multiple genes into plant cells. In addition, transformation of more exotic plant species is not straightforward. With the advent of direct DNA transfer procedures such as particle bombardment, it now becomes possible to embark on the genetic engineering of such plants and also to begin considering introduction of multiple genes into plants, taking advantage of co-transformation experiments. This makes the genetic engineering of multi-step pathways a feasible proposition. In this chapter, we will discuss some of the issues involved in such experiments. We will use tobacco as a model to illustrate advantages of particle bombardment in metabolic pathway engineering and we will also discuss experiments involving additional medicinal plants which are now targets for the genetic engineering of secondary metabolism.

Introduction

The development of procedures in cell biology to regenerate plants from single cells and organized tissue and the discovery of methodology to transfer genes to plant cells enabled practical applications of plant genetic engineering to take place. These techniques have given us the opportunity to create, characterize and select plant cultivars which cannot be obtained by traditional breeding methods.

Direct DNA transfer procedures, particularly particle bombardment, offer unique advantages over conventional means such as *Agrobacterium* for many reasons. The ability to deliver foreign DNA directly into regenerable cells, tissues, or organs appears to provide the best method, at present, for achieving truly genotype-independent

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transformation in many agronomic crops, bypassing *Agrobacterium* host-specificity and tissue culture-related regeneration difficulties. Instrumentation offers high tunability and fine control allowing precise targeting of particles carrying DNA to specific cell layers within organised tissue. By being able to bypass *Agrobacterium* host specificity, we are now able to broaden the range of transformable important medicinal plants and deliver genes effectively in a relatively short period of time. This opens up the opportunity for introducing multiple genes directly eliminating the need for stepwise re-transformation, a very time consuming and tedious approach, requiring different selectable/screenable markers at each step. Our recent observations, discussed below, that multiple unlinked genes are integrated at one genetic locus in the host genome make pathway engineering not only feasible but also practical. This observation has important implications in engineering multistep pathways. Another advantage of particle bombardment-based transformation methods is that input DNA size is not a limitation. Unlike conventional *Agrobacterium*-based transformation vectors, direct DNA transfer procedures do not require special constructs which would have genes of interest within the T-DNA borders.

Microprojectile bombardment employs high velocity metal particles to deliver biologically active DNA into plant cells. The concept has been described in detail by Sanford.[1,2] Following the original observation by Klein *et al.*,[3] that tungsten particles could be used to introduce macromolecules into epidermal cells of onion with subsequent transient expression of enzymes encoded by these compounds, it was demonstrated that the process could be used to deliver biologically active DNA into living cells, and result in the recovery of stable transformants.[4] Combining the relative ease of DNA introduction into plant cells with an efficient regeneration protocol, avoiding protoplast or suspension cultures, we have the most optimum system in place for transformation. Important advancements and refinements in the process described subsequently, using soybean[5,6] and rice[7] as model systems for dicotyledonous and monocotyledonous species, respectively, demonstrated the power and versatility of the technique.

Particle bombardment: general methodology

In order to address as many of the critical variables identified as being crucial to the transformation process as possible, a mechanism was developed that permitted maximum flexibility for tunability, targeting and cell penetration (for review see McCabe and Christou[8]). By varying the intensity of an electric discharge through a water droplet (thus creating a shock wave accelerating the DNA-coated gold particles) penetration of the target tissue can be controlled very accurately (Fig. 1). Using this mechanism the majority of the particles carrying the DNA can be directed to a specific cell layer. The instrument can be viewed as a shock wave generator. The shock wave produced by the initial electric discharge appears to be responsible for the particle acceleration. This realization led to new designs that no longer resemble "guns". In the current design the accelerating force is generated in a two chamber enclosure separated by a partition. One side contains two steel arc points which generate the initial motive force. The other

side acts as a reflection chamber. The sheet along with the beads is accelerated as it absorbs energy from the shock wave. It is then stopped by a retaining screen to allow the beads to proceed to the target alone. Since the shock wave carries no hot exhaust gases or debris it is possible to place the target tissue relatively close to the gun without fear of incineration. The entire assembly may be partially evacuated to reduce drag and tissue damage, however, too high a vacuum (less than 200 millibar) will cause the tissue to lose moisture rapidly with subsequent reduction in cell viability.

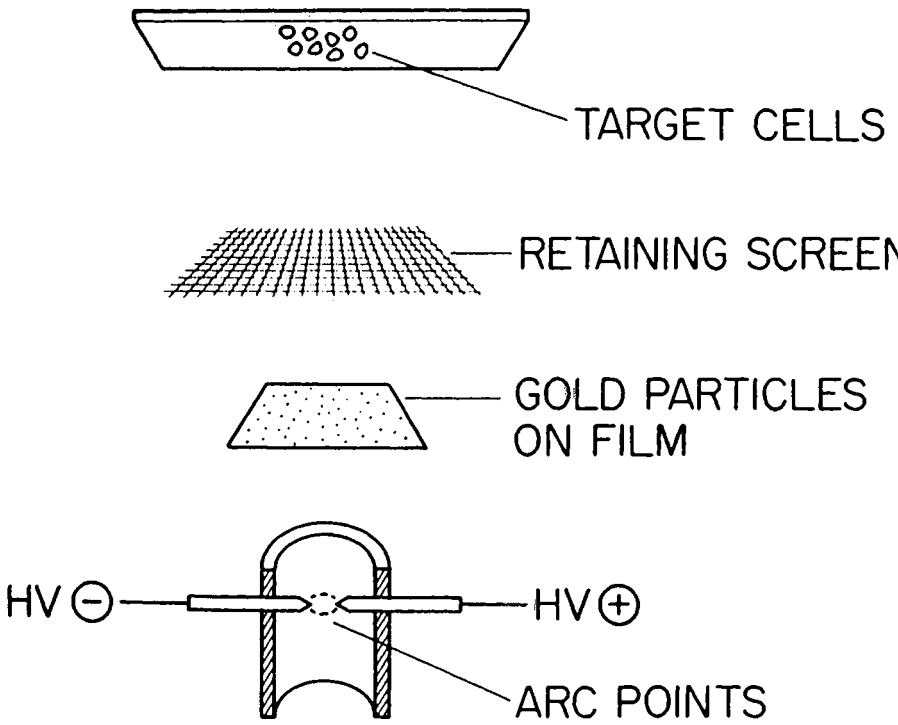

Figure 1. Operating mechanism of electric discharge particle bombardment instrument.

DNA is typically loaded onto 1.5-3 μm gold beads (Alpha Chemicals Inc.) at a rate of up to 40 μg DNA/mg of gold using $CaCl_2$ and spermidine[3] to precipitate the DNA onto the gold. The coated beads are gently centrifuged and resuspended in 100% ethanol, then pipetted onto the carrier sheets (18x18 mm squares of 1/2 mil metallized Mylar; Dupont 50MMC). After a brief period of settling, the ethanol is drained away and the sheet dried. The 'gun' is loaded by placing a 10 μl drop of water between the points and covering the spark chamber with the reflecting cap. The carrier sheet is laid over the top of the reflection chamber and the retaining screen put in place. The target is prepared in a way that allows the desired area to be exposed as it is inverted above the retaining screen. The assembly is then evacuated to 600 millibar before the discharge is activated.

Engineering of secondary metabolic pathways in plants

GENERAL CONSIDERATIONS

The modulation of secondary metabolic pathways to increase yields of desired natural products has been one of the goals of classical phytochemistry for many years. The work has been driven for several reasons: (i) many products of secondary metabolic pathways in plants are commercially and pharmaceutically important; (ii) target molecules are often accumulated in trace amounts in the plant due to tight developmental and cell-specific regulation; (iii) the targets are often complex and chiral molecules which are difficult, expensive or impossible to synthesise chemically.[9] Although some very suitable targets have been identified, the yields of these compounds have in many cases remained low or unaltered despite considerable efforts which focussed primarily on plant cell, tissue and organ culture.[10,11] With the advent of techniques for the introduction of genes into plants, together with the elucidation of a number of secondary metabolic pathways and the continual availability of an increasing number of genes[12,13,14,15,16,17,18,19] modulation and diversification of plant secondary metabolism in transgenic plants and cell cultures in a targeted, rational manner utilizing gene transfer methodologies, presents a powerful approach to tailoring plant secondary metabolism. Targets include rate limiting steps in metabolic pathways, or the complete introduction of multi-gene enzyme cascades in medicinal plants. Mechanisms include redirecting metabolite flow in ramified pathways or increasing enzyme concentrations controlling rate limiting steps.

DeLuca summarized problems that need to be addressed in the expression of heterologous genes in medicinal plants:

- The heterologous protein must avoid proteolysis
- Proper folding is required for activity
- Assembly for multiple subunits of a target gene product may be necessary for activity
- Prosthetic groups, if required must be accessible
- Gene product must be targeted to appropriate subcellular compartments for substrate use

Metabolic engineering can be used to modify existing pathways in the host plant, or produce transgenic plants or cell cultures with novel secondary metabolite chemistries by combining genes encoding secondary metabolic pathway enzymes from organisms that have evolved along separate phylogenies. Combinatorial gene approaches have been used very successfully in microorganisms to produce novel 'hybrid antibiotics' in industrial microorganisms.[20] Several examples of the transfer of a *single* heterologous gene to alter terminal portions of secondary pathways in the host plant have been reported.[21,22,23,24,25,26] A few examples of overexpression of a *single* early pathway enzyme in transgenic plants to modify metabolic flux through existing pathways with a consequent increase in downstream metabolites have also been reported.[27,28,29,30] However, the regulatory architecture of a specific plant secondary metabolic pathway will not necessarily allow such single-gene strategies to succeed, as seen from attempts to increase flux into alkaloid biosynthesis.[31,32] Although overexpression of a rate-limiting

enzyme may permit increase in metabolic flux down a partial segment of the pathway, further downstream steps may become limiting preventing the flow of flux to the desired end-product. Indeed, it is becoming clear that complex metabolic pathways are often controlled at multiple steps by several enzymes (reviewed by Stitt and Sonnewald[33] and refs therein), subsets of which may be regulated both spatially and temporally in a highly coordinated manner.[33,34] The engineering of complex metabolic pathways to alter yields of desired end-product will, in many cases, require the introduction of multiple genes, as will the engineering of multi-step extensions of pathways to create novel products.

INTRODUCTION OF TWO CONSECUTIVE GENES OF INDOLE ALKALOID BIOSYNTHESIS INTO TOBACCO

As a first step to establish important parameters for the engineering of metabolic pathways by the introduction of *multi-gene* cassettes into target medicinal plants by particle bombardment, we created a large number of transgenic tobacco plants transformed with a cointegrate vector carrying two unselected genes of terpenoid indole alkaloid (TIA) synthesis encoding tryptophan decarboxylase (TDC) and strictosidine synthase (STR1) and a selectable marker (Fig. 2). The plasmid was introduced into tobacco using particle bombardment[8] to establish a range of molecular and biochemical diversity in a large transgenic population to permit a study of the effects of individual integration events on steady state mRNA levels and TDC and STR1 enzyme activities. Tobacco provides a facile system for a detailed molecular and biochemical analysis of large numbers of transgenic plants. Studies focussing on tobacco may lead to a fundamental understanding of features which confirm coordinated expression in more complex situations using additional genes and recalcitrant (medicinal) species.

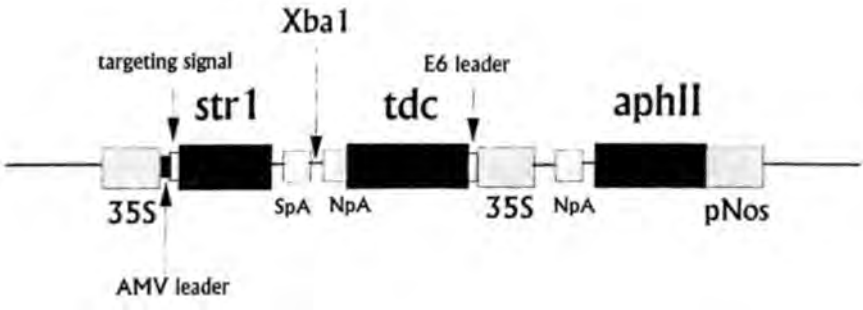

Figure 2. Schematic representation of plasmid TSK. The *tdc* and *str1* cDNAs are driven by the CaMV 35S promoter and the *aphII* gene by the nos promoter.

The cDNAs encoding TDC and STR1 were isolated from *Catharanthus roseus*.[35,36] Tryptophan decarboxylase converts tryptophan into tryptamine, thereby channelling primary metabolites into indole alkaloid biosynthesis. Strictosidine synthase is the first enzyme committed to TIA biosynthesis in *C. roseus*, catalysing the stereospecific condensation of tryptamine and secologanin to produce strictosidine. In our early experiments using transgenic tobacco, the *tdc* and *str1* products were targeted to the cytosol and the vacuole, respectively, because TDC and STR1 are localised in these compartments in *C. roseus*;[37] the *str1* cDNA used in our study includes an endogenous vacuolar-targeting signal.[36] Over 150 primary transformants were analysed for integration patterns of *tdc* and *str1*. Steady state mRNA levels for each transgene were also measured. Seed from 20 selected primary transgenic tobacco plants was germinated to provide material for enzymatic analysis in F1 populations.[38]

Firstly, then, particle bombardment must facilitate the introduction of multiple genes into the host plant. A DNA gel-blot analysis of 150 independent transgenic plants showed a 100% co-integration frequency of the *tdc* and *str1* transgenes and that the transforming plasmid generally integrated as a complete unit. Similar observations were reported for rice and wheat plants transformed with multi-gene transforming units in our laboratory.[39,40] Particle bombardment, therefore, fulfills the potential for the cointegration of multiple genes. Unpublished experiments from our laboratory demonstrated that plasmid constructs, engineered to contain several genes and introduced into plants using particle bombardment, show cointegration of each transgene in the majority of the transgenic population. No preferential expression of the individual genes is seen provided that the genes lie in a specific orientation with respect to one another within the transforming plasmid.

An analysis of the 150 independent transgenic tobacco plants for steady-state *tdc* and *str1* transcripts revealed a range of accumulated levels for each specific transgene. We observed expression from the *tdc* and *str1* transgenes in plants showing single as well as up to 12 multi-insertion events. No clear correlation between the number of integration events of the specific transgene and the levels of the accumulated transcript was observed, supporting the notion that variation in transgene expression can be attributed to effects other than differences in gene copy number. Again, these data correlated well with data from transgenic rice and wheat plants generated using particle bombardment with a plasmid containing three genes and similarly, no correlation between transgene copy number and expression was observed for any of the three transgenes.[39,40]

The diversity we observed in integration patterns and transcript accumulation in the transgenic tobacco plants was also reflected in a variation at the level of functional enzyme detected in extracts from F1 seedlings of the different lines for TDC and STR1. We observed a 24- and 110-fold variation in TDC and STR1 activities, respectively, in the 20 lines we analysed. The 24-fold variation in the expression of TDC observed among the 20 individual *tdc* and *str1* transformants is similar to that previously reported for TDC activity in transgenic tobacco (generated by *Agrobacterium* infection[41,42]), and for other genes driven by the CaMV 35S promoter.[43] However, we obtained plants that accumulated tryptamine to significantly higher levels than previously reported in transgenic tobacco; over a 5-fold increase above that reported previously.[42] In the case of STR1 activity we found levels of STR1 activities which were dramatically

higher than those previously reported for transgenic tobacco cell cultures or plants. Tobacco plants expressing the *str1* cDNA from *C. roseus*, showed STR1 activities of 3-22 times higher activity of than levels found in *C. roseus;*[44] transformed suspension cultured tobacco cells showed STR1 activities ranging from 28-104 pkat/mg protein.[45] We obtained transgenic plants showing STR1 activity up to 918 pkat/mg protein, representing a 150-fold increase in activity over the maximum levels reported previously.[44] In our study, and that of McKnight *et al.*[44] the STR1 was targeted to the vacuole of transgenic tobacco plants.

ENGINEERING SECONDARY METABOLIC PATHWAYS AND GENE SILENCING

The variation in expression of transgenes in independent transformants engineered with the same construct is a common phenomenon and reports have shown expression levels varying up to 100 fold or more.[46,47,48,49,50] Consequently, large numbers of transgenic plants or cell cultures may have to be analysed to establish a complete spectrum of the effects of introducing a foreign gene(s) on metabolic flux through a target pathway.

Much of the variation in transgene expression is likely to be due to chromosomal effects around the site of integration of the transgene.[50,51] Alternatively, multiple copies of the transgene may lead to ectopic pairing of homologous DNA leading to transcriptional[52,53,54] or post-transcriptional gene silencing (reviewed in Baulcombe and English[55] and Baulcombe[56]) as well as rearrangements of the inserted transgene following gene delivery *via* particle bombardment.[5,39,57] As yet, very little work has been reported on the likely impacts of gene silencing on the engineering of secondary metabolism in transgenic plants. A major condition for long term use of transgenic plant lines in breeding and commercial exploitation is transgene stability and expression over generations. Introduction of transgenes that display significant identity to endogenous genes in the host genome may lead to gene silencing through homology-dependant gene silencing mechanisms. For example, overexpression of a L-phenylalanine ammonia lyase (*PAL)* gene from *Phaseolus vulgaris* in transgenic tobacco showed some plants with severely reduced expression of the endogenous *PAL* genes.[58] Although reversal of sense suppression was observed in F1 plants,[59] the sense-suppressed state of the transgene was inherited over a number of generations, gradually decreasing until a wild type expression of the endogenous gene was obtained.[60,61] Gene silencing phenomenon, however, will not be limited exclusively to homology-dependant gene silencing mechanisms and will also occur with the introduction of transgenes that show no identity to endogenous genes present in the host genome. For example, the tobacco genome does not normally contain copies of either the *tdc* or the *str1* gene. However, analysis of the 150 primary tobacco transgenic plants described above in section 3.2 showed that in 26% of the plants, both the *tdc* and *str1* transcripts were undetectable by northern analysis and 41% of the plants demonstrated undetectable levels of **either** the *tdc* **or** the *str1* transcript. Neither transgene appeared to show preferential susceptibility to silencing effects. The transgenic tobacco population we have generated will prove useful in studying the mechanisms of silencing and patterns of transgene silencing in successive generations.

3.4 ENGINEERING OTHER MEDICINAL PLANTS

The dimeric indole alkaloids vinblastine and vincristine, produced in *Catharanthus roseus* plants have been the target of metabolic engineering for decades due to their medicinal value and rarity. The molecules are accumulated to just 0.00025% dry weight of the plant and the cost of the extraction and purification of these compounds from the plant is high.[62] Efforts to produce these compounds in cell culture systems have failed. Although high catharanthine-accumulating lines were produced[62,63] vindoline was absent from these lines. Further studies have shown that vindoline biosynthesis is not lost, but only repressed in cell cultures, since vindoline accumulation reappears concomitantly with shoot regeneration.[64]

The biosynthesis of dimeric TIAs in *Catharanthus* is complex: the pathway is long with more than 25 enzymatic steps and is highly compartmentalised within the plant cell and parts of the plant. Vindoline biosynthesis, which occurs mainly in the leaves, involves the chloroplast, vacuole, a postulated alkaloid vesicle, in addition to the cytoplasm. Catharanthine is synthesized largely in the cytoplasm. The production of precursors and also dimeric products, is highly regulated in a tissue-, developmental- and environment-specific fashion. Despite difficulties with the complex enzymology and protein purification several genes encoding key enzymes in monomeric alkaloid synthesis have been cloned.[19,35,36] Although transgenic cell cultures of *C. roseus* have been produced, using either *Agrobacterium* infection or particle bombardment[32,65] and promoter analysis of three genes involved in TIA synthesis in *C. roseus*, *tdc, str1* and *Cpr*[66,67,68] have been carried out in transgenic tobacco carrying promoter-reporter gene fusions, the absence of an effective means to recover transgenic *Catharanthus* plants has delayed gene expression and regulation studies in the homologous system.

As a first step in generating transgenic *C. roseus* plants expressing transgenes of secondary metabolism, we developed a system for the introduction of marker genes into regenerable tissues. The explants of choice are embryo axes isolated from *Catharanthus* seeds (Fig. 3). The general area of the meristem was subjected to bombardment using the *gus* gene as a marker to evaluate effectiveness of gene delivery.[69] Under the influence of appropriate hormones, *de novo* shoots can be induced easily as shown in Figure 3b. Transient expression analysis two weeks post bombardment indicated effective gene delivery into regenerable tissue (Fig. 3e). Prolific shoot formation leads to plantlet regeneration as shown in Figure 3c. In preliminary experiments, destructive GUS assays indicated the development of chimeric and a number of clonal shoots, as shown in Figure 3e and 3f. The system did not appear to be selectable and therefore, we developed a screening system to rescue putatively transformed plants. This was done relatively easily by observing stained tissue carefully following incubation with the appropriate GUS-substrate. When blue colour began to develop, it was possible to stop the reaction and wash the explant(s) thoroughly with sterile distilled water to remove any residual chemicals in the incubation mix. By following this procedure, it was possible to recover plants which were subsequently rooted and potted into soil. Molecular analysis indicated the stable integration and expression of the *gusA* gene. Obviously for the system to be useful in the context of metabolic engineering, it is essential that efficiencies are improved significantly and that chimeric plants are identified

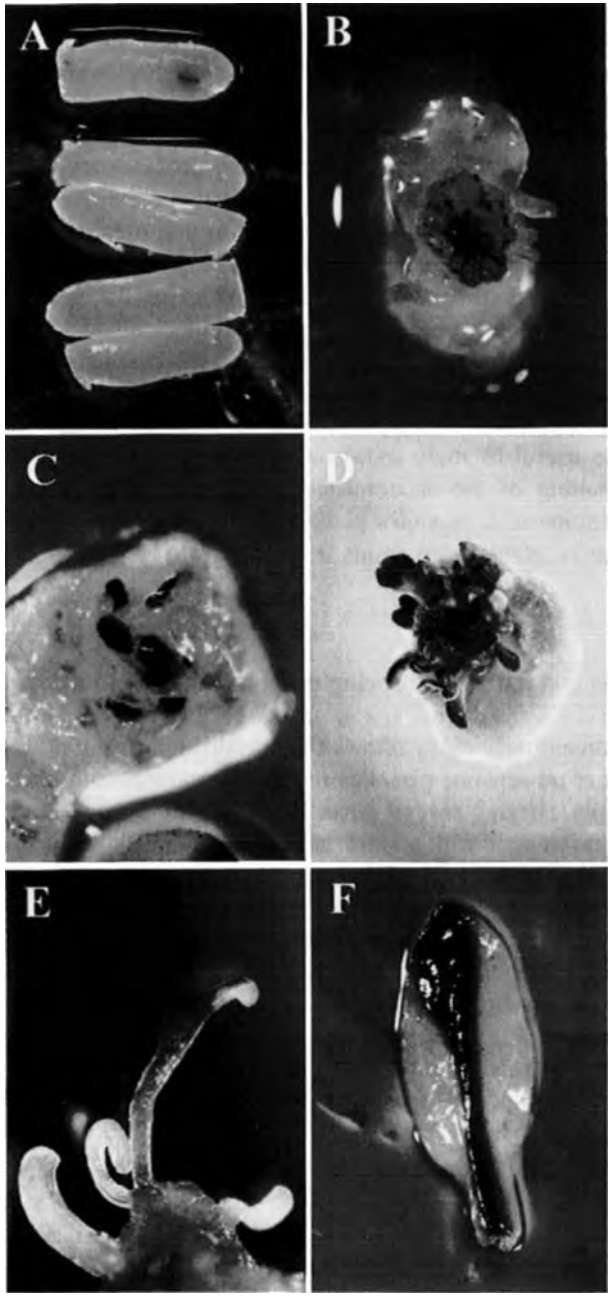

Figure 3. Developing a gene transfer system for *Catharanthus roseus.*
A. Embroyos excised from mature seeds of *C. roseus*
B. Induction of shoot meristems
C. Transient expression of GUS activity after particle bombardment
D. regenerating shoots after bombardment
E & F. Expression of GUS activity in regenerating shoots and leaves after bombardment of meristems

and eliminated at an early stage. We believe the system is amenable to such optimisation and work is currently in progress in our laboratory to achieve these ends.

Similarly, for *Lonicera tartarica* particle bombardment was shown to be effective in generating transgenic callus derived from bombarded leaves (Fig. 4). Like most members of the Caprifoliaceae family, *L. tatarica* accumulates secologanin to high levels.[70] Secologanin provides the terpenoid moiety in the synthesis of TIAs and the introduction of genes encoding suitable enzymes will permit the conversion of the accumulated secologanin to more useful TIAs. The yield of transgenic callus from bombarded leaves is relatively low and the recovery of transgenic callus in larger numbers is currently being optimized by bombarding callus derived from various explants. Although cell cultures suffice as biochemical factories to produce predicted end-product resulting from the activity of enzymes encoded by transgenes, provided callus are fed with a precursor for secologanin biosynthesis,[71] a whole plant regeneration system would be useful to study transgene regulation, activity and the potential for metabolic channelling of the accumulated secologanin down transgenic pathways. Although regeneration of *L. japonica* plants from callus has been described[72] attempts to obtain *L. tatarica* plants from callus in our laboratory have so far met with little success.

4 Multigene metabolic engineering using particle bombardment

Particle bombardment technology allows the introduction of multiple genes, either on a single plasmid or on separate plasmids (co-transformation) into plant cells. We have constructed vectors carrying several genes other than those of secondary metabolism. The transgenes co-integrate with a 100% efficiency at a single locus in most transgenic plants analysed. Furthermore, work in our laboratory has shown that introduction of several plasmids utilizing particle bombardment, with each plasmid carrying a different gene, results in over 90% of the plants analysed carrying all co-transforming plasmids. The introduction of 12 different plasmids into soybean callus similarly resulted in high co-transformation efficiencies.[73] Generally, we find no preferential uptake and integration of any of the co-transforming plasmids. The high efficiency of co-transformation using multiple plasmids into a single locus has been obtained for all plant species, both dicot and monocot, currently used in our laboratory. Co-transformation experiments utilising *Agrobacterium* generally utilise two different strains carrying T-DNA plasmids containing up to three different genes. The co-transformation efficiency is relatively lower than for particle bombardment.[74] Introduction of further transgenes requires re-transformation with vectors carrying an additional selectable marker. However, in conjunction with an enhanced system for *Agrobacterium*-mediated plant transformation, a new binary bacterial artificial chromosome (BIBAC) vector has been reported that is capable of transferring at least 150kb of foreign DNA into a plant nuclear genome.[75] Furthermore, the codelivery of T-DNA substrates and virulence genes may well add further flexibility to the *Agrobacterium* system for the transfer of multiple genes.[76]

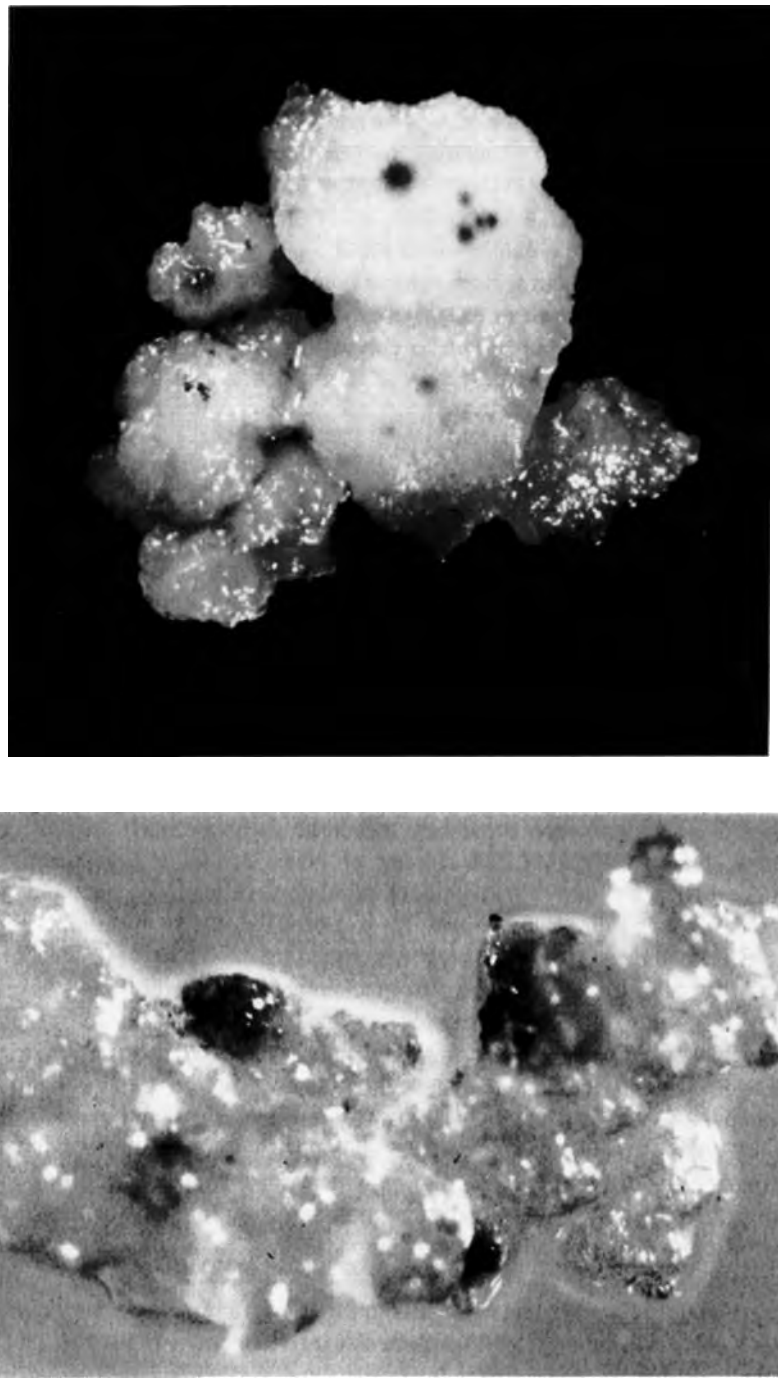

Figure 4. GUS-expressing selected transformed callus of *Lonicera tatarica*.

In contrast to *Agrobacterium*-mediated transformation, which often results in single-copy or low-copy integration of full-length T-strands carrying intact copies of the transgenes, the delivery of DNA into plant cells using particle bombardment can result in the foreign genes tending to integrate as multiple copies which, in some cases have been found to be fragmented and rearranged.[39,77,78] Such complex insertion events were thought to be unstable. However, we have found no evidence to support such a notion. Transgenic monocot or dicot plants produced in our laboratory show no correlation between transgene copy number and expression levels of the transgenes or multi-copy transgene instability during meiosis through subsequent generations. Both particle bombardment and *Agrobacterium*-mediated gene delivery, however, result in the majority of plants with integration events occurring at a single locus, which is important to ensure that the integrated locus is inherited as a single unit, maintaining the expression of all transgenes and thus the metabolic alterations, through subsequent generations.

5 Concluding remarks

Because of the complex organisation, regulation and metabolic compartmentalization of secondary metabolic pathways,[79] the engineering of such pathways is not an easy task. Several molecular tools will have to be combined to manipulate successfully the desired metabolic pathway. Metabolic pathways have multiple control points at rate-limiting steps.[33] Directed changes to a target pathway requires not only a transformation system and genes, but also suitable promoters both constitutive and those to allow cell- and tissue-specific expression, and effective targeting signals to direct the recombinant protein to it's final destination.

Although genes encoding secondary metabolic pathway enzymes will continue to be cloned, the identification and cloning of genes encoding components of signal transduction systems and transcriptional regulators of pathway enzyme genes will be a powerful addition to the repertoire of genes for use in metabolic engineering. The constitutive overexpression of genes encoding regulatory proteins of pathway enzymes may lead to an uncoupling of biochemical from cellular and developmental differentiation, thus overriding restricted synthesis of specified desired products in the engineered material. Furthermore, it is becoming evident that different transcription factors operating in a single pathway may effect the coordinated expression of subsets of genes encoding secondary metabolic pathway enzymes.[80,81,82,83] In *C. roseus*, the *tdc* and *str1* genes demonstrated a coordinated regulation by auxin and elicitors in cell cultures of *C. roseus*[84] suggesting the molecular regulatory mechanisms controlling these genes in response to specific environmental stimuli may be similar. By manipulating the expression of one or two single transcription factors, it should be possible to effect the expression of several coordinately regulated pathway enzyme genes.[85] However, a cautionary note to the outcome of manipulating transcriptional regulators was reported by Lloyd *et al.*[86] Although the constitutive expression of a transcriptional regulator of maize anthocyanin biosynthesis in tobacco and *Arabidopsis* led to an increase in anthocyanin pigmentation, in both species pigment production was still restricted to normally pigmented tissues.

One potential limitation of the use of *Agrobacterium*-mediated gene delivery is host range specificity. Although the number of plant species amenable to *Agrobacterium* infection is continually expanding, including both dicots and monocots, host range may limit the number of target medicinal plants that can be engineered using *Agrobacterium*. The use of particle bombardment avoids the host-range limitations that may be associated with *Agrobacterium*. Gene delivery using particle bombardment, however, is no panacea. Although engineered cell cultures may be sufficient for production of desired compound, in some cases engineered whole plants will be the target for increasing yields in harvestable biomass. One of the greatest challenges facing the genetic engineering of whole plants is the difficulty often encountered in setting up efficient regeneration systems. For example, other than the system reported here in section 3.3, there is only two additional reports of plant regeneration for *C. roseus*[86,87] which are limited by the time consuming or labourious tissue culture procedures or low frequency of shoot regeneration. Furthermore, some plants chosen for genetic manipulation may be woody species which, like *Lonicera sp.*, are notoriously difficult to regenerate and problems are often encountered in tissue culture, particularly when using selection agents, due to the generation of toxic phenolic compounds upon cell death. Transgenic whole plants, however, will be a great asset towards furthering our understanding of the molecular mechanisms that control cell-, developmental- and environmental-specific regulation of secondary metabolic pathways and to study the capacity of transgenic plants to increase biosynthesis and storage of these compounds.

The future, however, looks bright. There are exciting times ahead for secondary metabolic research and it's manipulation. There remains enormous opportunity for unravelling intriguing and exciting fundamental knowledge of the complex plant secondary metabolic pathways and their regulation and the application of new knowledge and understanding to the engineering of applied target pathways and molecules. An increased understanding of the enzymology and regulatory mechanisms of pathway flux, and the cloning of corresponding genes together with the application of versatile gene transfer techniques such as particle bombardment to a broader spectrum of target plants, provides a potent and exciting box of tools for the effective manipulation of secondary metabolic pathways.

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CHAPTER 5

MODULATION OF PLANT FUNCTION AND PLANT PATHOGENS BY ANTIBODY EXPRESSION

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Abstract

The expression of antibodies or antibody fragments in plants that bind to functional domains of plant or pathogen derived proteins, is a novel approach to elucidate and alter primary or secondary metabolism in plants and also to engineer pathogen resistance by inactivating targets inside the plant cell through immunomodulation. The feasibility of this approach either to interfere in plant metabolism or in plant pathogen infections has been shown for several antibodies that bind to key plant metabolites or to viral target proteins. Questions such as how cellular targeting alters the expression and accumulation of these proteins/molecules in plants remain to be answered before antibody technology can be used commercially. Alternatively, plants provide an excellent source for biomass production making it feasible to produce high amounts of valuable recombinant antibodies for diagnostic and therapeutic applications. This approach has yet to be evaluated in terms of expression levels, genetic stability in the field and downstream processing.

1. Introduction

Since Hiatt and co-workers showed in 1989 that plants can express functionally active antibodies (1), several research groups in plant sciences have started to make use of the highly specific antibody-antigen interaction. The suitability of the plant system to express recombinant antibodies or recombinant antibody fragments (rAbs) in various plant cell compartments (2-4) has led to many applications, including large-scale production of correctly folded rAbs or rAb fragments with high conformity at low cost. These rAbs are used for applications in bioprocessing, diagnosis and therapy of animal and human diseases, modification of plant functions by inhibiting enzymes or key substances involved in growth, differentiation and metabolism or the development of novel approaches to immunomodulate host-pathogen interactions and to elucidate structure-function relationships *in vivo*.

Full-size antibodies, Fab-fragments and single chain fragments (scFv) have successfully been expressed in leaves and seeds of tobacco or *Arabidopsis* plants, reaching expression levels as high as 6.8% of total soluble protein. Furthermore, antibodies have been

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targeted to different subcellular compartments of plants including the endoplasmic reticulum, chloroplasts and the intercellular space (full-size, Fab-fragments and scFv) as well as the cytosol (scFv) (3).

Intracellular expression of rAbs has only been successful for scFv fragments, because these do not require the post-translational processing required for full-size antibodies or Fab-fagments, such as assembly of heavy and light chain, formation of intrachain disulfide bonds and glycosylation. Nevertheless, in the majority of transgenic plants containing a scFv construct, cytosolic scFv levels were found to be very low or undetectable (5-8). This observation could be attributed either to the reducing environment or lack of chaperones in the cytosol, which results in the instability of scFvs. Although there are reports in which cytosolic scFvs have reached levels of up to 1.0% of total soluble protein in plants, (9), this is still an exception. Stabilizing rAbs in a way that allows their expression and biological activity within this cell compartment is one of the major tasks that have to be met before the potential of rAbs, especially for metabolic engineering of plant cells, i.e. by inhibiting enzymes or key substances involved in growth, differentiation and metabolism, can be fully exploited.

The unique properties of antibodies might also be of great importance for future plant breeding, in particular to increase the plants' defense repertoire against pathogens or to reduce anti-nutritional factors. Although several viral proteins have been inactivated in cells by other methods including gene disruption or gene replacement, these approaches do not confer permanent resistance, are confined only to single viral strains, and bear several risks for the environment (10-12). Antibodies binding to conserved functional domains of viral coat proteins, movement proteins, or replicases/helicases are an alternative approach to obtain broad-spectrum viral resistance by inactivating these targets in the cell through immunomodulation.

Generation of recombinant antibodies

Since the development of hybridoma technology in 1975 (13), monoclonal antibodies (mAbs) have been widely used for disease diagnosis and therapy. Gene cloning is not limited to naturally occuring, native immunoglobulin genes. Cloned genes can easily be modified by replacing or deleting codons using site directed mutagenesis or PCR. Cloned recombinant antibodies can be converted into Fab- or $F(ab')_2$ -fragments by introducing stop codons at the appropriate site (Fig. 1). Effector functions can be eliminated by deleting the C_H2 and C_H3 domain and new, or better effector functions introduced by converting mouse immunoglobulins into chimeric mouse/human polypeptides. Furthermore, alteration of the hinge region, carbohydrates or the inactivation of Fc-receptor binding sites can enhance performance, biodistribution and stability of rAbs. Monovalent single-chain antibodies (scFv) and bispecific single-chain fragments can be generated, reducing the original mAb to its variable region while maintaining similar specificity and affinity. Fusion proteins can be generated by synthetic coupling of antibody gene fragments to a ligand for example, by keeping the antibody variable domains but replacing the constant domains with toxins, biological response modifiers, enzymes, prodrugs or radionucleotides. Finally, the development of phage display technologies (14), as described extensively

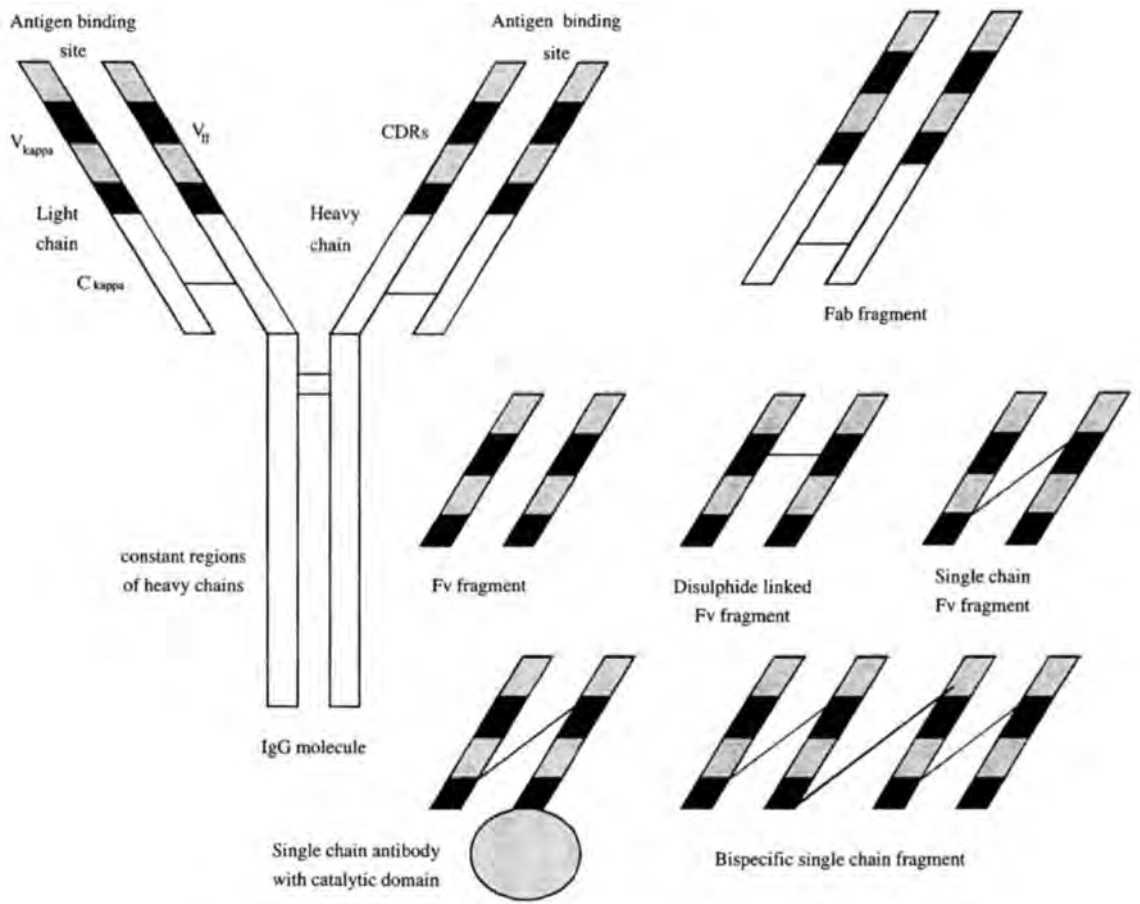

Figure 1. IgG molecule and recombinant derivatives thereof.

below, not only enables the engineering of improved antibody characteristics and kinetics but also the conversion of valuable therapeutic murine antibodies into humanized versions for use in cancer treatment.

GENERATION OF TARGET PROTEINS FOR IMMUNISATION

Target proteins for generation of rAbs can be purified from the organism they are naturally occuring in using standard procedures and protocols. If the natural source of protein is of limited amount or difficult to obtain, recombinant protein can be used as a source of antigen. Therefore, different strategies have been developed in the past, mainly using *E. coli,* as the host for foreign gene expression. Other suitable hosts for heterologous protein expression include yeast, animal and insect cells as well as plant cells or whole plants.

While most available expression and purification systems for recombinant protein expression require either the use of harsh conditions, *e.g.* denaturing chemicals, during purification or sophisticated purification protocols, the GST-system is based on a glutathione-S-transferase gene from *Schistosoma japonicum* as a fusion partner for expressing and affinity purifying the recombinant protein or peptide. This system offers two major advantages in comparison to conventional expression strategies. First, because of the high solubility of the GST-protein in the *E. coli* cytosol, most fusion proteins remain soluble, even if the respective fusion partner to the GST-protein showed insolubility in an alternative expression approach. Second, based on the availability of the enzyme's natural substrate, glutathione, and the highly specific binding of the GST to its substrate it is easy to develop a single-step purification procedure yielding homogenous GST-fusion proteins.

GENERATION OF MONOCLONAL ANTIBODIES

After immunizing an animal, triggering the immune response towards the desired antigen, the spleenocytes containing the antibody-producing B-cells are isolated. The fusion of an antibody-producing B-cell with a myeloma cell results in an immortal hybridoma cell line secreting a monoclonal antibody of defined specificity. However, due to the low frequency of fusions that occur between myeloma and B-cells, an average fusion only yields between 1000 and 5000 different clones. After several rounds of limiting dilution, 5 to 10 different clones secreting a specific monoclonal antibody can be obtained. To isolate and to clone the antibody-encoding genes, tedious steps of mRNA isolation, cDNA generation and PCR, as well as extensive sequencing have to be conducted until the corresponding heavy- and light-chain gene can be cloned. After completing the cloning, the antibody genes have to be modified before they can be expressed in *E. coli*, yeast, insect, mammalian or plant cells.

GENERATION OF ANTIBODIES BY PHAGE DISPLAY

Although hybridoma technology enables production of highly specific monoclonal antibodies, their generation is labor intensive and requires the use of animals, animal

tissue cell culture and expensive equipment. Many molecular biology laboratories have neither the facilities nor the experience to generate monoclonal antibodies. Moreover, as described above, hybridoma technology does not allow immediate isolation and cloning of immunoglobulin-encoding genes. In contrast, from its invention in the early 1990s, phage display of recombinant antibodies on the surface of filamentous bacteriophages (15) has revolutionized engineering of monoclonal antibodies specific for almost any given antigen, including self antigens (16,17). The main advantage of phage display lies in the fact that phage derived "monoclonal" antibodies can be produced in virtually any molecular biology laboratory using basic recombinant DNA techniques. During library generation, all cloned heavy- and light-chain gene fragments are recombined thus generation of novel specificities occurs, which cannot be found in the original donor animal. Additionally, while selection for the desired antibody specificity is performed, the recombinant antibody genes are co-selected, thus the tedious cloning of the antibody-encoding genes from hybridoma cells is avoided.

Several groups have shown the feasibility of phage display by isolating antibodies with nanomolar affinity from single pot libraries from immunized sources but also by completely bypassing the immune system using naïve libraries (17). Low affinity clones can be used to construct secondary phage libraries which can be subjected to affinity-maturation *in vitro* until suitable affinities in the nano- or even picomolar range are obtained by several approaches. These include methods such as mutagenesis, chain shuffling, CDR randomisation and gene shuffling followed by panning and screening to identify higher affinity clones (18-21). It is also possible to select for desired on- and off-rates that prolong or reduce the interaction time of a rAb with its respective target. Therefore, phage display technology has brought the ability to generate "monoclonal" antibodies tailored to specific needs and with any desired specificity in almost every molecular biology laboratory.

Generation and screening of phage display libraries

The most crucial step in generating antibodies by phage display is the set-up of the initial library, which determines the diversity of the library. Current state of the art involves RT-based PCR cloning of immunoglobulin heavy- and light-chain variable regions. During that procedure a flexible linker is integrated so that a functional scFv is assembled in fusion to the M13 gene III and is displayed on the phage surface (Fig. 2). Alternatively a bicistronic setup enables the display of Fab-fragments on the phage surface. These scFv- or Fab-displaying phages are then screened with the antigen of interest, whereby specific antibody fragments are enriched. This can either be done on solid phase antigen (ELISA plates, antigen-columns), or in solution using biotinylated antigen and streptavidin coated magnetic beads, on cell surfaces or on histochemical sections (19,20).

Using naïve libraries or libraries from immunized sources

Currently, two different approaches are employed for the establishment of phage display libraries. In the first approach variable light- and heavy-chain genes are amplified from unimmunized "naïve" sources and combined to create very large libraries, assuming that every possible mature VH-VL combination is represented. Currently, such libraries

contain more than $1x10^{10}$ different phages. The second approach uses cloned VH-and VL-gene fragments from immunized animals, shifting the representation of VH and VL genes toward those encoding specific antibodies binding to the desired antigen. These libraries usually contain $1x10^6$ to $1x10^7$ different clones (17,18). As the first approach circumvents immunisation and the resulting immune response, one library can be used for screening of many different antigens. Unfortunately, affinity-maturated antibodies are not included in a naïve library, therefore rAbs generated by this method usually are of a lower affinity compared to those isolated from an immunized library. Libraries from immunized sources, on the other hand, can deliver antibodies of high specificity but are necessarily restricted for use with the antigen against which they were generated and require more labour.

MODIFYING AND TAILORING ANTIBODIES FOR SPECIFIC USE

Gene technology is important to antibody engineering because immunoglobulin genes can be modified to create recombinant antibody fragments or fusion proteins. This progress, together with improvements in expression technologies, allows generation of antibodies against almost any antigen, fine-tuning of the antibodies for improved performance and their expression in a variety of hosts. Furthermore, incorporation of synthetic affinity tags has simplified purification of heterologously expressed recombinant proteins.

Bispecific antibodies

To broaden the potential use of antibodies in medical and biological applications, bispecific antibody molecules, which have two independent binding sites for two epitopes are often desirable. Bispecific antibody molecules are unique therapeutic agents that can crosslink two different antigens, and this property can be exploited to recruit cytotoxic T cells to a tumor cell during cancer therapy (23,24).

Various strategies have been used to generate bispecific antibodies. The first bivalent bispecific full-size antibodies or $F(ab')_2$ fragments were produced *in vitro* by chemical cross-linking of two different antibodies (25-27). In addition, bispecific $F(ab')_2$ fragments have been created by heterodimerization of *E. coli* expressed Fab fragments through cysteine residues (28) or leucine zippers (29). Bispecific antibodies have been produced *in vivo* using the hybrid hybridoma (quadroma) technology (30), but a limitation of this procedure is the low yield of bispecific antibody of the desired dual specificity. Protein engineering has permitted the design of even smaller bispecific fragments based on scFv fragments, such as the scFv heterodimer diabody, which is formed *in vivo* by non-covalent association of two single chain fusion products (31). Alternatively, the two different binding specificities can be combined within a single polypeptide using a flexible linker peptide to form a bispecific single chain antibody (biscFv). Functional biscFvs were first expressed in *E. coli* in 1994 (32). Since then several biscFv antibody molecules have been successfully expressed in microbes (33-35) and mammalian cells (36,37). However, expression levels are often low, or in case of *E. coli* expression inclusion bodies are produced containing mis-folded recombinant antibodies, which require labor intensive *in vitro* refolding procedures to produce functional biscFv (38,39).

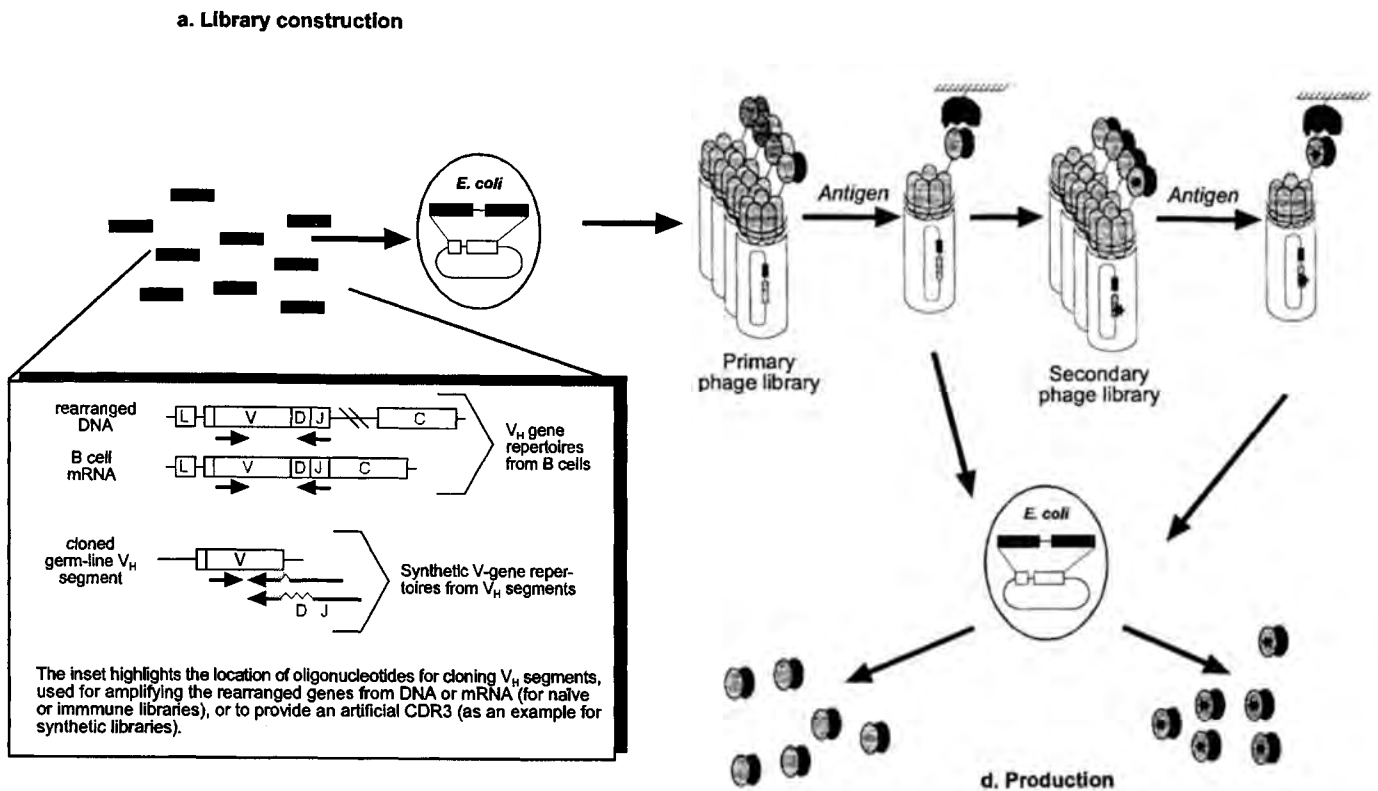

Figure 2. Overview of the phage display method (a) Library construction: V-genes (red and blue boxes) are cloned in phage(mid) vectors for display on phage. (b) From these libraries, antigen specific antibodies are enriched using affinity selection. (c) If necessary, the selected antibodies are affinity maturated, by diversifying their sequence, yielding a secondary library from which the highest affinity clones are enriched. (d) Finally, soluble antibody fragments may be produced by expression in *E. coli*, yeast or other recombinant expression systems (22).

To overcome these problems, we engineered biscFvs for production in plants. We selected biscFv2429, which contains the well characterized TMV-specific scFv fragments scFv24 and scFv29, recognizing epitopes present only on the surface of intact TMV particles or TMV coat protein monomers, respectively (8). Our data demonstrate that functional biscFv2429 could be expressed in different plant cell compartments (cytosol, apoplast, ER) with a maximum yield of 1.65% of total soluble protein when expressed in the ER of transgenic plants. Affinity purified biscFv2429 was bispecific for both epitopes, and had similar binding characteristics and affinities compared to its parental single chain antibodies.

Enzyme linked scFv's

Another way to modify rAbs is to create fusion proteins where the fusion partner encodes an enzyme with a desired activity toward a target molecule. One approach describes the linkage of a toxin to a cell type specific scFv fragment to create an immunotoxin capable of killing tumor cells (40,41). A second approach involves linking RNAses and proteases to scFv fragments which are subsequently guided to their target by the scFv specificity.

Cytosolic antibodies

Cytosolic antibodies can be used to modulate cellular physiology and metabolism (5,42,43) or to inactivate pathogens (6-8) and are a viable alternative to other methods of gene inactivation such as antisense RNA, dominant negative mutants and targeted gene disruption (10). In certain circumstances, cytosolic antibodies are the only option when the desired target has not been cloned or is a metabolite, carbohydrate or nucleic acid. Specific rAbs can be raised against these target molecules.

Single chain antibodies (scFv) (44) or single domain antibodies (dAbs) are the preferred molecules for cytosolic expression since they consist of a single polypeptide that does not require *in vivo* assembly or complex folding. Binding of such cytosolic antibodies may be used to 1) block or stabilize macromolecules (*e.g.*, by protein-protein or protein-nucleic acid interactions); 2) modulate enzyme function by blocking the active site, fixing the enzyme in an active or an inactive conformation or by blocking its substrate and 3) divert proteins from their usual compartment.

Unfortunately, cytosolic expression levels of scFv fragments derived from monoclonal antibodies in plant cells are low and rarely exceed 0.1% of total soluble protein (7). It is likely that the lack of protein disulfide isomerase and specific chaperones in the cytosol results in scFv misfolding and contributes to enhanced proteolysis (45). However, a recent report demonstrated that scFv fragments isolated using phage display reached 0.3 % and 1% of total soluble protein in the cytosol of *Petunia* leaves or petals, respectively (9), indicating that phage display can be used for enrichment of more stable antibody scaffolds that tolerate the absence of disulfide bonds. It is worth mentioning that the presence of the target molecule in the cytosol appears to stabilize a cytosolic antibody (unpublished results).

Expression levels of cytosolic recombinant proteins in prokaryotes have been enhanced by the addition of a stabilizing fusion protein, and antibody stability in plants has been improved by the fusion of peptide sequences to the antibody C-terminal.

Schouten *et al.* demonstrated that addition of a C-terminal KDEL sequence significantly increased scFv protein levels in the plant cytosol (46,47), indicating that this may protect the scFv fragment from proteolytic degradation. In *E. coli*, linkage to N-terminal fusion partners such as thioredoxin (TRX), glutathione S-transferase (GST) or the maltose binding protein improved the solubility and stability of heterologous proteins (48-50).

The success of protein stabilization with fusion partner proteins in the *E. coli* system prompted us to evaluate the suitability of using fusion partners to stabilize scFv fragments in the plant cytosol. We used the TMV-specific scFv24, which has a high affinity towards epitopes present on intact virions and confers virus resistance to transgenic plants. scFv24 has been successfully expressed to high level in the apoplast of tobacco plants but expression only reaches low levels in the cytosol (8). Our rationale was to improve cytosolic expression levels by linking scFv24 to small cytosolic proteins. scFv24 was fused to GST from *S. japonicum*, TMV coat protein (CP), thioredoxin from tobacco (TRXt) or thioredoxin from *E. coli* (TRXe) and these scFv-fusion proteins were expressed in the ER or the cytosol of tobacco leaves. The results demonstrated that all four scFv24 fusion proteins were functional but their expression levels were dependent on the fusion partner. The highest levels of fusion protein expression were observed for the ER targeted GST-scFv24 fusion protein, whereas in the cytosol only the CP fusion gave a significantly increased expression level. Cytosolic levels of CP-scFv24 could be further increased by the addition of a C-terminal KDEL sequence. Whether this approach leads to improved resistance in plants needs to be investigated.

Antibody expression in plants

A key aspect of modern plant biotechnology is the ability to increase the production levels of recombinant proteins to meet the market demand. Transgenic plants (51) offer advantages for pharmaceutical antibody production (52,53), for plants can be grown on an agricultural scale and the antibody harvested from them. Plants can produce high levels of safe, functional recombinant proteins and can be easily expanded to agricultural levels to meet industrial demand (54-56). Current applications of plants in biotechnology include the production of recombinant antibodies, enzymes, hormones, cytokines, plasma proteins and vaccines. In addition, chimeric plant viruses are used for the production of small recombinant proteins including vaccines. Plant based production of foreign proteins requires only a virus-infected or a transgenic plant, sunlight and mineral salts, either from the soil or fertilizers, and water. Furthermore, modern agricultural practice enables the ease in scale up, rapid harvesting and processing of large quantities of leaves or seeds.

Plants are easy to transform and grow and have similar pathways of protein synthesis, secretion, folding and post-translational modifications to animal cells. Heterologous proteins accumulate to high levels in plant cells and plant-derived antibodies are essentially indistinguishable to those produced by hybridomas. A major concern of antibody production in animal cell culture systems, co-purification of blood-borne pathogens and oncogenic sequences, is entirely avoided during downstream processing of recombinant proteins from plants, which gives high yields of homogenous, safe products for therapeutic applications.

GENERATION OF STABLE TRANSGENIC PLANTS

Transfer of a foreign gene into the plant cells can be achieved using *Agrobacterium*-mediated transformation (57,58), particle bombardment (59), electroporation of protoplasts (60) or viral vectors (61). While transgenic *N. tabacum* cv. Petite Havana SR-1 are regenerated from transformed leaf-discs, the *N. tabacum* BY-2 suspension cell line can be directly transformed by co-cultivation of suspension cells and Agrobacteria (62), because of its unique growth characteristics. Transient expression of the foreign gene can be detected within 2-3 days after co-cultivation, the selection of transgenic callus via Kanamycin-resistance takes approximately three weeks and the initiation of suspension cell lines from this callus can be performed within less than two months.

Stable transformation is characterized by the integration of the target gene into the host plant genome. The generation of transgenic plants often uses two core technologies, *Agrobacterium* mediated gene transfer to dicots, such as tobacco and pea, or biolistic delivery of genes to monocots, such as wheat and corn (58,59). However, rice can be transformed by *Agrobacterium* and methods are being developed for transforming other monocots.

TRANSIENT PROTEIN EXPRESSION IN PLANTS

Transient gene expression in plants has advantages over the generation of stably transformed transgenic plants and it seems particularly suited for verifying that the gene product is functional and stable before moving onto to large scale production in transgenics (63). The generation of stably transformed transgenic plants (51) requires a considerable investment in time before the expressed proteins can be analysed. In contrast, transient gene expression systems are fast, flexible and are not influenced by positional effects that potentially bias gene expression levels (63,64). Heterologous gene expression can be induced at an advanced developmental stage, which avoids any potential negative effects on the developing plant and can optimize the yield of the desired product. Furthermore, using viral vectors for transient protein expression (64) has attracted commercial interest because it can rapidly produce high yields of protein on a field scale. Currently, there are commercial field trials underway for producing recombinant protein using viral vectors.

Transient expression systems use various approaches including biolistic delivery of 'naked DNA', infiltration with recombinant Agrobacteria (Agroinfiltration), or infection with modified viral vectors. DNA can be efficiently introduced into the plant cells by biolistic methods, evacuated plant leaves take up large amounts of recombinant Agrobacteria upon rapid vacuum release and infection of whole plants can be achieved by mechanical inoculation with viral vectors. The overall level of transformation differs greatly between these three systems. 'Naked DNA' usually reaches only a few cells and for transcription it has to reach the nucleus of the cell (59). Agroinfiltration targets many more cells in a leaf and the T-DNA harboring the gene of interest is actively transferred into the nucleus by the bacterial proteins (63). A virus will systemically infect most cells in a plant and transcription of the introduced gene in RNA viruses is achieved by viral replication in the cytosol, which generates many transcripts of the gene of interest.

ANTIBODY PRODUCTION IN TRANSGENIC PLANTS

Antibodies are an ideal model for the expression of therapeutic or diagnostically important proteins in plants (65-68). Different forms of recombinant antibody fragments can be expressed in plants (69) and sub-cellular targeting of the protein is an important consideration for high level expression. There are technical considerations to bear in mind when planning transgenic plant expression of proteins (70). The pattern of codon usage in plants is different to that of animals but altering the composition of the heterologous cDNA to meet the plant pattern can increase the expression level.

Functional rAbs were first expressed in transgenic plants in 1989 (1) and 1990 (71). Functional full-size antibodies (71-76), Fab fragments (77) and scFv fragments (5-7,43,78-80) can be expressed in leaves and seeds of plants without loss of binding specificity or affinity. These antibodies are essentially identical to those produced by a parental monoclonal. Expression levels of different antibodies vary, with expression of full size IgG under the control of the 35S promoter ranging from 0.35% (81) to 1.3% of the total soluble protein (TSP) in tobacco leaves (1). This does not appear to be an upper limit, since transgenic plants have been identified with expression levels of single chain antibodies in leaves reaching 6.8% of the TSP (42) and up to 500 μg of secretory IgA per gram leaf material (52,76,82).

Seeds are plant storage organs that are often rich in protein and can be stored almost indefinitely (3,4,83). This can be exploited by biotechnologists to generate a storage container for recombinant proteins, since single chain antibodies can reach up to 4.0% of the TSP (42). Potato tubers have also been used as storage vessels for recombinant antibodies with levels reaching 2% TSP and cold storage for 18 months resulted in only a 50% loss of functional antibody (84).

ANTIBODY TARGETING IN TRANSGENIC PLANTS

Recombinant antibodies have been targeted to the following compartments of plant cells: intercellular space, chloroplasts and endoplasmic reticulum (3,43,71-75,78,79). Intracellular expression of rAbs in the cytosol has only been achieved using scFv fragments, (5,7,8,78) and in the majority of transgenic plants containing a scFv construct, cytosolic scFv levels were found to be very low or undetectable (5,6,8,47,78).

The most attractive approach for protein expression for in field production is to target the protein to the ER and if long term storage is required, to target it for seed specific expression with seed specific promoters. Transformation of major crop plants is now becoming more straightforward. Crop based expression systems (wheat, rice, corn) will become more popular because crop plants produce fewer toxic compounds than model species like tobacco and there is an existing infrastructure for their cultivation, harvesting, distribution and processing.

OPTIMISATION OF ANTIBODY PRODUCTION IN TRANSGENIC PLANTS

Plant bioreactors can be optimised by exploiting the innate protein sorting and targeting mechanisms plant cells use to target host proteins to organelles. Significant increases

in recombinant antibody yield have been observed when antibodies are targeted to the secretory pathway. Retaining expressed proteins within the endoplasmic reticulum currently gives the highest yields of functional protein but targeting them for secretion to the intercellular space beneath the cell wall (apoplast) also leads to significant levels of expression. Compared to expression in the secretory pathway, ER retention can give 10 to 100 fold increases in target protein yield (3). This strategy may keep scFvs in the presence of the molecular chaperones for longer, so lead to efficient and accurate protein folding. Signal peptides are cleaved from ER targeted scFvs, and scFvs have been localized to ER, ER vesicles and the nuclear envelope and these antibodies have high antigen binding activity. Interestingly, transgenic plant leaves expressing ER retained scFvs can be dried and stored for more than three weeks without losses of antigen binding activity or specificity (85). Targeting to the chloroplast and the vacuole could also be an interesting alternative as they comprise major compartments of the plant cell. Targeting of rAb fragments to plant cell membranes via heterologous mammalian membrane anchors is also feasible and practical (Schillberg *et al.*, unpublished results).

Furthermore, recombinant antibody expression can be optimized by the use of stronger promoters, improvement of transcript stability and translational enhancement with viral sequences and optimisation of codon usage (86,87).

Application of antibodies in plants

EXPRESSING ANTIBODIES IN PLANTS FOR DISEASE CONTROL

Disease control and generation of resistant plant lines protected against a specific disease, such as a viral and fungal infection, has been achieved in the past using conventional breeding technologies based on crossings, mutant screenings and backcrossing. Many approaches in this field have failed or the resistance obtained has been rapidly broken by the pathogen. A further limitation of conventional breeding is that it is time consuming, several generations of plants have to be screened and crossed before a stable line with the desired phenotype can be obtained. A more attractive approach is to produce transgenic plants that are intrinsically resistant to pathogens, which can be achieved by the expression of intracellular recombinant antibodies.

Engineering virus resistance

The recent progress in plant molecular virology has enhanced the understanding of plant viral disease. Fundamental knowledge on viral genomic organisation, gene function and plant-pathogen interactions have permitted development of novel strategies for controlling viral diseases of plants (10,11). Despite all these advances, there are still many gaps in our knowledge of the events leading to viral infection, replication, movement and vector transmission. Progress toward understanding many of these details is essential for developing virus-resistant transgenic plants and controlling virus transmission.

The analysis of viral proteins and enzymes involved in pathogenesis is a difficult task because of low viral expression levels and the difficulty of purifying plant viruses. However, basic information about functions and interactions of both structural and non-structural viral proteins *in vivo,* and their interaction with host factors or vector components, could aid the design of viral-protein derivatives that interfere with pathogenic functions of the invading virus. Although several viral proteins had been inactivated in infected cells by gene disruption or gene replacement, these methods do not confer permanent resistance, are confined to single viral strains and bear substantial risks to the environment (11,12,88). Antibodies binding to conserved functional domains of viral coat proteins, movement proteins, or replicases are an alternative approach to obtain broad-spectrum resistance and to reduce these risks by inactivating the targets inside the cell through immunomodulation. The feasibility of this approach has been recently shown for both animal (89-92) and plant viruses (6-8,75). The major disadvantage of using viral coat proteins as targets is their structural diversity. This restricts the effect of the engineered antibody to a small range of viruses and under selective stress, the viral coat protein sequence can alter without loss of function. This problem can only be overcome if antibodies against evolutionary conserved domains are generated and selected. Alternatively, plants could be protected against viral infections by expression of movement protein- or replicase/helicase-specific scFvs. Virus movement is one of the two processes that are fundamental to almost all plant viral infections, the second being viral genome replication. Antibodies directed against functional, essential, domains of these proteins should provide more potent, broad resistance against viral pathogens. Surface expression of virus-specific scFv fragments is a novel approach to shield the plant cell from an invading pathogen. Combining this strategy with cytosolic expressed scFvs specific for conserved viral functional domains, such as movement proteins or replicases/helicases, should generate plants that have durable resistance in field.

Microbial pathogen and insect pest control

An emerging application for immunomodulation is the control of microbial, fungal and insect attacks on plants. These pathogens have been controlled in the past by the use of fungicides or insecticides. Chemical controls have many disadvantages compared to the generation of plant cultivars that are intrinsically resistant to pathogens. Pesticides and fungicides are expensive, their production is limited to the industrialized world and extensive use promotes the appearance of resistant pathogen strains.

Recombinant antibodies and antibody fusion proteins offer a promising approach for controlling fungal or insect pests. The scope of antibodies used range from mycelia or elicitor specific anti-fungal antibodies to nematode-specific antibodies blocking the feeding of the nematode on the crop plant. Antibodies raised against harmful salivary secretions of a nematode did not protect the transgenic plant from nematode attack when the full-size antibody was secreted (72). An explanation for the lack of protection could be that the nematode saliva is injected directly in the cytoplasm and expression of a cytosolic expressed rAb may be required for effective protection against the pathogen.

IMMUNOMODULATION OF ENZYMES

In addition to their intrinsic value as tools for pathogen control and medicine, antibodies offer the possibility to manipulate plant metabolism. rAbs could be used to inhibit or to stabilize the activity of endogenous enzymes, thus providing an alternative to antisense and ribozyme technology. This approach has been used successfully to inhibit the reverse transcriptase activity of murine leukemia virus (92) and HIV in animal cells (91).

To date, antibody modulation of secondary metabolism has not been used in plants although many applications could be envisaged. For example, *Catharanthus roseus* cell cultures have been used to produce the alkaloid vinblastine, a potent antineoplastic agent (93-95). However, this high-value product is present only in trace amounts within the plant cells, and to-date, no reports of significant increases in vinblastine production by conventional approaches (modifications of culture conditions etc.) are available. Transgenic approaches could be used to manipulate the pathway leading to the production of the target molecule in plant cell cultures by introducing genes encoding enzymes to overcome rate-limiting steps in the biosynthetic pathway. Alternatively (or additionally), antibodies could be designed that inhibit key enzymes responsible for the branching of pathways and, increase vinblastine production by downregulating metabolic side pathways *in vivo*. This approach will require precise control of antibody targeting to defined locations within the cell together with improved strategies to maintain the antibodies' activity and affinity in these compartments.

MODULATION OF PLANT PHYSIOLOGY AND DEVELOPMENT

Single-chain Fv antibodies synthesized by plant cells and targeted against a particular host antigen can be used to locally block the biological functions of the selected endogenous molecule and to specifically interfere with cell growth and metabolism. This approach, defined as intracellular immunization, is particularly suited for inhibiting functions of small regulatory molecules, because only slight reduction of their activity may lead to the dramatic changes in plant development. Immunomodulation of the activity of a photoregulatory protein phytochrome using a cytosol-targeted scFv was shown by Owen *et al.* (5). Phytochrome-dependent promotion of germination and photocontrol of hypocotyl elongation were both aberrant in transgenic plants, providing a "proof of principle" for the immunomodulation of plant targets.

This approach has been shown to also be applicable to small non-protein regulatory molecules, such as the phytohormone abscisic acid (ABA). ABA regulates a variety of developmental and physiological processes, including seed dormancy, leaf water relations and adaptation to environmental stresses. Accumulation of an scFv antibody binding to ABA and targeted into the lumen of the ER in transgenic tobacco plants caused symptoms of ABA deficiency previously only described for ABA-deficient or -insensitive mutants. Ubiquitous expression of anti-ABA scFv led to the wilty phenotype and increased transpiration (43). Transgenic plants expressing anti-ABA scFv under control of a strong seed-specific promoter were phenotypically similar to wild-type plants apart from their seeds. Embryo development and seed maturation

of these plants differed from that of wild-type, the usual maturation programme was blocked and a vegetative programme was initiated. Anti-ABA scFv expressing embryos developed green cotyledons containing chloroplasts and accumulated photosynthetic pigments but produced less seed storage proteins and oil bodies. Anti-ABA scFv seeds germinated precociously and had reduced desiccation tolerance (42). Interestingly, despite the high levels of total ABA (up to 10 times higher than in wild-type plants), the amount of anti-ABA scFv antibodies produced in transgenic leaves and seeds (up to 6.8% and up to 4% of total soluble protein, respectively) was sufficient to prevent accumulation of free ABA. The mechanism proposed to explain the observed physiological effects in anti-ABA scFv transgenic plants is that capture of abscisic acid by binding to scFv antibodies in the ER may prevent its transport and interaction with ABA receptors. Moreover, the studies of the specificity of the parental monoclonal antibody for ABA analogues have shown that the structure of the side chain of ABA is important for both its activity and the antibody binding. Therefore, ABA molecules bound by the anti-ABA scFv antibodies are most likely to be biologically inactive.

Immunomodulation of ABA demonstrates the potential of this approach for the manipulation of desired features in plants, in tissue and temporally specific manner. The prerequisite for successful application of this technology is generation of recombinant antibodies highly specific for their targets, such antibodies can be now selected *in vitro* from large combinatorial libraries.

IMPROVEMENT OF FOOD QUALITY

A wide area of applications yet to be evaluated is the use of antibodies to enhance food quality by altering plant metabolism to reduce unwanted components or by expressing antibodies that have benefits for the consumer. Antibodies specific for key enzymes of plant secondary metabolism could be generated and expressed to reduce specific plant components *e.g.* a toxin, a flavonoid or bitter compounds. Also, plants could be used to produce antibodies that protect consumers from disease, either by oral immunisation (96) or by directly acting on a pathogen (97), for example by delivering a sIgA that binds to and inactivates bacteria causing dental caries (76).

Further directions and perspectives for antibodies in plants

ANTIBODIES FOR DISEASE CONTROL

Antibodies have proven their potential as vital and important agents for long term disease control in plants, especially for controlling diseases where classic breeding technologies for generation of resistant plants have failed. The antibodies discussed here are first generation antibodies directed against accessible epitopes within a clearly defined context. Although they have often been effective in controlling a single disease they do not provide the broad range resistance that is needed. Second generation antibodies are under development that aim at conserved structures or epitopes involved in pathogen function. These include evolutionary conserved viral movement protein motifs, which are necessary

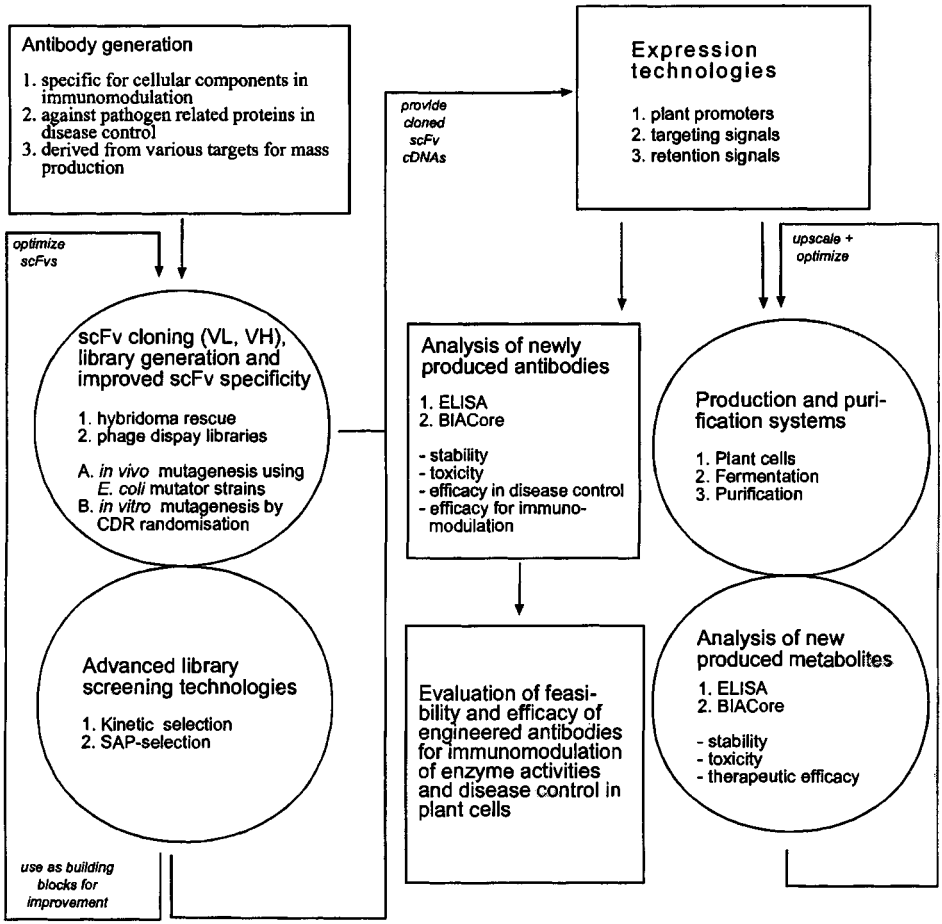

Figure 3. Expressing recombinant antibodies in plants for immunomodulation, disease control or mass production for therapeutics and diagnostics.

for virus spread within the plant, and viral replicases or helicases, which are important for viral replication as well as proteases necessary for virus maturation. This approach should lead to broad spectrum resistance and provide plants with durable protection against a variety of viruses. However, it will require the development of rAbs and rAb-fusion proteins that are stable in the plant cytosol. Such rAbs can be generated by phage or ribosome display instead of cloning hybridoma derived antibody genes or by selection of scFvs lacking cysteines by DNA shuffling followed by phage display. Furthermore, instead of expressing rAbs with a single defined specificity, the generation of biscFvs (98) and diabodies (31) binding to two different targets simultaneously, *i.e.* a viral replicase and a movement protein, will improve antibody mediated resistance significantly. A similar effect could be obtained by expressing 2-5 scFvs with different target specificities simultaneously in the same transgenic plant, either in the same or in different compartment(s).

Improving antibodies for the use in disease control also involves the directed expression of antibodies in certain compartments or tissues which can be achieved by the use of targeting sequences and membrane anchors. Anchoring sequences will lead to an increased concentration of rAbs at cellular sites where replication, assembly and movement takes place, instead of freely floating rAbs in the cytosol that might not reach the critical threshold to inactivate an invading pathogen. Moreover, tissue specific or inducible promoters which also reduce potential side effects of expressing antibodies continuously throughout the entire plant need to be considered for second and third generation rAbs. This could also include the use of pathogen inducible promoter(s) expressing the specific antibody only at the time of infection.

ANTIBODIES AS THERAPEUTICS AND DIAGNOSTICS

A second major focus of research on antibody expression in plants lies in the production of antibodies for special purposes, for example for use as therapeutics or diagnostic tools. Several successful strategies for large scale expression and downstream processing of recombinant antibodies from plants have been developed and open the field for cost effective production of high-quality rAbs (Fig. 3). These efforts are currently being evaluated by several groups in terms of genetic stability, level of expression, ease of downstream processing and quality control of the product. So far, first generation antibodies have fulfilled several key expectations, such as maintained specificity, stability in the plant environment, genetic stability and the maintenance of expression throughout several generations.

The production of second generation recombinant antibodies that can not be made in hybridomas, such as scFvs, fusion proteins and even autoantibodies, will extend the uses of plants as bioreactors for producing antibodies even further.

Microbial and animal cell cultures have proven to be important for production of therapeutic macromolecules, such as recombinant antibodies (99,100). However, these expression systems are cost intensive, in particular for removal of oncogenic DNA, pathogenic viruses (in particular HIV and hepatitis) prions and endotoxins during downstream processing of the raw cellular material. Despite the existing strong bias for expression in microbes and animal cells, alternative expression systems - such as plants - are required for large scale production of recombinant proteins at low cost with high consistency and no loss of specificity or efficacy.

Plants offer advantages over existing systems with respect to biomass build-up, product storage and distribution and low cost of production. Plants are easy to grow and, in contrast to bacteria or animal cells, their cultivation does not require specialist equipment or toxic chemicals. High level accumulation of recombinant proteins in plants results in low production costs and avoids ethical problems associated with transgenic animals. Furthermore, protein synthesis, secretion and chaperonin-assisted protein folding plus the post-translational modification processes such as signal peptide cleavage, disulfide formation, and initial glycosylation are very similar between plant and animal cells (53,101,102). These features justify the use of plants as alternative or even better source for producing recombinant proteins at low cost while eliminating disadvantages associated with microbial or animal cell systems (1,100). Plants seem ideal

for producing recombinant proteins which are too expensive to produce in conventional systems or which require significant investment to remove potential contaminants (54,56). In many cases the production of such proteins in animal cells can accumulate to cost in the range of $10,000 to $100,000 per gram of protein. Additionally, plant genetic material is readily stored in seeds or tubers, which are extremely stable, require no special maintenance and have an almost unlimited shelf life.

Plants produce recombinant proteins that are essentially indistinguishable from the original. Protein synthesis, secretion and chaperonin-assisted protein folding together with the post-translational modification processes such as signal peptide cleavage, disulfide bond formation and initial glycosylation, are very similar between plant and animal cells. To our knowledge only minor differences in further processing of complex glycans exist between animal and plant derived glycoproteins, which include the presence of xylose and α1-3 fucose in plant glycoproteins and the absence of terminal sialic acid residues. In addition, plants are currently the premier heterologous production system for secretory IgA antibodies. Importantly, genetically stable seed stocks of recombinant protein producing plants can be isolated and stored indefinitely at low cost and the seed stock can be converted into a harvest of almost unlimited amounts of the desired recombinant protein within one growing season. This allows flexibility in responding to market demand and has a significant advantage over transgenic animal technology because it allows both the recombinant protein and the production system to be stored until required.

Summary

Although recombinant antibodies can be generated against almost any target molecule, designing a neutralizing or modulating antibody is not an easy task. Interfering in plant pathogenesis, primary and secondary metabolism, morphogenesis or development depends on the binding affinity, the off- and on-rate kinetics and the stability of the engineered antibody, as well as a detailed understanding of the function of the target molecule(s) *in vivo*. Recent developments in antibody engineering, including phage display and expression technologies are valuable tools to overcome these problems as the plant produced antibodies need to effectively bind their target(s) *in vivo* in plant cell compartments. These developments are particularly important for cytosolic antibodies that have the potential to be used to either disrupt protein function, enhance/stabilize protein-protein or protein-nucleic acid interactions or perform catalytic activities themselves. Since the Fc-portion of an antibody is not required for binding, biscFvs, scFvs and scFv fusion proteins could be used to alter plant metabolism and development, confer pathogen resistance or to change the appearance of the plant.

Beyond the uses of antibodies to modulate plant metabolism, we anticipate that there will be a rapid expansion in using plants as production systems for recombinant antibodies in the near future. There is an increasing demand for mass production of safe recombinant antibodies (103) and antibody fusion proteins for disease diagnosis and disease therapy. The relative ease of creating transgenic plants that express rAbs and the emerging technology for recombinant antibody production in crop plants points to the molecular farming (104) of pharmaceuticals becoming a commercial reality within the next decade.

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CHAPTER 6

TRANSCRIPTIONAL REGULATORS TO MODIFY SECONDARY METABOLISM

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Introduction

Prospects to modify alkaloid production *via* genetic engineering of a single or a few enzymatic steps have been reviewed previously.[1] The aim of this contribution is to discuss opportunities to modify secondary metabolism using transcriptional regulators. Transcription factors are proteins that regulate gene expression by binding to specific sequences in the upstream region or promoter of a gene and by stimulating the activity of the RNA polymerase II-containing transcription complex. Single transcription factors can regulate complex pathways involving numerous target genes. A notable example is muscle differentiation in animals, where either one of a set of myogenic bHLH transcription factors (MyoD, myogenin, Myf5, MRF4) in combination with the MADS-domain transcription factor MEF2 induce muscle cell differentiation and switch on numerous muscle-specific genes.[2] Other examples include single homeodomain transcription factors in the fruit fly, that regulate complex pathways resulting in the determination of segment identity.[3,4] In *Arabidopsis thaliana*, overexpression of the transcription factor CBF1 resulted in coordinate upregulation of a set of cold-regulated genes, and in increased freezing tolerance.[5] These examples illustrate that single transcription factors can act as master regulators of complex developmental pathways, and can control the expression of a large number of target genes in a coordinate fashion. A general characteristic of secondary metabolic pathways is that the expression of the structural genes is coordinately regulated depending on cell type or in response to environmental stimuli. This coordinate control is most likely due to master regulators, and one can envisage that secondary metabolism can be modified by engineering the activity of such master switches. The following sections will briefly review the transcriptional regulation of the well-studied phenylpropanoid pathway and its flavonoid branch, and the less well-studied terpenoid indole alkaloid biosynthetic pathway. In the conclusions, perspectives for practical applications of transcription factors in engineering secondary metabolism will be discussed.

Regulation of the Phenylpropanoid and Flavonoid Pathways

The phenylpropanoid pathway is the source of a large number of compounds that are derived from the amino acid phenylalanine (Fig. 1). The flavonoid branch is responsible

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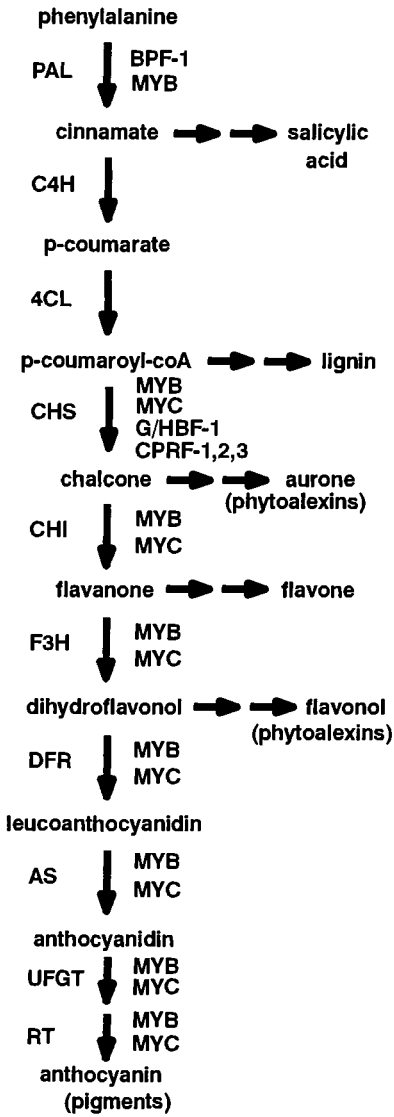

Figure 1. Simplified diagram of the phenylpropanoid and flavonoid biosynthetic pathways. Enzymes catalysing the reactions are placed on the left hand side, and transcription factors on the right hand side of the arrows. Both transcription factors for which their control over the enzymatic steps has been genetically proven, as well as transcription factors that were shown to interact with promoters of the structural genes, are shown.

PAL: phenylalanine ammonia-lyase; C4H: cinnamate 4-hydroxylase; 4CL: 4-coumaroyl-coenzyme A ligase; CHS: chalcone synthase; CHI: chalcone-flavanone isomerase; F3H: flavanone 3β-hydroxylase; DFR: dihydroflavonol 4-reductase; AS: anthocyanin synthase; UFGT: UDP glucose-flavonol glucosyltransferase; RT: anthocyanin rhamnosyltransferase.

for the production of the anthocyanin pigments, the UV-absorbing flavones and flavonols, and the anti-microbial phytoalexins. Other branches produce lignin precursors and soluble phenolics such as the signalling compound salicylic acid. A large number of structural genes have been cloned from many plant species.[6] The best studied aspect of transcriptional regulation regards the formation of the anthocyanin pigments, due to the fact that they provide a convenient visible marker. Another well-studied aspect is the stress- and pathogen-induced biosynthesis of phytoalexins. Both classes of endproducts share a large part of the biosynthetic pathway.

The structural flavonoid genes are subject to different regulatory mechanisms within a single plant depending on the cell type. Many *Arabidopsis* transparent testa (*tt*) mutants affected in the colour of the seed coat have been isolated. Some, such as *ttg*, have a general effect on anthocyanin accumulation in the whole plant, while others have a more restricted role in the seed. All the *tt* genes encode positive regulators of seed pigmentation. Their mutation reduces pigmentation. In contrast, the banyuls (*ban*) mutation results in an increased pigmentation of the seed coat,[7] indicating that the BAN protein is a negative regulator. Fusca (*fus*) mutations result in anthocyanin accumulation in *Arabidopsis* developing embryos and seedlings. The *FUSCA* genes are negative regulators, since their mutation results in enhanced pigmentation. In contrast to the *tt* and *ban* mutants, the *fus* mutants have a pleiotropic phenotype. Some *FUS* genes are allelic to constitutive photomorphogenic (*cop*) and de-etiolated (*det*) mutations described as defective in light-induced responses but without effect on anthocyanin accumulation. *FUS* genes are thus negative regulators of light responses,[8] with anthocyanin biosynthesis being one of the multiple light responses. Anthocyanin accumulation is a light-inducible trait that increases quantitatively with higher light intensity. The *fusca* genes are severely mutated alleles, and the loss of repressor function is thought to mimic the action of extremely intensive white light. The *fusca* alleles are lethal after the seedling stage, indicating that the *FUS* loci are essential for adult plant development. Experimental evidence indicates that the COP8/FUS8, COP9/FUS7, and COP11/FUS6 proteins form a nuclear complex, while other COP/DET/FUS proteins may regulate the activity of the complex.[8] The COP9 complex contains 12 subunits, some of which may correspond to other COP proteins. The COP1/FUS1 protein is nuclear-localized in the dark, and cytoplasmic in the light in wild-type plants, but not nuclear-localized in dark-grown mutants defective in the COP9 complex. The COP1 protein has structural features that indicate that it may act as a regulatory protein, which include a ring-finger zinc-binding domain, and WD-40 repeats which are also found in the β subunit of heterotrimeric G proteins.[9] The C-terminal half of COP1 bears significant homology with the $TAF_{II}80$ subunit of Drosophila TFIID, a central component of the basal transcription machinery.[10] COP1 directly interacts with the bZIP (basic-leucine zipper) transcription factor HY5.[11] The *Arabidopsis* HY5 mutant lacks many responses to light, indicating that HY5 is a positive regulator of light responses. HY5 is required for light-responsive expression of the *Chs* gene, and can directly bind to a G-box present in the light-responsive element unit 1 (see below) in the *Chs* promoter.[11] COP1 also directly interacts with CIP7 (COP1 interactive protein).[12] Downregulation of CIP7 mRNA levels *via* antisense expression lowered *Chs* expression and anthocyanin levels, indicating that CIP7 is a positive regulator of anthocyanin

biosynthesis. Although CIP7 does not show similarity to known transcription factors, it has transcription activating properties in tobacco when fused to the GAL4 DNA-binding domain, indicating that CIP7 may be a novel transcription factor.[12] Taken together, the results indicate that in the dark, COP1 acts as a repressor of light-regulated processes by directly binding to transcription factors that regulate light-responsive genes. Nuclear localization of COP1 is due to the action of other COP proteins acting in a complex in the dark, and is abolished upon illumination. The role of COP1 and COP9 as specific regulators of light-induced processes has been challenged by the observation that in the mutants also gene sets that are normally pathogen-induced are de-repressed.[13] Therefore, instead of viewing anthocyanin accumulation in the *fus* mutants as a result of the disturbance of light-regulated events as argued in references 8 and 9, it could also be due to a disturbance of defense responses. Postulating that (some of the) COP proteins are not specific regulators of light responses would also solve another paradox. According to pharmacological studies using a photomixotrophic soybean cell suspension (discussed in more detail below), it was concluded that the signal transduction pathways for *Lhcb* (encoding a light-harvesting chloroplast protein) and *Chs* expression are mutually exclusive.[14] Nevertheless, the *cop1* and *cop9* mutants have derepressed expression of both genes.[13] One explanation for this apparent paradox is the assumption that COP1 and COP9 are general repressors of plant responses instead of being specific for light. This would also explain that the human genome encodes proteins that show high similarity to COP9 and COP11,[15] although obviously human development is not light-regulated.

Complementary information on the effect of light on anthocyanin biosynthesis was obtained using the tomato *aurea* mutant, which lacks the photoreceptor phytochrome and fails to synthesize anthocyanins. Anthocyanin synthesis was restored by microinjection of different signalling compounds.[16,17] Combining the microinjection results with a pharmacological dissection of the light activation pathway for *Chs* expression in a photomixotrophic soybean cell suspension, it was concluded that phytochrome acts *via* heterotrimeric G proteins to increase the cellular cGMP concentration, which then activates anthocyanin biosynthesis.[14,17] Where cGMP signalling should be placed relative to the action of the FUS/COP/DET proteins remains an open question. As discussed before, (some of) the FUS proteins should maybe not be placed in the light signal transduction pathway at all.

The COP1 protein represses the flavonoid structural gene *Chs via* the modulation of the activity of the transcription factor HY5 and the CIP7 protein. The flavonoid structural genes including *Chs* are also controlled by a combination of two distinct transcription factor species, one of which has homology to the protein encoded by the vertebrate proto-oncogene c-*Myb*, and the other with the vertebrate bHLH protein encoded by the proto-oncogene c-*Myc*. These transcription factors bind to specific sequences in the promoters of the target genes.[18,19,20] The DNA-binding domain of plant MYB proteins consists of two, or for some of them one, imperfect repeats.[21] The DNA-binding specificity of plant MYB proteins varies considerably between themselves.[21] The MYC proteins have a bHLH-type (basic helix-loop-helix) DNA-binding domain and recognize variants of the sequence CANNTG. In particular, the G-box (CACGTG) has been reported to interact with a MYC-related factor.[22] The G-box is also a target for bZIP

transcription factors,[23] including the previously mentioned HY5 protein. Plant MYB and MYC proteins can physically interact with each other.[24] About seven enzymes are involved in the biosynthesis of anthocyanins starting from coumaroyl-coA (Fig. 1). In maize, the entire set of genes encoding these enzymes are thought to be regulated coordinately by the *Myc* gene *R* and the *Myb* gene *C1* in the aleurone (epidermal layer of the kernel endosperm), and by homologous genes in other parts of the plant.[6,25] In dicotyledonous plants, anthocyanin control appears to be more complex. Although all the structural genes in the anthocyanin branch of the pathway are coordinately regulated during flower development, the earlier and later parts of the biosynthetic pathway are thought to be controlled independently.[25] Nevertheless, introduction of the maize *R* and *C1* regulators in *Arabidopsis* intensifies pigmentation in normally pigmented tissues and induces pigmentation in plant tissues that are normally unpigmented.[26] Overexpression of the maize *Lc* gene, which encodes a MYC-type regulatory protein, in *Petunia* upregulated the whole flavonoid biosynthetic pathway starting from *Chs* and including the earlier and later genes, resulting among others in intensely pigmented leaves.[27] The latter result is clearly at odds with the theory proposed in refs. 25 and 28, stating that the earlier and later parts of the anthocyanin pathway are independently regulated in *Petunia*.

The expression of the *Myb* and *Myc* genes coincides with the structural genes that they regulate.[25,29] The mere presence of MYB and MYC in a cell switches on the anthocyanin structural genes. Although the DNA-binding affinity of an *Antirrhinum* MYB protein was negatively affected by phosphorylation,[19] the activity of MYB and MYC proteins does not appear to be regulated by cell type-specific mechanisms involving protein modification. This would explain why additional regulators of flower colour have been scarcely identified. One notable example is the Petunia *an11* mutant.[30] The expression of the *Dfr* structural gene in the *an11* mutant can be rescued by expression of the MYB protein AN2, indicating that the AN11 protein acts upstream of MYB. In contrast to the *Myb* gene *An2*, *An11* is ubiquitously expressed. Except for a WD-repeat (which is also present in the COP1 protein) the AN11 protein shows no notable homologies to other proteins. Since the *An2* mRNA level is normal in the *an11* mutant, it was suggested that the AN11 protein regulates MYB protein activity.[30] Another example is the maize *viviparous1* mutant, which does not produce anthocyanin in seeds, while other plant parts are normally pigmented. The *vp1* mutation also affects seed maturation, resulting in shoots and roots that emerge from the seeds while the kernel is still attached to the ear. Certain *vp1* alleles prevent anthocyanin biosynthesis but produce normal, nonviviparous seed, suggesting that control of the anthocyanin pathway is at least partially separable from regulation of embryo maturation. The *vp1-R* mutant fails to express the *C1* gene in developing seed tissues,[31] and the VP1 protein can transactivate a reporter gene driven by the *C1* promoter,[32] by specifically binding to the Sph sequence.[33] Therefore, VP1 is a transcription factor that acts upstream of the MYB protein *C1* by regulating its expression *via* direct binding to the promoter.

The fact that so few mutants affected in regulation of MYB and MYC activity have been found in plants is surprising in view of the multitude of protein modifications and protein-protein interactions reported for their mammalian counterparts. Mammalian MYC is active only when complexed with the bHLH protein MAX.[34] MYCs potential to

activate transcription is suppressed by association with p107, an Rb-related tumor suppressor.[35] Phosphorylation stimulates transactivation by MYC.[36] Phosphorylation[36,37] or reversible oxidation[38] of MYB reduces its DNA-binding affinity. The interaction of MYB with the general co-activator CREB binding protein (CBP) is required for transactivation.[39,40] It can be expected that similar interactions and modifications play a role in the regulation of the activity of plant MYB and MYC proteins. The fact that no mutants in these regulatory processes have been found, may be due to the fact that the genes involved are redundant or absolutely essential.

Many phenylpropanoid compounds that are constitutively expressed in certain cell types and/or during development, can be induced in other tissues by various stress-related signals.[41] Bean *Pal* and *Chs* genes are rapidly and coordinately induced by elicitors.[41] UV light or white light induces the transcription of the parsley *Chs* gene.[42,43] In the promoters of phenylpropanoid biosynthetic genes from various plant species common sequence motifs can be identified. The H-box [(T/A) CT(C/A) ACCTA(C/A) C(C/A)] and box P [CCA(C/A) C(A/T) AAC(C/T) CC] are present in the promoters of *Chs*, *Pal* and *4-cl* genes in different plant species, and have been associated with regulation of gene activation by stress stimuli such as UV light or elicitors.[42,44,45,46] In the *Chs* gene from parsley[42,47] and *Arabidopsis*[48] a light-responsive unit (LRU) has been identified, which is necessary and sufficient to confer gene expression upon UV-B and UV-A/blue light illumination. The LRU is composed of a H-box, also called MYB recognition element (MRE) and an ACGT element. In the parsley *Chs* gene the ACGT element forms part of a G box (CACGTG), which is a conserved element in plant promoters.[23] The ACGT element has been found to interact with the parsley bZIP class proteins CPRF-1, 2 and 3.[47] CPRF-1 transcripts are induced by UV light with much faster kinetics than *Chs* induction,[47] suggesting that CPRF-1 is responsible for UV-responsive expression of the *Chs* gene. As disscussed above, the *Arabidopsis* bZIP protein HY5 also interacts with the G-box in the LRU.[11] The H- (or MRE) box has been found to interact with DNA-binding proteins of the MYB class.[49,50] The parsley MYB protein *Pc*MYB1 transactivates expression of an MRE-containing reporter construct.[50] In contrast to *Chs* (and the CPRF-1 gene), *PcMyb*1 expression is not light-regulated. In addition, the H- (or MRE) and G-boxes in the bean *Chs* gene were shown to interact with a bZIP class protein with relaxed DNA-binding specificity called G/HBF-1.[51] While G/HBF-1 transcript and protein levels do not increase during the induction of phenylpropanoid biosynthetic genes, the DNA-binding affinity of the G/HBF-1 protein is enhanced by elicitor-induced phosphorylation, suggesting that G/HBF-1 is responsible for elicitor-responsive expression of the bean *Chs* gene. However, there are no *in vivo* expression studies that prove that the H and G-boxes are involved in elicitor-responsive expression of the bean *Chs* gene. The P-box in the parsley *Pal* gene was shown to interact with BPF-1, a protein with some similarity to MYB transcription factors.[52] The expression of the gene encoding BPF-1 is induced by fungal elicitor with slightly faster kinetics than *Pal* expression.[52]

The functional identification of these transcription factors is based on more or less specific recognition of stress-responsive elements in the promoters of the structural genes, and sometimes their stress-induced expression. Definitive proof of their function awaits (reverse) genetic evidence. Although the conservation of the binding sites in different structural genes

suggests that these stress-related transcription factors coordinate the expression of several genes, experimental evidence is lacking. It is unknown how stress-induced expression of phenylpropanoid biosynthetic genes ties in with their tissue-specific control by MYB and MYC proteins. Since MYB and MYC can induce pigmentation in otherwise unpigmented tissues without stress signals, it appears that they overrule stress signalling pathways. On the other hand, the structural genes can be induced by stress signals in tissues that appear to lack the appropriate tissue-specific MYB and/or MYC proteins. Whether there is cross-talk between tissue-specific MYB/MYC regulation and stress-induced signalling remains an open question. Stress-induced expression of phenylpropanoid biosynthetic genes is transient in nature. The signal transduction pathway becomes de-sensitized, which results in a temporary refractory state where cells are no longer sensitive to stress signals. Therefore, it remains to be seen whether stress-regulated transcription factors are suitable for metabolic engineering, since their activity appears to depend on protein modification, such as for instance phosphorylation, *via* a signal transduction pathway that is only transiently active. Tissue-specific transcription factors may be more suitable to modify metabolism, and, as discussed in the next section, the tissue-specific regulators *C1* and *R* have been successfully used to modify flavonoid biosynthesis.

Modification of Flavonoid Metabolism Using Transcription Factors

Myb and *Myc* genes have been ectopically expressed in the plant species from which they originated, as well as in heterologous plant species. All studies described here used the Cauliflower Mosaic virus (CaMV) 35S RNA promoter, which is highly active in most tissues of most plant species, to express the transcription factors.

Introduction of the *Delila* (*del*) gene from *Antirrhinum*, encoding a MYC-type transcription factor, in tomato strongly increased pigmentation in vegetative tissues.[53] In tobacco, *del* only intensified pigmentation of flowers, whereas in *Arabidopsis*, *del* had no phenotypic effects.[53] This indicates that DELILA is active in some, but not all, heterologous plant species, and recognizes its orthologous target genes. Overexpression of the maize *Lc* gene encoding a MYC-type regulatory protein in *Petunia* upregulated the whole flavonoid biosynthetic pathway starting from *Chs* and including the earlier and later genes, resulting among others in intensely pigmented leaves.[27] The expression of the general phenylpropanoid genes *Pal* and *C4h* was not affected by *Lc* overexpression, indicating that *Lc* only regulates structural genes in the flavonoid branch. Ectopic expression of the MYC-type transcription factors DELILA and *Lc* led to increased pigmentation only in tissues that are normally pigmented, or that can become pigmented under stress. The ectopically expressed MYC proteins probably rely on endogenous MYB proteins to activate their target genes.

Introduction of the maize *R* and *C1* regulators in *Arabidopsis* intensified pigmentation in normally pigmented tissues and induced pigmentation in plant tissues that are normally unpigmented.[26] In maize cell suspension, ectopic expression of *C1* and *R* led to the accumulation of anthocyanins and a number of other related 3-hydroxy flavonoids.[54] In addition, six anthocyanin structural genes that are targets for *C1/R* were expressed at high levels in the transgenic cell line.

These experiments demonstrate that the ectopic expression of transcription factors is a viable method for engineering secondary metabolism in plants and plant cells.

The Terpenoid Indole Alkaloid Biosynthetic Pathway

Research on the terpenoid indole alkaloids (TIA's) is mainly primed by the pharmaceutical applications of several of the compounds. The monomeric alkaloids serpentine and ajmalicine are used as tranquilizer and to reduce hypertension, respectively. The dimeric alkaloids vincristine and vinblastine are potent antitumor drugs. Putative functions in the plant include antimicrobial activity and protection against UV light and herbivores. Terpenoid indole alkaloids are found in a limited number of plant species. The best progress on molecular characterization of the pathway has been made with *Catharanthus roseus* or Madagascar periwinkle, a member of the Apocynaceae family. *Catharanthus roseus* cells have the genetic potential to synthesize over a hundred terpenoid indole alkaloids (TIAs). Their biosynthesis is induced by fungal elicitors,[55] jasmonates[56,57] and auxin starvation.[57] Many monomeric TIAs are found in all plant organs, but vindoline and vindoline-derived dimeric alkaloids are only found in chloroplast-containing plant tissues.[58] Vindoline biosynthesis is under the control of phytochrome.[59]

TIAs consist of an indole moiety provided by tryptamine, and a terpenoid portion provided by the iridoid glucoside secologanin (Fig. 2). Tryptamine is derived from primary metabolism by a single enzymatic conversion of the amino acid tryptophan, a reaction catalysed by the enzyme tryptophan decarboxylase (TDC - *Tdc* gene).

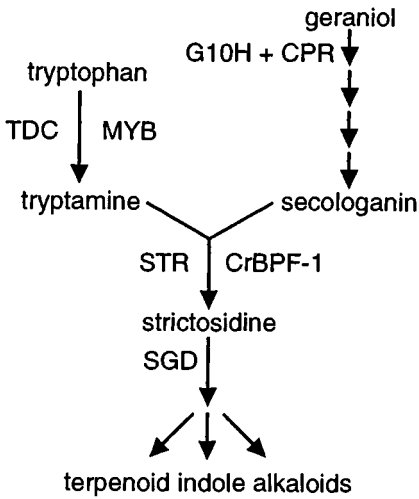

Figure 2. Simplified diagram of the terpenoid indole alkaloid biosynthetic pathway. On the left hand side of the arrows, enzymes catalysing the reactions are shown, and on the right hand side transcription factors that interact with promoters of the structural genes. TDC: tryptophan decarboxylase; STR: strictosidine synthase; SGD: strictosidine β-D-glucosidase; G10H: geraniol 10-hydroxylase; CPR: NADPH:cytochrome P450 reductase.

The biosynthesis of secologanin requires a number of enzymatic conversions of which the first committed step is the hydroxylation of geraniol by the enzyme geraniol 10-hydroxylase (G10H). Tryptamine and secologanin are condensed by the enzyme strictosidine synthase (STR - *Str* gene) to form strictosidine, which is the general precursor of all terpenoid indole alkaloids found in plants. The first enzymatic conversion of strictosidine is performed by strictosidine β-D-glucosidase (SGD).

cDNA clones encoding strictosidine synthase from *Rauwolfia serpentina*[60] and from *Catharanthus roseus*,[61,62] and genomic sequences from *R. serpentina* and *R. mannii*[63] and *C. roseus*[64] have been isolated. A *Tdc* cDNA[65] and corresponding genomic clone[66] have been isolated from *C. roseus*. *Str* and *Tdc* mRNA accumulate in suspension-cultured cells after auxin[62,67] or phosphate[68] starvation, exposure to fungal elicitors[62,69] or (methyl) jasmonate (Menke FLH, Parchmann S, Mueller MJ, Kijne JW and Memelink J; unpublished results), and their distribution in the plant is developmentally regulated with the highest levels in the roots.[62] In leaves *Tdc* and *Str* are induced by a UV-B light pulse (Ouwerkerk PBF and Memelink J, unpublished results). Since TDC and STR are encoded by single-copy genes in *C. roseus*[62,66], all these signals act on the same promoter regions. The chinese tree *Camptotheca acuminata* on the other hand, contains at least two distinct *Tdc* genes, that are differentially controlled.[70] Accumulation of *Tdc*1 mRNA is developmentally regulated, whereas *Tdc*2 mRNA is induced by fungal elicitor and methyl jasmonate.

In addition, cDNA clones for NADPH:cytochrome P450 reductase (CPR),[71] which is essential for the geraniol 10-hydroxylase catalysed reaction, and strictosidine β-D-glucosidase (Geerlings A, van der Heijden R, Memelink J and Verpoorte R, unpublished results) have been isolated. *Cpr* mRNA accumulation is rapidly induced by fungal elicitor,[71] and the *Cpr* promoter is elicitor-responsive in transgenic tobacco.[72]

The observations that *Tdc* and *Str* mRNAs coordinately accumulate in response to fungal elicitors, (methyl) -jasmonate, UV light, auxin depletion, phosphate depletion, and have similar tissue-specific distributions, clearly indicate that the *Tdc* and *Str* genes are controlled by common regulators. The possibility exists that these regulators control additional TIA structural genes, and maybe large parts or all of the biosynthetic pathway.

A search for promoter elements that dictate elicitor-responsive gene expression has revealed promoter regions that contribute to a high level of expression (Fig. 3). In addition, a true elicitor-responsive region was identified in the *Str* promoter. Its deletion resulted in an undetectable expression level without further stimulation after elicitation (unpublished results), indicating that it binds a transcription factor that is activated after elicitation. Interestingly, the *Tdc* promoter contains in a similar position a region that has elicitor-responsive activity in transgenic tobacco.[73] These observations raise the possibility that TIA structural genes are coordinately uninduced by an elicitor-responsive transcription factor.

Using *Tdc* promoter fragments in a yeast one-hybrid screen, we have isolated several transcription factors from *Catharanthus roseus* including a MYB-related factor. Interestingly, the *Tdc* promoter contains a G-box-like sequence (Fig. 3), that may be a target for a MYC-related factor. In addition, the *Str* promoter contains at a similar position a G-box that can interact *in vitro* with a MYC-related factor from *Arabidopsis* (unpublished results). These observations raise the possibility that TIA structural genes may be controlled by a combination of MYB and MYC proteins. Several plant species

have been shown to contain a large number of *Myb* genes, and a sizeable number of *Myc* genes. It is possible that subsets of these factors are dedicated to control certain metabolic pathways. The possibility that structural genes in the TIA pathway are controlled in a developmental or tissue-specific fashion by members of the MYB and MYC families clearly deserves scientific attention. In addition, MYB transcription factors may be involved in stress responses. MYB factors have been associated with responses to gibberellic acid,[74] salicylic acid,[75] and light.[50,76] Using a part of the *Str* promoter in a one-hybrid screen, a periwinkle homologue of the MYB-like factor BPF-1 from parsley was isolated (unpublished results). Like its parsley counterpart,[52] transcription of periwinkle CrBPF-1 is induced by fungal elicitor with kinetics that are somewhat faster than for *Str* induction (unpublished results).

Taking these results together, the regulation of the TIA pathway shows many similarities to the regulation of the much better studied phenylpropanoid pathway. In both cases, the transcription of the structural genes is coordinately regulated in plant development and in response to stress signals. In addition, although the picture for the TIA structural genes is still very sketchy, similar transcription factors appear to interact with the promoters of the structural genes. More research is necessary to identify central regulators of TIA biosynthetic genes. Overexpression of single biosynthetic genes *Tdc*[77] and *Str* [78] *per se* did not lead to increased alkaloid levels. Only in combination with changes in culture conditions did *Str* overexpression lead to increased alkaloid levels,[78] indicating that other biosynthetic steps besides STR are limiting. Overexpression of a central regulator may overcome all limiting steps.

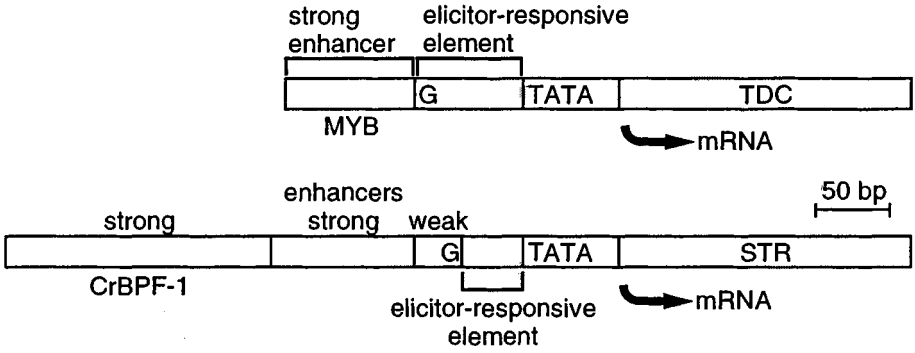

Figure 3. Functional structure of the *Tdc* and *Str* promoters. A G-box sequence is indicated with G.

Conclusions

Experiments with the MYB and MYC genes have demonstrated that flavonoid biosynthesis can be engineered using transcription factors. The MYB and MYC factors are active over species borders and faithfully recognize their orthologous target genes in heterologous species. In this respect the MYB and MYC proteins behave as master regulators of the flavonoid pathway, although maybe not in all plant species to a similar

extent. Studies described in reference 54 demonstrate that ectopic expression of *C1* and *R* constitutes a viable strategy for engineering anthocyanin production in plant cell cultures. Other metabolic pathways await the identification of transcriptional regulators. We have made some progress in isolating transcription factors that interact with promoters of TIA structural genes. It is clear that extensive molecular and reverse genetic studies are required to elucidate the transcriptional control of secondary pathways in plants. Even the current understanding of transcriptional regulation of anthocyanin biosynthesis shows large and fundamental gaps, despite the for the plant field relatively large research effort and the use of integrated (reverse) genetic and molecular approaches. Future research on other secondary pathways, including the TIA pathway, should answer the question whether the paradigm for regulation of phenylpropanoid biosynthesis holds true for other pathways. The first glimpse into TIA regulation indicates that this might be the case, but clearly further studies are needed.

In using central regulators to engineer secondary metabolism, the use of inducible promoters deserves some attention, because constitutive expression of a pleiotropic regulator, or a high constitutive level of certain secondary metabolites may not be compatible with cell viability. In addition, transcriptional regulators may be engineered to make them dominant and independent of the normal control of their activity. This maybe especially important for central regulators that are activated by stress signals. The two approaches may be combined by constructing hybrid transcription factors, that consist of the DNA-binding domain to direct them to their target genes, a strong transactivation domain, and a inducible domain that silences the activation domain in the uninduced state. Such an approach was used to produce anthocyanins in the *Arabidopsis ttg* mutant that lack anthocyanins. Expression of a fusion between the DNA-binding domain of the maize MYC protein *R* and the steroid-binding domain of the rat glucocorticoid receptor restored anthocyanin production in a steroid hormone-dependent manner.[79]

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CHAPTER 7

PLANT COLOUR AND FRAGRANCE

K.M. DAVIES

Introduction

The major pigments responsible for plant colour are the flavonoids, carotenoids, chlorophylls and the taxonomically restricted betalains. This chapter concentrates on flavonoids and carotenoids, as from a metabolic engineering point of view these are the most studied and most amenable to modification of the plant pigment groups. A general background to the biochemistry and molecular biology of their biosynthesis is given, along with the progress and prospects for genetic modification of their production. The betalain biosynthetic pathway, and its possible genetic modification is covered in the chapter of Prof. Zrÿd, and thus is not discussed here.

Flavonoids are water-soluble pigments which often account for pink, red, orange, scarlet, purple, blue, blue-black, and some yellow colours. In addition, they provide 'depth' to most white or cream colours. Carotenoids are lipid-soluble pigments which alone provide yellow, orange, red and purple colours. In many species both carotenoids and flavonoids contribute to the range of available colours, in combination producing orange, yellow, scarlet and brown/black. The betalains produce yellow, orange, red and purple colours in the floral tissues of only a few plant species, being restricted to the Caryophyllales. Chlorophylls have an obvious role in providing the green colour of photosynthetic tissues. In this role they are directly responsible for the colour of some fruits, such as apples, kiwifruit (*Actinidia deliciosa*) and bell peppers (*Capsicum annuum*), and a few cases of green flowers (*e.g. Helleborus*). They also influence colour as background pigments.

As the presence or absence of the coloured flavonoids is visually apparent, and their loss is non-lethal, they are particularly suitable subjects for research, and some of the first studies of genetics were on the anthocyanins.[1] Much early genetics was carried out in petunia (*Petunia hybrida*), snapdragon (*Antirrhinum majus*) and maize (*Zea mays*), and this has been carried through into molecular studies and transgenic experiments with these species. The first plant genes that encoded transcription factors to be isolated were genes regulating flavonoid production in maize (see[2,3,4]), and the first reports of both antisense RNA[5] and cosuppression[6,7] in plants concerned floral pigmentation in petunia. The latter are just two examples of the many reports of successful metabolic engineering of the flavonoid biosynthetic pathway. Until recently the prospects for metabolic engineering of the production of the coloured carotenoids were much poorer than for flavonoids. However, recent years have seen rapid progress in carotenoid molecular biology and the first reports of the engineering of their production in transgenic plants.

R. Verpoorte and A.W. Alfermann (eds), Metabolic engineering of plant secondary metabolism, 127-163.

Much of the research into flavonoid chemistry and biosynthesis has concentrated on flower systems, and indeed, the flavonoids are the most widespread and important of the flower pigments. In fruit, flavonoids and carotenoids are of equal importance, and much early molecular research into carotenoid biosynthesis has used fruits as the systems of study. Recently, arabidopsis (*Arabidopsis thaliana*) has become a prevalent model species for both flavonoid and carotenoid research.

Colour is also a key influence in the choices customers make from products in the marketplace, particularly for ornamental crops. Thus there is a drive to develop new colour phenotypes in commercial species. As genetic modification approaches may allow the introduction of colours outside the range available through traditional breeding, colour modification programmes have been set up by several research organisations and biotechnology companies. These companies have identified cDNA clones for commercially important flavonoid and carotenoid biosynthetic enzymes, and have now released their first novel commercial cultivars. More recently, genes encoding enzymes involved in the generation of fragrance volatiles have been isolated, raising the prospect of also engineering plant scent.

The flavonoids

THE BIOSYNTHESIS OF FLAVONOIDS

The basic flavonoid structure is a 15-carbon (C_{15}) nucleus composed of two aromatic rings (called the A and B rings) joined by a three-carbon unit (which usually forms a third ring called the C-ring; Fig. 1). The various classes of flavonoids are determined by the degree of oxidation of the C-ring, whilst the individual aglycone compounds within each class are determined by the extent of hydroxylation, or other substitution, of the A and B rings. They are generally water-soluble and stored in the vacuole.

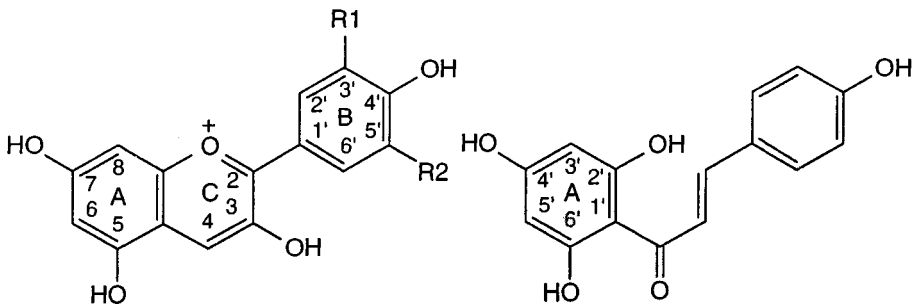

Figure 1. Structures of the main anthocyanidins (left) and a chalcone (right). The numbering of some of the carbons for the anthocyanidins (and the majority of flavonoids) and chalcones, and the lettering of the carbon rings are shown. Substitutions of R1 and R2 determine the various anthocyanidins as; R1 and R2 = H, pelargonidin; R1 = OH and R2 = H, cyanidin; R1 and R2 = OH, delphinidin; R1 = OCH_3 and R2 = H, peonidin; R1 = OCH_3 and R2 = OH, petunidin; R1 and R2 = OCH_3, malvidin.

Flavonoids are synthesized from phenylalanine as part of the larger phenylpropanoid pathway. Figure 2 depicts the phenylpropanoid pathway leading to the formation of various flavonoid types, including aurones, flavones, flavonols, and the simplest anthocyanins. A full description of this large, highly branched pathway is beyond the scope of this review, and only an outline of the specific steps of flavonoid biosynthesis is given here. Several excellent reviews of the biochemistry and genetics of the flavonoid, and general phenylpropanoid pathways have been published recently,[2,3,8,9,10] and the reader is referred to these for further information.

The first dedicated step to flavonoid biosynthesis is the formation of the C_{15} structure by the condensation of one hydroxycinnamic acid-CoA ester unit, usually *p*-coumaroyl-CoA, with three units of malonyl-CoA, a reaction catalyzed by the enzyme chalcone synthase (CHS). In most plants, the product of CHS is the 2',4',6',4-tetrahydroxylated naringenin chalcone. This is the substrate for the formation of most of the common flavonoid types, including anthocyanins and flavonols. It is also the substrate for many other derived chalcones. Narigenin chalcone, and its derived chalcones, are collectively known as 6'-hydroxychalcones. In a few species, primarily in the Leguminosae, chalcones without a hydroxyl at the 6' position (6'-deoxychalcones) are produced. These are substrates for the biosynthesis of the 5-deoxyflavonoids, which are generally involved in plant-microbe interactions.[11,12] The 6'-deoxychalcones are formed when a second enzyme, chalcone reductase (CHR), coacts with CHS. CHR is thought to reduce the enzyme-bound polyketide intermediate of the chalcone synthase reaction.[13]

Chalcones, yellow themselves, may also be converted to the brighter yellow aurones. The reactions involved in aurone biosynthesis have not been characterised, although enzyme preparations able to convert chalcones into the respective aurones have been studied.[14] More commonly, chalcones are converted to flavanones. This isomerisation reaction may occur spontaneously, but is generally catalyzed by the enzyme chalcone isomerase (CHI). *In vitro* and *in vivo* evidence suggests that CHI displays specificity with regard to the possible 6'-hydroxychalcone or 6'-deoxychalcone substrates.

Flavanones are converted to dihydroflavonols by flavanone 3-hydroxylase (F3H). The flavanones and dihydroflavonols are also substrates for flavone and flavonol formation, respectively, through the action of flavone synthase (FNS) and flavonol synthase (FLS). While occasionally these compounds are directly responsible for the perceived colour, they more commonly exert their influence through formation of molecular complexes with anthocyanins, a process called copigmentation.[15] Reduction of dihydroflavonols at the 4 position, catalyzed by dihydroflavonol 4-reductase (DFR), yields the corresponding leucoanthocyanidins. For most plants investigated DFR will reduce the three major dihydroflavonols types that occur. However, in some species strong substrate preference is shown (see Section 2.4.4).

Leucoanthocyanidins are the direct precursors of anthocyanidins and generally do not accumulate in plant cells. The enzyme reactions involved in the conversion of leucoanthocyanidins to anthocyanidins have not been clearly defined. The anthocyanidin synthase (ANS), a 2-oxoglutarate-dependent dioxygenase, does act at this step. It is not clear whether a second activity is required for anthocyanidin formation. Biochemical evidence suggests both a hydroxylase and dehydratase may be involved.[16] However, no appropriate cDNAs have been identified from differential screening approaches.[17,18]

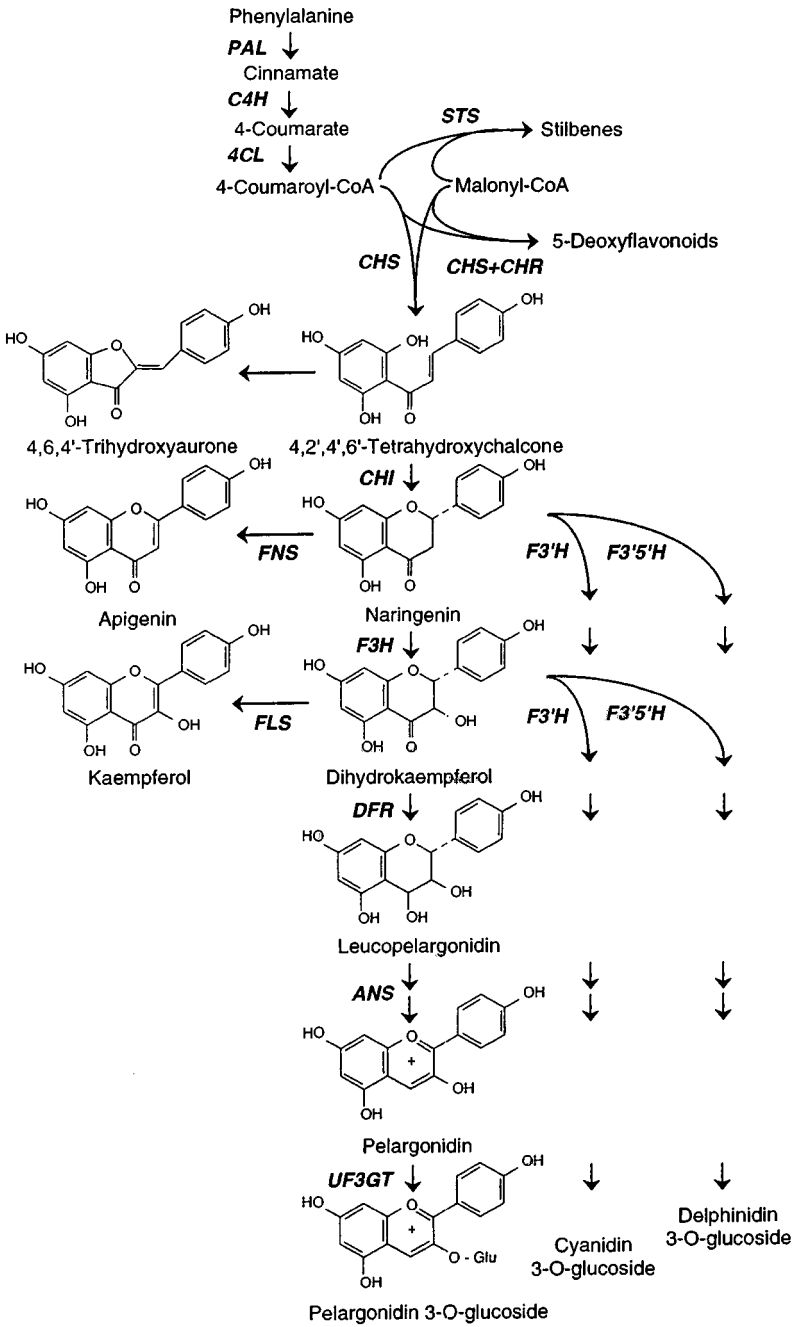

Figure 2. Schematic representation of a section of the phenylpropanoid biosynthetic pathway leading to the production of various flavonoid types, including the anthocyanins, anthocyanidins, flavonols, flavones, aurones, stilbenes and 5-deoxyflavonoids. Enzyme abbreviations are as given in the text.

Clarification of this question may come from transgenic approaches introducing the biosynthetic genes into acyanic backgrounds.[19] The product of the *Incolorata*1 loci from antirrhinum may also act at this biosynthetic step.[2]

Anthocyanidins are unstable under normal physiological conditions and do not accumulate. They are normally stabilised to form the corresponding anthocyanins by the addition of a sugar residue(s), usually at the 3 and/or 5 position. These reactions are carried out by the UDP-glucose: flavonoid 3-*O*-glucosyltransferase (UF3GT) and UDP-glucose: flavonoid 5-*O*-glucosyltransferase (UF5GT), respectively.

Many flavonoids, including anthocyanins, are located in the vacuole. The mechanism by which they are transferred has recently been defined.[20] A glutathione-S-transferase, encoded by the *Bronze2* gene in maize, transfers a glutathione residue to the flavonoid to form a glutathione-S-conjugate. The modified flavonoid may then be transported into the vacuole by a glutathione S-conjugate pump, with the glutathione being subsequently removed.[21]

The three major anthocyanidin types, pelargonidin, cyanidin and delphinidin differ only in the extent of B-ring hydroxylation, being 4', 3', 4' and 3', 4', 5' hydroxylated respectively (Fig. 1). The 4'-hydroxyl group is incorporated during formation of the flavonoid precursor 4-coumarate by the cinnamate 4-hydroxylase (C4H), and is common to most flavonoids. The addition of the 3' and 5' hydroxyl groups is generally catalyzed by the flavonoid 3'-hydroxylase (F3'H) and flavonoid 3', 5'-hydroxylase (F3', 5'H). Usually, the presence or absence of the different anthocyanidin types can be accounted for by the activity of these two enzymes. It is also possible that the 3'- and 3', 5'-hydroxyls could be introduced by the use of caffeoyl-CoA or other cinnamic acids by CHS. However, except for a few notable exceptions, this route is probably of minor, or no importance.[16]

From the basic flavonoid structures an enormous range of derivatives can be formed through glycosylation, acylation, methylation and even sulphation. For example, methylation of the B-ring hydroxyls of the anthocyanins leads to the common derivatives, peonidin, petunidin and malvidin glycosides (Fig. 1). Enzymes involved in secondary modification reactions have been characterised from several species,[9] and many related genetic loci are known.[8] Besides the common flavonoids, a wide range of less common flavonoid derivatives are known that vary from the typical substitution pattern of the A- and C-rings, including the 6-hydroxyanthocyanins, 6- and 8-hydroxyflavonols and 3-deoxyanthocyanins.[9,22]

Genomic and/or cDNA clones are available for all of the enzymes carrying out the early steps of phenylpropanoid biosynthesis: phenylalanine ammonia lyase (PAL), cinnamate 4-hydroxylase (C4H) and 4-coumarate:coenzyme A ligase (4CL). In addition, they have also been obtained for most of the major biosynthetic enzymes that form the common flavonoid types, including; CHS, CHR, CHI, F3H, DFR, FLS, ANS, UF3GT, F3'H and F3', 5'H (see[8,10,22] for reviews). Also available are cDNAs for some of the flavonoid modification enzymes, including the UDP rhamnose: anthocyanidin 3-glucoside rhamnosyltransferase (3RT),[23,17] anthocyanin 3', 5'-*O*-methyltransferase (A3', 5'MT),[24] a range of anthocyanin acyltransferases[25,26] and a cDNA with UF3,5GT activity.[27] Thus most of the genes required for genetic modification of both flavonoid amount and type are available. Notable enzymatic steps for which no cDNAs have

been published are for FNS and many of the enzymes which produce the less common flavonoid types, such as aurones and those with differing A- or C-ring hydroxylation. Little is known about many of these activities, making cDNA identification difficult. However, a cDNA encoding a flavanone 2-hydroxylase possibly involved in flavone biosynthesis, has been isolated.[28] Also, recessive alleles for the FNS locus of antirrhinum (*Daphne*) are known,[29] which may aid in cloning of a FNS cDNA by differential approaches. A number of cDNAs have also been isolated which encode biosynthetic activities modifying flavonoids involved in other plant processes besides colour.[12,30,31]

FLAVONOIDS AS PIGMENTS

Of the common flavonoids only chalcones and anthocyanins have significant colour, and of these anthocyanins are by far the most common and important pigments. Only three basic anthocyanin groups are commonly found, derivatives of pelargonidin, cyanidin or delphinidin. In general, pelargonidin-derived pigments produce orange, pink or red colours; cyanidin-derived pigments produce red or mauve colours; and delphinidin-derived pigments produce purple, blue, or blue-black colours. Thus, these three anthocyanin types can produce a wide range of colours. Several factors are important in determining the final colour produced, the most important ones being the extent of oxygenation of the anthocyanin, its molecular environment and the pH of the vacuole. Surprisingly, when placed *in vitro* under conditions similar to those commonly found in vacuoles, the anthocyanins alone do not exist in a stable coloured form. To achieve stability, hydration of the anthocyanin must be prevented, and this is usually achieved through molecular interactions. Anthocyanin molecules with much secondary modification may be able to complex around the anthocyanin core, preventing hydration. Alternatively, anthocyanins may complex with other anthocyanins or other flavonoids, the latter being termed 'copigments'. Further information on the role of copigmentation and the various forms that anthocyanins may exist in can be found in previous reviews.[15,32,33]

Genetic engineering of flavonoid biosynthesis was first reported over ten years ago, with the production of novel anthocyanin types in petunia through the introduction of a gene from maize.[34] Since then successful transgenic experiments targeting flavonoid biosynthesis have been reported on numerous occasions and in a wide range of species. These fall into three main groups, which form the next sections of this review: reduction of the activity of the biosynthetic genes; introduction of novel enzyme activities; and manipulation of the regulation of the pathway.

REDUCING BIOSYNTHETIC GENE ACTIVITY

A reduction in the production of all flavonoids, or only specific types, can be achieved by three approaches: individual biosynthetic steps can be targeted using antisense RNA or sense-suppression to reduce gene activity; substrate can be diverted away from sections of the pathway by introducing or increasing competing activities; and regulatory genes can be used to repress biosynthetic gene expression. All three approaches have been proven effective in transgenic plants. The targeting of individual

phenylpropanoid biosynthetic steps by antisense RNA or sense-suppression approaches is covered in this section. In antisense RNA, the coding sequence for the target gene is placed in the reverse physical orientation following a gene promoter, so that an antisense RNA copy is produced (reviewed in[35,36]). Sense-suppression refers to the discovery that the expression of additional sense copies of the target gene, usually under a strong promoter such as the Cauliflower Mosaic Virus (CaMV) 35S, can suppress the expression of the endogenous and/or transgene (see[35,36,37,38]). When both the endogenous and transgene are suppressed, this has been referred to as cosuppression. The use of sense transgenes for down-regulation of gene expression has been patented, and developed as the Transwitch™ technology.

The mechanisms for both antisense and sense-suppression have not yet been defined. While anthocyanin biosynthesis has provided an excellent model system for investigating possible mechanisms, a full review of this area is beyond the scope of this chapter. Thus, a brief outline with reference to some recent reviews is all that is given. Both antisense and sense-suppression represent sequence specific mechanisms for suppressing gene expression, and both operate post-transcriptionally.[39,40] However, it is still not clear whether they share a common mechanism. By analysis of large numbers of petunia CHS transgenics, Jorgensen *et al.*[41] showed that sense constructs produce a number of types of floral patterning not seen from antisense constructs, which suggests at least some aspects of the processes differ. There is evidence to suggest antisense involves formation of RNA duplexes and a specific degradation pathway.[40] A number of mechanisms have been suggested for sense-suppression[39,41,42,43,44,45], but most suggest the involvement of an antisense RNA intermediate.[39,42] How the suppression state is triggered is also unknown. It is possible that when transcript levels exceed a certain 'threshold' a specific transcript degradation mechanism is engaged.[37,38,41,43] Alternatively, antisense RNAs may be generated directly by plant promoters adjacent to the transgene, or from aberrant transgene transcripts.[39,40] In addition to these mechanisms, transgenes which share promoter homology may also become silenced through a process involving methylation.[40,46,47,48,49,50]

Enzymes of early phenylpropanoid biosynthesis

Several antisense and sense-suppression studies have targeted the activity of the enzymes carrying out the early steps of phenylpropanoid biosynthesis, from phenylalanine to *p*-coumaroyl-CoA. While production of substrates for flavonoid biosynthesis and thus plant colour may be affected in these plants, flavonoids have not been the main focus of the studies. Rather they have mostly examined other phenylpropanoid compounds, in particular the substrates of lignin formation. Transgenics have been produced with reduced levels of gene and enzyme activity for all three of the early biosynthetic steps; PAL,[51,52,53] C4H[53] and 4CL.[54,55] In addition to a reduction in general phenylpropanoid levels, of lignin in particular, pleotropic effects on aspects such as plant defense[51,56] have been observed.

Chalcone synthase and dihydroflavonol reductase

Most experiments to date have targeted CHS as the first committed step in flavonoid biosynthesis, or DFR - the first step specific to anthocyanin biosynthesis. The first use

of an antisense RNA construct to reduce gene expression was for CHS in petunia.[5] Introduction of an antisense transgene into a purple-flowered line led to a variety of novel flower phenotypes, from completely white to a range of pigment patterns. A specific reduction in transcript levels for CHS occurred, with no change in levels for DFR or CHI. However, the transcription rate for CHS was unaffected in these plants, indicating a post-transcriptional mechanism. The same antisense approach has subsequently been used to down-regulate CHS, and thus anthocyanin biosynthesis, in other model or commercial species, including gerbera (*Gerbera hybrida*),[57,58] chrysanthemum (*Dendranthema xgrandiflorum*),[59,60] rose (*Rosa hybrida*)[61] and lisianthus (*Eustoma grandiflorum*).[62,63]

The first demonstration of the cosuppression effect was for CHS and DFR in petunia.[6,7] Introduction of petunia CHS or DFR cDNAs driven by the 35S CaMV promoter back into petunia, resulted in transformants with greatly reduced flower pigmentation. The extent of reduction varied from completely white flowers (for CHS), to a range of pigmentation patterns. In the white petal sections of CHS transformants, transcript levels for both the endogenous CHS and the CHS transgene were greatly reduced. However, the transcription rate for CHS was unaffected, as found with other examples of cosuppression,[39,40] suggesting a post-transcriptional mechanism.

Sense CHS constructs have since been used to obtain a range of lines with reduced flower colour intensity from a deep-red coloured rose, cv. "Royalty",[59] and white lines from cyanic-flowered chrysnthemum[59,60] and *Torenia hybrida*[64] cultivars. DFR has also been targeted by antisense or sense-suppression in a range of species in addition to petunia, including gerbera[65] and *Torenia*.[64] In all three species, plants with much reduced levels of anthocyanin biosynthesis in the flowers were obtained, but no white lines. The partial reduction of pigmentation observed with DFR, and some CHS, suppression experiments may represent incomplete reduction of the target gene activity, perhaps because only some members of the CHS or DFR gene families are inhibited.

Ordered and erratic floral pigmentation patterns have been obtained from sense and antisense experiments, for both CHS and DFR experiments in petunia[5,6,7] and CHS antisense in lisianthus.[62,63] In both petunia and lisianthus, many of the transgenic patterns resemble closely those obtained by traditional breeding.[40,63] No floral patterning was observed from antisense or cosuppression experiments with chrysanthemum,[60] rose[59,61] or gerbera,[57] all species that traditionally lack patterned varieties. In lisianthus and petunia, the loss of pigmentation occurs in both an ordered manner, associated with features such as the petal veins, junctions and edges, and also in an apparently random manner. Considerable variation in patterning may even be observed among flowers on a single plant.[41,62] The type of pattern prevalent may also change during development,[66] and in response to the light environment or application of gibberellin.[67] Several distinct classes of pigmentation phenotype have been defined for CHS cosuppression experiments in petunia, and by the study of a large population of CaMV 35S-CHS petunia transgenics, a relationship was established between the occurrence of a particular type of pattern and the structure of the transgene locus or loci.[41] Single copy transgenes resulted in loss of pigmentation around the petal junctions; dispersed multiple transgenes produced patterns based both on the petal junctions and veins; and inverted repeats of the transgene were able to generate complex patterns, including

disordered (erratic) pigmentation. The somatic instability of some cosuppression phenotypes is thought to be related to the influence of developmental and morphological parameters, acting through abundance levels of transcription factors for the endogenous gene.[37,38,41] These morphological influences may act non-cell autonomously, both during formation of the floral cells in the meristem, and also in mature structures such as the corolla. Indeed, it has recently been shown that the cosuppression state can be triggered by grafting plant parts from silenced stocks to non-silenced scions.[44]

Chalcone isomerase

Chalcones provide yellow pigmentation in the flowers of several ornamental species (reviewed in[15]) and in tomato fruit of some lines.[68] In yellow-flowered varieties of carnation (*Dianthus caryophyllus*),[69] *Callistephus chinensis*,[70] and cyclamen (*Cyclamen persicum*),[71] chalcone accumulation occurs as a result of loss of CHI activity associated with recessive mutations. Thus inhibiting CHI activity may provide a route for producing yellow and orange colours in species that lack them. However, attempts to inhibit the activity of CHI in petunia by antisense or sense-suppression approaches have proved unsuccessful.[35,40] More than sixty petunia transformants were generated containing antisense CHI transgenes, without any phenotype observed. This lack of suppression in petunia may be due to the genomic location of the *chi*A gene, as a sense CHI transgene introduced into an antisense CHI line was successfully suppressed.[40]

One of the problems associated with enhancing chalcone accumulation by reducing CHI activity may be that even if CHI activity can be inhibited, chalcones may not accumulate. Chalcones with unsubstituted 2'- and 6'-hydroxyl groups (see Fig. 1) spontaneously isomerise to flavanones under *in vitro* conditions similar to those thought to occur in the vacuole,[72] and this may occur *in vivo* since carnation lines with no detectable CHI activity still synthesize some anthocyanins.[69] Chalcone stability is increased if the 2'-hydroxyl is substituted, preventing isomerisation. Thus, chalcones glycosylated at the 2' position generate yellow flower colours in carnation, *Helichrysum, Paeonia* and *Callistephus*.[73,74,75] If chalcones are to be useful for generating yellow flower colours, the presence of the 2'-glycosylation activity may be required. Unfortunately, if this activity is not present in the target species, it cannot currently be introduced, as no cDNA clones for the corresponding enzyme have been reported. An alternate route for modifying chalcone biosynthesis is discussed in 2.4.3.

Flavonol synthase

Flavonols are generally colourless flavonoids found in many plant tissues, being especially abundant in flowers and fruit. They provide 'depth' to acyanic flowers, and as common copigments, they have an important influence on the colour resulting from accompanying anthocyanin pigments. In short, the presence of flavonols may reduce the hydration of the anthocyanin molecule, causing a shift towards bluer colours.[15,32,33] Conversely, the loss of flavonol production may cause a shift towards orange-red colours.[76,77] In some species they may also be required for protection of the plant from damage by UV-B light[78] and for male fertility.[79,80]

Antisense expression of the petunia FLS cDNA in transgenics of the same species led to a marked reduction in flavonol levels and a shift in flower colour towards

red, thought to be a result of altering the copigmentation.[81] Introduction of the same antisense FLS construct into tobacco increased the level of anthocyanins in the flowers, indicating that in this species there is a competition for substrate supply between anthocyanin and flavonol production.[81] Similar results have been obtained with antisense FLS constructs in lisianthus.[63] A more marked change was obtained from the introduction of an antisense petunia FLS construct into a acyanic-flowered petunia line, *anthocyanin*2. Flavonol levels were reduced and anthocyanin production occurred in the petals, resulting in pink flower colours (the authors' and co-workers' unpublished data). Thus, reduction of the synthesis of side-branches of the flavonoid biosynthetic pathway can affect copigmentation and may also provide a means by which anthocyanin levels in acyanic or weakly pigmented flowers can be increased.

Flavonoid B-ring hydroxylases

Many species only produce anthocyanins derived from one or two of the three major anthocyanidin types, pelargonidin, cyanidin and delphinidin. Except in Solanaceous species, this is most probably due to the presence or absence of the F3'H and F3', 5'H activities. These activities tend to be dominant, so that naringenin and/or dihydrokaempferol substrate is completely converted to the 3', 4'-hydroxylated or 3', 4', 5'-hydroxylated form before it becomes committed to the formation of pelargonidin. Thus, in varieties in which the F3'H and/or F3', 5'H activities are lacking, pelargonidin-derived pigments may accumulate rather than the usual cyanidin or delphinidin-derived anthocyanins.[8] In some species, such as chrysanthemum,[82] cyclamen,[83] azalea (*Rhododendron simsii*)[84] and apple,[85] however, such lines are either absent or rare. The availability of the F3'H and F3', 5'H cDNA clones allows the possibility of developing mutant lines in species that lack them by sense or antisense inhibition of the corresponding gene activities. *In vivo* chemical inhibition experiments have demonstrated the applicability of this approach to chrysanthemum.[82] When tetcyclacis was introduced into intact chrysanthemum flower buds using wick feeding, pelargonidin pigments were formed.

UDP rhamnose: anthocyanidin 3-glucoside rhamnosyltransferase

All anthocyanins have at least one sugar residue attached, usually at the C-3 position, and commonly have two or more. The sugar residues themselves may then be modified by the addition of additional sugars or other moieties. In petunia, the anthocyanins may be glycosylated with glucose and rhamnose at the C-3, and glucose at the C-5 positions. They may also be methylated at the 3' and 5' positions. Brugliera *et al.*[23] and Kroon *et al.*[17] inhibited the activity of the 3RT gene in petunia by introducing antisense RNA constructs. A change in colour shade was observed in some of the transgenic lines, including in some cases patterning of pigmentation.[17] The change in colour reflected a shift in the petals to the production of less methylated anthocyanins, based on delphinidin rather than petunidin[17] or malvidin.[23] Presumably, the anthocyanin 3'5'-*O*-methyltransferase, which acts after the addition of the sugar and acyl residues, was unable to act on the simpler anthocyanin structure resulting from the prevention of rhamnosylation.

INTRODUCING NOVEL ACTIVITIES AFFECTING FLAVONOID BIOSYNTHESIS

By introducing novel enzyme activities into target species, using either cDNA or genomic clones, both the type and levels of flavonoids produced have been changed in transgenic plants.

Tryptophan decarboxylase

Altering early steps in the larger shikimate pathway can reduce flux into the phenylpropanoid branch. Overexpressing tryptophan decarboxylase from *Catharanthus roseus* in potato reduced the levels of tryptophan and phenylalanine in the transgenics by 40-50%.[86] The reduction in phenylalanine in turn led to reduced levels of both soluble and wall bound phenylpropanoids, and also increased susceptibility to fungal infection. The reduction in phenylalanine represents an elegant example of pathway regulation *via* biochemical intermediates. Both phenylalanine and tryptophan come from the common substrate chorismate. Normally, the presence of tryptophan limits its own synthesis, through feedback inhibition of anthranilate synthase, and promotes the production of phenylalanine, by activating chorismate mutase. When the flow through tryptophan is increased by raising the level of tryptophan decarboxylase, this feedback regulation promotes more tryptophan production at the expense of phenylalanine.

Stilbene synthase

Stilbene synthase is directly responsible for the production of resveratol type phytoalexins in species such as grape and peanut. The enzyme from grape uses the same substrates as CHS, malonyl-CoA and 4-coumaroyl CoA. Thus, if both CHS and STS are active in the same tissue they will compete for substrate. Fischer *et al.* introduced a cDNA encoding the grape STS under the control of the CaMV 35S promoter into tobacco. Flowers of the transgenics were near white rather than the usual dark pink, due to reduced anthocyanin levels. They were also male sterile, presumably due to reduced flavonol levels.[79,80] Thus, the introduction of STS offers a route for controlled reduction of flavonoid biosynthesis.

Chalcone reductase

Chalcones provide yellow colours to a range of flowers and also some fruit. However, as mentioned in section 2.3.3, the common 6'-hydroxychalcones with unsubstituted 2'- and 6'-hydroxyls may spontaneously isomerize to flavanones, making it difficult to engineer their accumulation. The 6'-deoxychalcones are produced in some legumes, in which they are substrates for 5-deoxyflavonoid biosynthesis. The isomerisation rate of 6'-deoxychalcones is much lower than that of the comparable 6'-hydroxychalcones,[72] perhaps due to the association of the 2'-hydroxyl with the carbonyl group of the carbon link,[88] and aglycones, and glycosides of 6'-deoxychalcones accumulate in some yellow-flowered varieties of *Cosmos, Dahlia*, Coreopsis and Bidens.[74,75] Therefore, modification of plants to produce 6'-deoxychalcones could provide a way of producing stable chalcones to give yellow colours. Welle *et al.*[89] and Ballance and Dixon[90] have reported the isolation of cDNA clones for CHR, making it is possible to introduce this activity into species that lack it.

In petunia, only the 6'-hydroxychalcones are synthesized, and except in the pollen of some genotypes, they are ephemeral intermediates in flavonoid metabolism. Davies *et al.*[91] introduced a CHR cDNA from alfalfa (*Medicago sativa*), under the control of the CaMV 35S promoter, into acyanic- or cyanic-flowered lines of petunia, resulting in a change in flower colour from either white to pale yellow or deep purple to pale purple. Lines were generated that accumulated up to 60% of their petal flavonoids as a range of 6'-deoxychalcones, including a novel plant chalcone, butein 4-*O*-glucoside. The results confirm *in vitro* observations,[9] that the petunia CHI is unable to use 6'-deoxychalcones as a substrate, so that 6'-deoxychalcones accumulate in petunia. The CHR enzyme provides a method to redirect the flavonoid pathway into chalcone production, in order to modify colour or to reduce the biosynthesis of other flavonoid types.

Dihydroflavonol 4-reductase

DFR preparations from plants of the Solanaceous genera, *Petunia*, *Lycopersicon* and *Nicotiana*, cannot use dihydrokaempferol,[9,92] thus preventing pelargonidin production. In the first case of engineering of flower colour, Meyer *et al.*[34] overcame this biosynthetic block in petunia by introducing the maize *A1* cDNA encoding DFR. The *A1* cDNA was placed under the control of the CaMV 35S promoter and introduced into a petunia line (RLO1) that accumulated dihydrokaempferol. The flowers of the resultant transgenics produced pelargonidin-derived pigments. Subsequent crosses of the transgenic lines resulted in a range of F2 lines that had flowers containing either pelargonidin-derived pigments, cyanidin-derived pigments, or a mixture of both.[93,94,95] Some lines had orange flower colours novel to petunia (see[93] for colour photographs of the F2). The gerbera[65] and rose[96] DFRs have also been introduced into dihydrokaempferol accumulating petunia lines, with similar results with regard to novel pelargonidin production in petals and pollen.

In some circumstances DFR may be a rate-limiting step for anthocyanin biosynthesis. Flavonols are often produced in tissues also producing anthocyanins, and depending on the timing of production and substrate preferences, DFR will compete with FLS for substrate. While anthocyanins accumulate in some vegetative tissues of *Forsythia*, such as the stems, petioles, leaf veins and sepals, only flavonol glycosides are found in petals. In an attempt to generate anthocyanins in the petals, Rosati *et al.*[19] introduced the antirrhinum DFR cDNA, under the control of the CaMV 35S promoter, into *Forsythia* x *intermedia*. Transgenic plants had increased levels of anthocyanins in tissues which already produced some, demonstrating that DFR is rate limiting for anthocyanin biosynthesis in these tissue. However, no anthocyanins were produced in petals, indicating a further biosynthetic block after DFR. Current research is targeting the introduction of transgenes for the next biosynthetic enzyme, ANS. Results from other systems suggest that substrate competition between DFR and FLS may be a common controlling factor in flavonoid biosynthesis. The introduction of a CaMV 35S DFR transgene into an acyanic-flowered line of petunia enabled substrate to be redirected into anthocyanin synthesis, resulting in production of pink flowers (the authors' and co-workers' unpublished results). Reduction of FLS activity in petunia and tobacco also led to increased anthocyanin production (see Section 2.3.4).

Flavonoid 3', 5'-hydroxylase

There is an excellent correlation between blue flower colour and the presence of delphinidin-derived anthocyanins. Several of the most popular ornamental species, particularly within the Asteraceae, do not produce delphinidin and lack blue colours. Thus, an obvious approach for biotechnology is to introduce the F3', 5'H into these species. Such experiments are in progress for rose, carnation and chrysanthemum,[36,97] and indeed, transgenic carnations containing delphinidin-derived anthocyanins are now commercially available in Australia. The presence of delphinidin-derived anthocyanins, however, does not necessarily result in a blue flower colour, and the current transgenic carnations are violet rather than blue in colour. Similarly, tulip, *Impatiens*, cyclamen, lisianthus and *Pelargonium* all produce delphinidin derivatives yet lack (true) blue flower colours. Further, in a few examples, blue flower colours are known to be derived from other pigments besides delphinidin, *e.g.* the main pigment in the blue cornflower, *Centaurea cyanus*, is an acylated cyanidin 3,5—diglycoside, and in blue-flowered Ipomoea is peonidin with six molecules of glucose and three molecules of caffeic acid attached.[33]

Chemical studies on several blue-flowering species have suggested that other factors besides the presence of delphinidin-derived anthocyanins are usually involved in producing blue. The major factors are vacuolar pH, intramolecular interactions and copigmentation, all of which are thought to affect the hydration of the anthocyanin molecule, which in turn affects the form the anthocyanin molecule assumes. The efficiency of intramolecular interaction or copigmentation in generating blue colour from the anthocyanin depends on the type of copigments present and the secondary modifications of both the anthocyanins and copigments. For example, the flavone C-glycosides seem particularly effective copigments for generating blue colours.[98,99] Excellent reviews of the effects of these various chemical and structural factors on the colours resulting from anthocyanins have been published.[32,33]

From these studies it may be concluded that the generation of a blue flower requires not only the production of a suitable anthocyanin (usually delphinidin-derived) but also the presence of an appropriate copigment and/or extensive modifications to the basic anthocyanin, in particular aromatic acylation. Further, a suitable vacuolar pH is required, and indeed, it is possible to obtain good blue colours in the absence of some of these other factors if the pH is sufficiently high. Successful generation of novel blue colours by introduction of the F3', 5'H will depend on either the selection of a recipient cultivar with a suitable chemical background, or subsequent further genetic modification of the secondary factors. At present no reports on the manipulation of these factors have been published. However, significant progress has been made recently, with the isolation of cDNAs encoding flavonoid acyltransferases,[26] and the identification of a cDNA which may be involved in flavone biosynthesis.[28] Technology for modifying vacuolar pH is also a current target. To date, the genes isolated that modify floral pH also regulate anthocyanin biosynthesis.[4,24,100,101]

Secondary modification activities

Methylation is a common modification of anthocyanins, and has a reddening effect. The isolation of a cDNA clone for an anthocyanin *O*-methyltransferase from petunia

has been reported,[24] but no transgenic results for this cDNA have been published. Methyltransferase genes could potentially be used to introduce novel activities into other species, however, the substrate usage of the enzymes may be highly specific.[9]

Genomic or cDNA clones have been isolated corresponding to various flavonoid glycosylation activities, including UF3GT, UF3,5GT and 3RT (see Sections 2.1 and 2.3.6). These might be used to increase, or introduce *de novo*, these activities into other species. This has been demonstrated successfully in lisianthus.[102] Flowers of lisianthus predominantly accumulate anthocyanins and flavonols that are 3-galactosylated, although a small amount of 3-glucosylated flavonols also occur.[103,104] Further, 50% of the galactosylated flavonols are acylated at the 4-hydroxy position of the galactose. Novel anthocyanins glucosylated at the C-3 position were produced in petals of transgenic plants expressing the antirrhinum UF3GT cDNA under the control of the CaMV 35S promoter. In addition, a decrease in the amount of acylated flavonols occurred, which correlated with an increase in the amount of glucosylated flavonols. Although copigmentation occurs in lisianthus between the anthocyanins and the flavonols,[103] no change in flower colour resulted. In general, it is the position the sugar residue occupies on the flavonoid, rather than the nature of the sugar, that has a more direct effect on the perceived flower colour. However, the nature of the sugar can impact indirectly on colour by affecting subsequent modifications such as methylation, acylation and further glycosylation, as illustrated in the petunia 3RT antisense transgenics (Section 2.3.6).

Other biosynthetic activities

There are uncommon types of flavonoid, differing in the oxygenation of the A- or C-rings, that can result in strong red or orange colours. However, the enzyme activities involved in the production of these compounds are not well characterized. The 3-deoxyanthocyanins provide orange-red or orange-yellow colours in flowers of some species of the Gesneriaceae, including *Sinningia cardinalis, Gesneria cuneifolia, Kohleria eriantha* and *Columena hybrida*.[105] The related phlobaphenes are also produced in maize pericarp, in which the subset of genes required for their synthesis is regulated by the P locus.[106,107] The 3-deoxyflavonoid precursors are formed by the flavanone 4-reductase through a reaction closely related to that of DFR.[108,109] Indeed, in some species, DFR may carry out the reduction of both dihydroflavonols and flavanones. Certainly in maize the same DFR gene is involved in both anthocyanin and phlobaphene production.[107] The introduction of the flavanone 4-reductase into other species might enable 3-deoxyanthocyanins to be produced, although the substrate specificities of the ANS and glycosyltransferases of the host species would need to be considered.

The presence of 6-hydroxypelargonidin produces striking tangerine colours in *Impatiens aurantiaca*.[110] The analogous 6-hydroxycyanidin and 6-hydroxydelphinidin have been found in red and pink-purple flowers, respectively, of *Alstromeria*.[111] The aurones, in some cases in combination with the related chalcone, are responsible for bright yellow flower colours in a few species. They occur in particular in the Asteraceae (*e.g. Cosmos, Coreopsis, Zinnia*), Scrophulariaceae (*Linaria* and *Antirrhinum*) and Plumbaginaceae (*Limonium*).[74,75] The ability to confer aurone or 6-hydroxyanthocyanin biosynthesis in novel species would be of considerable commercial interest. At present,

however, little is known about the biochemistry and genetics of aurone biosynthesis, nor have the enzyme(s) involved in the 6-hydroxylation reaction been characterised. Thus approaches to gene cloning using homology to known flavonoid enzymes would be difficult. The biosynthesis of aurones and the rarer anthocyanidin types is a research area that warrants more attention.

Many members of the Bromeliaceae produce red or orange-red floral colours, despite the predominance of cyanidin as the major anthocyanidin. This may be due to the occurrence of unusual cyanidin glycoside types in these species, which carry a glucose at the 3' position.[112] Four different cyanidin derivatives with 3'-glucosylation were identified, all bright scarlet in colour. No studies have been published on the presumed 3'-glycosyltransferase, but it may well share homology with other flavonoid glycosyltransferases already isolated.

Over 1500 different flavonol and flavone derivatives have been reported,[113] some of which are highly coloured. In particular, quercetin 7-glycosides, quercetin 4'-glucosides, 3'-methoxy-quercetin glycosides, 3',5'-dimethoxy-myricetin glycosides, and 6- or 8-hydroxy derivatives of kaempferol or quercetin are known to provide yellow pigments in a range of ornamentals.[114] Unfortunately, although the isolation of cDNA clones has been reported for the FLS and some glucosyltransferases, none are available for the enzymes involved in the biosynthesis of the more complex derivatives. However, several O-methyltransferases acting on a variety of different flavonoids have been isolated,[24,30] and these may provide an avenue to cloning further genes by sequence similarity.

REGULATION OF FLAVONOID BIOSYNTHESIS

Activation

The production of anthocyanins in a particular tissue is determined by the activity of the biosynthetic genes, which in turn is controlled by the expression of specific regulatory genes. Progress on characterisation of such genes has been covered in some excellent previous reviews.[2,3,4,115] In this chapter, except for a brief review of isolated genes, only their potential use for the engineering of flavonoid biosynthesis with regard to plant colour is considered.

Two types of regulatory products directly regulating flavonoid biosynthesis have been studied in detail. These are classified according to their homology to transcriptional factors from yeast and animals, as either Myb type (having N-terminal basic domains and C-terminal acidic domains homologous to Myb oncoproteins) or Myc/bHLH type (having an acidic N-terminal region and a basic helix-loop-helix domain characteristic of Myc oncoproteins). The structural aspects of Myb and bHLH, and their possible interactions, are covered in details in other publications[116,117] and reviews.[4] Genes encoding Myb and bHLH type factors were first identified in maize, in which they are encoded by the *C1/Pl* and *R/B* gene families respectively (Table 1). Subsequently, flavonoid regulatory genes have been isolated from other species, most of which also encode proteins with homology to either Myb or bHLH proteins. In petunia, these include *Anthocyanin* (*An*) 2 and *An4*, which encode Myb type proteins, and *An1* and *Jaf13*, which encode bHLH proteins. Loss of function mutations for *an2*, *an4* or *an1* results in reduction of pigmentation of flower petals or pollen. In *Antirrhinum*, *Rosea*

encodes a Myb type protein[118] and *Delila* encodes a bHLH type protein.[119] From studies of mutant lines, both genes are known to regulate anthocyanin biosynthesis in the flower.[2,3,118] Similar genes which may also regulate flavonoid biosynthesis have now been reported for several species (Table 1), and a new type of plant regulatory factor, encoded by the *An11* gene of petunia, has recently been cloned.[120] In addition to these cloned genes, a number of other loci have been identified from mutation studies that are also thought to encode regulatory factors (Table 1).

Table 1. Genetic loci and isolated genes encoding factors that regulate flavonoid biosynthesis.

Species	Gene	Cloned[a]	Product type	Reference[b]
Antirrhinum	*Delila*	yes	bHLH	119
	Rosea	yes	Myb	118
	Eluta	no	-	2, 3
	Myb305	yes	Myb	139, 193
	Myb340	yes	Myb	193
Petunia	*An1*	yes	bH*LH*	4
	An2	yes	Myb	4, 101
	An4	yes	Myb	4
	An11	yes	WD-40[c]	4, 120
	jaf13	yes	bHLH	4, 101
	mybPh3	yes	Myb	194
Maize	*R, S, Sn, Lc*	yes	bHLH	195, 196, 197, 198
	B, C1, Pl	yes	Myb	199, 200, 201, 202
	Vp1	yes	-	128, 129
	P	yes	Myb	106
	In1	yes	bHLH	133
Arabidopsis	*Ttg*	no	-	130
	Tt8	no	-	130
	icx1	no	-	131
	Banyuls	no	-	132
Black spruce	*C1* orthologue	yes	Myb	203
Gerbera	*gmyc1*	yes	bHLH	123, 204
Perilla frutescens	*C1* orthologue	yes	Myb	25
	Lc orthologue	yes	bHLH	25

a - cloning of cDNA or genomic clone

b - reference to gene cloning or loci description

c - protein containing the WD-40 repeat *motif*

In maize, the simultaneous expression of one member from each of the *C1* and *R* gene families is both necessary and sufficient to induce anthocyanin biosynthesis. The Del, *An1*, *An2*, *An4* and Rosea proteins seem to function analogously to *C1* and *R* in

antirrhinum and petunia, as mutations in any of these genes cause loss of anthocyanin biosynthesis. However, different groupings of the anthocyanin biosynthetic genes are regulated by the various proteins in their respective species, perhaps reflecting the presence or absence of other *trans*-acting factors and/or differences in the promoter regions of the target genes.

As the activity of the target anthocyanin biosynthetic genes appears primarily determined by the expression patterns of the regulatory genes, altering these patterns may allow the temporal and spatial modification of anthocyanin biosynthesis. This prospect has been tested by placement of the *C1* and *Lc* cDNAs from maize under the control of the CaMV 35S promoter and introduction of these constructs into tobacco and arabidopsis.[121] The expression of *Lc* alone increased anthocyanin production in both species, particularly in the flowers of tobacco, but did not alter where in the plant anthocyanins were made. Alone, *C1* had no effect. *Arabidopsis* plants containing both *Lc* and *C1* had both increased anthocyanin levels and novel tissue distribution patterns, with anthocyanins appearing in the petal, root and stamens. Similar phenotypes were obtained with a CaMV 35S *Lc* construct in petunia.[122] The petunia *Lc* transgenics had greatly increased anthocyanin biosynthesis in vegetative tissues and an associated increase in transcript levels for several flavonoid biosynthetic genes. Introduction of the same CaMV 35S *Lc* construct into lisianthus had no effect on vegetative or floral pigmentation or flavonoid biosynthetic gene expression (the authors' and co-workers' unpublished data).

Few reports of the use of genes other than *Lc* or *C1* in transgenics have been published. Constitutive expression of the *Delila* cDNA under the CaMV 35S promoter produced altered pigmentation in gerbera,[123] tomato (*Lycopersicon esculentum*)[124] and tobacco.[124] In tomato and gerbera, pigmentation in vegetative tissues was increased, but in tobacco an increase was only visible in flowers. In arabidopsis no consistent or strong phenotypic effect of *Delila* was observed.[124] Robbins and Harbord,[125] introduced a similar *Delila* construct into petunia. Although pigmentation of vegetative tissues increased, anthocyanin production in the corolla of the cyanic line V26 was reduced. One gerbera *Delila* transgenic line also displayed reduced floral pigmentation.[123]

Evidently, the availability of defined flavonoid regulatory genes provides tools for modulating both the amount and distribution of anthocyanins in floral and vegetative tissues. The data to date, however, also highlight the different effects the same factor(s) may have in transgenics of different species: for example, quite different phenotypes are obtained with *Lc* in tomato, arabidopsis, tobacco, petunia and lisianthus. This variation may reflect differences in both the promoter sequences of the endogenous flavonoid biosynthetic genes, and the presence or absence of endogenous transacting factors. Furthermore, although Del has extensive amino acid identity to Lc, and both proteins generally promote anthocyanin biosynthesis in their own species, in transgenic plants they produce quite different phenotypes. Obviously, similar anthocyanin regulatory proteins may differ in their target sequences, their DNA binding strength, and in how they interact with other transacting factors.

Most of the transgenic experiments to date have stably overexpressed cDNAs encoding regulatory factors under the control of the strong CaMV 35S promoter. Although these experiments have provided useful information, caution should be practised when

drawing conclusions on regulatory function. For example, proteins with bHLH domains may form heterodimers with other bHLH proteins, altering their activity. Also, producing large quantities of a particular transacting factor may allow it to out-compete other factors in situations in which it would not normally. For these reasons, the range of mutant lines available in species such as petunia, antirrhinum and maize are valuable assets for studying regulatory gene function. The advent of gene targeting in arabidopsis, and directed transposon tagging in petunia,[126] may also help in the development of more sophisticated studies. Caution is also needed when drawing conclusions from experiments using the biolistic 'gene gun'. This approach has been used extensively to introduce gene constructs of regulatory genes into plant tissues for transient assays of gene function[4,24,127] While these experiments have provided very useful data on the function and interactions of the regulatory factors, extrapolating the results to protein function in intact plants has proved more problematic. For example, bombardment of CaMV 35S *Lc* into petunia leaves failed to produce red anthocyanin spots,[4] yet constitutive expression of CaMV 35S *Lc* in transgenic plants caused high levels of anthocyanin production in leaves.[24,122] Also, results from transient assays suggesting the complementation of the *an1* and *an2* mutations with *C1* and *Lc*, respectively,[24] did not concur with later data on the *An1* and *An2* cDNAs.[4,24] Perhaps the wounding associated with the particle delivery can activate further interacting proteins that are not expressed in the equivalent tissue in intact plants.

Little is known about the next level of control - the regulation of the regulatory factors. Few transacting factors that directly regulate the flavonoid specific Myb and bHLH genes have been characterized. *Viviparous1* is known to control C1 expression during embryogenesis in maize,[128,129] and *An11* has been shown to act upstream of *An2* in regulating floral pigmentation in petunia, as the introduction of constitutively produced *An2* can overcome the *an11* phenotype.[120] Knowledge of the control of flavonoid biosynthesis in arabidopsis is increasing rapidly.[130] Several arabidopsis loci have been identified that may encode flavonoid regulatory factors (Table 1 and next section), and some of these may represent a higher level of pathway regulation.[4,131]

Repression

Two mutations have been identified in arabidopsis that may encode negative regulators of flavonoid biosynthesis, *icx1*[131] and *Banyuls*.[132] Anthocyanin levels are increased in lines recessive for either gene, and in *icx1* mutant lines transcript levels are increased for at least CHS, CHI and DFR.[131] These genes may provide tools for regulating flavonoid production in transgenic plants. However, to date, no clones have been published for either loci. The *Intensifier1* gene of maize, which may also repress flavonoid biosynthesis, has been cloned.[133] The encoded product shares homology to the anthocyanin activators *R* and *B*, being a bHLH type protein.

In addition to these genes, the *Fusca* complex of arabidopsis also acts to repress anthocyanin biosynthesis. The *fusca* series of mutations alter the activity of a complex which may normally suppress several aspects of seedling development in the light, including anthocyanin biosynthesis.[4,134,135,136] While this complex may not directly regulate anthocyanin biosynthetic genes, it may aid in identifying specific components of the downstream signalling pathway.

The genes that normally activate flavonoid, or early phenylpropanoid, biosynthetic genes may also be used to repress sections of the pathway. A natural mutation of the maize *C1* gene, *C1-I*, may reduce the transcriptional activation function of the C1 protein, but not its binding ability.[137] In *C1-I* plants the modified protein acts as a dominant, competitive repressor of flavonoid biosynthesis, presumably by binding to target promoters and reducing the access by wild-type C1 protein. A similar effect may also account for the phenotype of tobacco plants expressing the antirrhinum *myb305* cDNA under the control of the CaMV 35S promoter.[138] *Myb305* transgenic tobacco have greatly reduced levels of the phenolic compounds derived from 4-coumaroyl CoA, and reduced transcript levels for 4CL. Myb305 has been demonstrated to activate the promoters of early phenylpropanoid biosynthetic genes *in vitro*,[139] and it is possible that when overexpressed in transgenics it inhibits access to the 4CL gene promoter of other, more strongly activating, transacting factors.

The carotenoids

Carotenoids are an ubiquitous group of plant pigments differing greatly from flavonoids in structure and compartmentation. They are hydrophobic, lipid-soluble pigments with a structure normally based on a forty-carbon chain derived from the general isoprenoid pathway (Fig. 3). While their major role is to protect photosynthetic tissues from photo-oxidation, they can also provide brightly coloured pigments in non-photosynthetic cells, such as fruit and flower petals, where they act as attractants for pollinators or dispersal agents. To provide strong colours in such organs, high levels of carotenoids need to accumulate, and specialized structures form to sequester these large amounts.[140] These are the chromoplasts, carotenoid rich plastids differentiated from chloroplasts or non-photosynthetic plastids. In tomato and bell pepper fruit, the chloroplast to chromoplast transition is a highly ordered process, and mutations are known that affect both chlorophyll loss and carotenoid synthesis.[141]

A great diversity exists in carotenoid type, and approximately 600 naturally occurring carotenoids have been isolated and identified to date.[142] The initial products formed in the biosynthetic pathway are linear hydrocarbon carotenoids, such as lycopene (Fig. 3). Complexity is increased with the formation of the cyclic carotenoids, which have rings at the ends of the linear structures, the initial products being α-carotene or β-carotene. The rings may then be further modified by addition of hydroxy, carbonyl or epoxy groups, to produce the xanthophylls. These modifications may result in unusual carotenoid types in particular species, such as the keto-carotenoids, capsanthin and capsorubin, in bell pepper fruit.

Carotenoids are synthesized as part of the larger isoprenoid pathway, which encompasses a wide range of compounds derived from the common precursor of mevalonate.[143,144,145] Mevalonate is converted through a series of reactions to isopentenyl pyrophosphate (IPP) and its isomer dimethylallyl pyrophosphate (DMAPP), both C_5 compounds. These compounds form the C_5 building blocks for geranyl pyrophosphate (GPP, C_{10}), farnesyl pyrophosphate (FPP, C_{15}) or geranylgeranyl pyrophosphate (GGPP, C_{20}). Thus GGPP is formed from three IPP units and one DMAPP unit, by the enzyme GGPP synthase (GGPPS). GPP and FPP are the precursors for mono- and sesquiterpenes,

respectively. GGPP is the direct precursor not only of the first carotenoids, but also diterpenes, gibberellic acid and other compounds,[143,144,146] and thus represents an important branch point in terpenoid biosynthesis. A detailed discussion of carotenoid and general isoprenoid biosynthesis is beyond the scope of this chapter, and recent reviews[143,144,146,147,148,149] are recommended for further details.

The first committed step in carotenoid biosynthesis is the formation of phytoene from two molecules of GPP. This two-step reaction occurs *via* the intermediate prephytoene pyrophosphate and is catalyzed by phytoene synthase (PS, Fig. 3), which is associated with the stroma of the plastid.[150,151] Terpenoids up to and including the first carotenoid, phytoene, are colourless. The carotenoid chromophore is formed as a conjugated series of seven or more double bonds, introduced by four successive desaturations. Thus, the first coloured carotenoid is formed by the membrane-bound enzyme phytoene desaturase (PDS), which carries out the first of a series of desaturations. PDS from higher plants carries out two desaturation reactions to produce the yellow compound ξ-carotene, *via* the intermediate phytofluene. ξ-carotene is converted *via* two further desaturations to the bright red lycopene *via* the intermediate neurosporene, by the enzyme ξ-carotene desaturase (ξCD). In bacteria, PDS can carry out all four desaturations, to produce lycopene directly from phytoene. Lycopene is the direct precursor for α- and β-carotene, which are formed by the ε- and β-lycopene cyclases. The carotenes are the first of the wide range of xanthophylls that are formed by further modification of the rings at the ends of the structures.

Progress on the molecular biology of carotenoid biosynthesis was much slower than that for flavonoid biosynthesis. It was not until 1991[152] that a known cDNA for a carotenoid biosynthetic enzyme was published, although a cDNA for PS had been isolated from tomato previously[153], but not identified as such. Since then progress has been rapid, particularly in arabidopsis and the model fruit systems of tomato and bell pepper. The first cDNAs to be identified were for the early biosynthetic enzymes, GGPPS[154] and PS,[153,155] and the enzymes involved in the desaturation reactions, PDS[152] and ξ-CD.[156,157,158] However, plant cDNAs are now available for a number of the enzymes of cyclic carotenoid biosynthesis, including lycopene β-cyclase (LβC),[159,160] lycopene ε-cyclase (LεC),[160] b-carotene hydroxylase,[161] capsanthin/capsorubin synthase (CCS),[162] xanthophyll/zeaxanthin epoxidase[163,164] and violaxanthin de-epoxidase.[165] Only the earliest reports of the cDNA cloning are presented here. A more comprehensive list of clones isolated prior to 1995 can be found in Bartley and Scolnik.[149] Another useful review of the molecular biology of plant carotenoid biosynthesis can be found in Bartley *et al.* [141] There are still no plant cDNA clones identified for a number enzymes involved in the later stages of carotenoid biosynthesis, particularly for production of some of the species-specific carotenoid types. Much molecular research has been carried out on green algae and carotenoid producing bacteria, and some of the novel activities identified in these may be useful for engineering carotenoid biosynthesis in plants.[141,147,166,167]

Two common groups of carotenoids associated with colour are the carotenes and xanthophylls, which are often associated with orange and yellow colours respectively.[168] Some flowers are very rich in the abundance or diversity of their carotenoids. For example, in daffodils (*Narcissus* spp.) β-carotene levels can reach 16.5% dry weight in the flower corona,[169] and in approximately 40 yellow *Rosa* varieties and species,

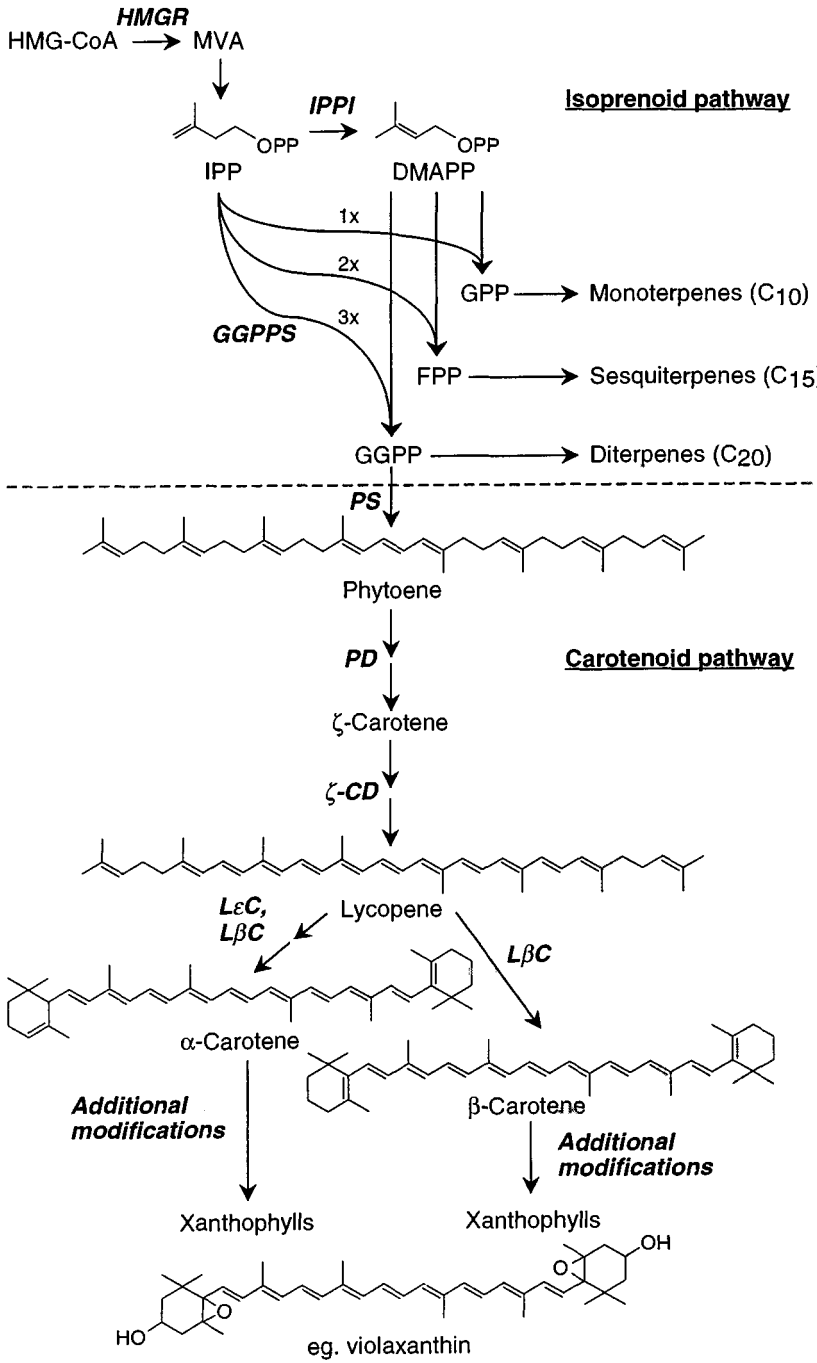

Figure 3. Schematic representation of sections of the isoprenoid and carotenoid biosynthetic pathways of plants. Enzyme and compound abbreviations are as given in the text, except for: mevalonic acid (MVA), HMG-CoA reductase (HMGR) and IPP isomerase (IPPI). OPP is used to represent a diphosphate group.

about 75 different carotenoids have been identified.[170] Generally, carotenoid-producing flowers will synthesize predominantly either the hydrocarbon type, the highly oxidized xanthophylls, or highly species-specific pigments, rather than a mix of these.[171]

The following section looks at individual biosynthetic enzymes and the results to date, or future prospects for, modifying their activity in transgenics.

PHYTOENE SYNTHASE

The first report on metabolic engineering of carotenoid biosynthesis, was the introduction of an antisense construct of PS (pTOM5) from tomato into plants of the same species. The fruit of transgenics was changed from orange-red to yellow in appearance, and the flowers had reduced pigmentation.[172] The phenotype was correlated with reduced PS mRNA levels and greatly reduced carotenoid levels.[172,173] The yellow colour of the fruit was due to presence of the flavonoid naringenin chalcone. Fray and Grierson[174] used the same PS cDNA to complement two *yellow flesh* (*r* and *ry*) mutant lines of tomato, and demonstrated the mutant phenotype was due to aberrant PS mRNA. The transgenics also accumulated lycopene ectopically, in the abscission zones and in fruit prior to the usual ripening stage. In some transgenic lines, carotenoid accumulation was further suppressed below the mutant levels in fruit, foliage and flowers, leading to photobleaching of leaves and fruit. The phenotype was thought to be due to cosuppression of other PS genes normally expressed in green tissues. Some of the PS sense transgenics also showed a reduction in height.

Fray *et al.*[175] studied ectopic pigment production and reduced stature further, introducing the CaMV 35S sense PS construct into wild type tomato lines. Highly increased levels of phytoene, lycopene and ξ-carotene were present in the unripe fruit of the transgenics, and increased pigmentation occurred in the seed coat, hypocotyl, and in a few cases, in cotyledons and young leaves. In the dwarf phenotype, there was an up to 30-fold reduction in the level of gibberellin A1, suggesting that reducing the pool of GGPP reduced the substrate available for the initial enzyme of gibberellin biosynthesis, *ent*-kaurene synthase A. Phytol, required for chlorophyll formation, is also produced from GGPP, and there was evidence of chlorophyll deficiency in the transgenics. Thus, flux through GGPP is an important step for controlling the levels of different isoprenoid products, and altering flow rate into one branch of the pathway, *e.g.* carotenoids, can have a major impact on the production of other derivatives.

PS over-expression has also been targeted as a means of modifying carotenoid biosynthesis for improved nutritional quality. Rice (*Oryza sativa*), one of the world's major staples, lacks β-carotene, and is thus a poor dietary source for vitamin A precursors. Introduction of a PS cDNA from daffodil (*Narcissus pseudonarcissus*) into rice led to production of phytoene in the endosperm, raising the prospects for introducing β-carotene synthesis by introducing the subsequent biosynthetic steps.[176]

PHYTOENE DESATURASE

Misawa *et al.*[177,178] introduced the gene encoding PDS from *Erwinia uredovora*, linked to the CaMV 35S promoter and the chloroplast transit peptide of ribulose-1,5-bisphosphate

carboxylase small subunit, into tobacco plants. The PDS enzyme from *Erwinia* differs from those of plants by carrying out four desaturation reactions, converting phytoene directly to lycopene. There was a change in both the quantity and quality of carotenoids in the foliage of the transgenic, in particular increases in β-carotene and its xanthophyll derivatives. This result is encouraging with regard to using genes from carotogenic bacteria to modify carotenoid biosynthesis in plants.

β- AND ε-LYCOPENE CYCLASE

Carotenoids with cyclic end groups are important pigments of fruit and flowers, as well as essential components of photosynthetic membranes. Cyclisation of lycopene represents an important branch point in carotenoid biosynthesis. Both β-rings or ε-rings are commonly formed in plants. Formation of β-rings at both ends of lycopene produces β-carotene, which is the precursor of xanthophyll pigments such as zeaxanthin, violaxanthin and neoxanthin, which are commonly found in flowers and fruit. Formation of one β-ring and one ε-ring produces α-carotene, the precursor of lutein, an important pigment in photosynthetic tissues.

Cunningham *et al.*[160] isolated cDNA clones from arabidopsis for both LβC and LεC. By expressing these in a lycopene accumulating strain of *E. coli*, they were able to show LβC alone produced β-carotene, while the action of LεC alone produced σ-carotene from lycopene, a monocyclic carotenoid with a simple ε-ring. Interestingly, σ-carotene rather than lycopene is accumulated in the *delta* mutant of tomato, producing yellow/orange rather than red fruit. Thus, the availability of the β and ε-cyclases raises the possibility of modifying lycopene, β-carotene, α-carotene and σ-carotene production, and therefore plant colour.

CAPSANTHIN-CAPSORUBIN SYNTHASE

The CCS enzyme of bell pepper catalyzes the formation of the unusual keto-ring from the modified β-ring present in antheraxanthin and violaxanthin, to give the red pigments capsanthin and capsorubin, respectively. Bouvier *et al.*[164] reported the isolation of a cDNA encoding CCS, and analyzed its expression in various fruit colour mutants of pepper. CCS transcript was absent in green and yellow fruit mutants, but was present in red fruited lines. The CCS has high sequence identify (55%) to LβC from pepper, and indeed when expressed in *E. coli* could also form β-carotene from lycopene.[179] Induction of CCS during pepper fruit ripening may provide a strong drive, in conjunction with the constitutive expressed LβC, for the synthesis of both β-carotene and its derivatives capsanthin and capsorubin.[179] The availability of the CCS cDNA provides a valuable tool for increasing levels of these compounds in heterologous target species.

REGULATION OF CAROTENOID BIOSYNTHESIS

Except for the genes involved in the production of the precursor mevalonate, very little is known regarding the regulation of carotenoid biosynthesis. Carotenoid biosynthetic genes are known to be under developmental control in some tissues such as bell pepper[180]

and tomato fruit,[181] and indeed, PS mRNA does not accumulate, or only accumulates at reduced levels, in a range of ripening mutants, such as *Never-ripe*, *ripening inhibitor* and *non-ripening*. However, these mutations are pleotropic, affecting many facets of ripening, such as softening, as well as carotenoid production. Indeed, *Never-ripe* has been shown to encode a protein involved in the early stages of ethylene perception.[182] To date, no carotenoid specific regulatory loci have been published, and this is an area for future investigation. One possible candidate is the *y* gene of bell pepper, which, in the recessive form, produces yellow fruit which lack mRNA for CCS and another protein, ChrA.

SUMMARY OF PROSPECTS FOR MANIPULATING CAROTENOID BIOSYNTHESIS

Rapid progress is being made in the molecular biology of carotenoid biosynthesis, particular in the model species of tomato, bell pepper and arabidopsis. Most of the plant genes required for engineering carotenoid production have now been isolated, and more genes are available from non-plant sources. These might be used to increase the biosynthesis of specific novel carotenoids, or to increase the flow through a rate limiting step, as demonstrated in tomato with PS. Alternatively, these cDNAs might be used in antisense RNA or sense-suppression approaches to interrupt the biosynthetic pathway, to either eliminate colour or to encourage accumulation of highly coloured types, such as lycopene.

There are, however, a number of points to consider when undertaking such modification experiments. For example, the gene product must be located to the plastids, requiring the addition of appropriate target sequences to potentially useful bacterial genes. In addition, the carotenoid precursors, and the chromoplasts themselves (or potential chromoplasts), must be present in the target tissue. These precursors are also involved in the synthesis of a wide range of other plant products, *e.g.* gibberellins and monoterpenes, and it has already been demonstrated that altering carotenoid biosynthesis can affect the plant phenotype in other ways.[175] Furthermore, as carotenoids serve an essential protective function in green tissues, preventing photo-oxidation, the perturbation of their biosynthesis in non-target organs could have undesirable effects on the plant.

Fragrance

The flowers of many plants emit volatiles as a means of attracting pollinators. These fragrances are also important aspects of the appeal of garden plants and cut flowers. Similar volatiles are also components of many of the plant extracts used as essential oils.[145] Scent is often a complex mixture of low molecular weight compounds, particular combinations of which give a plant its characteristic fragrance. The compounds are usually terpenoids, benzenoid compounds or acyl lipid derivatives, and several thousand different types have been identified.[145,183] In vegetative tissue, the major source of essential oils, they are often associated with glandular trichomes.[145]

In flowers, a variety of specialised structures of the petal epidermis may be involved in the production and release of the scent volatiles.

Many plant volatiles are mono-, sesqui- or diterpenes, three groups of isoprenoids derived from the same precursors, IPP and DMAPP (Fig. 3).[145,146] Most studies on the biosynthesis of these isoprenoids have concentrated on their production with regard to either essential oils or as plant defence agents. Work on essential oil biosynthesis has concentrated on members of the mint family (spearmint, *Mentha spicata*, and peppermint, *Mentha X piperita*) and other herb species, *e.g.* fennel (*Foeniculum vulgare*), sage (*Salvia officinalis*) and sweet marjoram (*Majorana hortensis*).[145] Floral volatile biosynthesis has just begun to be defined. The prospects for the metabolic engineering of the isoprenoid biosynthetic pathway have recently been reviewed in detail.[145]

The monoterpenes are perhaps the best defined class of floral volatiles.[146] They are produced by a class of enzymes termed monoterpene synthases, which catalyze the cyclisation of GPP to the various monoterpene carbon skeletons. From the basic carbon skeletons a vast array of compounds can be derived by secondary modification.[145,146] Several monoterpene synthases involved in essential oil production have been characterized biochemically,[145] and genomic or cDNA clones are now available for a few of these.[143] In particular 4S limonene synthase, which catalyses the cyclisation of 4S-limonene from GPP, has been studied in detail in spearmint, and the encoding gene isolated.[184] The enzymes involved in the production of other isoprenoids involved in scent are less well defined. However, related enzymes involved in the production of isoprenoid plant defence compounds have been studied in detail.[145]

The biosynthesis of fragrance volatiles in flowers is not well characterised, with the exception, however, of *Clarkia breweri*. The strong sweet scent of *C. breweri* is made up of eight to twelve volatiles, including both monoterpenes and phenylpropanoids (benzenoids).[185] One of the major monoterpene volatiles is S-linalool, which is a common component of many scents.[183] S-linalool is produced from GPP by the enzyme S-linalool synthase (SLS). Dudareva *et al.*[186] isolated a cDNA for SLS, and showed that in *C. breweri* SLS is highly expressed in various cell types, including the epidermal cells of the petals, whereas in the non-scented *C. concinna*, SLS is expressed only in the stigma. Unlike many monoterpene producing species, the flowers of *C. breweri* do not have specialised structures for the release of the scent volatiles.

Pichersky *et al.*[185] have examined the production of fragrant phenylpropanoid volatiles by *C. breweri* flowers. The bulk of the phenylpropanoid emission from flowers is from petals, and may include at least four phenylpropanoids types (eugenol, isoeugenol, methyleugenol, and isomethyleugenol). Some lines of *C. breweri* produce all four phenylpropanoids, while others produce only eugenol and methyleugenol. Isoeugenol and isomethyleugenol are produced from eugenol and methyleugenol, respectively, by the enzyme S-adenosyl-L-methionine: (iso) eugenol O-methyltransferase (IEMT), which catalyzes the methylation of the para-4'-hydroxyl. A *C. breweri* cDNA clone encoding IEMT has been isolated,[187] and shown to use eugenol and isoeugenol as substrates. IEMT mRNA and activity was detected only in lines that emit all four phenylpropanoid compounds, and was most abundant in petal tissue.

A number of the genes and cDNAs now isolated, particularly those for monoterpene cyclases, offer good opportunities for metabolic engineering approaches, as they enable

the introduction of new activities, *e.g.*, for SLS. SLS uses the ubiquitous isoprenoid precursor GPP to produce S-linalool, which is then released without the need for specialised flower structures. GPP would be produced in significant quantities in petals coloured by carotenoids, although, as it is mainly located in chromoplasts, it might not be readily available to the introduced enzyme. In any approach for introduction of isoprenoid production *de novo*, the sub-cellular location of the substrates and the possible need for secretory structures needs careful consideration. It may also be possible to alter the range of isoprenoid types produced in a plant by modifying the activity of known terpene cyclases using amino acid substitution or domain swapping. It has been shown that it may be possible to engineer both the flux into the major terpene groups,[188] and activity of specific terpene cyclases[189,190] by these approaches.

An important point with regard to manipulation of isoprenoid metabolism for scent, is the assignment of the specific compounds to particular scents. Some individual C_{13}-norisoprenoid aroma compounds have been defined with regard to the associated scent, and small differences in chemistry may have marked effects. For example, individual stereoisomers of a single compound have been classified as 'intense blackcurrant' scent compared to 'naphthalene like'.[191] If the aim is to modify the production of C_{13} volatiles, it may be important to steer production into only one type of stereoisomer. Similar stereochemical effects may affect the production of other volatile compounds.[192]

Concluding remarks

The prospects for the genetic engineering of the flavonoid pathway for altering plant colour are very good. Many years of work on the chemistry, biochemistry and genetics of flavonoid biosynthesis have formed a substantial knowledge base from which molecular strategies can be developed. Genes have been isolated corresponding to most of the important biosynthetic enzymes, including the F3'H and F3'5'H. Colour has been successfully modified in transgenic plants of petunia, gerbera, lisianthus, tobacco, rose, *Forsythia*, chrysanthemum, *Torenia* and carnation. In the case of the DFR petunia[34] and F3'5'H carnation these experiments resulted in production of colours novel to the species. In addition, the isolation of genes which regulate flavonoid biosynthesis has allowed the control of not only the absolute amount, but also the temporal and spatial distribution of anthocyanin production in vegetative and floral tissues of several species. However, whilst the amount or basic type of anthocyanin formed may be modified, the final colour obtained in plants depends on many other factors, such as copigmentation and vacuolar pH. The technology for modifying these is still at an early stage. Neither have cDNA clones been obtained for many of the enzymes involved in the biosynthesis of the rarer, but highly coloured, flavonoid types.

The TDC[86] and STS[87] experiments highlight the complexities of the regulation of the phenylpropanoid pathway, and the unexpected outcomes that may still arise from targeted metabolic modification approaches. In the transgenic tobacco expressing a tapetum-specific STS construct, male fertility could be restored by adding the product of STS, resveratrol, presumably through feedback inhibition of the STS activity. In the case of the TDC, reducing the level of tryptophan also reduced flow into the phenylpropanoid pathway.

Molecular knowledge on the biosynthesis of carotenoids has increased very rapidly, and carotenoid biosynthesis has been successfully modified in fruit and flowers of tomato. It is likely that similar experiments in other species will soon be reported. However, detailed knowledge is still lacking on the carotenoid profile of some of the potential target species, the supply of carotenoid precursors in different tissues, and the regulation of the biosynthetic genes.

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CHAPTER 8

METABOLIC ENGINEERING OF CONDENSED TANNINS AND OTHER PHENOLIC PATHWAYS IN FORAGE AND FODDER CROPS

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Introduction

Despite their name, secondary compounds are of primary importance in forage and fodder crops. But before we discuss specific compounds in particular crops perhaps we should focus on some of the characteristic features of forages and fodders. These crop species are either fed directly to animals (forages) or are used for feed after cutting or for conservation as silage (fodders). If we consider typical forages such as grasses and clovers, these crops are often grown for years on end in medium and long-term leys. It is not surprising therefore that such crops accumulate a wide range of secondary products which function to protect against pathogens, pests and other environmental challenges.[1]

Table 1 outlines some of the chemical end products reported in common temperate and tropical forage crops. In many cases it is clear that these compounds are biologically active and, for example, one can envisage strategies aimed at modifying such pathways and improving durable resistance to pathogens and pests.[2] However, another consequence of the accumulation of secondary compounds relates to the effects that these end products have upon grazing ruminant livestock and this chapter will focus upon these issues. It is our contention that, in many cases, the primary productivity of forage crops is compromised by non-optimal levels of secondary compounds and that there are opportunities for significant improvements based upon genetic manipulation.[3] In fact it can be said that the value of a forage crop really does depend upon its chemical composition.

Condensed tannins are one particular class of secondary products and these are critically important in forage crops. These biopolymers are highly biologically active and are a focus of work at IGER. There are a range of forage legumes where alterations in tannin content would be desirable. Briefly, two types of required intervention can be considered. In the case of highly tanniferous species such as Desmodium, high tannin content reduces palatability and nutritive value.[4] For these crops, which are mainly of tropical origin, strategies are required which will result in gross reductions in tannin content. It is interesting to note that temperate forages such as *Lotus corniculatus* (bird's foot trefoil)[5] and *Onobrychis viciifolia* (sainfoin) can also accumulate high levels of condensed tannins under some environmental conditions.

R. Verpoorte and A.W. Alfermann (eds), Metabolic engineering of plant secondary metabolism, 165-177.

Table 1. Some examples of secondary compounds found in common crops used for animal feed

Crop species	Secondary metabolite
Feed Seed	
Rapeseed (*Brassica napus*)	Glucosinolates, free and esterified phenolic acids.
Soybean (*Glycine max*)	Saponins, oestrogenic isoflavonoids, pterocarpans, coumestans, C-glucosyl flavones.
Oats (*Avena sativa*)	Saponins, free and esterified phenolic acids, C-glycosyl flavones, aminoalkyl phenols, phenolic amides, feruloyl n-alkanols, avenacins (feruoyl sterols), avenanthramides.
Faba bean (*Vicia faba*)	Condensed tannins, leucoanthocyanins, pterocarpans.
Temperate Forage/Fodder	
White clover (*Trifolium repens*)	Oestrogenic isoflavonoids, pterocarpans, coumestans, cyanogenic glycosides.
Alfalfa, Lucerne (*Medicago sativa*)	Saponins, coumestans, oestrogenic isoflavonoids, cyanogenic glycosides, pterocarpans, isoflavans.
Red clover (*Trifolium pratense*)	Oestrogenic isoflavonoids, pterocarpans, coumestans.
Subteranean clover (*Trifolium subteraneum*)	Oestrogenic isoflavonoids, deoxybenzoins.
Birds-foot trefoil (*Lotus corniculatus*)	Condensed tannins, cyanogenic glycosides, coumestans.
Big trefoil (*Lotus uliginosus*)	Condensed tannins, cyanogenic glycosides, coumestans.
Saifoin (*Onobrychis viciifolia*)	Condensed tannins, 2-aryl benzofurans.
Crown vetch (*Coronilla varia*)	Condensed tannins, C-glucosyl flavones.
Lespedeza (*Lespedeza cuneata*)	Condensed tannins, C-glucosyl flavones, dihydrochalcones.
Cowpea (*Vigna sinensis*)	Isoflavonoids, pterocarpans.
Rygrass (*Lolium multiflorum, Lperenne*)	Cell wall esterified monomeric and dimeric hydroxycinnamic acids.
Fescue (*Festuca arundinacea, F pratense*)	Cell wall esterified monomeric and dimeric hydroxycinnamic acids.
Maize (*Zea mays*)	Hydroxamic acids, proanthocyanidins, coumarins, phenolic amides, cell wall esterified monomeric and dimeric hydroxycinnamic acids.
Tropical forage/browse	
Sorghum (*Sorghum vulgare*)	Condensed tannins, proanthcyanidins, aminoalyl phenols (Dhurrin).
Brachiaria (*Brachiaria decumbens*)	Flavone c-glycosides, cell wall esterified monomeric and dimeric hydroxycinnamic acids.
Cenchrus (*Cenchrus cilliaris*)	Flavone c-glycosides, cell wall esterified monomeric and dimeric hydroxycinnamic acids.
Desmodium (*Desmodium ovalifolium*)	Condensed tannins, isoflavones.
Acacia (*Acacia cyanophylla*)	Condensed tannins, flavans, leucoanthocyanidins, chalcones.
Leucaena (*Leucaena leucophala, L. pallida*)	Condensed tannins.
Sesbania (*Sesbania seban*)	Condensed tannins.
Flemengia (*Flemengia macrophylla*)	Condensed tannins, isoflavones, dihydrochalcones.
Khejri (*Prosopis cineraria*)	Condensed tannins, procyanidin dimers, C-glucosyl flavones.

With regard to temperate forage legumes, such as *Trifolium repens* (white clover) and *Medicago sativa* (lucerne), alternate strategies are required. In these species, foliar tissues are tannin-negative and the consequence of this is a high initial rate of fermentation when these crops are consumed by ruminant livestock. This scenario results in high rates of microbial deamination in the rumen and protein foaming also occurs which can result in symptoms of bloat. However, these effects are ameliorated by the presence of low to moderate levels of tannin[6] and therefore we are interested in methods for transactivating tannin biosynthesis in leaf and stem tissues of white clover.

This chapter will concentrate on topics relating to the genetic manipulation of condensed tannin biosynthesis in forage legumes. Recent data is reported and some future strategies for engineering this pathway are described. One additional point to bear in mind is that strategies that prove successful for the condensed tannin pathway in legumes are likely to be of general applicability to food crops where tannins pose nutritional or processing problems[7] as well as being relevant for modifying other secondary pathways in forage and fodder crops.

Current research

For fundamental work on the genetic engineering of condensed tannin pathways it is important to have a convenient model organism. However a number of typical transgenic plant systems; tobacco, tomato and *Arabidopsis* do not accumulate tannins in vegetative tissues. For this reason we have developed *Lotus corniculatus* as a transgenic test system.[8] In fact, *L. corniculatus* has a range of useful characteristics; it is readily co-transformable using *Agrobacterium rhizogenes* and can therefore be analysed in root culture or shoot format and as regenerated plants. Leaf, stem, root and flower tissues constitutively accumulate condensed tannins and these polymeric molecules accumulate in specific tannin-containing cells. A photographic summary outlining tannin cell distributions in *Lotus* tissues is shown in Figure 1. Another advantage of this model species is that there is a wealth of information about the chemical composition of this plant and its value as a forage crop.[8] Therefore *Lotus* is particularly desirable in being both a model transgenic system and also a *bona fide* forage crop and this will be of value when testing the agronomic performance of *L. corniculatus* transgenics in animal performance trials.

A good place to start a consideration of current work is to examine the pathway to condensed tannins in *L. corniculatus* and this is shown in Figure 2. This is a major secondary pathway in *Lotus* which can account for up to 12% dry weight. The pathway utilises coumaroyl CoA as a carbon skeleton which is derived from the shikimate pathway and malonyl CoA units that are used to construct the basic flavonoid units. After an initial condensation reaction catalysed by chalcone synthase (CHS) a series of hydroxylations and reductions produce catechin 4-ol and catechin monomer units. These units are then polymerised together to produce condensed tannin polymers which are stored in the vacuole. The terminal steps of the pathway, here described as polymerising enzyme and condensing enzyme may in fact relate to transporters which import catechin 4-ol and catechin units into the vacuole for non-enzymic polymerisation.

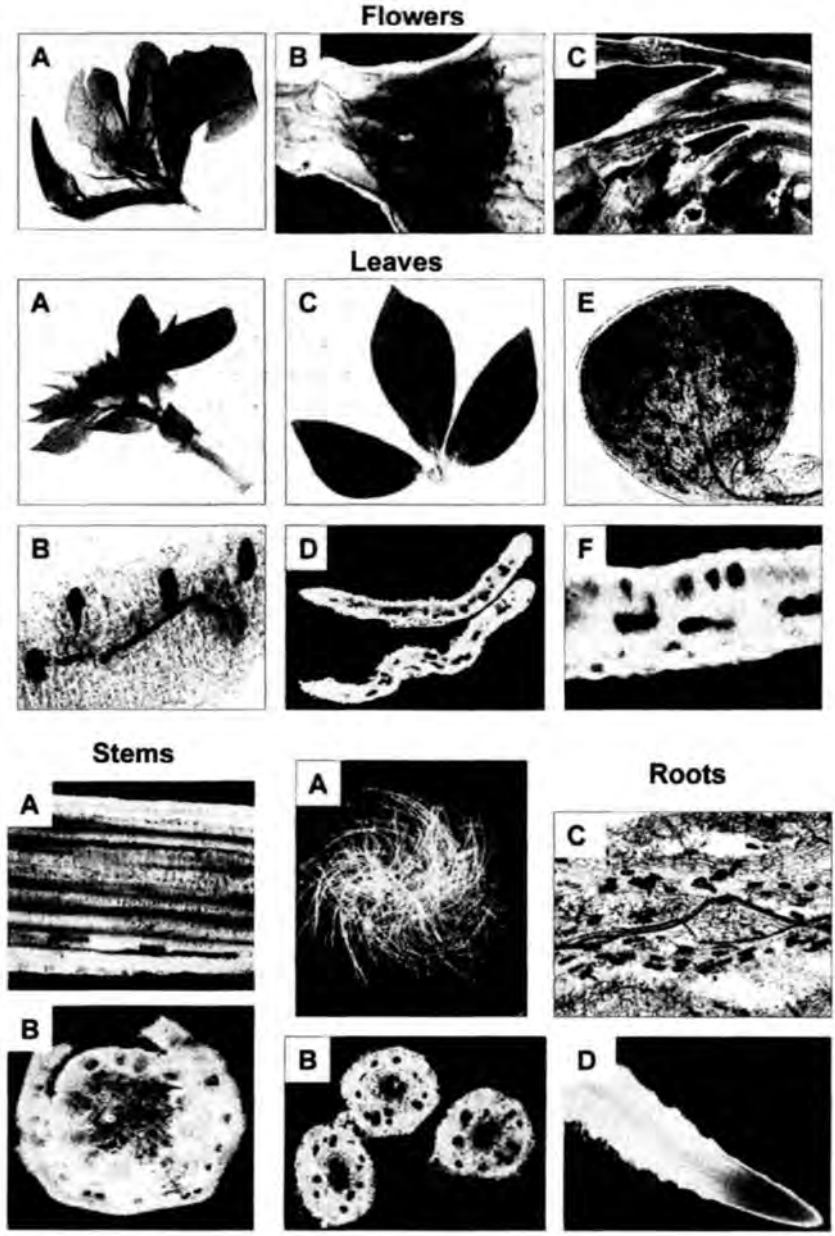

Figure 1. Distribution of condensed tannin cells in different tissues of *Lotus corniculatus*. Flowers: A- petals, B- longitudinal section of base of floral stem, C- stamens (vanillin HCl). Leaves: A- Shoot meristem with terminal leaves (vanillin/HCl), B- transverse section though base of terminal leaf (DMACA), C- mature trifoliate leaf (vanillin/HCl), D- transverse section through tip of trifoliate leaf (DMACA), E- difoliate leaf (DMACA), F- transverse section through difoliate leaf (DMACA). Stems: A- longitudinal section through mature stem (DMACA), B- transverse section through mature stem (DMACA). Roots: A- Hairy root cultures, B- transverse section through mature hairy root (DMACA), C- longitudinal section through mature hairy roots, (vanillin/HCl), D- root tip of hairy root. (DMACA).

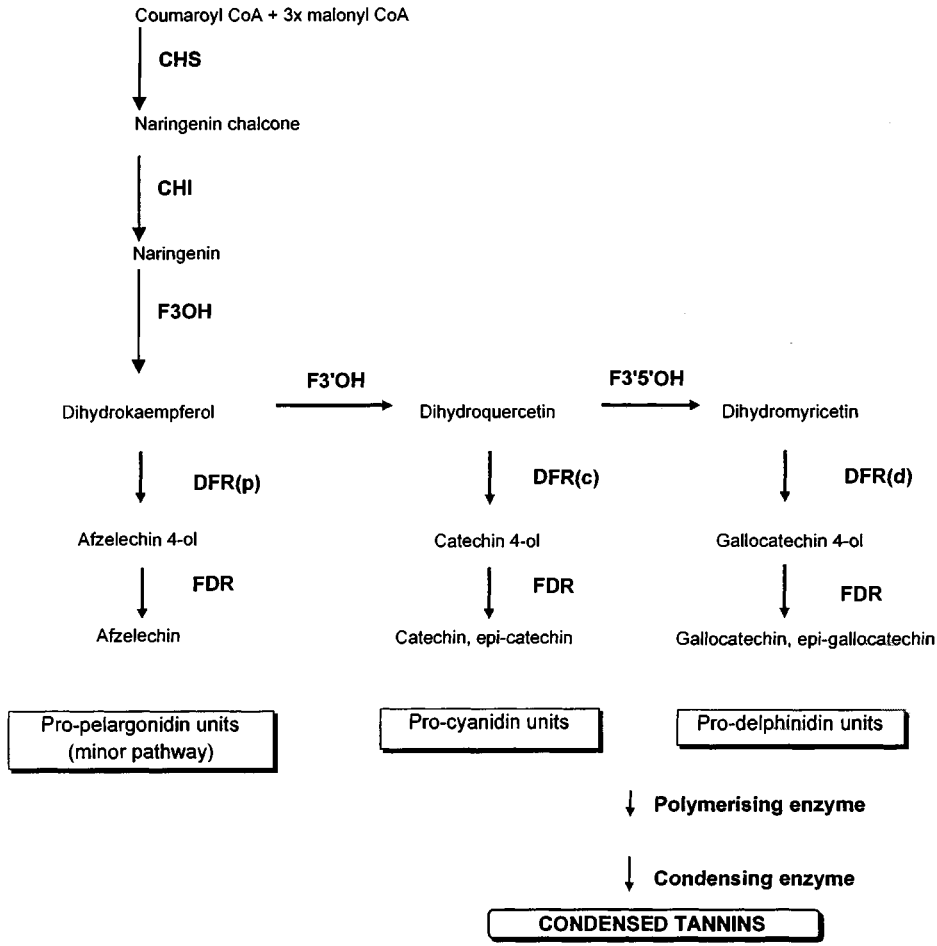

Figure 2. Enzymic steps involved in the biosynthesis of condensed tannin polymers in higher plants. CHS [chalcone synthase], CHI [chalcone isomerase], F3OH, [flavonone 3-hydroxlase], F3'OH [flavonoid 3'-hydroxylase], F3'5'OH [flavonoid 3'5'-hydroxlase], DFR (p, c, d) [dihydroflavonol reductase; with specificities for pelargonidin, cyanidin and delphinidin], FDR [flavan 3,4 diol reductase].

A fortunate circumstance for those of us studying condensed tannins is that this pathway is common with the anthocyanin pathway up to flavan 3,4 diol reductase (FDR) and therefore a number of useful common genes have been cloned from developing flower tissues. Another point to note is that while tannins are polymers as described before, the hydroxylation of the polymer depends upon the hydroxylation state of the constituent monomer units. The majority of condensed tannins so far described contain pro-cyanidin units derived from dihydroquercetin and more highly hydroxylated pro-delphinidin units derived from dihydromyricetin.[9] There is currently very little information on the molecular mechanisms which control polymer molecular weight which is nonetheless an important feature of condensed tannin structure and activity.

So, given this background knowledge, an initial question relates to whether it is possible to make directed changes in this metabolic pathway. In particular can one alter the levels of condensed tannins and can one alter the degree of hydroxylation of condensed tannin polymers?

MODIFICATION OF LEVELS OF CONDENSED TANNINS

When first looking at a pathway it is difficult to predict the exact result of any given intervention. Explanations for this depend upon the possibility of multiple pathways, branch points to other end products and due to a limited understanding of metabolic control analysis. These factors may help to explain problems encountered when attempting to manipulate primary and secondary metabolic pathways.[10] Therefore the approach that we have taken is to select genes which we surmise from prior knowledge may be important in controlling pathway flux into condensed tannins

The first gene that we selected was dihydroflavonol reductase (DFR) which encodes an enzyme which performs the first reductive step in the pathway using NADPH as a co-factor. When this gene was antisensed in clonal genotypes of *L. corniculatus* novel transgenic lines were produced.[11] When genotypes that biosynthesised low or medium levels of condensed tannins were analysed, antisense lines were noted which showed an expected phenotype ie. a reduction in condensed tannins. A summary of typical results is shown in Figure 3. Using the 5' half of an *Antirrhinum majus* cDNA, reductions in the order of 0.3 mg/gFW were noted. Larger effects may have been noted if a homologous (*L. corniculatus*) gene had been used in these experiments. However, one conclusion from these experiments might be that not only is DFR unequivocally involved in condensed tannin synthesis in this species but that some degree of pathway control is exerted at this step. A comparison of DFR enzyme activities and condensed tannin levels would help to clarify this issue.

In subsequent experiments we antisensed a chalcone synthase (CHS) gene from *Phaseolus vulgaris*.[12] In this case the predicted phenotype is a reduction in condensed tannin levels. After all, suppressing DFR or CHS has been effective in reducing anthocyanin levels in the flowers of higher plants ([13] and chapters in this book). However typical results from our experiments are shown in Figure 4. In addition to null phenotypes, lines were noted with significant increases in tannin levels. While it is convenient to have uncovered a method for increasing levels of condensed tannins in higher plants this was not an obvious result from this genetic intervention. Molecular analysis confirmed the presence of antisense transcript but interestingly the levels of endogenous CHS transcripts were elevated in up-regulated lines. The hypothesis that we put forward is that this AS-CHS construct has been responsible for down regulating member(s) of this gene family and that there has been a resultant over-compensation in CHS expression mediated by other members of the gene family. We describe this phenomenon as 'differential modulation of a multigene family' (DMMF)[12] and it will be interesting to see whether there will be other examples of this effect in genetic engineering experiments involving other pathways. In this context, we note that similar effects have been found when manipulating the expression of HMGCoA reductase in *Arabidopsis*[14] so this phenomenon may not be restricted to polyploid species such as *Lotus*.

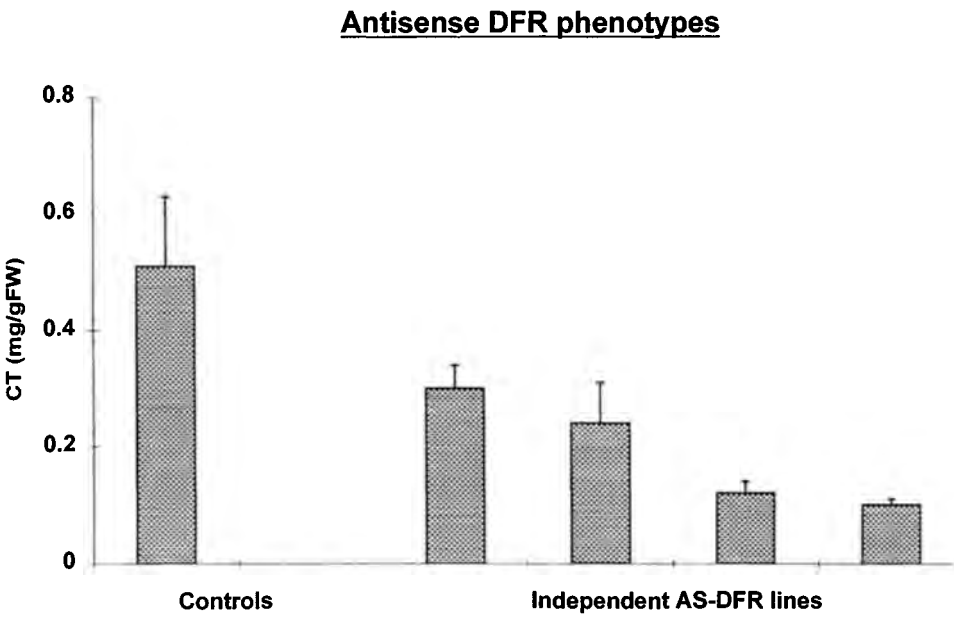

Figure 3. Condensed tannin levels in *Lotus* root cultures harbouring antisense dihydroflavonol reductase constructs.

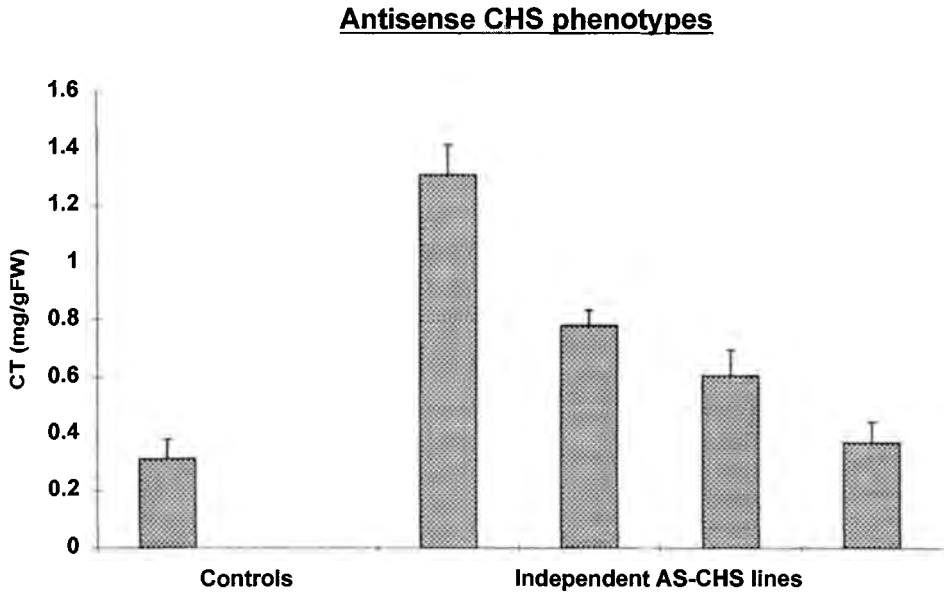

Figure 4. Condensed tannin levels in *Lotus* root cultures harbouring antisense chalcone synthase constructs.

MODIFICATION OF CONDENSED TANNIN STRUCTURE

As described previously we have a reasonable description of the control of tannin hydroxylation. Basically this process appears to be controlled by the levels of dihydroflavonol intermediates and by the subsequent routing *via* DFR. Certainly in anthocyanin biosynthesis, DFR appears to exert significant control over the relative content of pelargonidin, cyanidin and delphinidin pigments in flower tissues.[15] In order to elucidate the role of DFR in tannin biosynthesis we introduced a sense *A. majus* DFR cDNA into *Lotus*.[16] The enzyme encoded by this gene favours the synthesis of pelargonidin from dihydrokaempferol, while most condensed tannins have units synthesised *via* dihydroquercetin (pro-cyanidin pathway) or *via* dihydromyricetin (pro-delphinidin pathway).

Typical results from this intervention are shown in Figure 5. In our experiments we determined monomer composition using high pressure liquid chromatography (HPLC) analysis of hydrolysed polymers and found low levels of pelargonidin units in condensed tannins from control lines. However, pelargonidin levels were greatly enhanced in a line where we over-expressed *A. majus* DFR and which showed elevated tannin levels [Line 3 in Fig. 5]. This increased routing into the pelargonidin pathway can be compared with equivalent experiments carried out on the anthocyanin biosynthetic pathway.[15] We also found lines with no significant change in tannin amounts but with elevated proportions of cyanidin units or elevated proportions of delphinidin units relative to control lines [Lines 1 and 2 in Fig. 5]. Once again we have an intervention which has produced an unpredicted tannin phenotype. In order to try to explain the high cyanidin and high delphinidin polymers we should go back to look at the pathway outlined in Figure 2.

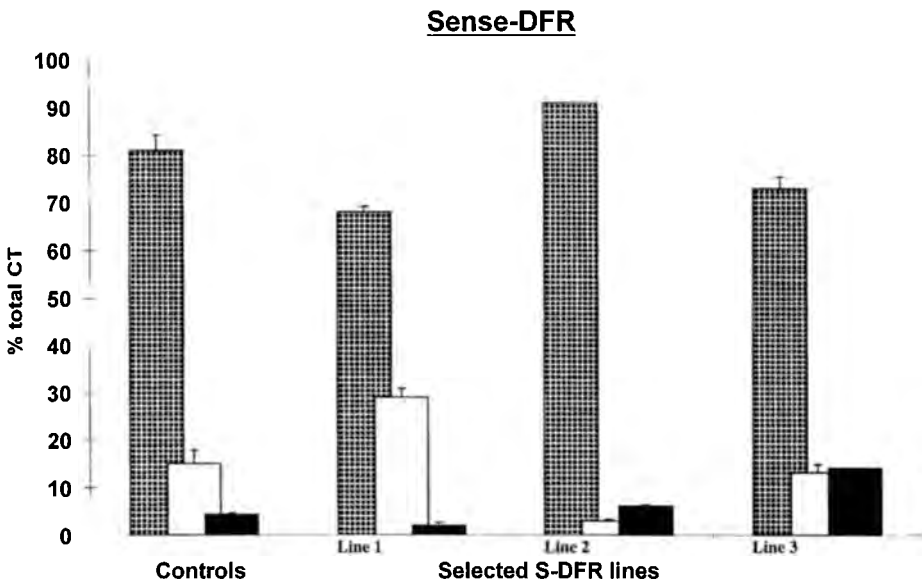

Figure 5. Hydroxylation patterns of condensed tannin polymers in *Lotus* root cultures harbouring an *A. majus* sense dihydroflavonol reductase construct. Hatched bars; procyanidin units, white bars; prodelphinidin units and black bars; propelargonidin units.

If one assumes that there are normally at least two classes of DFR genes with differing substrate specificities being expressed in *Lotus*, an explanation is possible. Therefore if a gene encoding a DFR enzyme primarily involved in cyanidin biosynthesis (DFRc) is suppressed one would expect to note high delphinidin polymers. By contrast, if a DFR(d) gene were suppressed this could result in the biosynthesis of high cyanidin polymers. Interestingly two other sense DFR lines were noted with reduced levels of condensed tannin and with normal cyanidin and delphinidin ratios; perhaps these correspond to events where both DFR(c) and DFR(d) were suppressed. Southern analysis has indicated multiple DFR genes in *Lotus*[16] but the substrate specificities of these DFR genes has yet to be determined. In order to begin to gain a molecular understanding of the mechanisms giving rise to these novel tannin hydroxylation patterns it will be important to clone expressed members of the DFR gene family and to assess the substrate specificity of the enzymes encoded by the different family members.

Anticipated developments and future applications

Therefore, based upon our experiments outlined in the previous section using antisense and sense genes, the condensed tannin pathway appears to be highly manipulable in *L. corniculatus* and hopefully in other tannin-positive species. As regards future target genes for the pathway, a number of interesting prospects present themselves. Firstly there are additional untested strategies for altering the gross structure of condensed tannins. With regard to altering hydroxylation, in addition to existing strategies using DFR it may be possible to modify the degree of hydroxylation of dihydroflavonol intermediates directly using flavonoid 3' hydroxylase (F3'OH) and flavonoid 3'5' hydroxylase (F3'5'OH) genes. We are currently trying to overexpress a F3'5'OH cDNA in *L. corniculatus* in an attempt to increase polymer hydroxylation.

Another aspect which has yet to be discussed relates to the degree of polymerisation of condensed tannin molecules and their molecular weight distribution. A wide range of tannin molecular weights has been reported in different plant species but factors that control this important parameter are ill understood. Polymer length may be controlled by the relative levels of catechin 4-ol and catechin units ie. high levels of catechin 4-ol extension units may result in high molecular weight tannins while increasing the relative levels of catechin units may increase polymer termination and produce lower molecular weight polymers. If this model is correct then experiments aimed at modifying the relative expression of DFR: FDR (flavan 3,4 diol reductase) may be of interest. In this context we are carrying out work to determine changes in molecular weight in *Lotus* lines with modified levels of DFR expression. Furthermore, our previous work has suggested that DFR, at least partly, regulates pathway flux so engineering FDR might alter polymer molecular weight without altering tannin levels. Unfortunately FDR is as yet uncloned, but note a putative candidate sequence which has been cloned from the seed coat of Arabidopsis[22]. The final two enzymes in the pathway, condensing enzyme and polymerising enzyme, have not been cloned either. One could postulate that modifying the relative expression of these genes might also be predicted to alter polymer length.

As regards increasing or decreasing levels of condensed tannins, in addition to existing strategies based upon the modulation of CHS and DFR, other targets have yet to be explored. CHI, F3OH and F3'OH are all essential genes in the pathway and are interesting candidate genes but there are other possibilities. Modifying co-factor supply has been reported to alter lignin levels in transgenic plants[17] and by analogy it may be possible to perform similar modifications to the condensed tannin pathway. DFR and FDR are both reductases and both use NADPH for reducing reactions, therefore any genes that modify the supply of NADPH might have an effect on the overall expression of the condensed tannin pathway. The pathway also includes two P450 hydroxylases (F3'OH and F3'5'OH) and the genetic manipulation of genes co-acting with such hydroxylases[18] might be predicted to have a knock-on effect that alters flux in the metabolic pathways of condensed tannin biosynthesis. Finally, it may be possible to alter levels of condensed tannins by the introduction of genes that degrade these highly stable macromolecules. The identification, cloning and introduction of a microbial or plant-derived tanninase gene driven by suitable developmental promoters has been recently discussed[19] and is being actively pursued.

REGULATORY GENES AND PATHWAY TRANSACTIVATION

As discussed previously, for crops such as white clover and lucerne the major challenge for genetic engineers is the introduction of the condensed tannin pathway into the foliar tissues of these species. The background to this is that these species express the pathway in floral tissues but shoot tissues only contain flavanone, isoflavone, flavanol (and anthocyanin) end products. Therefore one can speculate that desired interventions are those which alter the tissue-specific expression of the tannin pathway. Modifying structural genes may not be useful for modifying tannin-negative tissues and studies on higher order regulatory genes may be of most interest for engineering white clover and lucerne.

From work on anthocyanin biosynthesis evidence suggests that both myb and myc regulatory genes are important in pathway regulation[20]. Similarly P, a myc, has been shown to be important in transactivating phlobophene accumulation in maize[21]. Up until now, no mybs or mycs involved in tannin biosynthesis have been reported. However in recent work, Damiani has reported that introducing Sn (a maize anthocyanin myc) into *L. corniculatus* results in the co-suppression of the condensed tannin pathway. The observations of Damiani and colleagues would appear to suggest that there is at least one myc homologue in *Lotus* that regulates the expression of this pathway[23].

If one continues to consider the potential transactivation of condensed tannins in forage legumes there are major questions which relate to how one can clone pathway regulator genes and then test them. We present here a scheme which indicates a possible approach to this problem and this is shown in Figure 6. The basic concept is to find a convenient test system in order to identify genes that are responsible for controlling the tissue-specific expression of the condensed tannin pathway. Putative genes could be existing transactivators or genes of unknown function that have been cloned using subtractive, mutational or tagging methodologies. It is possible that a number of these candidate genes have no relationship with the condensed tannin pathway whereas

other(s) may be intimately associated with pathway regulation or be involved in controlling cell-specific tannin accumulation (see Fig. 1). This issue can be clarified rapidly by preparing short sense or antisense constructs, co-transforming them into *L. corniculatus* and then screening resultant lines for loss-of-tannin phenotypes. Gene(s) that dramatically alter tannin accumulation are then prime candidates for being genes that will successfully transactivate the pathway. Genomic sequences can then be obtained and introduced both individually and in combination into species such as white clover. The prediction is that the introduction of one or more of these sequences will result in the successful transactivation of this pathway.

Source genes

a) Existing transactivators eg. anthocyanin mybs and mycs
b) Genes from subtractive hybridisation eg. cloned from high vs. low tannin genotypes
c) Putative transactivators cloned by other methods eg. from mutant and gene tagging experiments

⇓

Functional assessment in *L.corniculatus*

Take candidate genes and express in antisense or short sense in clonal genotypes of *Lotus corniculatus*

⇓

Analyse resultant lines. Measure levels of condensed tannins and determine which gene(s) result in a gross reduction in condensed tannins ie. a loss-of-function phenotype.

⇓

Application of functionally assessed genes

Isolate genomic sequences of functionally assessed gene(s) and introduce into white clover and lucerne. Screen resultant plants for transactivation of condensed tannin biosynthesis.

Figure 6. A scheme for the functional assessment of higher order regulatory genes relating to the condensed tannin biosynthetic pathway.

FINAL CONCLUSIONS

We hope that this chapter gives an indication of some future perspectives relating to the genetic manipulation of condensed tannins. As stated before, similar methodologies may be used to modify other secondary pathways in forage and fodder crops and tannins in food crops. As a final thought, it occurs to us that this technology may allow us to go beyond the traditional uses of forage crops. Our work on altering tannin structure opens up the possibility of 'designer tannins' being produced from forage crops such as *Lotus*. For example one can envisage a time when forages and fodders may be used as a source of speciality tannins for industrial applications.

Acknowledgements

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CHAPTER 9

METABOLIC ENGINEERING OF CROPS WITH THE TRYPTOPHAN DECARBOXYLASE OF *CATHARANTHUS ROSEUS*

V. DE LUCA

Introduction

A wide variety of plant families produce aromatic and indole amines *via* the decarboxylation of their respective amino acids. These reactions which are catalyzed by aromatic amino acid decarboxylases are commonly the first steps in the biosynthesis of many thousands of different plant alkaloids, which have a number of important physiological effects. In order to create crops that make useful alkaloids, genetic engineering methods for the production and accumulation of aromatic and indole amines need to be developed. This report describes the results of several transformation experiments to express the tryptophan decarboxylase gene in different species of plants.

Molecular cloning of aromatic amino acid decarboxylases

An important characteristic of plant aromatic amino acid decarboxylases is their high specificity for particular substrates[1-12] (Table 1). The TDCs of *Catharanthus* and *Camptotheca* show complete specificity for *L*-tryptophan, whereas those of *Papaver* and *Thalictrum* show specificity for tyrosine and DOPA. The enzymes from *Sanguinaria* and *Cytisus* only accept DOPA as a substrate. This survey suggests that aromatic amino acid decarboxylases with a particular spectrum of substrate specificities could be selected from the natural variability already occurring in plants. Other substrate-specific decarboxylases could, therefore, be cloned and used for genetic engineering of plants which would accumulate particular aromatic amines.

TDC from *Catharanthus roseus*[1] was the first aromatic amino acid decarboxylase from plants to be cloned. A full length cDNA clone was isolated by antibody screening. This clone showed significant amino acid similarity to the dopa decarboxylase of *Drosophila melanogaster* and to other animal amino acid decarboxylases. A second TDC from *Camptotheca*[5] was cloned recently, as were Tyrosine/dopa decarboxylases (TYDC) from *Papaver*[6,7], *Petroselinum*[8] *and Arabidopsis*[9]. The amino acid sequence similarities of these decarboxylases to TDC from *Catharanthus* were also very high, confirming the presence of a conserved gene family in plants and animals.

It is also interesting that in species such as *Catharanthus* a single TDC gene could be found (Table 1) whereas in *Camptotheca*[5] there may be 2 or 3 TDC genes. The parsely[8] and opium poppy gene[6] families may contain 4 and up to 14 TYDC genes, respectively.

R. Verpoorte and A.W. Alfermann (eds), Metabolic engineering of plant secondary metabolism, 179-194.

The presence of gene families in some species of plants and not in others, raises interesting questions concerning their biological roles. In opium poppy, this family of genes is responsible for the biosynthesis of a large diverse group of tetrahydroisoquinoline alkaloids and cell wall associated hydroxycinnamic acid amides which are produced in different plant organs. In situ RNA hybridization techniques have revealed that expression of two subgroups of differentially expressed TYDC genes comprising this family, are restricted to metaphloem and protoxylem of vascular tissues in mature stems and roots[13]. The differential and tissue-specific expression of various members of this gene family could, therefore, define which tissues would be active in biosynthesis and which products would be made. The restriction of TYDC expression to particular cells or tissues illustrates the strict control to which these genes are subjected and suggests that genetic engineering aromatic amine production in plants should probably take this factor into account.

Table 1. Biochemical and molecular properties of plant aromatic amino acid decarboxylases*

Species	Mr (kDa)	Substrate specificity (%) **			Gene #
		Tryp	Tyr	DOPA	
Catharanthus roseus ***	56	100[3,4]	0	0	1[1,2]
Camptotheca acuminata[5]	55.5	100	0	0	2
Papaver somniferum[6]	57-50	0	90	100	12-14
Petroselinum crispum[7]	57	100	100	28	4
Arabidopsis thaliana[8]	-	-	-	-	1
Thalictrum rugosum[9]	56.3	0	100	74	-
Eschscholtzia californica[9]	56.3	0	100	-	-
Syringa vulgaris[10]	-	0	100	63	-
Sanguinaria canadensis[10]	-	0	3	100	-
Hordeum vulgare[10]	-	0	33	100	-
Cytisus scoparius[11]	-	0	0	100	-

** Relative enzyme activities for each species are expressed as a% of the maximal acivity observed for the best substrate.

** Tryp, Tyr, and DOPA are *L*-tryptophan, *L*-tyrosine and *L*-dihydroxyphenylalanine, respectively.

*** Uppercase numbers represent references from which the information was obtained.

BIOCHEMICAL AND MOLECULAR PROPERTIES OF *CATHARANTHUS* TDC

The importance of TDC as a rate limiting step in indole alkaloid biosynthesis is based on the role of this enzyme in diverting primary metabolites into secondary pathways[14]. Early biochemical studies in cell suspension cultures[14-16] and with developing seedlings[17-19] have demonstrated that under all conditions tested, the

expression of TDC enzyme activity is transient. Studies with cell suspensions have shown that the half-life of TDC activity is 21 h under culture conditions which activate alkaloid biosynthesis[15]. Similar results were obtained when the inducing stimulus was elicitor treatment[16], but evidence was also presented to suggest that the cellular concentration of tryptamine could regulate the expression of TDC.

Studies with *Catharanthus* seedlings have shown that expression of this enzyme is also under developmental control[17]. TDC activity appears transiently for a brief 48 hr period during seedling development. The appearance of enzyme activity coincides with the transient production of TDC protein[17], followed by the accumulation of indole alkaloids[19] and by the production of TDC proteolysis products[18,19]. These chemical and biochemical studies suggest that the pathway of indole alkaloid biosynthesis is coordinately activated to mobilize tryptamine into alkaloids. Biochemical evidence also indicates that TDC may exist in the cell in equilibrium between a stable active dimer and a monomer which is susceptible to irreversible inactivation followed by proteolysis[18]. The process of TDC breakdown appears to involve ATP-mediated binding of ubiquitin and proteolysis by the ubiquitin-pathway[20]. This cytosolic pathway for protein breakdown is well known to select for proteins with short half lives[21], and suggests that TDC may indeed be in a dynamic state of turnover, ready to respond to cellular requirements for tryptamine.

Studies at the molecular level have corroborated the transient and regulated the expression of the TDC[3,22-24] and TYDC[6-9,13] genes (Table 1) in cell suspension cultures and in intact plants. Gene expression can be activated transiently as a result of elicitor treatment[5,8,9,22-24], auxin starvation[23,24], jasmonate treatment[5] or as a result of tissue-specific developmental expression in intact plants [3,13,23]. It is also relevant to note that auxin treatment activates rather than represses TDC activity in *Catharanthus* seedlings[25]. The multilevel biochemical and molecular regulation observed for expressing aromatic amino acid decarboxylases in plants raise important questions concerning genetic engineering of pathways for the production of aromatic amines.

Expression of TDC in transgenic tobacco plants

A full length TDC cDNA clone isolated from *Catharanthus roseus* and driven by the CaMV 35S promoter was introduced into tobacco[26-28]. Several dozen independent transgenic tobacco plants which had 4 to 45 times the TDC activity of nontransformed controls were initially produced in 1990[26]. The experiment was repeated in another laboratory in 1994[28] and essentially identical results were obtained. Tryptamine accumulated in transgenic TDC expressing tobacco plants to levels which correlated with both enzyme activity and transcript levels[26,28]. Plants with the highest TDC activities accumulated up to 1% of their dry weight as tryptamine (Table 2), but did not appear to have any unusual phenotype as a consequence of its accumulation. Several correlative studies have suggested that tryptamine is a biosynthetic precursor of the plant hormone, indole acetic acid (IAA). Measurements using a monoclonal antibody to IAA failed to detect any differences in the levels of this auxin in high tryptamine accumulating transgenic plants compared to non-transformed plants[23].

Table 2. Comparison of TDC activity compared to tryptamine and tyramine content in leaf tissues control and CaMV 35S-TDC transformed tobacco plants *

Plant	TDC activity (pmol/μgprotein/h)	Tryptamine (μg/g fresh wt)	Tyramine (μg/g fresh wt)
Control	1.3	4	40
T-150 **	5.8	247	897
T97	13.4	344	690
T-183	13.6	357	347
T-105	15.1	497	221
T-186	17.2	480	63
T-180	19.2	310	38
T-210	21.4	667	43
T-128	25.2	345	447
T-118	29.8	637	22
T-157	32.3	401	1200
T-148	40.9	1077	108
T-162	41.6	906	105
T-201	64.3	1085	65

* Data reproduced and modified from References 26 & 27.

** The different transgenic lines are from separate transformation events.

These surprising results prompted more detailed studies[28] to determine how increased requirements for tryptophan are accommodated by the shikimate pathway of TDC expressing transgenic tobacco plants. Anthranilate synthase and chorismate mutase regulate the amount of chorismate being incorporated into tryptophan or phenylalanine and tyrosine, respectively (Fig. 1). Assays of these branch enzymes revealed that their activities were not affected by the increased requirements of TDC expressing plants for tryptophan. These results were interpreted to mean that normal tryptophan biosynthesis was sufficient to supply the needs for this essential metabolite in TDC expressing transgenic tobacco. The results[26,28] also raised hopes that transformation of any plant species with a particular decarboxylase would create an instant supply of the related amine, since the amino acid requirements could easily be met by the plant. These amines could in turn be used as precursors for biosynthesis of related secondary metabolites, including alkaloids.

The L-aromatic amino acid decarboxylases which have been isolated to date (Table 1) from plants appear to have rather distinct specificities for their aromatic substrates. TDC from *Catharanthus roseus* will only catalyze the decarboxylation of tryptophan, but not of DOPA, phenylalanine or tyrosine. Transgenic tobacco plants which express TDC were, however, also reported to accumulate high levels of tyramine[27] (Table 2). Interestingly, it was reported that 65% of TDC transformed tobacco plants also accumulated tyramine in addition to tryptamine. The apparent contradiction between the reported

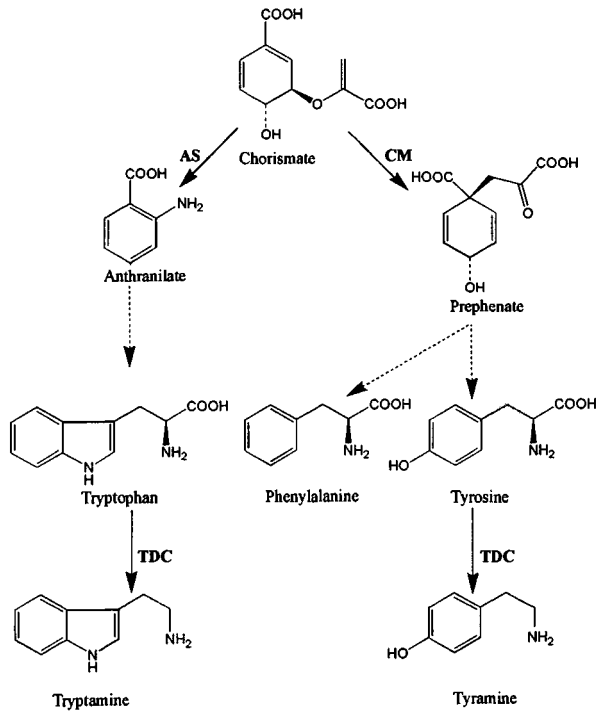

Figure 1. Branch points of the shikimate pathway leading to the biosynthesis of aromatic amino acids and to aromatic amines. Anthranilate synthase (AS), chorismate mutase (CM), tryptophan decarboxylase (TDC).

specificity of TDC for tryptophan (Table 1) and the accumulation of tyramine in TDC expressing tobacco plants, has yet to be explained.

We have attempted to repeat these studies with the selfed progeny of transgenic TDC expressing tobacco lines in Table 2. These lines clearly accumulate tryptamine to the levels reported previously[26], but we have been unable to detect tyramine levels beyond those occurring in non-transformed plants. The results shown in Table 2 clearly display the relationship between TDC enzyme activity and tryptamine content, whereas lines with low TDC activity (T-97, T-183) appear to accumulate high levels of tyramine. This suggests that if these lines are producing tyramine, it may not be due to TDC activity, but to an inducible endogenous TYDC[29]. Previous studies have shown that tobacco plants infected with tobacco mosaic virus[29] accumulate high levels of tyramine as a result of induction of TYDC. In addition, healthy tobacco plants also accumulate some tyramine in stems and in large amounts in flowers. One biological function of tyramine in tobacco appears to be in the biosynthesis of phenolic amides which become structural cell wall components of vascular tissues and of infected cells[29-31].

COMPARISONS BETWEEN TDC EXPRESSION IN CANOLA, POTATO AND TOBACCO PLANTS

After transformation and selection on kanamycin, 66 canola and 22 potato plants were regenerated, respectively. Unlike untransformed control plants, 7 canola and 13 potato plants showed varying levels of TDC activity which accumulated corresponding amounts of tryptamine. Stability of transformation with the TDC gene was tested over 3 generations for transgenic tobacco and canola plants. All tobacco transformants retained their respective TDC enzyme activities and tryptamine accumulating capabilities. The canola transformants completely lost their TDC activities by the 3rd generation, but they retained their resistance to kanamycin. The TDC-expressing transgenic potatoes which were propagated vegetatively through tuber production, maintained their respective TDC activities and tryptamine accumulation over a 3 year period.

The transformation of three different crop species with a TDC driven by the CaMV 35S promoter, resulted in plants with a range of enzyme activities and tryptamine accumulation capabilities. Tobacco appeared best suited to accommodate the highest levels of TDC activity (Table 3) with line T-201-1 having the highest specific activity and accumulating over 1 mg of tryptamine per gram fresh weight of tissue. Compared to this, the highest TDC expressing potato line M-9-D-21 only showed 30% of the specific activity of tobacco and it accumulated less than 8% of the tryptamine found in tobacco line T-201. The highest TDC-expressing canola line, ST-062, showed only 9% and 2%, respectively, of the TDC activity and tryptamine accumulation found in tobacco line T-201-1. In contrast, non-transformed control plants had neither TDC activity nor did they accumulate tryptamine (Table 3).

When transgenic plants with similar TDC specific activities from each of the three species were compared, they clearly accumulated different levels of tryptamine (Table 3). Although canola line St-062, potato line M-9-D-23 and tobacco line T-150-3 had a similar TDC specific activity varying between 5.4 to 6.3 units, the tobacco line accumulated 4.5- and 10.5- fold more tryptamine than the potato and canola line, respectively. These results raise important questions about the capabilities of different plant species to accommodate novel pathways where the supply of substrate may be limiting.

Transformation of canola and potato lines were also confirmed by the detection of TDC mRNA in all transformed plants (Fig. 2)[32] but not in wild type canola (Fig. 2 lane 14, row 2), potato (Fig. 2, lane 14, row 3) or tobacco (Fig. 2, lane 14, row 4). The relative levels of TDC mRNA in each species were consistent with the specific activities and the levels of tryptamine accumulation of individual plants. TDC mRNA was most abundant (Fig. 2, lanes 9-13, row 4) in tobacco where the highest enzyme activities and tryptamine accumulation occurred (Table 3), whereas it was correspondingly less abundant in transgenic potato (Fig. 2, lanes 1-13, row 3) and canola (Fig. 2, lanes 1-8, row 2) lines, respectively. It is interesting to note that the mRNA from *Catharanthus roseus* which has the highest TDC enzyme activity (Table 3) was not detected under the conditions used in this experiment (Fig. 1 lane 14, row 1), but prolonged exposure of slot blots resulted in detection of TDC mRNA from *Catharanthus* tissue. These results suggest that although TDC mRNA levels were significantly higher in all transgenic plants tested, this alone was not sufficient to produce the high TDC enzyme activities observed in *Catharanthus* tissues.

Table 3. Comparison of TDC activity and tryptamine content in young leaves of wild-type and CaMV 35S-TDC transformed canola, potato and tobacco plants.

Species Lines	Plant Coordinates*	Blot Activity**	TDC (ug/g fw)	Tryptamine
Canola	ST-004	1	1.9	1
	T-005	2	2.2	10
	ST-045	3	3.6	15
	ST-067	4	4.1	15
	ST-053	5	4.3	18
	ST-029	6	4.3	15
	ST-062	7	5.4	19
Wild type	14	0.9	0	
Potato	M-9-D-17	1	3.3	35
	M-9-D-02	2	3.5	22
	M-9-D-05	3	5.2	44
	M-9-D-23	4	6.3	45
	M-9-D-11	5	8.5	73
	M-9-D-24	6	8.7	49
	M-9-D-12	7	9.3	54
	M-9-D-13	8	10.1	48
	M-9-D-10	9	12.7	85
	M-9-D-08	10	15.6	43
	M-9-D-16	11	16.0	58
	M-9-D-25	12	16.9	95
	M-9-D-21	13	17.2	80
Wild type	14	1.1	0	
Tobacco	T-150-3	9	6.2	201
	T-105-1	10	17.8	510
	T-157-2	11	28.9	604
	T-162-3	12	50.1	850
	T-201-1	13	57.6	1040
Wild type	14	1.2	4	
Catharanthus roseus	Wild type	14	124.7	0

* The blot coordinates refer to Figure 2 which displays a block diagram of densitometric scans of total RNA slot blots of transgenic and nontransformed plants. The numbers are related to the those of the x-axis in Figure 2. values i are the relative% densities of each plant in relation to tobacco cell line T-201-1 which has the value of 100%.

** pmol tryptophan converted to tryptamine/ug protein/h

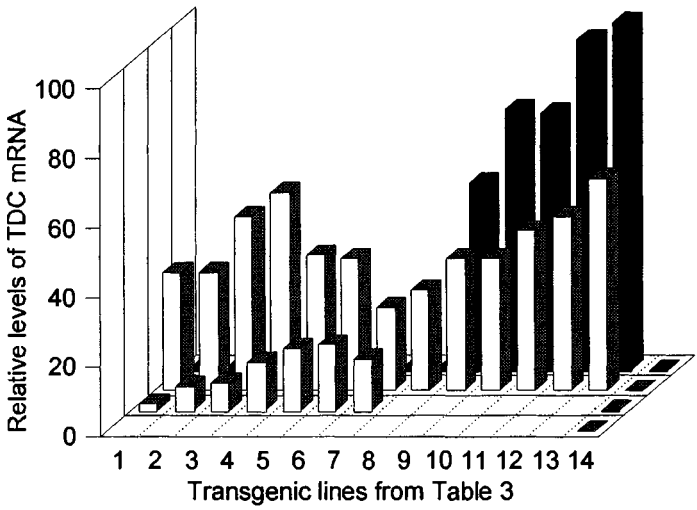

Figure 2. Comparison of relative TDC mRNA levels in Catharanthus roseus (first row, lane 14), wild-type canola (second row, lane 14), potato (third row, lane 14) and tobacco (fourth row, lane 14) compared to those in 7, 13 and 5 transformed canola, potato and tobacco lines, respectively. The densitometric scans of an RNA slot blot probed with ^{32}P-labeled 1.6 kb EcoR 1 TDC cDNA fragment are expressed as a % of the most intense blot obtained for tobacco line T-201-1. The data in this figure which has been modified from is related to the lines reported in table 3.

IMMUNOLOGICAL DETECTION OF TDC IN TRANSFORMED PLANTS

Initial studies were performed with crude extracts of transgenic canola, potato and tobacco. Tissues were processed for immunoblotting as described by Fernandez *et al.*[17] but no TDC antigen was detected with this procedure. Using this procedure for *Catharanthus roseus* seedlings, TDC immunoreactive protein is easily detected in immunoblots from protein extracts representing as little as 0.3 seedlings (Fig. 3, lane 7). In order to detect TDC on immunoblots of these extracts from canola, tobacco and potato, the enzyme was purified 50-fold by gel filtration and anion exchange column chromatography. Partially purified protein extracts were processed for SDS PAGE, electrophoretic transfer and immunoblotting with anti-TDC antibodies[17]. Extracts from each transgenic plant now revealed the presence of an immunoreactive protein identical in size to that of *Catharanthus roseus* TDC (Mr 56 kDa) (Fig. 3, lane 7) in extracts from transgenic canola, potato and tobacco (Fig. 3 lanes 1, 3 & 5 respectively). In contrast, no immunoreactive proteins were detected in partially purified extracts of non-transformed canola, potato and tobacco (Fig. 3, lanes 2, 4 & 6, respectively). These results are consistent with those reported above. High mRNA levels in transgenic plants don't necessarily produce high TDC protein levels or enzyme activities. It is not surprising in this context that *Catharanthus roseus*, which is the source plant for TDC makes best use of this gene.

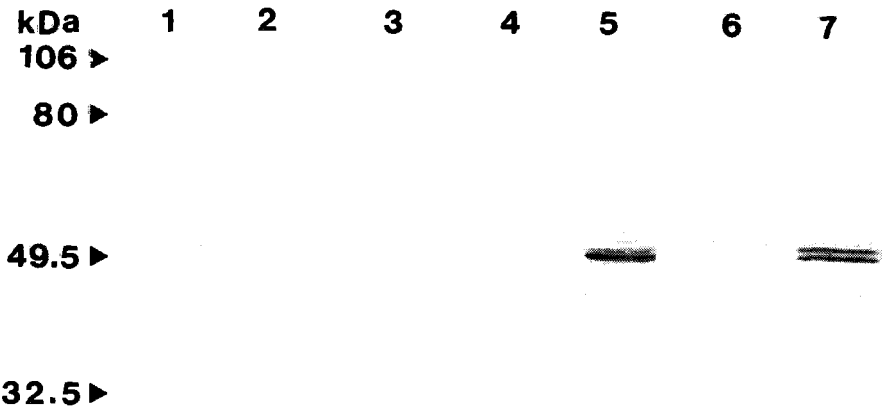

Figure 3. Immunoblot of TDC antigens in: partially purified extracts in transformed canola (lane 1), potato (lane 3), tobacco (lane 5); partially purified extracts of non-transformed canola (lane 2), potato (lane 4), tobacco (lane 6); crude extracts of *Catharanthus roseus* (lane 7).

TRYPTAMINE CONTENT OF TRANSGENIC PLANT PARTS

Individual organs of each transgenic plant were analysed for tryptamine content (Fig. 4). The levels of tryptamine were highest in all organs of transgenic tobacco where it was 2 orders of magnitude higher than in potato and 3 orders of magnitude higher than in canola. Tryptamine was notably absent in canola and tobacco seeds as well as in potato tubers, whereas the skin of potato tubers contained 14 μg tryptamine/gram fresh weight of skin.

Other effects of metabolic engineering plants with TDC

The multi-level regulation of aromatic amino acid decarboxylases described above suggests that plants exercise clear control over which stage of plant development synthesis will occur, the cellular sites of production and the amounts of amine to be produced for a given purpose. It is not surprising, therefore that expression of TDC under the control of the constitutive CaMV 35S promoter in different plants appears to have a number of interesting but unpredictable metabolic effects. For example plants with constitutive TDC activity may exercise continual pressure on the tryptophan pool and it is not clear how this would affect regulation of the shikimate pathway. In a different context, would expression of this gene enhance tryptamine-based product formation?

REDIRECTION OF TRYPTOPHAN

Brassica napus (canola) is a commercially important oil crop which is also an important source of protein meals after extraction of oil from the seed. These meals contain high levels of indole glucosinolates which decrease the quality and commercial value of the canola protein meals. Expression of the TDC gene in transgenic canola plants resulted in greenhouse-grown plants which accumulate tryptamine rather than tryptophan-derived indole glucosinolates[34] (Fig. 4). The successful competition for tryptophan by the TDC transgene was especially evident in transgenic canola seeds which only accumulated 3% of the indole glucosinolates normally found in control seeds. The establishment of a successful competition for a common substrate reflected the potential value of this technique to divert metabolism away from a secondary metabolite normally accumulating in the plant to a totally novel artificial product. The experiment also reflected the limited availability of tryptophan in canola since the transgenic plant was unable to accommodate the competing demands of the natural and the artificial metabolic sink for this substrate. The ability of TDC to compete for tryptophan was also tested when transgenic canola lines were grown under field conditions in 1994 in Saskatchewan, Canada[35]. Under these conditions, indole glucosinolate levels were identical in both transgenic and non-transformed seedlings. Although the discrepancies between plants grown in a greenhouse and those grown in the field remain to be elucidated, it is clear that plants submitted to environmental stress may behave very differently than those raised in the greenhouse.

COMPLEMENTING A RATE-LIMITING STEP IN A BIOSYNTHETIC PATHWAY

Cell cultures of *Peganum harmala* which synthesize and accumulate β-carboline alkaloids and serotonin may lose this ability under certain culture conditions. Serotonin is synthesized *via* a 2 step pathway involving decarboxylation and hydroxylation (Fig. 5). Biochemical studies have shown that loss of TDC rather than hydroxylase activity was responsible for cessation of serotonin biosynthesis[36]. In order to prove this, the TDC gene from *Catharanthus* was also expressed constitutively in cell cultures of *Peganum harmala*[37]. Transformed cell cultures which expressed up to 8 times more TDC activity than untransformed control lines did not produce increased levels of tryptamine. Instead they accumulated 10-fold more serotonin compared to non-transformed cultures. These results show that in *Peganum harmala*, TDC can catalyzes a rate limiting step in serotonin biosynthesis. Furthermore, by feeding tryptophan to transgenic cell lines, they accumulated up to 40-fold more serotonin than untransformed cultures which were also fed tryptophan. These results show that complementing one rate-limiting step may be replaced by a new bottleneck, in this case the supply of tryptophan substrate.

The role of TDC as a bottleneck enzyme was also studied in transgenic crown gall tissues of *Catharanthus roseus* over expressing this gene[38]. Transformed tissue clearly displayed a several-fold increase in TDC protein, enzyme activity and tryptamine accumulation, whereas no increases in complex monoterpenoid indole alkaloid accumulation were observed. The contrasting results obtained in *Peganum*[37] and in *Catharanthus*[38] outline the unpredictability of metabolic engineering experiments when multi-step pathways are involved.

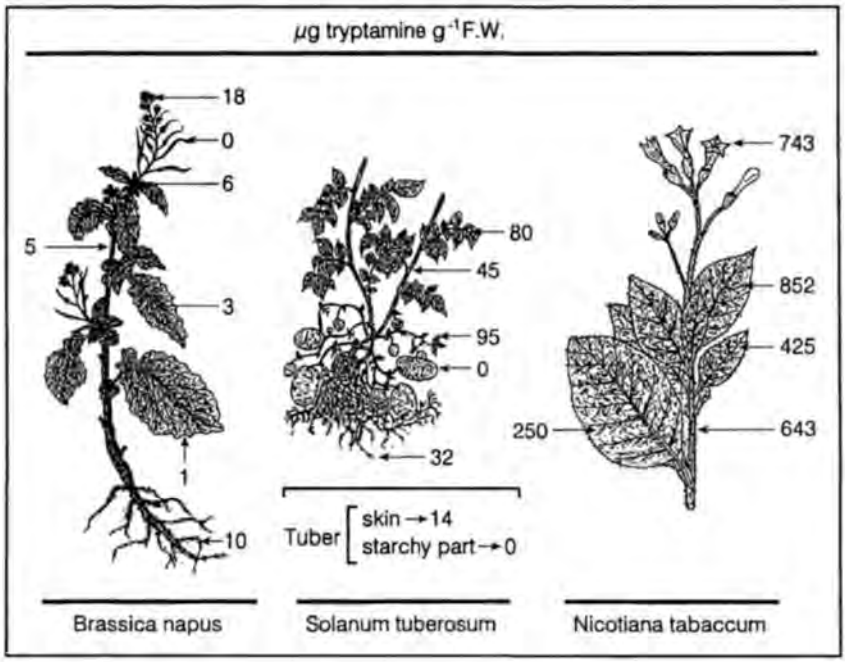

Figure 4. Tryptamine content of individual organs in transgenic canola line St-062, potato line M-9-D-21 and tobacco line T-201-1.

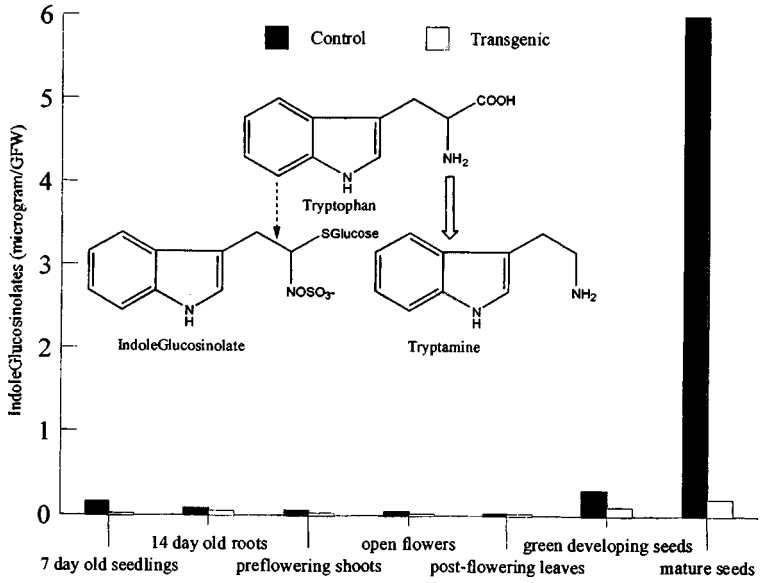

Figure 5. Indole glucosinolate content in different organs of TDC-expressing Canola line St 062 compared to wild-type plants. Reprinted with permission from Chavadej S, Brisson N, De LucaV. Proc Nat Acad Sci 1994; 91: 2166-2170.

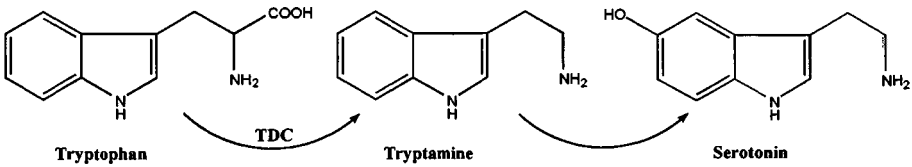

Figure 6. Pathway leading to serotonin biosynthesis from tryptophan in *Peganum harmala.*

Transgenic tobacco cell suspension cultures expressing both TDC and strictosidine synthase were also produced recently[39]. Although transgenic tobacco plants expressed both TDC and strictosidine synthase activities, they only accumulated small amounts of tryptamine. Strictosidine synthase catalyzes the condensation reaction between tryptamine and the terpenoid, secologanin, to yield the monoterpenoid indole alkaloid, strictosidine. Feeding studies with secologanin demonstrated that transgenic tobacco expressing both enzyme activities accumulated strictosidine in the medium. It was concluded from these studies that expression of foreign genes from a common pathway could function cooperatively in tobacco to make reaction products if the necessary substrates are present.

COMPLICATIONS OF METABOLIC ENGINEERING WITH TDC

The creation of a TDC artificial metabolic sink in potato revealed even more striking and subtle features concerning the regulation of the shikimate pathway[40]. Studies conducted with transgenic potato tubers showed that they contained tryptophan levels which were approximately 30% lower than non-transformed tubers. The pools of phenylalanine in transgenic tubers were at least 50% lower than in control tubers, whereas tyrosine pools were the same in both types of tubers. The meaning of these results is difficult to explain since only the peels of potato tubers appear to have any TDC enzyme activity (Fig. 4). Expression of TDC activity in the potato peel or completely outside the tuber appears to affect in an unknown manner the pool sizes of phenylalanine and tryptophan inside the tuber.

The maintenance of low tryptophan pools in transgenic tubers in relation to controls was dramatically increased when they were wounded, since 72 h after wounding the levels of tryptophan in transgenic tubers were at least 10-fold lower than in control wounded tubers. The altered tryptophan pool size could be directly attributed to wound-induced activation of tryptophan decarboxylase and the resulting increased competition for tryptophan occurring in the cell. In contrast, the phenylalanine pools did not change either in wounded control or in transgenic tubers. The phenylalanine levels in control wounded tubers did, however, decrease to those occurring in transgenic tubers after treatment of tubers with arachidonic acid elicitor, an activator of the hypersensitive reaction in potato.

Chlorogenic acid which is the major soluble phenylpropanoid which accumulates in wounded potato tubers (Fig. 7), is produced from caffeic and quinic acid precursors that

are derived from shikimate pathway intermediates phenylalanine and dehydroquinate, respectively. The levels of wound-induced accumulation of chlorogenic acid was found to be 2- to 3-fold lower in wounded transgenic tubers compared with non-transformed controls (Fig. 7) and this also affected the ability of transgenic tubers to produce polyphenolic compounds such as lignin. Treatment of wounded tubers with arachidonic acid led to a dramatic accumulation of soluble and wall-bound phenolic compounds in tubers of untransformed plants but not in transgenic tubers.

As a result of weakened cell walls, transgenic potato appeared to be more susceptible

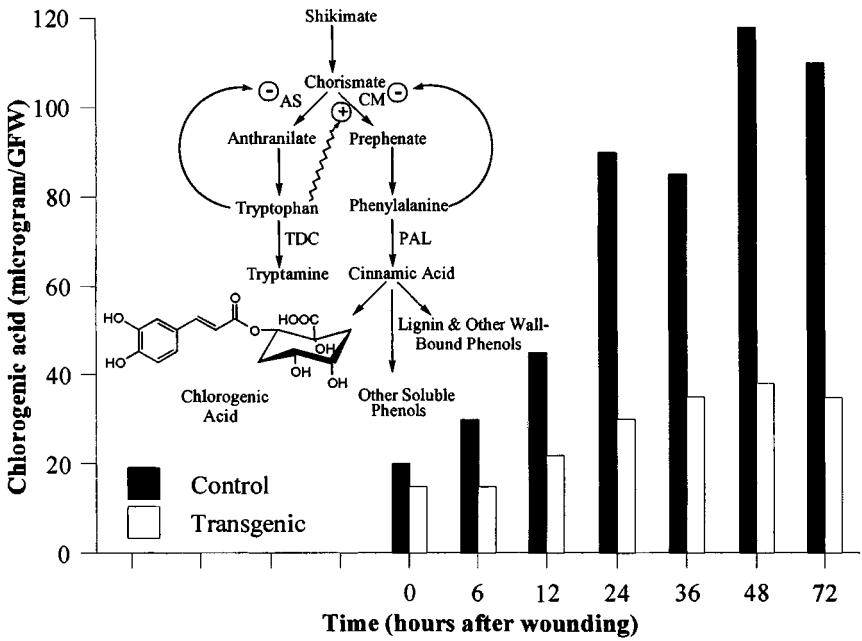

Figure 7. Time course of chlorogenic acid accumulation in wounded transformed (line M-9-D-25) and wild-type potato tubers. Reprinted with permission from Yao K, De Luca V, Brisson N. Plant Cell 1995; 7:1787-1799.

to the fungal pathogen, *Phytophthora infestans* than non-transformed tubers. This increased susceptibility to *Phytophthora* with transgenic tubers dramatically illustrates how altering the availability of tryptophan also changes the supply of phenylalanine, thus creating serious and unexpected consequences.

It is interesting that the reduced tryptophan and phenylalanine pools observed in transgenic tubers appear to affect their ability to produce wound-induced antimicrobial defense compounds such as chlorogenic acid. It is well known that tryptophan inhibits its own synthesis through feedback inhibition of anthranilate synthase and feedback activation of chorismate mutase (Fig. 1 and 7)[41]. These biochemical properties suggest that a decrease in the concentration of tryptophan increases the flux of chorismate through the tryptophan branch of the pathway and decreases the flux of chorismate through the phenylalanine branch of the pathway[40]. *In vivo* labeling experiments with ^{14}C-shikimic

acid have provided experimental support that expression of the TDC gene in transgenic tubers increased the flux of the tryptophan branch of the shikimate pathway[40].

Numerous physiological studies in the past have provided correlative evidence concerning substrate-based regulation of metabolism to explain why the quantity of phenylpropanoids produced did not always match the level of phenylalanine ammonia lyase activity[42]. The studies with TDC expressing transgenic plants also infer that the shikimate pathway is in part regulated by the availability of aromatic amino acid substrates. It is now clear that a firm understanding of the mechanisms by which plants supply aromatic amino acids for secondary metabolite production is required. This information could help to define the parameters which must be fulfilled before a particular cell can be harnessed to produce secondary metabolites. These requirements may include development-, cell- or organ-specific gene expression of the transgene in locations where substrate supply is not limiting. Our knowledge concerning these parameters is still quite primitive and offer many new opportunities for research.

Conclusions

The present overview describes what we have learned from attempts to constitutively express TDC into potato, canola, tobacco, *Peganum* and *Catharanthus*. A number of guidelines can be inferred from the results obtained. The high degree of regulation under which plant aromatic amino acid decarboxylases seem to operate suggest that it is a mistake to try to express these genes under the control of constitutive promoters like CaMV. While many of the results obtained in these studies are interesting, a more rational approach to expression of TDC is required for potential commercial applications. This includes knowledge of the sites in the plant where the amino acid substrate is not limiting and coupling of this information with cell- or tissue-specific expression of TDC in order to assimilate the substrate for product formation. Additionally, efforts should be made to develop crop plants which accumulate desired substrate in particular target tissues in order to facilitate metabolic engineering.

Acknowledgements

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CHAPTER 10

GENETIC ENGINEERING OF ENZYMES DIVERTING AMINO ACIDS INTO SECONDARY METABOLISM

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Introduction

At the end of the 70ties, plant tissue cultures were expected to become an alternative source to field grown plants for commercially important compounds (secondary metabolites). The idea was to grow suspension cultures of medicinal plants in huge bioreactors and to isolate from their biomass the desired compounds, for example morphinanes, quinine or cardiac glycosides. It was assumed that one would produce the compounds under better controlled conditions, independent of the climate and could react more flexible to changing demands of the market. For several reasons this ambitious goal has not yet been acchieved. The most serious problem is the fact that nearly all commercially attractive candidates for this technology are produced only in low amounts or are even lacking in biotechnologically relevant tissue cultures. Biotechnologically relevant means that product formation occurs or is stably inducable in rapidly growing cultures. Though all available conventional techniques (screening, selection, production media, biotic and abiotic elicitors) have been applied to these cultures, their productivity could not be improved distinctly. Presently, we have to accept that only some compounds of minor or no commercial impact can be produced by tissue cultures in high levels so that a commercial production could be envisaged (see for review[1]). It is more and more believed that the application of new, especially genetic techniques is required to express the really interesting pathways in cultured cells.

Evidently, some pathways are well and spontaneously expressed in cultured cells while others remain repressed. This phenomenon is not surprising if one looks at the intact plant. Some compounds are biosynthesized only in leaves but not in roots, others are synthesized in roots but not in flowers etc. Morphologically undifferentiated cells represent a special state which allows the expression of some but evidently not of all pathways. Clearly, the initiation of many secondary pathways depends upon the morphological differentiation of the plant cell. This has nicely been proven when non-producing undifferentiated suspension cultures were changed into shoot or root cultures. Such redifferentiated cultures synthesized a compound pattern similar to that found in the corresponding organ of the intact plant. Thus, the undifferentiated non-producing cell usually contains the genetic information for the production of the compounds but does not express the signal(s) for turning on their synthesis. Although some pathways can be induced by stress factors and elicitors, we are still far away from knowing which specific internal signals make, for example, a tobacco root cell

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produce the alkaloid nicotine. Expression of signals turning on desired pathways in all cells independent upon their morphological state would greatly improve the standing of the tissue cultures as producers and may be required for a broad breakthrough of this technology to commercialization.

Although the search for specific signals leading to the expression of whole pathways seems to be most logical, a straightforward approach for going this way is not yet possible. However, the easiness of establishing nowadays stable hairy root cultures by transforming the plant with *Agrobacterium rhizogenes* has made more pathways available for such studies. Comparison of suspension cultures in which the target pathway is repressed and of root cultures where the pathway is well expressed may become a suitable system for detecting such pathway inducing signals. Today we can make only rather small steps towards improving the production of secondary compounds in cultured cells by genetic engineering.

Improvement of secondary product levels by genetic engineering - some general considerations

Metabolic engineering of pathways in microorganisms and higher plants has become an area of great interest during the last 5 years and various strategies have been applied.[2-6] Our goal has been to look for systems where the genetic modification will lead to increased production of a target compound in all probability. Therefore this chapter is restricted to only the approach which seems presently to be the most promising for our aim. We have decided to overexpress enzymes which are limiting in a pathway. It seems to be logical that overexpression of a limiting enzyme activity leads to overproduction of the limiting metabolite. The overproduced metabolite is expected to pass through its natural pathway(s) so that the levels of the final product(s) might be greatly enhanced. We were aware that this was a very simplified view and we accepted that the number of plant secondary pathways might be rather low to which this approach might successfully be applied. However, we believed that a genetically engineered overproduction of a target compound will give new ideas and clues how to further improve productivity.

If one wants to start metabolic engineering of a pathway only when the chances for increasing the levels of a target compound look good, one has to analyze especially critically which systems are presently suitable for this purpose. Therefore we would like to develop here how we found our systems described below. For establishing a rational project for production improvement according to our approach, we have to find out which enzyme is the limiting one. How can flux limiting enzymes be identified? If all enzymes of a pathway are known and fully characterized, one may be able to recognize a limiting enzyme from that knowledge. However, this does not yet seem to be an applicable approach for secondary pathways. In contrast to enzymes of primary metabolism the enzymes of secondary pathways are often not that well characterized or sometimes are even unknown. This limitation excludes presently several commercially attractive secondary pathways from flux enhancing metabolic engineering approaches. In addition, synthesis and turnover rates of the individual enzymes may change in

an unpredictable way upon the physiological state of the cell. Up to now, nobody engaged in the engineering of plant secondary metabolism has used the above approach for enhancing the carbon flow through a pathway. Therefore it remains undecided whether metabic control analysis or flux analyis[6] might today successfully be applied to secondary pathways of higher plants. A more convenient way for identifying limiting sites is to look for changes of enzyme activities when the biosynthesis of a secondry pathway is induced. Such inductions have nicely been studied in plant cell culture systems. One may assume that an enzyme activity is probably limiting which is greatly stimulated when product formation is induced in the cells, for example, by the addition of an elicitor. However, most often the activities of several enzymes of a pathway are greatly induced when a biosynthetic pathway is activiated. Thus, it is not that easy to identify in such cases which enzyme has the most limiting position. It is also questionable whether overexpression of one of these induced enzymes would be sufficient to effect product levels of that pathway. The easiest and best manifested way to recognize the limiting character of an enzymatic step is to demonstrate by feeding that the product of the enzyme reaction is limitinmg in that pathway. Unfortunately, intermediates of a pathway are not always available, feeding may be disturbed by artificial side reactions, uptake rates can be poor or channelling may prevent entrance of the fed intermediate into the pathway. Nevertheless, if it works it is the best proof.

After having identified the limiting step of a pathway, one has to look whether the corresponding gene is available. This is presently quite often not the case. Plant molecular biology studies of secondary metabolism have mainly been concentrated to a few pathways and these studies are rarely intended for obtaining production improvements. For example, many genes involved in the phenlypropanoid metabolism including lignin, flavonoids and anthocyanins have repeatedly been isolated from several sources by various researchers.[7] and literature cited therein For other secondary pathways just one, two or three cDNAs have been cloned, sometimes not a single gene is available. Momentary, metabolic engineering of plant secondary pathways is still hampered by the lack of suitable genes. As metabolic engineering of secondary pathways is still underdeveloped, one may prefer to perform the first metabolic engineering steps with genes which are not restricted to only one specific pathway. That is perhaps one reason why genes linking primary and secondary metabolism have quite often been cloned.

It seems also to be important to know where the enzyme is located in the cell. The engineered enzyme has to be supplied with substrate. As we cannot assume that the substrate is freely floating through the cell, we should try to target the overexpressed enzyme to the compartment where the natural enzyme is located. The tissue where the enzyme is overexpressed should be able to synthesize the target compound. These are in our eyes the first important questions to be answered when one starts production improvement of a culture system by genetic engineering. If the transformation results in the overexpression of the limiting enzyme it can be predicted that the levels of the target compound are enhanced.

Branching point enzymes

As pointed out the identification of a flux limiting enzyme is not always easy. Therefore one may also ask where such enzymes are likely located in a pathway. Although a simple answer fitting to all pathways cannot be given, it seems to be reasonable that enzymes located at branching points are potential candidates. There are two types of branching points - first, the site where primary metabolites are diverted into secondary pathways and second, branching site(s) within a pathway. Indeed secondary pathways are often branched and many branches are under developmental control. In the first view such enzymes seem to be the most interesting ones. Enzyme activities of these enzymes are often only detected after morphological differentiation/specialization and thus their absence prevents the production of the desired compounds in undifferentiated cells. A good example is putrescine N-methyltransferase involved in the biosynthesis of tropane and pyrrolidine alkaloids in the roots of a number of Solanaceae (see Fig. 5). The biosynthesis of these alkaloids depends upon its presence.[8] If it is proven that only the enzyme at the internal branching point, for example putrescine N-methyltransferase, is lacking, one could express the corresponding gene under a constitutive promoter permanently and could expect to produce the compounds of this branch (in this case: nicotine) in undifferentiated cell. However, we do not know of an example where simple overexpression of an internal pathway branching enzyme would have this effect. The problem is quite often that not only the branching enzyme but also the whole branch is under developmental control. This means that one would have to do the metabolic engineering in cells of the morphological background where the pathway is active. For example, one would have to overexpress putrescine N-methyltransferase in root cells for increasing tropane alkaloids or nicotine. Trying to overexpress this enzyme in roots would, however, only be useful if its limiting character has been proven.

Some enzymes are not specific for only one pathway. A good example for this are the three enzymes of general phenylpropanoid metabolism (Fig. 1) which are involved in the biosynthesis of flavonoids, anthocyanins, lignanes, lignin, coumarins etc. Some of these pathways require a specific morphological background to become expressed. Even if it is shown that one of the three general phenylpropanoid enzymes is limiting in a specific pathway, product increases may only be seen if the overexpression occurs in the morphological background where the specific pathway is active. The question arises whether these enzymes of general phenylpropanoid metabolism which are active in specialized cells, are all under the same regulatory control. This is indeed rather unlikely. The fact that several enzymes of secondary pathways are members of gene families and that some of them react differently to external factors (*e.g.* biotic and abiotic elicitors), allow the conclusion that these seemingly "unspecific" enzymes are also under developmental control. They are often co-activated together with branch-specific enzymes. If a pathway is activated only as a complete unit, an enzyme at a branching site (according to textbook drawings) would only be a member of a biosynthetic chain without branching. This should make clear that the impact of apparent branching points within a pathway has to be analyzed in each case for its limiting character and position.

The branching point between primary and secondary metabolism seemed thus to be the "better" candidate for starting a metabolic engineering program for increasing

product levels. In our understanding a secondary pathway begins at the site where primary metabolites (*e.g.* amino acids) are diverted into the secondary pathway. Why did we believe that these enzymes were good choices for our approach? First of all, we felt that it is generally most useful to start with modifications at the beginning of a pathway - except there is good information that another enzyme within the pathway holds the limiting role. Secondly, it is known that growth and secondary product formation are often inversely correlated. Thus, the activities of enzymes diverting primary metabolites into secondary pathways are often low or lacking during the growth phase and are greatly induced when secondary product formation starts. Thus, these enzymes are evidently regulatory control sites. To be honest, the above concept has heavily been rejected by prominent colleagues. However, criticism of the concept of starting genetic engineering approaches at this site would only be justified if one over- or misinterpretes its meaning. It is evident that the site or the inducibility of an amino acid decarboxylase does **not** mean that it is indeed rate-limiting in a pathway. Examples where amino acid decarboxylases are not limiting will be given below. As outlined above, it is also evident that overexpression of a linking enzyme will not induce a repressed pathway. Despite all these reservations, it is nowadays, after the first results have been published, even more justified to state: **It seems to be in general useful to start genetic manipulations aimed to enhance the flux through a pathway, at its beginning whenever other information do not withstand.** This conclusion holds even true when the first enzyme will later be identified as not being the main rate-limiting one. If, for example, overexpression of an amino acid decarboxylase activity leads only to increased accumulation of the reaction product or an intermediate further down the pathway but not of the desired end product, one may assume that the enzyme responsible for the conversion of the accumulated product is the limiting one. Thus, overexpression of such enzymes helps to identify control points further down the pathway. One can walk along a pathway. We assume that the earlier in the pathway one can start the better are the chances to clarify the most limiting steps. However, it must also be clear that a critical analysis whether the above concept is applicable to a target pathway is not only recommended but necessary for avoiding disappointments. In the meantime several groups have overexpressed genes encoding enzymes linking primary and secondary metabolism - so that the points discussed here can be checked by experimental data.

Overexpression of enzymes linking primary and secondary pathways

In the following we will analyze systems where overexpression of enzyme activities diverting amino acids into secondary metabolism was done or may be done in the near future. Not all of the metabolic engineerings using corresponding genes were performed for increasing secondary product levels. In addition, the classification "enzyme linking primary and secondary metabolism" is sometimes not that clear. Arginine and ornithine decarboxylases are, for example, involved in the biosynthesis of primary (vitally important) polyamines putrescine, spermidine and spermine and in the biosynthesis of secondary metabolites (*e.g.* alkaloids).

Naturally, researchers interested in secondary products may look for utmost product increases in the engineered plant cells. However, for readers it may be even more interesting to see whether knowledge obtained from physiological studies can help to make predictions whether or not production increases can be achieved by overexpression of a specific enzyme. Especially under our view that flux manipulation of a pathway should at best be started when good chances for a succesful outcome are most likely given, we will try to provide sufficient background information so that one can understand why one system reacts in this way, the other in a different way. Indeed, we should presently not stare too much to product level increases, as high increases may only be obtained when more than one enzyme of a pathway are overexpressed.[6]

Phenylalanine ammonia lyase and cinnamic acid derived metabolites

Higher plants synthesize from phenylalanine a great variety of natural products - *e.g.* cinnamic acid conjugates, stilbenes, flavonoids, anthocyanins and lignin (Fig. 1). Due to its importance the general phenylpropanoid pathway and therefrom derived branches belong to the best studied secondary pathways. Many genes of these widely branched pathway have been cloned and transgenic plants overexpressing some of these genes have been established. However, most of these studies were not intended to enhance the flux of the pathways. The main interest has been to modify the colour of flowers by introducing genes of anthocyanin biosynthesis[9], to alter polymerization of lignin[10] or to produce new phytoalexins[11]. In addition, molecular pathway analyses have given first insight into regulatory aspects.

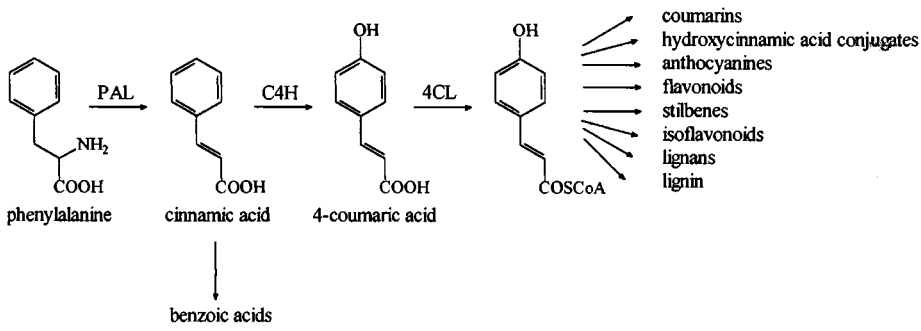

Figure 1. The initial enzymic reactions common to the "independent" biosynthetic pathways of different classes of phenylpropanoids. PAL = phenylalanine ammonia-lyase; C4H = cinnamic acid 4-hydroxylase; 4-CL = 4-coumaric acid:CoA ligase. Each class of the phenylpropanoids may use its specifific PAL, C4H or 4-CL enzyme for biosynthesis.

Though, the phenylpropanoid pathway seems to be the best understood secondary pathway, surprisingly little efforts have been made to alter its flux with the aim of enhancing product levels. Manipulations within the pathways (*e.g.* at chalcone synthase and dihydroflavonol 4-reductase) affected the product pattern but did not

lead to enhanced product levels.[9,10] The bridging enzyme between primary and secondary metabolism is phenylalanine ammonia-lyase (PAL). PAL is encoded by a small gene family of differently regulated genes and *pal* cDNAs have been isolated from a variety of higher plants. Indeed *pal* could have been the first plant specific gene used for flux analyses of secondary pathways. Up to now only the groups of C. Lamb and R.A. Dixon established *pal*-transgenic plants by engineering the bean *pal2* gene into tobacco.[12-14] They observed abnormal plant development and surprisingly a down-regulation of phenylpropanoid biosynthesis, as the accumulation of transcripts encoded by the endogenous *pal* genes was suppressed (sense-suppression).[12] In some transgenic plants the levels of chlorogenic acid and rutin were reduced by 60- and 10-fold, respectively. However, when the transgenic plant was selfed a recovery of PAL-activity was noted in the homozygous progenies. In one case it took four to five generations before PAL activity returned to wild-type levels. In one other tobacco plant sense-suppression was already overcome in plants derived from seeds of the T_0 generation and due to the overexpression of the bean PAL the plants had indeed significantly higher PAL activity than the wild-type.[13] A direct relationship between PAL avtivity and chlorogenic acid accumulation indicated that PAL activity almost exclusively controls chlorogenic acid accumulation in leaves. Chlorogenic acid levels were enhanced roughly 3-fold due to the higher PAL activity. In the case of the flavonoid rutin PAL activity is an important factor but one or more steps of that branch must also be involved in the overall control. Therefore rutin levels were not significantly enhanced by the higher PAL activity.[14] Interestingly, some other phenolic compounds hardly detectable in wild-type extracts were greatly enhanced. They were identified as p-coumaric acid and its glucoside suggesting that p-coumaric acid converting enzymes may have become limiting. Although this assumption has to be confirmed, the authors conclude - in agreement with our general statement - that overexpression of PAL activity in transgenic plants can help to reveal control points for the flux into phenylpropanoid biosynthesis.[14] As next step one could try to make transgenic tobacco plants overexpressing PAL and chalcone synthase (CHS) activity, which may help to increase both chlorogenic acid and flavonoids.

From our point of view it is also interesting to know how wild-type and *pal*-transgenic cultures behaved. Evidently the sense-suppression of PAL activity in leaves was not noted in callus cultures. The *pal*-transgenic callus cultures showed substantially higher PAL activity and consequently substantially higher levels of phenolic compounds.[13] Evidently, the biochemical characteristics of transgenic cells depend not only upon the efficiency of transcription and translation of the foreign gene but also upon the physiological state of the cells or the overall tissue control. Thus, the effects of metabolic engineering observed in cultured cells may or may not become visible in regenerated plants derived therefrom.

Tryptophan decarboxylase and tryptamine derived metabolites

Indole alkaloids (*e.g.* monoterpene indole alkaloids) are widespread in the plant kingdom and some of them are of great commercial interest; for example, quinine,

reserpine, vinblastine. The biosynthesis of most indole alkaloids starts with the decarboxylation of tryptophan to tryptamine (Fig. 2). Thus, tryptophan decarboxylase activity is needed for the biosynthesis of most indolic compounds. As induction of tryptophan decarboxylase activity and indole alkaloid accumulation seemed to correlate quite well in some culture systems, this enzyme was regarded as a good site for genetic manipulations. Successful cloning of a DNA from *Catharanthus roseus* coding for tryptophan decarboxylase activity was soon reported by the groups of Vincent deLuca at Montreal and Harry Hoge at Leiden.[15,16] In the meantime the tryptophan decarboxylase gene has been overexpressed in tobacco, potato, canola, *C. roseus* and *Peganum harmala.* In our context, only the expressions in *C. roseus* and *P. harmala* are of interest because they were intended to increase tryptamine derived metabolites.

Cell cultures of *C. roseus* have been for many years a favored system for groups interested in the biotechnological production of secondary metabolites by tissue culture (see for review [17a,17b]). Target compounds were the dimeric monoterpene indole alkaloids vinblastine and vincristine, rather expensive antitumor compounds, as well as ajmalicine and serpentine. Although, one can state today that the realization of a commercial process was not and will not be reached in the near future, a lot of information was obtained from physiological and biochemical studies. For example, it was shown that TDC activity was absent in non-producing cells but increased greatly when cells were transferred from a growth medium to a production medium by which ajmalicine, serpentine or catharanthine biosynthesis and accumulation was initiated. Another known enzyme of that pathway, strictosidine synthase (Fig. 2), behaved variably. It was found in some non-producing cultures; in some lines its activity did not change in a production medium, in others it increased distinctly. Thus, if one compared the behavior of these two enzymes in producing and non-producing cultures one would guess that TDC rather than strictosidine synthase might represent a limiting factor of the alkaloid biosynthesis. However, when cells were transferred to a production medium to which increasing levels of phosphate were added, one saw an induction of TDC activity but the alkaloid formation declined with increasing levels of phosphate. This meant, one could induce TDC activity without producing alkaloids which made the limiting role of TDC questionable. As consequence of TDC induction without effects on alkaloid levels, one observed accumulation of tryptamine. Thus, tryptamine was evidently not a limiting metabolite for indole alkaloid biosynthesis. Indeed several groups demonstrated that feeding of tryptamine had no effect on alkaloid levels. Consequently, overproduction of tryptamine by overexpression of tryptophan decarboxylase should have no effect on alkaloid levels in *C. roseus.* This was exactly found when transgenic *C. roseus* cultures were analyzed which overexpressed an engineered *tdc* coding sequence under the control of the *CaMV35S*-promoter.[18] Strictosidine synthase needs tryptamine and secologanin as substrates for performing the next step in the biosynthesis (Fig. 2). Evidently, a shortage of secologanin prevented formation of more strictosidine and/or alkaloids. Indeed, there is some evidence that geraniol-10-hydroxylase, an enzyme of secologanin biosynthesis, is more limiting than TDC. The induction of this enzyme in a production medium was shown to be inhibited by phosphate. Search for the corresponding gene is underway in several laboratories.

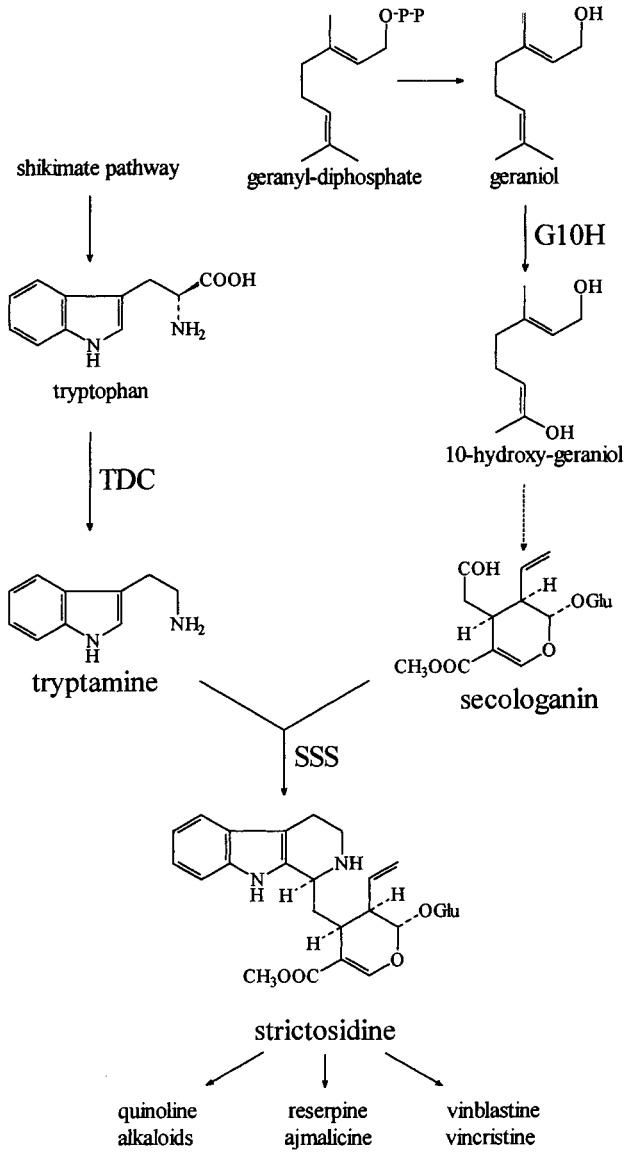

Figure 2. The initial steps of the biosynthesis of monoterpene indole alkaloids. TDC = tryptophan decarboxylase; G10H = geraniol-10-hydroxylase; SSS = strictosidine synthase.

If this gene is available and can be overexpressed as active enzyme at the correct site in *C. roseus*, it seems to be possible to increase the monomeric indole akaloids which normally accumulate in the suspension cultures (ajmalicine, serpentine or catharanthine). Shortage of tryptamine should be no problem when cells are transferred to a production medium. Only if this first step is successful, one may also look for

further steps which may include transgenic cells overexpressing more than one enzyme (geraniol-10-hydroxylase plus TDC and/or strictosidine synthase). The predictions/ suggestions given here are based on our present knowledge from physiological studies using *C. roseus* cell culture systems. This knowledge tells also that overexpression of strictosidine synthase and tryptophan decarboxylase in one cell will **not** effect levels of monomeric monoterpene indole alkaloids. Assumptions that vinblastine or vincristine levels would be increased in such transgenic cells are speculations without a scientific basis.

Overexpression of tryptophan decarboxylase in *C. roseus* and tobacco cells demonstrated that TDC activity and in consequence tryptamine levels can be increased. The overexpressed enzyme is evidently supplied with tryptophan when it is located in the cytoplasm. Our goal, however, is to show that the overproduced tryptamine leads also to increased levels of a target end product. A suitable and instructive system for demonstrating this are cell cultures of *Peganum harmala* (see for review[19]). This plant produces two groups of metabolites derived from tryptophan - β-carboline alkaloids (*e.g.* harmine, harmalol) and serotonin (Fig. 3). It is assumed that the initial step of both compounds is the decarboxylation of tryptophan to tryptamine.

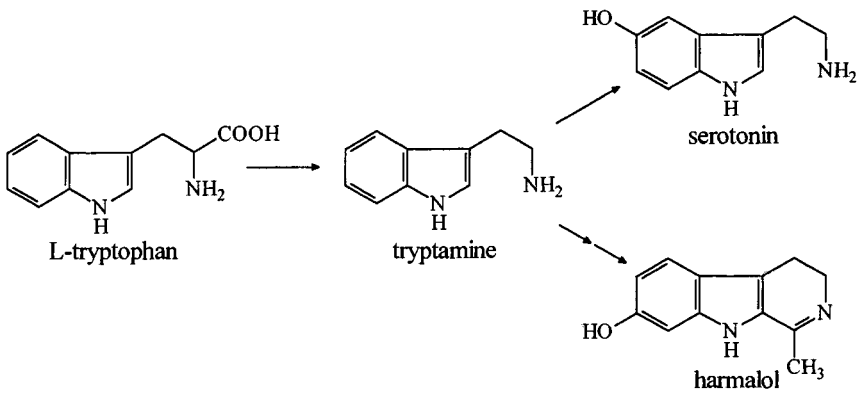

Figure 3. Biosynthesis of ß-carboline alkaloids and serotonin in *Peganum harmala.*

Cell cultures of *P. harmala* accumulate initially β-carboline alkaloids and serotonin. When they are maintained as suspension cultures, one selects automatically for the more rapidly growing cells and these are cells with a poorly expressed secondary metabolism. The non- or poorly producing fine suspension cultures lacked tryptophan decarboxylase activity. When these cells were transferred to a production medium, tryptophan decarboxylase activity was stimulated and production of β-carboline alkaloids and serotonin was resumed. However, the inducibility of TDC activity was eventually lost. Such observations suggested that the lack of TDC activity is the reason why fine cell suspensions can no longer produce β-carboline alkaloids and serotonin. Therefore, the seemingly

limiting metabolite tryptamine was fed to the cultures. Surprisingly, β-carboline alkaloid levels were not stimulated which showed that tryptamine should not be the main limiting component in that pathway. However, all tryptamine was converted to serotonin which clearly demonstrated that the lack of tryptamine formation prevents serotonin biosynthesis. A short look at the biosynthetic pathway of serotonin (Fig. 3) tells that the lack of TDC activity must be the only reason for the interruption of this pathway in *P. harmala* cell suspension cultures. We have transformed seedlings of *P. harmala* using an *A. tumefaciens* strain containing a plant transforming vector carrying the coding sequence of the *tdc* gene of *C. roseus* under the control of the *CaMV35S*-promoter.[20] The vector contained as selective marker a gene coding for neomycin phosphotransferase II, making genetically transformed cells resistant against the antibioticum kanamycin. Among the kanamycin-resistant calli we found several with high TDC activity and serotonin levels of up to 2% dry mass. As freshly initiated cell cultures were shown to contain TDC activity and produce also serotonin, one had to be cautious. However, comparisons of control lines and *tdc*-transgenic lines in Southern (DNA), Northern(mRNA) and Western(protein) analyses made it likely that the overproduction of serotonin was due to the action of the engineered *tdc* gene. In addition, it was shown that the TDC activity remained high in *tdc*-transgenic cell cultures over the whole growth cycle. In control cultures TDC activity was induced after transfer to fresh medium and declined then to hardly measurable values during the next 5-6 days. Due to this, feeding tryptophan to control cell suspension cultures of *P. harmala* did not enhance levels of serotonin. In contrast, feeding tryptophan to the *tdc*-transgenic lines enhanced levels from 2 to up to 8% dry mass. The overexpressed TDC could evidently convert much more tryptophan than is made available by the cells. We have overcome the main limiting step of serotonin biosynthesis by the overexpression of TDC activity and we have identified tryptophan supply as the second limiting step of serotonin biosynthesis. Thus, if the cells are to make more serotonin, one has to make *tdc*-transgenic cells produce more tryptophan. Two ways seem to be possible - a) biochemical selection or b) genetic engineering.

Anthranilate synthase (AS) is regarded as regulatory enzyme in tryptophan biosynthesis in higher plants. It is feedback controlled by tryptophan. Selection for 5-methyltryptophan-tolerance has been shown to yield cell lines with a feedback-insensitive anthranilate synthase and these lines had greatly enhanced levels of tryptophan.[21] Thus, we selected 5-methyltryptophan-tolerant cell lines from the *tdc*-transgenic cell lines of *P. harmala*. However, the result was negative and should be regarded as warning. First, of all none of our 5-MT-tolerant cell lines overproduced tryptophan. This was not that surprising to us, as selection for tolerance to other tryptophan-analogs did neither yield tryptophan-overproducers. More surprising was that all tolerant lines had greatly reduced levels of TDC activity and produced lower levels of serotonin than the parent line. We did not analyze why the 5-MT-resistant lines had lower TDC activities. However, one should realize that during biochemical selections the genetically engineered trait might get lost or might be lowered in selected cell lines. As a consequence, we favor now the genetic engineering approach. The plant transformation vector should insert at the same time the gene of an anthranilate synthase and the gene of a tryptophan decarboxylase. As AS is located in plastids, one should clone the *as* gene behind a leader targeting the enzyme into this compartment. Coding sequences for AS enzymes have been isolated

from *Ruta graveolens*[22] and *Arabidopsis thaliana*.[23] If the assumption is correct that AS is the second limiting factor one could imagine to make the cells produce distinctly more serotonin than 2% dry mass.

As mentioned above we could not repair the loss of β-carboline formation by feeding tryptamine. This indicated that the loss of TDC activity and tryptamine supply was not the only reason for the inability of morphologically dedifferentiated cells to synthesize the alkaloids. Probably the whole pathway was repressed under these conditions. The biosynthesis of β-carboline alkaloids occurs naturally in root cells. As long as some root-like structures are present or can be induced, β-carbolines are formed in reasonable levels. Alkaloid levels of 2% dry mass were found in normal and transformed root cultures. Normal root cultures are often formed spontaneously during culture initiation, but they are rather unstable. Transformed root cultures are obtained by infecting seedlings with *A. rhizogenes*. These root cultures are stable and some of lines have produced β-carboline alkaloids now for nearly 10 years. Thus, root cells seemed to be a suitable system to analyze the role of TDC in β-carboline alkaloid biosynthesis. We established *tdc*-transgenic root cultures by inserting the vector into a Ri-plasmid (root inducing) instead of the above Ti-plasmid (tumor-inducing). We did this, although we were quite sure that tryptamine overproduction would not improve β-carboline levels in root cultures. Feeding of tryptamine to root cultures enhanced serotonin levels dramatically but did indeed not alter alkaloid levels. The analyses of many *tdc*-transgenic root cultures confirmed the feeding experiments. All *tdc*-transgenic root cultures with good levels of TDC activity had distinctly enhanced levels of serotonin.[20] However, β-carbolines were not improved in any of these lines. Feeding of tryptophan to the *tdc*-transgenic roots enhanced serotonin but not the alkaloids. Although both pathways are claimed to use tryptamine, only one is affected by TDC overexpression. The clear cut conclusion is that TDC activity and tryptamine supply are not limiting β-carboline biosynthesis. Indeed, we speculate that free tryptamine is not a natural intermediate in β-carboline biosynthesis in *P. harmala*. Our results suggest to reconsider the initial steps of β-carboline biosynthesis in higher plants.

Tyrosine decarboxylase and tyramine derived metabolites

Tyrosine is the primary precursor of many important alkaloids (morphinanes, isoquinolines, benzophenanthridines, protoberberines) in higher plants (Fig. 4). It is, for example, estimated that more than one million kilograms opium is produced annually from which morphine and codeine are extracted. Protoberberines and benzophenanthridines belong to the secondary pathways which are well expressed in cell cultures. Usually tyrosine is either hydroxylated to dopa and then decarboxylated or is decarboxylated directly to tyramine before being further metabolized in various pathways or branches. Tyrosine decarboxylase might thus be an interesting enzyme for increasing alkaloid levels. It is an inducible enzyme similar to tryptophan decarboxylase and there is some correlation between induction of this enzyme activity and protoberberine or benzophenanthridine accumulation in cell cultures.[24,25] However, it has not yet been shown that tyramine or dopamine are indeed limiting components for these compounds. Therefore claims

that overexpression of a tyrosine decarboxylase will enhance benzophenanthridine and protoberberine alkaloids would be rather speculative. Though genes encoding tyrosine decarboxylase have been cloned from *Petroselinium crispum*[26] and *Papaver somniferum*[27] it seems to be worthwhile to clarify, before any genetic engineering experiments are initiated, whether tyramine overproduction will enhance alkaloid levels with a great likelihood. The finding of an organ-dependent accumulation of benzophenanthridine and morphine alkaloids and the detection of two differently regulated tyrosine decarboxylase like transcripts indicates a coordinated, developmentally controlled regulation of the alkaloid biosynthesis in *P. somniferum*.[28] This means that alkaloid increases (provided tyrosine decarboxylase is a limiting factor) may only be obtained when the gene is expressed in the specialized cells where the target pathway is active. In the case of benzophenanthridine or protoberberine biosynthesis undifferentiated cell cultures could represent the specialized cells needed for the manipulation of these pathways.

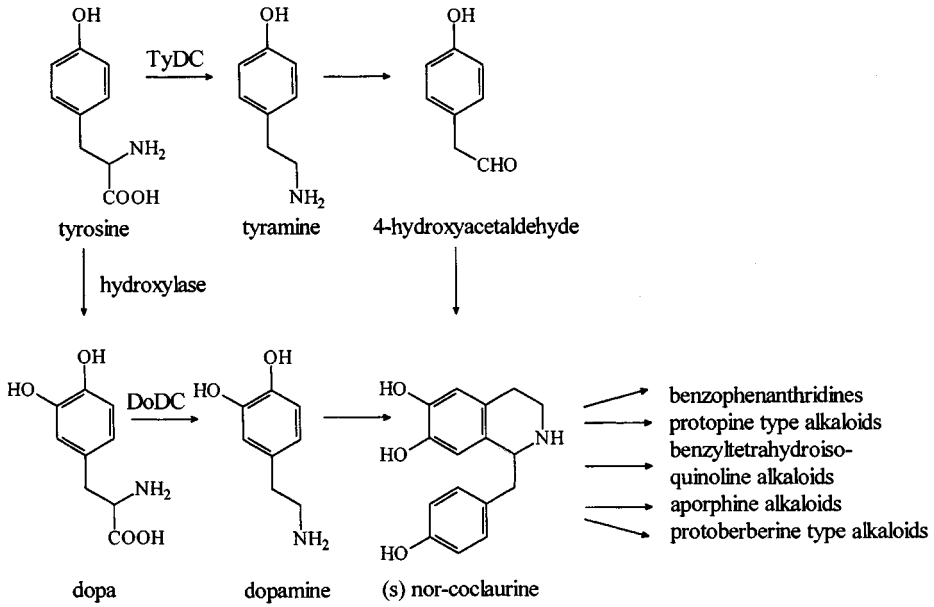

Figure 4. The initial biosynthetic steps of tyrosine-derived isoquinoline alkaloids. TyDC = tyrosine decarboxylase; DoDC = dopa decarboxylase.

Ornithine, arginine and lysine decarboxylases and diamine derived metabolites

Putrescine is precursor for a great number of alkaloids in higher plants. For example, the nicotine or tropane alkaloids are derived from putrescine (Fig. 5). In the case of putrescine it is difficult to decide whether it is a primary or secondary product. As it

is present in all cells and has a regulatory function in plant development we should regard it as a primary metabolite. When used for the biosynthesis of nicotine, tropane alkaloids or conjugates, one would name it a secondary metabolite. As we do not look here for the primary functions of the polyamines, we regard putrescine in our context as secondary metabolite and the corresponding amino acid decarboxylases as enzymes linking primary and secondary metabolism. Putrescine can be formed in higher plants from ornithine or from arginine *via* agmatine.

The first report that overexpression of an amino acid decarboxylase in higher plants can increase the levels of derived secondary metabolites came from Hamill and colleagues in Norwich.[29] They overexpressed a yeast ornithine decarboxylase (ODC) under the control of the *CaMV35S*-promoter in tobacco root cultures. As the construct did not contain any sequence encoding a signal or transit peptide, the encoded ODC was likely located in the cytoplasm. In contrast to controls, the *odc*-transgenic roots contained high levels of ODC activity over the whole growth cycle. The levels of nicotine and hydroxycinnamoylputrescines (HCPs) were up to 2-fold increased. If measured in absolute values, this would mean rather high product increases because the basic levels of nicotine and HCPs are usually quite high in root cultures. The given data seem to support the conclusion of the authors that ODC overexpression improved secondary metabolite levels. Nevertheless, a number of questions remain open. First of all, to all what was known about nicotine biosynthesis, putrescine has never been regarded as limiting factor. Feeding putrescine has never been shown to have a strong affect on alkaloid levels and/or conjugates. Why should putrescine overproduction have a different effect? A very useful experiment to avoid critical arguments would have been feeding of higher levels of ornithine to control and *odc*-transgenic root cultures. If ODC is the limiting factor in nicotine biosynthesis one would expect to see strong effects on putrescine and alkaloid levels in the transgenic lines, while in the control root culture only minor effects on alkaloid levels should be noted. Nicotine and HCP levels of transformed root culture lines of another tobacco variety differed in our hands up to a factor three in individual transformants, although the levels of these compounds were not effected by the introduced gene.[30] Therefore, it is suggested that metabolic engineering experiments aimed to improve product levels should include analyses which proof unambigously that the engineered step is the main cause for the altered product yields. Feeding experiments may also give hints whether the engineered enzyme is sufficiently catered with substrate or whether the enzyme should be targeted into a different compartment.

Putrescine overproduction has also been obtained by overexpression of mouse ODC cDNA in tobacco and carrots.[31,32] In tobacco leaves putrescine levels were only up to 2-fold increased. Callus cultures derived from these transgenic plant contained 4-12-fold more putrescine than untransformed callus cultures. The extent of overproduction depends not only upon the activity of the introduced enzyme but also upon the physiological state of the engineered cell and the actual cellular control in a specific tissue. The authors did not check whether putrescine derived secondary pathways were affected, as the authors were mainly interested to study the morphological consequences of putrescine overproduction.

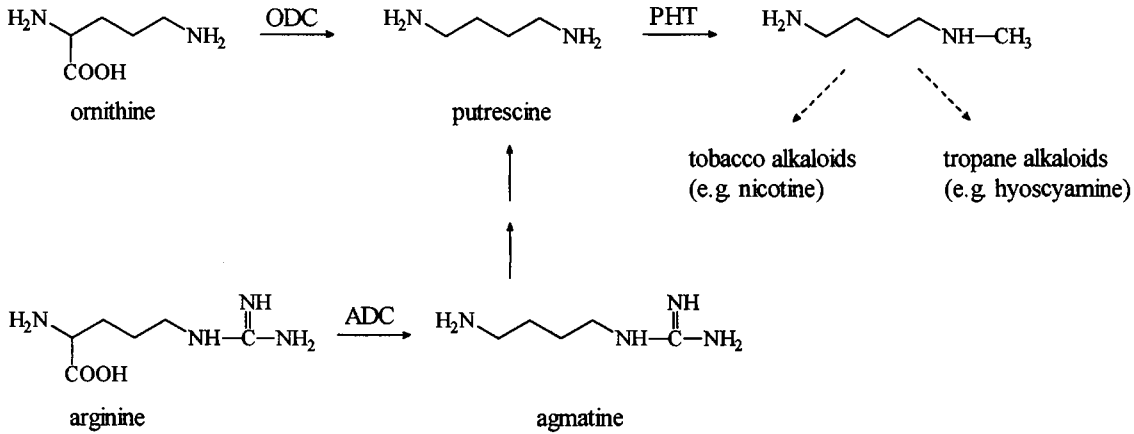

Figure 5. Putrescine biosynthesis in higher plants and its channelling into alkaloid biosynthetic pathways. ADC = arginine decarboxlase; ODC = ornithine decarboxylase; PMT = putrescine N-methyltransferase.

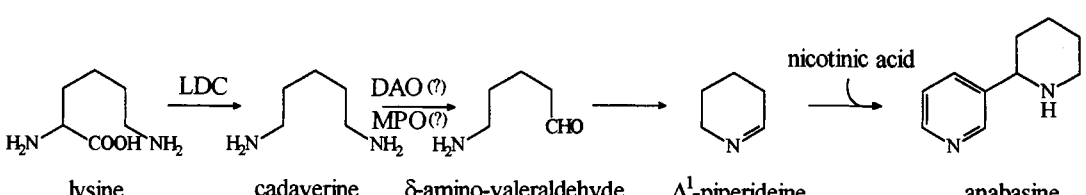

Figure 6. Biosynthetic pathway of anabasine. LDC = lysine decarboxylase; DAO = diamino oxidase; MPO = N-methylputrescine oxidase. The latter two enzymes are able to oxidize cadaverine to δ-amino-valeraldehyde.

In contrast to mammalian cells, bacteria and higher plants produce putrescine also *via* arginine decarboxylase (ADC). Indeed it is speculated that primarily ADC provides putrescine for nicotine. Several cDNAs encoding *adc* have been isolated from higher plants.[33] However, only a monocot oat cDNA has so far been used for genetic transformation of dicot plants.[33,34] Two different promoter systems have been tested to study metabolic effects caused by the overexpression of ADC in tobacco. In the first case, tobacco plants were established carrying the oat *adc* gene under the control a tetracycline-inducible promoter.[34] The main intention of the authors was to study effects of altered polamine metabolism on plant development. The advantages of an inducible promoter system for such studies are evident. Controls of transgenic cells carrying a gene under a constitutive promoter such as the *CaMV35S*-promoter are transformants lacking the gene. Thus, controls of the latter system result always from independent transformation events. Transgenic plant cells carrying an inducible promoter system use the same plant source as control. Thus, the problem of variation among individually transformed cell lines is minimized. Therefore this system could also be interesting for researchers trying to improve secondary product levels by overexpression of enzymes linking primary and secondary pathways.

Although ADC enzyme activity was at least 10-20-fold increased compared to controls, the levels of putrescine and other polyamines were increased only by 50-100% by the best plant.[34] Even total levels of agamatine, the direct product of arginine decarboxylation, remained quite low. So far only leaves of the *adc*-transgenic plants have been analyzed for changes of polyamine levels. If, as in the case of *odc* overexpression in tobacco, overproduction depends not only upon the enzyme activity but also upon the environment where the enzyme is active, one could imagine that in other tissues higher increases and total levels of putrescine may be found. Naturally ADC of higher plants is located in the plastids. As an obvious targeting signal was not found, it might be possible that the monocot oat ADC was not transported into chloroplasts of dicot tobacco. If the engineered ADC is located in the cytoplasm, it might be that it is not supplied with sufficient arginine. Or, is there a counter regulation against too much putrescine? Are disturbing levels of putrescine diverted into secondary pathways? Nicotine or HCP levels have not yet been analyzed in the induced plants. An answer may be obtained by feeding arginine to see to what extent putrescine may be increased in induced and control plants. The feeding solution should be absorbed *via* the roots and especially the roots should be analyzed for changes of nicotine and HCP after induction of the heterologous ADC activity. Prelimary experiments with transgenic plants of Tiburcio's group[34] in our laboratory gave, however, no indication that nicotine or HCP levels are enhanced when the oat-ODC is induced in tobacco roots (unpublished).

In the second system, the oat *adc* cDNA was under the control of the *CaMV35S*-promoter.[33] The authors analyzed only the leaves of the *adc*-transgenic plants. Their findings are rather dischanting with respect to improving nicotine levels by overexpression of ADC. The leaves of the second generation contained 10-20-fold higher ADC activity and 20-40-times more agmatine. Levels of putrescine, spermine, spermidine as well as those of nicotine and HCPs were not affected by the overproduction of the precursor agmatine.

Presently, we have to conclude that transgenic plants and root cultures have not yet provided clear evidence that secondary products such as HCPs and nicotine levels can be improved by overexpression of heterologous ADC or ODC activity.

Cadaverine is a polyamine which has attracted less attention because it does not seem to have a role as modulator of growth related responses. Cadaverine levels in higher plants are often low and hardly to detect. It may thus be regarded as secondary metabolite formed by the decarboxylation of lysine (Fig. 6). Cadaverine is precursor of several groups of alkaloids - *e.g.* quinolizidine alkaloids in lupins. Indeed, the interest in lysine decarboxylase (LDC) resulted from the idea to endow lupin cells with high LDC activity and to increase by this quinolizidine alkaloids. However, we rejected this idea when we had a closer look to this seemingly simple 2-step pathway.[35] Our unpublished studies told us that cadaverine is not the main limiting factor in the biosynthesis. The pathway is well expressed in leaves, but poorly in cultured cells. Transformation of lupins with genetically constructed strains of *Agrobacterium* sp. and regeneration of intact plants are not trivial. Therefore we looked for an an attractive alternative to use the *ldc* gene we had cloned from the enterobacterium *Hafnia alvei,* in metabolic engineering experiments.

Anabasine is a structural analog of nicotine in which the putrescine-derived N-methylpyrrolinium ring is replaced by a cadaverine-derived Δ^1- piperidinium ring (Fig. 6). In most tobacco varieties anabasine is a minor alkaloid, often hardly detectable. The reason for this is evidently the lack of cadaverine. Feeding of cadaverine to hairy root cultures of various tobacco ssp. greatly increased the levels of anabasine.[36] Despite the fact that the way is not fully elucidated how cadaverine is incorporated into anabasine, it is clear that cadaverine is limiting in anabasine biosynthesis. Consequently, overexpression of LDC in *ldc*-transgenic tobacco should result in the overproduction of anabasine in tissues where nicotine formation occurs. In addition, one could also expect that novel hydroxycinnamoylcadaverines (HCCs) might be formed in cadaverine-overproducing cultures, as feeding of cadaverine led to the formation of these compounds.[37] The enzyme hydroxycinnamoyl-CoA:putrescine hydroxycinnamoyltransferase accepts also cadaverine as substrate.[38] Thus, if it works as anticipated, one would have a system where a) two pathways are affected by the overexpression of LDC and b) due to the almost complete absence of the target compounds in controls, product increases are easily recognized. In the following we report only about those *ldc*-transgenic cultures which led to significantly enhanced product levels of anabasine and HCCs. We can conclude from all our experiences that high expression of the *ldc* gene requires a) a leader sequence and b) a targeting signal for import into plastids. The LDC must in addition be present in tissues where the pathways of the target compounds are active.

For fulfilling the above demands, an expression cassette was constructed in which the *ldc* gene fused to the coding region of a *rbcS* (small subunit of ribulose biphosphate carboxylase) transit peptide (*tp*) was under the control of the *CaMV35S*-promoter. This was cloned into a binary vector and introduced by triparental mating into an *A. tumefaciens* strain with an Ri-plasmid. Tobacco seedlings were transformed with this *Agrobacterium* strain and many *ldc*-transgenic root cultures were obtained. These lines were screened for those having high LDC activity. We had to screen a larger number

of individually transformed lines to find the superior ones. Our best line had 3-times higher LDC than the second best and 10-times moore than average lines. All lines carrying the *35S-tp-ldc* insert had distinctly higher LDC activity than lines without the targeting signal. The next step was to analyze the various lines for their levels of cadaverine, anabasine and HCCs. There was a rather good correlation between LDC activity, cadaverine and anabasine levels. The best line SSR68-2* with its very high LDC activity (ca.30 pkat/mg protein) had accumulated ca. 5 mg cadaverine and anabasine/g dry mass, respectively. This were two times higher product accumulations than found in the next best lines.[39] Naturally, total and specific yields varied in independent analyses performed during a period of 4 years. However, all *35S-tp-ldc*-transgenic lines had under all circumstances significantly higher anabasine and cadaverine levels (3- to 10-times) than controls or lines transformed with a *ldc* expression cassette lacking the sequences encoding the transit peptide. The positive effect of the coding region of the *rbcS* transit peptide might be explained by the observation that various leader sequences have been shown to improve the expression of procaryotic genes in plant cells. In addition, import of LDC into its natural compartment plastid, which is also the site of lysine biosynthesis, seems to be necessary to sufficiently supply the enzyme with substrate. All root cultures of tobacco accumulate usually HCPs. As discussed above cadaverine overproducing cell lines might also accumulate novel HCCs. Therefore all lines were inspected for alterations of the HCP pattern. However, only one line showed a pattern indicating the accumulation of HCCs and this was, as one would have expected, line SSR68-2*.

Taking the data obtained from the analyses of the different ldc-transgenic tobacco cells, we can state that the higher the LDC activity the higher were the changes of the target compounds. However, one cannot make a simple calculation that so much overproduced LDC activity results in so much increased secondary product level. This was best demonstrated by feeding of lysine to a 35S-ldc and a 35S-tp-ldc transgenic line.39. The two lines differed in their LDC activity by a factor 30-60 and the 35S-ldc line had at least 10-fold lower levels of cadaverine and anabasine. When lysine was given over a period of 4 weeks, both lines had converted nearly the same portion of the amino acid into secondary metabolites despite their great differences in LDC activity. This means that the ldc-transgenic lines with low activity might produce much more cadaverine and derived products when a better substrate supply is given. The poor rate of decarboxylation by the 35S-ldc lines is thus not only due to the low enzyme activity but also to its localization in the cytoplasm. On the other hand, when the enzyme is imported into plastids substrate supply does not seem to be saturating. Product levels can be doubled by feeding lysine to 35S-ldc-tp lines. The same holds true for HCC synthesis. Feeding of lysine SSR68-2* greatly enhanced HCC levels by a factor 2-3.[30]

Though we had directed the LDC into the correct cell compartment, the transgenic lines could have produced more of the target compounds if more substrate had been available. In the case of lysine we have two possibilities to make the cells synthesize more lysine. One could, for example, select aminoethylcysteine(AEC) -tolerant tobacco cell lines. Such cell lines may have a less feedback-sensitive (lysine) dihydrodipicolinate synthase (DHPS) and accumulate higher levels of lysine.[40] We did this.

However, it was a rather discouraging experience. For selection we had to change first the root culture SSR68-2* into a suspension culture. We selected several lines able to grow on 1.5 mM AEC which accumulated up to 20-times more lysine than the parent line. However, LDC activities in all AEC-tolerant lines was only 5-10% of that found in unselected cultures. Cadaverine and HCC levels were greatly reduced compared to controls. The consequences on anabasine levels could not be measured, as the cultures could not be returned to the root state. Thus, the first way (combination of genetic transformation followed by biochemical selection) is not very suitable in overcoming a second limiting step. The better way is probably to make double transformants overexpressing DHPS and LDC both targeted into plastids. Lysine overproduction by overexpression of DHPS has been shown several times. Interestingly, lysine overproduction was highest when DHPS and asparte kinase were both overexpressed in the same plant.[41] This result encourages to establish transgenic root cultures overexpressing DHPS and LDC activity for further increasing anabasine production.

Outlook

The aim of this chapter was to summarize experiments aimed to improve target product levels by the overexpression of enzymes diverting amino acids into secondary pathways. Up to now not too many examples can be given where the insertion of such enzymes led unambigously to end product increases. More often it has been shown that genetically engineered overexpression of an enzyme activity can lead to increased accumulation of the direct reaction product. Thus channelling of the overproduced metabolite into target pathways has to be optimized. This requires that more emphasis is put into the understanding of the pathway architecture - identification of limiting sites, knowledge about the location and transport of the metabolites etc. In addition, overexpression of one enzyme activity will rarely lead to dramatic product increases due to complex regulatory control mechanisms in most pathways. Even in cases where the main limiting site can be overexpressed, one may see only great effects if the difference between the first and the second limiting step is large. We identified two systems where after overcoming the first limiting step the second one became limiting. Therefore the next generation of transgenic lines will deal with the overexpression of two limiting enzyme activities. If we succeed in overcoming limiting steps of a pathway and reach by this distinctly higher product improvements than obtained by now, genetic engineering of secondary pathways may attract a broader biotechnological interest. We would recommend to use initially for such studies simple systems where clear cut answers can be obtained. Serotonin and anabasine biosynthesis could become suitable model systems.

The overexpression of limiting enzymes seems to be the most direct way of improving product levels. Whether this is in the long run the most promising way that may be questioned. Basic studies on regulatory controls of pathways will undoubtedly give new clues and may open new (better?) opportunities how to enhance the production of interesting secondary metabolites in higher plants.

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CHAPTER 11

MODIFICATION OF PLANT SECONDARY METABOLISM BY GENETIC ENGINEERING

R. HAIN *and* B. GRIMMIG

Introduction

Plants have evolved a complex secondary metabolism for the production of an enormous range of secondary metabolites which comprises three major classes of natural products - the phenylpropanoids, isoprenoids and alkaloids. Many of these fulfil important functions in the plant's interaction with the environment and were used from human as drugs, colour pigments and fragrances. During the past years, considerable progress has been made in identifying the genes underlying their synthesis and understanding how their synthesis is regulated. Elaborate gene transfer tools have been developed and are applicable for a wide range of plants. Therefore, genetic engineering approaches for the manipulation of plant secondary metabolism are now feasible.

Major interest has been focussed on the phenylpropanoid metabolism which plays important functions in plants, since it provides the precursors for a range of antibiotic substances including furanocoumarin [1], pterocarpan [2] or stilbene phytoalexins [3], for the reinforcement and lignification of the cell wall [4], and for the diverse group of flavonoids playing an important role as flower pigments, UV-protectants and in the interaction with pathogenic and symbiotic microorganisms. Genetic engineering has been used to alter the level of expression of particular genes. Silencing of genes can be achieved by antisense or cosuppression strategies and has proven successful in manipulating production of several secondary metabolites e. g. modification of lignin content and composition [5, 6] whereas overexpression can be achieved by introducing additional copies of the particular gene or placing them under a strong promoter. Branch point intermediates can furthermore be redirected by the introduction of new enzyme activities. The recent identification and cloning of transcriptional regulator genes acting in signal transduction pathways has opened the possibility of manipulating expression of complete biosynthetic pathways. These means offer fascinating perspectives for the introduction of new traits in plants such as expanding the plant's colour palette, enhancing the resistance of plants to microbial pathogens or improving the nutritional quality and composition of crop products. This paper describes recent approaches in the modification of secondary metabolism. We focus on our own work on stilbene phytoalexin expression which, not least, provides valuable lessons for genetic engineering.

R. Verpoorte and A.W. Alfermann (eds), Metabolic engineering of plant secondary metabolism, 217-231.

Genetic engineering of plant secondary metabolism using the stilbene synthase technology

INTRODUCTION OF STILBENE PHYTOALEXINS IN TRANSGENIC PLANTS

The strategy of engineering pathogen resistance by the introduction of novel phytoalexins is to date limited by the availability of only a few biosynthetic genes [7]. In most cases such genes are members of small gene families [8-10] whose individual transfer may not lead to the level of expression required for enhanced disease resistance in the foreign plant. This is particularly so, because due to association with their original promoters expression patterns differ among gene family members with respect to developmental control, tissue-specificity, inducibility and promoter strength [9]. So far, stilbene synthase (STS) genes have been studied most intensively. STS have been isolated from several unrelated species such as peanut, grapevine and pine [8, 11, 12]. In most cases the biosynthesis of stilbenes with phytoalexin properties is induced by pathogens and under environmental stress conditions like ozone and UV-light [13-16]. Stilbene formation specifically requires the sole presence of the key enzyme STS which can be further classified according to its substrate preference. The STS enzymes from peanut and grapevine uses malonyl-CoA and 4-coumaroyl-CoA as substrates to form the trihydroxystilbene phytoalexin resveratrol [17-19] (Fig. 1). Tobacco plant does not have a resveratrol synthase, though the precursor molecules, which are also used by chalcone synthase, the key enzyme of flavonoid biosynthesis, are present throughout the plant kingdom. Tobacco plants carrying a STS gene from peanut showed an inducible *de novo* formation of the phytoalexin resveratrol [17]. Moreover, transfer of two stilbene synthase genes from grapevine which were expressed under the control of their natural promoters resulted in increased disease resistance of the transgenic tobacco plants [8]. However, the amount of STS mRNA accumulated in transgenic tobacco after induction was only 5% or less compared to the amount found in grapevine.

Transfer of the whole STS gene family of grapevine to a foreign plant could surmount this limited expression but is yet impracticable. Therefore, we tried to optimise the expression of single STS genes by using heterologous promoters and enhancer elements, which promises strong transcriptional activity. Our first approach was to increase the expression of a grapevine STS gene in tobacco by the insertion of transcriptional enhancer elements (Fig. 2), yet maintaining the natural regulation defined by the STS promoter. It is known that upstream sequences of the 35S RNA promoter from Cauliflower Mosaic Virus (CaMV) are able to stimulate transcription of heterologous promoters [20, 21], although their properties do not strictly meet the definition of enhancers as being active in an orientation independent manner at distances greater than 1 kb up or downstream of the transcription start site [22, 23]. In our approach we used a tetramer of the -343 to -90 region which had been successfully employed as a transcriptional enhancer in T-DNA tagging experiments [24]. In addition, an almost linear correlation between the enhancing activity of the -209 to -46 fragment and its multimerization of up to four copies had been previously described [25]. Insertion of the tetrameric enhancer 1.5 kb upstream of the transcription start site of the STS gene (VE5+; Fig. 2) led to a significant increase in STS mRNA accumulation without

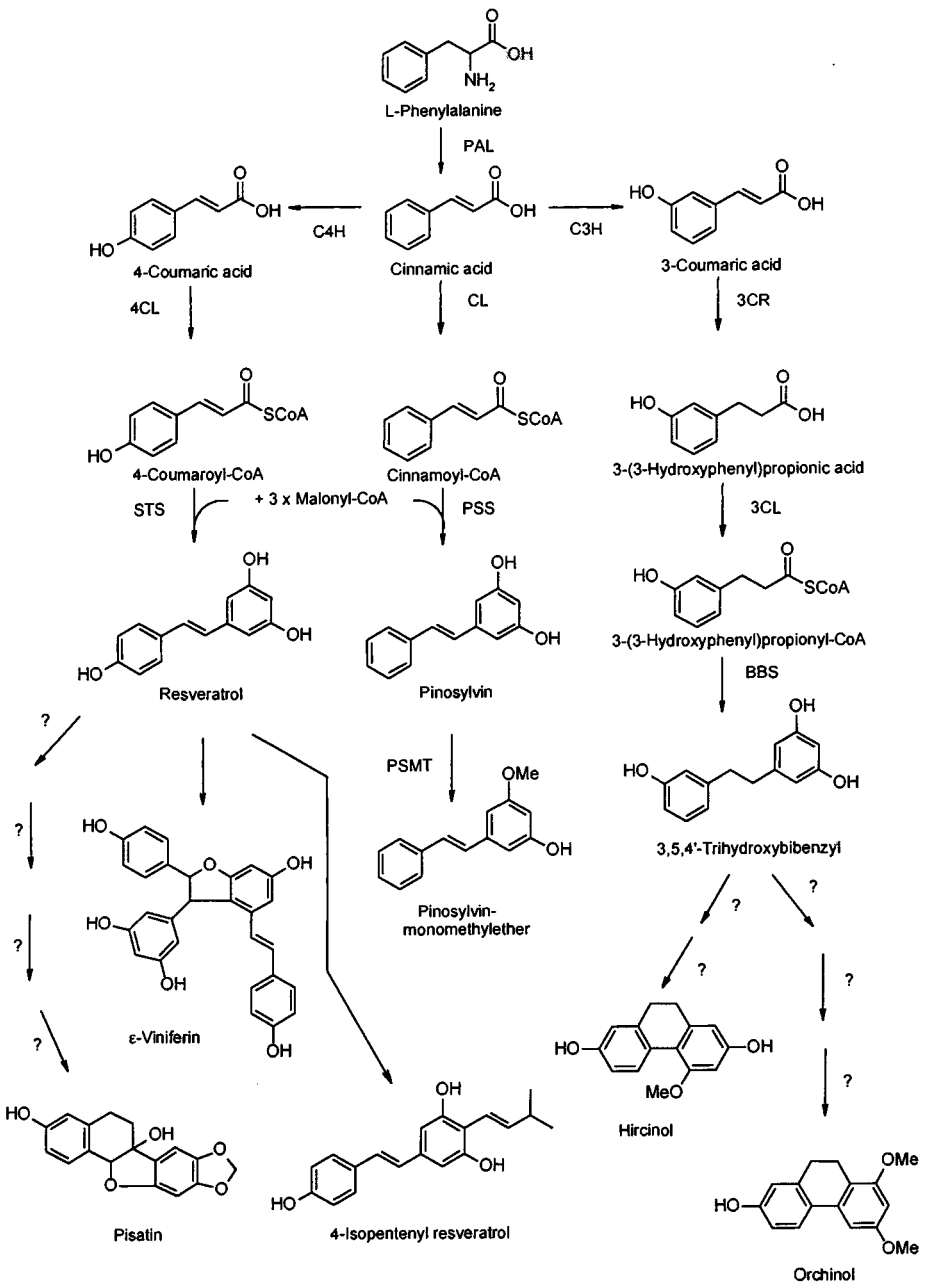

Figure 1. Stilbene phytoalexin biosynthesis in peanut, pea, grape, pine and orchid. Abbreviations: PAL, phenylalanine ammonia-lyase; C4H, cinnamate 4-hydroxylase; C3H, cinnamate 3-hydroxylase; 4CL, 4-coumarate:CoA ligase; CL, cinnamate:CoA ligase; 3CR, 3-coumaroyl-CoA reductase; STS, stilbene synthase; PSS, pinosylvin synthase; 3CL, cinnamate 3-hydroxylase; PSMT, pinosylvin monomethyltransferase; BBS, bibenzyl synthase.

altering the kinetics of induction. This enhancement was striking in transgenic plants derived from *Agrobacterium tumefaciens*-infected leaves (about 14-fold) and in shoots regenerated from protoplasts after direct gene transfer (about 4-fold). The enhancing property was lower when the tetrameric enhancer was inserted in the inverse orientation, supporting earlier observations [21].

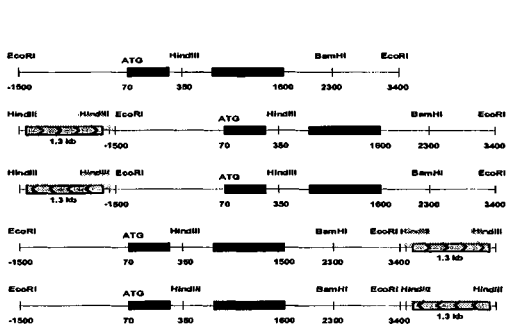

Figure 2. Comparison of STS expression in fungal elicitor-treated leaf discs from tobacco plants harbouring different STS/enhancer constructs. *Left side*) Schematic representation of the STS/enhancer plasmid constructs pVst1, pVE5+/-, pVE3+/-. The multiple enhancer elements (bars with arrows) were orientated at all positions in the original forward orientation as found in the 35S RNA promoter (+ -orientation) as well as in the inverse orientation (- -orientation) at different positions in the context of the *Vst1* gene. Black bars represent the *Vst1* coding regions. *Right side*) STS transcript accumulation in the different constructs was compared to the expression of the 'wild-type' *Vst1* gene (n-fold amount) and corresponding STS enzyme activities [pmol resveratrol x mg^{-1} protein x min^{-1}] are given.

The tetrameric enhancer element was, however, less active when it was inserted downstream of the STS coding region at a distance of 1.8 kb from the stop codon. Here only a 5-fold stimulation was achieved confirming previous observations [21, 25] and, in contrast to the upstream position, the activity was independent of the enhancer orientation. Insertion of the 1.3 kb enhancer fragment into the intron of the STS gene, turned out to have a negative impact on transcription efficiency, presumably due to interference with the RNA processing. In biological trials conducted in the greenhouse VE5+ enhancer plants were reproducibly less susceptible towards *Botrytis cinerea* than control plants. In comparison to line Vst1 expressing the unmodified grapevine STS gene, line VE5+ exhibited an additional attenuation of disease incidents of approximately 20%. Thus, the level of disease resistance based on the inducible synthesis of an additional phytoalexin can be influenced positively by modulation of the corresponding promoter strength.

APPLICATION OF THE PHYTOALEXIN TECHNOLOGY IN PLANT PROTECTION AND PLANT BREEDING

STS genes have been transferred to a number of plants including tobacco, tomato, potato, oil seed rape, rice as well as in grapevine plants. Besides the synthesis of the foreign phytoalexin, enhanced disease resistance against *Botrytis cinerea* has been demonstrated in tobacco [17]. Experiments in transgenic tomato plants, expressing STS genes from

grapevine revealed an increase in resistance against *Phytophthora infestans* [26]. Data with transgenic potato plants indicate enhanced resistance to *Phytophthora infestans* and *Fusarium sulfureum* (Stahl pers. com). Furthermore, STS expression in rice resulted in enhanced disease resistance against rice blast (*Pyricularia oryzae*) [27]. In the meantime, STS has also been expressed in wheat resulting in induced resveratrol synthesis and first phytopathological experiments have shown promising results [28]. Surprisingly, the transfer of a grape STS under control of a heterologous inducible promoter back into grape resulted in very high accumulation of resveratrol and preliminary results indicated enhanced disease resistance of grape plants against the greymould (*Botrytis cinerea*) [29].

Taken together, these results imply a more general relevance of the STS system as tool for engineering disease resistance. For enhanced disease resistance, the particular pathogen has to trigger foreign phytoalexin biosynthesis in the host plant. Molecular analysis of the expression of STS genes show that these genes can be induced in heterologous plants at least by perthotrophic fungi [17]. Resveratrol synthesis in tobacco is triggered by several fungal plant pathogens such as *Botrytis cinerea*, *Alternaria longipees*, and *Phytophthora infestans* in tomato and potato plants. It might be of importance in terms of agricultural relevance whether the foreign phytoalexin biosynthesis is also induced by biotrophic fungal pathogens (*e.g. Erysiphe graminis*, *Bremia lactuca*, *Plasmopara viticola*, *Puccinia* spp.) in the respective target plant.

The regulatory pattern of STS expression which has evolved is of general relevance for disease resistance reactions of plants. The antimicrobial compound is only transiently induced at the site of necessity. This reflects one of the most sophisticated means in plant protection generated by nature. Strategies for enhancing disease resistance should therefore make use of the regulatory pattern of such inducible defence genes to avoid constitutive production of substances also potentially toxic to plants. Such constantly active pathways could trap intermediates needed for other important biosynthetic pathways as we will discuss in the following chapter.

MODIFICATION OF STILBENE SYNTHASE EXPRESSION PATTERN CAUSES FLOWER COLOUR CHANGES AND MALE STERILITY IN PLANTS

In another strategy, the effects of constitutive stilbene synthesis was studied. A rather common approach to enhance the expression of a foreign gene makes use of the 35S RNA promoter which ensures constitutively high rates of transcription. In the case of phytoalexin genes we considered that the permanent synthesis of the antimicrobial compound was possibly undesirable and could negatively impact metabolic profiles in the transgenic plant. To study the effects of permanent STS expression in plants, the *Vst1* gene from grapevine was expressed under control of the cauliflower 35S promoter strengthened by the duplication of its enhancer region (see Fig. 3). The effects of high foreign phytoalexin gene expression was examplified in tobacco and petunia. Interestingly, very high constitutive STS expression has a dramatic impact on flower colour and pollen development in tobacco plants. A very high constitutive level of STS expression caused white and male sterile flowers, while the remaining organs did not reveal any changes [30].

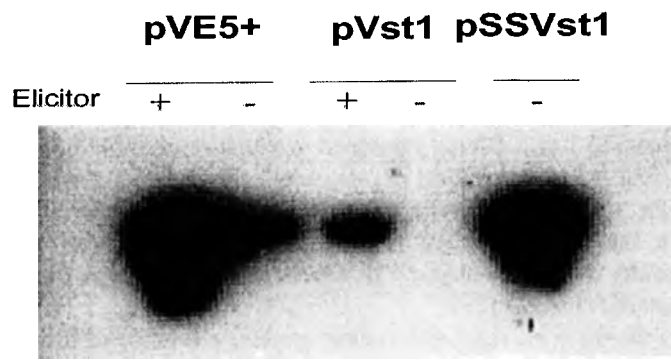

Figure 3. STS transcript accumulation in leaves from transgenic tobacco plants harbouring the STS coding sequence *Vst1* under control of the natural *Vst1* promoter (pVst1), *Vst1* promoter/enhancer fusion (pVE5+), or a doubled version of the CaMV 35S RNA promoter (pSSVst1). The leaves were incubated in LS medium for 4 h in the absence (-) or presence (+) of fungal elicitor (25 µg/ml Pmg, cell wall preparation from *Phytophthora megasperma*) prior to RNA extraction for Northern Blot analysis.

Table 1. Effect of STS gene expression on the resistance of *Nicotiana tabacum* towards greymould. Average disease reduction (%) compared to SR1 wild-type tobacco is presented. All experiments were done with F1 generation plants being hemizygous (67%) or homozygous (33%) for the respective STS gene. After inoculation with *Botrytis cinerea* spores (2×10^5 spores/ml) and subsequent incubation for 4 to 6 days, infection density and symptom development were evaluated using six leaves. In every trial 30-40 plants for each construct were tested. All trials (A-G) represent data of biological tests with transgenic tobacco plants expressing the inducible grapevine STS gene *Vst1*.

	Vst1	**VE5+**
Trial A	19.4	63.7
Trial B	21.9	42.2
Trial C	37.1	32.3
Trial D	47.3	60.8
Trial E	44.7	62.2
Trial F	38.3	55.3
Trial G		36.0

However, in petunia additional effects on the phenotype resulted from STS over-expression. Petunia plants expressing STS constitutively to a very high level developed smaller and curly leaves, shorter internodes as well as smaller flowers and earlier senescence [31]. Analysis of several transgenic lines demonstrated that the regulation

and the level of STS expression directly determined pollen development and flower hue. Diminished coloration as well as deficient microsporogenesis seems to result from a marked reduction of flavonoid biosynthesis. In plants expressing the STS gene under control of the tapetum-specific promoter of *Antirrhinum majus* only pollen development was impaired while the flower pigmentation as well as the phenotype remained unchanged [9, 31].

Nuclear male sterility has been genetically engineered by different means [32-37] and is useful for hybrid seed production in crop plants. Here, we propose a new strategy for engineering flower pigmentation as well as male sterility in plants exploiting the direct competition of STS and chalcone synthase (CHS) for their precursors (Fig. 4). A constant high level of STS expression depletes the metabolic pools of malonyl-CoA and 4-coumaroyl-CoA which are no longer available in sufficient amounts for competing pathways like the flavonoid branch of the phenylpropanoid pathway. Similar approaches to male sterility are also connected with an alteration of flower pigmentation [38, 39]. Usually, the inhibition of flavonoid biosynthetic genes (*e.g.* CHS or dihydroflavonol 4-reductase) is accomplished by antisense-inhibition or cosuppression. Taken together these experiments support the notion that flavonoids not only determine the pigmentation of flowers, but also play an essential role in the development of fertile male gametophytes.

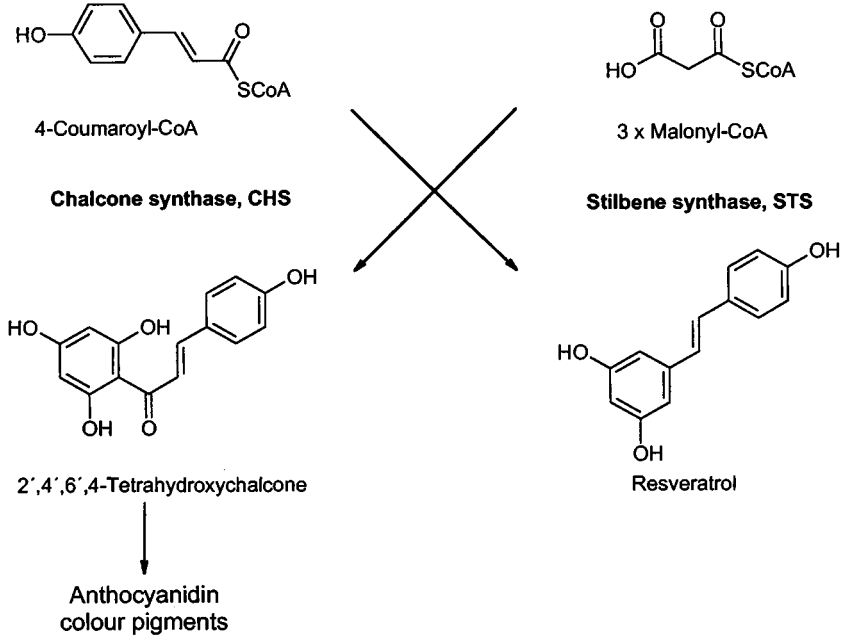

Figure 4. Competition for common substrates by chalcone- and stilbene synthase in the synthesis of anthocyanidin colour pigments or stilbene phytoalexins.

Preliminary biological tests in the greenhouse have shown that symptom expression after inoculation with greymould was slightly lower on male sterile plants expressing

STS constitutively high in comparison to control plants. Nevertheless, disease resistance was not as pronounced as in plants expressing the inducible grapevine STS gene. Taking into account that after fungal attack the viral 35S RNA promoter used in these experiments was even down-regulated at the level of transcription, this observation was not surprising. This down-regulation of the 35S RNA promoter after fungal infection was observed in several independent experiments [40]. This phenomenon could be explained by a silencing of viral promoters through pathogen-induced defence mechanisms. Regulatory sequences in 35S RNA promoter had been identified in the activation sequence-1 (*as-1*). The *as-1* motif constitutes a class of *cis*-regulatory promoter elements characterised by the TGACG consensus sequence. This class of *cis*-acting elements can respond to auxin and salicylic acid treatments [41]. Nuclear DNA-binding activities for the *as-1* element (ASF-1, TGA1) was found in monocots and dicots [40, 42]. These factors interacts not only with the 35S RNA promoter but also with other plant promoters containing *as-1*-like sequences such as glutathione S-transferase and several pathogenesis-related proteins [42, 43]. Therefore, silencing of the 35S RNA promoter after pathogen attack is most likely the result of competition for a limited amount of cognate transcription factors which is supported by competition experiments and insertion of additional *as-1* elements into the 35S RNA promoter [44, 45]. Another explanation could be a negative influence of the depletion of malonyl-CoA and 4-coumaroyl-CoA needed for other plant defence mechanisms using phenylpropanoid pathway metabolites.

So far, our observations led to the conclusion that in terms of engineering disease resistant, the enhancement of the natural STS expression pattern is more favourable than the constitutive STS expression. Perhaps the transient expression of phytoalexin genes could be further optimised by multimerization of corresponding pathogen-responsive *cis*-elements or similar improvements of signal transduction. Moreover, it remains to be clarified whether the systemic impairment of flavonoid biosynthesis in our male sterile plants may influence their tolerance against environmental stresses (*e.g.* UV-B). Such possible disadvantages can be avoided by tissue- and/or developmentally restricted STS expression since we have demonstrated that a tapetum-specific STS expression was sufficient for male sterility in tobacco.

These findings open further possibilities to exploit stilbene synthase genes for disease resistance and male sterility - in the same transgenic crop. Future experiments will be needed on restoring fertility in our male sterile plants for the development of a novel hybrid seed system exploiting STS gene expression.

FUNCTIONAL FOOD BY MODIFICATION OF SECONDARY PLANT METABOLISM

Functional food is defined as unprocessed or processed (plant) nutrition, which provides additional health benefits to consumers or animals. Nutriceuticals are natural bioactive ingredients or supplements to overcome nutritional deficiencies or to prevent disease. The markets for medical nutrition and health food will certainly increase in the future.

Plant secondary metabolites are nutritional components of our daily diet. Phenolics such as flavonoids and stilbenes occurring in fruits, vegetables, grains, tea, and wine

are consumed in quantities up to several hundred milligrams each day. As more is known about their medicinal and pharmacological properties their nutritional relevance is becoming apparent. Experiments have shown that several flavonoids possess anti-allergenic, anti-inflammatory, antiviral and antioxidant activities and can even help combating cancer [46].

Resveratrol, the product of STS expression, was recently described as a potential phytopharmaceutical [47]. It is a component in red wine and seems to be responsible for the so-called French paradox, describing the observation that French people have a relatively low incidence of heart attack. In addition *in vitro* and animal experiments have shown that resveratrol possesses additional biological activities: Protection against arteriosclerosis by its antioxidant activity, inhibition of platelet aggregation, modulation of hepaticapolipoprotein and lipidsynthesis and the production of pro-artherogenic eicosanoids by human platelets [47].Therefore, expression of STS genes could be even a promising tools for the production of functional food and forage by metabolic engineering of beneficial natural nutriceuticals and phytopharmaceuticals or by eliminating toxic (allergic) plant constituents [48].

Pharmascience is a leader in the production of pharmaceutical grade resveratrol shipped under the trade name of Resverin™. This company provides grant programs for research on resveratrol and states that resveratrol is a powerful antioxidant in wine and grapes and has been demonstrated to have important activities on the cardiovascular system, on cancer chemoprevention and neuroprotection.

Thus, future uses of STS genes to enrich human diet with the phytopharmaceutical resveratrol can be envisioned. On the one hand resveratrol could be isolated from plants overexpressing STS and be applied as a natural food supplements in the same way as synthetic material, or the STS gene could be inserted into the crop plant leading to induce the biosynthesis of resveratrol prior to processing the crop to a health food product.

Disease resistance by engineering phytoalexin pathways

There are several genes involved in phytoalexin biosynthetic pathways whose regulation and inducibility have been investigated intensively in the plant of origin and which might provide potential in engineering improved disease resistance. One proposed means of increasing the plant's tolerance spectrum is the modification of phytoalexins to increase their toxicity or rendering pathogens unable to degrade them [49]. As a general but not absolute rule, toxicity is correlated with lipophilicity and many phytoalexins *e.g.* coumarins, stilbenes and isoflavonids are prenylated to increase their toxicity. Therefore, transfer of an isoflavonoid specific prenyltransferase in plants which does not contain prenylated isoflavonoids should enhance resistance of these plants as it was suggested for alfalfa which obviously does not contain a prenyltransferase specific for alfalfa isoflavonoids or pterocarpans. A bean pterocarpan prenyltranserase could meet this demand [48]. There is evidence that the fungitoxic properties of stilbenes are also related to their hydrophobicity [50, 51]. Pinosylvin synthase (PSS), a stilbene synthase in *Pinus sylvestris*, forms the 3,5-dihydroxystilbene, pinosylvin, which is

more hydrophobic than resveratrol [52]. A cDNA clone for PSS has been isolated and intergeneric gene transfer has been performed from scots pine to tobacco. PSS has been expressed under control of the 35S RNA promoter of CaMV and a STS promoter from grapevine. Pinosylvin synthase activity has been demonstrated in crude extracts of tobacco. The antimicrobial activity of the pine phytoalexin expressed in tobacco to different pathogens has to be evaluated. However, in the case of PSS it might be necessary to provide higher amounts of the precursor cinnamic acid. Therefore, further flux of cinnamic acid into phenylpropanoid pathway should be prevented by downregulation of endogenous cinnamate hydroxylases. The same result might be obtained by overexpressing cinnamate-CoA-ligase (CL, Fig. 1). This development opens the possibility to synthesise a second foreign phytoalexin besides resveratrol and might increase disease resistance and broaden the spectrum of antimicrobial activity of such novel plants. Methylation is a further approach to increase the hydrophobicity of phytoalexins. Therefore, the transfer of genes like resveratrol methyltransferase or pinosylvin monomethyltransferase (PSMT) to new host plants could result in the production of more fungitoxic resveratrol or pinosylvin monomethylether, respectively (see Fig. 1).

A feasible approach to escape detoxification by pathogens may be the alteration of stereochemistry. Some enzymes, associated with stereoisomerism in plants, have been studied intensively [53, 54]. Pisatin, the major phytoalexin in pea, differs from most known pterocarpanoid phytoalexins by its (+) optical stereochemistry. The enzyme responsible for this step, an isoflavone oxidoreductase, has been isolated and characterised [55]. Since it is known that some pathogens are more sensitive to the (-) enantiomer than to the (+) enantiomer of a phytoalexin, or *vice versa* [56], transfer of genes responsible for stereospecificity from one plant to another could broaden the spectrum of fungal resistance. The corresponding isoflavone oxidoreductase from chickpea has already been identified and a cDNA clone has been isolated [57]. This is the first step in synthesising more potent fungitoxic compounds in plants which cannot be degraded by the pathogen.

Since most phytoalexins are produced by complex multistep pathways, there are limited opportunities to introduce novel phytoalexins in plants by transfer of single genes. Throughout the family of Orchidaceae bibenzyls, 9,10-dihydrophenanthrens and phenanthrens occur as phytoalexins. Very recently, bibenzyl synthase (BBS), catalysing the committed step in the biosynthesis of these phenolic constituents (see Fig. 1), has been purified from the orchid *Bletilla striata* [58]. The corresponding cDNA has been isolated. With such genes the synthesis of dihydrophenanthrene phytoalexins such as hircinol and/or orchinol appears feasible in foreign plants provided that the sufficient substrates of BBS are present or inducible in the target plant. So far, enzymatically active BBS has been expressed in tobacco. However, the product of bibenzyl synthase was not yet detectable in crude extracts of transgenic tissue or plant material. Most likely the precursor 3-(3-hydroxyphenyl)propionyl CoA is not present in tobacco in sufficient amounts and thus it can be envisioned that cinnamate 3-hydroxylase (C3H), the 3-hydroxycinnamate reductase (3CR) as well as the corresponding CoA-ligase (3CL) gene have to be transferred in addition to BBS for supply of substrate. Moreover, it is not clear whether 3,5,4'-trihydroxybibenzyl can be converted into hircinol in

tobacco without the addition of further genes. This is an example for a more complex situation and shows that metabolic engineering of new phytoalexin pathways might have limitations. Nevertheless, there are other enzymes like naringenin-7-O-methyltransferase (NOMT) which has been identified in rice, and which is responsible for the synthesis of the rice phytoalexin sakuranetin [59] biotransformed from naringenin, (Fig. 5). The gene has not yet been cloned but it can be envisioned that this gene could be used in many plants for experiments to enhance disease resistance.

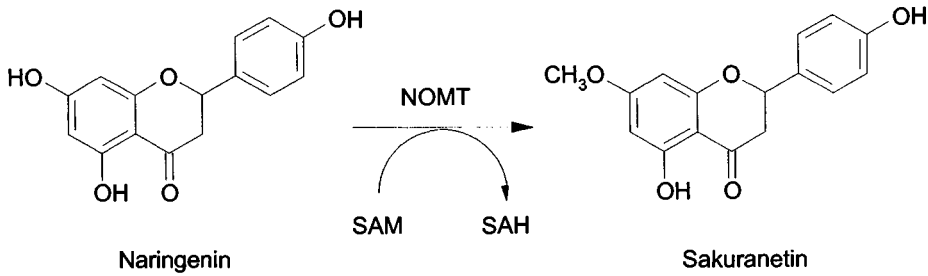

Figure 5. Biosynthesis of sakuranetin in rice. Abbreviation: SAM, S-adenoyl-L-methionine; SAH, S-adenosyl-L-homocysteine; NOMT, Naringenin-7-O-methyltransferase.

Summary and conclusions

Plant secondary metabolism produces an extravagant variety of chemicals that fulfil important functions in plant biology and have entered human life in manifold ways. They were used for diverse purposes as drugs, pharmaceuticals, food and flower colours and, not least, brings scent in our life. For these reasons, plant secondary metabolism has been a subject matter of intensive biochemical and genetic research. Many biosynthetic pathways of plant secondary metabolites have already been unravelled and together with the advent of sophisticated gene transfer technologies the genetic engineering of plant secondary metabolism becomes reasonable.

Considerable progress in enhancing resistance of plants to fungal pathogens by transfer of foreign genes has been made in model systems. However, none of the strategies seem to have resulted in acceptable resistance under field conditions. Further investigations with existing tools and/or the combination of defence mechanisms might become necessary [60]. The intriguing strategy of engineering pathogen resistance by the modification of phytoalexin biosynthetic pathways is to date limited by the availability of suitable biosynthetic genes like STS which gives rise to novel phytoalexins in a single step from common metabolic intermediates. Nevertheless, the expression of foreign antimicrobial natural compounds will undoubtedly become a successful strategy in molecular plant breeding in the future. With increasing knowledge of the molecular basis of host/pathogen interactions, increasing number of identified phytoalexin pathway genes, and expanding insight in signal transduction pathways and transcription

factors controlling metabolic pathways manipulation of plant secondary metabolism for manifold purposes becomes reasonable. We have shown that depending on the regulation of foreign phytoalexin genes biosynthetic profiles can be modified in ways which even lead to changes of plant phenotypes. The engineering of secondary metabolism will certainly not only open further possibilities to synthesise antimicrobial metabolites but also to express flavours, phytopharmaceuticals, chemical intermediates, nutriceuticals or even natural crop protection agents in plant cells or in cell culture bioreactors. The example of STS gene transfer may encourage such endeavours leading to novel and beneficial agricultural practices.

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CHAPTER 12

EXPRESSION OF THE BACTERIAL *UBIC* GENE OPENS A NEW BIOSYNTHETIC PATHWAY IN PLANTS

L. HEIDE

Introduction

Genetic engineering of plant secondary metabolism may be achieved by overexpression of secondary metabolic genes of the respective plant itself, intended to cause an increase or decrease in the content of a certain secondary metabolite. Instead of the plants' own genes, bacterial genes catalyzing the same reaction may be used, and other chapters of this book describe such approaches.

Even more challenging is the introduction of *new* biosynthetic reactions into a plant, which may lead to new, artificial secondary metabolites. This requires, however, that the plant cell is able to cope with the presence of the resulting new, potentially toxic substances. The new reaction may also open a new biosynthetic route to a secondary metabolite which was previously produced by other reaction sequences.

In order to study such processes, we decided to genetically engineer the biosynthesis of 4-hydroxybenzoate (4HB) in plants. This compound is produced as a primary metabolite both by plants and by bacteria for ubiquinone biosynthesis, and is also involved in the secondary metabolism of some plants.[1-3]

THE BIOSYNTHESIS OF 4-HYDROXYBENZOATE

Using feeding experiments with [1,7-$^{13}C_2$]shikimic acid (Fig. 1)[4] we have shown that the production of 4HB in plant cell cultures of *Lithospermum erythrorhizon* proceeds exclusively *via* phenylpropanoid intermediates, and it has been proposed that most benzoic acids as well as ubiquinones are derived from the phenylpropanoid pathway in plants.[5] Furthermore, the conversion of the phenylpropanoid precursors to 4HB was recently characterized,[3] showing that the reaction sequence from chorismate to 4HB in plants involves up to ten successive enzymatic reaction steps in cell cultures of the plant *Lithospermum erythrorhizon*.

E. coli, on the other hand, possesses a much simpler biosynthetic route to 4HB, which involves the direct conversion of chorismate to 4HB by chorismate pyruvate-lyase, the gene product of *ubi*C (Fig. 1b). If *ubiC* were expressed in a plant cell compartiment which contains chorismate, this should give rise to the formation of 4HB. We have therefore cloned the *ubiC* gene.[6,7] The encoded protein was overexpressed, purified and characterized, and shown to be a soluble protein of 19 kDa. It shows a k_m value for chorismate of 6.1 μM, a pH optimum at 7.5 and does not require cofactors.

R. Verpoorte and A.W. Alfermann (eds), Metabolic engineering of plant secondary metabolism, 233-251.

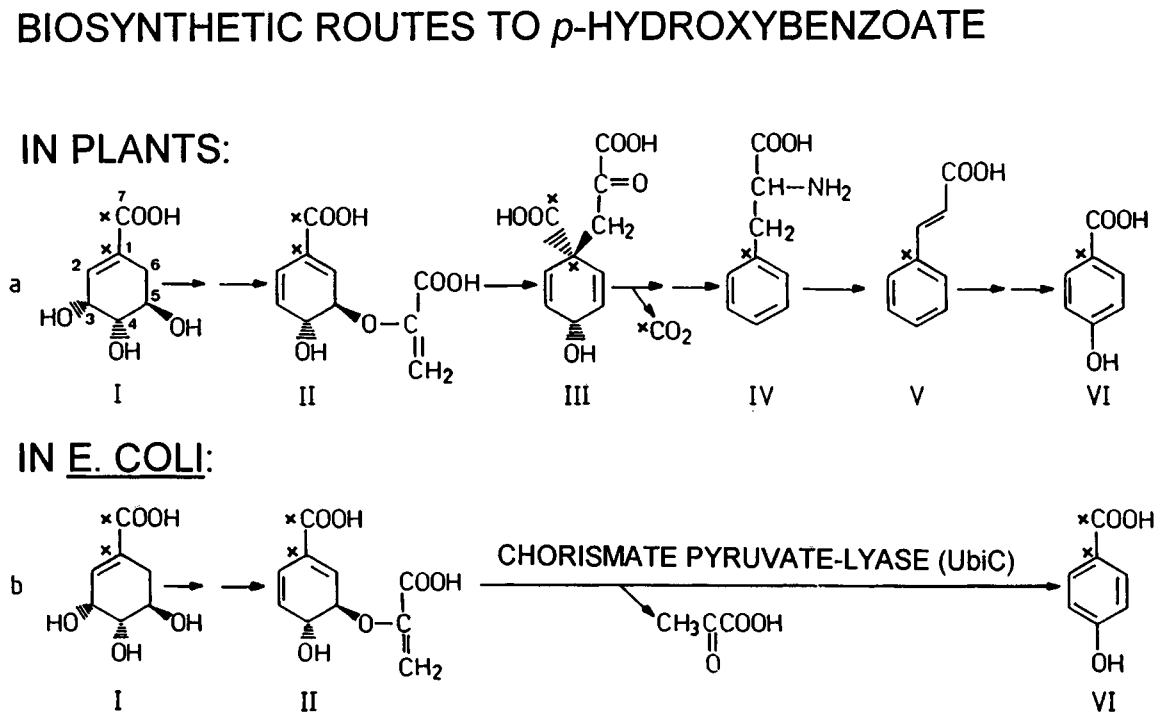

Figure 1. Pathway for biosynthesis of *p*-hydroxybenzoate in a) plants and b) *E. coli*. I, shikimic acid; II, chorismic acid; VI, *p*-hydroxybenzoic acid. The positions used for ^{13}C labeling (see text) are marked with x.

INTRACELLULAR LOCALIZATION OF SECONDARY METABOLISM IN PLANTS

In contrast to the genetic engineering of bacteria, careful consideration of the intracellular compartmentation and transport of proteins and metabolites is of essential importance in the genetic modification of plant metabolism. In this context, the plastids are a compartment of prime interest, since they appear to be the intracellular locus of important metabolic pathways both of primary metabolism (*e.g.* the shikimate pathway)[8] and secondary metabolism (*e.g.* monoterpene, diterpene and alkaloid biosynthesis).[9]

In contrast, the cytosol is the locus of the phenylpropanoid pathway, which leads from phenylalanine and tyrosine *via* hydroxycinnamic acids to lignin, flavonoids, hydroxybenzoic acids etc.[10] Whether the aromatic amino acid precursors for this pathway are provided entirely from the plastids, or whether an additional shikimate pathway exists in the cytosol, is controversial.[11] So far, all published gene sequences for shikimate pathway enzymes in plants contain sequences for transit peptides for plastidic import.[8]

Chorismate is an intermediate of the shikimate pathway and therefore localized in the plastid. If the transformation of a plant with the *ubi*C gene is intended to increase the production of 4HB, the gene product should be targeted to the plastid. Such targeting of the *ubiC* gene product to the plastids can be achieved by fusion of the N-terminus of the bacterial structural gene to the sequence of the plastidic transit peptide of the small subunit of ribulose-1,5-bisphosphate carboxylase (rubisco)[12]. The transit peptide will ensure uptake of the recombinant protein into the plastids, and will be removed by the action of a stromal protease. The cleavage site of this protease reaction is known.[13] We therefore devised a *ubiC* construct for plastidic targeting, TP-UbiC, which contained the transit peptide of the rubisco small subunit fused to *ubi*C.

USE OF AN INDUCIBLE PROMOTER

Expression of *ubiC* may have detrimental effects on a plant cell, *e.g.* due to toxicity of the phenolic compound 4HB, or due to the diversion of chorismate from its normal metabolic flow. If so, transformation events leading to a high expression of *ubiC* may not allow the regeneration of viable transgenic plants. To avoid such a selection against high *ubiC* expression, we decided to use, besides the constitutive 35S promoter, an inducible promoter. We chose the tetracycline-inducible Triple-Op (TOP) promoter,[14] which is tightly repressed during the regeneration of transgenic plants and which can be induced at any desired time point by the addition of tetracycline.

Constitutive expression of *ubi*C in tobacco

CONSTRUCTION OF BINARY VECTORS FOR *UBIC* EXPRESSION IN PLANTS

To achieve expression of the bacterial *ubiC* gene in plants, the gene was fused to a plant promoter, a plant terminator and to the above mentioned transit peptide (TP) for plastid targeting (Fig. 2). The gene construct TP-ubiC was therefore transferred into the binary

vector pROK1,[15] which contains a 35S promoter, a *nos* terminator for foreign gene expression, and a kanamycin resistance gene as a selectable marker.[16]

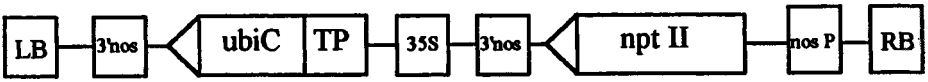

Figure 2. Binary vector p35S-TP-*ubiC* for the expression of the bacterial ubiC gene in tobacco. TP, transit peptide sequence of the small subunit of ribulose 1,5-bisphosphate carboxylase; nptII, neomycine phosphotransferase gene; 35S, CaMV 35S promoter; nosP, *nos* promoter; 3'*nos*, *nos* terminator; LB, left border; RB, right border.

UBIC EXPRESSION IN TRANSGENIC PLANTS

The binary vector construct p35S-TP-ubiC was used for *Agrobacterium tumefaciens* transformation of leaf disks of *Nicotiana tabacum* cv. Petite Havanna (SR1). In first experiments, four transgenic plants (I - IV) were regenerated under a selection pressure for antibiotic resistance, with no phenotypic changes observed in comparison to the untransformed control. These plants were examined by PCR and by genomic DNA gel blot analysis, showing the presence of the bacterial *ubiC* gene, which was absent in the untransformed control.[16] Seeds of the transgenic plants, obtained by self pollination, were germinated under sterile conditions in the presence of kanamycin, and were examined for segregation of the antibiotic-resistance phenotype. A segregation of approximately 1 (sensitive) to 3 (resistant) was observed, as expected for a monogenic dominant trait. Therefore it was concluded that the resistance gene had been stably integrated into the genome as a single copy.

RNA gel blots showed, upon hybridization with a *ubiC* probe, a single band of the expected size (approx. 1100 bases), whereas no signal was observed with SR1 control plants. The transgenic plants were investigated for chorismate pyruvate-lyase (CPL) activity, and the results are shown in Table 1. The transformants had high enzyme activity, exceeding those of a wild-type *E. coli* by a factor of up to 50. As expected, different transformants showed different activities, presumably due to integration into different positions of the genome.

Immunoblot analysis of the transgenic plants showed a single band at the appropriate position for UbiC, rather than for the TP-UbiC fusion, suggesting that the TP-UbiC fusion protein had been processed by the stromal protease of the plastid. In addition, cell fractionation experiments with cell suspension cultures obtained from the transgenic tobacco indicated that CPL activity correlated with the plastid marker enzyme shikimate dehydrogenase rather than with the cytosolic marker enzyme alcohol dehydrogenase (Table 2).

Table 1. Chorismate pyruvate-lyase activity in *N. tabacum* after expression of *ubiC* gene constructs.

Transformant		Chorismate pyruvate-lyase activity (pkat/mg protein)	Content of 4HB derivatives in leaves (mmol/g dry wt)
E. coli: pUC18a		2.7 ± 0.8	-
N. tabacum SR1		<1.0	<0.02
N. tabacum transgenic plants:			
	I	207.8 ± 21.3	17.2 ± 2.0
	II	148.4 ± 12.7	15.0 ± 4.7
	III	138.6 ± 10.1	14.6 ± 4.6
	IV	38.4 ± 8.8	5.9 ± 3.8

[a] empty vector. The value obtained with pUC18 can be expected to correspond to the activity in a wild-type *E. coli*.

Table 2. Fractionation of a crude enzyme extract from tobacco plants transformed with *ubiC*. Data represent mean values of two incubations.

Fraction	alcohol dehydrogenase (nkat/mg protein)	shikimate dehydrogenase (nkat/mg protein)	chorismate pyruvate-lyase (pkat/mg protein)
crude extract	12.9 ± 0.3	2.65 ± 0.05	35.5 ± 0. 5
cytosol	**18.2 ± 0.3**	3.45 ± 0.05	32.0 ± 1.0
orgnellar extract	3.0 ± 0.1	**9.40 ± 0.03**	**68.0 ± 2.0**

ACCUMULATION OF 4-HYDROXYBENZOATE DERIVATIVES IN THE TRANSGENIC PLANTS

The expression of active chorismate pyruvate-lyase in the plastids should give rise to the formation of 4HB. Leaves of the transgenic plants were therefore examined for their 4HB content. Transgenic plants accumulated up to 17.2 µmol 4HB/g dry weight, compared to <0.02 µmol/g dry weight in the untransformed control, and the total content of 4HB detected after acid hydrolysis in the different transgenic plants correlated with the CPL activity of these transformants (Table 1).

Without acid hydrolysis very little 4HB (0.2 μmol/g dry weight) was detected in the transgenic plants, showing that the plants accumulate 4HB mainly in the form of derivatives. To identify these 4HB derivatives fresh leaves were extracted with methanol. Two compounds were detected which were present in the transgenic plants but not in the untransformed control. These compounds were isolated by HPLC and identified as the phenolic glucoside of 4HB, *i.e.* 4-O-(1-β-D-glucosyl) benzoic acid (Fig. 3, 4HBOG) and as the ester glucoside of 4HB, *i.e.* 4-hydroxybenzoic acid 1-β-D-glucosyl ester (Fig. 3, 4HBCOOG).

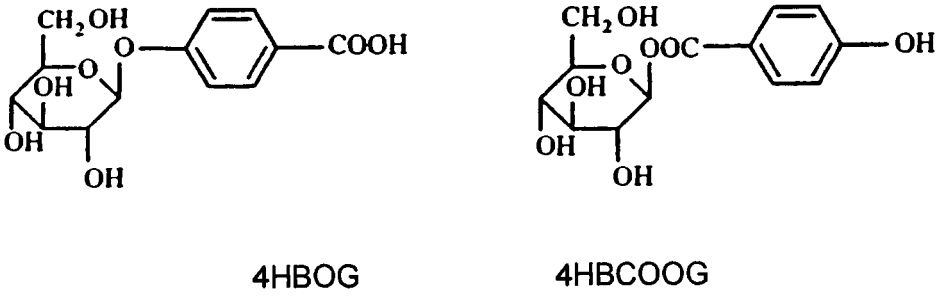

Figure 3. 4-Hydroxybenzoate derivatives accumulated in tobacco plants expressing the bacterial *ubiC* gene. 4HBOG = 4-O-(1-ß-D-glucosyl)benzoic acid.

The total amount of 4HB derivatives, calculated as glucosides, was approx. 0.52% of dry weight. Of this total 4HB, the phenolic glucoside **4HBOG** represented approx. 69%, the ester glucoside **4HBCOOG** approx. 26%. Free 4HB accounted for only 1.2% of the total, while another 1.7% was bound to the cell wall and could be released by acid or alkaline hydrolysis. The ratio between phenolic and ester glucoside was essentially the same in all four transformants.

INCORPORATION OF [1,7-$^{13}C_2$]SHIKIMIC ACID INTO 4-HYDROXYBENZOATE IN THE TRANSGENIC PLANTS

To quantify to which extent the 4HB in our transgenic plants was a product of the artificially introduced CPL reaction or of the endogenous phenylpropanoid pathway, we carried out feeding experiments with [1,7-$^{13}C_2$]shikimic acid, analogous to published experiments with *E. coli* and *Lithospermum erythrorhizon*.[4,7] Feeding studies were carried out with suspension cell cultures, which were established from the transgenic tobacco (transformant II). These cell cultures were shown to accumulate the same 4HB derivatives as in the intact transgenic plant. After feeding of the ^{13}C-labelled precursor the phenolic glucoside of 4HB was isolated, crystallized, subjected to ^{13}C NMR analysis. Both labelled carbons were incorporated (1.9% enrichment) into 4HB in the transgenic plants (see Fig. 1). After calculating the integrals of the peaks and the accuracy of the measurement, we concluded that at least 95% of the 4HB, found as phenolic glucoside in the transgenic plants, was produced by the CPL reaction that had been introduced by genetic engineering.

DETOXIFICATION OF 4-HYDROXYBENZOATE

For the introduction of new biosynthetic reactions by methods of genetic engineering, it is of importance that the transformed plant is able to tolerate the formation of the resulting new, potentially toxic secondary metabolites. We therefore wanted to study the reaction of plant cells to the genetically altered secondary metabolism, and to examine the detoxification mechanisms which may be employed. Since the expression of the bacterial *ubi*C gene in tobacco lead to a more than 1,000-fold increase of the content of 4-hydroxybenzoate (4HB) derivatives in the plant,[16] this system presented a very suitable object for such studies.

4HB GLUCOSYLTRANSFERASE ACTIVITIES IN TRANSGENIC AND WILD-TYPE TOBACCO CELLS

The observed conversion of free 4HB into its glucosides may represent a detoxification mechanism of this potentially toxic substance. An enzymatic glucosylation of 4HB was readily detected in cell-free extracts from the transgenic tobacco cell cultures, which formed 4HB by expression of the bacterial gene *ubi*C. The formation of the two enzymatic products, *i.e.* 4HBOG and 4HBCOOG, was dependent on the presence of 4HB, UDP-glucose and active enzyme; the enzyme activities forming 4HBOG and 4HBCOOG were soluble, *i.e.* they were found exclusively in the supernatant of a 100,000 x *g* centrifugation.

Since 4HB is a new, artificial secondary metabolite in the transgenic cell cultures, it may be expected that the 4HB glucosyltransferase activities in these cultures are induced in response to 4HB formation. However, comparison of the 4HB glucosyltransferase activities found in transgenic and in wild-type (SR1) tobacco cell cultures showed very similar activities in both cultures. Even upon addition of up to 2 mM external 4HB to the cell cultures, no increase of glucosyltransferase activity was observed, neither in transgenic nor in wild-type tobacco. Therefore, detoxification of the artificial secondary metabolite 4HB is carried out by constitutively expressed glucosyltransferases, and these enzymes are not induced neither by endogenously nor by exogenously supplied 4HB.

We investigated the time course of the glucosyltransferase activities forming 4HBOG and 4HBCOOG over the culture period. The enzyme activity forming 4HBCOOG increased 3.5-fold within one day after inoculation, and decreased rapidly to its original level thereafter. In contrast, the enzyme activity forming 4HBOG changed after inoculation only by 50%.[17] This indicates that the two reactions are catalyzed by different enzymes, which show differences in their regulation.

RATIO OF 4HBOG AND 4HBCOOG FORMATION UNDER DIFFERENT CONDITIONS

In transgenic tobacco cell cultures, the ratio of 4HBOG and 4HBCOOG accumulated was approximately 3:1. This ratio remained constant at different growth stages of the cell cultures and was also found in the intact transgenic plants. In contrast, the enzyme activities observed *in vitro* showed a ratio of approximately 1:3 for

4HBOG and 4HBCOOG formation. When we investigated the pH dependence of both glucosyltransferases using extracts of transgenic cell cultures, the glucosyltransferase forming 4HBCOOG had a higher activity than that forming 4HBOG at all pH values. We examined whether the presence of a glucosidase hydrolyzing 4HBCOOG may explain the low levels of 4HBCOOG accumulated *in vivo*. However, glucosyltransferase activity greatly exceeded glucosidase activity at all pH values examined. Also chemical decomposition of 4HBCOOG could be excluded as a reason for the low accumulation of 4HBCOOG *in vivo*.

The different ratios of glucoside formation *in vitro* and *in vivo* could finally be explained when cell-free extracts were incubated with UDP-glucose and different concentrations of 4HB. At low 4HB concentration (8 μM), 4HB was converted mainly to 4HBOG, and the resulting ratio of the two glucosides resembled the ratio found *in vivo*. With increasing 4HB concentration (up to 5 mM), however, the rate of 4HBCOOG formation increased more rapidly than that of 4HBOG formation, and 4HBCOOG became the dominant product.[17] These data indicate that the glucosyltransferase forming 4HBCOOG has a lower affinity, *i.e.* a higher k_m value for 4HB than the enzyme forming 4HBOG, and that the former enzyme shows a higher total activity in our extracts than the latter. A similar dependence of the ratio of glucoside accumulation on the concentration of 4HB was observed *in vivo* after feeding 4HB at various concentrations to the transgenic cells.[17]

FURTHER METABOLIZATION OF 4HBCOOG: INCORPORATION OF 4HB INTO CELL WALLS

After external feeding of 4HB to transgenic cell cultures, the accumulation of 4HB glucosides was monitored over the entire culture period (Fig. 4). This revealed an unexpected effect: whereas 4HBOG was apparently stable, and its content increased constantly during culture, 4HBCOOG showed only a transient accumulation, and its content subsequently diminished quickly. After feeding 4HB at 2 mM, the decrease of 4HBCOOG content from day 2 to day 15 was much faster than the increase of 4HBOG content. Examination of the culture medium showed that 4HBCOOG was not excreted by the cells. These observations raised the question to which compound 4HBCOOG was metabolized after its transient accumulation in the cell.

Glucose esters *e.g.* of hydroxycinnamic acids present an activated form of these acids, and the acyl moiety can be transferred to hydroxy or amino groups[18-21]. In the same way, 4HBCOOG may act as an acyl donor, transferring the 4-hydroxybenzoyl moiety to an alcohol or amine. HPLC analysis of methanolic extracts of our tobacco cells using UV detection at 254 nm, however, showed no evidence of the formation of other 4HB derivatives than the two glucosides 4HBOG and 4HBCOOG. Likewise, after feeding of [U-^{14}C]4HB, no other radioactive products were observed in the methanolic extracts besides 4HBOG and 4HBCOOG. In elicited cultures of *Daucus carota,* 4HB has been found in the cell wall,[1] and similarly in *Populus spp.*, 4HB was found to be esterified to lignin.[22,23] In order to investigate the possible incorporation of 4HB *via* 4HBCOOG into cell walls, we fed [U-^{14}C]4HB externally to the tobacco cell cultures and measured its uptake from the medium and its incorporation into 4HBOG,

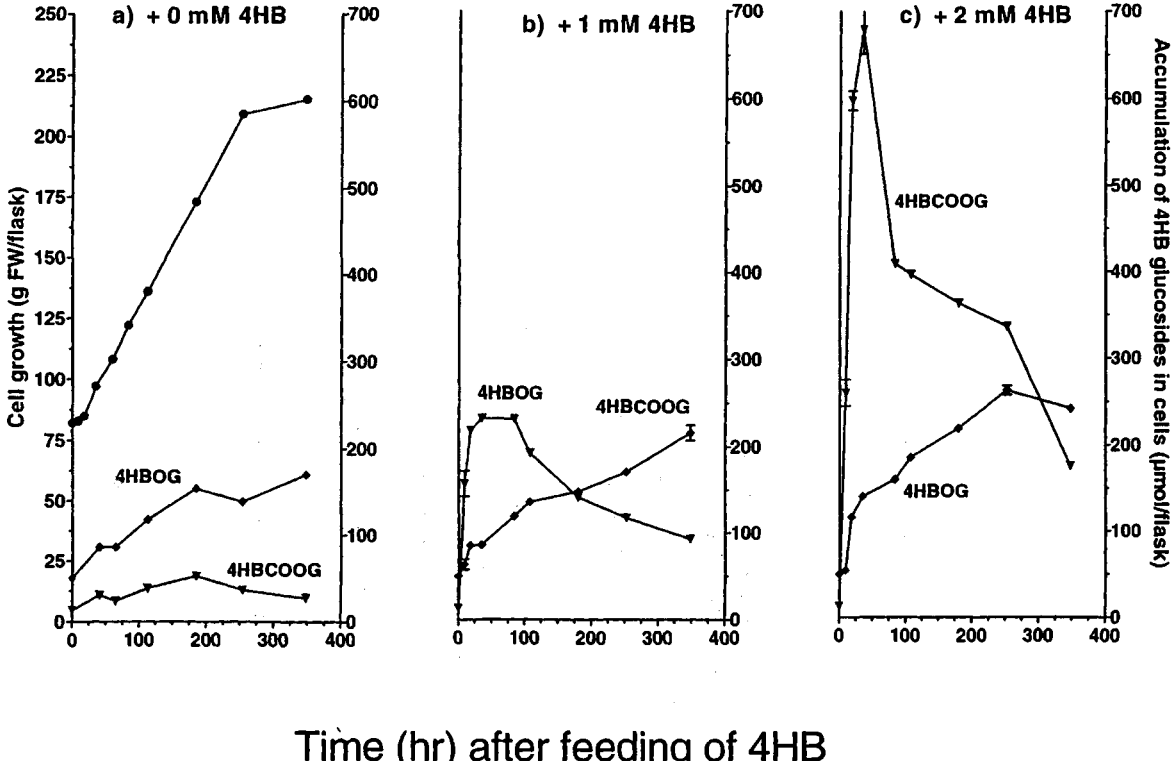

Figure 4. Time course of accumulation of 4HBCOOG and 4HBOG in transgenic tobacco cell cultures after feeding of 0, 1 or 2 mmol 4HB/l medium, respectively. Cells were cultured in 300 ml liquid medium. 4HB was fed two days after inoculation.

4HBCOOG and into the cell wall. The results are shown in Table 3. Three days after feeding, 4HBCOOG constituted 45% of the total radioactivity found in the cultures. This value thereafter decreased to 28%, while the radioactivity in cell walls increased from 28% at day 3 to 42% at day 15. The incorporation of 4HB in its intact form into cell walls was confirmed by feeding of [U-^{14}C]4HB to the cells, alkaline hydrolysis of the purified cell wall fraction and HPLC analysis of the resulting solution; 4HB was the only radioactive compound detected under these conditions. A significant part (50-75%) of the radioactivity in the cell walls, however, was not released by alkali treatment, possibly indicating that 4HB was attached to the cell wall not only by ester bonds but also by C-C bonds formed by phenol coupling with the aromatic moieties of lignin.

Table 3. Incorporation of [U-^{14}C]4HB into 4HB glucosides and into the cell wall in cell cultures of transgenic tobacco 4HB was fed at a concentration of 0.67 mmol/l medium.

time after feeding	distribution of total radioactivity recovered (%)			
	medium	4HBOG	4HBCOOG	cell wall
3 d	12.3 ± 1.8	14.6 ± 1.3	45.0 ± 2.3	28.1± 3.7
6 d	2.6 ± 0.5	21.0 ± 2.8	43.9 ± 3.9	32.5 ± 5.5
12 d	1.2 ± 0.1	27.8 ± 3.3	32.8 ± 1.6	38.2 ± 1.8
15 d	0.3 ± 0.0	29.2 ± 0.6	28.2 ± 0.8	42.3 ± 1.4

The results given above indicate that 4HBCOOG is an activated form of 4HB and that the acyl moiety of 4HBCOOG can be transferred to cell wall components. This transfer may either occur intracellularly, *e.g.* in Golgi vesicles, or in the apoplast under catalysis of extracellular enzymes. The latter hypothesis could be excluded, however, by feeding of [U-^{14}C]4HB, [U-^{14}C]4HBCOOG and [U-^{14}C]4HBOG to the cell cultures. No significant uptake of the intact glucosides neither into cells nor into cell walls could be shown under these conditions.

A demonstration of the transfer of the acyl moiety of 4HBCOOG to cell wall components in cell-free extracts is difficult since it is not known which cell wall precusor may serve as the acceptor of the acyl group. When crude cell wall preparations were incubated with cell-free extracts of transgenic tobacco cells and with [U-^{14}C]4HBCOOG, a low incorporation of radioactivity into the cell wall fragments could be detected (0.6% incorporation in 2 hrs). The incorporation obtained with [U-^{14}C]4HBCOOG as substrate reproducibly exceeded that obtained with [U-^{14}C]4HB, at least by a factor of 2, and no significant incorporation was observed with [U-^{14}C]4HBOG. However, overall incorporation rates were too low to allow a detailed investigation of this reaction.

EXPRESSION OF UBIC UNDER CONTROL OF AN INDUCIBLE PROMOTER

Vector construction and transformation of tobacco

The recombinant plant promoter Triple-Op contains the constitutive 35S plant promoter with a tetracycline-inducible repressor/operator element from a bacterial transposon. This promoter is normally tightly repressed in plant cells, but specifically induced by the addition of tetracycline or chlorotetracycline.

The bacterial gene *ubiC,* combined with a chloroplast transit peptide, was fused to the inducible Triple-Op promoter. Using *Agrobacterium tumefaciens*, the resulting construct was transformed into leaf disks of *Nicotiana tabacum* W38 TET plants, which express the TET repressor of the bacterial Tn10 transposon.[24] Intact plants were regenerated, and three cell lines, called Tipo I, II and III and representing different transformation events, were established from the transgenic plants. After induction of the Tipo lines with chlorotetracycline, Northern blot analysis clearly showed the *ubiC* transcripts. No mRNA was detected in the absence of chlorotetracyline. In the genomic DNA of the transgenic cell cultures, the presence of the *ubiC* gene constructs was confirmed by Southern blotting and by PCR analysis.[25]

Induction with different amounts of chlorotetracycline

Induction with chlorotetracycline resulted in the formation of 4HB glucosides in the transgenic cell cultures. Whereas no induction of 4HB formation was detected with 0.1 µg chlorotetracycline/l medium, a concentration-dependent formation of 4HB was observed between 1 and 1000 µg chlorotetracycline/l. The data indicate that a half-maximal induction is achieved by approx. 20 µg/l. Raising the chlorotetracycline concentration above 1000 µg/l did not increase the 4HB levels any further, and 10.000 µg/l and more reduced cell viability.

Time course of the induction of mRNA and of enzyme activity

After addition of 2 mg/l chlorotetracycline, the time course of *ubiC* mRNA formation, CPL enzyme activity and 4HB glucoside accumulation was observed.

ubiC mRNA was detectable already after 15 min and reached a maximum about 3-5 hours after induction, and the level remained constant thereafter for at least 3 days. In contrast, enzyme activity required approximately 8 days to reach the plateau level (Fig. 5), and the accumulation of the 4HB glucosides reached its maximum only after several weeks. These data suggest that interaction of chlorotetracycline with the tet repressor occurs very quickly, and maximum transcription rates may be achieved within minutes after addition of the inducer. A steady state between mRNA synthesis and mRNA degradation is reached after 3 to 5 hours, allowing a maximum rate of synthesis, transport and processing of the protein. Apparently, the enzyme is rather stable in the plastids, since only after approximately 8 days an equilibrium between protein synthesis and protein degradation is reached.

After 13 weeks of subculturing in the continous presence of chlorotetracycline, TipoI cells were transfered to medium without chlorotetracycline. CPL activity dropped rapidly, indicating a degradation of the enzyme protein. The decrease of the 4HB glucoside content per g dry weight was slower and may be explained simply by the continous cell growth in the absence of 4HB synthesis.

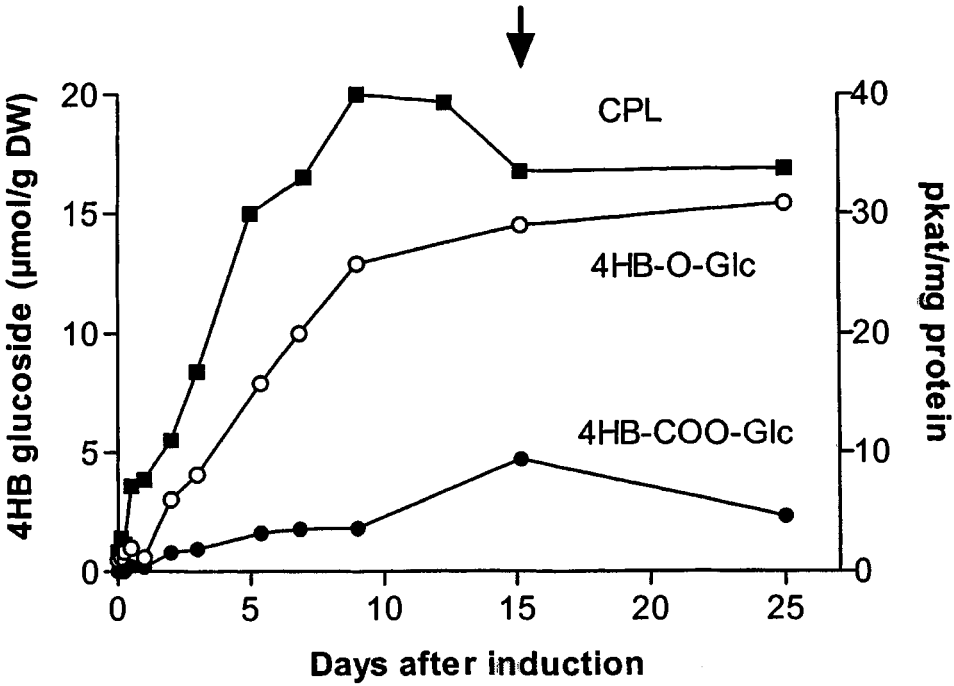

Figure 5. Time course of CPL activity and accumulation of 4HB glucosides after induction of TipoI suspension cultures with chlorotetracycline (2 mg/l). The arrow indicates the time of subculturing.

In conclusion, suspension cultures transformed with the *ubi*C gene present a very suitable system to study induction processes regulated by the recombinant, tetracycline-inducible plant promoter Triple-Op.[14] Within minutes after application of the inducer, the transcripts of the *ubiC* gene could be detected, followed later by an increase of CPL enzyme activity and of 4HB accumulation. The time required to reach maximum enzyme levels, however, was comparatively long (8 days), and the biotechnological application of similar protocols for the production of useful compounds would require a shortening of this time, *e.g.* by the development of promotors which cause higher transcription rates. The increase of enzyme activity and especially of secondary product formation appears slow at a first glance. It has to be considered, however, that data were measured in a fast-growing cell culture, where secondary product content is continously diluted by an exponential growth of cell mass. If the inducer were added to a single batch culture towards the end of the exponential growth phase, maximum productivity may be reached considerably faster. The use of recombinant inducible promoters may become possible for the secondary metabolite production in plant cell cultures, and this method may become a useful biotechnological tool, as it is already in industrial microorganisms.

TRANSFORMATION OF *LITHOSPERMUM ERYTHRORHIZON* CELL CULTURES

Cell cultures of *Lithospermum erythrorhizon* (Boraginaceae) are capable of producing large amounts of the red naphthoquinone pigment shikonin and its derivatives, when cultured in the production medium M9.[26,27] Shikonin shows antibacterial,[28] anti-inflammatory, wound-healing [29] and anti-tumor[30,31] activities and became the first plant-derived pharmaceutical produced industrially by plant cell cultures.[32]

The two key precursors for the biosynthesis of shikonin are 4-hydroxybenzoate (4HB) and geranylpyrophospate (GPP) (Fig. 6). 4HB is derived from cinnamate,[33,34] and Löscher *et al.*[3] have shown that the degradation of the side chain of cinnamate proceeds *via* p-coumaroyl-CoA which is cleaved to 4-hydroxybenzoyl-CoA and acetyl-CoA, in analogy to the β-oxidation of fatty acids. The entire reaction sequence from chorismate to 4HB in plants therefore involves up to 10 successive enzymatic reaction steps. In contrast, the direct formation of 4-hydroxybenzoate from chorismate in a single step is catalyzed by the gene product of *ubi*C (Fig. 1).

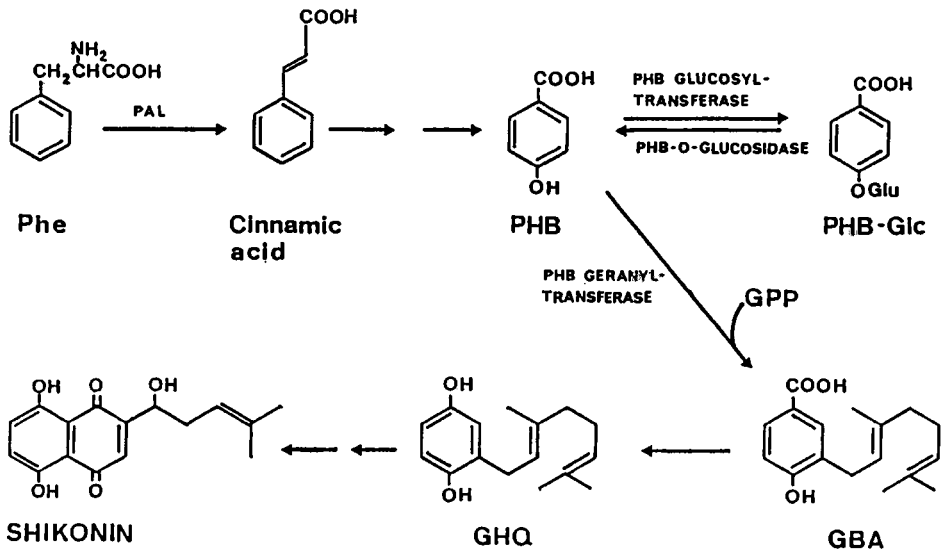

Figure 6. Biosynthesis of shikonin.

Expression of the *ubiC* gene in *Lithospermum erythrorhizon* cell cultures should present an elegant way to genetically engineer the biosynthesis of the biotechnological product shikonin. Introduction of this gene may shorten the biosynthesis of shikonin precursor 4HB by several reaction steps, circumventing the need for phenylpropanoids intermediates and influencing the quantity, the regulation and the stability of secondary metabolite production in these cell cultures.

Hairy root cultures from *Lithospermum erythrorhizon* have been shown to produce large amounts of shikonin.[35] Therefore we used *Agrobacterium rhizogenes* in order to

establish *ubiC*-transformed *hairy root* cultures from *L. erythrorhizon* and investigated the effect of this transformation on the secondary metabolism in these cultures.

UBIC EXPRESSION IN TRANSGENIC HAIRY ROOT CULTURES

Transformation was carried out with the p35S-TP-ubiC construct shown in Fig. 2, and with the pTOP-TP-ubiC construct containing the Triple-Op (TOP) promoter.[14] In the absence of the *tet* repressor protein, the TOP promoter functions as a constitutive plant promoter.

In genomic DNA of the transformed *hairy root* cultures, the presence of the *ubiC* gene could be detected both by PCR and by southern hybridization. RNA blots showed, upon hybridization with a *ubiC* probe, a single band of the expected size of the *ubiC* transcript (approximately 1100 bases). Transcription and translation of the *ubiC* gene resulted in the formation of active chorismate pyruvate-lyase. As expected, enzyme activity varied between different culture lines, most likely due to integration at different positions in the genome.

CONTRIBUTION OF THE GENETICALLY INTRODUCED CHORISMATE PYRUVATE-LYASE REACTION TO SECONDARY METABOLITE FORMATION IN *L. ERYTHRORHIZON*

Suspension cultures of *L. erythrorhizon* form shikonin in the production medium M9. In the presence of ammonium ions (*e.g.* in MS or MSV medium), however, shikonin formation is suppressed and 4HB-O-glucoside is accumulated instead (Fig. 6).[2,36,37] The hairy root cultures (both control and *ubiC* transformed) reacted in the same way.

In order to provide unequivocal proof that the 4HB formed *via* the CPL reaction is used as a precursor for secondary metabolites in the *ubiC*-transformed *L. erythrorhizon* cultures, a feeding experiment with [1,7-$^{13}C_2$]shikimic acid was carried out, similar to the above mentioned experiments with *ubiC*-transformed *N. tabacum*.[4,7,16] 10 days after feeding, 4HBOG was isolated and analysed by ^{13}C NMR. As expected incorporation of label was detected both in the carboxy group and the neighbouring ring position, with 3.4% and 16.7% enrichment of ^{13}C, respectively. Therefore, about 20% of the accumulated 4HBOG is produced by the newly introduced pathway to 4HB *via* CPL.

The contribution of the CPL reaction to the formation of shikonin was assessed by addition of the PAL inhibitor AIP[38] to transgenic and control cultures in the shikonin production medium M9. AIP blocks 4HB biosynthesis *via* the phenylpropanoid pathway and therefore completely inhibited shikonin formation in the control culture. The *ubiC* transformed culture, however, retained 36% of its shikonin formation even in the presence of 4HB.

INFLUENCE OF UBIC-TRANSFORMATION ON THE TOTAL AMOUNT OF SECONDARY METABOLITES IN *L. ERYTHRORHIZON* CELL CULTURES

A comparison between 9 different lines of control *hairy root* cultures, 11 different lines of TOP-TP-UbiC-transformed cultures and 4 different lines of 35S-TP-UbiC-

transformed cultures showed high clonal variation between lines (Fig. 7). Both in control and in *ubiC*-transformed cultures, low producing and high producing lines can be found, and overall no statistically significant differences in the secondary metabolite formation of the three groups was detected in the examined number of lines.

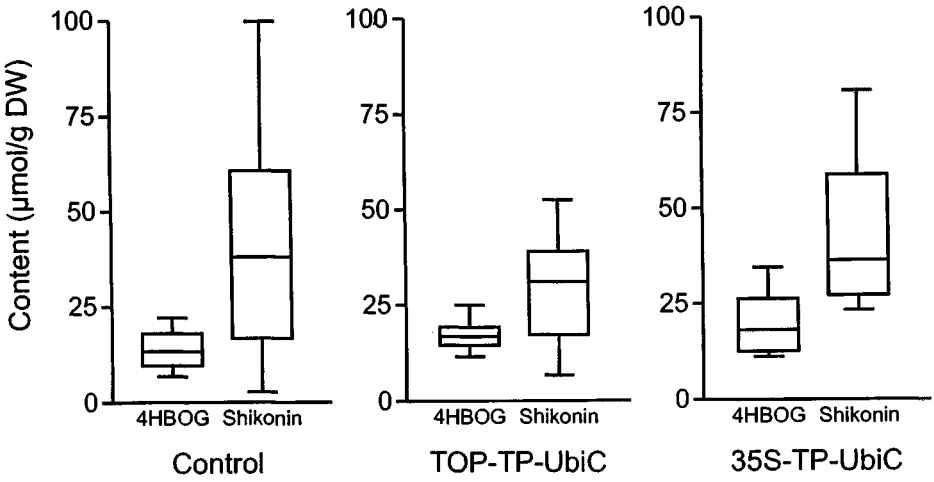

Figure 7. Variation of 4HBOG and shikonin contents in different lines of control cultures, and *ubiC* transformed cultures. Control: 9 different lines; TOP-TP-UbiC: 11 different lines; 35S-TP-UbiC: 4 different lines. All measurements were carried out in duplicate. Box plot diagrams show: minimum, first quartile, median, third quartile, maximum.

DISCUSSION

Genetic engineering of secondary metabolism in plants may open the possibility to improve the disease resistance of plants[39] or the production of useful secondary metabolites such as pharmaceuticals. Expression of the bacterial *ubi*C gene in plants, described in this chapter, allowed the introduction of a new biosynthetic pathway, which led to the formation of 4HB glucosides as new, artificial secondary metabolites in tobacco.

This conversion of chorismate to 4HB presents a certain diversion from the natural flow of the shikimate pathway, producing now a secondary metabolite instead of aromatic amino acids. However, the transgenic plants did not show visible phenotypic changes, suggesting that this pathway can be accomodated without seriously affecting other metabolic processes involving shikimate pathway intermediates.

Tobacco cells transformed with the bacterial *ubi*C gene are challenged with the formation of high amounts of 4HB, a phenolic metabolite which is not normally accumulated in tobacco and which is potentially toxic. Cells respond to this challenge by a rapid glucosylation of 4HB to 4HBOG and 4HBCOOG, *i.e.* the phenolic and the ester glucoside of 4HB. This glucosylation of 4HB is achieved by constitutively expressed glucosyltransferases which are found in transgenic and wild-type tobacco cells in the same activity.

We could show that the metabolic fates of 4HBOG and 4HBCOOG are very different. 4HBOG appears to be accumulated as a stable, artificial secondary metabolite in the cell. Cell cultures of *Lithospermum erythrorhizon* accumulate this glucoside as a natural secondary metabolite in the vacuole,[44] and the same compartiment may be used in the transgenic tobacco cells for the storage of this compound. 4HBCOOG, in contrast, is an activated intermediate, showing a similar metabolization as described for the ester glucosides of hydroxycinnamic acids;[18-21] *i.e.* it appears to be used for the acylation of cell wall components, such as lignin.

Cell wall-bound 4HB may play a role as phytoalexin, *i.e.* in the plant defense against pathogens. It has been shown that the formation of cell wall-bound 4HB in *Daucus carota* cell cultures is induced by fungal elicitors.[40,41] In addition, it has been reported that 4HB stimulates the production of pathogen-related proteins in *Nicotiana tabacum*, although to a considerably lower extent than salicylic acid.[42] Therefore, it would be of considerable interest to examine whether *ubi*C expression in plant cells may influence the pathogen resistance of the transgenic plants.

By expression of *ubi*C in cell cultures of *Lithospermum erythrorhizon* we have also modified the biosynthetic pathway to shikonin, a biotechnologically produced pharmaceutical. Introduction of the bacterial *ubiC* gene created a 'shunt' from the shikimic acid pathway (*i.e.* from chorismate) to 4HB, which is a central intermediate of the shikonin biosynthesis (Fig. 6). The newly introduced pathway contributed about 20% of the entire biosynthesis of 4HB derivatives, as shown by feeding experiments. Since the *ubiC* gene in our study had been fused to a sequence for a chloroplast transit peptide, the expression of these constructs in *L. erythrorhizon hairy roots* should lead to the formation of 4HB in the plastids. In contrast, the native site of 4HB formation in the secondary metabolism in *L. erythrorhizon* is the cytosol and the endoplasmatic reticulum.[43] 4HBOG is accumulated in the vacuole.[44] The incorporation of CPL-derived 4HB into 4HBOG and shikonin therefore indicates transport of 4HB through the plastidic membrane either by diffusion or by a specific transporter system.

The transformation with *ubiC* appeared to cause an increase of the production of 4HBOG (data not shown), but not of shikonin (Fig. 7). This confirms earlier experiments which showed that HMG-CoA-reductase[45] and 4HB geranyltransferase[37] are of central importance for the regulation of shikonin biosynthesis. An increase of the production of shikonin may not be achieved by overexpression *ubi*C alone but rather by a simultaneous overexpression of several activities, such as chorismate pyruvate-lyase, HMG-CoA-reductase and 4HB geranyltransferase.

Several studies have reported an increase of secondary produced formation by methods of genetic engineering. *E.g.*, a 20% increase of anthraquinone production was reported to result from an expression of bacterial isochorismate synthase in cell cultures of *Rubia peregrina*.[46] Figure 7 of our study shows that somaclonal variation of secondary product formation is high, not only between the transgenic lines but also between control lines. Even if certain transgenic lines contain *e.g.* twice as much secondary metabolites as a given control, only a careful statistical evaluation of high numbers of transgenic lines and appropriate control lines can determine whether the increased content is indeed due to introduced enzyme activities.

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CHAPTER 13

REGULATION OF TROPANE ALKALOID METABOLISM IN PLANTS AND PLANT CELL CULTURES

K-M. OKSMAN-CALDENTEY *and* R. ARROO

Introduction

Tropane alkaloids are among the oldest drugs in medicine. Besides the medicinal use they possess hallucinogenic and poisonous properties. Many plants belonging to the Solanaceae family have been used for centuries because of their active principles, hyoscyamine and scopolamine. The name hyoscyamine is derived from *Hyoscyamus niger* from which these two tropane alkaloids also for the first time were isolated.[1,2] Later on Dunstan and Brown[3] detected hyoscyamine in *H. muticus*. Also other Solanaceous plants belonging to *Atropa*, *Datura*, *Duboisia* and *Scopolia* produce these valuable alkaloids.

Atropine, hyoscyamine and scopolamine affect the parasympathetic nervous system. They are competitive antagonists of acetylcholine and other muscarinic agonists in organs innervated by postganglionic cholinergic nerves. In medicine they find important applications in ophthalmology, anaesthesia, and in the treatment of cardiac and gastrointestinal diseases. In addition to their peripheral anticholinergic effects they also act on the central nervous system and are used to relieve the symptoms of Parkinson's disease, and as antidotes for the anticholinesterases such as organophosphates. Scopolamine has a stronger effect on the central nervous system at low therapeutic doses, but causes fewer side-effects, and is used therefore for the treatment of motion sickness and the production of derivative drugs for gastric disorders.[4] Cocaine, which has strong stimulant effects on the central nervous system and is used as a topical anaesthetic, is also a tropane alkaloid but is found outside Solanaceae in *Erythroxylum coca*. The total tropane alkaloid content varies from 0.01 - 3% depending on the plant species. In some cases plants have been cultivated that produce over 4% of scopolamine only[5] (M. Keil, personal communication).

Although a lot of information is available on the pharmacological effects of tropane alkaloids, surprisingly little is known about how the plants synthesize these substances and almost nothing is known about how synthesis is regulated. Progress in the elucidation of the biosynthetic pathways of plant secondary products has long been hampered by lack of good model systems. In the past two decades plant cell and tissue cultures have proven to be invaluable tools in the investigation of plant secondary metabolite biosynthetic pathways.[6] Plant cell and tissue cultures have also been widely studied in order to obtain alternative production systems of tropane alkaloids.[7,8,9] The main problem has usually been a lack of sufficient amount of alkaloids and/or instability of the production.

R. Verpoorte and A.W. Alfermann (eds), Metabolic engineering of plant secondary metabolism, 253-281.

Many cultures have shown a decrease in productivity with time. Moreover, the bioreactor cultivation of the plant cells or differentiated tissues is complicated and more expensive than that of microbes.[10] Thus, a better understanding of the function of the regulatory genes in the biosynthesis of tropane alkaloids is essential to be able to improve the productivity in plants or in plant cell cultures by genetic engineering.

In this review, recent achievements in tropane alkaloid biosynthesis especially at enzymatic level are described. Moreover, the present possibilities and future prospects of genetic engineering in the production of tropane alkaloids are discussed.

Importance of tropane alkaloids

The medicinal importance of tropane alkaloids, hyoscyamine and scopolamine, is demonstrated by the range of their pharmaceutical applications as described in introduction. Both alkaloids have (+)- and (-)- forms but only (-)-hyoscyamine and (-)-scopolamine are biologically active. Atropine is a racemic mixture of (-)- and (+)-hyoscyamine and the racemization usually occurs during its isolation from plants. Scopolamine seems to be more resistant to racemization.[7] As the chemical synthesis of scopolamine and hyoscyamine have proved to be difficult and not economically feasible, these alkaloids are excusively produced by plants. Presently the most important commercial sources are a *Duboisia* hybrid, which is cultivated mainly in Australia, and *Atropa belladonna* L.[11] The world demand for scopolamine is estimated to be about 10 times larger than that of (-)-hyoscyamine and atropine combined. The price of scopolamine as pure substance (HBr salt) is about $ 27/g according to the producer (Sigma, 1999). Although the market volume for tropane alkaloids is not very high there are no other classes of compounds that can be substituted for these plant-derived drugs and therefore the demand for them will continue.[12]

Calystegins constitute a novel and unique subgroup of the tropane alkaloid class.[13] They are characterized by the absence of an *N*-methyl substituent and a high degree of hydroxylation. Trihydroxylated calystegins are summarized as the calystegin A-group, tetrahydroxylated calystegins as the B-group, and pentahydroxylated derivatives form the calystegin C-group.

Calystegins were originally discovered in roots and root exudates of *Calystegia sepium*, *Convolvulus arvensis* (Convolvulaceae), and *Atropa belladonna* (Solanaceae).[14] The authors tested another 102 plant species, amongst which a number of Solanaceae, but were unable to detect any further calystegins. It was concluded then that the synthesis of these compounds is rare in the plant kingdom. More recently however, calystegins have been described in a number of plant species, notably Convolvulaceae and Solanaceae (Table 1).

Polyhydroxy alkaloids, in general, have been found to be competitive inhibitors of various glycosidases, presumably by virtue of structural similarities to carbohydrates. Calystegins represent a novel structural class of polyhydroxy alkaloids possessing potent glycosidase inhibitory properties next to longer known classes of the monocyclic pyrrolidones (*e.g.* dihydroxymethyldihydroxy pyrrolidine) pyrrolines and piperidines (*e.g.* deoxynojirimycin), and the bicyclic pyrrolizidines (*e.g.* australine) and indolizidines

(*e.g.* swainsonine and castanospermine).[15,16] Glycosidases are involved in several important biological processes, such as intestinal digestion, the biosynthesis of glycoproteins, and the lysosomal catabolism of glycoconjugates. Glycosidase inhibitors are potentially useful as antidiabetic, antiviral, antimetastatic, and immunomodulatory agents.[17]

Table 1. Occurrence of different calystegins in plant kingdom.

Plant species	**Presence of calystegins**	**Reference**
Convolvulaceae		
Calystegia sepium	A_3, B_1, B_2	13
Convolvulus arvensis	A_3, B_1, B_2	13
Ipomoea sp. *Q6*	B_2	19
I. polpha	B_2, C	19
Moraceae		
Morus alba	A_3, B_1, B_2, C_1	16
M. bombycis	B_1	22
Solanaceae		
Atropa belladonna	A_3, B_1, B_2	13
Capsicum sp.	A_3, B_1, B_2, C_1	23
Datura wrightii	B_2	18
Duboisia leichhardtii	B_1, B_2, B_4, C_1, C_2	24
Hyoscyamus albus	A_3, B	25
H. aureus	A_3	25
H. muticus	A_3	25
	A_3, B_1, B_2	26
H. niger	A_3	25
	A_3, A_5, A_6, B_1, B_2, B_3, N_1	27
H. pusillus	A_3	25
Lycium chinense	A_3, A_5, A_6, A_7, B_1, B_2	
	B_3, B_4, B_5, C_1, C_2, N_1	28
Lycopersicum esculentum	A_3, B_1, B_2,C_1	23
Mandragora officinarum	A_3, B_1,B_2	29
Nicandra physaloides	B_1-glucoside	30
Physalis alkekengi	A_3, A_5, B_1, B_2, B_3	31
Scopolia carniolica	A_3, B_1, B_2	29
S. japonica	A_3, A_5, B_1, B_2, B_3, B_4	32
Solanum dimidiatum	B_2	18
S. dulcamara	B_2	18
S. kwebense	B_2	18
S. melongena	B_2	18
S. tuberosum	A_3, B_2	18
	A_3, B_2	29

The regulation of calystegin biosynthesis, however, may be of agricultural rather than of pharmaceutical interest. A number of plants containing calystegins have been reported to cause degenerative neurological disorders in cattle. In a recent survey of the occurrence of calystegins in edible fruits and vegetables, calystegins A_3, B_1, B_2 and C_1 were detected in sweet and chilipeppers, potatoes, eggplants, tomatoes, *Physalis* fruits, sweet potatoes, and mulberries. The possibility was raised of toxicity in humans consuming large amounts of plants that contain calystegins.[18,19,20]

The biological role of calystegins in the plant species which produce them is not fully understood at the present time. It has been shown that they are specifically catabolized by the soil microorganism *Rhizobium meliloti* strain 41, thus providing a source of carbon and nitrogen.[14,15] Also, it has been established that calystegin-catabolizing bacteria associate with plants that produce calystegins.[21] These data strongly suggest that calystegins influence rhizosphere ecology through nutritional selection. Thus, in addition to homoserine and opines, they constitute a third class of natural products associated with Rhizobiaceae nutritional factors.[13,15]

Biosynthesis and enzymology of tropane alkaloids

Edward Leete (1928-1992) has pioneered the biosynthetic studies of tropane alkaloids since 1950's using whole plants and isotope labels.[33,34] His experiments have indicated the outline of the biosynthetic pathway to these alkaloids. Also a lot of information has been obtained using plant cell cultures and feeding experiments.[35-37] In spite of intensive studies, the biosynthesis of tropane alkaloids is not totally understood, especially at the enzymatic level.

The first indication that the roots are the site of alkaloid biosynthesis was given by reciprocal grafting between tropane alkaloid producing and non-producing plants.[38] Later on this idea has been proven by other experiments.[39-41] Tropane alkaloids are then transported to the aerial parts of the plant through xylem.[41,42] These alkaloids can be found in different amounts and ratios in stems, leaves, fruits and seeds depending on the plant species.[43]

Figure 1 shows the still partly hypothetical metabolic pathway of tropane alkaloids. The tropane alkaloids have a pyrrolidine and a piperidine ring, which share the nitrogen atom and two carbon atoms.[34] Tropane alkaloids, hyoscyamine and scopolamine, are esters of tropic acid and tropine, and tropic acid and scopine, respectively. The bicyclic tropane ring is derived from L-ornithine and / or L-arginine *via* tropinone whereas the tropic acid moiety is synthesized from phenylalanine. Tropinone is stereospecifically reduced to form either tropine which is incorporated into hyoscyamine, or on the other hand into pseudotropine which is thought to be a precursor of calystegins, a recently discovered new group of tropane-related alkaloids.[13] Hyoscyamine is converted to scopolamine *via* intermediate 6β-hydroxyhyoscyamine. In the following review, emphasis is given on the known enzymes which catalyze different steps in the biosynthesis of tropane alkaloids.

ORNITHINE (EC 4.1.1.17) AND ARGININE (EC 4.1.1.19) DECARBOXYLASES

It is confirmed by radiolabelled feeding experiments that the pyrrolidine part of tropane alkaloid structures is derived from ornithine or arginine *via* agmatine. The results of Hashimoto and co-workers[35,36] provided strong evidence that the tropane alkaloids in *H. albus* root cultures are synthetized from both ornithine and arginine *via* putrescine (Fig. 2). The experiments performed with transformed root cultures of *Datura stramonium* and *A. belladonna* show that L-arginine can act directly as a source of hyoscyamine, since the label from agmatine is readily incorporated.[44] Moreover, they showed that δ-*N*-methylornithine as a formerly accepted intermediate[33] is improbable. However, it is possible that different plant species may operate *via* two different pathways.

Ornithine decarboxylase (ODC) catalyzes the formation of putrescine from ornithine. This enzyme has been isolated from *Hyoscyamus albus.*[35] Arginine decarboxylase (ADC) is an enzyme involved in the biosynthesis of agmatine from arginine leading *via* *N*-carbamoylputrescine to putrescine[45] (Fig. 2). The activity of ADC in the root cultures of *H. albus* is twice that of ODC.[35] To demonstrate the importance of these two flux-limiting enzymes the activity of either ADC or ODC was suppressed *in vivo* by using the specific inhibitors, DL-α-difluoromethylarginine (DFMA) or DL-α-difluoromethylornithine (DFMO), respectively. The suppression of ADC in *Datura* resulted in severe decrease in free and conjugated putrescine and other intermediates of hyoscyamine biosynthesis. In contrast the inhibition of ODC increased the activity of ADC and caused only minimal loss of metabolites.[46] According to these results it can be concluded that at least in *Datura* two routes to form putrescine are possible and ADC activity is more important than that of ODC one (Fig. 2).

The activity of ADC is limited to bacteria and plants whereas ODC is found in all living organisms. ODC activity is closely associated with cellular proliferation and is essential for normal cell growth. The cDNA of ODC has been derived from root cultures of *D. stramonium* and expressed in *E. coli.*[47] The longest cDNA insert contained 1576 bp. The deduced amino acid sequence encoded by the open reading frame a peptide of 431 amino acids, and ODC of *Datura* is highly similar to other prokaryotic and eukaryotic ODCs and ADCs. Furthermore, there was evidence that ODC may be represented by more than one gene copy in this plant.[47]

The cDNA of ADC has been obtained from many plants but so far not from tropane alkaloid-producing ones. Primary structures of the *E. coli* and plant ADCs have been found to be similar.[48] Overexpression in transgenic tobacco plants of an oat-derived cDNA coding for ADC resulted in accumulation of agmatine, the direct product of ADC. No increase in the levels of putrescine or other polyamines, or of the alkaloid nicotine could be detected.[49] The results indicate that putrescine biosynthesis is tightly regulated and that the capacity of ADC alone to influence alkaloid biosynthesis thus seems to be limited.

PUTRESCINE *N*-METHYLTRANSFERASE (EC 2.1.1.53)

Tropane and nicotine alkaloids share the same biosynthesis route from ornithine to *N*-methylpyrrolinium cation (Fig. 1). Putrescine and the *N*-methylated diamine

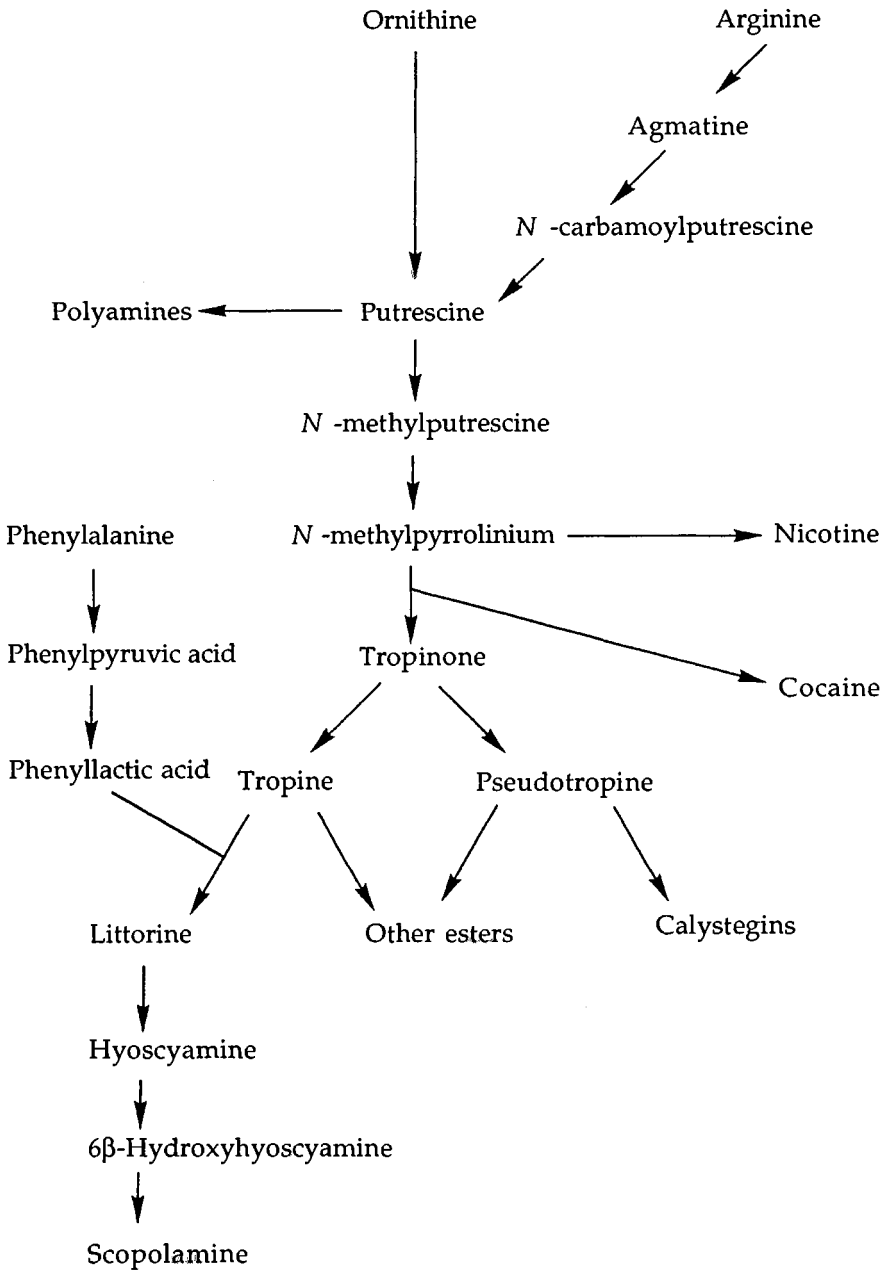

Figure 1. Biosynthetic pathway of tropane alkaloids.

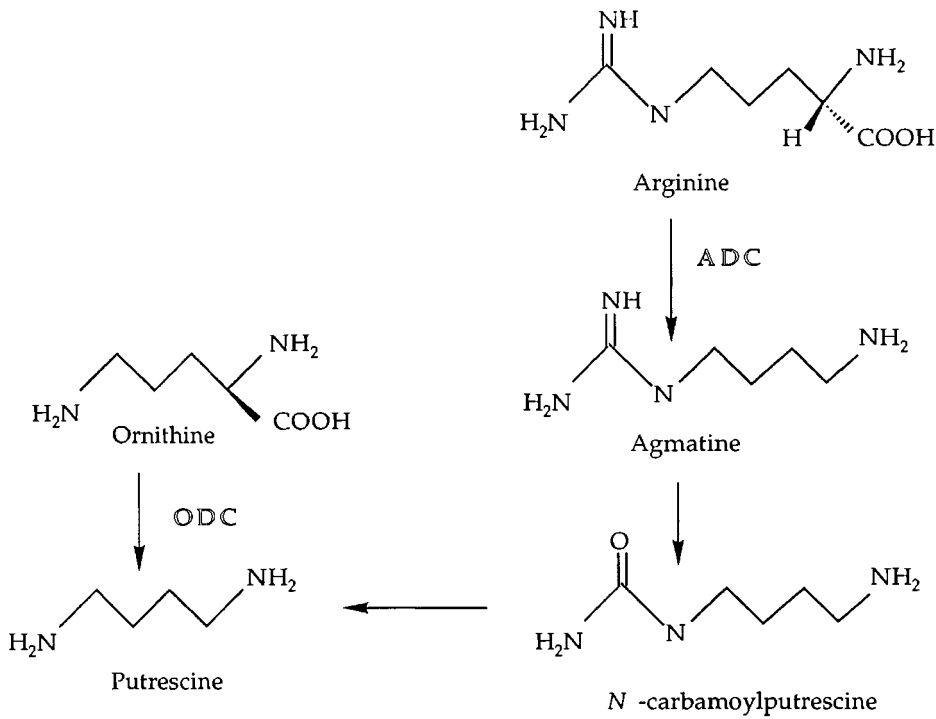

Figure 2. Formation of putrescine from amino acids arginine and/or ornithine.

N-methylputrescine are the two important intermediates. On the other hand putrescine serves also as a precursor for higher polyamines (Fig. 3), spermidine and spermine.[36] Polyamines are often conjugated with cinnamic acid and its derivatives. In Solanaceous plants and transformed root cultures (*e.g. H. muticus* and *N. tabacum*) putrescine conjugates form the major part of the total polyamines.[50,51] Earlier studies indicated that *N*-methylputrescine could not be formed from putrescine because feeding labelled ornithine in *Datura* plants resulted in nonsymmetric labelling in the pyrrolidine ring of hyoscyamine.[33] Later on several experiments especially using root cultures of *H. albus*, have however supported the putrescine pathway[36,44] showing the route to be similar to the biosynthesis of nicotine.[52,53]

The enzyme which catalyses the formation of *N*-methylputrescine from putrescine (Fig. 3) is putrescine *N*-methyltransferase (PMT). This is the first committed step in tropane alkaloid biosynthesis.[54] The enzyme has been isolated from *N. tabacum*, *D. stramonium*[55] and *H. albus* [54] and it catalyzes the transfer of the methyl group from *S*-adenosyl-L-methionine to an amino group of putrescine. The molecular weight of *H. albus* PMT as determined by gel filtration is 62.1 kDa. A cDNA coding for this enzyme has been isolated and partially purified and characterized from *H. albus* by Hibi and co-workers.[54] High activity of PMT was found in branch roots and cultured roots of several Solanaceous plants especially in *H. albus*, *H. bohemicus* and *A. belladonna*.

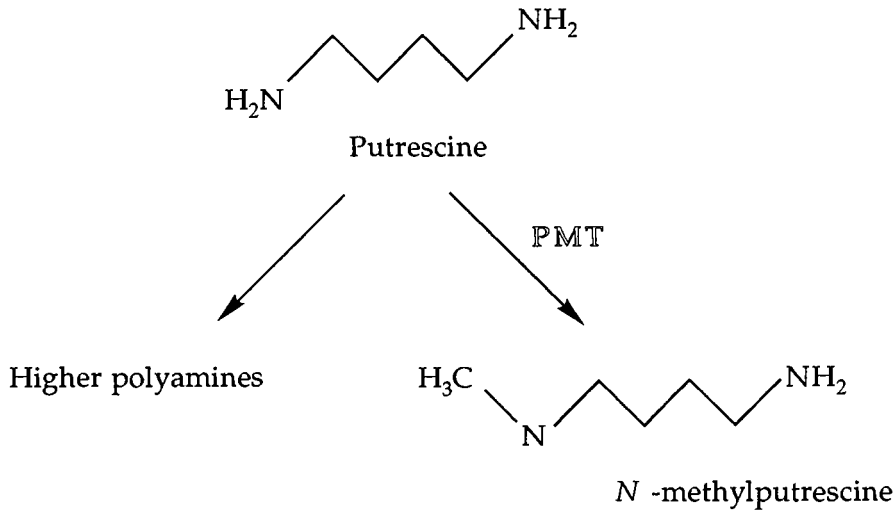

Figure 3. Formation of N-methylputrescine from putrescine.

Monoamines, *e.g. n*-butylamine or cyclohexylamine, were shown to be good competitive inhibitors of PMT by experiments in which the root cultures of *H. albus* and *D. stramonium* were fed with these monoamines. A drastic decrease in *N*-methylputrescine and hyoscyamine contents occurred, free and conjugated putrescine levels increased and the biosynthetic flux towards the polyamines was notable. The inhibitory effect of these monoamines are similar to their effects on spermidine synthase which catalyses the transfer of the aminopropyl moiety of decarboxylated *S*-adenosyl-L-methionine to putrescine and further to polyamines.[54] PMT seems to be the key-enzyme in the biosynthesis of tropane and nicotine alkaloids and is the first enzyme that can direct the synthesis away from the direction to polyamines. The amino acid sequence of PMT was found to have high homology with human and mouse spermidine synthase and over 50% similarity to spermidine synthase from *E. coli*. However, PMT does not have spermidine synthase activity and *vice versa*.[56]

N-METHYLPUTRESCINE OXIDASE (EC 1.4.3.6)

All the experimental evidence shows that *N*-methyl-Δ^1-pyrrolinium salt is a precursor of the tropane nucleus.[34] *N*-methyl-Δ^1-pyrrolinium cation is formed from *N*-methylputrescine *via* 4-methylaminobutanal and the reaction is catalysed by the diamine oxidase enzyme, *N*-methylputrescine oxidase (MPO) (Fig. 4). Diamine oxidases are widely spread in nature and they have been isolated from several plants and animals. MPO has been isolated and partially purified from cultured roots of *H. niger*. The molecular weight of MPO as determined by gel filtration is 135 kDa. The enzyme is most probably a dimer, and is not specific for *N*-methylputrescine but uses it as a precursor

in preference to putrescine and cadaverine.[57] MPO has also been isolated from the conventional or hairy root cultures of *D. stramonium*[37] and *N. tabacum*.[58,59]

DFMO and DFMA, the two specific inhibitors of tropane and nicotine alkaloid biosynthesis, did not affect the levels of MPO contrary to the other enzymes ODC, ADC and PMT as described above.[46] Furthermore, it was shown that exogeneously applied plant growth regulators changed the root morphology towards undifferentiated stage, and at the same time the activities of ODC, ADC, PMT and MPO diminished and alkaloid content drastically decresed. The level of PMT activity was declined most rapidly, about 80% being lost within 12h, and totally absent from fully dispersed cultures. MPO activity was also severely diminished by plant growth regulator treatment but never completely lost from the system. MPO was also substantially restored by removal of growth regulators, whereas PMT was not.[60] Robins and co-workers[37] showed that the activities of both enzymes (PMT and MPO) decreased when the cultures were fed with tropine which might indicate the strong feed back mechanism.

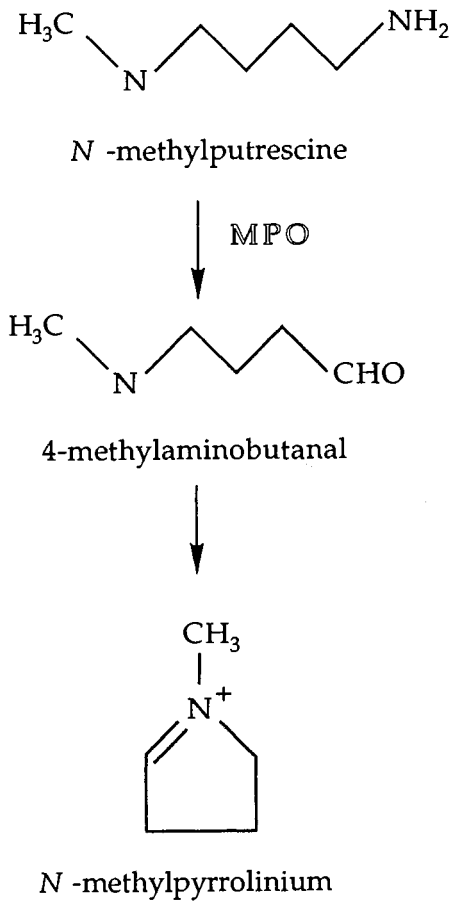

Figure 4. Formation of N-methylpyrrolinium ion from N-methylputrescine.

The levels of MPO activity were found to be higher in cultured roots of *H. albus* and *H. niger* than in shoots. However, the activity in *H. albus* suspension cultures was more than twice as high as in the roots of the both species, and no correlation was found between alkaloid production and MPO activity in these three systems.[57] The biosynthesis of tropane alkaloid is apparently not regulated directly by the MPO activities at least in *Hyoscyamus* species.

For a long time it was believed that the formation of tropane ring from *N*-methyl-Δ^1-pyrrolinium cation occurs by condensation reaction with acetoacetyl-CoA to yield the β-keto acid which then undergoes decarboxylation to form hygrine.[33] Hygrine was thought to be converted to tropinone *via* the 5-acetonyl-1-methyl-Δ^1-pyrrolinium salt (dehydrohygrine) by Mannich reaction in which cyclization occurs and two hydrogens are removed.[34] However, after feeding experiments with *Datura innoxia*, Abraham and Leete[61] presented a revised hypothesis for the biosynthesis of the tropane moiety. They could not detect any incorporation of (*R,S*) -[2',3'-$^{13}C_2$]hygrine into scopolamine whereas high incorporation of doubly labelled ^{13}C was shown after feeding ethyl (*R,S*) -[2,3-$^{13}C_2$,3-^{14}C]-4-(1-methyl-2-pyrrolidinyl) -3-oxobutanoate. These results were confirmed by Robins *et al.*[62], who in addition showed that ^{13}C-label from [1,2-$^{13}C_2$]acetate was incorporated symmetrically into the C-2 and C-4 positions of (-) -hyoscyamine. The authors conclude that, most probably, the acetate derived carbon atoms are not incorporated stepwise, but rather as a single acetoacetate unit which reacts *via* its C-4 position with *N*-methyl-Δ^1-pyrrolinium salt to give 4-(1-methyl-2-pyrrolidinyl) -3-oxobutanoate. This intermediate would then favour cyclization to give 2-carboxytropinone (Fig. 5) with tropinone being formed after decarboxylation.

No enzyme has been found so far which would catalyse the formation of tropinone from *N*-methyl-Δ^1-pyrrolinium cation. However, it is also possible that this above mentioned reaction would happen totally without enzymes.[63]

TROPINONE REDUCTASES (EC 1.1.1.236)

Whereas tropine has been established as a precursor of hyoscyamine and scopolamine as early as 1972[64], tropinone long remained a hypothetical intermediate in tropane alkaloid biosynthesis. It was only in 1990 that, by feeding [*N*-methyl-^{14}C]tropinone to *Datura innoxia* plants, the incorporation of this ketone into the tropane esters was first described.[65] Nevertheless, the first report of tropinone reduction in a cell-free system obtained from a root culture of *D. stramonium* was published by Koelen and Gross.[66] The authors reported that tropine was the exclusive product of tropinone reduction. Later reports described the enzyme-catalyzed reduction of tropinone to pseudotropine[67] or to both tropine and pseudotropine[68] in crude enzyme extracts. It has now been demonstrated that two separable proteins exist, tropinone reductase I (TR-I) and tropinone reductase II (TR-II), that stereospecifically reduce tropinone into tropine (3α-hydroxytropane) or pseudotropine (3β-hydroxytropane), respectively (Fig. 6).[69,70]

An internal amino acid sequence was found in purified TR-I (273 amino acids) and TR-II (260 amino acids) and an oligonucleotide probe corresponding to this sequence was used to screen cDNA libraries derived from cultured roots of *D. stramonium*[71] and *H. niger*.[72] Isolated cDNA clones coding for TR-I and TR-II, respectively, were

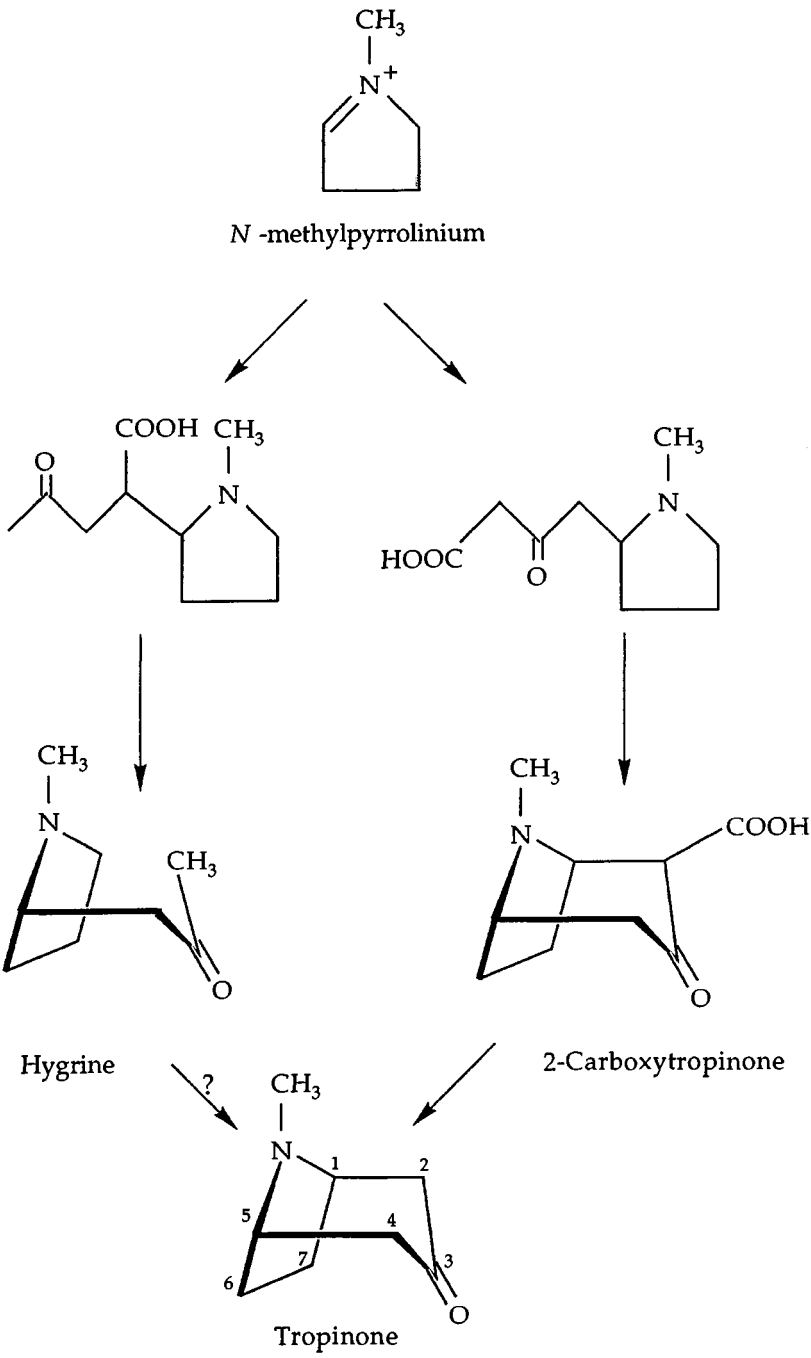

Figure 5. Formation of tropinone from N-methylpyrrolium ion.

expressed in *E. coli*. The recombinant TRs showed the same strict stereospecificity as that observed for the native TRs that had been isolated from plants. Sequence analysis of the cDNA clones lead to the deduced primary structures of the enzymes. It was concluded that tropinone reductases belong to the short-chain dehydrogenase/reductase family that utilizes NAD(P) (H) as a cofactor.[71]

The tropinone reductases were further characterized by constructing sixteen chimeric TR enzymes which were expressed in *E. coli*. Stereospecificities, substrate specificities, and K_m values for tropinone of the chimeric enzymes were compared with those of wild-type enzymes.[73] The results indicated that the stereospecificity of TR is determined by the orientation of tropinone at the substrate-binding site, which is composed mainly of the carboxyl-terminal half region. The amino-terminal half region of the protein constitutes the NADPH-binding site. Recently, in a collaborative effort between the Nara Institute of Science and Technology and the Institute for Chemical Research of Kyoto University, the structural basis for the different reaction stereospecificities of TRs were clarified by determination of the crystal structures of the two enzymes at 2.4 and 2.3 Å resolution.[74] The binding sites for the cofactor and the positions of the active sites are well conserved between the two TRs. The substrate binding site is composed mostly of hydrophobic amino acids in both TRs but the presence of different charged residues confers different electrostatic environments on TR-I and TR-II. A modelling study has indicated that these charged residues play a major role in controlling the binding orientation of tropinone in the substrate-binding site, thereby determining the stereospecificity of the reaction product.

The activity of TR-I is not believed to be rate-limiting in the biosynthesis of tropine-derived alkaloids (Fig. 6). Thus, increase of TR-I activity or reduction of TR-II activity by means of genetic engineering is not expected to result in increased biosynthesis of hyoscyamine and scopolamine in transgenic medicinal plants.[69]

The role of TR-II in tropane alkaloid biosynthesis was not immediately clear since high levels of enzyme activity could be measured while pseudotropine-derived esters only comprised a small fraction of the total of tropane alkaloids that accumulate in Solanaceae. It has been suggested that some pseudotropine metabolites have escaped detection by conventional extraction and analysis methods of typical tropane alkaloids.[69,24,25] A family of recently discovered polyhydroxy nortropanes, the calystegins, appears to be the most likely fate of pseudotropine metabolites (Fig. 6).

The discovery of calystegins is an example of serendipity. In an attempt to isolate plant secondary metabolites which might serve as selective agents for specific rhizosphere bacteria, Tepfer *et al.*[14] used extraction and detection methods designed to assay opines. This led to the discovery of calystegins in root exudates of *Calystegia sepium*. Elucidation of the structures of these compounds by ^{1}H and ^{13}C NMR spectroscopy showed their strong similarity to tropane alkaloids.[13] Biosynthetic relationship with tropane alkaloids is suggested by the incorporation of [1,4-^{14}C]putrescine[13] and [^{15}N]tropinone[25] into calystegins. Furthermore, it has been shown that the presence of 8-thiabicyclo[3.2.1]octan-3-one (TBON), a sulfur analogue of tropinone that competitively inhibits the formation of tropinone[75,76] depresses hyoscyamine accumulation whereas calystegin production significantly increases.[25]

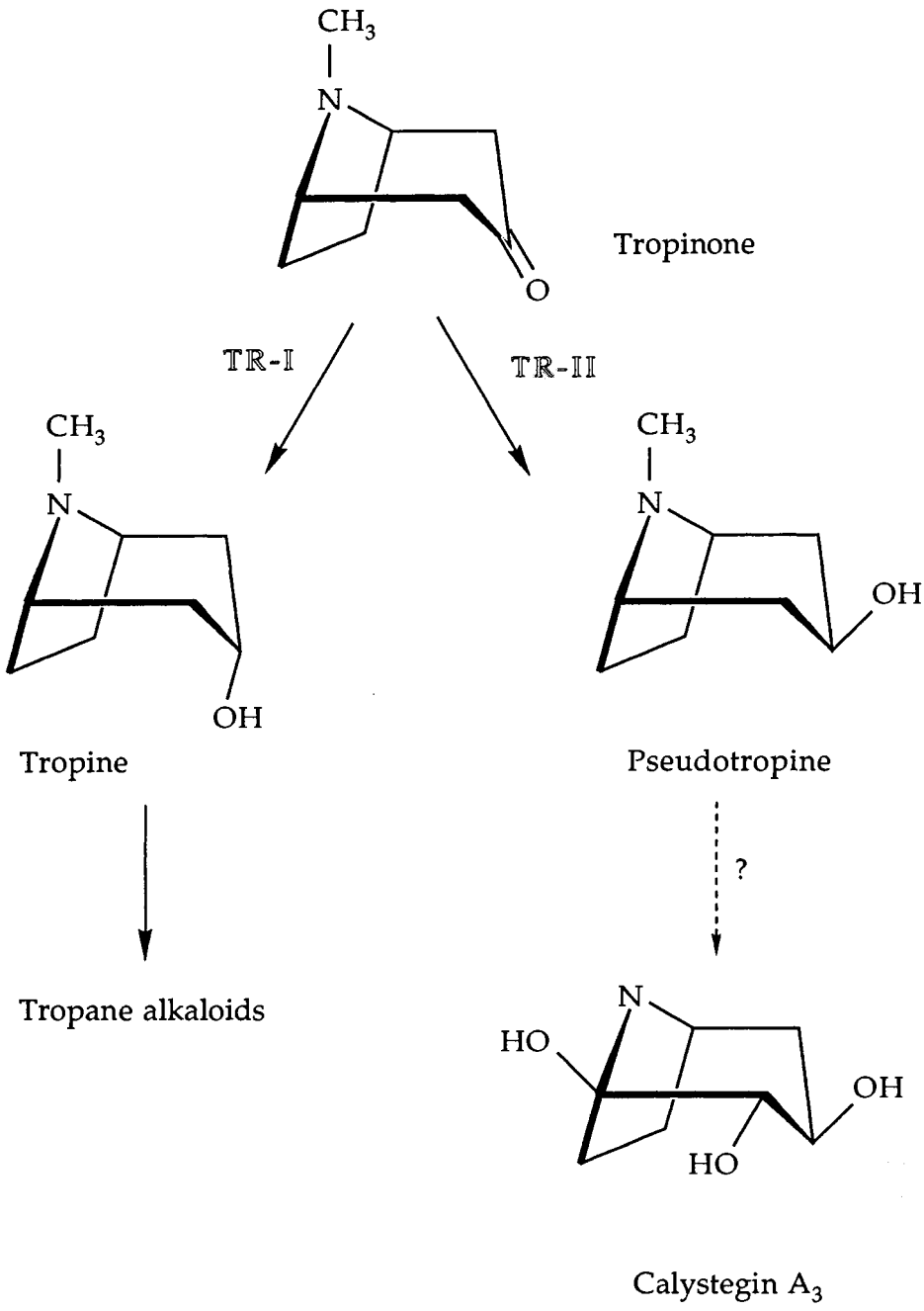

Figure 6. Formation of tropane alkaloids and calystegins from tropinone.

The reduction of tropinone constitutes a branch-point in tropane alkaloid biosynthesis.[71,77] Formation of tropine leads to the accumulation of the major tropane alkaloids hyoscyamine and scopolamine, and formation of pseudotropine results in the accumulation of calystegins (Fig. 6). Moreover, the two tropines are precursors of various minor tropine and pseudotropine esters.[78,79] The conversion of 2-methoxycarbonyl-3-tropinone to methyl ecgonine, in which the hydroxyl-group at C-3 is β as in pseudotropine, is not believed to be mediated by TR-II.[68]

ESTERIFICATION REACTIONS IN TROPANE ALKALOID METABOLISM

Several Solanaceous genera produce a wide range of both aliphatic and aromatic esters of tropine or pseudotropine. Several acyl transferases that mediate the formation of minor acetyl-, phenylacetoyl and tigloyl esters have been reviewed recently by Robins *et al.*[78] The aromatic moiety of hyoscyamine and scoplamine is (*S*)-(-)-tropic acid. However, free tropic acid is not believed to be an intermediate in the biosynthesis of these major alkaloids of pharmaceutical interest[80] (Fig. 7).

Some correlation has been shown between the ability of Solanaceae to accumulate aliphatic tropine esters and the *in vitro* activity of acyl transferases.[80] In contrast, the ability to form pseudotropine esters shows little correlation with the accumulation of these products. The significance of pseudotropines for tropane metabolism in general was questioned by the authors, especially since feeding experiments showed that pseudotropine is also esterified in a number of species that do not form tropane alkaloids at all. It was suggested that the wide spectrum of minor bases observed in alkaloidal extracts is simply due to low selectivity of the enzymes for acyl-CoA thioesters. At present, only tigloyl-CoA:pseudotropine acyl transferase is believed to be an enzyme specific for pseudotropine conversion. The protein has been purified to near electrophoretic homogeneity, about 330-fold, from root cultures of *D. stramonium*.[79]

Free tropic acid has long been thought to be an intermediate in the biosynthesis of hyoscyamine but recently, in a series of elegant biochemical experiments, this was shown not to be the case.[80-87] It has long been known that phenylalanine is a precursor of the tropic acid moiety of hyoscyamine and scopolamine[88] (Fig. 7). Feeding experiments with phenyl[1,3-$^{13}C_2$]alanine led to the appearance of contiguously labelled centres located by satellite signals in the NMR spectra thus showing that the rearrangement of the side chain is an intramolecular process.[89] In a search for the intermediate acid that undergoes the rearrangement process, phenyl[1,3-$^{13}C_2$]lactic acid was fed to *D. stramonium* plants.[86] Examination of the ^{13}C-NMR spectra of isolated hyoscyamine and scopolamine again showed spin-spin coupling from the newly formed contiguous centres, indicating that tropic acid is formed by an intramolecular rearrangement of phenyllactate (Fig. 7). The results were confirmed by Chesters *et al.*[83] who, in a feeding experiment with *D. stramonium* root cultures, demonstrated that the ^{13}C-^{2}H bond of (*RS*) -3-phenyl[2-^{13}C, 2-^{2}H]lactate is incorporated intact into the hydroxymethyl group at C-3' of the (*S*) -tropoyl moiety. The presence of an excess of (*RS*) -tropic acid has little influence on the specific incorporation of label from (*RS*) -phenyl[1,3-$^{13}C_2$]lactic acid into the acidic moiety of the tropane esters. It was concluded that free tropic acid is not an intermediate in hyoscyamine biosynthesis but rather that the arrangement of phenyllactic acid occurs subsequent to its

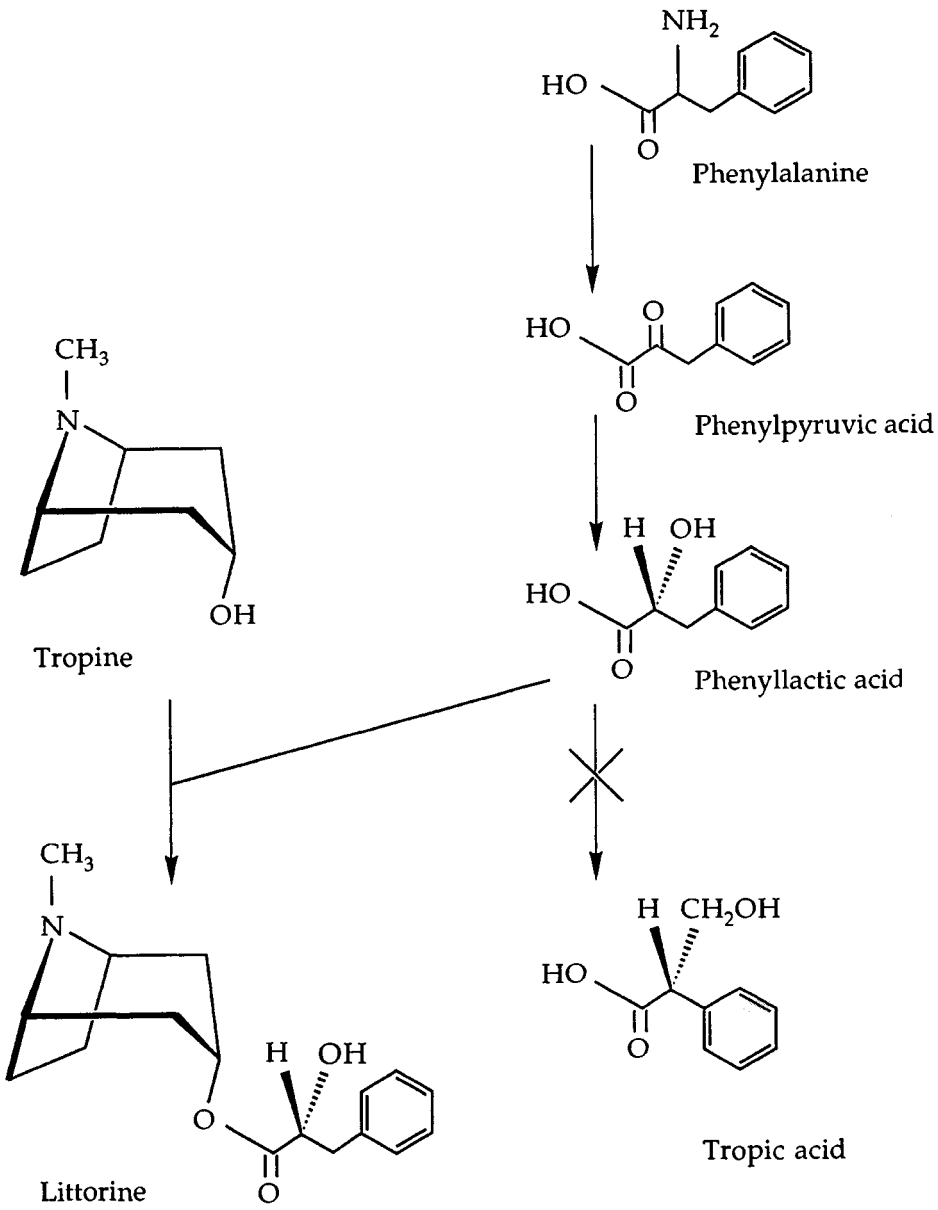

Figure 7. Esterification of tropine with phenyllactic acid leads to littorine. Phenyllactic acid is formed from amino acid phenylalanine.

esterification.[80] Thus, littorine, the (*R*) -(+) -phenyllactoyl ester of tropine would be precursor to the other aromatic tropane alkaloids (Fig. 7). The direct synthesis of hyoscyamine from littorine has been demonstrated by feeding (*RS*) -phenyl[1,3-$^{13}C_2$]lactoyl[methyl-$^{2}H_3$]tropine

(littorine) to root cultures of *D. stramonium*.[81] The quintuply labelled precursor was incorporated intact in the aromatic tropane alkaloids.

The revision of this part of the biosynthetic pathway has important consequences for the further study of the regulation of hyoscyamine and scopolamine biosynthesis. It is now believed that the pathway involves the activation of phenyllactic acid, likely through the formation of phenyllactoyl-CoA.[80] The acyl group of phenyllactoyl-CoA could then be transferred to tropine to form littorine. The next step, *i.e.* the intramolecular rearrangement of littorine to form hyoscyamine, is then processed by a mutase[82,84] (Fig. 8). At present, neither phenyllactoyl-CoA:tropine transferase nor the littorine mutase activity has been shown *in vitro*, and the presence of these enzymes thus remains hypothetical.

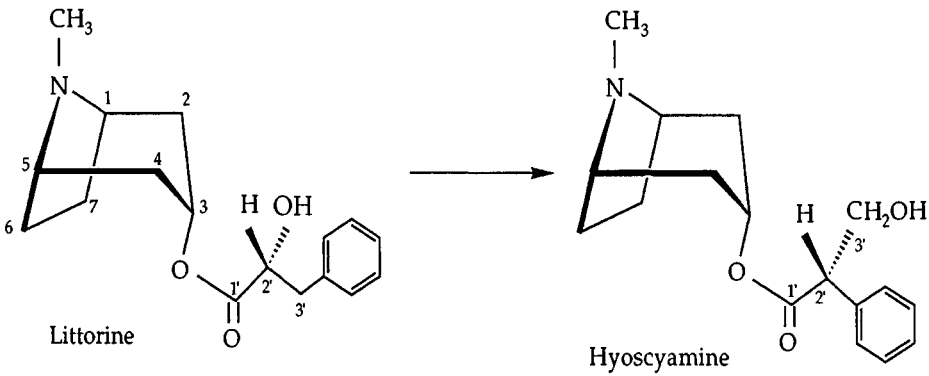

Figure 8. Hyoscyamine is formed from littorine.

HYOSCYAMINE 6β-HYDROXYLASE (EC 1.14.11.11)

Scopolamine, which is the 6,7β-epoxide of hyoscyamine, is formed from hyoscyamine *via* 6β-hydroxyhyoscyamine (Fig. 9). Both reactions are catalysed by hyoscyamine-6β-hydroxylase (H6H) which is a bifunctional enzyme. The H6H is a 2-oxoglutarate-dependent dioxygenase that requires alkaloid substrate, 2-oxoglutarate, Fe^{2+}, molecular oxygen and ascorbate for catalysis. The enzyme has been purified using several steps to homogeneity and characterized from cultured roots of *H. niger*. It is a monomer of 37.7 kDa as determined by SDS-PAGE.[90,91] The amino acid composition has shown no peculiarities except the low number of arginine residues compared to the average occurrence of amino acid residues in proteins. The pI value of the hydroxylase enzyme is 4.6 as determined by nondenaturing isoelectric focusing.[91]

H6H catalyses two consecutive reactions that oxidize hyoscyamine to scopolamine.[92] It is localized at the pericycle of the root and is especially active in cultured roots but is absent in aerial parts of the plant.[90,41,93] Hydroxylation of hyoscyamine gives 6β-hydroxyhyoscyamine which is further epoxidized to scopolamine by dehydrogenation of 7β-hydrogen[94] (Fig. 9). It has been demonstrated that the hydroxylase activity of H6H is 40 times stronger than its epoxidase activity.[92] This was shown by expressing H6H in

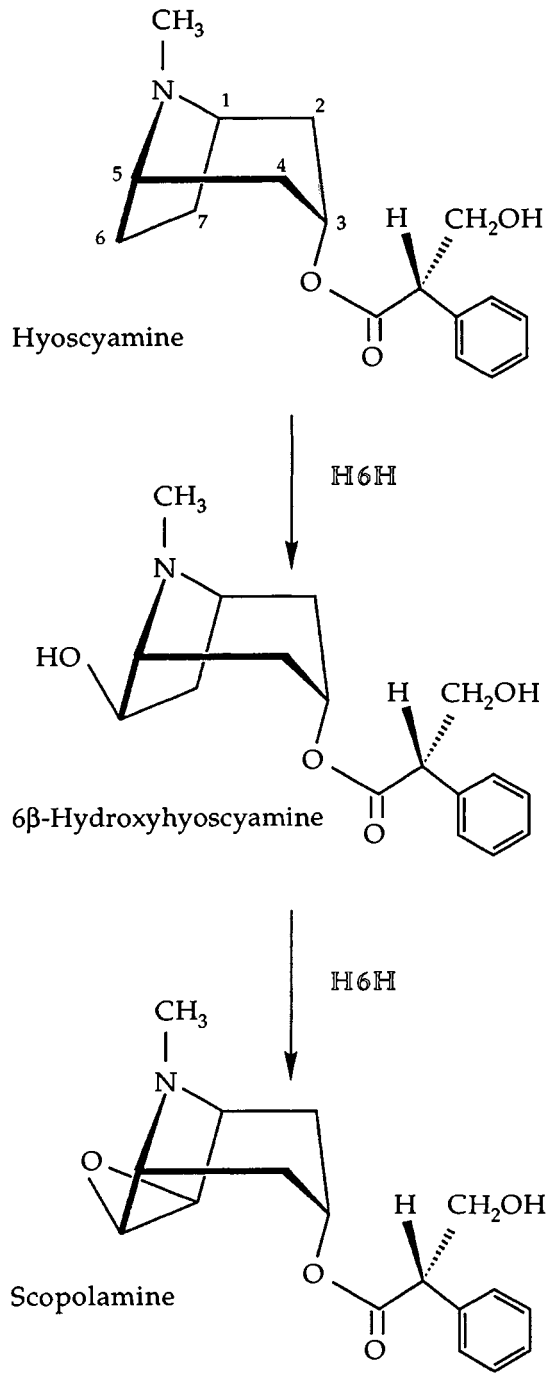

Figure 9. Scopolamine is formed from hyoscyamine via 6β-hydroxyhyoscyamine.

E. coli as a fusion protein with maltose-binding protein and feeding of hyoscyamine to the recombinant bacterium. Hashimoto *et al.*[94] have proposed the model that explains how a single enzyme can catalyze two types of reactions. Most probably a highly reactive ferryl enzyme intermediate which is common to all 2-oxoglutarate-dependent dioxygenases homolytically cleaves a nearby carbon-hydrogen bond. Even though the epoxidase activity of H6H is weaker it seems not to be the rate-limiting factor in the biosynthesis of scopolamine.[95] Furthermore, 6β-hydroxyhyoscyamine usually does not accumulate in scopolamine-producing plants.[90]

H6H is encoded by the *h6h* gene. The nucleotide sequence of the H6H cDNA has been reported, and it reveals an open reading frame that encodes 344 amino acids.[93] When comparing the amino acid sequence of H6H with other proteins relatively high homology was found with the polypeptides encoded by several related genes which are induced in ripening of fruits, with two synthases involved in the biosynthesis of β-lactam antibiotics in micro-organisms, and maize *A2* gene product that catalyzes the formation of anthocyanin from flavan-3,4-*cis*-diol.[93] The expression levels of *h6h* gene is shown to be cell-specific in a species-dependent manner, and is controlled by the 5' flanking region of the gene.[42] Furthermore, the pericycle-specific expression of *h6h* gene is speculated to be very important in the translocation process of tropane alkaloids from roots to aerial parts through xylem.[41]

Production of tropane alkaloids in hairy root cultures

For the past few decades considerable interest has been shown by a number of research groups all over the world to produce a variety of speciality chemicals by plant cell and tissue cultures, among the others tropane alkaloids (for reviews see[8,9,96]). Undifferentiated callus and suspension cultures have failed to accumulate interesting levels of these alkaloids.[39,97,98] Moreover, the production has often been unstable. Roots are the main site of the tropane alkaloid biosynthesis and hence also cultured roots are capable of accumulating higher contents of the metabolites.[40] Several studies indicate that the metabolism of secondary products is correlating with the degree of organization of cell structures.[99,100,60] Although the production of tropane alkaloids is much better in differentiated cultures conventional root cultures show slow growth rate which makes them not attractive production systems.

Hairy root cultures can be obtained by transformation with virulent strains of *Agrobacterium rhizogenes*, and they produce high contents of secondary metabolites characteristic to the mother plant.[101,102] However, the stability of *e.g.* tropane alkaloid formation is highly dependent on the maintenance of root organization.[103] Transformed roots are fast growing *in vitro*, the growth being comparable to the suspension cell cultures, and they are genetically and biochemically stable. The roots are laterally highly branched, and they can be cultivated in hormone-free medium because the genes in the Ri T-DNA regulate the balance of endogeneous hormones.[104,105] In Solanaceous plants, the production of tropane alkaloids by hairy root cultures has been reported for several genera including *Atropa*, *Datura*, *Duboisia*, *Hyoscyamus* and *Scopolia* (*e.g.*[8,102,106] and references therein). Table 2 summarizes the highest contents of tropane alkaloids in hairy

roots in various Solanaceous plants. There are substantial differences in the alkaloid production depending on the plant species. Often, however, a high content of product is associated with poor growth and thus the real productivity (mg/l) remains low. In most cases hyoscyamine is the major alkaloid and the contents often are at the same level as in the plant or even higher. The highest scopolamine content (32 mg/g) so far has been obtained in *Duboisia myoporoides* by repeated selection.[107]

Table 2. Secondary metabolite production of the hairy root cultures of some Solanaceous plants

Plant species	**Tropane alkaloid**	**Content (mg/g d.w.)**	**Reference**
Atropa belladonna	atropine	3.7	122
	atropine + hyoscyamine	9.5	123
	scopolamine	3.0	95
Brugmansia candida	hyoscyamine	8.2	124
Datura candida	scopolamine	5.7	125
	hyoscyamine	1.1	125
D. innoxia	hyoscyamine	14	126
	scopolamine	1.9	126
D. metel	hyoscyamine	3.1	106
D. stramonium	hyoscyamine	6.4	127
	scopolamine	5.6	128
Duboisia hybrid	hyoscyamine	2.1	129
	scopolamine	2.5	129
D. leichhardtii	scopolamine	18	110
D. myoporoides	hyoscyamine	8.6	130
	scopolamine	2.5	130
	scopolamine	32	107
Hyoscyamus albus	hyoscyamine	12	115
	scopolamine	4.6	129
H. aureus	hyoscyamine	6.6	106
	scopolamine	0.6	106
H. x györffyi	hyoscyamine	8.8	126
	scopolamine	0.4	126
H. muticus	hyoscyamine	12.2	103
	scopolamine	1.0	135
H. niger	hyoscyamine	12.5	129
	scopolamine	1.3	106
Scopolia carniolica	hyoscyamine	0.2	132
	scopolamine	0.02	106
S. jabonica	hyoscyamine	13	133
	scopolamine	5.0	133
S. tangutica	hyoscyamine	0.52	129
	scopolamine	0.2	129

Protocols used for establishing of hairy root cultures vary, as well as the susceptibility of plant species to infection by *Agrobacterium*.[108,109] It is known that the *Agrobacterium* strain used for transformation has a great influence on root morphology and the degree of tropane alkaloid accumulation in hairy root cultures.[109] It has also been shown that it is possible, by systematic clone selection *e.g. via* protoplasts, to find high-yielding, stable, and from single cell derived-hairy root clones. This is possible because the hairy root cultures possess a great somaclonal variation.[110,103] Since each clone is different also the basic nutrient requirements for each clone may vary, and thus growth and production conditions should be optimized individually.[111]

Tropane alkaloid production in hairy roots is the highest in the late stationary phase; in shake flasks usually after three to four weeks of incubation (*e.g.* see references[105,111]). In normal growth conditions only less than 5% of the total tropane alkaloid content is usually found in the medium.[112,113] Hairy roots are not as readily manipulated by altering culture conditions as are suspension cultures. However, addition of some auxins (IAA, NAA) or chitosan have increased hyoscyamine content two-fold and five-fold, repectively, in the hairy roots of *H. muticus*.[114,113] Also elicitation with abiotic elicitors, such as Cu^{2+}, has stimulated hyoscyamine production about 40% in the hairy roots of *H. albus*.[115] It is possible that after elicitation some novel compounds (phytoalexins) not typical for the hairy roots can be formed[116,9] or elicitors enhance the permeability of the cells thus leading to the product release into the medium.[112,113,117]

Much work has been carried out with designing bioreactors and developing processes for plant cell cultures. Hairy root cultures, however, are more difficult to cultivate in large scale since mechanical agitation is seldom suitable because they are susceptible to shear stress that causes disorganization and callus formation which subsequently lowers the productivity. Therefore air-lift reactors or droplet reactors, in which the medium is sprayed over the roots and periodically sucked out, are found to be better for root growth.[118] The modified mist spray reactor seems to be the most promising bioreactor type in which the productivity is comparable to that in the shake flasks.[119]

Transgenic plants regenerated from hairy root cultures have been reported for several plant species but only a few medicinal plant species,[102] and until now very little information on their secondary metabolite production has been available.[8,9] In our own laboratory we have established a protocol to regenerate plants from the hairy roots and hairy root-derived protoplasts of *H. muticus*. The transgenic plants differed from the controls in their morphology, fertility and growth pattern.[120,121] These studies also comprised for the first time the analysis of tropane alkaloids in various stages of plant development in a high number of transgenic plants.[26] The high production of tropane alkaloids had a clear negative correlation with *rol* genes of the *Agrobacterium*. Interestingly, these transgenic plants, however, produced large quantities of calystegins which are present only as traces in control plants. Thus it is possible that, during the regeneration process, some severe genetic rearrangements occur which might lead the biosynthetic pathway towards an unusual direction (see Fig. 1) with this system.

Genetic engineering of tropane alkaloid producing plants and tissue cultures

Several Solanaceous plants contain hyoscyamine as the main tropane alkaloid and only such species as *Duboisia myoporoides* and *Hyoscyamus niger* produce scopolamine much more than hyoscyamine. However, scopolamine is more valuable of these two alkaloids, and there has been an increasing interest to obtain plants with enhanced contents of scopolamine. Whether the expression of hyoscyamine-6β-hydroxylase (*h6h*) in those plants which accumulate hyoscyamine instead of scopolamine could alter the alkaloid pattern towards scopolamine production has recently been investigated in two plant species. The hydroxylase gene under the control of the cauliflower mosaic virus 35S promotor has been introduced to *Atropa belladonna* and *Hyoscyamus muticus via Agrobacterium* transformation.[134,95,135,131] Until very recently the lack of understanding of the regulation of secondary metabolite pathways and cloned genes have limited the general use of metabolic engineering of the medicinal plants.

The experiments of Yun *et al.*[134] provided the first example how pharmaceutically important plants can succesfully be altered by genetic engineering. Their research focused on *A. belladonna* which is a typical hyoscyamine-rich plant. The chimeric 35S-*h6h* gene was introduced by a leaf-disk transformation system into the target plant, and a total of almost 30 kanamycin resistant transformants were further screened. One transformant which expressed H6H very strongly was grown to maturity and self-pollinated to give 10 progenies. All the progenies contained the introduced 35S-*h6h* gene, detected by Southern blot hybridization. Also strong expression of H6H polypeptide (38 kDa) and H6H enzyme activity was detected in leaves, stems and roots. The transformed *A. belladonna* plants showed no peculiarities in their morphology compared to the control plants. The alkaloid pattern in the primary transformant and its progenies showed clearly elevated scopolamine contents. In three-month-old transformants and in mature plants after seed formation the percentage of scopolamine out of total tropane alkaloids in the leaves was >70% and 97%, respectively.[134] These experiments elegantly demonstrate that transfer of the *h6h* gene and its expression in hyoscyamine-rich plant efficiently converted the composition of the transgenic plants towards the more valuable alkaloid.

The enhanced scopolamine levels cannot only be achieved in transgenic plants but also in transformed hairy root cultures.[95,135,131] The hydroxylase gene from *H. niger* has been introduced under the control of 35S promoter to *A. belladonna* by a binary vector system using *A. rhizogenes*. The seven transformed root clones showed elevated levels of H6H activity and the best clone contained 5-fold higher concentrations of scopolamine than the wild-type hairy roots.[95] To study the influence of *h6h* gene in other Solanaceous species, and in a larger population of the clones, the hydroxylase clone was introduced to *H. muticus* the hairy roots of which normally produce high contents of hyoscyamine but very low levels of scopolamine (see the previous chapter). Two different *A. rhizogenes* strains were used, 15834 and LBA9402, both carring the *h6h* gene, and altogether 68 new hairy root clones were obtained.[135,131] A remarkable somaclonal variation was observed in the morphology, hyoscyamine and scopolamine contents between the clones. A total of 43 clones were positive to the transgene as determined by PCR, but only 40% of the clones had elevated levels of the scopolamine

compared to the wild type hairy root clones[135]. Thus the expression levels of the transgene may vary from clone to clone. The best clone produced about 100 times more scopolamine than the control clones, and the production has been stable over the following transfer passages.[131] However, contrary to the results of Hashimoto and co-workers[95] hyoscyamine contents in the *H. muticus* hairy roots containing the 35S-*h6h* transgene were not considerably reduced despite of the enhanced scopolamine contents, and hyoscyamine remained still the main alkaloid.[131, 135] This finding shows that there is no strong feed back mechanism present in these clones.

The above described results would indicate also that the different conversion mechanisms for scopolamine might be possible in various plant species. The feeding experiments with 6β-hydroxyhyoscyamine did not increase the scopolamine contents in wild-type hairy roots of *A. belladonna*.[95] The same phenomenon was observed when wild-type tobacco plants were fed either with hyoscyamine or 6β-hydroxyhyoscyamine, but when the tobacco plants that constitutively expressed the 35S-*h6h* gene were fed with those two precursors they were rapidly converted to scopolamine.[134] Thus the *A. belladonna* plant has very low natural epoxidase activity, and H6H alone seems to be responsible for the two step oxidations of hyoscyamine to scopolamine *via* 6β-hydroxyhyoscyamine.

Future aspects

The study of tropane alkaloid biosynthesis is active in various research groups in Europe and Japan, and more genes will be cloned in the near future which might lead to better productivity of the tropane alkaloids in transgenic cultures and/or plants. Even though the scopolamine contents in the transgenic tissue cultures or plants of *Atropa belladonna*[134,95] and *Hyoscyamus muticus*[131,135] do not yet compete with the present production system of *Duboisia*, the metabolic engineering procedure reported in these experiments clearly shows that when the rate-limiting steps of the biosynthetic pathway are completely known, and the respective genes cloned, the exact regulation towards desired medicinal product will be possible in the near future. With the help of metabolic engineering it might also be possible to make arrays of new compounds in plants or alternatively in cell and tissue cultures by introducing foreign genes coding for enzymes which may convert various compounds which already are produced by the plant or even open new pathways.

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