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Review

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Pollen wall development in flowering plants

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Summary

The outer pollen wall, or exine, is more structurally complex than any other plant cell wall, comprising several distinct layers, each with its own organizational pattern. Since elucidation of the basic events of pollen wall ontogeny using electron microscopy in the 1970s, knowledge of their developmental genetics has increased enormously. However, self-assembly processes that are not under direct genetic control also play an important role in pollen wall patterning. This review integrates ultrastructural and developmental findings with recent models for self-assembly in an attempt to understand the origins of the morphological complexity and diversity that underpin the science of palynology.

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I. Introduction

Pollen development excites a great deal of interest because of its fundamental importance in plant reproduction, its unique interplay between the diploid and haploid generations, and its potential as a model system for the study of cell polarity, patterning signalling and cell fate. Meiosis in anthers gives rise to haploid microsporocytes that develop into mature pollen grains within the environment of the anther locule and the diploid, sporophytic tissues of the anther. The highly resistant outer wall (exine) of the pollen grain, which is the focus of this review, thus forms around a haploid cell but includes material derived from the sporophytic tapetum. Structurally, the exine is the most complex plant cell wall and its vast morphological

diversity is the basis of the discipline of palynology, with its numerous branches and applications (Blackmore, 2007). An important component of this diversity is the arrangement of germinal apertures, predetermined spaces in the exine that allow for the emergence of pollen tubes and play an important part in pollen–stigma interactions. The value of pollen morphological characters in understanding plant phylogeny has long been recognized, and the evolutionary pattern of aperture configurations, in particular, is highly congruent with the recent molecular phylogenies (discussed in APG II, 2003), in which basally branching dicotyledons and monocotyledons are characterized by monosulcate (or monosulcate-derived) pollen and the eudicot clade by tricolpate pollen (Blackmore & Crane, 1998; Furness *et al.*, 2002). Sporopollenin, the biopolymer that makes up the majority of the material of the exine, played a pivotal role in the conquest of land by plants (Chaloner, 1976). In addition, pollen development, which Heslop-Harrison (1972) aptly called 'morphogenesis in miniature', is of current interest because it involves both tightly controlled gene-determined processes and epigenetic phenomena.

Molecular genetic studies, especially in *Arabidopsis thaliana*, have dramatically increased our understanding of the development of the male gametophyte (Honys & Twell, 2003; McCormick, 2004; Ma, 2005). It is timely to attempt to integrate these recent findings with earlier observations from electron microscopy and conceptual models for pattern generation and substructural organization of the pollen wall. Such a synthesis should provide new insights into the diversity of form encountered in angiosperm pollen. This review focuses on the events most relevant to pollen wall development, from meiosis through to pollen mitosis, and follows the terminology of Punt *et al.* (2007), with the division of the pollen wall into ectexine and endexine rather than the alternative terminology of sexine and nexine (Fig. 1). Although many of the processes of pollen wall development occur concurrently, and there are significant variations in relative timing between species, a number of distinct stages can be recognized (Fig. 2). The most familiar of these are probably the 12 stages of pollen development described by Owen & Makaroff (1995) in *Arabidopsis*.

As our primary interest is in understanding the complexity and diversity of pollen walls, and because *Arabidopsis* has a

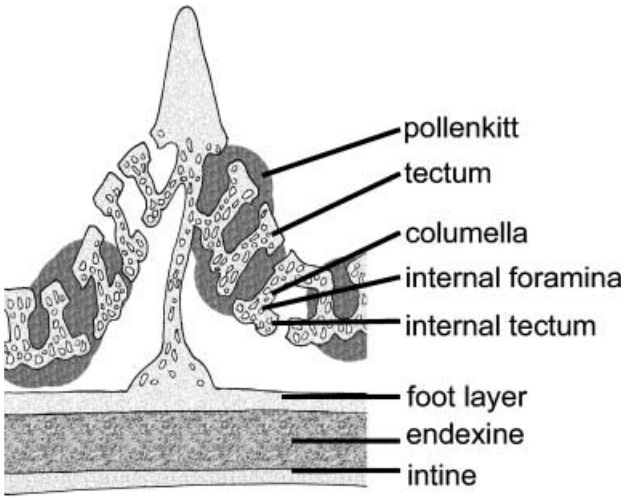

Fig. 1 The terminology used in this article distinguishes two major wall layers: the outer exine and the inner intine. Two layers of exine are distinguished, namely ectexine and endexine. Ectexine may be solid or contain internal foramina and comprises tectum (tegillum), columellae (bacula) and foot layer. In the example illustrated, *Catananche caerulea*, an internal tectum, is present below the outer layer of columellae.

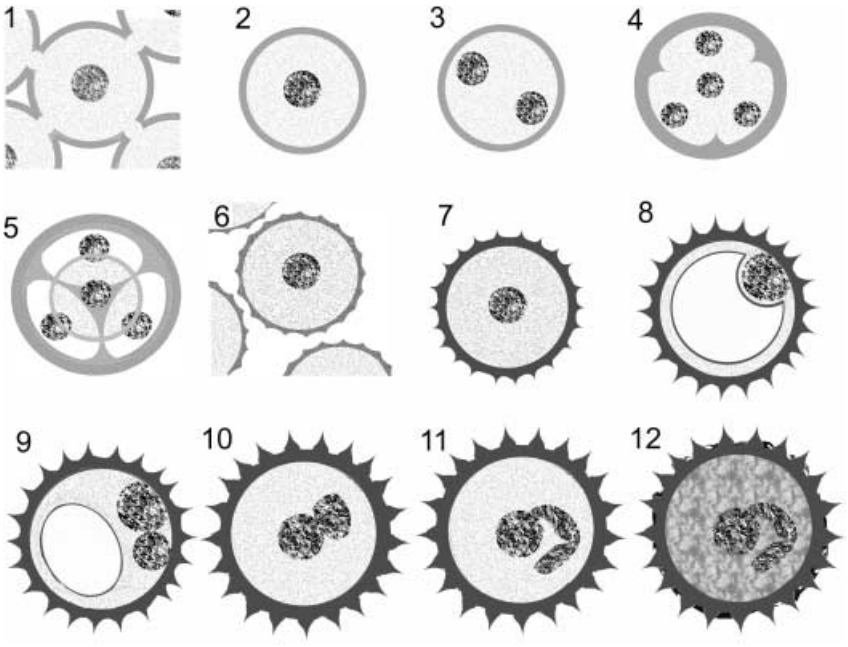

Fig. 2 Stages of pollen development, following the system of Owen & Makaroff (1995). (1) Premeiosis I: microsporocytes connected by cytomictic channels. (2) Premeiosis II: a microsporocyte surrounded by the callose special cell wall. (3) Meiosis: division underway in a microsporocyte. (4) Meiosis complete: before cytokinesis. (5) Tetrad: callose special cell walls are present around the microspores. (6) Released microspore I. Microspores are surrounded by differentiating exine. (7) Released microspore II: further differentiation of the exine. (8) Ring-vacuolate microspore: with a large vacuole causing the characteristic signet ring appearance. (9) Bicellular pollen I: asymmetric mitosis gives rise to the vegetative cell surrounding the peripheral generative cell. (10) Bicellular pollen II: with the generative cell central. (11) Second mitotic division: forming the male germ unit. (12) Mature pollen: with storage products accumulated in the cytoplasm and surface tryphine.

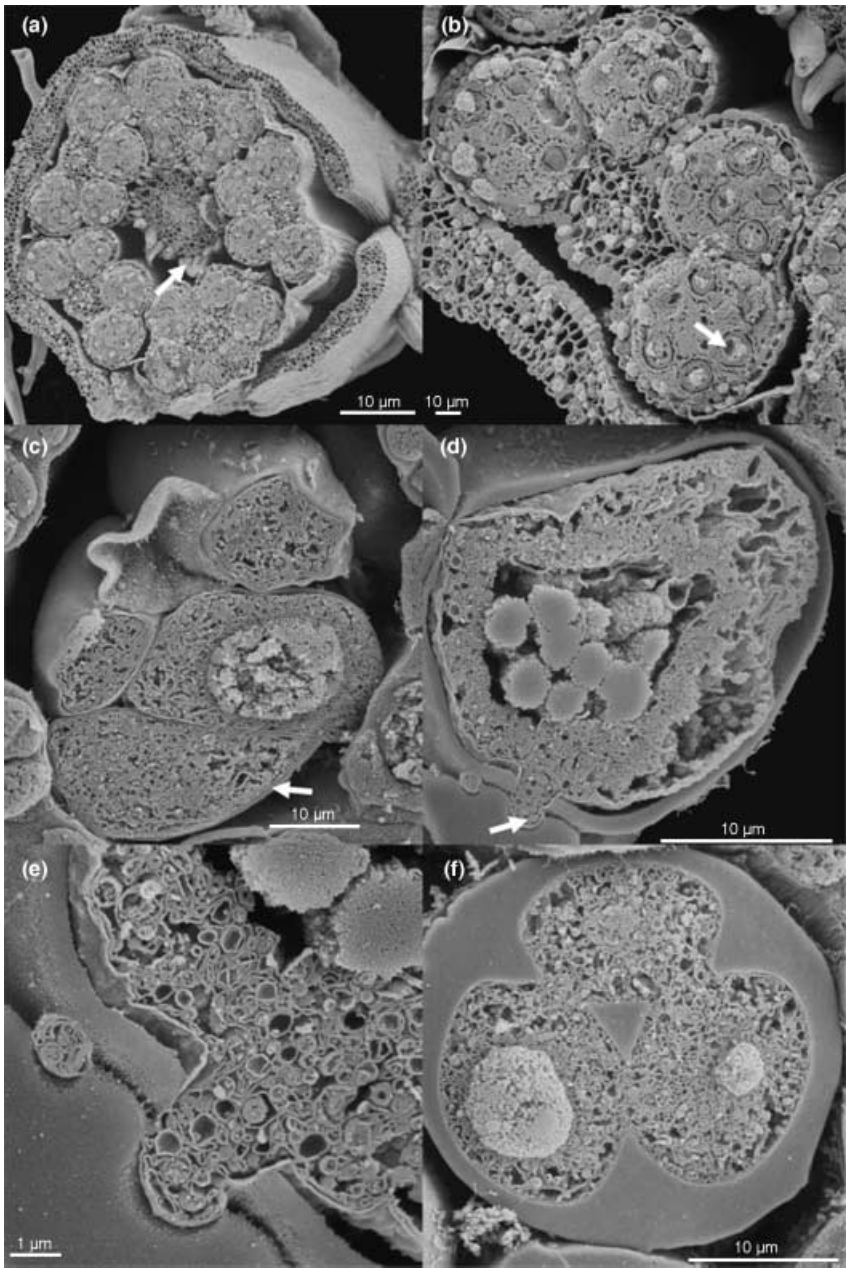

Fig. 3 Early stages of pollen development in *Catananche caerulea* (Compositae: Cichorieae). (a) A developing floret forms as a fused cylinder with anthers surrounding a central style and stigma covered in stigmatic papillae (arrowed). (b) A single anther showing individual microspores surrounded by the plasmodial tapetum at the vacuolate stage (vacuole arrowed). (c) Microspore mother cells (MMCs) showing early stages of callose deposition at the outer wall only (arrowed). (d) A single MMC with condensed chromosomes, showing connecting cytomictic channels (arrowed) passing through the callose layer. (e) Detail of a cytomictic channel surrounded by callose. (f) A tetrad with tetrahedral symmetry undergoing simultaneous cytokinesis. Apertures will form at the last points of continuity between the shared cytoplasm.

structurally simple pollen wall, we take most of our examples from the family Compositae. This is the largest family of flowering plants, with 30 000 species (Funk *et al.*, 2005) and, relevant to the purposes of this review, includes some of the most complex exine structures known (Stix, 1960; Skvarla & Larson, 1965; Skvarla *et al.*, 1977; Blackmore, 1984). The capitulate inflorescence of Compositae, comprising few to many individual florets (Fig. 3a), develops centripetally, making it possible to sample all stages of pollen development within a single inflorescence. The anthers are laterally fused to form a cylinder around the style arms (Fig. 3a,b). The tapetum is initially parietal, but becomes amoeboid, forming a plasmodium around the microspores after they are released from tetrads. Heslop-Harrison (1969a) showed that in *Cosmos*, *Ambrosia* and *Tagetes*, the plasmodial tapetum secretes a sporopollenin membrane, which is external to the tapetum and lines the inside of each loculus. Such membranes are widely documented in other plants (e.g. *Pinus*; Dickinson, 1970a).

II. Progress of research on pollen wall development

The study of pollen development and, in particular, pollen wall development, has progressed from purely microscopic

observations to an emerging understanding of the molecular and genetic processes involved. Some remarkably detailed early studies were undertaken using optical microscopy (Gates, 1920; Gates & Rees, 1921). However, it was through transmission electron microscopy (TEM) that the fundamentals of pollen development were elucidated and the basic sequence of events was established (Fig. 2). Of paramount importance are a series of classic papers by Heslop-Harrison (1963, 1964, 1966, 1968, 1969a,b, 1971, 1972, 1976). Some of the key findings from this pioneering phase were as follows.

1 Before meiosis, the microsporocytes form a coencytium connected by cytomictic channels thought to enable the synchronisation of division. The deposition of a callose special cell wall (SCW) around each microsporocyte closes the cytomictic channels. There follows a cytoplasmic reorganization, involving the elimination of organelles before the start of the haplophase (Heslop-Harrison, 1966, 1968; 1974; McKenzie *et al.*, 1967; Dickinson & Heslop-Harrison, 1976).

2 During meiosis, the configuration of the spindle is the primary determinant of polarity and symmetry in both the tetrad and the individual microspores (Heslop-Harrison, 1968).

3 Exine development is initiated through the deposition of a glycoclayx-like fibrillar polysaccharide material, called primexine, at the microspore surface. Subsequently, precursor components of sporopollenin are accumulated at specific places within the primexine, forming the principal components of the pollen wall (Heslop-Harrison, 1968). Skvarla & Larson (1966) had previously described the primexine as the 'exine template' because of the organized manner in which it accumulates sporopollenin at specific sites.

4 In the majority of plants, the germinal apertures are formed in positions on the microspore surface where 'apertural shields' of endoplasmic reticulum block the deposition of primexine (Heslop-Harrison, 1963, 1968, 1972).

5 After the release of the microspores from the SCW at the end of the tetrad stage, sporopollenin is incorporated into the developing pollen wall, both from within the haploid microspore and from the surrounding tapetum (Dickinson & Heslop-Harrison, 1968; Heslop-Harrison, 1968; Dickinson & Potter, 1976).

6 The positioning of spines and other features of pollen ornamentation could not be linked with the arrangement of specific organelles in the microspore cytoplasm but were shown to involve 'space filling' epigenetic phenomena of pattern formation not under direct genetic control (Heslop-Harrison, 1969b). As early as 1935, Wodehouse (1935), who had been greatly influenced by D'Arcy-Thompson's ideas about pattern formation (Thompson, 1917), had suggested that many aspects of pollen symmetry and patterning reflected physical interactions within and between developing microspores.

During the same period, other researchers were exploring the subtle differences in development that give rise to pollen morphological diversity. For example, Rowley (1975) described instances in which aperture position was not determined by an apertural shield, and studied pollen and spore development in a wide range of plants, including *Artemisia vulgaris*, in the Compositae (Rowley & Dahl, 1977; Rowley *et al.*, 1981a, 1999). In particular, he investigated the role of the microspore glycoclayx in ectexine development and the substructural organization of the exine (Rowley & Dahl, 1977). Several different models of exine substructure have been proposed, on the basis of either ontogeny or the chemical disassembly of mature exines (Rowley *et al.*, 1981a,b, 1999; Southworth, 1985a,b, 1986; Blackmore & Claugher, 1987; Blackmore, 1990). Despite the considerable interest in exine substructure, it has been difficult, until recently, to begin to integrate these different models into a single scheme. For this reason, exine substructure is discussed in detail here.

With growing interest in the relationship between evolution and development, which has matured into the 'evo-devo' research agenda, comparative approaches to pollen ontogeny led to the development of predictive models (Blackmore & Crane, 1988, 1998) for the origin of specific structures, such as exine pattern (Sheldon & Dickinson, 1983, 1986; Dickinson & Sheldon, 1986; Takahashi, 1986, 1989, 1991; Takahashi & Kouchi, 1988). In Compositae, Blackmore & Barnes explored the factors involved in the development of echinolophate (i.e. with spines borne on a specific pattern of ridges), rather than echinate pollen (Blackmore & Barnes, 1985, 1987, 1988; Barnes & Blackmore, 1986, 1987, 1988). Such studies reinforced the view that pollen wall patterning is determined during and soon after meiosis, but the precise mechanisms of pattern formation remained obscure, as will be discussed in Section IV.

Molecular genetics can provide causal explanations for events observed during ontogeny, and much progress has been made concerning the development of flowers (Jack, 2001; Ma, 2005), anthers (Goldberg *et al.*, 1993; Ma, 2005), meiosis in anthers (McCormick, 2004; Scott *et al.*, 2004), pollen wall development (Piffanelli *et al.*, 1998; Scott *et al.*, 2004) and processes relating to cell fate, pollen maturation, pollination and germination (McCormick, 1993; Edlund *et al.*, 2004). Many of these studies have focused on *A. thaliana*, where the baseline of normal pollen development has been carefully documented (Owen & Makaroff, 1995), as have the occurrence of a growing number of developmental mutations (summarized in Section V in relation to particular stages of development). *Arabidopsis* has now become the focus of transcriptome analyses that have provided an enormous increase in our knowledge of gene expression (Becker *et al.*, 2003; Honys & Twell, 2003) and have shown that, remarkably, more than 17 000 genes are expressed during male gametophyte development. Of these, Honys & Twell (2003) thought that approx. 800 might prove to be pollen-specific genes. Subsequently, they undertook transcriptome profiling at four different stages of development in *Arabidopsis* pollen: uninucleate microspores; bicellular pollen; immature tricellular pollen; and mature pollen grains (Honys & Twell, 2004). This

revealed 13 977 genes that were expressed during at least one of the four stages, with the majority expressed in early development (11 565 in microspores compared with 7235 in mature pollen). Transcriptome analysis enabled Pina *et al.* (2005) to investigate gene expression in relation to pollen germination and pollen tube growth, and now clearly provides a platform for analysing the genetic basis of many aspects of gametophyte development and biology.

This review describes the developmental sequence of the male gametophyte generation within the flowering plant life cycle with respect to pollen wall formation. It emphasizes three processes central to the generation of morphological diversity in pollen grains: the establishment of organizational symmetry, or polarity, during meiosis; the deposition of the complex, multilayered exine made up of tectum, columellae and foot layer and the less elaborate endexine (see Fig. 1); and the substructural level of organization within the elements of the exine. It takes account of the current understanding of the biosynthesis and polymerization of sporopollenin which, throughout the pioneering phase of electron microscopy in pollen ontogeny, was mistakenly thought to be a polymer of carotenoids and carotenoid esters (see Scott, 1994). The macromolecule, sporopollenin, is now known to be a polymer of relatively uniform composition, made up of chains of small, straight-chain (aliphatic) organic monomers (Meuter-Gerhards *et al.*, 1999; Bubert *et al.*, 2002). Dominguez *et al.* (1999) proposed a detailed hypothesis for the chemical structure of sporopollenin, supported by more recent studies (Bubert *et al.*, 2002), as a network with a high proportion of carboxylic acid groups, unsaturated carbon chains and ether bonds, based on Fourier-transform infrared spectroscopy analysis. This led Dominguez *et al.* (1999) to hypothesize a role for unsaturated fatty acids in the formation of sporopollenin, which was confirmed using a combination of spectroscopic methods by Ahlers *et al.* (2000). It has been recognized since the early 1990s that the chemical structure of sporopollenin can differ, not only between species but between stages of development (Hemsley *et al.*, 1993; Meuter-Gerhards *et al.*, 1995). de Leeuw *et al.* (2006) recently reviewed the types of sporopollenin that are now known to occur. They conclude that the sporopollenin of extant pollen consists primarily of oxygenated, aromatic monomers, particularly *p*-coumaric and ferulic acids, whereas fossil sporopollenin has a higher aliphatic content, which might reflect different biosynthetic pathways, the effects of fossilization or treatment methods (de Leeuw *et al.*, 2006). As Scott (1994) pointed out, it is now apparent that sporopollenin belongs to a family of plant cell wall materials, including cutin and suberin, that function to prevent water loss or movement and mechanically to reinforce cell walls.

A further topic of long debate in the field of pollen development has been the nature of the highly distinctive 'white line centred lamellae' or 'tripartite lamellae' (TPL) (Rowley & Southworth, 1967), primarily observed in the endexine, but also reported in the early stages of primexine differentiation (Dickinson, 1976). Such structures are characterized by two electron-dense layers, 5–6 nm thick, lying either side of an electron-lucent layer 4–8 nm thick. They are present in spore and pollen walls of all major groups of land plants and in some of their nearest algal relatives (Blackmore, 1990) and, in evolutionary terms, represent the most ancient and fundamental mode of sporopollenin deposition. A complex, structured ectexine, derived by sporopollenin accumulation within a primexine, is a more recent innovation found within seed plants (Blackmore, 1990; Blackmore & Crane, 1998), which is presumed to be associated with more complex reproductive biology involving, for example, incompatibility systems. Scott (1994) noted that the participation of the plasma membrane is not a prerequisite for the formation of lamellae in the case of cutin and possibly of suberin because the monomers are capable of self-assembly into lamellae. This might equally apply to sporopollenin, which is sometimes polymerized at considerable distance from the microspore plasma membrane, for instance in the case of supratectal features formed late in development, such as the spines of *Hibiscus* pollen (Takahashi & Kouchi, 1988).

III. The developmental role of the special cell wall

Before the onset of meiosis (in stage 1, 'premeiosis I' of Owen & Makaroff, 1995), microspore mother cells (MMCs) are interconnected by cytomictic channels (Fig. 3c–e). Heslop-Harrison (1966, 1968, 1974) considered these cytoplasmic connections to function in the synchronization of developmental events in the interconnected MMCs. The cytomictic channels are closed by the deposition, around each MMC, of a SCW composed of callose, a β-1,3-glucan marking the beginning of 'premeiosis II' (Owen & Makaroff, 1995). Sequentially deposited concentric layers of callose form the 'common SCW' (Waterkeyn, 1962) and later, following cytokinesis, 'individual SCWs' are formed around each microspore (Figs 3f and 4a). This process was exquisitely illustrated in both successive and simultaneous meiosis by Longly & Waterkeyn (1979). The SCW persists until the end of the tetrad stage (stage 5 of Owen & Makaroff), when it is broken down by callase.

A variety of important functions have been ascribed to the SCW (reviewed by Barnes & Blackmore, 1986; Paxson-Sowders *et al.*, 1997; Boavida *et al.*, 2005; Dong *et al.*, 2005; Enns *et al.*, 2005). Waterkeyn (1962) proposed that the SCW serves to prevent cell cohesion between developing microspores, and this idea is consistent with the observation that the SCW is much reduced or lacking in taxa, such as certain Juncaceae and Cyperaceae, with pollen dispersed in permanent tetrads or polyads (reviewed by Blackmore & Crane, 1988). Heslop-Harrison & Mackenzie (1967) suggested that the SCW formed a molecular filter to isolate the haploid microspores (Fig. 4b) from the surrounding tissues of the diploid sporophyte. This idea is controversial because of more recent evidence that even relatively large molecules can pass through it (Scott *et al.*,

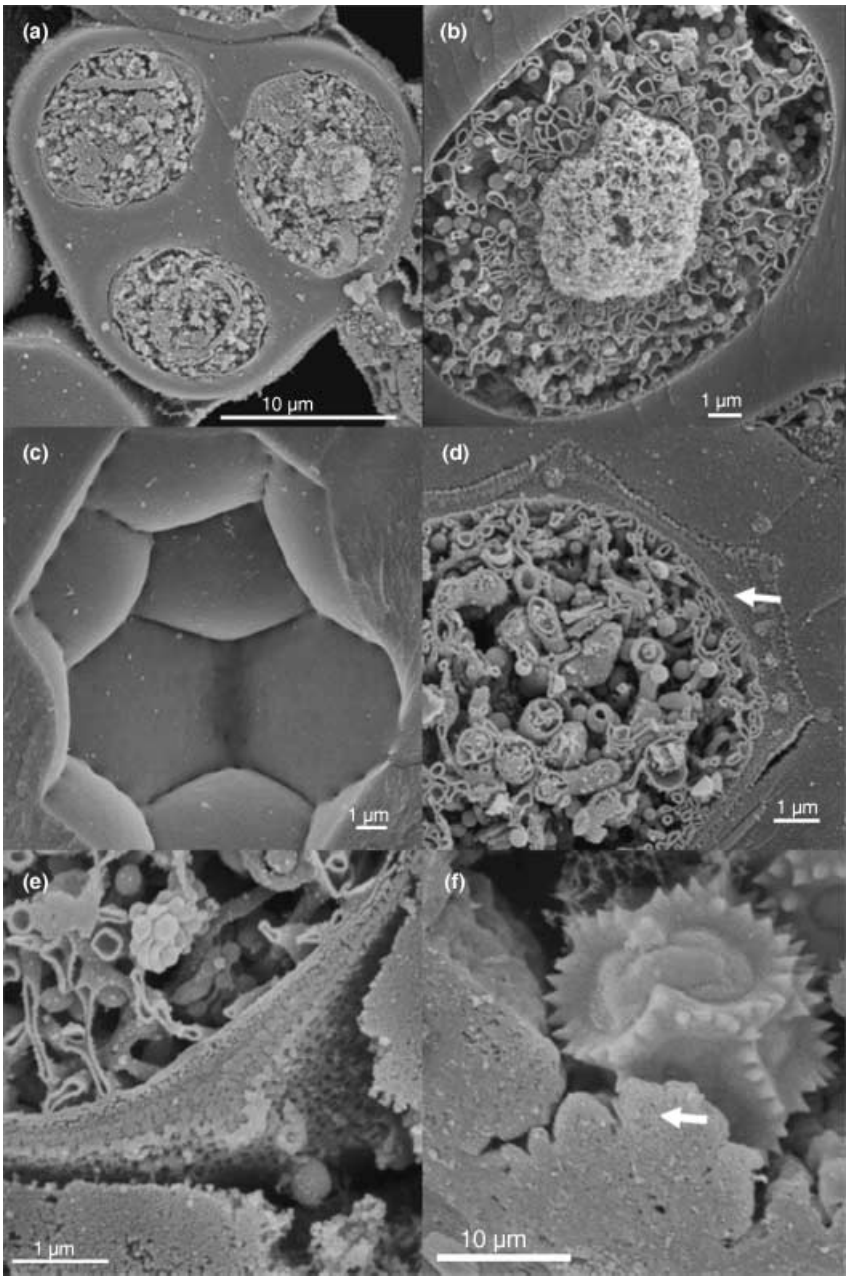

Fig. 4 Compositae pollen development during the tetrad to free microspore stages. (a) *Cosmos bipinnatus* tetrad showing shared and individual special cell walls (SCW) around microspores. (b) *Catananche caerulea* microspore with complete SCW immediately before primexine deposition. (c) Detail of an abortive tetrad of *Scorzonera hispanica* in which the callose wall shows the pattern of spines and ridges present in mature pollen (compare with Fig. 6d,f). (d) *S. hispanica* microspore showing formation of primexine (arrowed) in ridges beneath the SCW. (e) *S. hispanica* microspore showing differentiation of the outer primexine to form a reticulate tectum as the SCW starts to break down. (f) *Tragopogon porrifolius* young free microspore with low conical spines, showing tapetum beginning to become invasive (arrowed).

2004). The involvement of the SCW in microspore surface pattern generation has been much discussed since it was suggested that it acts as a template or a negative stencil, regulating the deposition of the primexine in *Ipomoea* (Goodwin *et al.*, 1967; Waterkeyn & Bienfait, 1970). In Compositae pollen, the inner surface of the individual SCWs (Fig. 4c) also shows the characteristic pattern of spines and/or ridges present in the particular taxon (Barnes & Blackmore, 1986; Blackmore & Barnes, 1987). However, this does not necessarily mean that the SCW imparts a pattern on the primexine. Both callose and primexine are deposited at the microspore surface through processes mediated by the plasma membrane. Consequently, it is the plasma membrane that should be regarded as mediating this pattern, and in the transition from callose to primexine deposition, a pattern will be generated if this transition takes place at different times in different places on the microspore surface (Fig. 4c,d). Several different types of pattern have been described on the inner surface of the SCW. In *Ipomoea*, the pattern takes the form of repeated polygons, mostly hexagons, evenly distributed over the microspore surface (Scott, 1994). Like the regularly and geometrically spaced spines of *Tagetes* (Heslop-Harrison, 1969b) such patterns suggest the operation of space-filling physical effects of a reaction-diffusion model (Turing, 1952). In *Tagetes*, the

developing spines are arranged to maximize the distance between them, which creates a hexagonal pattern with a spine at each angle and one in the centre of each hexagon. Spacing patterns of this kind are widespread in nature and are indicative of epigenetic effects, rather than precise patterning directly mediated by genes. Heslop-Harrison (1972) cautioned against assuming that genetic control extended to such details of patterning, despite the difficulty we may have in understanding how physical forces can generate such patterns.

The biosynthesis and molecular genetics of callose deposition (Kudlicka & Brown, 1997; Dong *et al.*, 2005; Enns *et al.*, 2005), and subsequent digestion of the SCW at the end of the tetrad stage, are becoming better understood. Scott *et al.* (2004) pointed out that the absence of a callose SCW is not necessary fatal and indeed it is well known that some species lack a SCW (see Blackmore & Crane, 1988). However, in taxa where the presence of a SCW is normal, its premature dissolution is associated with male sterility (Izhar & Frankel, 1970; Worall *et al.*, 1992). Reduced callose can have significant effects on reproduction. For example, Dong *et al.* (2005) showed that the *CalS5* gene is responsible for callose synthesis in the SCW and that reduced fertility occurs in *Arabidopsis* pollen with the *cals5* mutant. Enns *et al.* (2005) demonstrated that *glsl5* homozygous mutants (*cals5* and *glsl5* being synonymous) produced aberrant pollen grains with deformed shapes, missing apertures and smaller lumina in the reticulate tectum.

IV. Meiosis and the establishment of microspore symmetry

Meiosis (stage 3 of Owen & Makaroff, 1995), a conserved process of cell division essential for eukaryotic sexual reproduction (Ma, 2005), is a gene-directed process (Golubovskaya, 1979) that involves a different cytokinetic apparatus from mitosis (Brown & Lemmon, 1992a,b). Meiosis marks the start of haplophase and is a key stage of ontogeny, during which both the polarity of microspores and the initial establishment of surface pattern take place. Thus, the beginnings of the great morphological diversity of pollen grains are found during meiosis. This was appreciated as long ago as the 1930s by Wodehouse (1935), who remarked on the close correlation between simultaneous or successive cytokinesis and pollen polarity expressed in terms of aperture number and position, although he considered that there were too many exceptions for this to be a rule. However, these apparent exceptions reflect the fact that the simplistic distinction of two kinds of meiosis is inadequate (Blackmore & Crane, 1998) and in most cases aperture position and pollen polarity are indeed determined by meiosis, as Heslop-Harrison (1968, 1971) and Dover (1972) demonstrated. Confusion can arise unless meiosis is observed directly, because its characteristics cannot be reliably inferred from the arrangement of the microspore tetrad. Tetrahedral tetrads formed by simultaneous cytokinesis are, for example, often indistinguishable from decussate tetrads formed by successive cytokinesis with a distinct dyad stage. Not only is it important to observe the timing of cytokinesis but also the mode of partitioning, whether by centripetal furrows, as in the Compositae (Fig. 3f) and most other eudicots, or by centrifugal cell plates, as in some Proteaceae (Blackmore & Barnes, 1995). It is important to note that in 'modified simultaneous' cytokinesis found, for example, in some, but not all, species of *Magnolia* (Farr, 1916), both centripetal furrowing and centrifugal cell plates are involved in partitioning the cytoplasm. The latter are ephemeral, as they are in some orchids (Brown & Lemmon, 1991), but they have the same effect as a dyad wall in isolating two cytoplasmic domains within which the second meiotic division takes place. In most cases, apertures are formed at the last points of cytoplasmic contact between the meiotic products (Blackmore & Crane, 1988). Exceptions to this include pantoporate pollen grains with large numbers of spirally or geometrically arranged apertures. Because such aperture patterns are not keyed into the symmetry of the microspore tetrad, Blackmore & Crane (1988) hypothesized that their arrangement might reflect a space-filling self-patterning process, perhaps related to the radial system of microtubules present in the early tetrad stage immediately after cytokinesis.

Much evidence for the role of the cytoskeleton in pollen development comes from experiments, using centrifugation or colchicine, to disrupt the meiotic spindle (Heslop-Harrison, 1971; Sheldon & Dickinson, 1983, 1986; Dickinson & Sheldon, 1986). Heslop-Harrison (1971) established that centrifugation at different stages of meiosis yielded cell fragments and whole cells that exhibited three types of induced anomalies – imperfectly separated microspores, misplaced apertures and pattern anomalies affecting the reticulate pattern of the exine and the underlying bacula or columellae. The major conclusion drawn from these experiments was that the microspore cytoplasm is autonomously able to carry out exine patterning by the time that tetrads are formed, because the 'essential spore-wall information has already been transferred to the cytoplasm by the end of the meiotic prophase. No longer can one seek to explain events in terms of sequential gene activation; the onus of control must lie rather with factors acting at the level of translation or later.' (Heslop-Harrison, 1971: 296). A number of meiotic mutants have now been described (reviewed by McCormick, 2004; Boavida *et al.*, 2005), which produce remarkably similar effects to the results of colchicine treatment or centrifugation.

The *STUD* (*STD*) and *TETRASPORE* (*TES*) genes were shown by Spielman *et al.* (1997) to be essential for cytokinesis, so that *stud/tes* mutants undergo nuclear but not cytoplasmic division during meiosis, resulting in large microspores with four nuclei, some of which progress to maturity as mature pollen grains containing up to four pairs of sperm cells. Although the structure of the exine is normal in such pollen grains, they differ in organizational symmetry and the arrangement of apertures. More recently, *STD* has been shown to be allelic to

TES 3, the *TES/STD* locus cloned and the protein it encodes found to be a putative kinesin involved in the organization and stability of the spindle (Yang *et al.*, 2003). The *quartet* (*qrt*) mutant (Preuss *et al.*, 1993) exhibits reduced SCWs and produces pollen in permanent tetrads. This mutant conforms to the model predicted by Blackmore & Crane (1988), although it is now known that microspore cohesion involves pectin components of the SCW (Rhee *et al.*, 2003) and not simply a reduction of callose. Several nonallelic qrt mutants are now known, and Francis *et al.* (2006) have shown that *QRT1* encodes a pectin methylesterase expressed in anthers after meiosis that is responsible for the separation of tetrads into monads. They also demonstrated *QRT1* expression elsewhere in the plant and suggest that the *QRT1* gene plays a wider role in plant cell wall loosening. The *tardy asynchronous meiosis* (*tam*) mutant in *Arabidopsis* (Magnard *et al.*, 2001) causes a lack of synchronicity and delays in cytokinesis, resulting in dyads and aberrant tetrads, including polyads and non-tetrahedral tetrads. By constructing *tam/qrt1* double mutants, Magnard *et al.* (2001) were able to prevent the meiotic products from separating after dissolution of the SCW. This demonstrated that the second meiotic division is often not completed before callose dissolution in *tam* mutants but can continue to completion after this event. This gives an interesting insight into the possibilities for variation in the relative timing of developmental events within microsporogenesis, which could lead either to successive or simultaneous cytokinesis and, in evolutionary terms, to phenomena of heterochrony.

V. The origins of the exine during the tetrad stage

Following the completion of cytokinesis (at the start of the tetrad stage, stage 5 of Owen & Makaroff, 1995), wall deposition within the microspore tetrad switches from callose to primexine (Heslop-Harrison, 1963). By this time, the organizational polarity of the future pollen grains, in terms of aperture number, type and position, has already been determined, as described in the previous section. In early tetrads of most species, an apertural shield of endoplasmic reticulum is thought to have the effect of restricting primexine deposition to nonapertural regions. Primexine (Fig. 4d,e) is a microfibrillar material composed largely of cellulose, which functions as an elaborate glycoclayx in which the patterned accumulation of sporopollenin precursors and their subsequent polymerization takes place (Rowley & Dahl, 1977). As the polymerization of sporopollenin progresses, the developing exine becomes less elastic and increasingly resistant to acetolysis (Heslop-Harrison, 1971).

Although much has been written about the conversion of primexine into exine through sporopollenin accumulation (recently reviewed by Edlund *et al.*, 2004; Scott *et al.*, 2004) the process remains the subject of as-yet unconfirmed models. TEM established a familiar sequence of events (Heslop-Harrison, 1963, and later papers) in which electron-dense materials, corresponding to probaculae and protectum, form within the primexine as sporopollenin accumulates. This sequence of events has been confirmed and illustrated with greater clarity in studies using freeze-substitution TEM. In *Brassica campestris*, Fitzgerald & Knox (1995) described the deposition of a primexine matrix or glycoclayx 200 nm thick, outside the plasma membrane. Slightly later, the plasma membrane becomes undulating in section because of the patterned deposition of ellipses of further material, which Fitzgerald & Knox called 'spacers', occupying the depressions of the plasma membrane. The raised areas around each spacer are the point at which probaculae are formed in a more or less hexagonal pattern, corresponding to the undulating pattern of the plasma membrane. Similar results were obtained by Takahashi (1986, 1991) in other taxa.

Scanning electron microscope (SEM) studies of primexine deposition and sporopollenin accumulation gave a different, but consistent, perspective (Blackmore & Barnes, 1985, 1987, 1988). The primexine initially appears uniform, but progressively, from the outer surface inwards, some regions become less solid and eventually disperse, leaving solid elements corresponding first to the tectum (Fig. 4d,e) and then to the columellae (Fig. 5a–d). This temporal sequence is a universal aspect of ectexine development. The outermost part of the primexine, which generally corresponds to the tectum, is defined and accumulates sporopollenin first, followed by the infratectal elements, such as columellae or a granular infratectum, and finally by the foot layer, if one is present.

Sheldon & Dickinson (1983) suggested that reticulate patterning in *Lilium* is generated by protein-filled coated vesicles fusing randomly with the plasma membrane and forming pattern-specifying structures by self-assembly. The model for exine patterning proposed by Fitzgerald & Knox (1995), involving 'spacers' that establish separation between probaculae, is completely consistent with the self-assembly process invoked by Dickinson & Sheldon. Such ideas were further built upon by Southworth & Jernstedt (1995), who proposed a self-patterning model that can account for pattern induction in the primexine reacting against the SCW as a consequence of tensegrity (Ingber, 1993). They proposed that 'exine pattern is generated by the physical properties of: the callose shell; the matrix and primexine; and conditions in the microspore that generate osmotic pressure and cytoskeletal tension' (Southworth & Jernstedt, 1995: 86). This 'Tensegrity Model' provides a possible explanation for the generation of echinolophate pattern in Compositae pollen. The large lacunae between ridges form a variety of patterns, of considerable systematic significance (Blackmore, 1986). During development, secretion of callose continues for longer in areas corresponding to lacunae (Fig. 4c) than in areas corresponding to spines and ridges. Thus, there is evidence, from a range of plant taxa, that the SCW is involved in pollen surface pattern generation, but as part of a system involving both the SCW and primexine.

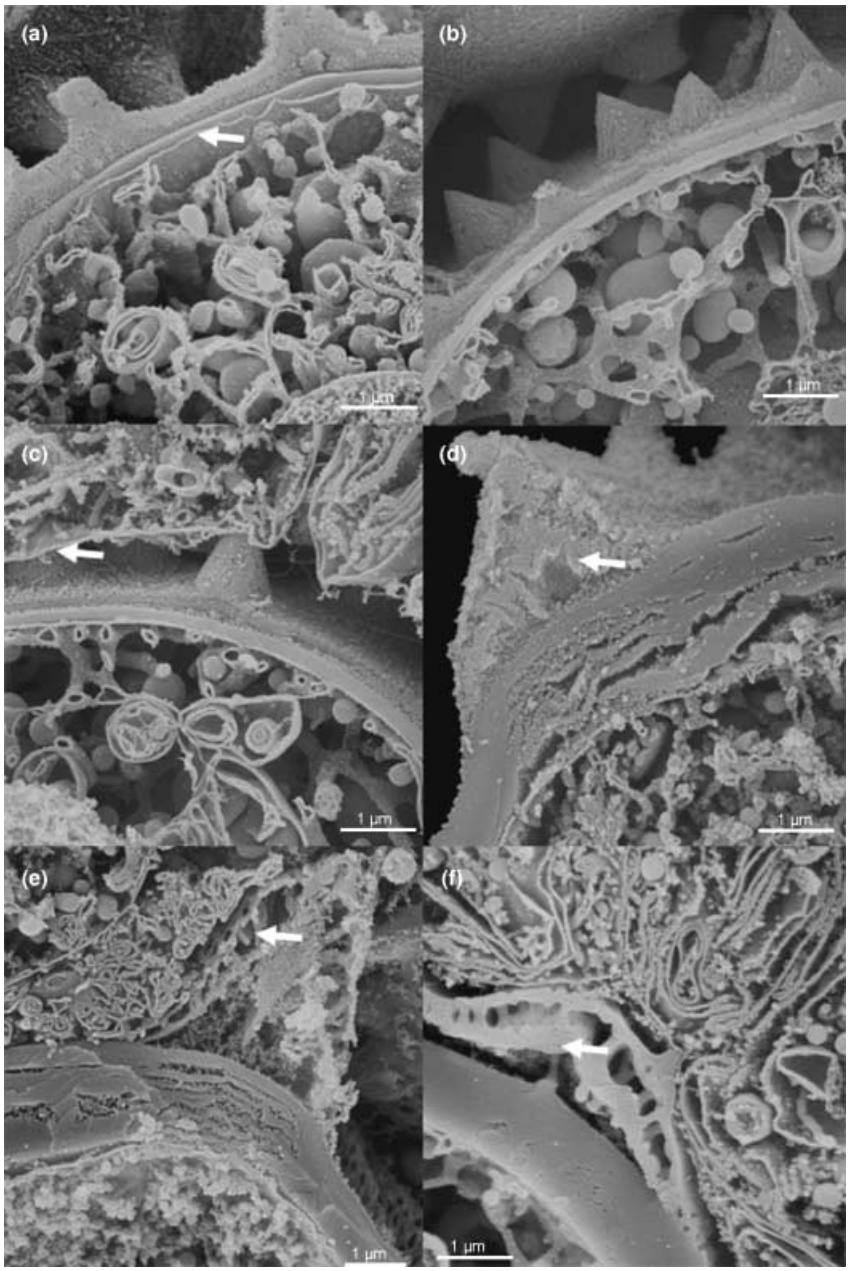

Fig. 5 Development of pollen in *Catananche caerulea* and *Tragopogon porrifolius* during the free microspore stage. (a) *C. caerulea* with blunt conical spines and the endexine beginning to form (arrowed). (b) *C. caerulea* microspore with undifferentiated primexine. (c) *C. caerulea* at a later stage showing thicker endexine and invasive tapetum bounded by plasma membrane (arrowed). (d) *T. porrifolius* showing early differentiation of the ectexine into a system of hollow tubes corresponding to columellae (arrowed). The lamellate nature of the endexine is apparent. (e) A slightly later stage of *T. porrifolius* showing removal of nonsporopollenin receptive matrix of the primexine and recognizable columellae (arrowed). (f) *C. caerulea* microspore with almost mature ectexine organization with internal foramina visible (arrowed) and distinct voids between ectexine elements.

Once the microspores are released from the callose SCW (Fig. 4f), further sporopollenin deposition on top of the tectum frequently occurs. In Compositae, for example, the spines of microspores, immediately after release from the tetrad, are low, conical structures (Fig. 5a–c). The spines subsequently become longer and more acute (Fig. 5d–f) through the accumulation of sporopollenin (without the participation of the primexine matrix). The addition of supratectal sporopollenin is widespread, and the results of such deposition can be important determinants of pollen surface pattern. In Malvaceae, for example, Takahashi & Kouchi (1988) demonstrated that the large spines, so characteristic of mature pollen grains, are lacking in microspores immediately after release from the tetrad, and are instead formed by the accumulation of sporopollenin during the free microspore stage. In contrast to the reticulate pollen grains of *Lilium* and *Arabidopsis*, mature pollen of Compositae is generally spiny, with the tectum having a microreticulate or microperforate pattern, which first becomes apparent as sporopollenin accumulates in the primexine (Fig. 4e). At this stage of development, the spines and microspore surface are covered with cylindrical structures corresponding to 'tufts'. Tufts, one of the first interpretations proposed for exine substructure, are units 60–100 nm in diameter that consist of helically coiled subunits 10 nm in

diameter (Rowley & Dahl, 1977). Strikingly similar observations of tufts on the surface of microspores have been made in a wide variety of taxa, including *Canna* (Rowley & Skvarla, 1975) and *Centrolepis* (Rowley & Dunbar, 1990). A more recent interpretation of the exine substructure is based on the properties of surfactant molecules dispersed in a liquid colloids. According to this model, amphiphobic molecules within the glycoclayx form micelles of various shapes, including spheroids, cylinders and bilayers (Hemsley *et al.*, 2003; Gabarayeva & Hemsley, 2006; Hemsley & Gabarayeva, 2007). This concept extends an earlier model for the formation of TPL, in which the electron-lucent central layer was interpreted as the hydrophobic regions of long-chain fatty acids and the electron-dense layers as their hydrophilic ends in complex with phenolic compounds (Scott, 1994). By demonstrating that, depending on whether the liquid medium is aqueous or lipidic, such a mechanism can generate either cylindrical structures or hollow tubes (as in Fig. 5d). Gabarayeva & Hemsley (2006) established a model for the formation of tufts and for the tendency of tufts to cluster together into the hexagonally repeating structures observed by Scott (1994) and others. Gabarayeva & Hemsley (2006) showed how Rowley's concept of groups of tufts, displaying characteristic hexagonal patterns in thin section, could originate through the self-assembly of micelles in the glycoclayx. As in TPL, the electron-dense part of such subunits would correspond to the hydrophilic end of long-chain fatty acid molecules, and their progressive accumulation of phenolics compounds would reflect this pattern as the sporopollenin component of the primexine becomes progressively polymerized. Early in their development, the micelle surfaces form a 'boundary layer' defining the organization of ectexine elements (Blackmore, 1990). As sporopollenin accumulates within boundary layer-defined structures, the structure of the exine becomes more solid and increasingly acetolysis resistant (Fig. 5e,f). Some forms of oxidative treatment can recover the boundary layer from within mature exines (Blackmore & Claugher, 1987; Blackmore, 1990). Regions of the primexine in which sporopollenin precursors do not become polymerized are gradually dispersed in a manner resembling the dissolution of the SCW (Fig. 6a,b). The process of sporopollenin accumulation with the primexine progresses from the outer surface inwards. The tectum therefore forms from the oldest part of the primexine, adjacent to the SCW, and in Compositae it typically has a microreticulate surface that is apparent even before infratectal elements of the ectexine, such as columellae, are distinguishable. If the temporal sequence is read from the end of callose deposition to the onset of the primexine glycoclayx through to its completion, it can be seen that the complex, multilayered pollen walls of Compositae appear to reflect changing patterns of micelle formation through time. Viewed in the same way, the onset of endexine deposition represents a switch in the conditions for micelle formation, resulting in the formation of TPL.

A number of mutants have been identified that affect the tetrad stage. In *Tetraspore* (*tes*) mutants (Spielman *et al.*, 1997), primexine is deposited around the surface of the undivided cytoplasm containing four nuclei. Those pollen grains that reach maturity form an exine with normal structure but displaced or irregular apertures. Paxson-Sowders *et al.* (1997) showed that in *dex1* mutants, pollen development follows the same programme as the wild type until the early tetrad stage when the normal undulating pattern of the plasma membrane (cf. Fitzgerald & Knox, 1995) is not seen. Sporopollenin becomes randomly deposited outside the plasma membrane, resulting in an exine surface that lacks a normal reticulate pattern. Paxson-Sowders *et al.* (1997) concluded that the mutation blocks the normal invagination of the plasma membrane, confirming the importance of the plasma membrane in determining the pattern of the exine. Later, Paxson-Sowders *et al.* (2001) isolated and characterized the novel plant protein, DEX1, which is expressed throughout the plant, although *dex1* plants do not display morphological defects other than in the pollen wall. Using rapid freezing followed by freeze substitution, they improved upon earlier observations to show that in addition to the failure of the plasma membrane to develop undulations, primexine deposition is delayed and altered, that 'spacers' (Fitzgerald & Knox, 1995) do not form, that sporopollenin deposition is random and that fibrillar material is not present within the primexine. The DEX1 protein was predicted to be a membrane-associated protein that may span the plasma membrane and contains several calcium-binding zones. Paxson-Sowders *et al.*, 2001) offered three possible roles for DEX1: as a linker protein that attaches sporopollenin or its precursors to the plasma membrane; as a component of the primexine involved in the polymerization of sporopollenin; or as a component of the rough endoplasmic reticulum involved in transport of primexine precursors. The authors favoured the second of these roles and highlighted the fact that the DEX1 protein may cause changes in Ca^{2+} ion concentrations within the primexine. Just such changes in ionic concentration would provide precisely the kind of variables that would determine the shape of micelles formed in the primexine, according to the self-assembly model of Gabarayeva & Hemsley (2006), which emphasizes the colloidal properties of the glycocalyx and early primexine (Hemsley & Gabarayeva, 2007). Changing ionic concentrations, through time within the primexine, would generate different patterns of micelles as development through the tetrad stage progresses. The self-assembly of the pattern, exhibited in even the most complex exine morphologies found in the Compositae, could be generated in this way.

Another pollen pattern mutant, *Faceless pollen-1* (Ariizumi *et al.*, 2003), produces pollen with an almost smooth surface without a prominent reticulate pattern. TEM observations showed that the pattern of the reticulate muri in these mutants was modified relative to wild-type *Arabidopsis* (appearing more or less regulate), and that the smooth surface was the

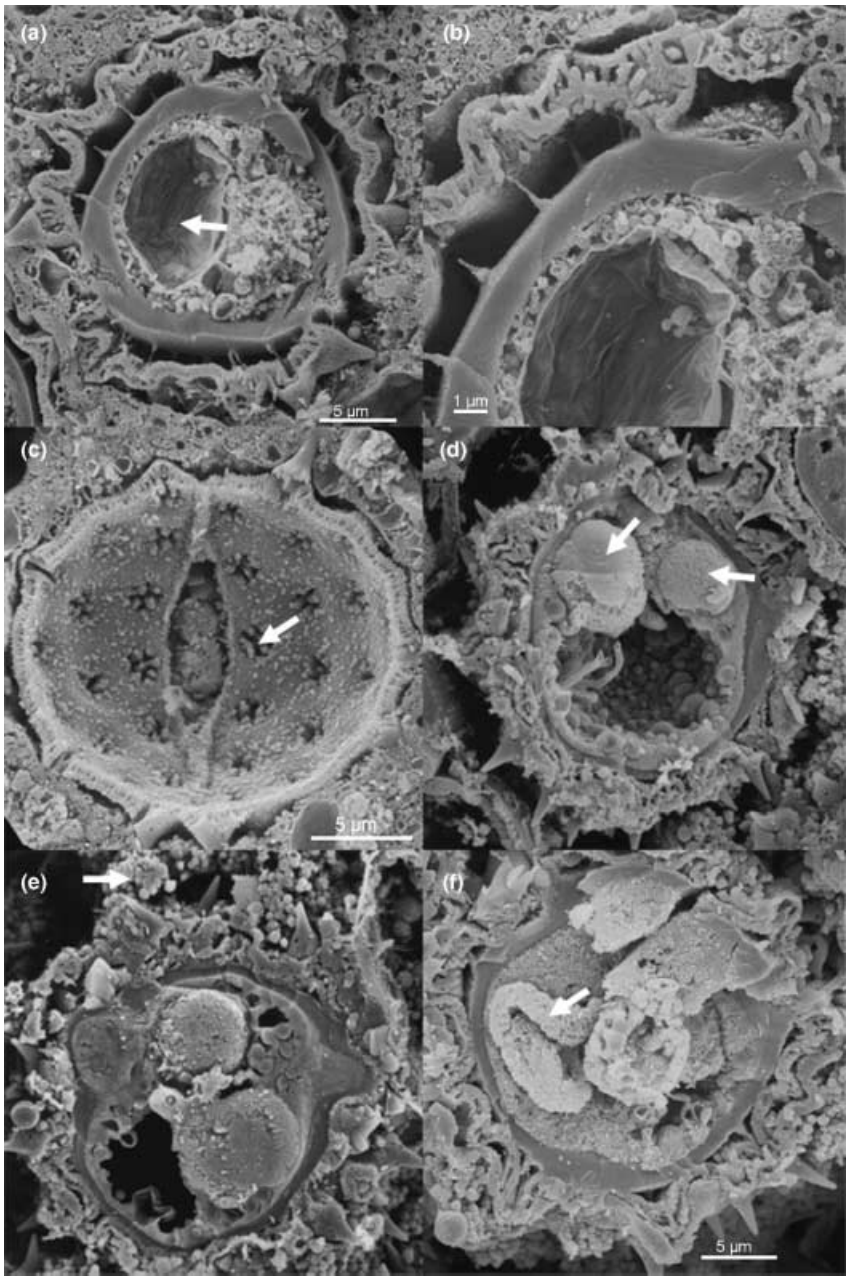

Fig. 6 Compositae pollen development through the vacuolate stage to the male germ unit. (a) *Catananche caerulea* microspore at the vacuolate stage (vacuole arrowed) with more mature exine. (b) Detail of the vacuole in *C. caerulea* showing the solid endexine layer and distinct caveate space within the ectexine. (c) Internal view of the outer ectexine of the *Cosmos bipinnatus* microspore at the vacuolate stage showing a hexagonal arrangement of channels leading through to the regularly spaced spines (arrowed). (d) *Catananche caerulea* pollen grain after the first mitotic division, nuclei of the vegetative (left) and generative (right) cell (arrowed). The invasive tapetum is degenerating. (e) *C. caerulea* at the bicellular stage showing invasive tapetum breaking down (arrowed). (f) *C. caerulea* pollen grain with the male germ unit (arrowed) and spaces within the ectexine occupied by pollenkitt.

result of excessive amounts of lipidic tryphine droplets covering the pollen surface. Acetolysis resistance is reduced in pollen grains of the *flp1* mutant. Ariizumi *et al.* (2003) concluded that either sporopollenin precursors synthesized in the tapetum are not transferred correctly, or that the precursors are not polymerized within the primexine causing aberrant exine stratification and reduced resistance to acetolysis. The latter interpretation would be highly consistent with the observation (Heslop-Harrison, 1969b) that early exines exhibit great elasticity and are capable of significant increases in size (Dickinson, 1970b; Rowley & Dahl, 1977), whereas fully polymerized, mature exines are not. Interestingly, the *flp1* gene was also shown to be involved in the biosynthesis of cuticular waxes, supporting the concept of shared biosynthetic pathways for cutin, lignin and sporopollenin (Scott, 1994).

The *MS1* gene, which is expressed in the tapetum and encodes a protein belonging to the plant homeo domain (PHD)-finger family of transcription factors, regulates tapetal gene expression (Wilson *et al.*, 2001; Ito & Shinozaki, 2002; Wilson & Yang, 2004). The effects of *MS1* on primexine formation and pollen development have been studied at the ultrastructural level, in the *hackly microspore* (*hkm*) mutant, by Ariizumi *et al.* (2005), who demonstrated that the *HKM* gene is identical to *MS1*. Undulations of the plasma membrane in

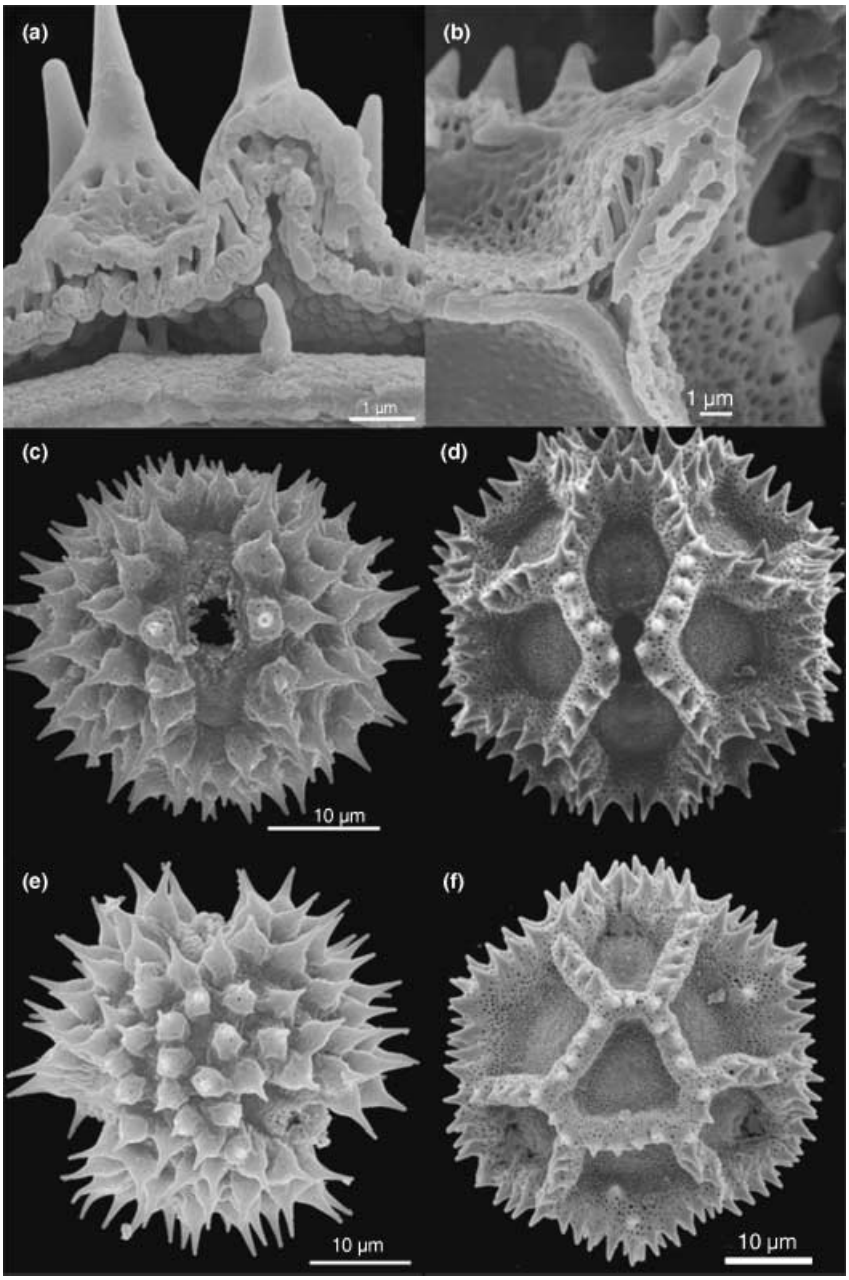

Fig. 7 Mature grains of echinate (*Catananche caerulea*) and lophate (*Scorzonera hispanica*) Compositae pollen treated by acetolysis to remove pollenkitt and cytoplasm. (a) Section of the mature exine of *C. caerulea*. (b) Section of the mature exine of *S. hispanica*. (c) *C. caerulea* mature pollen grain in an apertural view. (d) *S. hispanica* mature echinolophate pollen grain (apertural view). (e) *C. caerulea* mature pollen grain (polar view). (f) *S. hispanica* mature pollen grain (polar view).

hkm mutants are more pronounced than in the wild type, but the primexine is much reduced in thickness and probaculae are not apparent. In the free microspore stage, sporopollenin deposition is random, forming an unstructured exine, but a foot layer and intine are not developed. Mutants are male sterile, and Ariizumi *et al.* (2005) showed that the *hkm* mutation occurs within the *MS1* gene, which they interpreted as evidence that critical processes of primexine formation are under sporophytic control, because the *MS1* gene is expressed in the tapetum.

In terms of pollen morphology, by the end of the tetrad stage the key characteristics of aperture configuration and exine structure have already been determined, although additional sporopollenin may be added during later stages. Towards, or soon after, the end of the tetrad stage, endexine deposition commences on TPL. Blackmore (1990) interpreted TPLs, which occur in all groups of embryophytes (land plants) and in their algal nearest relatives, as the most plesiomorphic form of exine deposition. He pointed out two key evolutionary innovations: a change in the relative timing of TPL deposition (before meiosis in algae, after meiosis in embryophytes) and the multiplication of TPLs in the thicker walled spores of early land plants. Subsequently, Scott (1994) proposed an elegant model for TPL self-assembly that is fully

consistent with the extended and more recent model of self-assembly proposed by Gabarayeva & Hemsley (2006). Scott's model also provides explanations for both the short, radial TPLs sometimes observed within the differentiating primexine and for the observation that although the endexine of angiosperms develops on TPLs, they are rarely visible in mature pollen grains.

VI. The free microspore stage to pollen maturation

The free microspore stage (Figs 4f and 5a–f) extends from the end of the tetrad stage, marked by the dissolution of the SCW, until mitosis, after which the microspore becomes a microgametophyte or pollen grain. By the early free microspore stage, the morphology of the exine is essentially complete and therefore, in this review, the later stages of pollen ontogeny are only briefly summarized. In most flowering plants, only two components of the pollen wall are deposited between the free microspore stage and maturity: the pecto-cellulosic intine; and the tryphine or pollen coat.

The tapetum is highly active during the free microspore stage (Stages 6–8 of Owen & Makaroff, 1995) and displays considerable variety of behaviour in different groups of angiosperms (Pacini, 1997). In the Compositae, the tapetal cells enlarge and engulf the microspores (Fig. 5e,f), although they retain a plasma membrane until microspore mitosis takes place. Although the free microspore stage is characterized by the active synthesis of sporopollenin precursors in the tapetum and their incorporation into specific sites within the differentiating ectexine and on its surface, this does not generally involve the initiation of new exine patterning. There are cases, for example, in *Hibiscus* (Malvaceae), where the large and characteristic spines are formed during the free microspore stage on the surface of a columellate ectexine, which originates from primexine deposited in the tetrad stage (Takahashi & Kouchi, 1988). In most flowering plants that have an endexine (it is usually absent in monocotyledons), the majority of this is formed on TPL (Fig. 5d) during the free microspore stage (Fig. 5e).

Although the post-tetrad stages are less important for pollen wall formation, they are the period during which developing microspores undergo mitotic divisions to form the male gametophyte. Before pollen mitosis, a single large cytoplasmic vacuole (Fig. 6a–d) forms by the coalescence of smaller vacuoles (Yamamoto *et al.*, 2003), giving rise to the characteristic 'signet ring' appearance (Stage 8 of Owen & Makaroff, 1995), in which the cytoplasm and nucleus are entirely peripheral. Pollen mitosis is an asymmetric cell division that gives rise to the vegetative cell and the much smaller generative cell (Fig. 6d,e). Immediately after mitosis, the generative cell has a thin callose wall (Boavida *et al.*, 2005) and remains peripheral, surrounded by the cytoplasm of the generative cell. In the *Arabidopsis* mutant, *Sidecar* (*scp*), a premature, symmetrical microspore mitosis is followed by the assymetrical division of one of the daughter cells to form the sperm cells (Boavida *et al.*, 2005; Ma, 2005). In the *two-in-one pollen* (*tio*) mutant, cytokinesis fails during the first mitotic division (Twell & Howden, 1998), whereas in the *limpet pollen* (*lip*) mutant, the migration of the sperm cells away from the periphery is blocked and the temporary callose wall, which normally forms around the generative cell, is not broken down (Howden *et al.*, 1998). In the normal course of events, the large vacuole gradually disappears and the generative cell migrates to a more central position before the second mitosis of the generative cell. In the Compositae, *Arabidopsis* and other plants with tricellular pollen, mitosis results in the formation of male gametes: the two sperm cells. These are elongated, with little cytoplasm, and are linked together (Fig. 6f). One of them is also connected to the nucleus of the generative cell so that they form a remarkable structure, which Dumas *et al.* (1985) called the male germ unit (MGU) (Lalane & Twell, 2002). Two classes of mutants affecting the formation of the MGU have been described: *male germ unit displaced* (*mud*) and *germ unit malformed* (*gum*) (Lalane & Twell, 2002).

Before pollen grains are shed from the anthers, three important processes take place: the accumulation of starch or lipids as storage materials within the pollen grain; the addition of pollenkitt or tryphine to the exine from the degeneration of the tapetum; and the dehydration of the cytoplasm. The deposition of pollenkitt (as it is generally known in palynological literature), or tryphine (as developmental studies tend to term it), on the surface and within the chambers of the exine is the only one of these processes that affects the pollen wall. Pollenkitt has several important functions, including increasing the resistance of the pollen wall to desiccation and conveying sporophytic incompatibility substances derived from the tapetum (Ma, 2005). Dehydration of the pollen cytoplasm reduces its volume and causes harmomegathic changes in the appearance of the pollen wall (Wodehouse, 1935). Each of these three events is of profound importance in the biology of the pollen grain and worthy of a review in its own right.

VII. Conclusions

Recent research involving molecular genetics and the analysis of mutants means that many of the principal events in pollen wall development are increasingly well understood. Despite the enormous progress this represents, some of the earlier mysteries still remain and there is much scope for innovative research. We have seen how the structure of the SCW and the processes of meiosis lay the foundations for the diversity of morphological patterns exhibited by pollen exines (e.g. Fig. 7). We now have some persuasive models of how self-assembly might generate the vast array of complex nano-scale features encountered in pollen grains. The exciting opportunity, now within reach, is the possibility of these models being thoroughly

challenged by experimentation. Two completely different scales of self-assembly are opening up to experimental investigation. First, the phenomenon of cellular tensegrity and how it might define large-scale surface patterns; and, second, the formation of micelles at the cell surface. Both offer different challenges and both have the potential to inform our wider understanding of differentiation at the cellular level in other biological contexts.

The prospect for the near future is a full understanding of Heslop-Harrison's insight, from the 1970s, that pollen wall patterning is not directly coded for by genes but involves an interplay between the products of gene expression and the influence of physical forces. Well-characterized genes, such as *DEX1* (Paxson-Sowders *et al.*, 2001), could serve as an immediate starting place to begin to modulate the patterns of self-assembly in the primexine, for example, by inducing changes in calcium ion concentrations through the tetrad stage. With the rapid progress of transcriptome analysis, and the increasingly sophisticated analysis of gene expression at different stages of pollen development, we can be confident that other candidate genes for such experiments will be identified. Whereas during the pioneering era of investigation of pollen development it was possible to observe and document the temporal sequence of events, we can now look forward to an entirely new understanding of cause and effect underlying 'morphogenesis in miniature'. The experimental tools available are infinitely more subtle than colchicine treatment or centrifugation!

We are on the brink of understanding the basis of evolutionary diversity in the finely tuned systems of plant reproduction on which food production, biodiversity and so much else depends. If that was not enough, discoveries that can be made in the model system of pollen development will have direct relevance to other areas of plant science.

Acknowledgements

This paper is dedicated to the memory of John Heslop-Harrison (1920–98) and Bruce Knox (1938–97), two giants of the microscopic universe of pollen development.

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