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REVIEW: PART OF A SPECIAL ISSUE ON PLANT NUTRITION

Comparative physiology of elemental distributions in plants

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• *Background* Plants contain relatively few cell types, each contributing a specialized role in shaping plant function. With respect to plant nutrition, different cell types accumulate certain elements in varying amounts within their storage vacuole. The role and mechanisms underlying cell-specific distribution of elements in plants is poorly understood.
• *Scope* The phenomenon of cell-specific elemental accumulation has been briefly reviewed previously, but recent technological advances with the potential to probe mechanisms underlying elemental compartmentation have warranted an updated evaluation. We have taken this opportunity to catalogue many of the studies, and techniques used for, recording cell-specific compartmentation of particular elements. More importantly, we use three case-study elements (Ca, Cd and Na) to highlight the basis of such phenomena in terms of their physiological implications and underpinning mechanisms; we also link such distributions to the expression of known ion or solute transporters.
• *Conclusions* Element accumulation patterns are clearly defined by expression of key ion or solute transporters. Although the location of element accumulation is fairly robust, alterations in expression of certain solute transporters, through genetic modifications or by growth under stress, result in perturbations to these patterns. However, redundancy or induced pleiotropic expression effects may complicate attempts to characterize the pathways that lead to cell-specific elemental distribution. Accumulation of one element often has consequences on the accumulation of others, which seems to be driven largely to maintain vacuolar and cytoplasmic osmolarity and charge balance, and also serves as a detoxification mechanism. Altered cell-specific transcriptomics can be shown, in part, to explain some of this compensation.

Key words: Cell-specific, element, vacuole, transcriptome, ionome, plant nutrition, nutrient storage.

INTRODUCTION

Nutrients are often acquired in excess of the immediate needs of the plant and as a result they must be stored within plant tissue until required. Whilst taking-up essential micro- and macronutrients, and despite sophisticated exclusion mechanisms, plants inadvertently acquire elements that are toxic. Therefore, compartmentalization and/or separation of particular elements, especially in their soluble ionic form, are required by the plant for optimal function (Leigh, 1997). This review reports commonly observed elemental distributions within plant tissues, explores the potential reasons for these distributions and examines the processes that may be involved in these organ-, tissue- and cell-type distributions.

Plant vacuoles play a major role in elemental storage (including compartmentation of toxic substances) and in the maintenance of nutrient availability within the cytosol (Leigh, 1997). Well-documented examples include mechanisms for cytosolic homeostasis of calcium (Ca^{2+}) and nitrate (NO_3^-), and the ability of cells to limit cytosolic sodium (Na^+) accumulation (Miller and Smith, 2008; Munns and Tester, 2008; McAinsh and Pittman, 2009). Plant organs may have very different concentrations of elements within tissues which may relate to properties of long-distance transport (xylem vs. phloem), sites of complexation (for many heavy metals or charge-dense ions), or tissue- or cell-specific transport processes such as ion accumulation within vacuoles (Walker and Pitman, 1976; Marschner, 1995; Leigh, 1997; Tester and Leigh, 2001; Britto *et al.*, 2006). In fact, the vacuole of a particular cell may have a very different elemental profile compared with that of a neighbouring cell of the same cell-layer or more commonly of different cell types (Karley *et al.*, 2000*a*; Karley and White, 2009). As the central vacuole occupies most of the cell volume it is ideally suited for a role in elemental storage (Wagner, 1982; Canut *et al.*, 1993; Andreev, 2001). It is specifically these differences in vacuolar element content on which this review will be focused.

We propose that elemental distributions in plants can be explained by (*a*) transport pathways that elements take through plants and (*b*) storage properties of cells that constitute or border the elemental transport corridor. To illustrate this theme the compartmentalization of Ca^{2+}, an essential plant macronutrient, and cadmium (Cd^{2+}) and Na^+, two 'toxic' cations in plant leaf and root tissues, will be examined in detail. As potassium ions (K^+) are the major cationic osmoticum in plants, K^+ compartmentation with respect to the aforementioned elements will also be touched upon throughout, but for recent detailed reviews on K^+ homeostasis, refer to Shabala and Cuin (2007), Luan *et al.* (2009) and Karley and White (2009).

There have been over 150 published reports in the past 20 years of cell-type-specific element profiling which have been mainly of a phenomenological nature; however, there has been a resurgence in such reports in the past 5 years (over

50) following technological developments in the molecular interrogation of single cells or cell types. Much of this resurgence has arisen through an interest in bio-fortification of staple foods and phytoremediation/phytoextraction of contaminated sites, which requires a need for an understanding of the physiology of specific-cell types and cell-type compartments in response to enhanced storage of particular ions. By focusing on Ca^{2+}, Cd^{2+} and Na^{+}, and predominantly plant leaf tissue, other equally important elemental distributions, interactions or relevant processes will not be explored in depth. For instance, seed-loading and seed cell-type-specific nutrient compartmentation is an emerging topic of scientific study but will not be touched upon (refer to Zhang *et al.*, 2007; Kachenko *et al.*, 2009; Punshon *et al.*, 2009; Tauris *et al.*, 2009; White and Broadley, 2009).

The role of particular solute transporters located on the plasma membrane and tonoplast of particular cell types with respect to the accumulation of particular elements within cells will also be discussed. However, the role of transport proteins located on other membranes and indeed membrane processes such as endocytosis or membrane trafficking may play a role in ion compartmentalization but will not be discussed in detail here. For recent reviews on these subjects, the reader is referred to articles such as those by Amtmann and Blatt (2009) and McAinsh and Pittman (2009).

TECHNIQUES FOR PLANT ELEMENTAL PROFILING

The term 'ionome' is commonly and perhaps erroneously used to describe the total concentration (i.e. all forms) of selected elements in a sample of plant tissue (Salt *et al.*, 2008). Ionic concentrations (or activities) of elements are more difficult to measure accurately, but are usually considerably less than the total elemental content. For instance, the calcium content of certain *Arabidopsis thaliana* leaf vacuoles may be as high as 80 mM but the Ca^{2+} activity ('free' Ca^{2+} not complexed or irreversible bound) has been measured at 12 mM (M. Gilliham and T. Cuin, unpubl. res.). Therefore, the terms 'ionome' and 'ionomics' (the study of elemental content of plants) should be considered with caution.

Many techniques are available for investigating elemental or ionic content of plant tissues with resolution from the whole plant to the sub-cellular level. Techniques covered here (see Table 1), and used extensively in studies reviewed below, can be broadly separated into those based on: fluorescence or luminesence of indicator macromolecules (e.g. protein 'biosensors', chemical-based fluorescent indicators); ionophores (e.g. ion-selective electrodes); X-ray fluorescence detected directly from elements (e.g. X-ray microanalysis); mass-spectroscopy [e.g. inductively coupled plasma–mass spectroscopy (ICP-MS) and stable isotopes] and radioactive emission from tracers. These techniques vary in their throughput, sample preparation, cost, detection limits and ability to discriminate forms of the element. There have been few reviews of each of these techniques in a comparative light (Ortega, 2005; Lobinski *et al.*, 2006), yet each individual method has again been detailed recently in a review by Salt *et al.* (2008). As a result, we will only address limitations of these with respect to current research findings detailed in the sections below.

Techniques to sample elemental content of plant tissues

Whole plant tissue analysis has been used to identify genetic factors that directly or indirectly influence elemental content of plants. ICP-MS is a common technique for elemental profiling of whole plants or explants (Table 1). Application of ICP-MS to a mutagenized population of 6000 arabidopsis plant lines uncovered 51 mutants with altered elemental profiles (Lahner *et al.*, 2003). Baxter *et al.* (2008) identified 'elemental signatures' which were used as a diagnostic when analysis of the target element alone may be a poor indicator of nutritional status, such as altered iron (Fe) and phosphorous (P) homeostasis. The use of 'elemental signatures' reduces the proportion of plants incorrectly identified as having altered elemental accumulation patterns and takes advantage of the many interactions that occur between acquisition, redistribution and storage of mineral elements (see following sections on particular elements). A recent improvement on the throughput of ICP-MS (Table 1) was achieved by Hansen *et al.* (2009) who developed a miniaturized version (called micro-ICP), involving microwave processing of smaller tissue samples (1–20 mg), in greater number and equivalent accuracy to traditional ICP spectroscopy.

Plants contain approx. 40 different cell types (Martin *et al.*, 2001), each varying in transcript and protein complement and abundance (Kehr, 2003; Roy *et al.*, 2003; Brandt, 2005; Galbraith and Birnbaum, 2006), physiological role (e.g. Tester and Leigh, 2001) and, potentially, individual elemental composition (Karley *et al.*, 2000*a*). Therefore, whole tissue analysis has its limitations and can blur the individual contribution of each cell type to the elemental profile. As such, methods have been developed to analyse cell-specific element profiles (Table 1).

Except for secondary ion mass spectroscopy (SIMS) and laser ablation ICP-MS (LA-ICP-MS), the multi-element profiling techniques summarized in Table 1 are based on excitation of electrons within ions with measurement of the characteristic and commonly element-specific X-ray emissions. SIMS and LA-ICP-MS involve the physical disturbance of the sample surface to generate charged particles, which are subsequently measured by mass spectroscopy. The variations between methods are the identity of the incident beam (laser, X-ray, electron beam, ion beam), the emission type (X-rays, fluorescent X-rays, plasma/charged ions) and also the detector (EDX or MS). Furthermore, these techniques can be extended by scanning the incident beam across the surface of the sample to generate a two-dimensional ion quantification map, used to great effect in various species looking at numerous ions simultaneously (Storey and Leigh, 2004; Küpper *et al.*, 2000; Kerton *et al.*, 2009). However, X-ray microanalysis (XRMA)-based measurements have a resolution based on electron beam energy which limits the minimum size of compartment that can be assayed without contamination from adjoining compartments. For instance, in cryo-frozen biological tissues with an excitation voltage of 15 keV this approximates to approx. 5 μm^2 (Echlin, 1992) so cytoplasmic and cell-wall compartments cannot generally be quantified reliably and depth of the sample must always be considered.

TABLE 1. *Techniques for quantifying elemental (and ionic) content within plant cells: the main spatially resolved analytical techniques for chemical element imaging, quantification and speciation in plant samples*

Basis of measurement	Technique/ indicator	Detection limit/ range (μg g^{-1})	Spatial resolution (μm)	Selectivity	Quantification	Analytical depth (μm)	References
Protein sensors	Aequorin	0·004–4·0 (0.1–10 μM)	5–10	Calcium	Ratiometric	Tissue depth (<300)	Rutter *et al.*, 1996; Fricker *et al.*, 1999; Plieth, 2006
	pHluorin	pH 5·5–7·5	5–10	pH (H^+)	Ratiometric	Tissue depth (<300)	Miesenbock *et al.*, 1998
	CAMeleon	0·0004–0·17 (0.01–4.3 μM)	5–10	Calcium (variants with less H^+ sensitivity)	Ratiometric	Tissue depth (<300)	Miyawaki *et al.*, 1999
Fluorescent indicators	Fura-2	0·0056 (140 nM) (kDa 0·14)	5–10	Calcium	Ratiometric	Cell depth	Mühling and Läuchli, 2002*a*, *b*;
	SBF1	87·4 (K. 3·8 mM)	5–10	Sodium	Ratiometric		Halperin and Lynch, 2003;
	PBF1	199·4 (kDa 5·1 mM)	5–10	Potassium (interfering Na^+)	Ratiometric		Mazel *et al.*, 2004; Kader and Lindberg, 2005
Ion-selective electrodes	ETH 157	46 (2 mM)	1–2	Sodium (interfering H^+, K^+)	Quantitative (ion activity)	Relative to tip dimensions and penetrance (>5)	Fluka Chemicals
	ETH 129	0·0004 (10 nM)	1–2	Calcium (interfering K^+, H^+, Mg^{2+})	Quantitative (ion activity)		
	Valinomycin	3·9 (100 μM)	1–2	Potassium (Ca^{2+}, NH^+)	Quantitative (ion activity)		
Measures of total elemental content	Inductively coupled plasma–mass spectroscopy (ICP-MS)	≥0·001	N/A	Multi-elemental (all Z)	Quantitative	N/A	
	X-ray microanalysis (SEM-XRMA)	~100 (dehydrated)–1000 (hydrated)	0·5–5	Multi-elemental ($Z \geq 6$)	Semi-quantitative (commonly, but can be quantitative)	0·1–1	Echlin, 1992; Ortega, 2005
	Ion beam microprobe (μ)PIXE	1–10	0·2–2	Multi-elemental (all Z)	Quantitative	10–100	
	Synchrotron radiation microprobe (XRF)	0·1–1	0·1–1	Multi-elemental and isotopic	Semi-quantitative	>100	
	Laser ablation inductively coupled plasma–mass spectroscopy (LA-ICP MS)	0·01	15–50	Multi-elemental and isotopic	Semi-quantitative	200	
	Secondary ion mass spectrometry (SIMS)	0·1	0·05	Multi-elemental and isotopic	Quantitative	0·1	Metzner *et al.*, 2008

The values shown are representative of a variety of elements ranging from transition metals to macronutrients, within a biological matrix such as an isolated cell or a tissue section (adapted from Ortega, 2005).

Synchrotron X-ray fluorescence microprobes (μXRF) are becoming a favoured technology for obtaining (semi-) quantitative spatial data in plants and is an improvement on many current 'mechanical' means (Metzner *et al.*, 2008; Punshon *et al.*, 2009). These advantages include (with certain modifications) its non-destructive nature, important for precious samples and living material; it permits three-dimensional reconstruction of accumulation patterns and can also distinguish between ionic valencies, critical for accumulation of toxic forms of various ions, including thallium in the hyperaccumulator *Iberis intermedia* (Scheckel *et al.*, 2007).

Additional dimensions to these techniques have been developed to increase throughput or broaden the scope for analysis of specific samples. One method is single cell sampling and analysis (SiCSA) which involves the insertion of a fine microcapillary into living tissue, for the isolation of cellular sap, which is subsequently dried on membranes (e.g. Pioloform) and analysed by XRMA (Malone *et al.*, 1989). SiCSA has a greater sensitivity and permits a more accurate quantification of elements than hydrated or non-hydrated XRMA. As summarized in Table 1, XRMA requires sufficiently high element concentration to distinguish them from background radiation. Furthermore, as the extracted sap contains all cellular components below 0·5–1 μm (tip diameter) it has been used for the simultaneous detection/quantification of carbohydrates, proteins and gene transcripts by RNA amplification and microarray/qPCR (reviewed in Brandt, 2005). As SiCSA does not require the extensive preparation required with

fixation/freezing of laser microdissection, LMD or protoplasting, for cell sorting, SiCSA is both rapid and facilitates repeated measurements on the same individual plant throughout the lifecycle, or to following responses to and recovery from stresses (Fricke *et al.*, 1994*b*; Tomos and Sharrock, 2001). However, although arguably more invasive and suffering from a greater number of potential artefacts, protoplasting and LMD can sample a greater number of cells and therefore provide a better average signal across cell or tissue types (Meyers *et al.*, 2004; Galbraith and Birnbaum, 2006; Kralj *et al.*, 2009).

Techniques for measuring ion activities or transport

A greater number of techniques exist for the profiling, not total element concentrations, but solute or ion activities within tissue, and transport of ions across membranes. Such information is useful in indicating elemental content of individual cells or subcellular compartments or interrogating ionic homeostasis mechanisms. These include the use of indicator molecules, complementation of yeast, electrophysiological measurements of current through native membranes or of proteins expressed in heterologous systems and spectrophotometer-based measurements of transport across isolated membranes (Gilliham, 2007).

Processes with degrees of invasiveness are used to introduce light-emitting or -absorbing indicators such as chemical probes or recombinant biomolecular indicators [e.g. for the measurement of pH 2′,7′-bis-(2-carboxyethyl)-5-(and-6)-carboxyfluorescein (BCECF) vs. green fluorescent protein (GFP) derivative pHluorin]; once present kinetic and spatial information regarding ion distributions in living specimens can be obtained (Plieth, 2001; Fricker *et al.*, 2006). Both luminescent and [single or dual excitation (or emission) wavelength] fluorescent indicators are commonly used. However, use of such indicators to measure ion activities relies on the binding and therefore 'buffering' of the target species so can directly interfere with the intended measurement (Fricker *et al.*, 1999).

Single wavelength excitation chemical-based indicators are available for many ions, including Na^+ (e.g. CoroNa green) and Ca^{2+} (e.g. Ca-green, Fluo-3), but studies using these indicators often fail to include loading controls so data cannot be interpreted quantitatively (Fricker *et al.*, 2006; Oh *et al.*, 2009). Dual-excitation ratiometric indicators (which can be quantitative) are shown in Table 1; however, disadvantages of these indicators still remain, including a poor specificity in targeting the indicator to particular compartments or the inability to discriminate between the target species and interfering ions (Plieth, 2001). Bioluminescent recombinant aequorin can be used to quantify changes in $[Ca]_{cyt}$ within plant tissues in response to various stimuli or following circadian rhythms (Knight, 2000; Welch, 2002; McDowell, 2003; Plieth, 2006; Dodd *et al.*, 2007; Morris *et al.*, 2008). The ability to target recombinant proteins easily to discrete intra- or extra-cellular compartments, membranes or specific cell types along with recent improvements in indicator proteins to detect a whole range of metabolites and ions including H^+, Ca^{2+} and glutamate to sensitivities equivalent to traditional chemical indicators, means these biosensors are a powerful tool for dynamic, sub-cellular ionic quantification at varying concentrations in plants with a capacity for stable transformation (Kiegle *et al.*, 2000; Lalonde *et al.*, 2005). Ion activities can also be measured directly within plant tissues using ion-selective electrodes which have excellent spatial and temporal resolution but are extremely low throughput having only been used successfully by a handful of research groups (Miller and Wells, 2006; see review by Miller *et al.*, 2001).

Radioactive and non-radioactive tracers have been used for over 50 years to measure elemental fluxes or distribution within plant compartments using analogues or tracers (e.g. ^{45}Ca, Sr and ^{90}Sr for Ca; ^{15}N and ^{13}N for N; ^{22}Na for Na) in combination with radiography for plant distribution (Britto *et al.*, 2004; Loveland *et al.*, 2005; Kiser *et al.*, 2008; Kronzucker *et al.*, 2008) or chromatography for flux measurements (MacRobbie, 1971; Sherma and Van Lenten, 1971; Hirsch *et al.*, 1998; Fernie *et al.*, 2005) or SIMS (Metzner *et al.*, 2008). Compartmental analysis by tracer efflux (CATE) has been useful in estimating vacuolar and cytoplasmic pool sizes of elements and fluxes across membranes (Walker and Pitman, 1976; Britto *et al.*, 2006). But this technique has been questioned with regard to solutes with pool sizes that rapidly turnover (such as Ca^{2+}) and also gives different results from techniques that measure ion activity leading to different interpretations of results (for further discussion, see Carden, 2003; Kronzucker *et al.*, 2006).

In summary, compartmentation of elements and transcripts has been detected by multiple techniques, each with their own caveats or limitations. The only way to overcome such problems is by using techniques in combination, or several techniques in parallel (Gilliham, 2007). The following section summarizes data from these many different techniques, drawing together consistent data but highlighting differences that may be due to both technical and biological issues.

CELL-SPECIFIC ELEMENTAL COMPARTMENTATION IS COMMONLY OBSERVED

Organ (leaf and root) and cell-specific vacuolar concentrations of leaf elemental distributions (Ca, K, P, Cd and Na) between the Poales and the majority of eudicot species studied have been summarized in Fig. 1, with a broader dataset presented in Table 2. Generalizations can be drawn which will be expanded on below in case studies, including: (*a*) elements that are transported exclusively in the transpiration stream will accumulate to greater concentrations in highly transpiring organs, e.g. Mn and Ca; (*b*) Ca and P are never co-localized together when soluble; (*c*) many heavy metals are excluded from the shoot by complexing in the root apoplast; (*d*) K^+ is a major osmoticum and compensates for, among others ions, Ca^{2+}, Cd^{2+} and Na^+ accumulation in conditions of deprivation or excess; (*e*) expression profiles of specific genes (particular ion transporters) can be correlated with elemental content.

CALCIUM

Calcium nutrition

To plants, Ca^{2+} has both pivotal structural and essential signalling roles (Hirschi, 2004). Therefore, control of its distribution is of vital importance to physiological function (White and Broadley, 2003; McAinsh and Pittman, 2009).

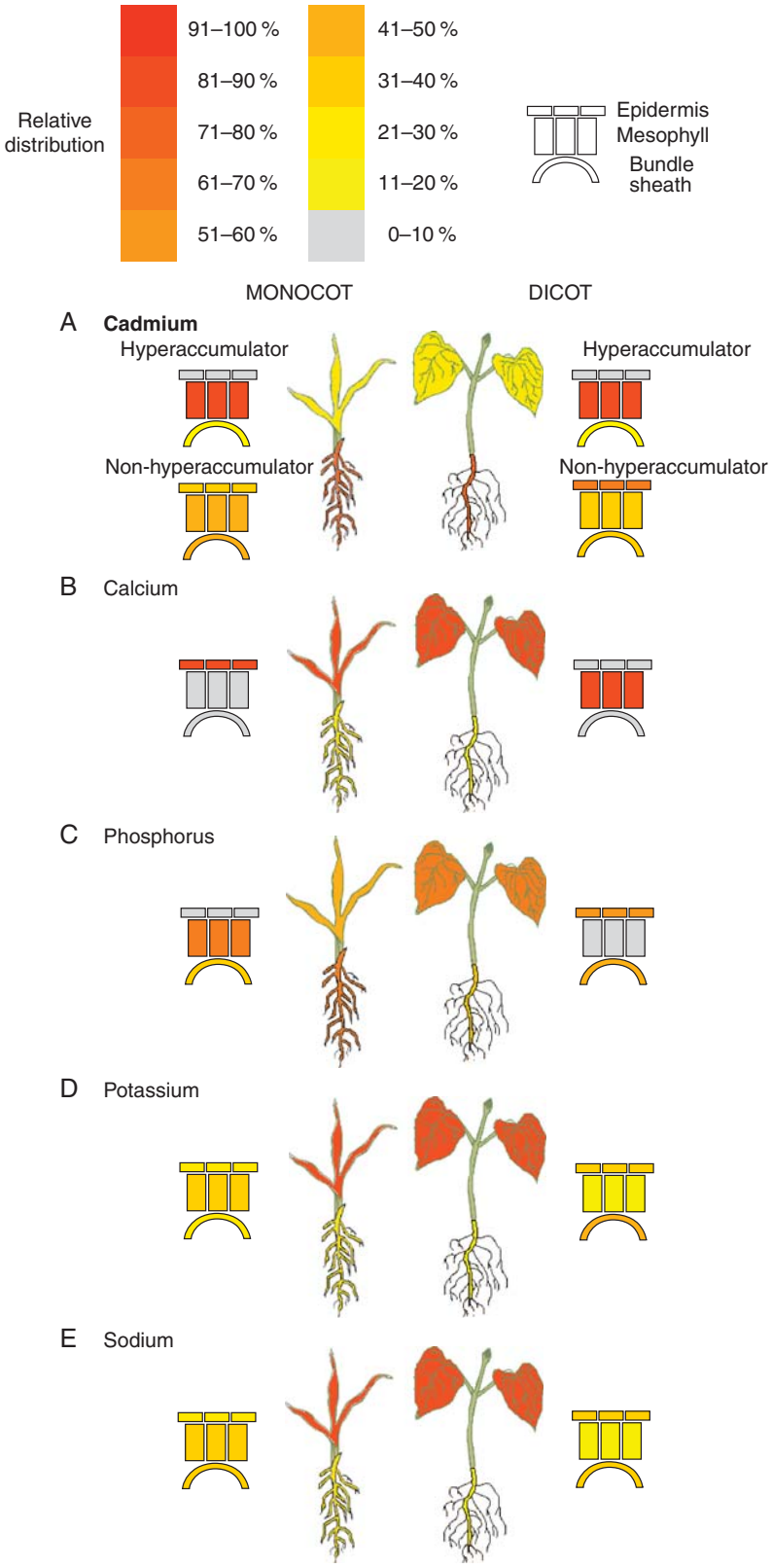

FIG. 1. Relative accumulation profiles of the elements Cd (both hyperaccumulators and non-hyperaccumulators), Ca, Cd, K, P and Na at the organ level and cell-specific levels within the leaf. Model monocot (left side of each figure) and dicot (right) shown with shoot and root concentrations (independent of root/shoot mass) converted into proportion within the plant and then averaged for $n = 2–12$ plant species. Stylized leaf cross-section shown for both monocot and dicot, again represented as a heat map showing relative proportion of [ion] *within* the leaf. Despite prevalence of all ions within trichomes, they are excluded from images due to lack of reporting on trichome prevalence, or dimensions.

TABLE 2. *Ion distribution within plants reveals distinct, cell-specific patterns that contrast between monocot (Poales*) and eudicot† orders*

Element	Ion concentration (mM)			References
	Epidermis	Mesophyll	Bundle sheath	
Poales				
Ca	27·5 ± 5·0 (15–37)	4·0 ± 0·8 (2–6)	3·2 ± 0·5 (2–5)	Malone *et al.*, 1989, 1991; Dietz *et al.*, 1992*b*; Fricke *et al.*, 1994*a*, *b*, *c*, 1996; Brune *et al.*, 1995; Koroleva *et al.*, 2000
Na (2 mM)	31 ± 12·8 (1–51)	18 ± 3·1 (3–29)	17 ± 1·8 (5–19)	
K	211 ± 18·8 (169–249)	258 ± 11·8 (216–299)	185 ± 23·8 (180–225)	
Mg	5 ± 1·5 (2–10)	17·1 ± 4·3 (12–28)	N/A	
S	0·8	2·4	N/A	
P	3·8 ± 1·8 (0–10)	54·3 ± 8·4 (25–75·5)	28	
Cl	106 ± 20 (61·8–160)	26 ± 7 (16–40)	34	
NO_3	161 ± 36 (55–220)	151 ± 46 (47–226)	160	
Mn	High	Undetectable	Undetectable	
Ni (100 μM)	0·0021 ± 0·0006	0·0094 ± 0·0013	N/A	
Cd (100 μM)	0·0303 ± 0·0057	0·0423 ± 0·0106	0·040 ± 0·0077	
Malate	160 ± 31	30 ± 14·1	32 ± 17·2	
Eudicots				
Ca	10·1 ± 8·2 (0–45)	68 ± 14 (15–88)	5·2 ± 2·6 (~2–9·6)	Mesjasz-Przybylowicz *et al.*, 1994, 2001*a*, *b*; Küpper *et al.*, 1999, 2000, 2001; Zhao *et al.*, 2000; Ager *et al.*, 2002, 2003; Bidwell *et al.*, 2004, Epimashko *et al.*, 2004; Fernando *et al.*, 2006; Kerton *et al.*, 2009
Na	15	5	14	
K	106 ± 34 (40–154)	86 ± 25 (10–120)	128 ± 22 (75–180)	
Mg	25	8	N/A	
S	20	30	N/A	
P	60·2 ± 20·9 (20–101)	20·2 ± 8·5 (0–42)	47 ± 15 (20–80)	
Cl	60·6 ± 10·4 (31–82)	11·6 ± 1·4 (5–21)	N/A	
NO_3	n.d.	n.d.	N/A	
Mn	Undetectable	High	Undetectable	
Ni (100 μM)	185 ± 36 (154–213)	59 ± 12 (25–82)	26	
Cd (100 μM)	0·0502 ± 0·010	0·0202 ± 0·008	0·0252 ± 0·009	
Malate	N/A	30·9 ± 2·3 (midday)	N/A	

* Poales includes barley and wheat, with data presented for older leaves, ≥4 d after full expansion.

† Eudicots include tomato, tobacco, arabidopsis, poplar, coriander and *Mesambranthemum* with Ni data presented for the Ni hyperaccumulators *Berkheya coddii*, *Hybanthus floribundus* and *Senecio anomalochrous*.

Data are presented as mean ± standard error ($n = 5–16$ reports for each ion) for all reports under their standard growth conditions (concentration range among all species shown in brackets).

N/A, data was not available.

Plant productivity and tolerance to biotic and abiotic stresses is also linked to plant Ca status; plants deficient in Ca are often more susceptible to pathogens and stresses such as salinity (Bangerth, 1979; Marschner, 1995). Ca deficiencies in humans (e.g. osteoporosis, rickets, eclampsia) and animals (e.g. milk-fever, lambing sickness) are also common and may be reduced by maximizing Ca intake in diets (Welch, 2002; McDowell, 2003; Morris *et al.*, 2008). In populations with staple diets containing only cereals or low dairy intakes, increasing crop Ca bioavailability may be an important part of this strategy (Morris *et al.*, 2008). Therefore, understanding the mechanisms of Ca transport and storage in plants is fundamental to maintaining plant productivity and animal health.

Calcium ions (Ca^{2+}) are carried through the plant mainly through the apoplastic (extracellular) pathway from where they are taken into cells via protein-mediated transport (Marschner, 1995; White and Broadley, 2003). Ca^{2+} is transferred from root-to-shoot in the xylem, with the rate of deposition driven by transpiration rate and soil Ca^{2+} concentration ($[Ca^{2+}]$) (Smith, 1991; White, 2001). Ca^{2+} is ostensibly phloem immobile; once deposited in the shoot it is not readily redistributed (White and Broadley, 2003). Debate surrounds whether the endodermis within the root forms an impermeable barrier to the continuous extracellular movement of Ca^{2+} to the shoot and whether two membranes must be traversed, once prior to the endodermis and once within the stele, before Ca^{2+} can enter the xylem and be delivered to the shoot (Clarkson, 1991; White, 2001; Cholewa and Peterson, 2004). The differences detected in the permeability of the endodermis to Ca^{2+} appear to be species-specific. For instance, in arabidopsis most Ca^{2+} follows an apoplastic-dominated pathway (White, 2001; Hayter and Peterson, 2004; Baxter *et al.*, 2009) whereas in other species, such as onion, suberization of the endodermis requires that most Ca^{2+} transport to the xylem has a symplastic component (Cholewa and Peterson, 2004). It has been proposed that monocots have a tighter control apoplastic loading of Ca^{2+} through the presence of an intact endodermis (Chino, 1981), but there are obvious exceptions to this with rice which has a significant proportion of delivery in the shoot through the apoplast-dominated pathway (Yadav *et al.*, 1996). Regardless, once loaded into the xylem there is a substantial flux of Ca^{2+} from plant roots to shoots carried in the transpiration stream which is then distributed through the apoplast, taken up by cells and deposited in vacuoles for long-term storage (Clarkson, 1984; Leigh, 1997; White and Broadley, 2003; Storey and Leigh, 2004; Karley and White, 2009).

Accumulation of Ca in different organs

The majority of Ca^{2+} taken into the root system is transported to the shoots (Fig. 1). Given the relative phloem immobility of Ca^{2+}, the levels accumulated within lowly transpiring organs, such as fruits or seeds, are very low, generally <1 % of the total plant Ca (Starck *et al.*, 1994; Bermudez *et al.*, 2008). Broadley *et al.* (2003) performed a literature survey on over 200 angiosperm species regarding shoot Ca accumulation, summarizing the variance within and across plant species and orders. It was shown that, while much variance was at the level of the plant orders, a highlight was the divergence between commelinoid monocots, accumulating relatively less shoot calcium than most eudicots. This may be in part due to the lower cation exchange capacity of roots or the greater endodermal barrier to apoplastic loading of Ca (Chino, 1981; Marschner, 1995). However, it may also be related to the lower Ca storage capacity of most commelinoid monocots, as elaborated on later.

Within transpiring leaves, cellular uptake of Ca^{2+} or assimilation into cell wall pectate complexes is crucial to control free Ca^{2+} in the apoplast, $[Ca^{2+}]_{apo}$, which must be kept below approx. 0·05 mM to prevent Ca^{2+}-induced stomatal closure (Smith, 1991; de Silva *et al.*, 1996). This may also be achieved by sequestration into trichomes, which accumulate large quantities of Ca^{2+} (and phosphorous), into crystal-containing idoblasts and/or into Ca oxalate crystals, or by secretion into cells close to guard cells (de Silva *et al.*, 1996; Franceschi and Nakata, 2005; Broadley *et al.*, 2003; Sanders *et al.*, 2002). In contrast, lowly transpiring organs may suffer from Ca deficiencies and this has major horticultural implications in terms of crop spoilage as susceptible plant parts include fruits and leaf tips [e.g. blossom end-rot in tomato (Ho and White, 2005) and leaf burn in lettuce (Barta and Tibbitts, 2000)].

Cell-specific accumulation of Ca

Within cells, concentrations of free Ca ions $[Ca^{2+}]$ are spatially regulated (Sanders *et al.*, 2002; Hetherington and Brownlee, 2004). This appears, in part, due to the presence of particular transporters present on each (sub-) cellular membrane with varying affinities for Ca^{2+}, which can be regulated by a host of transcriptional and post-translational factors. Transport of Ca^{2+} across membranes regulates $[Ca^{2+}]_{cyt}$ between nM and low μM levels temporally during 'resting' and signalling events that can be specific to particular physiological responses (reviewed in McAinsh and Pittman, 2009). Despite its ubiquitous functions, Ca appears to be stored differentially between vacuoles of different cell types, with only certain cell types exceeding $[Ca]_{vac} > 5$ mM.

Root apoplastic $[Ca^{2+}]$ is consistently greater than root vacuolar $[Ca^{2+}]$, XRMA studies of root cell vacuoles from a range of species have found Ca to be below the level of detection [for cryo-scanning electron microscopy (cryo-SEM)/XRMA, i.e. <10 mM] in all root cell types, while other ions were found to be measurably and disproportionately accumulated (Storey *et al.*, 2003*a*). These species include *Arabidopsis thaliana* (M. Gilliham, unpubl. res.), *Thlaspi caerulescens* (Wójcik *et al.*, 2005), *Lactuca sativa* (lettuce) (Lazof and Läuchli, 1991); *Zea mays* (maize) (Chino, 1981), *Phaseolus vulgare* (kidney bean) (Chino, 1981), *Populus alba* (poplar) (Cocozza *et al.*, 2008), *Triticum aestivum* (wheat) (Hodson and Sangster, 1989) and *Hordeum vulgare* (barley) (Huang and Van Steveninck, 1988). Few studies have looked at cell specificity in Ca accumulation along the length of the root. However, one study in poplar showed similar cellular levels in all root cells across a transverse section, yet a longitudinal reduction in total vacuolar Ca from the highest level in the root tip (Cocozza *et al.*, 2008). Meristematic cells of *Vitis vinifera* (grapevine) roots

accumulated Ca in raphide crystals (proposed to be calcium oxalate) in specialized cortical cells which are hypothesized to play a role in regulating Ca delivery to the xylem stream (Storey *et al.*, 2003*b*). In contrast to roots, Ca is frequently observed to be compartmentalized within leaf vacuoles.

In barley, Ca has been found in the leaf epidermis but is below detection limits within the mesophyll (<10 mM; see Table 1 and Fig. 1) (Dietz *et al.*., 1992*b*; Fricke *et al.*., 1994*a*; Karley *et al.*, 2000*a*). Furthermore, different epidermal cells vary in their vacuolar [Ca], with interstomatal cells (those epidermal cells next to stomata) accumulating much greater Ca (>300 mM) than epidermal ridge or trough cells further away from the peristomatal cavity (Fricke *et al.*, 1995, 1996; Fricke, 2004). All commelinoid monocots so far studied, including wheat and *Sorghum bicolor* (millet) (Boursier and Läuchli, 1989; Läuchli and Boursier, 1989; Dietz *et al.*, 1992*b*; Leigh and Storey, 1993; Williams *et al.*, 1993; Hodson and Sangster, 1998; Karley *et al.*, 2000*a*, *b*) accumulate Ca within the epidermis.

In contrast, most eudicots studied, accumulate Ca predominantly in the mesophyll, as observed in *Vicia faba* (broad bean), *Lupinus luteus* (yellow lupin), *Leontodon hispidus*, *Citrus jambhiri* (rough lemon), *Thalapsi precox* and *Coriandrum sativum* (coriander) (Fig. 1) (Outlaw *et al.*, 1984; Treeby *et al.*, 1987; de Silva *et al.*, 1996; Storey *et al.*, 2003*a*; Vogel-Mikuš *et al.*, 2008*a*, *b*; Kerton *et al.*, 2009). Given that mesophyll cells generally contribute >50 % of the total leaf area, and the epidermis <15 % in eudicots and ≤28 % in barley (Pyke *et al.*, 1991; Dietz *et al.*, 1992*a*; Klich, 2000) the distinct patterns of Ca accumulation between commelinoid monocots and eudicots is likely to, at least in part, underpin the lower leaf [Ca] in the monocots (see above; Broadley *et al.*, 2003).

The role, and mechanism, for preferential accumulation of elements is currently unknown but may be, in part, linked to maximizing elemental availability to the plant. For instance, Ca and P are never localized within the same compartment in high concentrations when soluble and bioavailable (Fig. 1 and Table 1) (Hepler and Wayne, 1985; Leigh and Tomos, 1993; Grusak and DellaPenna, 1999; Brinch-Pedersen *et al.*, 2002). Another consequence of cell-preferential vacuolar ion accumulation is that only certain ion combinations contribute to osmotic balance in specific cell types. For instance, vacuolar concentrations of total Ca ($[Ca]_{vac}$) can be enhanced over that reported under standard growth conditions (Table 2), increasing from approx. 20 mM to 100 mM in shoots of K-deficient barley, suggesting a role of Ca^{2+} in charge and/or osmotic balance (Leigh *et al.*, 1986). Ca/K interplay is observed to occur in a cell-specific manner in barley plants grown under low [Ca] conditions, where epidermal K compensates for the loss of Ca (Fricke *et al.*, 1994*b*). Furthermore, an arabidopsis T-DNA insertion line with enhanced root suberization, termed *esb1*, was shown to have a 50 % reduction in shoot Ca, but a 30 % increase in K^+ and a 40 % increase in Na^+ (Baxter *et al.*, 2009). Interestingly, Ca^{2+}-deficiency down-regulates expression of vacuolar-targeted Na^+/H^+ antiporters of the NHX family, including *AtNHX1*, *AtNHX2* and *AtNHX5* in roots (Maathuis *et al.*, 2003). This appears to correlate with the increased Na^+-content of shoots under such conditions which may result from the reduced ability of roots to sequester Na^+ (also see the section entitled 'Cell-specific compartmentation of Na in leaves').

Does differential expression of Ca^{2+}-transporters underpin cell-specific Ca distribution?

Previously it has been suggested that it is the transport routes that Ca^{2+} takes that determines where Ca^{2+} is stored; in cereals, vein extensions from the xylem are proposed to expose the epidermis to Ca^{2+} first, and in most eudicots the xylem unloads to the mesophyll first (Karley *et al.*, 2000*a*). However, if this is the predominant mechanism determining leaf cell-specific accumulation, why is Ca not accumulated within the bundle sheath cells of species that accumulate Ca in the mesophyll? Furthermore, leaves that have been fed unphysiological levels of Sr^{2+} (50 mM), which is a reliable tracer for Ca^{2+}, accumulate Sr^{2+} in the apoplast around all cells but only within vacuoles of cells that normally take up Ca^{2+} (Storey and Leigh, 2004). However, it was also shown in barley that the influx of $^{45}Ca^{2+}$ across the plasma membrane of mesophyll and epidermal protoplasts was equal despite the clear differences in storage capacity of the vacuole (Karley *et al.*, 2000*b*). As a result, it is likely that the different transport characteristics of the epidermal and mesophyll tonoplast control differential Ca accumulation.

There are several gene families reported as capable of transporting Ca^{2+} within plants, including Ca^{2+}-permeable ion channels (GLRs, CNGCs, annexins and TPC), Ca^{2+}-transporting P-type-ATPases (ACA and ECA) and divalent cation-H^+ antiporters (CAX, CCX and CHX) (Maser *et al.*, 2001; Shigaki and Hirschi, 2006; McAinsh and Pittman, 2009). A study by Carter *et al.* (2004), on the tonoplast proteome of arabidopsis mesophyll cells, identified key members of these families. Whilst channels will move Ca^{2+} passively down its electrochemical gradient (into the cytoplasm), only tonoplast-localized transporters capable of catalysing Ca^{2+} uptake against its electrochemical gradient into vacuoles could perform the role of preferential Ca^{2+} accumulation under physiological conditions (Pottosin and Schönknecht, 2007; McAinsh and Pittman, 2009).

'Calcium channels' do not directly control calcium compartmentation within arabidopsis vacuoles

Although non-selective cation channels (NSCC) have frequently been characterized using radioactive tracers and electrophysiology, the molecular identity of NSCCs is not clear (Demidchik and Maathuis, 2007). As related proteins encode ion channels in other kingdoms, likely NSCC candidates include members of both cyclic-nucleotide gated channel-like proteins (CNGCs) and glutamate receptor-like proteins (GLRs) (Demidchik and Maathuis, 2007; Roy *et al.*, 2008). Misexpression of individuals from both gene families have been shown to alter Na^+, K^+, Cs^+ or Ca^{2+} fluxes into and accumulation within tissues, or the sensitivity of plants to Na^+, K^+ or Ca^{2+} (Hampton *et al.*, 2005; Gobert *et al.*, 2006; Ali *et al.*, 2007; Guo *et al.*, 2008).

Most arabidopsis plants so far characterized with misexpression of *AtGLR* have Ca-related phenotypes. For instance, *AtGLR3·3* and *AtGLR3·4* have been implicated Ca^{2+}-entry

into cells gated by selected amino acids, and while the latter is speculated to have a role in abiotic stress signalling both have been implicated in sensing soil N status (Meyerhoff *et al.*, 2005; Stephens *et al.*, 2008). *AtGLR3·1* is important in stomatal guard cell closure mediated by apoplastic Ca^{2+} (Cho *et al.*, 2009). Over-expression of *AtGLR3·2* did not alter total shoot [Ca], but induced symptoms of Ca deficiency (leaf tip necrosis, hypersensitivity to Na^+ and K^+), that could be alleviated by increasing [Ca^{2+}] in the growth medium (Kim *et al.*, 2001). Whereas, antisense *AtGLR1·1* plants were hypersensitive to supplemental Ca^{2+} but not K^+ or Na^+ (Kang and Turano, 2003). Due to its vascular localization, it was proposed that *AtGLR3·2* has a role in xylem unloading of Ca^{2+} (Kim *et al.*, 2001; Turano *et al.*, 2002). However, in general, it is not entirely clear how misexpression of any *AtGLR* can cause Ca-$^{2+}$-sensitive phenotypes. Furthermore, in arabidopsis no T-DNA insertion line for the GLRs have a perturbed Ca^{2+} accumulation phenotype from the public PiiMS database (http://www.ionomicshub.org/home/PiiMS) and there is no clear expression pattern of the 20 members of the GLR family in the leaf epidermis or mesophyll (Ca-poor and Ca-rich cell types, respectively) except that *AtGLR3·7* was present in all cell types (Roy *et al.*, 2008). Whether this was due to stoichastic variation in detection of transcripts (Raser and O'Shea, 2004) or a flexible complementation between family members is unknown. However, with such information it is difficult to delineate the precise role of *AtGLRs* in cell-specific Ca accumulation in arabidopsis.

The channel properties of *AtCNGC*s appear to be a little more secure than those of *AtGLR*s. Heterologous expression of certain members resulted in detection of Na^+-, K^+- and Ca^{2+}-permeable ion-channel activity and *in planta* study of mutants lacking expression of *AtCNGC1*, *AtCNGC2*, *AtCNGC11* and *AtCNGC12* revealed Ca^{2+}-related phenotypes (Hampton *et al.*, 2005; Chin *et al.*, 2009). For instance, *cngc1* mutants show reduced Ca^{2+}-uptake into plants, while the *cngc2* lines are hypersensitive to Ca^{2+} in the growth medium (Chan *et al.*, 2003; Ma *et al.*, 2006) and in standard growth medium are transcriptionally similar to high Ca^{2+}-grown plants (Chan *et al.*, 2008). Mining the PiiMS database, indicated that two independent mutations in *AtCNGC2* showed a 5–15 % increase in shoot [Ca] and a 10 % decrease in shoot [K] compared with the wild-type background. The cell type in which this additional Ca is stored is unknown and needs to be established to see if the poor growth phenotype of these plants is related to defective Ca^{2+}-signalling or -storage (Chan *et al.*, 2008). In summary, the roles for *AtCNGC* and *AtGLR* in cell-specific Ca accumulation are not well established and it is likely, from current evidence, that many of these genes play more of a role in Ca^{2+}-signalling than in Ca storage.

Richardson *et al.* (2007), in the search for epicuticular wax genes compared the transcriptomes of barley leaf epidermis (the Ca-accumulating cell type) against that of the remaining leaf. Mining their dataset, we found almost seven times the number of transcripts for Ca^{2+}-transporters and Ca^{2+}-binding proteins enriched within the Ca-rich epidermis. Among these included homologues of the auto-inhibited Ca-ATPase (ACA) family, the cation exchanger (CAX) family, the annexins and numerous calcium-dependent/calmodulin interacting protein kinases (CDPK/CIPK) and calmodulins. Homologues have also been found to be enriched in the mesophyll (where Ca is accumulated) in the eudicot arabidopsis by transcriptomic (Yang *et al.*, 2008) and proteomic methods (Carter *et al.*, 2004). Those candidates detected in all three studies and reported to be tonoplast targeted will be discussed here.

The role of ACA in cell-type-specific Ca accumulation

There are ten members in the ACA type-P2A Ca^{2+}-ATPase family in arabidopsis. In general, ACA proteins are likely to be high-affinity Ca^{2+} pumps ($K_M = 0{\cdot}4–10$ μM; Hayter and Peterson, 2004), stimulated by calmodulin, and have been implicated in adjusting $[Ca^{2+}]_{cyt}$ within the nanomolar range (Shigaki and Hirschi, 2006). As a result, these have been suggested to be critical for control of the $[Ca]_{cyt}$ signature by returning $[Ca]_{cyt}$ to the pre-signalling level (Maeshima, 2001; Anil *et al.*, 2008). However, only *AtACA4* and *AtACA11* are both leaf and tonoplast specific (Geisler *et al.*, 2000; Baxter *et al.*, 2003; Carter *et al.*, 2004; Lee *et al.*, 2007*a*).

A mutant lacking both *AtACA4* and *AtACA11* was reported to have reduced shoot [Ca^{2+}] and a reduction in transpiration rate compared with parental plants in a wild-type background. Whether the reduction in shoot [Ca^{2+}] is a consequence or cause of the reduced transpiration rate is unknown. In addition, whilst ACA4 and ACA11 have been found in the Ca-rich mesophyll tonoplast of arabidopsis (Carter *et al.*, 2004; Yang *et al.*, 2008), it is not known whether the mesophyll cells of *aca4/aca11* plants are compromised in capacity to load Ca. Numerous hypersensitive response-like lesions were also associated with *aca4/aca11* mutation but could be suppressed by abolishing expression of the *sid2* gene (isochorismate synthase essential for SA biosynthesis), or over-expression of a salicylic acid-degrading enzyme, *NahG* (Boursiac *et al.*, 2007). Such a phenotype in *aca4/aca11* plants hints at a perturbed regulation of $[Ca]_{cyt}$ which is likely to include a delayed return to steady-state $[Ca]_{cyt}$ following any elevation.

The role of cation/H^+ antiporters (CAX) in cell-specific Ca accumulation

CAX proteins include low-affinity (CAX1; $K_M = 10–15$ μM) Ca^{2+}-transporters that use the proton-motive force generated by the vacuolar proton-pumping ATPase and pyrophosphatase to remove Ca^{2+} from the cytosol when elevated significantly above resting concentrations (i.e. after a signalling event or as a result of high Ca influx into the cell) (Hirschi *et al.*, 1996). In arabidopsis there are six CAX genes and four have been functionally characterized to some degree (Shigaki and Hirschi, 2006; Shigaki *et al.*, 2006). CAX proteins have also been implicated with a role in Ca accumulation within the leaf and given the lower expression of *AtCAX1* in roots and localization of the protein in mesophyll tonoplast preparations (Carter *et al.*, 2004); this may contribute to the very low root cell vacuolar [Ca^{2+}] and the higher accumulation of Ca^{2+} (and Mn^{2+}) in the mesophyll (Table 2) (Hirschi, 1999; Hirschi *et al.*, 2000; Cheng *et al.*, 2005).

Furthermore, over-expression of an N-terminally truncated form of *AtCAX1* (called sCAX1) which is constitutively

active in tobacco (Hirschi, 1999) and tomato (Park *et al.*, 2005) was associated with an increased leaf [Ca], although this vacuolar sequestration seemed to be irreversible, isolating Ca away from the apoplast, signalling pathways and pectin cross-linking (Park *et al.*, 2005) as plants also had symptoms of Ca-deficiency. As with the ACA genes, an arabidopsis mutant lacking two CAX family members, CAX1 and CAX3, sharing 77 % protein identity (Maser *et al.*, 2001; Cheng *et al.*, 2003) resulted in a significant reduction in shoot Ca, while the single CAX mutants have insignificant reduction in shoot [Ca^{2+}], but do exhibit other growth phenotypes (Cheng *et al.*, 2003; Zhao *et al.*, 2008). Studies on the cell-specific expression of these Ca^{2+}-transporters in arabidopsis and homologues in other plant species will be required to ascertain if their differential expression or regulation is involved in cell-specific Ca^{2+} compartmentation.

Calcium binding proteins can also affect Ca accumulation in specific cells and may help in biofortification efforts

Calcium-binding proteins (CaBP) such as calreticulin located in the endoplasmic reticulum, help retain Ca^{2+} within the endoplasmic reticulum and buffer $[Ca^{2+}]_{cyt}$ (Akesson *et al.*, 2005). CaBP are also on vacuolar membranes and are thought to reduce vacuolar Ca^{2+} activity (aCa^{2+}) and thereby increase Ca-storage capacity of the vacuole (Yuasa and Maeshima, 2001). Furthermore, while tomato plants overexpressing *CAX1* result in a Ca-deficiency phenotype, this is alleviated and [Ca] is further increased by co-expression of calreticulin. Whether the storage capacity of both the endoplasmic reticulum and vacuole is increased remains to be seen. Further indicating their potential role in buffering Ca^{2+}-availability, CaBP found in arabidopsis have so far only been localized to the plasma membrane and so are not likely to play a significant role in Ca-accumulation (Nagasaki *et al.*, 2008).

The role of signalling and transpiration in Ca accumulation

Although it is likely that the vacuolar Ca^{2+}-transporter complement may influence where Ca is stored in the leaf, the role of transpiration is not disputed in the supply of Ca; when transpiration was inhibited, Ca (or Sr) did not accumulate in leaves (Storey and Leigh, 2004). Lower transpiration was also shown to reduce the accumulation of Ca (Storey and Leigh, 2004; Kerton *et al.*, 2009; Fricke, 2004). Nevertheless, Ca still preferentially accumulates in the same cell types as under higher transpiration. This contrasts with nutrients such as Cl and K that do not require transpiration to accumulate to high levels in tissues, presumably due to the redistribution that occurs via symplast or the phloem (Fricke, 2004; Storey and Leigh, 2004).

We propose the importance of vacuolar Ca transporters in cell-specific accumulation patterns based on evidence from transcriptional profiling and the phenotypes of mutant plants, yet many of these transporters are also post-translationally regulated. However, as with the transporters, it is clear that protein kinases (CDPKs or CIPKs), and their interacting partners – calmodulins or calcineurin B-like proteins (CBLs) – may be expressed in a cell-specific manner (EFP browser; Winter *et al.*, 2007). As they are known to interact with, and regulate the activity of various ion transporters, including ACAs, transcriptomics may still provide a powerful tool in deciphering elements important in determining Ca accumulation (Hwang *et al.*, 2000*a*, *b*; Kim *et al.*, 2007; Luan *et al.*, 2009),

We have shown, and as will be seen in the following section on sodium, that Ca levels in cells can be perturbed under various conditons. The CBL-CIPK and calmodulin–CDPK networks have been implicated in signal transduction, thus providing a medium for bringing about the observed ion perturbations in a cell-specific manner (Luan *et al.*, 2009). While influencing transporter activity, these protein kinase networks may also contribute to wider transcriptional control, with leaf expressed members of the CDPK family known to bind and activate transcription factors (Choi *et al.*, 2005; Milla *et al.*, 2006; Yu *et al.*, 2007).

SODIUM COMPARTMENTATION IS AN IMPORTANT MECHANISM OF SALINTY TOLERANCE

Compartmentation of Na^+ maintains low $[Na^+]_{cyt}$

Sodium is a micronutrient for C_4 and CAM plants, where it plays a role in CO_2 fixation, but is not generally required for the growth of C_3 plants (Brownell and Crossland, 1972, 1974). Some halophytic land plants require high concentrations of Na^+ surrounding their roots (100–200 mM) for optimum growth but to most plants such concentrations would terminally interfere with growth and metabolism (Flowers and Dalmond, 1992; Tester and Davenport, 2003; Shabala and Cuin, 2007). For halophytes and glycophytes alike, compartmentalization of Na^+ away from the apoplasm and cytoplasm is thought to be one of a series of important mechanisms to tolerate high soil Na^+ (Flowers *et al.*, 1977; Flowers and Colmer, 2008; Munns and Tester, 2008). Cytosolic Na^+ is believed to interfere with cellular processes which require K^+ and high apoplastic [Na^+] will reduce turgor and the driving force for leaf growth (Flowers *et al.*, 1991). As Na^+ is carried in the transpiration stream, and there is limited evidence for Na^+ redistribution in the phloem leaves are particularly prone to injury by Na^+ (Munns and Tester, 2008).

Technical limitations have resulted in few measurements of plant tissue aNa_{cyt} and, to our knowledge, leaf aNa_{cyt} has never been measured directly. Debate remains surrounding the reliability of such measures which is not helped by comparison of measurements of total ion concentration ([i]) to activity (*a*i) (Kronzucker *et al.*, 2006). However, there is agreement that when aNa_{cyt} increases beyond 30–40 mM, or $[Na^+]_{cyt}$ beyond 100 mM, cellular function will be impared (even in halophytes) (Hajibagheri *et al.*, 1988; Halperin and Lynch, 2003; Flowers and Colmer, 2008; Munns and Tester, 2008). As such, more salt-tolerant cultivars have often been observed to minimize aNa_{cyt}, and retain greater aK_{cyt}, compared with less-tolerant cultivars (Flowers and Hajibagheri, 2001; Carden, 2003; Kader and Lindberg, 2005; Anil *et al.*, 2007).

Retention of vacuolar K may contribute toward the regulation of cytosolic [K^+] and [Na^+] under saline conditions

(Flowers and Hajibagheri, 2001; Carden, 2003; Läuchli *et al.*, 2008). At high external $a\mathrm{Na}^+$, Ca and K deficiency is induced by reducing membrane $a\mathrm{Ca}^{2+}$ which reduces selectivity of Na^+ transport into cells and reduces K^+ influx (Reid and Smith, 2000). Membrane Ca^{2+} also reduces Na^+-induced K^+ efflux (Shabala *et al.*, 2006). Both of these can be partially ameliorated by adding additional Ca^{2+} to salinized plants (Munns and Tester, 2008). So mechanisms to control compartmentation of K, Ca and Na are all important to salinity tolerance.

Roots compartmentalize Na in a specific cell type to reduce shoot Na load

Cell-specific and whole-organ compartmentation of Na within plant roots has been observed in many species, but variation exists between the cell type that accumulates the highest total Na according to the species or variety, root type, composition of the root bathing medium, the growth conditions and sampling time. Retention of Na within roots has been muted as a key Na tolerance mechanism of glycophytes which enables them to lower their turgor and continue to grow in the face of an increasingly negative soil water potential (Tester and Leigh, 2001; Munns and Tester, 2008). However, in barley and wheat, sensitive varieties have been observed to accumulate more Na in roots than tolerant varieties (Flowers and Hajibagheri, 2001; Läuchli *et al.*, 2008), although in tolerant varieties of barley the amount of root Na was greater in younger plants (Flowers and Hajibagheri, 2001; Carden, 2003).

XRMA studies of salinized arabidopsis, grapevine and wheat roots show interesting parallels (Storey *et al.*, 2003*b*; Läuchli *et al.*, 2008). In 2-week-old durum wheat (and arabidopsis) seedlings exposed to 50 mM NaCl the [Na] in seminal (or primary) roots is relatively high in the epidermal vacuoles and decreases towards the endodermis (Läuchli *et al.*, 2008). The vacuoles of root cortical cells are much like leaf mesophyll cells in that they have the highest volume and hence have the greatest storage capacity of each respective organ. It is hypothesized that the epidermis and cortex 'mops up' apoplastic Na^+ prior to reaching the inner part of the root. In all species and root types (e.g. seminal or lateral), the pericycle appears to play a major role in Na compartmentation in more tolerant varieties. Although xylem [Na] concentrations are likely to be higher in the less-tolerant varieties, the Na content of the xylem parenchyma vacuoles can differ (Läuchli *et al.*, 2008; Møller *et al.*, 2009). In durum wheat, [Na] increased in the pericycle vacuoles in both varieties when exposed to 50 mM NaCl but, in the less-tolerant durum variety, [Na] was also significantly higher in the xylem parenchyma cell vacuoles – possibly reflecting the higher xylem [Na]. This contrasts with the result with more salt-tolerant arabidopsis where xylem parenchyma cells had higher [Na] than the more Na-sensitive arabidopsis genotype (Møller *et al.*, 2009). Whether XRMA is the best technique to estimate ion concentrations in small cells or cells with small vacuoles such as xylem parenchyma cells, or the pericycle, is debatable (refer to 'Techniques for plant elemental profiling' section; Wegner and Raschke, 1994). In addition, positional and developmental effects are likely in different plant species or even across the same organ; therefore sections taken at different distances up the root may yield different results and, when it is not known if the whole root or only part of the root shares the same accumulation profile, [Na] results are difficult to interpret physiologically.

The role of HKT1;5-type genes in reducing shoot [Na] by retrieving Na^+ from the xylem is now well established (reviewed in Munns and Tester, 2008). Both the HKT1;5 homologous proteins in rice [*OsHKT1;5* (*SKC1*)] and arabidopsis (*AtHKT1;1*) are expressed in the stele (Sunarpi *et al.*, 2005). The homologues from bread wheat (*TaHKT1;5D*) and durum wheat line 149 (*TmHKT1;5A*) have also been proposed to share such a localization (Byrt *et al.*, 2007). By over-expressing *AtHKT1;1* selectively within stelar cells, Na^+-influx into such cells was increased over non-HKT1-expressing stelar cells, as was their [Na] content, whilst shoot [Na] was reduced (Møller *et al.*, 2009). Furthermore, highlighting the importance of such a mechanism in plants, transgenic plants over-expressing *AtHKT1* within stelar cells were also more salt tolerant.

In wheat, *Nax1* (*TaHKT1;4*) is the proposed candidate for another mechanism contained within durum wheat line 149 that keeps lower laminar [Na] by retrieving Na^+ into the leaf sheath (and the root) (Byrt *et al.*, 2007). However, although *Nax1* is likely to encode a Na^+-selective transporter, all attempts to clone and functionally characterize *HKT1;4* genes have so far failed.

A number of other genes, which may have a role in the accumulation of Na in particular root cells or in the regulation of Na^+ transport to the shoot, have been implicated from existing datasets (Birnbaum *et al.*, 2003; Dinneny *et al.*, 2008). In arabidopsis, *AtNHX4*, *AtNHX5* and *AtNHX6* all decrease in expression from epidermis to inner cortex, then peak again in the stele at various segments (stages I, II, III as per Birnbaum *et al.*, 2003) along the primary root (Birnbaum *et al.*, 2003; Nawy *et al.*, 2005) and increase in total expression levels along the vertical axis. Both AtNHX1 and AtNHX2, which are tonoplast located, and AtNHX5, which is of unknown membrane location, have all been implicated in Na compartmentation in the root vacuole (Yokoi *et al.*, 2002). However, arabidopsis mutants lacking expression of *NHX4* have recently been shown to increase salt tolerance and this enhanced tolerance was associated with reduced root and shoot [Na] (Li *et al.*, 2009). As such, it would be important to identify the membrane location of AtNHX4. It is possible that pleiotropic effects were also at work masking the true function of the gene (as occurs with many vacuolar Ca transporters, see above). However, little functional compensation has so far been reported between members of the NHX family; when AtNHX1 is knocked out, no other members change in expression (Sottosanto *et al.*, 2007). Interestingly, salt-tolerant varieties of wheat have greater transcript abundance of NHX transporters and the majority of other arabidopsis NHX T-DNA insertion lines have resulted in greater sodium sensitivity (Apse *et al.*, 2003; Saqib *et al.*, 2005). In addition, over-expression of members of the NHX family increases [Na] of most tissues and Na^+-tolerance in other species [*AtNHX1* in cotton (He *et al.*, 2005*a*), brassica (Zhang *et al.*, 2001) and in tomato (Zhang and Blumwald, 2001); *OsNHX1* in ryegrass (Wu *et al.*, 2005); *AtNHX3* in sugarbeet (Liu *et al.*, 2008); and *AtNHX7/SOS1* in arabidopsis (Shi *et al.*, 2003)]. Therefore, it is likely that many of these

genes do contribute to cell-specific accumulation of Na in the root or shoot; however, definitive information is limited on which particular gene or suite of genes is active in each cell type.

In arabidopsis, SOS1 (AtNHX7), an Na^+/H^+ antiporter, is located on the plasma membrane of root-tip meristematic cells and xylem parenchyma cells of the root and shoot (Shi *et al.*, 2002). SOS1 has been demonstrated to catalyse the movement of Na^+ out of cells and by doing so SOS1 has recently been proposed to protect cells of the elongation zone from accumulating Na^+ (Guo *et al.*, 2009; Oh *et al.*, 2009). In addition SOS1 activity is thought to keep the pericycle [Na] low which may be important in reducing symplastic flow toward the xylem (Oh *et al.*, 2009). Higher activity (i.e. not only salt-inducible but also in a cell-specific manner) of SOS1 in *Thellungeiella halophila*, a salt-tolerant relative of arabidopsis, is also thought to be a mechanism that allows the plant to grow in higher salinities than arabidopsis (Oh *et al.*, 2009).

Although *HKT2;1* has been implicated in Na^+ entry into some plants under low K^+ (Laurie *et al.*, 2002), the major entry point for Na^+ into the root cells (and the root symplast) is thought to be through NSCCs on the plasma membrane. Most of these NSCC have been characterized electrophysiologically in the cortical or epidermal cells and so would co-incide with sites of accumulation. However, it is not known whether similar conductances are present in pericycle cells where lower $[Na]_{vac}$ are found. Several *AtGLR*s may encode Na- (and Ca^{2+}-) permeable channels (Roy *et al.*, 2008) and several *AtGNGC*s such as *AtCNGC3* have been shown to be permeable to Na^+ (Demidchik and Maathuis, 2007). Also members of both families have altered expression during salinity (Maathuis, 2006; Maathuis *et al.*, 2003). The entry of Na^+ through NSCC depolarizes the membrane which precludes K^+-influx through inward K channels, such as AKT1, and can lead to substantial K^+-loss from cells through depolarization-activated K^+-channels, such as GORK, in less-tolerant species or varieties contributing to reduced K : Na ratios in cells (Shabala *et al.*, 2006; Chen *et al.*, 2007; Shabala and Cuin, 2007; Cuin *et al.*, 2008). A diagnostic to test for the NSCCs involved in Na^+ entry into cells may be their apparent block by polyamines (Shabala *et al.*, 2007); polyamines appear to reduce Na^+ entry into cells by a dual role of directly blocking NSCCs and increasing activity of H^+-ATPases in the plasma membrane which would increase membrane potentials and hence the driving force for K^+-entry through proteins such as AKT or high-affinity K^+/H^+ exchangers such as HAK/KUP or Na^+ removal through plasma membrane Na^+/H^+ exchangers such as SOS1 (Shabala and Cuin, 2007; Shabala *et al.*, 2007). Interestingly, another mechanism that *T. halophila* employs that results in higher Na^+ tolerance than arabidopsis appears to be a higher selectivity of K^+ over Na^+ in its root cell NSCCs which results in a smaller depolarization upon Na exposure of *T. halophila* allowing continued K^+ uptake, unlike in arabidopsis (Amtmann *et al.*, 2005; Volkov and Amtmann, 2006). Such a mechanism could be conferred by amino acid substitutions or regulatory differences of the same protein or the presence of completely different proteins catalysing Na^+ and K^+ influx.

Cell-specific compartmentation of Na in leaves

In grasses, there are contradictory data in regard to whether specific cell types differentially compartmentalize Na within leaves (Fricke *et al.*, 1994*a*, *b*, 1996; Karley *et al.*, 2000*a*). Depending on the techniques used, preferential accumulation of Na was detected in vacuoles of epidermal cells of barley grown in $[Na]_{ext}$ of <1 mM (using ICP of isolated protoplasts) or ≤ 150 mM Na (SiCSA/XRMA), but no preferential accumulation was observed using cryo-SEM/XMRA at low (25 mM NaCl) or high Na (≥ 200 mM NaCl) (Leigh and Storey, 1993; James *et al.*, 2006). Na appears to be accumulated at the expense of both Ca and K in all cell types of the leaf (Fricke *et al.*, 1996). Although XRMA is not very sensitive to changes in [Na] due to high background Bremsstrahlung radiation it is mystifying why preferential accumulation is reliably detected in roots but not the shoots as [Na] is usually greater in shoots than in roots of barley. It is possible, as the number of studies looking at leaf cell-specific [Na] are relatively few, that differences in growth conditions or genotypes are the explanation for the differences between studies; clearly more work would be needed to confirm the overriding pattern.

The accepted paradigm for many cereal crops (excluding bread wheat which shows some degree of tissue tolerance to Na) is that the severity of salinity (NaCl) stress is proportional to leaf [Na] (Munns and Tester, 2008). However, declines in photosynthetic rates are not necessarily associated with increases in [Na] in leaf mesophyll cells in plants grown at high $[NaCl]_{ext}$ (Fricke *et al.*, 1996; James *et al.*, 2006). Based on measurements of aK^+_{cyt} (Cuin *et al.*, 2003) and calculated $[K^+]_{cyt}$ (James *et al.*, 2006) both papers speculated that redistribution of K^+ from epidermal vacuoles to mesophyll cytoplasm was a more important mechanism in salinity tolerance than excluding Na from the mesophyll *per se*. Cuin *et al.* (2003) further speculated that unlike the mesophyll, the epidermis can maintain viability with a very low aK^+_{cyt}, as epidermal cells are not highly metabolically active, as opposed to mesophyll cells.

In contrast to Na^+, Cl^- does show cell-specific compartmentation in cereals accumulating preferentially within the epidermis (Fricke *et al.*, 1994*a*). Only above 100–150 mM $[NaCl]_{ext}$ does Cl begin to accumulate to appreciable levels within the mesophyll vacuoles and these increases are still only proportional to those within the epidermis. However, no strong or consistent link has been found with accumulation of Cl in the mesophyll with a reduction in photosynthesis and a much stronger correlation holds for Na (Leigh and Storey, 1993; Fricke *et al.*, 1996; James *et al.*, 2006). It appears that mesophyll vacuoles are also able to compartmentalize toxic $[Cl^-]$ away from the cytoplasm when cells start accumulating Cl. Furthermore, the distribution of nitrate (NO_3^-) mimics that of Cl^- and as many nitrate-permeable channels and/or transporters are permeable to both ions it is likely that both ions are secreted into the vacuoles through the same mechanism. The identity of that transporter which is likely to be more active in epidermal cells than the mesophyll and is located on the tonoplast has not yet been definitively determined but is likely to be CLCa (De Angeli *et al.*, 2009). A complicating factor in correlating nitrate transporters

with nitrate accumulation is that the activity of nitrate reductase will also determine nitrate distribution; as nitrate reductase is more active in mesophyll cells compared with the epidermis this may contribute toward the greater epidermal [NO_3^-], but not [Cl], within leaf epidermal cells (Roy *et al.*, 2003). In arabidopsis, both Na and Cl are selectively accumulated in the leaf epidermis under NaCl stress (M. Gilliham, unpubl. res.), opening the way for a more rapid identification of the molecular determinants of both Na and Cl storage in leaves.

CADMIUM COMPARTMENTATION DIFFERS BETWEEN CELL TYPES WITHIN AND BETWEEN HYPERACCUMULATOR AND NON-ACCUMULATOR PLANTS

Cadmium nutrition

The sensitivity of plants to heavy metals depends on an interrelated network of molecular and physiological mechanisms. These include; uptake and accumulation of metals through binding to extracellular exudates and cell wall; complexation of ions inside the cell by organic acids, amino acids, ferritins, phytochelatins (PCs) and metallothioneins; general biochemical stress defence responses such as the induction of antioxidative enzymes and activation or modification of plant metabolism to allow adequate functioning of metabolic pathways; rapid repair of damaged cell structures; and compartmentalization in the vacuole (Verkleij *et al.*, 1990; Rauser, 1999; Hall, 2002; Cho *et al.*, 2003). Cadmium (Cd) translocation and accumulation within the plant is intimately linked with transport through a variety of proteins with greater specificity for ions other than Cd^{2+}, including Ca^{2+}, Zn^{2+}, Mg^{2+}, Mn^{2+}, Pb^{2+}, Cu^{2+} and Ni^{2+} (Broadley *et al.*, 2001; Shigaki *et al.*, 2003; Yang *et al.*, 1996). As a result, it is not surprising that there are correlations in shoot content of Cr, Pb, Cu, Ni and Zn (Broadley *et al.*, 2001), yet the cell-specific accumulation patterns are not universally consistent and, as such, Cd is a good candidate to elaborate on in this review. The terms Cd hyperaccumulator and Cd-tolerant lines will refer to species able to accumulate large amounts of Cd in the shoot and able to grow in Cd-rich environments with limited effects on growth, while non-accumulators and sensitive lines are those that are significantly affected by high levels of Cd.

In plants, cadmium disrupts the homeostasis of essential metals by displacing them from metal-binding proteins and transcription factors (Clemens, 2006). This affects root growth and biomass production by inhibiting photosynthesis, respiration and mineral uptake and by disturbing the plant water status (Perfus-Barbeoch *et al.*, 2002). In addition, genotoxicity and ecotoxicity of food-borne Cd in animals is well-documented, so its accumulation in the edible portion of plants is disadvantageous (Peralta-Videa *et al.*, 2009; Chaney *et al.*, 2000). There is substantial genotypic variation underlying Cd accumulation in leaf and grain in maize (Zhang and Song, 2008), suggesting that genetic factors underlie differences in Cd accumulation. But, the ability to redistribute Cd and/or limit its uptake in agricultural crops to reduce the adverse effects to the plant and to human health are important strategies being adopted for the traditionally higher Cd-accumulating tobacco leaf (Wagner and Yeargan, 1986) and durum wheat grain (McLaughlin *et al.*, 1998*a*; Ozkutlu *et al.*, 2007).

Cadmium is preferentially partitioned in plant roots

A number of reports have demonstrated the preferential accumulation of Cd and other non-essential heavy metals within the root as opposed to the shoot (Puig and Peñarrubia, 2009; Verbruggen *et al.*, 2009). Work in maize and pea grown at a range of [Cd] within the soil demonstrates consistently 12–17 % of the total Cd in the plant is partitioned to the shoot, with the majority retained within the roots (Fig. 1) (Lozano-Rodríguez *et al.*, 1997). As Cd is phloem-mobile (Herren and Feller, 1997), this evidence implicates root-to-shoot translocation as a major hurdle to the accumulation of Cd within the aerial tissues (leaf and grain). This theory is supported by reports in rice correlating Cd allocation in the shoot and grain across a genetic diversity set (69 accessions) with Cd concentration in the xylem (Uraguchi *et al.*, 2009) and identifying associated QTL (quantitative trait loci) (Xue *et al.*, 2009). Given the toxicity of Cd^{2+} to plants and animals, it is critical to determine how Cd partitioning within the leaf is correlated with Cd tolerance and how it can be enhanced to reduce accumulation in the edible portion of plants (Hu *et al.*, 2009; Xue *et al.*, 2009).

Given that Cd does not accumulate to very high intracellular levels in most plants (20–50 μM), often being conjugated in the apoplast, a detailed investigation of methods of Cd tolerance, and optimization of plant-based strategies for phytoremediation/phytoextraction (Ishikawa *et al.*, 2006), has focused research on Cd hyperaccumulators, including *Potentilla griffithii* (Hu *et al.*, 2009), *Arabidopsis halleri* (Küpper *et al.*, 2000), black oat (*Avena strigosa*) (Uraguchi *et al.*, 2009) and *Thlaspi caerulescens* (Lombi *et al.*, 2000; Wójcik *et al.*, 2005; Küpper *et al.*, 2007*a*). Once internalized, it has been shown that the vacuole is the primary site of accumulation of a number of heavy metals including Cd (Ernst *et al.*, 1992; Hall, 2002).

Cadmium is distributed differently between cell types in hyperaccumulator and non-accumulator plant species

Cells accumulating Cd vary widely between and within plants. Within certain ecotypes of *T. caerulescens*, Cd is accumulated in the root apoplast (bound to cell wall components) and vacuoles of cortex parenchyma cells, endodermis, parenchyma cells of the central cylinder and xylem vessel, while accumulation within the leaf is predominantly in the leaf mesophyll vacuole, with less in the epidermis and none found in the apoplast by X-ray microanalysis (Fig. 1) (Wójcik *et al.*, 2005). This may be linked to the probable forms of Cd complexed with PCs, or organic acids, which can be taken into the xylem and then rapidly taken-up by leaf cells – which may contribute to the mesophyll cells (being the initial cell type exposed to the Cd–PC complexes from the xylem) accumulating more than epidermal cells (Hu *et al.*, 2009). *Arabidopsis halleri* accumulates 1500 mg kg^{-1} Cd in the shoot, predominantly in the trichome base (Hokura *et al.*, 2006) and mesophyll cells (Küpper *et al.*, 1999, 2000; Sarret *et al.*, 2002; Küpper and Kroneck, 2005; Ueno *et al.*, 2008).

It was shown recently, by ion-selective electrode measurement, that the majority of Cd in the xylem sap is in the ionic form, but the remaining Cd appears to be complexed in an organic, sulphur-rich form, e.g. Cd–PC complexes (Ueno *et al.*, 2008). We propose that free Cd^{2+} is not rapidly taken up by leaf cells and as a result accumulates at the base of the trichomes (as $CaPO_4$ fills the branches of the trichomes and the upper stem, restricting additional extracellular nutrients into the branch of the trichomes), while complexed Cd is taken up rapidly into the mesophyll. This is supported by evidence in the Cd-sensitive *A. thaliana*, which accumulates fewer PCs than *A. halleri*, where Cd is only found in the trichome base and stem (Lee *et al.*, 2003). Increasing the levels of PCs by over-expression of *AtPCS1* caused an increase in shoot Cd and Cd tolerance within *A. thaliana* (Ager *et al.*, 2002, 2003; Lee *et al.*, 2003).

Using X-ray microanalysis, Wójcik *et al.* (2005) also demonstrated mesophyll accumulation of Cd as electron-dense deposits in *Thlaspi caerulescens* with some localized in the epidermis, in a decreasing manner following the route of water flow. Vogel-Mikuš *et al.* (2008*b*) showed the slightly less-tolerant *Thlaspi praecox* accumulates less Cd in the mesophyll than in the epidermis. This evidence supports a partitioning role of Cd into the mesophyll vacuole to enhance Cd tolerance. The mesophyll cells were also shown to accumulate elevated concentrations of Mg^{2+}, interpreted as a defence against the substitution of Mg^{2+} in chlorophyll by Cd^{2+} (Küpper *et al.*, 2001, 2007*a*).

Thlaspi caerulescens can accumulate 25 000–30 000 mg Zn kg^{-1} dry weight and 2000 mg Cd kg^{-1} dry weight in the shoot without showing any toxicity symptoms or reduction in growth (Brown *et al.*, 1996; Shen *et al.*, 1997; Küpper *et al.*, 1999). The increase in $[Cd^{2+}]$, given it is primarily targeted to the mesophyll, is found to be associated with a proportionate reduction in other cations within the same cell, primarily K^+, and Ca^{2+} in *Thlaspi* (Wójcik *et al.*, 2005) and *A. halleri* (Küpper *et al.*, 2000) by comparison with closely related non-hyperaccumulators and by analysis of response to altered levels of Cd in the growth solution used. It has been shown that when *Trifolium repens* (Wang and Song, 2009) and barley (Elenany, 1995) plants are exposed to higher Ca : Cd ratios, plants show an enhanced tolerance to Cd. Given the differential accumulation profile of Ca and Cd the protective influence of higher [Ca] has been proposed to result from reduced absorption of Cd (McLaughlin *et al.*, 1998*b*; He *et al.*, 2005*b*), ability to form Ca/Cd crystals intracellularly and extracellularly (Choi *et al.*, 2001; Choi and Harada, 2005) and/or out-competition for transport by Ca-permeable channels (Perfus-Barbeoch *et al.*, 2002).

Role of Cd^{2+}-transporters in accumulation

Molecular approaches indicate that transporters for essential metals such as Zn^{2+} and Fe^{2+} can mediate Cd^{2+} uptake by root cells (Korshunova *et al.*, 1999; Nakanishi *et al.*, 2006). Xylem loading of Cd has been shown to be mediated by the P-type ATPase transporter AtHMA4 in *Arabidopsis thaliana* (Verret *et al.*, 2004) and its homologues in the Cd-hyperaccumulator plants *Thlaspi caerulescens* and *Arabidopsis halleri* (Papoyan and Kochian, 2004; Hanikenne *et al.*, 2008). Specifically, the higher expression of *AtHMA4* found in *A. halleri* is attributable to a combination of modified *cis*-regulatory sequences and an increase in copy number, in comparison to the non-accumulating sister species *A. thaliana* (Hanikenne *et al.*, 2008). Physiological experiments also demonstrated the existence of phloem-mediated Cd transport into seeds of various plants (Popelka *et al.*, 1996; Herren and Feller, 1997; Harris and Taylor, 2001; Tanaka *et al.*, 2003).

Cation transporters with broad substrate specificity are the likely entry point of Cd^{2+} into cells (Clemens, 2001). It is surprising therefore that the site of accumulation of Zn and Cd, while similar in the roots of *T. caerulescens*, differs in the shoot (Küpper *et al.*, 1999; Vázquez *et al.*, 1992, 1994). This possibly results from the preferential complexing with PCs prior to transport of Cd, but not Zn, or differences in leaf cell-type expression or specificities of respective transporters (Küpper *et al.*, 1999, 2000). Indeed in *T. caerulescens* fed high levels of Zn, with leaves studied by Synchrotron XAS, Zn (but not Cd) was again found in the epidermis, but not bound to any strong ligands (Küpper *et al.*, 2000, 2004). Adding to the complexity, *A. halleri*, previously known as *Cardaminopsis halleri* L. Hayek, is one species known to hyperaccumulate Cd and Zn within the same leaf cell types (Küpper *et al.*, 2000; Bert *et al.*, 2003).

The majority of proteins identified with affinity for Cd^{2+} and Zn^{2+} transport belong the ZIP (ZR, IRT-like protein), IRT (Korshunova *et al.*, 1999; Clemens, 2001), NRAMP (natural resistance-associated macrophage protein) (Cailliatte *et al.*, 2009; Thomine *et al.*, 2000), MTP HMA (P-type ATPase) families (Guerinot, 2000; Ramesh *et al.*, 2003; Puig and Peñarrubia, 2009). By complementation of a yeast Zn-transport-defective mutant with a *T. caerulescens* cDNA library, Lasat *et al.* (2000) cloned the *ZNT1* cDNA, which encodes a high-affinity Zn transporter. However, ZNT1 can also mediate low-affinity Cd transport (Lasat *et al.*, 2000). Based on the study of two *T. caerulescens* ecotypes, Lombi *et al.* (2000) suggested that Cd may be transported in the low Cd-accumulation ecotype via ZNT1 but, conversely, that Cd may be mediated in the high-accumulation ecotype via a high-affinity Cd transporter. This latter point was further strengthened given that the expression of *ZNT1* in high Cd-accumulating *T. caerulescens* by *in situ* hybridization was lower in the non-accumulating cell types in the leaf (Kupper *et al.*, 2007*b*).

Other proteins found to transport Cd^{2+} include the calcium exchanger (CAX) family in arabidopsis. AtCAX1 and AtCAX2, tonoplast localized calcium/proton antiporters, were first identified in a screen for rescuing a Ca^{2+} transport-deficient *Saccharomyces cerevisiae* line (Hirschi *et al.*, 1996). Over-expression of full-length and N-terminally truncated (constitutively active) versions of *AtCAX1* (Hirschi, 1999); *AtCAX2* (Hirschi *et al.*, 2000); *AtCAX2*, *AtCAX4* and *AtCAX11* (now called CCX5) in tobacco have been shown to increase the rate of Cd^{2+} (and Ca^{2+}) uptake by root tonoplast vesicles (Korenkov *et al.*, 2007). The expression levels of *AtCAX2*, *AtCAX4* and *AtCAX11*, but not *AtCAX1* or *AtCAX3*, are higher in the root than the shoot, and within the root the cell-specific expression levels mimic the Cd accumulation profile (EFP browser; data from Yang *et al.*, 2008). This

evidence suggests that these members of the so-called CAX2-like clade may be responsible for mediating vacuolar sequestration of Cd^{2+} (and other heavy metals), despite only being induced poorly by Cd^{2+} (Shigaki *et al.*, 2006; Edmond *et al.*, 2009). Another two pieces of evidence support the distinction between clades of the CAX family: (1) co-expression of *AtCAX2* and *AtCAX4* in tobacco using two root-selective promoters resulted in increased Cd accumulation in the root and 15–25 % reduced leaf Cd (Korenkov *et al.*, 2009); (2) expression of *AtCAX2* (Hirschi *et al.*, 2000), but not *AtCAX1* (Hirschi, 1999) under the 35S promoter leads to a significant increase in root, but not shoot [Cd]. It would be interesting to measure cell-specific [Cd] and [Zn] in the 35S::*AtCAX2* over-expressing line in the root and shoot, to see if this gene and its homologues in *T. caerulescens* contribute to the differential accumulation of these ions, or to see if they are post-translationally regulated.

A study in barley identifying changes in the tonoplast proteome after exposure to varying levels of Cd has affirmed theories regarding the aforementioned transporter families (Schneider *et al.*, 2009). Schneider *et al.* (2009) found *Cax1a* (implicated in Cd^{2+} transport), a member of the MRP family of ABC transporters (implicated in transport of Cd–PC complexes) and an NRAMP member to be up-regulated in the presence of 20 μM Cd^{2+}. Each of these transporters plays a different putative role in Cd detoxification, with their simultaneous up-regulation suggesting the importance of multiple strategies to enhance cellular tolerance.

Finally, a wheat gene, *LCT1*, was cloned by its ability to rescue a *S. cerevisiae* mutant deficient in K^+ uptake (Schachtman *et al.*, 1997). It was later shown that LCT1 is able to transport not only K^+ and Na^+, but also Ca^{2+} and Cd^{2+}, in yeast (Clemens *et al.*, 1998). Expression of this gene in tobacco resulted in lower shoot and root [Cd^{2+}], higher shoot [Ca^{2+}] and an increased Cd tolerance, but only when plants were grown in the presence of 1 mM Ca (Antosiewicz and Hennig, 2004). Heterologous expression of *LCT1* in the methylotrophic yeast, *Pichia pastoris*, also resulted in reduced endogenous Cd transport activity (Diatloff *et al.*, 2006). Together, LCT1 seems to be linked with Cd tolerance, via a Ca^{2+}-dependent pathway, but, with no known homologues in rice or arabidopsis, transcriptional profiling and protein interaction work will need to be performed in the future to determine the mechanism of reducing endogenous Cd uptake.

FUTURE RESEARCH AND CONCLUDING REMARKS

Within the limited scope of this review, we have found that cell-specific accumulation of ions (Na, Cd, Ca and K) is typically robust within an individual plant. However, the cell type in which ions accumulate may differ between plant species, the contrast between leaf Ca accumulation in the Poales and eudicots being a case in point (Table 2 and Fig. 1). Furthermore, Ca and K (and P) are never localized, when soluble, at high levels within the same leaf cell types, while Cd accumulation within the leaf differs between hyperaccumulators and non-accumulators. Na and Cd predominantly accumulate within roots, but Cd within the apoplast and Na with specific cell types, to prevent translocation to the shoot. When in the shoot, accumulation mechanisms are needed to exclude the toxic ions from the mesophyll cytoplasm.

Means of enhancing the tolerance of plants to heavy metals and toxic cations was outside the scope of this review, yet it is clear that the same protein can transport various ions. As a result, knocking out these genes may reduce accumulation of not just the toxic ion. Therefore, protein engineering strategies may be required to change the selectivity of certain ion transport proteins to alter toxic ion accumulation without perturbing overall critical ion homeostasis.

It is clear that a large body of work exists describing the cell-specific ion accumulation within a variety of plants using a number of different techniques for their quantification. Contemporary efforts to combine knowledge have emerged for the cell-specific transcriptome (EFP browser; Winter *et al.*, 2007), the proteome (Weckwerth *et al.*, 2008), the interactome (Geisler-Lee *et al.*, 2007) and the 'ionome' of various plants (PiiMS database; Baxter *et al.*, 2007). Integration of these 'omes' on a cell-specific basis would permit greater understanding of the control of the ion profile. Furthermore, among this expanse of knowledge may reside the links to enhancing the success of bio-fortification and phytoremediation strategies.

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