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Cell-specific compartmentation of mineral nutrients is an essential mechanism for optimal plant productivity—another role for *TPC1*?

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Vacuoles of different leaf cell-types vary in their capacity to store specific mineral elements. In *Arabidopsis thaliana* potassium (K) accumulates preferentially in epidermal and bundle sheath cells whereas calcium (Ca) and magnesium (Mg) are stored at high concentrations only in mesophyll cells. Accumulation of these elements in a particular vacuole can be reciprocal, i.e., as $[K]_{vac}$ increases $[Ca]_{vac}$ decreases. Mesophyll-specific Ca-storage involves *CAX1* (a Ca^{2+}/H^{+} antiporter transcript) and Mg-storage involves *MRS2-1/MGT2* and *MRS2–5/MGT3* (both Mg^{2+}-transporter transcripts), all of which are preferentially expressed in the mesophyll and encode tonoplast-localized proteins. However, what controls leaf-cell $[K]_{vac}$ is less well understood. *TPC1* encodes the two-pore Ca^{2+} channel protein responsible for the tonoplast-localized SV cation conductance, and is highly expressed in cell-types that do not preferentially accumulate Ca. Here, we evaluate evidence that *TPC1* has a role in maintaining differential K and Ca storage across the leaf, and propose a function for TPC1 in releasing Ca^{2+} from epidermal and bundle sheath cell vacuoles to maintain low $[Ca]_{vac}$. Mesophyll-specific Ca storage is essential to maintain apoplastic free Ca concentration at a level that does not perturb a range of physiological parameters including leaf gas exchange, cell wall extensibility and growth. When plants are grown under serpentine conditions (high Mg/Ca ratio), *MGT2/MRS2-1* and *MGT3/MRS2–5* are required to sequester additional Mg^{2+} in vacuoles to replace Ca^{2+} as an osmoticum to maintain growth. An updated model of Ca^{2+} and Mg^{2+} transport in leaves is presented as a reference for future interrogation of nutritional flows and elemental storage in plant leaves.

Introduction

The storage pool for mineral elements in the vacuoles of different leaf cell-types is compositionally distinct despite a ubiquitous need for all nutrients in each cell, or a constitutive toxicity for all cells when heavy metals and NaCl are accumulated at high concentrations.[1] For instance, it is generally observed that phosphate and calcium (Ca) do not co-localize to high concentrations in the same cell vacuole; if they did it would be expected that a large proportion of both elements would exist as insoluble calcium phosphate.[1] In contrast, magnesium (Mg), potassium (K), chloride and nitrate may share similar cellular locations but can be at very different concentrations in different cells.[1,2] Furthermore, the cellular location of a particular element is robust within an individual plant, but the cell-type that accumulates each element can vary between species.[1-3]

Until recently, the mechanisms and physiological role for element compartmentation in different cell-types was unknown. In two papers published in 2011, single-cell analysis and sampling (SiCSA) was used to reveal the genetic basis underpinning mesophyll-specific Ca and Mg storage in *Arabidopsis thaliana* leaves. In addition, a variety of physiological assays were used to uncover the fundamental importance of cell-specific nutrient compartmentation with respect to plant productivity.[4,5]

Cell-Specific Ca Storage in Leaves

In Conn et al.[4,5] Arabidopsis leaves were observed to preferentially store Ca in vacuoles of palisade and spongy mesophyll cells at concentrations ($[Ca]_{vac}$) over 60 mM, whereas epidermal and bundle sheath cell vacuoles accumulated a [Ca] of less than 10 mM. For Ca^{2+} to be accumulated in the vacuole it first enters the cell across the plasma membrane, presumably through Ca^{2+}-permeable channels. It must then be transported against an electrochemical gradient across the tonoplast because $[Ca^{2+}]_{cyt}$ is normally in the nM range. In an attempt to identify the mechanism underpinning the observed mesophyll cell-preferential compartmentation of Ca, adaxial epidermal and

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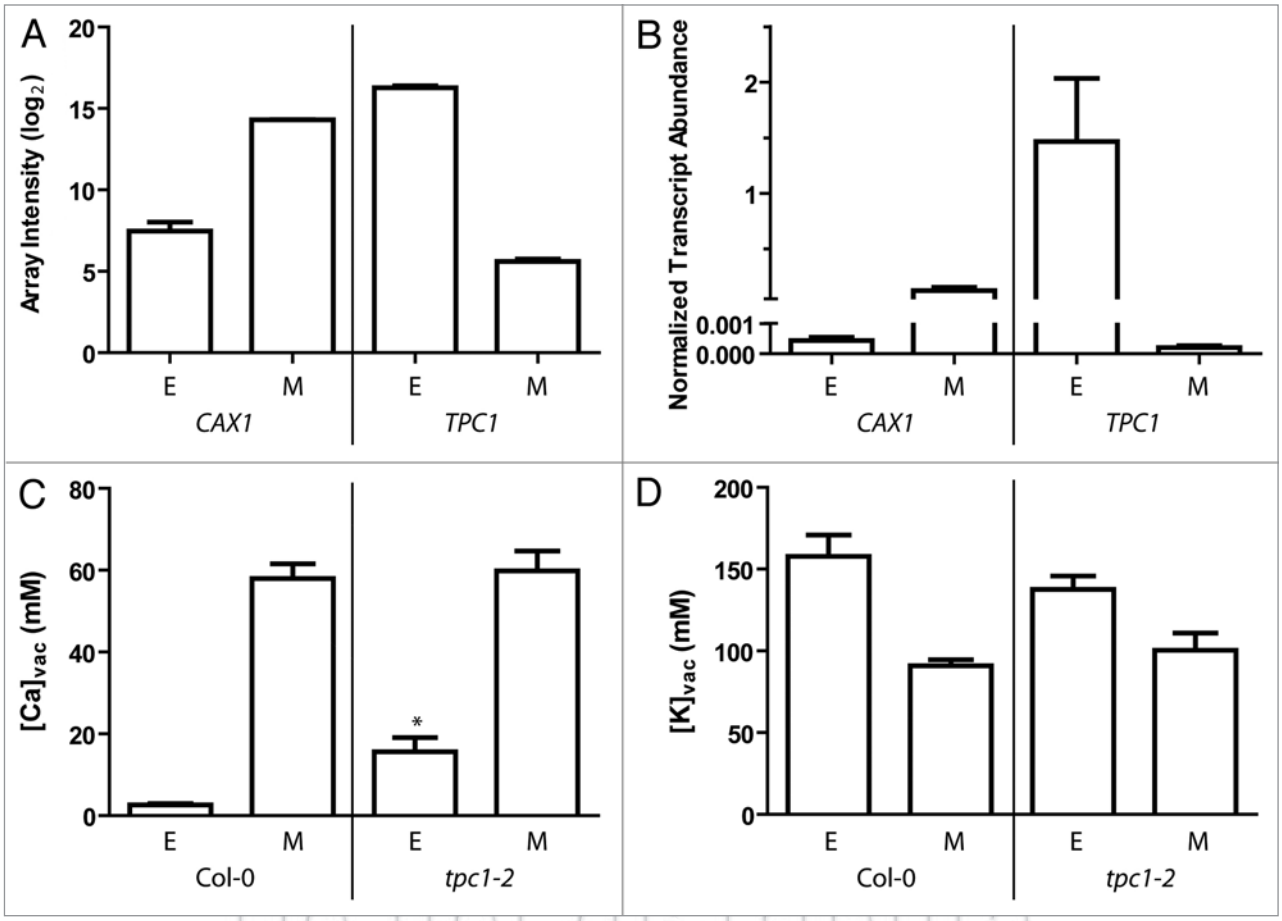

Figure 1. (A) Expression of *TPC1* and *CAX1* in epidermal (E) and mesophyll (M) cells. Sampled using SiCSA and subsequent microarray (mean $\log_2$ array spot intensity + SEM) or (B) qPCR (mean transcript abundance + SEM) normalized against *EF1*-α and β-*Tubulin5*; n = 3 for each cell-type isolated from three different plants. For methods see Conn et al.[4,5] Mean transcript abundance was statistically different between epidermal and mesophyll samples between genotypes for both genes ($p < 0.05$, using a One-way ANOVA and tukey's posthoc test). (C) Vacuolar [Ca] in Col-0 and *tpc2-1*, n = 7 per cell type across three plants. Asterisk indicates a statistically significant larger value $p < 0.01$ in epidermal [Ca] of *tpc1–2* compared with Col-0 using an unpaired t-test with Welch's correction (GraphPad Prism). (D) Vacuolar [K] in Col-0 and *tpc2-1*, n = 7 per cell type across 3 plants. No statistically significant differences were detected between cell-types.

palisade mesophyll cell transcriptomes were compared. Although no known or hypothesized Ca^{2+}-channels were differentially expressed between these cell-types it was revealed that certain Ca^{2+}-transporters were differentially expressed. A vacuolar-localized Ca^{2+}/H^+ exchanger (*CAX1*) was the most highly represented Ca^{2+}-transporter transcript present in the mesophyll and the most differentially expressed between epidermal and mesophyll cells, ~375-fold higher in the mesophyll (**Fig. 1A**).[4] The T-DNA insertional mutant of *CAX1* (*cax1-1*) did not have a $[Ca]_{vac}$ phenotype.[4] However, the T-DNA insertion mutant *cax1-1/cax3-1 (cax1/cax3)*,[6] lacking expression of both *CAX1* and *CAX3*, had 42% lower Ca stored in the palisade mesophyll but a 3-fold higher free apoplastic Ca concentration ($[Ca]_{apo}$).[4] *CAX3* is not normally highly expressed in leaves, however, the *CAX3* transcript encodes a tonoplast-localized Ca^{2+}/H^+-transporter closely related to *CAX1* that is pleiotropically upregulated in *cax1-1* leaves when CAX1 is not present.[4,6]

Despite the reduction in mesophyll $[Ca]_{vac}$ in *cax1/cax3* plants there was still a preferential Ca accumulation in the mesophyll over the epidermis. This was hypothesized to be due to the presence of other Ca^{2+}-transporters located on the tonoplast as there was an observed increase in transcript abundance for genes that encode tonoplast localized proteins (*CAX2*, *CAX4*, *ACA4* and *ACA11*).[4,6] However, in terms of Ca^{2+} accumulation, a principal role for the tonoplast localized Ca^{2+}-ATPases *ACA4* and *ACA11*, which are also highly expressed in the mesophyll, can be ruled out as single and double knockout plants did not have a reduced leaf Ca content.[4,7] Instead, *ACA4* and *ACA11* have been found to have a role in salicylic acid (SA) signaling and systemic acquired response.[7]

In *cax1/cax3* plants, in addition to an increase in transcript abundance for several genes that encode tonoplast localized Ca^{2+}-transporters, there was an increase in abundance of transcripts that encode Ca^{2+}-transporters that are expressed on other membranes.[4] Most notably this included ACA1 and ACA2 localized on plastid membranes and ACA10 on the plasma membrane.[4] This suggests that when the ability to deposit $[Ca^{2+}]_{cyt}$ into the vacuole is compromised, the cell may maintain a low $[Ca^{2+}]_{cyt}$ by

sequestering Ca^{2+} into other compartments including the apoplast. High apoplastic Ca^{2+} concentration ($[Ca^{2+}]_{apo}$) has been implicated in a range of physiological processes such as stomatal closure and cell wall modification,[8,9] therefore these processes were examined in the *cax1/cax3* mutant.[4]

Physiological Processes Regulated by $[Ca^{2+}]_{apo}$

Lower gas exchange and cell wall extensibility, and greater cell wall thickness and demethylesterified pectin content, was found in *cax1/cax3* leaves compared with those from the parental background Columbia-0 (Col-0) when grown under standard nutrient supply. All these parameters were hypothesized to underpin the slower growth rate of *cax1/cax3* compared with Col-0.[4] By growing plants in low Ca solution (LCS; a_{Ca} = 25 μM) compared with basal nutrient solution (BNS; a_{Ca} = 1 mM), free $[Ca]_{apo}$ in the leaf could be equalized between both genotypes. Under such conditions all of the underlying parameters mentioned above and growth were equalized between both genotypes.[4] A preliminary investigation into the transcriptional basis for the modification in cell wall strength elicited by changes in free $[Ca]_{apo}$ was also performed and results were consistent with the increase in growth of *cax1/cax3* when $[Ca]_{apo}$ was reduced. For instance, transcript abundance of genes from the *PECTINMETHYLESTERASE* family (*PMEPCRB*, *PME1* and *PME2*) were higher in *cax1/cax3* leaves than Col-0. Proteins of the PME family are believed to demethylesterify pectin in order for Ca^{2+} to bind, thereby strengthening the wall by crosslinking cellulose microfibrils. Also the transcript abundance of *EXPANSIN* genes (*EXP15* and *EXP16*), believed to be involved in cell wall expansion, and *POLYGALACTURONASE* (*PGA3*) believed to breakdown demethylesterified pectin were lower in *cax1/cax3* leaves than Col-0 in BNS.[4] However, for all the above cell wall-related genes, their expression in *cax1/cax3* plants grown in LCS was similar to Col-0 grown in BNS.[4] Such a result is consistent with the equalization of growth of the two genotypes under these conditions. Therefore, the importance of controlling free $[Ca]_{apo}$, and by extension the role of sequestration of apoplastic Ca^{2+} into mesophyll cell vacuoles, can be seen to be central to optimal leaf productivity.[4]

The Control of Passive Efflux of Ca^{2+} Out of Vacuoles

Despite the differences in Ca accumulation within the different cells of the leaf, all viable cells have an electrochemical gradient for Ca^{2+} across the tonoplast that will favor passive movement of Ca^{2+} out of the vacuole into the cytoplasm, even during Ca^{2+} signaling events when $[Ca^{2+}]_{cyt}$ rises to micromolar values. Therefore this process must be tightly controlled.[10] As such, in addition to a greater presence of *CAX1* it is possible that cells in which high $[Ca]_{vac}$ accumulates there are differences in the activity of Ca^{2+}-permeable channels in the tonoplast compared with cells that do not accumulate high $[Ca]_{vac}$. This may take the form of either differentially regulated tonoplast localized Ca^{2+} permeable channels or a different complement of Ca^{2+}-permeable channels.

Electrophysiological studies have identified at least four Ca^{2+}-permeable conductances in the tonoplast[11] but the SV conductance is the only one encoded by a known gene, *TPC1*.[12,13] TPC1 has been implicated in a number of roles including $[K^+]_{vac}$ homeostasis, Ca accumulation in the vacuole, extracellular Ca^{2+} sensing and stomatal closure, wounding and jasmonic acid signaling.[11-13] However, there is considerable conflicting data that demonstrates, at physiologically relevant tonoplast potentials and ion concentrations, the SV channel/TPC1 can either catalyze transport of alkali earth metals with an ionic radius the same size or smaller than Mg^{2+} (e.g., Ca^{2+}, K^+ and Na^+) or can only transport K^+ and Na^+ across the tonoplast.[11,13] For these ions there are interesting differences in the features of transport, for instance the rectification by luminal Na^+ is such that it can enter the vacuole via the channel, but interacts on the luminal side so that the channel is not able to transport Na^+ out of the vacuole.[14] In the case of Ca^{2+}, despite there being a measurable permeability to Ca^{2+} through the SV channel,[15] luminal Ca^{2+} shifts the voltage range of activation such that the open probability of SV channel opening is very low at physiological tonoplast membrane potentials.[11,13] It has been proposed that Ca^{2+} is prevented from leaking out of the vacuole through the SV channel via this mechanism (as summarized in ref. 13). However, in other reports it has been calculated that the fA range Ca^{2+}-current detected through single SV-channels would result in a physiologically relevant Ca^{2+}-efflux out of the vacuole even at very low open probabilities due to the high abundance of this protein.[11]

Interestingly, we found that *TPC1* was differentially expressed between epidermal and mesophyll cells, being enriched in the epidermis (**Fig. 1A and B**). In fact, *TPC1* was more highly expressed in the epidermis than *CAX1* was in the mesophyll (**Fig. 1A and B**). In addition, the differential transcript abundance between cell types of *TPC1* was >1,000-fold while *CAX1* was only ~375 fold more abundant in the mesophyll compared with the epidermis (**Fig. 1A and B**). This is considerably more than the ~5-fold difference found for *TPC1* between guard cell and mesophyll protoplast preparations.[16,17] As low $[K^+]_{vac}$ and high $[Ca^{2+}]_{vac}$ reduce the open probability of the SV channel/TPC1, and as the transcript abundance of *TPC1* in mesophyll cells is low, the activity of the SV channel in intact mesophyll cells is likely to be relatively low in these conditions.[11,17] However, in epidermal cells where the *TPC1* transcript is highly abundant and $[Ca]_{vac}$ is low, the SV channel is likely to be more active and could contribute to an elemental accumulation phenotype. As a result we quantified the $[K]_{vac}$ and $[Ca]_{vac}$ in epidermal and mesophyll cells of the T-DNA insertion line *tpc1–2* that lacks expression of *TPC1* [12] using SiCSA (as described by Conn et al.[4]).

Reconciling Phenotypes Associated with Altered TPC1 Function

Despite no visible phenotype in *tpc1–2*, epidermal $[Ca]_{vac}$ was higher than in Col-0 wildtype plants whereas no significant difference in $[K]_{vac}$ was found (**Fig. 1C and D**). Previously it was found that *fou2* plants, which harbour a D454N point mutation in *TPC1*, have a significantly lower mesophyll $[K]_{vac}$ and higher $[Ca]_{vac}$.[18] The *fou2* mutation in *TPC1* results in a SV conductance that is insensitive to, and active at, a higher $[Ca^{2+}]_{vac}$ when

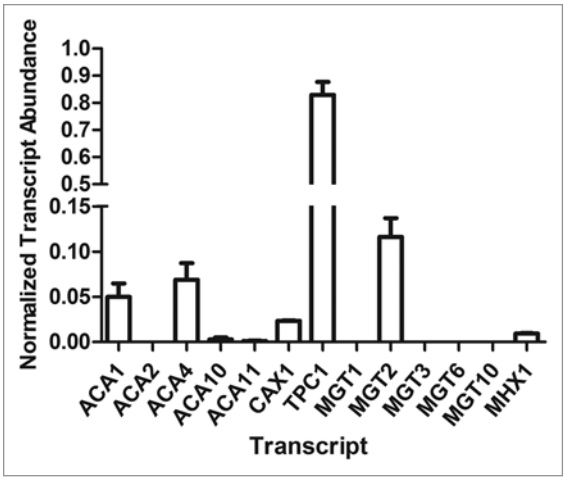

Figure 2. PCR of selected Ca^{2+} and Mg^{2+} transporters in vascular-associated tissue of *Arabidopsis thaliana* ecotype Col-0, prepared by LCM (as described in Conn et al.).[4] qPCR (mean transcript abundance + SEM.) normalized against *EF1-α* and *Actin2*, n = 3 (3 biological replicates with 3 technical replicates per qPCR reaction). For space constraints all Mg^{2+}-transporters have been referred to using their *MGT* nomenclature only: *MGT1/MRS2–10*; *MGT2/MRS2-1*; *MGT3/MRS2–5*; *MGT6/MRS2–4*; *MGT10/MRS2–11*.

compared with wildtype.[18] In an attempt to reconcile these phenotypes we have developed three hypotheses.

The K-shunt hypothesis. On the one hand, the *fou2* phenotype may suggest that the SV channel/TPC1 functions to facilitate accumulation of Ca^{2+}, and one proposal is that the SV channel provides a K^+ shunt conductance so that the V-ATPase proton pump and CAX-mediated Ca^{2+}/H^+ will have higher transport rates.[13] However, this explanation is inconsistent with our observations in that: (1) *TPC1* is expressed predominantly in cells that have low $[Ca]_{vac}$; and (2) *tpc1–2* plants have higher $[Ca]_{vac}$ in epidermal cells, a cell-type in which *TPC1* is normally highly expressed. Unless considerable pleiotropic effects are occurring in *tpc1–2*, these observations require that a different explanation is sought for how TPC1 influences vacuolar Ca^{2+} accumulation consistent with the phenotype of both *fou2* and *tpc1–2*.

The Ca leakage hypothesis. Conceivably, if TPC1 conducts passive movement of Ca^{2+} across the tonoplast, the epidermal preferential expression pattern of *TPC1* could help to sustain lower epidermal $[Ca]_{vac}$ in wildtype plants. This would occur if TPC1 allows leak of Ca^{2+} from the epidermal vacuoles into the cytoplasm down the large electrochemical gradient. This is consistent with the higher epidermal $[Ca]_{vac}$ phenotype we observed in *tpc1–2* plants, since the ability of epidermal vacuoles to lose Ca^{2+} in *tpc1–2* plants may be compromised (**Fig. 1C**). To explain the *fou2* phenotype the relatively high mesophyll $[Ca]_{vac}$ of *fou2* may result from excessive release of Ca^{2+} from epidermis leading to more available apoplastic Ca^{2+} for mesophyll cells to sequester. As *TPC1* is much more highly expressed in the epidermis, the *fou2* mutation will have a relatively larger effect in these cells compared with mesophyll cells. Furthermore, we investigated *TPC1* expression in other leaf cell-types that do not accumulate high $[Ca]_{vac}$ and found that in vascular-associated cells (including the bundle sheath) *TPC1* was highly expressed (**Figs. 1B and 2**). As $[K]_{vac}$ is very high in these cell-types this adds further weight to the "Ca leakage" hypothesis that TPC1 may be involved in preventing the build up of vacuolar Ca^{2+} in both the epidermis and the bundle sheath (**Fig. 2**). To further investigate the feasibility of this hypothesis it would be interesting to measure transcript abundance and activity of *ACA10* and other PM-Ca^{2+}-transporters in vascular-associated cells of *tpc1–2* (see **Figs. 2 and 3**) to see if they are more abundant and upregulated to cope with the release of Ca^{2+} into the cytoplasm. It would also be expected that apoplastic $[Ca^{2+}]$ of *tpc1–2* may be elevated, though perhaps not to the extent that was observed with the *cax1/cax3* phenotype and this may become more evident when high Ca^{2+} is supplied to the plant. A *fou2/cax1/cax3* mutant would be expected to be even more sensitive to high apoplastic $[Ca^{2+}]$ and the *tpc1/cax1/cax3* would be less sensitive than the *cax1/cax3* based on this hypothesis.

The Ca compensation hypothesis. Another explanation for the phenotype in *fou2* also exists. Hedrich and coworkers have presented considerable in vitro evidence that TPC1 cannot conduct Ca^{2+} currents at physiological potentials.[13] In light of this an alternative hypothesis for the above *fou2* phenotype could be the reduced inhibition of TPC1 by $[Ca]_{vac}$ leading to more K^+ release from the vacuole, and a greater build up of $[Ca]_{vac}$ to osmotically compensate.[17] This could only occur if a gradient for K^+ across the tonoplast could be maintained. To this end it would be informative to measure the $[K]_{vac}$ and $[K]_{cyt}$ of epidermal cells in *fou2* plants.

The "Ca compensation" hypothesis and "K-shunt" hypothesis could also be examined using the triple mutants suggested above since CAX1 would presumably be needed for accumulating Ca^{2+} within the vacuole. Although it is difficult to reconcile a K^+-based transport hypothesis with *tpc1–2* mutants, as no $[K]_{vac}$ phenotype was observed using cell-specific XRMA (**Fig. 1D**), it is possible there is an upregulation of other vacuolar K^+-transporters to maintain cellular K^+-homeostasis in these mutants. Regardless, further elemental analysis of both mutants under a variety of nutritional regimes is warranted to resolve these hypotheses.

Cell-Specific Mg Storage

Magnesium is also stored preferentially in mesophyll cells compared with epidermal cells and this is more apparent when plants are grown in LCS or high $[Mg^{2+}]_{ext}$ (HMS, a_{Mg} = 7 mM), or detached leaves are transpirationally-fed high $[Mg^{2+}]$.[5] Interestingly, *cax1/cax3* mutants also had higher mesophyll $[Mg]_{vac}$ than Col-0 plants.[5]

Roles of Cell-specific Mg Storage

Given the high concentration of Ca in the mesophyll vacuole it is likely to contribute significantly to the osmotic potential of the cell. The decrease seen in $[Ca]_{vac}$ in *cax1/cax3*, or for Col-0 in serpentine or low calcium growth conditions, is partially compensated by an increase in both $[K]_{vac}$ and $[Mg]_{vac}$.[5] The increase in $[Mg]_{vac}$ in the mesophyll is dependent upon presence of *MGT2/MRS2-1* and *MGT3/MRS2–5*, which encode vacuolar localized Mg^{2+}-transporters, and an increase in transcript abundance

of these genes under such conditions plays a key role in maintaining growth.[5] Interestingly, leaf cells of T-DNA insertional mutants of either *MGT2/MRS2-1* or *MGT3/MRS2–5* grown in LCS actually increased the osmotic potential of their tissues and this was found to be due to a hyper-accumulation of K in vacuoles and a reduction in growth.[5] Another consequence of knockout of *MGT2/MRS2-1* and *MGT3/MRS2–5* was a greater reduction in chlorophyll content under LCS conditions than in wildtype plants, but interestingly not in BNS. The reason for this is unknown but may be related to the lower $[Mg]_{vac}$ available for remobilisation and chlorophyll synthesis.[5] The significance of this work for Mg accumulation in plants and nutrition was summarized by Waters (2011) [19] in a commentary on the roles of the *MGT/MRS2* gene family in plants.

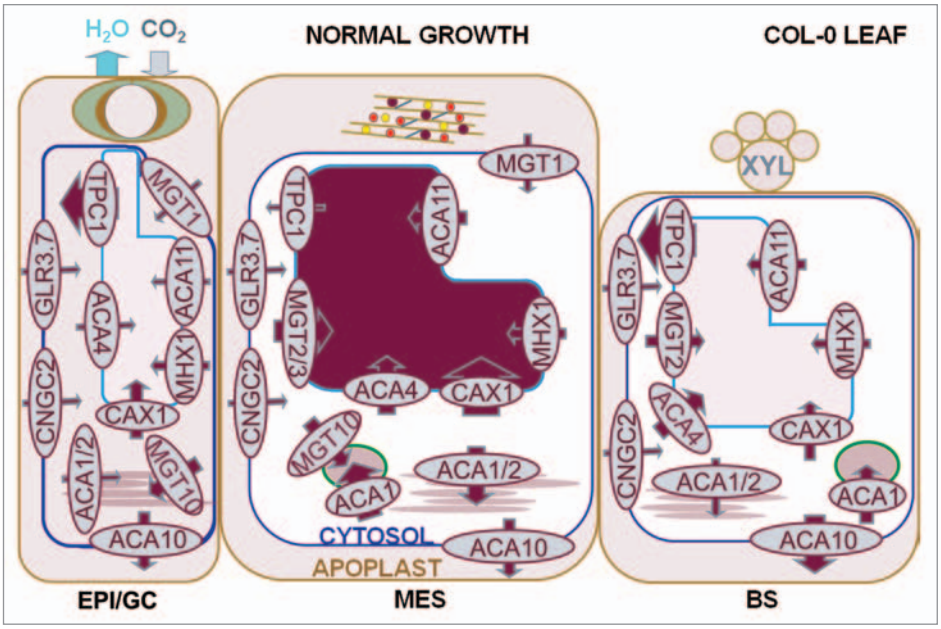

Figure 3. Proposed model for vacuolar control of Ca^{2+}- and Mg^{2+}-content through expression of key transporters. The sequestration of Ca^{2+} into the mesophyll vacuole is important to maintain low $[Ca^{2+}]_{apo}$ in order to maintain optimal gas exchange and growth rates. High $[Ca^{2+}]_{apo}$ will close stomata which reduces transpiration and carbon assimilation. In addition high $[Ca^{2+}]_{apo}$ will bind to demethylesterifed pectin within the cell wall crosslinking cellulose microfibrils and reducing extensibility. A reduction in either parameter could reduce growth. Magnesium is also sequestered selectively into the mesophyll vacuole and is required for maintaining turgor and growth especially when Ca^{2+} supply to the plant is low. (Data combined from Conn et al.[4,5] Figures 1 and 2 and data reviewed in text). Note plasma membrane-localized Ca^{2+}-and Mg^{2+}-permeable channels other than GLR3.7 and CNGC2 have not been included at this stage due to paucity of information. MGT6/MRS2–4 has not been included here but its respective protein is localized to the mitochondria and was detected as more highly expressed in the mesophyll than the epidermis (Conn S, unpublished results). Size of arrow indicates proposed size of flux through transporter, intensity of purple shading indicates concentration of Ca and Mg; transporters with M prefix are proposed to be Mg^{2+}-permeable and other transporters are Ca^{2+}-permeable. Model contains apoplast, chloroplast, cytoplasm, endoplasmic reticulum, vacuole, but not mitochondria, golgi or other major organelles until further information is acquired. Cell wall components indicated in apoplast of mesophyll (brown rods, cellulose; gray lines, rhamnogalacturonan II; purple circles, Ca-pectate; yellow circles polygalacturonase, red circles, expansins; guard cell shown in apoplast of epidermis). EPI, epidermis; GC, guard cells (shown in the top left corner in green); MES, mesophyll; BS, bundle sheath/vasculature. For space constraints, Mg^{2+}-transporters have been referred to using their *MGT* nomenclature only instead of *MRS2*: *MGT1/MRS2–10*; *MGT2/MRS2-1*; *MGT3/MRS2–5*; *MGT10/MRS2–11*.

Expression of Mg and Ca Transporters Within Vascular-Associated Cells

In addition to the mesophyll and epidermal transcriptomes gathered by SiCSA microarray and qPCR,[4,5] we have isolated vascular tissue cDNA (including the bundle sheath cells) of Col-0 *Arabidopsis thaliana* leaves via laser capture microdissection and performed qPCR for specific Ca^{2+}- and Mg^{2+}-transporter genes, as well as *TPC1* (**Fig. 2**). In vascular associated cells *CAX1* is relatively lowly expressed when compared with mesophyll cells and *TPC1* is highly expressed (**Fig. 2**). The negative correlation between cell-type specific *TPC1* abundance and $[Ca]_{vac}$, and the positive correlation between *CAX1* expression and $[Ca]_{vac}$, adds further weight to their respective proposed roles in cell-type specific Ca^{2+} leakage and Ca^{2+} accumulation. The only other two Ca^{2+}-transporter transcripts detected in vascular associated cells were *ACA1* and *ACA4*, albeit at relatively low levels; it was previously noted that these transcripts are widely expressed.[4]

In terms of Mg^{2+} accumulation, the tonoplast-localized Mg^{2+}/H^+ exchanger transcript, *MHX1*, which was equally expressed between all cell-types (**Fig. 2**; reviewed in ref. 5), is known to be inducible by increasing Mg^{2+} supply to plants and increases in expression in different ecotypes of Arabidopsis with an increase in Mg content of leaves.[5] It is likely that it contributes to Mg^{2+} accumulation in all cell-types whereas *MGT2/MRS2-1* and *MGT3/MRS2–5* contribute more toward mesophyll-specific Mg^{2+} accumulation.[5] *MGT10/MRS2–11* was found to be equally abundant in epidermal and mesophyll SiCSA samples but is reported to be predominantly chloroplastic.[5,20] However, Bräutigam and Weber[21] found MGT10/MRS2–11 highly abundant in protoplastids so may be present in other plastid membranes in the epidermis, therefore this appears similar to ACA1 in that it shares both a chloroplastic and unspecified plastid localization.[4]

Model of Ca and Mg Storage in Leaves

A model of Ca^{2+}-transport and storage in the epidermis and mesophyll of Arabidopsis leaves has been presented (see Fig. 6 in ref. 4), here we expand that model to include vascular tissues and also Mg^{2+}-transporters in each of these tissues (**Fig. 3**). This model provides a basis for further interrogation.

In the future, when the identity of additional Ca^{2+}-channels in plasma membranes and Ca^{2+}-permeable proteins of other organelles are better resolved, whole cell-type transport networks could be constructed and be used as the basis of models of cellular homeostasis across the leaf. Both glutamate receptor-like proteins (GLRs) and cyclic-nucleotide gated channels (CNGCs) have been implicated as plasma membrane Ca^{2+}-channels.[1,22] Interestingly, *cngc2* has a similar Ca^{2+}-sensitive growth phenotype to *cax1/cax3* so, as well as its well documented roles in pathogen defense signaling, may be a key entry point of apoplastic Ca^{2+} into cells for plant nutrition.[23,24] Additionally, a recent report has confirmed that GLR3.7 and GLR2.1 act as Ca^{2+}-channels in pollen tubes.[25] The expression of *GLR*s in epidermal and mesophyll cells was previously investigated in Arabidopsis and despite a preference of expression of 5–6 members per cell-type, all members lacked both discernible co-expression partner(s) and cell-specificity, with *GLR3.7* being the only one found in all cells.[26] Therefore, GLR3.7 is likely to form a plasma membrane localized Ca^{2+}-channel in all cell types of the leaf. Our data also shows no difference in *CNGC2* expression between cell-types and this data agrees with early studies that suggest that the Ca^{2+}-conductance properties across the plasma membrane are equal in all leaf cell-types.[2]

Final Remarks

The vacuole plays a key role in co-ordinating fluxes across the plasma membrane and controlling apoplastic solute concentrations, as such this represents a paradigm shift in the understanding of nutritional-related physiology of plants. Why Ca^{2+} and Mg^{2+} should be preferentially sequestered into the mesophyll in Arabidopsis and not other cell-types has not been directly tested, however it may be a consequence of the need to keep certain elements apart.[1] For example, phosphorous (P) is accumulated preferentially in the epidermal cell vacuoles of Arabidopsis. If both Ca^{2+} and inorganic P were sequestered into the same vacuole, CaP precipitate would form making both elements potentially less available to the plant. It is attractive to speculate that the mixed results obtained through attempting Ca biofortification of food crops through constitutive misexpression of transporters are due to the need to segregate certain elements and the mechanisms that control cell-preferential accumulation of solutes.[1,27] Future biofortification studies therefore may benefit from taking cell-specific approaches to improve nutrient content without being deleterious to plant physiology.[27] Water flow across membranes will also be affected by $[Ca^{2+}]_{apo}$ and must also be considered.[28]

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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