Running head: CAX1 and CAX3 in *Arabidopsis* seed

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The role of CAX1 and CAX3 in elemental distribution and abundance in Arabidopsis seed.

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ABSTRACT

The ability to alter nutrient partitioning within plants cells is poorly understood. In Arabidopsis thaliana, a family of endomembrane cation exchangers (CAXs) transports Ca\(^{2+}\) and other cations. However, experiments have not focused on how the distribution or partitioning of Ca and other elements within seeds are altered by perturbed CAX activity. Here we investigate Ca distribution and abundance in Arabidopsis seed from cax1 and cax3 loss of function lines and lines expressing deregulated CAX1 using synchrotron x-ray fluorescence microscopy. We conducted 7 – 10 µm resolution in vivo x-ray microtomography on dry mature seed, and 0.2 µm resolution x-ray microscopy on embryos from lines over-expressing deregulated CAX1 (35S-sCAX1) and cax1cax3 double mutants only. Tomograms showed an increased concentration of Ca in both the seed coat and embryo in cax1, cax3 and cax1cax3 lines compared to wild type. High resolution elemental images in the mutants showed that perturbed CAX activity altered Ca partitioning within cells, reducing Ca partitioning into organelles and/or increasing Ca in the cytosol, and abolishing tissue-level Ca gradients. In comparison with traditional volume-averaged metal analysis, which confirmed subtle changes in seed elemental composition, the collection of spatially-resolved data at varying resolutions provides insight into the impact of altered CAX activity on seed metal distribution and indicates a cell type-specific function of CAX1 and CAX3 in partitioning Ca into organelles. This work highlights a powerful technology for inferring transport function and quantifying nutrient changes.
INTRODUCTION

Transporters play fundamental roles in life, namely, the selective import or efflux of molecules through biological membranes. The vast majority of transporters are secondary, energized by the proton gradient and membrane potential. The plant cation/H+ exchangers (CAXs) are part of the ensemble of transporters that may coordinate the redistribution of various cations including calcium (Ca$^{2+}$) in exchange for the protons generated by H$^{+}$-pumps (Hirschi, 2004; McAinsh and Pittman, 2009). Elucidating CAX, and other transporter functions, is challenging because it is difficult to discern phenotypes among a multitude of transporters with similar putative functions.

The Arabidopsis thaliana genome contains six CAX open reading frames and the transporters predominantly reside on the vacuole (Shigaki et al., 2006; Martinoia et al., 2007). CAX1 is a high-affinity Ca$^{2+}$/H$^{+}$ transporter and is expressed strongly in the leaves (Cheng et al., 2003). The cax1 loss-of-function mutants have subtle phenotypes and microarray studies indicate compensatory expression of other transporters in the mutant (Cheng et al., 2005; Conn et al., 2011). The absence of strong phenotypes prompted the development of dominant gain-of-function CAX variants, namely a deregulated, N-terminally truncated sCAX1 (Diener and Hirschi, 2000). Recently, a role for CAX1 in controlling apoplastic Ca$^{2+}$ concentrations has been delineated (Conn et al., 2011). CAX3 is 77% similar to CAX1 and CAX3 can compensate for loss of CAX1 function (Cheng et al., 2005; Conn et al., 2011). When both CAX1 and CAX3 are perturbed, stunting and leaf chlorosis are observed; however, experimental tools to discern functional differences among these related transporters have not been readily available.

CAX1 and CAX3 are co-expressed only at specific stages during the Arabidopsis life cycle (Zhao et al., 2009; Conn et al., 2011) and may interact to form a transporter with distinct substrate transport characteristics (Cheng et al., 2005). Both genes are expressed during seed development. Figure 1A shows the relative expression levels of CAX1 and CAX3 during seed development using data from the Harada-Goldberg Arabidopsis LCM GeneChip Data Set (on the Seedgenenetwork website: http://estdb.biology.ucla.edu/seed/, GEO accession series GSE12404). These expression data were normalized to the highest expression value set to 1 and visualized using the
Genesis software (Sturn et al., 2002). CAX1 expression peaks during the earlier stages of seed development, whereas CAX3 expression is higher in the later stages. CAX1 is expressed in the chalazal endosperm at the preglobular stage, and CAX3 is expressed in the mature green seed coat. The expression of both CAX1 and CAX3 during seed development and germination provides a compelling rationale for examining the roles these transporters play in seed nutrient partitioning.

Evidence suggests diversity in the substrate range and function of the CAX transporters. In addition to Ca, CAXs may transport cadmium (Cd), manganese (Mn), and Zinc (Zn) (Hirschi et al., 2000; Kamiya and Maeshima, 2004). Studies have primarily focused on CAXs as drivers of Ca$^{2+}$ accumulation in plant tissues and engineering CAX is thought to have great potential in biofortification (Hirschi, 2009); however, how the distribution or partitioning of Ca and other elements in plant tissues are altered by perturbed CAX activity has not been systematically addressed.

The study of metal ion homeostasis in plants can benefit from spatially resolved metal analysis techniques, such as particle induced x-ray emission (PIXE) (Bhatia et al., 2003; Isaure et al., 2006) and nano secondary ion mass spectroscopy (nanoSIMS) (Moore et al., 2010; Smart et al., 2010). Synchrotron x-ray fluorescence (SXRF) microscopy has been used to characterize gene function in plants (Kim et al., 2006). SXRF can be used to show the quantitative elemental characteristics of plant tissues, frequently without sample preparation, on a sub-micron scale. This can be informative for characterizing genes that facilitate metal ion movement in and out of specific cells or organelles, such as those that encode for membrane transport proteins. The simultaneous multi-elemental information in SXRF is particularly useful in less-characterized gene families, where imaging can show tissue- or cell-level elemental localization and thereby focus searches for metal responsive genes expressed in those tissues. Likewise, multi-elemental analysis can reveal unexpected elemental co-associations and a high level of co-association can provide candidate elements for binding partners of metals of interest, which can be investigated using complementary techniques such as x-ray absorption spectroscopy.

SXRF microtomography allows the collection of elemental information from intact samples in the form of virtual cross sections (tomograms), removing the need for physical
sectioning and chemical preservation, and is well suited to small samples such as Arabidopsis seed.

In this study we used two synchrotron microprobes with different spatial resolutions to collect elemental images from seed of lines with altered CAX expression. Images suggested that overexpression of a CAX1 with a N-terminal truncation in the regulatory region (sCAX1), caused a disruption of selective Ca accumulation by cell types; rendering all layers equally Ca-rich, and that deletion of both CAX1 and CAX3 caused a disruption in Ca storage within the cell. The use of two different spatial resolutions (micron and sub-micron) not only demonstrates the capabilities of SXRF for analysis of biological tissue, but also allows for the comparison between sample preparation techniques. It also adds to the developing body of literature on elemental distribution within the plant cell, which finds application in the manipulation of membrane transporters for various purposes such as biofortification and the exclusion of toxic metals from edible plant parts.

RESULTS
Volume averaged analysis via ICP-MS

In order to gain a first approximation of the role of CAX transporters in mineral seed content, bulk analysis of soil-grown seed was performed on lines altered in CAX expression via ICP-MS (Table 1). Notable alterations in elemental composition were disrupted bulk Ca levels; statistically, there were higher Ca concentrations in cax1 and lower Ca in 35s-sCAX1; 22% higher, and 14% lower, respectively than Col-0. Among the micronutrient elements analyzed, Mn showed the greatest changes as a result of CAX gene disruption, mirroring the trend seen in Ca, with higher Mn in cax1 (32% higher than Col-0) and lower Mn in 35s-sCAX1 (14% lower than Col-0). This trend was also observed in bulk concentrations of Cu. Iron and Zn appeared minimally perturbed by CAX gene disruption.

Semi quantitative RT-PCR Gene Expression Analysis

As determined by semi quantitative RT-PCR, the transcript levels of CAX3 in 35S::sCAX1 and cax1-1 are about 0.5 fold and 1 fold, respectively, higher than that of
Columbia wild type (Figure 1B). For CAX1, the transcript was at low levels in all the cDNA samples and was undetectable after 30 cycles of amplification (data not shown). Even after 35 cycles, CAX1 bands were weak (data not shown). At 35 cycles, non-specific amplification also becomes a factor that severely decreases the accuracy the measurement of very low levels of transcripts of interest. Thus, no conclusion was drawn regarding the relative levels of CAX1 transcripts in cax3-1, Columbia wild type and 35S-sCAX1.

SXRF microtomography

We conducted SXRF microtomography to analyze the impact of CAX1 and CAX3 disruption on the spatial distribution of elements within mature seeds (Figure 2). Microtomography of intact seed showed no distributional anomalies at the tissue level, of either macronutrients (K and Ca) or micronutrients (Fe, Mn and Zn), nor was elemental allocation between seed coat and embryo disrupted (Supplemental Figure 1). There were differences in elemental abundances between lines, with higher K, Ca and Zn in cax1 and cax3, and lowest abundances of these elements in 35S-sCAX1.

Volume-averaged seed analysis via ICP-MS (Table 1) and spatially-resolved analysis via SXRF microtomography (Figure 2) agreed on the following trends: K and Ca were highest in cax1-1 and cax3-1, Mn was lowest in 35s-sCAX1 whereas Zn was highest in cax1-1 and cax3-1 and lowest in 35s-sCAX1. However, absolute values between these two datasets differ, and the extent to which K, Ca and Zn differ appears is less pronounced in volume-averaged data. We offer several explanations for this. First, volume-averaged data is the average elemental concentration of a large number of seeds (there are approximately 4000-5000 seeds per 50 mg aliquot), and considers the whole seed volume. This is compared with the elemental composition of a 10 µm-thick slice through a single Arabidopsis seed. In volume-averaged data, averaging over a large number of seeds will reduce between-seed elemental variability, whereas in spatially-resolved data, the inherently low replicate number enhances variability. Additionally, spatially resolved data is presented as maximum pixel abundance, indicating only the upper limit of the data, rather than the average.
For this reason, we have conducted region of interest (ROI) analysis on identified tissue layers to derive their mean elemental abundances. These tissues consisted of seed coat (Table 2a) and embryo (Table 2b), showing macronutrients K and Ca, and the micronutrients Fe, Mn, Cu and Zn. Table 2a shows that Ca abundances in the seed coat of cax1-1, cax3-1 and cax1cax3 are higher that wild type, and lower in 35S-sCAX1. The high standard deviations of Fe and Mn in the embryo are due to their discrete distribution in specific tissue layers, which were analyzed separately (Table 2c). Iron localizes to the endodermal cells (radicle) continuous with the bundle sheath cells (cotyledons) at abundances in the range of 4-17 µg cm⁻². Manganese localizes to the sub-epidermal layer of the abaxial side of the cotyledons, with abundances in the range of 0.6-3.4 µg cm⁻² (Table 2c). High standard deviations (60-70% of the mean) for Mn and Fe enriched cell layers suggest that the spatial resolution of this ROI analysis is not optimal, strongly indicating subcellular heterogeneity, consistent with varying numbers and sizes of vacuole.

**High resolution SXRF mapping of embryo sections**

We conducted higher resolution (sub-micron) spectroscopy to fully investigate the elemental distribution of subcellular compartments. High-resolution SXRF mapping (0.2 µm² beam) of the Ca distribution of whole embryo thick sections of wild type, 35s-sCAX1 and cax1cax3 mutants respectively are shown in Figure 3A, D and G. We chose the double knockout and lines expressing deregulated CAX1 (35s-sCAX1) with the assumption that elemental differences would be more pronounced than in the single mutants (Conn et al., 2011).

Calcium was present in all cells, both enclosed within numerous organelles at higher pixel abundances, and as a component of the cell walls at several orders of magnitude lower abundance (arrow, Figure 3C). The Ca abundance range typically spanned several orders of magnitude; therefore normalized fluorescence is shown on a logarithmic scale. The Ca abundance of the epidermal cell layer of the embryo was lower than the internal cell layers. This distribution was also observed for P (See Supplemental Figure 2). In wild type embryos, Ca abundance in cells of the endodermis (marked on Figure 3B) was comparatively greater than neighboring layers; the pericycle, protoxylem
and protophloem on the interior and cortical cells on the exterior (Figure 3B). The endodermal cells contain storage vacuoles rich in Fe (Roschztarttartz et al., 2009), which corresponded to enrichment of Fe, Ca, Mn and Zn. This tissue-level Ca gradient was less pronounced in 35s-sCAX1 (Figure 3D and E) and absent in cax1cax3 (Figure 3H). Given the Ca enrichment of the endodermis, and the likelihood that endodermal organelles are storage vacuoles, we chose to further analyze this layer for CAX phenotypes.

Looking closely at individual endodermal cells (Figure 3C, F and I), Ca appeared to be highly localized within the lumen of a subcellular organelle in wild type. This is indicated by the increased Ca abundance at the center of the body in comparison with a lower abundance at the margin. This contrasted with lines expressing an activated CAX1 (35s-sCAX1) where Ca was localized at higher abundances at the margin (arrows, Figure 3F). In this line, Ca appeared to be associated with numerous bodies of generally smaller size (Figure 3F) as well as with irregular shaped masses that could indicate its presence outside of the organelle. The cax1cax3 endodermal cell image (Figure 3I) was conducted at an identical analytical resolution (0.5s dwell, 0.15 µm step), and yet distinct organelles within the endodermal cells were difficult to discern. In cax1cax3, elements were localized in regions without a distinct margin, and do not resemble the large bodies seen in Col-0. Figure 3I shows both an endodermal (en) and cortical cell (co), and organelles are clearly visible in the cortical cell (Figure 3I, arrow). This suggests that the diffuse elemental distribution in the endodermis is not attributable to insufficient analytical resolution, (although insufficient fixation of tissue cannot completely be ruled out) because organelles with distinct margins are evident in neighboring cortical cells. This indicates that these elements were not confined within organelles in cax1cax3 endodermal cells in the same manner as wild type cells.

High resolution images were conducted at an energy (10 keV) and a resolution that made it possible to see other elements of biological interest within the cell, such as P, S, Fe and Cu. Given the novelty of subcellular resolution images of the seed, we examined the characteristic elemental distributions in these sections. We found common distributions of elements; namely that certain elements were either located within the lumen of subcellular organelles (e.g. in wild type P, Fe and Ca), outside of organelles (e.g. S) or associated with the cell wall or cell membrane (e.g. Cu and Ca) (Figure 4).
High resolution images were also collected of whole-seed sections to gain an understanding of elemental association with certain tissue types. Recent studies suggest that only certain cells have the ability to accumulate Ca (Conn et al., 2011), but this has not previously been imaged at this resolution in Arabidopsis seed for a range of biologically-relevant elements. Whole-embryo high resolution images of Ca in Col-0, 35s-sCAX1 and cax1cax3 (Figure 3A, D and G) show that Ca is present at maximal abundances (i.e. red on the colorbar) in the majority of cells expressing deregulated CAX1, in comparison with wild type. In the double mutant, it is the cytosol and cell membrane regions that dominate the Ca abundance. Given the changes we observed in Mn bulk and spatially resolved concentrations, we wanted to look at Mn in greater detail. We found in our earlier studies that Mn was strongly localized to a sub-epidermal layer of the cotyledons (Kim et al., 2006), whereas high resolution mapping of cax1cax3 whole embryo sections (Figure 5) showed a shallow Mn gradient. Iron was distributed solely within organelles of the endodermal cells of the radicle and around the vasculature of the cotyledons (Figure 5). Zinc was distributed uniformly throughout the cells of the embryo, strongly co-localizing with Ca.

**DISCUSSION**

1. **Planting the seeds of Synchrotron X-Ray Fluorescence in Plant Science**

Nutrient storage within cells is often compartmentalized, but the mechanisms underpinning this compartmentalization are not fully appreciated (Leegood, 2008; Conn et al., 2011). Ionomic measurements have fostered the concept of ion homeostasis networks (Salt, 2004), but this technology has yet to fully address spatial distribution of macro- and micronutrient elements within the various plant organs. Here we have used two synchrotron x-ray fluorescence microprobes to collect high resolution spatially resolved elemental images of how CAX transporters impact nutrient distribution within seeds.

For technical reasons, we focused our SXRF analysis on seeds. Seed is naturally dehydrated and has an extended stability during analysis and is therefore ideally suited to x-ray imaging studies. Numerous experiments have used transgenic approaches to alter
rice nutrient content (Lee et al., 2009) and SXRF can now be used to analyze the spatial
distribution of nutrients within seeds.

Some of our observations in seeds may not be applicable to other parts of the
plant (Vreugdenhil et al., 2004). For example, in seeds some nutrients may be in a
complex with the P containing compound phytate. Elemental distribution patterns may
vary among tissues and it is likely that the correlations observed here would not be found
in other tissues.

2. A closer look at CAX1 and CAX3

Imaging the cellular and subcellular distribution of Ca and other elements within
seed of CAX1 and CAX3 mutants has shown changes in seed Ca distribution as a result
of CAX gene disruption. On a cellular level, deletion of CAX1 and CAX3 has caused
relative increases in Ca outside organelles (Figure 3H); tentatively, different types of
storage vacuoles, and expression of deregulated CAX1 disrupted the selective Ca-
accumulation abilities of certain cell types, rendering all layers Ca-rich (Figure 3E) and
suggesting CAX1 is only operating in certain cell types. On a tissue level, significant
increases in Ca as a result of CAX1 and CAX3 deletion – observed in both volume
averaged and spatially resolved analytical techniques – are counter-intuitive. It suggests
either an over compensation of subsequently upregulated CAX3 in cax1-1, or the
enhanced expression of other Ca transport systems. Recent studies also suggest that
altering CAX activity can alter shoot to root phosphate signaling (Liu et al., 2011);
however, this does not appear to drastically alter the P levels in the seeds.

The distributional changes observed (increases in Ca outside of organelles) are
consistent with an altered ability for effective Ca storage. Further, high resolution
imaging of individual cells indicates that an overexpression of a deregulated version of
CAX1 and a deletion of cax1cax3 disrupts normal elemental distribution on a cellular
level. Cells expressing the deregulated CAX1 transporter appear replete with more
numerous, smaller organelles (in comparison with wild type cells), which have
comparatively greater Ca abundance at the margin than the lumen. By contrast, images of
a cell in which both CAX1 and CAX3 have been deleted showed no readily discernable
vacuoles (Figure 3I), despite an identical analytical resolution. In these cells, elements
appeared diffusely distributed. Future studies are needed to clarify these observations and
survey other cell layers for evidence of similar mislocalization with greater replication.

Our study demonstrates how physiological changes in the plant can impact the
ccentration and distribution of multiple elements. Although the seed ionome of CAX
mutants has not previously been measured, the vegetative phase shoot ionome of soil-grown cax1, cax3 and cax1cax3 via ICP-MS shows some perturbation of elements in
cax1 and cax3, which corresponded with our measurements in the seed (Cheng et al.,
2005); in particular the increases in Mn and Zn concentration in seeds of cax1, cax3 and
cax1cax3 (Figure 2).

Recent studies have established CAX1 and CAX3 as key regulators of apoplastic
Ca levels (Conn et al., 2011). Calcium moves apoplastically through the transpiration
stream, and is generally present at low levels in seeds (White and Broadley, 2003).
Although there is no active transpiration stream within cells of the mature embryo,
nutrient transfer between maternal and filial tissue is restricted to the apoplast (Patrick
and Offler, 2001), and therefore changes in apoplastic Ca levels of the maternal plant
may be reflected in the mature embryo or seed coat. During seed development the seed
cot is derived from maternal tissues (the integuments of the ovule), and in this study
differences in Ca concentration of the embryo and the seed coat were found in CAX
mutants.

The semi quantitative RT-PCR provides a snapshot of the transcript levels of
CAX1 and CAX3 in mature dry seed. At this stage, the level of CAX1 transcript is low,
which agrees with the Harada-Goldberg Arabidopsis LCM Genechip data set (Figure
1A). At the dry seed stage, CAX3 transcript was more abundant than CAX1 and was
enhanced in both the cax1-1 and 35S-sCAX1 lines. This fits previous reports showing
CAX3 expression is enhanced in cax1-1 lines (Cheng et al., 2003); however, in 35S-
sCAX1 lines, the marginal increase in CAX3 expression was unexpected. Since there is
evidence that CAX1 and CAX3 function as dimers (Zhao et al., 2009), there may be
coupled regulation of gene expression. The mRNA transcript levels reported here may
not reflect the transport activity of CAX1 and CAX3 at this stage of development. CAX
transcripts at earlier developmental periods may influence the transport activity that
mediates the nutrient distribution patterns shown here (Figure 2).
3. The future of SXRF in Plant Sciences

It is clear from this and other studies (Kim et al., 2006; Carey et al., 2010; Moore et al., 2010; Carey et al., 2011) that spatially resolved elemental mapping can be a powerful technique for analyzing transporter phenotypes. While some shared elemental network components can be predicted by binding and transport properties, in practice, living systems are so complex that most relationships cannot be predicted easily from chemical or biological principles and must be determined experimentally (Baxter, 2009). For SXRF to become a widely used experimental platform among plant biologists, certain limitations must be addressed. It remains necessary to stabilize hydrated tissue such as leaves and roots, which require extended analysis times to image micronutrient elements present at very low abundances.

Microtomographic analysis of the low atomic number (Z) elements (the macronutrient elements P, S and Ca) is hindered by self-absorption effects; where the low energy x-rays emitted by these elements are easily absorbed by the air path between the sample and detector, and by the sample itself. In this study we added an additional silicon drift detector at 180° geometry to the existing Ge multi-element array. With this new set up, as soon as self-absorption effects begin to dominate for one detector, the opposite detector is ideally placed to receive fluorescent x-rays from the sample, and count rates are doubled. This geometry effectively eliminates self-absorption effects and enhances detection of low abundance elements. Tomograms shown in Figure 2 were collected using this new configuration.

As SXRF develops under the continued presence of users from the life sciences, there is the potential to use elemental imaging to explore gene × environment interactions by imaging samples with altered gene expression under various environmental stresses. With advances in detector technology, the collection of in vivo data will remove the need for intrusive sample preparation in the majority of instances. However, even with faster detectors it is likely that radiation damage from x-rays will limit the possibility of in vivo imaging. Currently, SXRF remains a static image of a dynamic system, and consideration of time is particularly crucial in elements like Ca, where concentration gradients and
spatial distribution patterns are known to change rapidly in response to stimuli (White and Broadley, 2003).

Evidence has shown that nutrient localization is crucial in normal growth and development (Kim et al., 2006), therefore our working hypothesis is that plant growth and nutrient bioavailability is determined by the correct cellular and subcellular localization of macro- and micronutrient elements. For endeavors such as biofortification, it is necessary also to increase the bioavailable form of the nutrient rather than simply increasing the bulk amount. Our long-term goal is to combine ionomic and imaging approaches to identify the relationships between nutrient distribution and subsequent changes in the chemical forms of nutrients in the plant cell.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

*Arabidopsis thaliana* ecotype Columbia-0 was used in this study. Seeds from wild type and transgenic plants were surface-sterilized, germinated and grown on full strength B5 medium containing 0.5% sucrose and solidified with 0.8% agar. All plates were sealed with paper surgical tape, and incubated at 22°C under continuous cool-fluorescence illumination. Seedlings were transplanted to soil (3:1:1, Pro-Mix: vermiculite: perlite) and grown under a 16h light/8h dark cycle. Plants were frequently watered with nutrient solution (5mM KNO₃, 50 mM KPO₄, 2mM MgSO₄, 2mM Ca(NO₃)₂, 50 mM FeEDTA, 70 µM H₂BO₃, 14 µM MnCl₂, 0.5 mM CuSO₄, 1 µM ZnSO₄, 0.2 mM NaMoO₄, 10 mM NaCl, 0.001 mM CoCl₂).

Volume-averaged elemental analysis via inductively coupled plasma mass spectroscopy (ICP-MS)

cax1-1, cax3-1, cax1cax3 35S-sCAX1 and wild type plants were grown as described above, in the same growth chamber and harvested on the same day. Three replicates of approximately 50 mg aliquots of dry seed were digested in 2 ml Optima HNO₃ in Teflon vessels using a MARS5 EXPRESS microwave-assisted reaction system (CEM, Mathews, NC). A standard reference material (NIST 1573a, Tomato Leaves) and
a HNO₃ blank were included after every fifth sample. Sample volume was brought to 10 ml with DI water. The vessels were heated to 180°C in 10 minutes and held at that temperature for a further 10 minutes. After the samples had cooled, they were brought up to approximately 15 ml volume with DI water. Samples were analyzed for trace element concentrations using an Agilent 7500cx ICP-MS operating in collision mode at the Trace Element Analysis Core facility of Dartmouth College.

**Semi quantitative RT-PCR Gene Expression Analysis**

*CAX1* and *CAX3* transcripts levels were measured in the mature dry seeds of *cax1-1*, *cax3-1*, Columbia wild type and 35S::sCAX1. Total RNA was extracted from about 50 mg of seeds for each sample with Spectrum™ Plant Total RNA kit (Sigma-Aldrich, St. Louis, MO). First strand cDNA was synthesized from 1µg of RNA with Superscript® First-Strand Synthesis system for RT-PCR kit (Invitrogen, Carlsbad, CA) with oligo (dT)₁₂₋₁₈. The cDNA samples were then diluted to the equivalent of 20ng RNA per µl. PCR was performed with the following thermal program: 94 °C for 5 min to denature DNA; and then in each cycle, 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 60 s; 72 °C for a 7 min final extension. *UBQ10* was used as the internal reference gene. The number of cycles that was used in PCR for *UBQ10*, *CAX1*, and *CAX3* were 21, 30 and 35, respectively. These cycle numbers were determined to be within the exponential phase of PCRs for these 3 genes for the mature dry seed cDNA samples. For *CAX1*, forward primer was 5’- TTCCGGCCATTTCCTGCG -3’, and reverse primer was 5’-CACCACGGTTCTTGCTCC -3’. For *CAX3*, forward primer was 5’- ACAACTACGGTGCTCCGTT -3’, and reverse primer was 5’-GCGACATTTTGTAAATCATAGAGGTCG -3’. For *UBQ10*, forward primer was 5’-GATCTTTTGCCGAAAAACAATTGGAG -3’, and reverse primer was 5’-CGACTTTGTCATTAGAAGAAGAGATAACAG -3’. All amplicon sizes are around 500bp. DNA gel was imaged by the the Biorad (Hercules, CA) Gel Doc XR⁺ system with exposure time of 2 s. Relative densitometry of the the bands are analyzed with the Biorad Image Lab software.
SXRF microspectroscopy of intact seed

Tomograms were collected at the bending magnet beamline X26A at the National Synchrotron Light Source, Brookhaven National Laboratory (Upton, NY). X-ray fluorescence measurements were conducted using a 12 keV monochromatic x-ray beam. Due to spectral overlap with the abundant macronutrient K, fluorescence data could not be collected for Cd at the 12 keV excitation energy. Monochromatic x-rays were tuned using a Si (111) channel-cut monochromator, and focused to a beam size of 5 × 8 µm using Rh-coated, silicon Kirkpatrick-Baez microfocusing mirrors. Incident beam energy was monitored using an ion chamber upstream of the focusing optics. X-ray fluorescence spectra were collected with a Vortex-EX silicon-drift detector (SII Nanotechnology) with an active area of 50 mm². X-ray transmission through the sample was recorded simultaneously using a p-type, intrinsic, n-type (PIN) photodiode.

Individual mature, dry and unsectioned seeds were attached to a 100 µm diameter silica fiber using Devcon® 5-minute epoxy resin, with the micropyle uppermost. The fiber was inserted in to a Huber 1001 goniometer, mounted on a xyzθ stage and centered. Tomograms were collected from the mid-point of the seed. During fluorescence microtomography the seed samples were translated horizontally through the focused x-ray microbeam in step sizes ranging from 5-7 µm, and then rotated at intervals of between 0.8-1.1° angular steps and repeating the translation through a total of 180°. Full energy dispersive spectra were collected at each pixel with a dwell time of 2 seconds per pixel. Two dimensional sinograms (plot of intensity against θ) were computationally reconstructed using fast Fourier transform based Gridrec software developed by Brookhaven National Laboratory (Dowd et al., 1999), which is controlled by the Interactive Data Language (IDL) programming software (Research Systems, Inc.) to provide images of the cross-sectional internal metal distribution.

Abundance quantification and post-processing of SXRF tomography data

Elemental abundances (% weight fraction) were calculated for the fluorescence measurements, adapted from description by McNear et al (2005). Briefly, a thin-film standard reference material (SRM 1833) was measured prior to the collection of each data set to establish elemental sensitivities (counts per second per µg cm⁻²) for Fe. We
used an assumed object density of 1.2 g cm\(^{-3}\) for *Arabidopsis* seed, a measured voxel size of \(3.887 \times 10^{-8}\) cm\(^3\) (reconstructed pixel area \(\times\) beam height) and the average Fe response from the sample to calculate the Fe content of a whole tomogram. This Fe abundance was used as a fixed value for input into the NRLXRF program (Naval Research Laboratory X-ray Fluorescence) (Criss, 1977) from which abundances for potassium (K), Ca, Mn, iron (Fe), nickel (Ni), copper (Cu) and zinc (Zn) were calculated. The concentration precision is typically ±15% and ±10% (1σ) for individual and mean values respectively.

Following quantification, region-of-interest (ROI) analysis was carried out to investigate differences in elemental abundance and concentration of specific tissues. This was conducted using the freehand ROI capability of the beamline-specific imaging software (muplot_alpha4 running in the Interactive Data Language Virtual Machine, © ITTVIS). Separation of the seed from the background or air surrounding the sample prior to any data enquires was conducted as standard. ROI analysis was used to calculate nutrient allocation within the various tissues, expressed as the percentage of the total in the tomogram allocated to the seed coat or embryo. Concentration was expressed in ng \(\mu\)m\(^2\), using seed weights determined for each line (weight of 100 seeds/100, measured for each line).

### Preparation of samples for high resolution 2D SXRF raster scanning

The beam line set-up at the Advanced Photon Source 2-ID-D (Argonne National Laboratory, Argonne, IL), in particular the helium enclosure around the sample stage and detector, required that seed be sectioned for analysis, which necessitated a resin-embedding sample preparation step. *Arabidopsis* seed was imbibed on moist filter paper for two days to allow removal of the seed coat and release the embryo. This was carried out to ensure optimal infiltration of resin in to the embryo cells. Embryos were placed in fixative solution (a mixture of 3% glutaraldehyde and 4% paraformaldehyde in 0.4 M sodium cacodylate) under a gentle vacuum overnight. Embryos were rinsed in a solution of 0.2 M sodium cacodylate and 2.5 mM CaCl\(_2\), pH 7.2 followed by distilled water. Embryos were then dehydrated in an ethanol series (30, 50, 70, 95 and 100% for 30 minutes each) with the final step repeated three times over the course of one hour. Embryo samples were then immersed in three changes of 100% ethanol for ten minutes
each, followed by a LR White resin: ethanol mixtures of 1:3, 1:2 and 1:1 (twice) for one hour each, after which they were stored at 4°C in 1:1 LR White resin: ethanol overnight. Samples were warmed to room temperature and moved to a 2:1 LR White:ethanol solution over four hours, before immersing in two changes of 100% LR White solution for one hour each. Samples were stored at 4°C overnight, and then warmed to room temperature the following day before immersing in three changes of 100% LR White resin over a 4 hour period. Embryos were transferred to flat embedding moulds using a toothpick to achieve the correct orientation, before polymerizing for 24 hours.

Embedding moulds were constructed following the methods described in Palmieri and Kiss (Palmieri and Kiss, 2005). Custom molds were created to provide flat embedding chambers that exclude oxygen from contacting the embedding medium. We chose 0.254 mm (0.01@) and 0.381 mm (0.015@) polycarbonate films (McMaster-Carr, Cleveland, OH) because both satisfied our size requirements, were easy to work with, were low cost and withstood exposure to LR White. These polycarbonate film was cut using a scalpel to fit standard 25 3 75 mm² glass microscope slides. The slides were pre-cleaned with 100% ethanol and wiped with lint free Kim wipes. To facilitate specimen removal, the slide was treated with an anti-stick agent (Fluoroglide spray, EMS, Hatfield PA) prior to adhering the gasket to the slide. This was applied three times and then polished to remove lubricity. Silicone adhesive (EIS, Cincinnati, OH) was then added to one side of the gasket and pressed on to the slide so an airtight seal was formed. Silicone adhesive was cured at room temperature for 24h or in a 60°C oven for one hour and allowed to cool before use.

LR White and embryo specimens were added until the chamber was slightly over filled and the liquid formed at convex surface, and then an Aclar strip (Ted Pella) was cut to a slightly larger width than the slide and placed on top of the chamber to shield the LR White from oxygen. One end of the Aclar strip was placed on to the edge of the chamber and the rest the rest rolled down on to the resin so that any excess resin spilled over the side and prevented air bubbled from being trapped underneath. The specimens were polymerized at 60°C for 24 hours. After polymerization, the Aclar was removed and specimens were excised with a razor blade while the slide was still warm. Initial samples
were cut to between 1-5 µm thick with a microtome and a glass knife and allowed to adhere to silicone nitride windows

High Resolution SXRF microscopy

Scanning x-ray fluorescence microscopy was performed at Beamline 2-ID-D of the Advanced Photon Source at the Argonne National Laboratory (Cai et al., 2000). Incident x-rays of 10 keV were chosen to excite elements from P to Zn. A Fresnel zone plate focused the x-ray beam to a spot size of 0.2 × 0.2 µm on the sample, which was raster scanned (Yun et al., 1999) at resolutions of 1.25 µm step in the whole seed images, 0.5 µm step in whole-endodermis layer images and 0.15µm step in the single cell images, and dwell times ranging from 0.5-1 second per pixel. X-ray fluorescence from the sample was captured with an energy dispersive silicon drift detector. Data was collected over a number of scheduled beam time experiments, using samples that were prepared and sectioned separately and are therefore expressed as normalized fluorescence counts, and expressed on individual scales.

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Figure legends

Figure 1. A: Expression of CAX1 and CAX3 genes during seed development (Harada-Goldberg Arabidopsis LCM GeneChip Data Set, Seedgenenetwork website, http://estdb.biology.ucla.edu/seed/, GEO accession series GSE12404). These expression data were normalized to the highest expression value set to 1.0 and visualized using the Genesis software (Sturn et al., 2002). A color scale is used to represent variations in transcript abundance for each gene, in which red represents the highest expression and yellow the lowest expression. B: Semi quantitative RT-PCR measurement of CAX3 transcript level in the mature dry seeds of cax1-1, Columbia wild type (Col-0) and 35S-sCAX1. Top: Semi quantitative RT-PCR gel image. Bottom: Relative expression level to UBQ10 as determined by relative densitometry analysis.

Figure 2. Quantified SXRF tomograms of Arabidopsis seed, WT and cax1-1, cax3-1, cax1cax3 and transgenic line 35S-sCAX1 collected with a 7 × 10 µm beam. Tomograms for each element are scaled to the highest maximum pixel abundance, shown on the colorbar, expressed as moles, the minim is zero for all elements.

Figure 3. High-resolution SXRF elemental maps of Ca in WT, 35s-sCAX1 and cax1cax3 embryo sections. Data is expressed as normalized fluorescence on a logarithmic scale, with each image individually scaled. A, D and G: Whole embryo sections (0.2 µm beam, 0.3 µm step) oriented with the hypocotyl on the left and the cotyledons to the right. Boxes indicate the position from which higher resolution maps were collected. B, E and H: Endodermal layer of the hypocotyl. C, F and I: Single endodermal cell of the hypocotyl showing subcellular localization of Ca in vacuoles.

Figure 4. High resolution SXRF elemental maps showing normalized fluorescence of P, S, Ca, Fe, Cu, Zn and macronutrient and micronutrient overlay distribution in the endodermal cells of the embryonic hypocotyl in wild type and cax1cax3 lines.
Figure 5. High resolution SXRF maps of micronutrient distribution within whole Arabidopsis seed, showing Mn, Fe, Zn and overlays in wild type, 35s-sCAX1 overexpressor and cax1cax3 lines. Images are individually scaled.
Table 1. Mean (± standard deviation) elemental concentrations of *Arabidopsis thaliana* seed grown under standard soil conditions (µg g⁻¹ d.wt), where n=8-16. Pairwise comparison of means used Tukey-Kramer HSD statistical test. Means not connected by the same letter are significantly different (P<0.05).

| Genotype      | K    | Ca   | Mg   | Mn   | Fe   | Zn   | Cu   |
|---------------|------|------|------|------|------|------|------|
| Columbia-0    | 11,024±962 | 3,593±242 | 3,097±168 | 48.67±4.1 | 46.8±2.8 | 51.1±0.66 | 6.4±0.6 |
| cax1-1        | 11,241±571 | 4,393±516 | 3,124±98 | 63.82±4.5 | 41.5±1.2 | 59.9±4.44 | 8.8±0.5 |
| cax3-1        | 12,081±481 | 3,601±68 | 3,238±111 | 46.83±5.9 | 44.3±1.1 | 59.7±1.81 | 7.6±0.3 |
| cax1cax3      | 11,860±331 | 3,647±410 | 2,980±12 | 51.1±2.2 | 48.7±3.3 | 58.4±1.19 | 8.4±0.22 |
| 35s-sCAX1     | 10,592±316 | 3,088±193 | 2,741±91 | 41.7±1.0 | 51.9±4.2 | 48.7±0.75 | 6.1±0.3 |
Table 2a. Elemental concentration of the seed coat of Arabidopsis seed from *in vivo* SXRF microtomography analysis, as defined by Region of Interest analysis. Values are means and standard deviations of each user-defined region, expressed as µg cm⁻².

| Genotype       | K    | Ca   | Mn   | Fe   | Zn   | Cu   |
|----------------|------|------|------|------|------|------|
| Columbia-0     | 99.29 (±16.86) | 70.00 (±33.05) | 0.54 (±0.37) | 1.42 (±1.09) | 1.12 (±0.70) | 0.88 (±0.55) |
| cax1-1         | 205.15 (±75.17) | 137.79 (±54.61) | 0.53 (±0.32) | 1.76 (±1.07) | 1.61 (±0.86) | 0.93 (±0.37) |
| cax3-1         | 301.93 (±110.91) | 129.29 (±55.67) | 1.03 (±0.62) | 1.60 (±1.18) | 1.83 (±1.08) | 1.04 (±0.44) |
| cax1cax3       | 100.79 (±37.29) | 81.98 (±36.05) | 0.48 (±0.39) | 0.72 (±0.99) | 1.08 (±0.67) | 0.61 (±0.30) |
| 35s-sCAX1      | 160.49 (±73.82) | 46.00 (±22.07) | 0.34 (±0.28) | 0.45 (±0.54) | 1.19 (±0.82) | 0.34 (±1.15) |
Table 2b. Elemental concentration of the embryos of Arabidopsis seed from *in vivo* SXRF microtomography analysis, as defined by Region of Interest analysis. Values are means and standard deviations of each user-defined region, expressed as µg cm⁻².

| Genotype | K    | Ca    | Mn    | Fe    | Zn    | Cu    |
|----------|------|-------|-------|-------|-------|-------|
| Columbia-0 | 52.92 (±13.74) | 36.09 (±11.76) | 0.61 (±0.83) | 3.85 (±6.42) | 2.56 (±0.67) | 0.51 (±0.20) |
| cax1-1    | 104.12 (±38.07) | 85.88 (±25.43) | 0.46 (±0.46) | 3.03 (±4.02) | 3.93 (±1.05) | 0.68 (±0.24) |
| cax3-1    | 127.08 (±43.14) | 55.05 (±19.68) | 1.04 (±1.39) | 3.81 (±6.12) | 3.89 (±0.92) | 0.69 (±0.28) |
| cax1cax3  | 50.09 (±20.33) | 37.88 (±14.44) | 0.52 (±0.74) | 1.82 (±2.79) | 2.35 (±0.56) | 0.53 (±0.16) |
| 35s-sCAX1 | 69.25 (±35.60) | 34.13 (±13.11) | 0.30 (±0.30) | 1.01 (±1.67) | 1.97 (±0.71) | 0.23 (±0.49) |
Table 2c. Abundance of Fe in the endodermis-bundle sheath tissue layer, and Mn in the abaxial sub-epidermis of Arabidopsis seed from *in vivo* SXRF microtomography analysis, as defined by Region of Interest analysis. Values are means and standard deviations of a user-defined region, expressed as µg cm⁻².

| Genotype      | Mn   | Radicle | Fe   | Cotyledon |
|---------------|------|---------|------|-----------|
| Columbia-0    | 1.8  | 16.7    | 14.3 |           |
|               | (±1.3)| (±28.7) | (±24.6)|          |
| cax1-1        | 1.1  | 10.4    | 8.9  |           |
|               | (±0.7)| (±19.2) | (±16.4)|          |
| cax3-1        | 3.4  | 17.1    | 12.5 |           |
|               | (±2.0)| (±29.8) | (±21.8)|          |
| cax1cax3      | 1.8  | 8.5     | 6.3  |           |
|               | (±1.2)| (±11.6) | (±8.6)|          |
| 35s-sCAX1     | 0.6  | 4.8     | 4.1  |           |
|               | (±0.4)| (±8.1)  | (±7.1)|          |
A: Expression of CAX1 and CAX3 genes during seed development (Harada-Goldberg Arabidopsis LCM GeneChip Data Set, Seedgenenetwork website, http://estdb.biology.ucla.edu/seed/, GEO accession series GSE12404). Data normalized to the highest expression value, set to 1.0 and visualized using the Genesis software (Sturn et al., 2002). Color scale to represents variations in transcript abundance: red represents the highest expression and yellow the lowest.

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Figure 3. High-resolution SXRF elemental maps of Ca in WT, 35s-sCAX1 and cax1cax3 embryo sections. Data is expressed as normalized fluorescence on a logarithmic scale, with each image individually scaled. **A, D and G:** Whole embryo sections oriented with the hypocotyl on the left and the cotyledons to the right. Boxes indicate the position from which higher resolution maps were collected. **B, E and H:** Endodermal layer of the hypocotyl. **C, F and I:** Single endodermal cell of the hypocotyl showing subcellular localization of Ca in vacuoles.
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