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Project Title: From the Soil to the Seed: Metal Transport in Arabidopsis

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Executive Summary

Deficiencies of micronutrients such as Fe, Mn, and Zn commonly limit plant growth and crop yields. The long-term goals of our program are to understand how plants acquire metal micronutrients from the soil and distribute them while protecting themselves from the potential redox damage metals can cause to living tissues. Metals serve as important co-factors for photosynthesis and respiration, yet we still know very little about metal transport. Our approach combines experimental and computational tools from the physical sciences with biochemistry and molecular biology. Specifically, we combine mutant analysis with synchrotron X-ray fluorescence (SXRF) spectroscopy, a technique that allows us to image the elemental composition of living plant material in 3-D. By analyzing the phenotypes of lines carrying mutations in various metal transporters, we have identified the genes responsible for uptake of zinc from the soil as well as genes involved in loading the seeds with metal micronutrients. Several of these transporters affect the localization of metals in the seed without affecting the overall metal content. Understanding how seeds obtain and store nutrients is key to developing crops with higher agronomic and nutritional value.

Summary of project activities including a comparison of the actual accomplishments with the goals and objectives of the project

Synchrotron Imaging. Because so much of our work is informed by use of the National Synchrotron Light Sources, we begin with a general summary of our synchrotron imaging program and then briefly summarize progress on our original aims.

We had 'Super-User' status at beam line X26A of the National Synchrotron Light Source (NSLS), allowing us to contribute to end-station upgrades that directly impacted the quality of our data, and made major methodological advances for all other beamline users. Specifically, we added a second silicon drift detector at beamline X26A, which eliminated self-absorption for low atomic number elements, enhanced detection of the low-abundance micronutrient and contaminant elements (such as Fe and As), and facilitated the switch to flyscanning mode. We plan to continue our collaboration at the newly commissioned NSLS II.

Co-PI Punshon has been a first-experiment user at two newly commissioned beam lines: the hard X-ray microprobe at 13-ID-E of the Advanced Photon Source (APS, Argonne National Laboratory, Argonne, IL), now starting its third year of operation, and the sub-micron resolution X-ray Spectroscopy (SRX) beamline of the National Synchrotron Light Source-II (NSLS-II), which begins accepting users this year. The 13-ID-E beamline has a spot size smaller than 1 μm, and allows us to continue imaging plants without the risk of sample preparation artifacts. The improvement in sensitivity by a factor of 100 over bending magnet beamlines (e.g. X26A), translates to massively reduced analysis times, which is essential for analyzing fragile hydrated tissues such as young roots. At 13-ID-E, preliminary data from our group was used to develop superior reconstruction algorithms for use in microtomography [see (Gursoy et al., 2015)].

Our use of SXRF imaging has resulted in bi-directional technology transfer between the synchrotron and life sciences communities. We are producing the 'Atlas of Arabidopsis'; a searchable database of elemental images we have collected since 2005 from Arabidopsis transgenic lines and natural accessions, including roots, leaves and siliques as well as dry seed. When complete, the database will allow researchers to search a collection of over 2000 scalable elemental images by line name or metal. Translating the benefits of synchrotron-based imaging to the life sciences, we organized a workshop at the 2012 National Synchrotron Light Source's Annual User's Meeting entitled "Applications of Synchrotron Techniques in Plant Biology." This day-long workshop brought together researchers using a variety of synchrotron based techniques. Co-PI Punshon also presented 'Using SXRF in ionomics' at the 2012 Structural Molecular Biology Summer School (July 16-20, 2012) at SSRL. This course included two days of lectures covering basic theory, experimental considerations and applications of synchrotron X-ray techniques. Co-PI Punshon was invited to speak at a session at the 96th Canadian Chemistry Conference to speak in a session on "Metallomics - Metal Speciation within Living Cells," again speaking to how our work interfaces with the Physical Sciences. Co-PI Punshon was an invited plenary speaker at the 6th Synchrotron Environmental Sciences Conference held at the Advanced Photon Source (September 11-12, 2014), to describe the gene characterization approaches that have developed from the combination of SXRF and molecular genetics in this project [see (Punshon et al., 2013)]. Finally, Co-PI Punshon now serves as a member of the Proposal Review Panel of the Advanced Photon Source and as an academic spokesperson for the National User

Facilities Organization.

We have a number of exciting collaborations that while not explicit specific aims of this grant, represent important contributions to studies of the distribution of Ca in Arabidopsis seed (Punshon et al., 2012), arsenic in rice seed (Carey et al., 2011a; Carey et al., 2011b) and iron in the *opt3* mutant of Arabidopsis (Zhai et al., 2014). We have also written several reviews highlighting the use of synchrotron imaging (Punshon et al., 2009; Donner et al., 2011; Punshon et al., 2013).

Our grant had 5 specific aims. Progress on each is summarized below.

Specific aim #1. Test the hypothesis that metals stored in the embryo are quickly mobilized and re-distributed for use by the developing seedling.
We knew from our previous analysis of the *vit1* mutant (Kim et al., 2006) and from analysis of the *nramp3 nramp4* double mutant (Lanquar et al., 2005) that vacuolar Fe stores are essential for seedling development under Fe limiting conditions. We had imaged Fe in mature seed but encountered technical issues imaging tissues with higher water content. We spent a significant amount of time during the funding period developing synchrotron methods for analyzing living tissues including leaves (Figure 1) and roots (Figure 2) and then moved on to image developing seedlings.

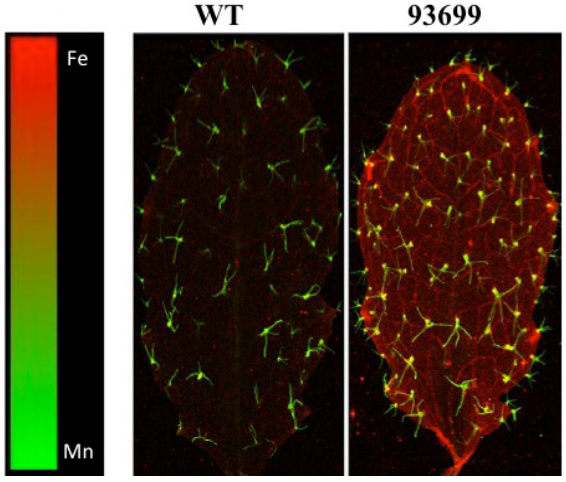


Figure 1. Images collected at SSRL of a wild type leaf compared to a mutant (93699) that is clearly accumulating Fe. Note the accumulation of Mn in the trichomes.

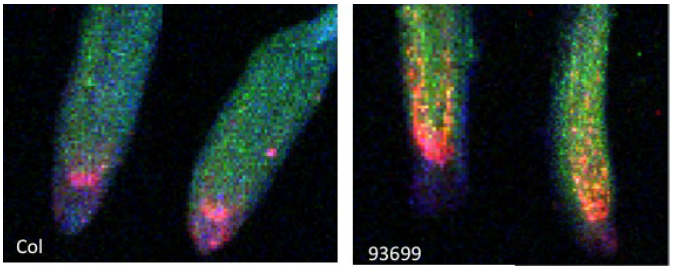


Figure 2. Images collected at SSRL of roots from wild type Col and a mutant (93699) that accumulates Fe. Note the accumulation of Fe in the root meristematic region. Here Fe is in red, Zn is green and Mn is in blue.

We were particularly interested in Mn which is stored in a particular set of mesophyll cells in the embryonic cotyledons but is quickly re-distributed in young cotyledons. We were able to identify the time window for Mn remobilization to the period between 48 and 72 hrs after germination (Figure 3). As we have previously documented, in a mature seed Mn is visible at the periphery of the embryonic cotyledons (specifically the sub-epidermal layer). At 72 hrs after germination, the Mn has remobilized and is localized throughout the entire cotyledon whereas the Fe is still

associated with the cells surrounding the vasculature. We are now focusing on the time period between 48 and 72 hrs to uncover which transporters are involved in the re-mobilization of Mn.

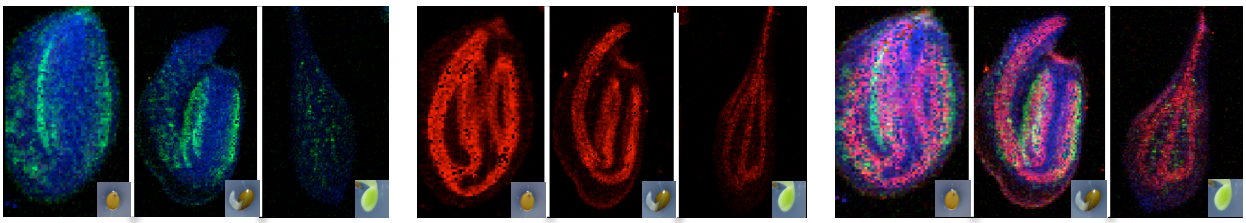


Figure 3. Time series of germinating seedlings. Each set of three images shows a dry seed, a seedling 48 hrs after imbibing where the radicle has emerged and a young cotyledon 72 hrs after germination. Insets depict the stages of germination that correspond to the SXRF images. Mn is in green, Zn is in blue and Fe is in red. Left: Mn and Zn, middle, Fe and right, composite image.

Specific aim #2. Test the hypothesis that VIT1 plays a role in Mn homeostasis.
In order to explore the role of VIT1 in Mn homeostasis, we took several approaches. First, we generated lines that overexpress *VIT1*. Although the loss of function mutant of *VIT1* did not show any changes in Mn concentration relative to wild type, overexpression of VIT1 leads to increased levels of Mn in the leaves as well as the seed. Most interestingly, although Fe also accumulated in leaves of plants ectopically expressing VIT1, seeds had lower concentrations of Fe than wild type. Not surprisingly, 35S-VIT1 seedlings grow less well than wild type on Fe limiting soil. The increase in leaf Fe is accompanied by increased Fe(III) chelate activity and increased levels of IRT1 protein, the high affinity Fe uptake components in Arabidopsis roots. The loss of *VIT1* attenuates the induction of IRT1 protein, resulting in delayed responses to high metal treatment. Taken together, these results suggest that the pool of Fe that is being sensed has been altered and that plants are responding accordingly. The simplest hypothesis is that in a *35S-VIT1* cell, there is less Fe in the cytosol and in a *vit1* loss of function mutant there is more Fe is the cytosol. Our results suggest that VIT1, by moderating vacuolar Fe content, plays a pivotal role in cellular Fe homeostasis.

Secondly, we looked more closely at whether Mn loading of the developing embryo is altered in a *vit1* mutant. For this analysis, developing siliques were imaged at BL2-3 (SSRL) via 2D raster scanning and computed tomography (for developing embryo cross sections), at 7 and 12 days after flowering (DAF) to analyze multiple developing embryos within the siliques. User-defined region of interest analysis compared elemental abundances of the whole silique, individual embryos and the vasculature between the two lines, and at the two time points. Overall, abundances increased in both lines between 7 and 12 DAF and there was no apparent difference in Mn loading.

Our third approach to the question of the role of VIT1 in Mn homeostasis builds on the observation by Sebastian Thomine's lab that a mutation in *VIT1* can suppress the growth phenotype of an *nramp3nramp4* mutant. We are investigating whether this is solely an Fe phenotype or may also be related to Mn homeostasis. Under Fe deficiency, a *vit1* mutant and an *nramp3 nramp4* mutant have the same phenotype: they germinate and then grow very poorly, presumably because they cannot mobilize the Fe from the vacuole. The *vit1* mutant does not put Fe in the vacuole and the *nramp3 nramp4* mutant cannot get Fe out of the vacuole. A suppressor

screen carried out in the Thomine lab looked for improved germination of *nramp3 nramp4* mutants under Fe deficient growth conditions and two of the suppressor lines carried mutations in *vit1*. A triple mutant of *vit1 nramp3 nramp4* confirmed that the mutation in *VIT1* was causing the observed phenotype in the suppressed lines. We know that the *nramp3 nramp4* mutant has a more severe phenotype than *vit1*. That is, it is worse to put Fe in the vacuole and then not be able to access it – as opposed to not putting in the vacuole in the first place and perhaps suffering from redox stress.

Specific aim #3. Determine the chemical binding form of Fe in Arabidopsis seed using SEC-ICP-MS.

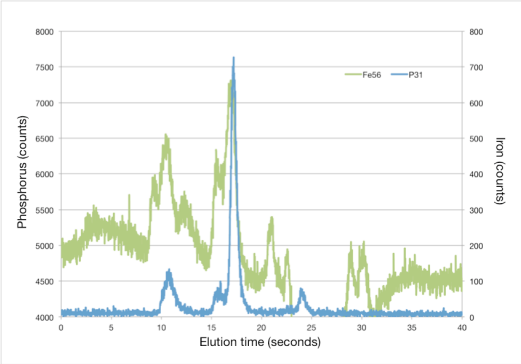


Figure 4. Size Exclusion ICP-MS chromatogram of Arabidopsis Col-0 seed extract, showing co-elution of Fe and P. Seeds were ground with liquid N in an anaerobic chamber to preserve the native oxidation state of Fe, followed by extraction with 0.0625 M TRIS-HCl (pH 6.8) 2% SDS, 5% β-mercaptoethanol and 10% glycerol. After sonication, the sample was centrifuged, with the supernatant analyzed via size-exclusion chromatography ICP-MS.

Using SEC-ICP-MS, we saw co-elution of Fe and P in extracts prepared from Col-0 seed, suggesting that Fe was bound to phytate. However, we were only able to extract less than 20% of the seed Fe. Although this extraction efficiency is typical of this technique, we decided to switch to a genetic approach to ask whether the spatial distribution of Fe would be disrupted in lines that lacked either of the possible major binding ligands for Fe, either by not being stored appropriately, or by being less stable during storage and migrating in to other cell layers. The most likely candidate to cause disruption was phytate, so we examined three *ipk* mutants. IPK1 and IPK2β catalyze various stages of the phytate synthesis pathway. The double mutant *ipk1ipk2*β contains 95% less phytate than Col-0 (Stevenson-Paulik et al., 2005). The alternate Fe binding ligand, ferritin, is encoded by four genes in Arabidopsis, but only *FER2* is expressed in the seed (Ravet et al., 2009).

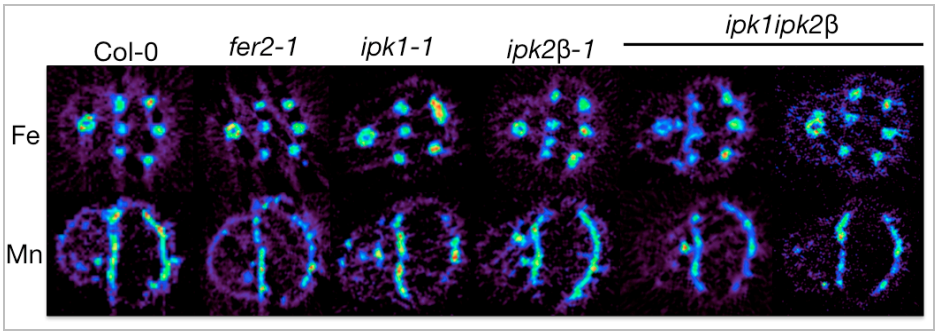


Figure 5. SXRF computed tomography of the *ipk* seed series *in vivo*. All seeds are shown with the radicle on the left and are individually scaled.

The analysis of Arabidopsis seed via SXRF-CMT indicated that Fe distribution was not disrupted as a result of a lack of either ferritin or phytate. However, closer observation of Fe

distribution in the *ipk1ip2*β double mutant (two replicates are shown, Figure 5) show Fe outside of its usual location: it is typically found only in the endodermal cell layer, and is not prone to the sporadic distribution often seen for Mn in the radicle. It is likely that the potential cellular damage caused by mislocalized Fe is the main driver of this tight localization. However, in the double mutant replicates, Fe appears (a) in the sub-epidermal cell layer of the abaxial surface of the cotyledons, and (b) in a cortical cell of the radicle. Both of these are common locations for Mn, which often shares membrane transport proteins with Fe (Pittman, 2005). We decided to investigate the double mutant further at higher resolution, and using a beamline with an energy range able to detect P, a major component of phytate. We tested whether P abundance was altered relative to Col-0, and whether co-localization between Fe and P was altered in *ipk1ip2*β embryos. Using a protocol for sample preservation and sectioning that we have demonstrated does not disrupt vacuole-bound metals such as Fe and Mn (Punshon et al., 2012), we collected high-resolution (0.25 µm beam) images of the embryonic radicle (Figure 6) from Col-0 and *ipk1ipk2*β embryos. We analyzed 1 µm sections of embryonic radicle at beamline 2-ID-D of the Advanced Photon Source (Argonne National Laboratory, IL).

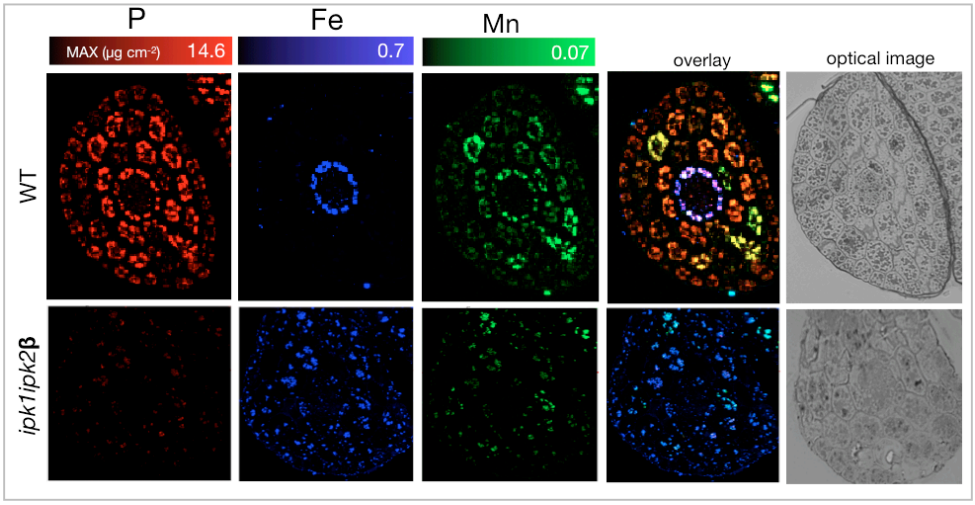


Figure 6. High-resolution SXRF images of P, Fe and Mn in sections of the embryonic radicle of Col-0 and *ipk1ip2*β. Data are quantified as µg cm^{-2}, scaled to the maximum abundance as shown.

High resolution analysis of *ipk1ip2*β contrasted strongly with data from *in vivo* analysis. Most notably, the typical pattern of Fe localization in the endodermal layer was completely missing, and Fe was observed throughout the radicle. After repeating the analysis to confirm the correct identity of the lines, and finding the same distribution, we concluded that Fe had been completely remobilized in *ipk1ip2*β during sample preparation. Region of interest analysis on the total content of Fe within the sample view shown in Figure 6 confirmed that total content in Col-0 and *ipk1ip2*β were very similar, suggesting that Fe had not been lost from the sample, but had been redistributed. Also of note in this image series was the lower P abundance in *ipk1ip2*β. The second hypotheses – to test Fe and P co-localization – showed a contrasting ratio between Col-0 and *ipk1ip2*β.

Taken together, the data collected for this aim suggest that Fe is bound to phytate in seeds. Surprisingly, unbound Fe (or Fe not bound to phytate) still localizes correctly. It is intuitive to assume that the form of a metal strongly influences the cellular and subcellular localization, although in this example that is not the case. Although widely regarded as having the potential to

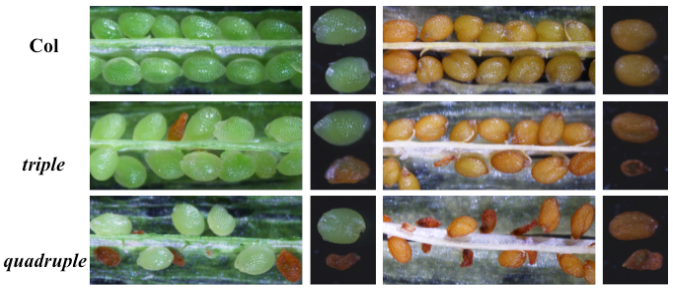
cause experimental artifacts, intrusive sample preparation was in this case able to demonstrate the weaker stability of Fe in *ipk1ip2*β in comparison with Col-0.

Specific aim #4. Determine how many Zn transporters participate in Zn uptake from the soil.

We feel this an important question to answer, especially in light of the fact that Arabidopsis seems to have only one major Fe transporter (IRT1) for uptake from the soil yet seems to have multiple Zn transporters. We are still working to identify the main root Zn uptake transporter(s). We have ruled out most of the ZIPs acting as the sole Zn transporter, including ZIP2 and ZIP5 that localize to the epidermis. *zip2* and *zip5* single mutants, as well as a *zip2 zip5* double mutant, behave like wild type plants. We have constructed a triple mutant of *zip2*, *zip5* and *zip9* as we now know that ZIP9 also localizes to the epidermis in the differentiated zone of the root when plants are grown under Zn deficiency.

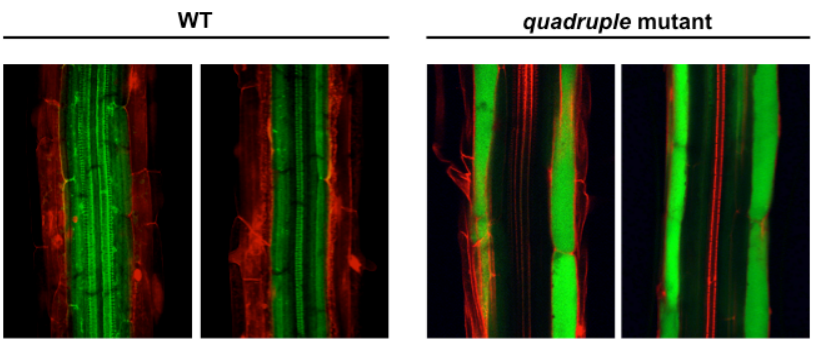
At this point, we have tissue localization information for IRT1, IRT2, IRT3, ZIP1, ZIP2, ZIP3, ZIP4, ZIP5, ZIP6, ZIP7 and ZIP9. ZIP8 is a pseudogene in Col-0. We will complete our analysis of the remaining family members (ZIP10, ZIP11 and ZIP12) to see if there is another epidermis-localized ZIP.

Specific aim #5. Test the hypothesis that ZIP4, ZIP9, IRT3 and ZIP6 all play a role in Zn metabolism of the developing embryo.



ZIP4, ZIP9, IRT3 and ZIP6 are all induced under Zn deficiency and all show high expression in the Zn hyperaccumulator *Arabidopsis halleri* (Talke et al., 2006). When expressed in yeast, all four ZIPs transport Zn but not Fe, Mn or Cd. Although none of the single or double mutants show obvious growth defects, a triple mutant of *zip4 irt3 zip6* as well as a quadruple mutant of *zip4 irt3 zip6 zip9* do show impaired growth under both Zn sufficient and Zn deficient conditions and have higher rates of seed abortion (see Figure above). The seed abortion can be rescued by supplying plants with high levels of Zn during seed fill. The triple and quadruple mutant seed have lower concentrations of Zn and show slower rates of germination. In addition, seedlings have much less Zn in their vasculature compared to wild type (see Figure below).

Here Zn is imaged using the Zn fluorophore Zinpyr1 that fluoresces green when bound to Zn. In the quadruple mutant, Zn is accumulating in cortical cells and is not accumulating in the vasculature. We know that each of these four ZIP proteins is localized to the plasma membrane and we have documented where



each of the genes is expressed using transgenic lines carrying GUS fusions. We are also examining other mutant combinations that may shed light on the role of the various ZIP transporters in Zn homeostasis. The paper describing the quadruple mutant should be submitted by the end of the year.

Literature Cited

Carey, A.M., Lombi, E., Donner, E., de Jonge, M.D., Punshon, T., Jackson, B.P., Guerinot, M.L., Price, A.H., and Meharg, A.A. (2011a). A review of recent developments in the speciation and location of arsenic and selenium in rice grain. . Anal. Bioanal. Chem. 402, 3275-3286.

Carey, A.M., Norton, G.J., Deacon, C., Scheckel, K.G., Lombi, E., Punshon, T., Guerinot, M.L., Lanzirotti, A., Newville, M., Choi, Y., Price, A.H., and Meharg, A.A. (2011b). Phloem transport of arsenic species from flag leaf to grain during grain filling. New Phytol. 192, 87-98.

Donner, E., Punshon, T., Guerinot, M.L., and Lombi, E. (2011). Functional characterisation of metal(loid) processes in planta through the integration of synchrotron techniques and plant molecular biology. Anal. Bioanal. Chem. 402, 3287-3298.

Gursoy, D., Lanzirotti, A., Newville, M.G., and De Carlo, F. (2015). Hyperspectral Image Reconstruction for X-ray Fluorescence Tomography. Optics Lett. In Press.

Kim, S.A., Punshon, T., Lanzirotti, A., Li, L., Alonzo, J.M., Ecker, J.R., Kaplan, J., and Guerinot, M.L. (2006). Localization of iron in Arabidopsis seed requires the vacuolar membrane transporter VIT1. Science 314, 1295-1298.

Lanquar, V., Lelievre, F., Bolte, S., Hames, C., Alcon, C., Neumann, D., Vansuyt, G., Curie, C., Schroder, A., Kramer, U., Barbier-Brygoo, H., and Thomine, S. (2005). Mobilization of vacuolar iron by AtNRAMP3 and AtNRAMP4 is essential for seed germination on low iron. EMBO J. 24, 4041-4051.

Pittman, J.K. (2005). Managing the Manganese: molecular mechanisms of manganese transport and homeostasis. New Phytol. 167, 733-742.

Punshon, T., Guerinot, M.L., and Lanzirotti, A. (2009). Using synchrotron x-ray fluorescence microprobes to study metal(loid) homeostasis in plants. Ann. Bot. 103, 665-672.

Punshon, T., Ricachenevsky, F.K., Hindt, M.N., Socha, A.L., and Zuber, H. (2013). Methodological approaches for using synchrotron X-ray fluorescence (SXRF) imaging as a tool in ionomics: examples from *Arabidopsis thaliana*. Metallomics 5, 1133-1145.

Punshon, T., Hirschi, K., Yang, J., Lanzirotti, A., Lai, B., and Guerinot, M.L. (2012). The role of CAX1 and CAX3 in elemental distribution and abundance in Arabidopsis seed. Plant Physiol. 158, 352-362.

Ravet, K., Touraine, B., Boucherez, J., Briat, J.F., Gaymard, F., and Cellier, F. (2009). Ferritins control interaction between iron homeostasis and oxidative stress in Arabidopsis. Plant J 57, 400-412.

Stevenson-Paulik, J., Bastidas, R.J., Chiou, S.T., Frye, R.A., and York, J.D. (2005). Generation of phytate-free seeds in Arabdiopsis through disruption of inositol polyphosphate kinases. Proc. Natl. Acad. Sci. U.S.A. 102, 12612-12617.

Talke, I.N., Hanikenne, M., and Kramer, U. (2006). Zinc-dependent global transcriptional control, transcriptional deregulation and higher gene copy number for genes in metal homeostasis of the hyperaccumulator *Arabidopsis halleri*. Plant Physiol. 142, 148-167.

Zhai, Z., Gayomba, S., Jung, H., Vimalakumari, N.K., Pineros, M., Craft, E., Rutszke, M.A., Danku, J., Lahner, B., Punshon, T., Guerinot, M.L., Salt, D.E., Kochian, L.V., and Vatamaniuk, O.K. (2014). OPT3 is a phloem-specific iron transporter that is essential for systemic iron signaling and redistribution ofiron and cadmium in Arabidopsis. Plant Cell 26, 2249-2264.

Publications resulting from the Award

Guerinot, M.L., I. Baxter and D.E. Salt. 2009. From the ionome to the genome: Identifying gene networks that control the mineral content of plants. *In* Plant Systems Biology. G. Coruzzi and R. Gutierrez (eds.), Wiley-Blackwell. Annual Plant Reviews 35: 290-303.

Punshon, T., A. Lanzirotti and M.L. Guerinot. 2009. Using synchrotron X-ray fluorescence microprobes to study metal(loid) homeostasis in plants. Annal. Bot. 103:665-672.

Palmer, C. and M.L. Guerinot. 2009. Facing the challenges of Cu, Fe and Zn homeostasis in plants. Nature Chem. Biol. 5:333-340.

Morrissey, J. and M.L. Guerinot. 2009. The good, the bad and the ionome. Chem. Rev. 109:4553-4567.

Carey, A-M., E. Lombi, E. Donner, M. D. de Jonge, T. Punshon, B. P. Jackson, M.L. Guerinot, A. H. Price and A.A. Meharg. 2011. A review of recent developments in the speciation and localization of arsenic and selenium in rice grain. Anal. Bioanal. Chem. 402: 3275-86.

Donner, E., T. Punshon, M.L. Guerinot and E. Lombi. 2011. Functional characterisation of metal(loid) processes *in planta* through the integration of synchrotron techniques and plant molecular biology. Anal. Bioanal. Chem. 402: 3287-98 (cover).

Punshon, T., K. Hirschi, J. Yang, A. Lanzirotti, B. Lai, M.L. Guerinot. 2012. The role of CAX1 and CAX3 in elemental distribution and abundance in Arabidopsis seed. Plant Physiol. 158: 352-362.

Hindt, M.N. and M.L.Guerinot. 2012. Getting a sense for signals: Regulation of the iron deficiency response. Biochim Biophys Acta 1823: 1521-30.

Yang, J., T. Punshon, M.L. Guerinot and K.D. Hirschi. 2012. Plant calcium content: ready to remodel. Nutrients 4: 1120-1136.

Schroeder, J.I., E. Delhaize, W.B. Frommer, M.L. Guerinot, M.J. Harrison, L. Herrera-Estrella, T. Horie, L.V. Kochian, R. Munns, N.K. Nishizawa, Y.-F. Tsay and D. Sanders. 2013. Using membrane transporters to improve crops for sustainable food production. Nature 497: 60-66.

Socha, A. and M.L. Guerinot. 2014. Mn-euvering manganese: the role of transporter gene family members in manganese uptake and mobilization in plants. Front. Plant Sci. 5:106. doi: 10.3389/fpls.2014.00106.

Zhai, Z., S. Gayomba, H. Jung, N.K. Vimalakumari, M. Pineros, E. Craft, M.A. Rutszke, J. Danku, B. Lahner, T. Punshon, M.L. Guerinot, D.E. Salt, L.V. Kochian, and O. Vatamaniuk. 2014. OPT3 is a phloem-specific iron transporter that is essential for systemic iron signaling and redistribution of iron and cadmium in Arabidopsis. Plant Cell 26: 2249-2264.

Kim, S.A. and M.L. Guerinot. Overexpression of the vacuolar iron transporter VIT1 increases metal accumulation in *Arabidopsis thaliana*. In revision for Plant Physiology.