



## Analysis of the *VIT1* Promoter Activity in Developing *Arabidopsis thaliana* Plants

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### ABSTRACT

Iron (Fe) deficiency in plants is one of the widespread problems limiting agricultural production. Generating crops more tolerant to Fe deficiency by genetic engineering or breeding is of great interest but challenging due to the knowledge gaps in general plant Fe homeostasis. Although several genes involved in Fe homeostasis have been identified, characterization of their roles is mainly limited to specific organs at specific developmental stages of the plant, where their mutants show the most striking phenotype. *Vacuolar Iron Transporter 1 (VIT1)* is a well-known gene that has been characterized for its function in the mature seed of *Arabidopsis thaliana*. *VIT1* is an Fe transporter that determines the correct distribution of Fe storage in this organ. The study aimed to explore new physiological functions for *VIT1*. As a first step, *Arabidopsis thaliana* plants that contain *PromoterVIT1: GUS* constructs were used to study the temporal and spatial expression of the gene throughout the plant's lifecycle. GUS histochemical staining revealed the *VIT1* promoter is active in the mature leaves and mature reproductive organs. *VIT1* promoter activity in the stamen increased developmentally and was limited to tapetum and guard cells in the pollen sac. In the female organ, *VIT1* promoter activity increased as the pistil developed into a silique. Although all the silique exhibited staining, staining density was higher in the peduncle, replum, and stigma regions. Inside the developing silique, funicles were heavily stained. Furthermore, *in silico* analyses of *VIT1* transcriptome and protein levels confirmed flower and the silique are hot spots for *VIT1* activity. Thus, the results may suggest a possible involvement of *VIT1* protein in several stages of the reproductive system, specifically in the flowering and in the fruit development.

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## Introduction

One of the widespread problems in agriculture is iron deficiency in plants. Plants often fail to retrieve enough Fe from the soil, posing a challenge in productivity and human health. Such plants cannot meet the Fe demand to achieve their full growth potential, causes a loss in yield (Abadía et al., 2011) and overaccumulation of harmful metals such as nickel and cadmium in their edible parts (Korshunova et al., 1999). Fe deficiency is especially prominent in calcareous soil, consisting of approximately one-third of all agricultural lands (Holloway et al., 2001). Therefore, to enhance plants in Fe uptake and storage, plant scientists have heavily explored Fe homeostasis, which led to the discovery of genes and physiological processes that involve them.

Fe is taken up from the soil by the root, distributed in the plant, and finally remobilized to the seed to give rise to the new generations. Root retrieves Fe from the soil with Iron-Regulated Transporter 1 (IRT1) protein on the plasma membrane of the outer root cells (Korshunova et al., 1999).

These cells transfer Fe to the inner root cells with the help of Natural Resistance Associated Macrophage Protein 1 (NRAMP1) (Castaings et al., 2016). In the vasculature, xylem and phloem transfer Fe associated with chelators, especially citrate for xylem, which is pumped into the xylem by Ferric Reductase Defective 3 (FRD3) and nicotianamine for phloem (Durrett et al., 2007; Schuler et al., 2012). Once translocated from root to shoot, Fe fuels various metabolic processes, notably those containing electron transport chain reactions in mitochondria and chloroplast of the leaves (Vigani et al., 2013). Part of the shoot Fe mobilizes from older tissues to the developing seeds in the siliques, in annual plants such as *Arabidopsis thaliana*, in a developmental process called senescence. Among the parts of the *Arabidopsis* seed, the majority of the Fe is diverted to the embryo (Eroglu et al., 2019). This Fe is built around the provasculature of the embryo with the help of Vacuolar Iron Transporter 1 (*VIT1*) (Kim et al., 2006). Consequently, plants that do not have a functional *VIT1* protein lacks Fe stores in

the provasculature (Kim et al., 2006). *VTI* stands out among the other Fe homeostasis proteins as it is one of a handful of proteins that has successfully been used for biofortification of crops (Connorton et al., 2017; Narayanan et al., 2015, 2019).

*VTI*'s structure has been extensively characterized. *VTI* was first identified by homology to yeast CCC1 family proteins (Kim et al., 2006). It is well conserved in the plant kingdom (Eroglu et al., 2019). *VTI* localized to the tonoplast and transported Fe and manganese when expressed in yeast (Kim et al., 2006). *VTI*'s crystal structure indicated *VTI* has five transmembrane domains and functions as dimers (Kato et al., 2019). *VTI*'s expression pattern, which was revealed by *PromoterVTI: GUS* lines, overlaps the localization of embryo Fe stores (Kim et al., 2006). Despite the extensive knowledge of *VTI*'s structure, exploration of its physiological function has mostly been limited in the seed/seedling stage of *Arabidopsis thaliana* life cycle. A common strategy in searching for a function of a protein is to start exploring where and when its gene is preferentially expressed, following an examination of how knock-out mutant lines of this gene may behave physiologically different from the wild type, especially at stages and tissues where the gene expression was most remarkable. Therefore, to set the stage for investigating *VTI*'s other physiological functions for future studies, *VTI* promoter activity in various tissues at various growth stages was examined by using *PromoterVTI: GUS* lines. The approach revealed that specific organs, tissues, and even cells show preferential *VTI* promoter activity, indicating an elusive function for *VTI*.

## Materials and Methods

### Plant Material and Growth Conditions

Two independent *PromoterVTI: GUS* lines were obtained from Prof. Mary Lou Guerinot (Dartmouth College, Hanover, USA) (Kim et al., 2006). To observe growth on soil, seeds were sown onto standard potting soil (Substrat 1; Klasmann-Deilmann GmbH, Geeste, Germany). Pots were placed in a growth cabinet (Sanyo, Japan) set to 22°C day and 19°C night temperature and a 16 h light period with a light intensity of 140  $\mu\text{mol photons m}^{-2} \text{s}^{-1}$ .

### Histochemical GUS Assay

To perform GUS histochemical staining, all samples were vacuum infiltrated for an hour and then incubated at 37°C in a GUS reaction buffer containing 0.4 mg mL<sup>-1</sup> 5-bromo-4-chloro-3-indolyl- $\beta$ -D-glucuronide, 50 mM sodium phosphate (pH 7.2), and 0.5 mM ferrocyanide, 0.5 mM ferricyanide and 0.1% Triton X-100 (Eroglu et al., 2017).

### In Silico Analyses

For in silico transcriptome analysis of *VTI*, Genevestigator software was used (Hruz et al., 2008). All *Arabidopsis* microarray datasets were included in the study, and among them, ten organs that show the highest *VTI* signal were sorted. Protein expression data is obtained from the Athena proteomics database (athena.proteomics.wzw.tum.de) (Mergner et al., 2020).

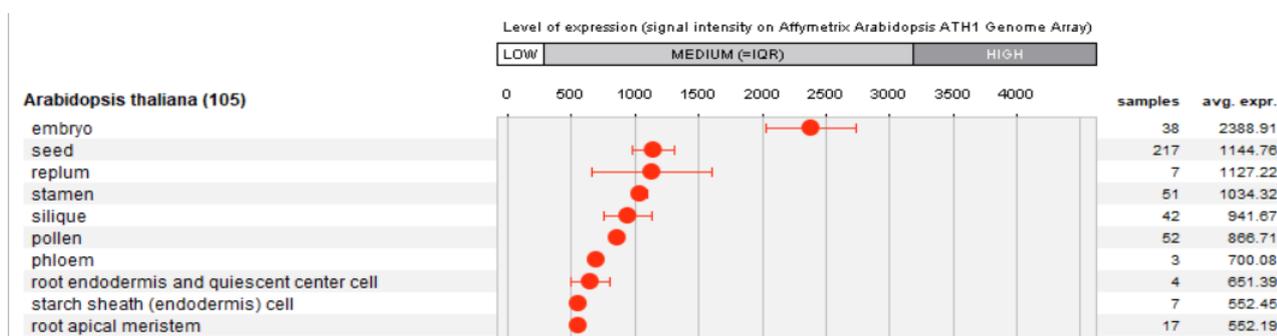


Figure 1. *VTI*'s abundance in *Arabidopsis thaliana*'s organs. Publicly available microarray analysis results were retrieved using Genevestigator software. Data show the top ten organs exhibiting the highest average *VTI* signal.

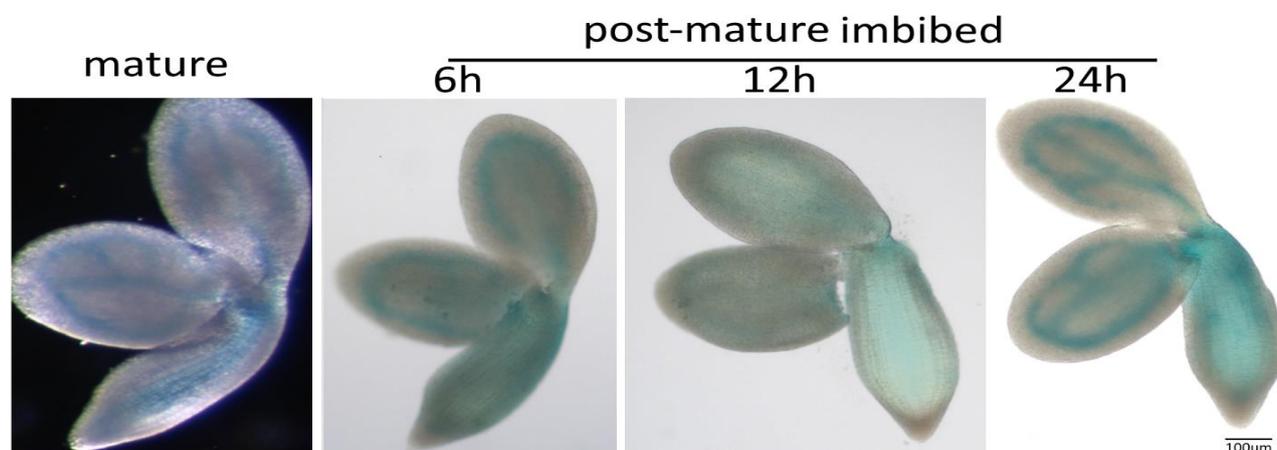


Figure 2. *VTI* promoter activity in imbibed embryos. Mature drying siliques of *PromoterVTI: GUS* lines were dissected, and embryos were isolated (mature). Post-mature, dry seeds of the same line were imbibed in water for 6, 12 or 24 hours and embryos were isolated. All embryos were incubated in GUS staining solution for two hours and visualized under a light microscope.

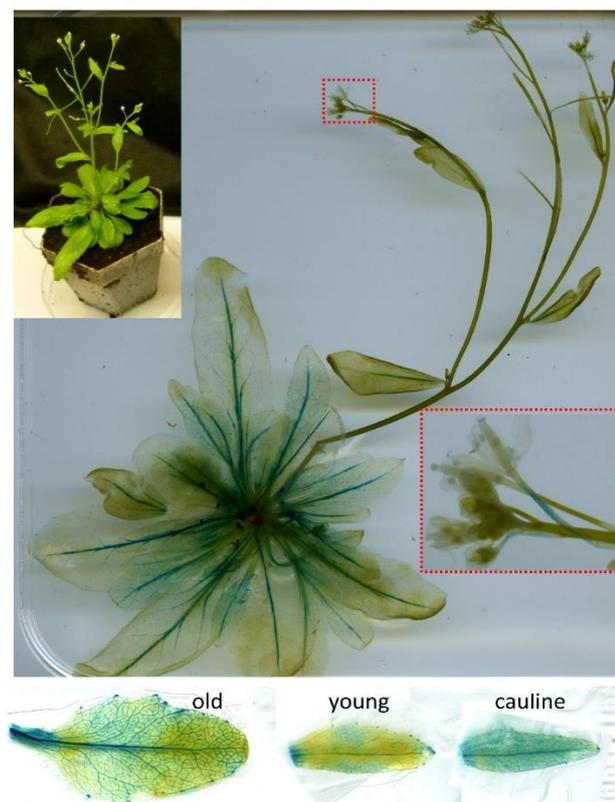


Figure 3. *VTI* promoter activity in the bolting plants. *PromoterVTI: GUS* line was grown until the bolting stage (top left photo) and was stained in GUS solution. The whole shoot was incubated in GUS for 12 hours (middle photo) or 24 hours (only the dissected parts in the bottom panel). Old and young leaves from the rosette and cauline leaves were examined and photographed separately from the whole shoot (bottom right photo). Red box, close up to flowers.

## Results

To investigate *VTI* promoter activity in plant tissues, microarray databases were used (Figure 1). The curated microarray experiments in Genevestigator software's database showed the embryo part of the plant expresses *VTI* highest, as expected. Besides embryo, flower organs express *VTI* in the second place. These organs included silique, stamen, and their subparts (i.e., replum and pollen). While the *VTI* signal was approximately two times that of the flower, the software still grouped them in the medium-range category. Thus, *in silico* analysis confirmed *VTI* in the embryo and revealed *VTI* in flower.

Previous investigations show *VTI* promoter activity in developing embryos but not in postmature, desiccated ones (Eroglu et al., 2017). Next, *VTI* promoter activity was investigated by histochemical GUS staining in the early phase of germination by imbibing seeds of *PromoterVTI: GUS* lines in a time course (Figure 2). The results showed that imbibed seed embryos express *VTI* similar to postmature embryos regarding the localization (Figure 2).

Next, *VTI* promoter activity in the whole shoot of bolting plants was assessed. After 12 hours of incubation, a bright blue color appeared on the vasculature region of the leaves (Figure 3). Among the leaves in the rosette, older leaves showed slightly higher staining than younger ones.

Twelve hours of incubation did not result in staining of the inflorescence, except the budding branches of the stem. The plant was also visualized after 24 hours of incubation. This time GUS staining was apparent on the minor veins of the leaf.

The promoter activity was also assessed also in the inflorescence after 24 hours of incubation, which resulted in heavy staining in older stamens (Figure 4). Stamens show staining only in the anthers. On top of the stamen, tissues inside the pollen sac, corresponding to tapetum exhibited staining. In contrast, pollens did not show staining (Figure 4E, black arrowheads). GUS staining accumulated in certain regions of the epidermis. The shape of the accumulation corresponded to the guard cells (Figure 4F, red arrowheads). In addition to stamen, younger branches showed staining. Therefore, the data suggest *VTI*'s preferential presence in developing flowers tapetum and guard cells of the pollen sac.

*In silico* analyses suggested *VTI*'s presence in the silique, but it was not observed in the carpel of the flower. *VTI*'s presence in the developing carpel/silique was examined (Figure 5A). The GUS staining result showed extensive staining in the developing siliques (Figure 5B), confirming the *in silico* expression analysis (Figure 1, Supplementary Figure 1). This staining was more intense in the internode/peduncle, replum, and stigma regions. Funicles showed intense staining inside the siliques (Figure 5C, D).

## Discussion

*VTI* is a tonoplast Fe transporter. It builds up Fe reserves in the seed, but whether it also contributes to any other physiological processes in *Arabidopsis thaliana* has not been investigated. As a first step to build a hypothesis for such an investigation *VTI* expression was assessed using transgenic lines that contain *PromoterVTI: GUS* constructs. Growth stages and organs that express *VTI* were identified. These included the stamen of the flower and the silique, indicating a potential role of *VTI* protein in these organs.

Elucidating spatial and temporal expression patterns of already characterized genes often leads to discovering new physiological functions for the same genes. For example, MTP8 protein serves under Fe deficient conditions in the *Arabidopsis* roots. Still, a later realization of its expression in the seeds led to discovering a new role for it building up the manganese reserves in the seed embryo (Eroglu et al., 2016, 2017). Previous studies have reported *VTI*'s critical role during the germination of seeds under stress conditions, while the results here may indicate an additional developmental role for *VTI* (Figure 4, 5).

The findings in this manuscript infer a role for *VTI* in the late stage of developing male and female organs of the plants. The male organ, anther, consists of a stamen and a filament that holds it. The male organ matures as the filament develops and stamen produces pollens inside the tapetum of the pollen sac. Mature stamens dehiscence to release the pollen and die soon after. The female organ, the pistil, receives the spores and gradually becomes a silique. *VTI* is expressed in the maturing stamen, specifically in the postmature stage at the tapetum and the guard cells of the stamen epidermis.

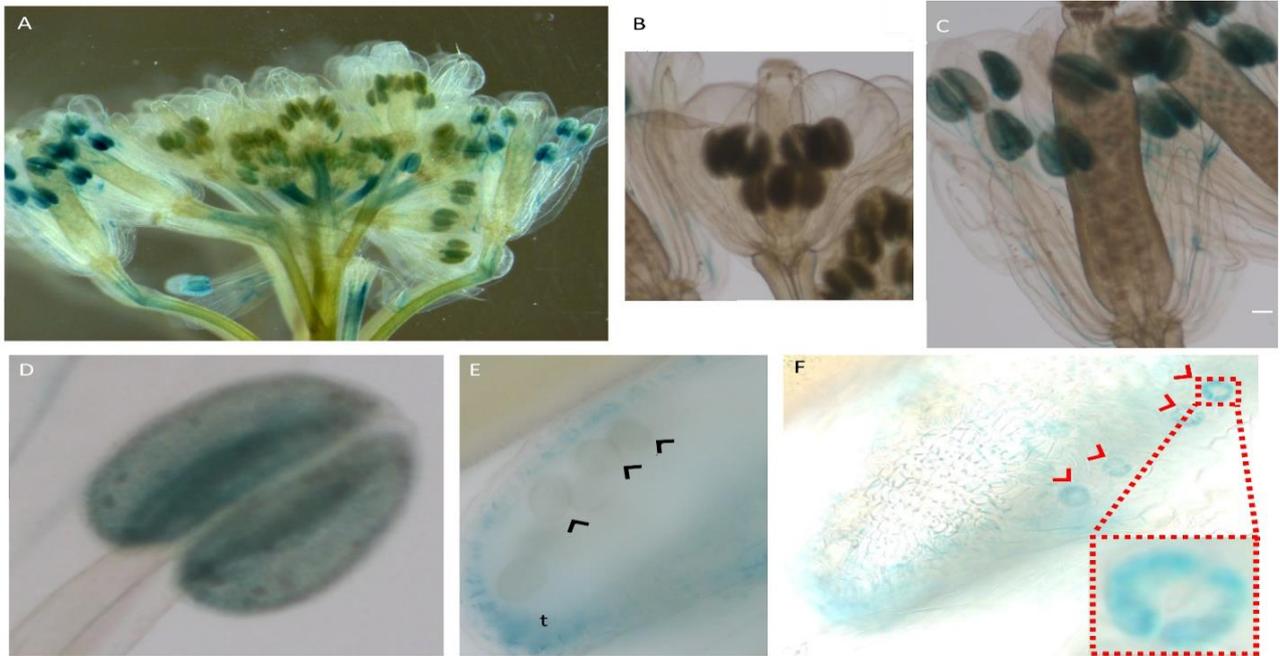


Figure 4. *VTI* promoter activity in the stamen: *PromoterVTI: GUS* line inflorescence was incubated in GUS solution for 24 hours and visualized under the light microscope. A, cluster of flowers, note that the flowers in the middle are younger than the ones surrounding. B, young unopened flower close up. C, mature flower, close up, one day after flowering. D-F, the stamen of a mature flower. t, tapetum of a stamen. Black arrowheads, pollens. Red arrowheads, guard cells. Red box, guard cell, close up.

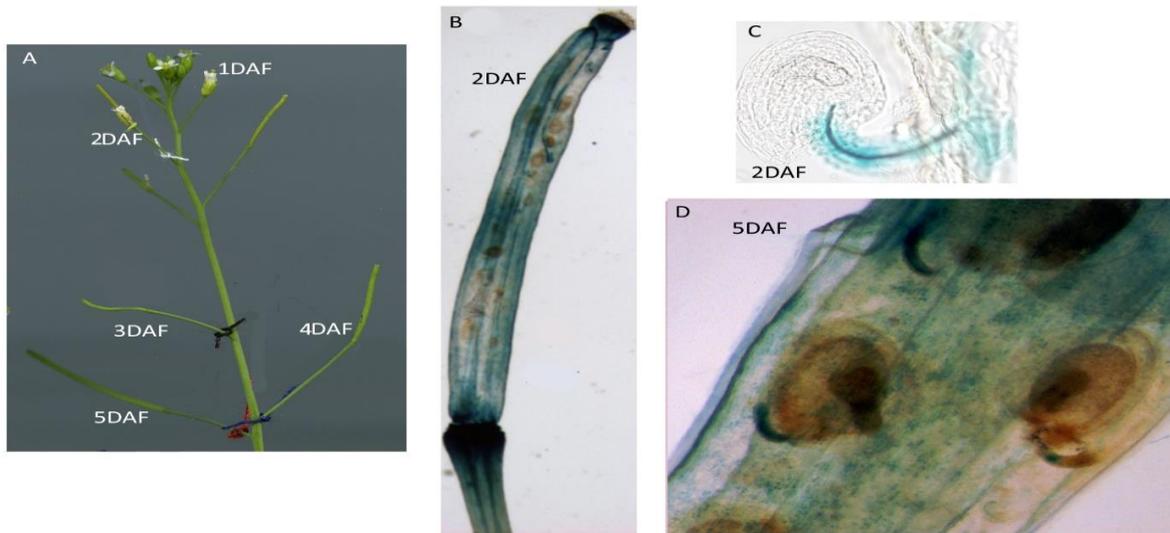


Figure 5: *VTI* promoter activity in the silique following pollination: *PromoterVTI: GUS* line's developing siliques were incubated in GUS solution for 24 hours and visualized under the light microscope. A, untreated plant with growing siliques. B, silique; C, ovule, and funiculus; D, silique close up, developing seeds, and their funiculus were under focused. DAF: Day after flowering.

Table 1. Summary of findings

	Sample	GUS level
Seed	Mature	H
	P. Dry	H
	P. Imbibed	H
Leaf	Rosette	M, more as the leaf ages, vasculature
	Cauline	M, vasculature
Flower organ	Petal	NA
	Carpel	NA
	Stamen	M, exclusive to tapatum and guard cells
	Silique	M, especially in peduncle, stigma, and funiculus

H, high; M, medium; NA, not available; P, postmature

While *in silico* analysis pointed out *VTI* expression in the pollens (Figure 1), the GUS staining method failed to confirm it (Figure 4). However, developing pollens may express *VTI* before dehiscence, but maybe the GUS solution could not infiltrate the intact pollen sac to reveal that. After the dehiscence, *VTI* might function for nutrient recycling as a part of senescence in the male organ before it dies. *VTI* is expressed in the pistil after pollination while pistil matures into the silique. The early developing embryos require Fe to meet their fast metabolisms' demand, which must be transported to the embryos via peduncle, the base of the pistil, to the silique, and from the silique to the funicle, then finally from funicle to the seed. *VTI* is active throughout this process, suggesting it might protect the cells from Fe-related ROS damage by sequestering excess cytoplasmic Fe in the middle of this Fe trafficking between the mature plant and the nascent seed. Excess cytosolic Fe is a common ROS inducer that must be enclosed in organelles or vacuoles (Briat & Lebrun, 1999). In summary, results revealed that *VTI* is active in maturing female and male organs of the flower, suggesting a putative developmental role for *VTI* in flower following pollination (Table 1).

Here in this manuscript, the *VTI* promoter activity was analyzed. Results suggested *VTI* expression is not limited to the embryo of the seed. Further studies such as the comparison of wild type and *VTI* loss of function mutants may reveal new physiological roles for the *VTI*, sealing some gaps in plant Fe homeostasis to generate Fe-fortified plants.

## References

- Abadía J, Vázquez S, Rellán-Álvarez R, El-Jendoubi H, Abadía A, Álvarez-Fernández A, López-Millán AF. 2011. Towards a knowledge-based correction of iron chlorosis. *Plant Physiology and Biochemistry*, 49(5): 471–482. <https://doi.org/10.1016/j.plaphy.2011.01.026>
- Briat JF, Lebrun M. 1999. Plant responses to metal toxicity. *Comptes Rendus de l'Académie Des Sciences - Serie III*, 322(1): 43–54. [https://doi.org/10.1016/S0764-4469\(99\)80016-X](https://doi.org/10.1016/S0764-4469(99)80016-X)
- Castaings L, Caquot A, Loubet S, Curie C. 2016. The high-affinity metal Transporters NRAMP1 and IRT1 Team up to Take up Iron under Sufficient Metal Provision. *Scientific Reports*, 6: 37222. <https://doi.org/10.1038/srep37222>
- Connorton JM, Jones ER, Rodríguez-Ramiro I, Fairweather-Tait S, Uauy C, Balk J. 2017. Wheat vacuolar iron transporter TaVIT2 transports Fe and Mn and is effective for biofortification. *Plant Physiology*, 174(4): 2434–2444. <https://doi.org/10.1104/pp.17.00672>
- Durrett TP, Gassmann W, Rogers EE. 2007. The FRD3-mediated efflux of citrate into the root vasculature is necessary for efficient iron translocation. *Plant Physiology*, 144(1): 197–205. <https://doi.org/10.1104/pp.107.097162>
- Eroglu S, Giehl RFH, Meier B, Takahashi M, Terada Y, Ignatyev K, Andresen E, Küpper H, Peiter E, Von Wirén N. 2017. Metal tolerance protein 8 mediates manganese homeostasis and iron reallocation during seed development and germination. *Plant Physiology*, 174(3): 1633–1647. <https://doi.org/10.1104/pp.16.01646>
- Eroglu S, Karaca N, Vogel-Mikus K, Kavčič A, Filiz E, Tanyolac, B. 2019. The conservation of *VTI*-dependent iron distribution in seeds. *Frontiers in Plant Science*, 10. <https://doi.org/10.3389/fpls.2019.00907>
- Eroglu S, Meier B, Von Wirén N, Peiter E. 2016. The vacuolar manganese transporter *mtp8* determines tolerance to iron deficiency-induced chlorosis in arabidopsis. *Plant Physiology*, 170(2): 1030–1045. <https://doi.org/10.1104/pp.15.01194>
- Holloway RE, Bertrand I, Frischke AJ, Brace DM, McLaughlin MJ, Shepperd W. 2001. Improving fertiliser efficiency on calcareous and alkaline soils with fluid sources of P, N and Zn. *Plant and Soil*, 236(2): 209–219. <https://doi.org/10.1023/A:1012720909293>
- Hruz T, Laule O, Szabo G, Wessendorp F, Bleuler S, Oertle L, Widmayer P, Gruissem W, Zimmermann P. 2008. Genevestigator V3: A Reference Expression Database for the Meta-Analysis of Transcriptomes. *Advances in Bioinformatics*, 2008, 1–5. <https://doi.org/10.1155/2008/420747>
- Kato T, Kumazaki K, Wada M, Taniguchi R, Nakane T, Yamashita K, Hirata K, Ishitani R, Ito K, Nishizawa T, Nureki O. 2019. Crystal structure of plant vacuolar iron transporter *VTI*. *Nature Plants*, 5(3): 308–315. <https://doi.org/10.1038/s41477-019-0367-2>
- Kim SA, Punshon T, Lanzirotti A, Li A, Alonso JM, Ecker JR, Kaplan J, Guerinot M. Lou. 2006. Localization of iron in Arabidopsis seed requires the vacuolar membrane transporter *VTI*. *Science*, 314(5803): 1295–1298. <https://doi.org/10.1126/science.1132563>
- Korshunova YO, Eide D, Clark WG, Guerinot M. Lou, Pakrasi, H. B. (1999). The IRT1 protein from Arabidopsis thaliana is a metal transporter with a broad substrate range. *Plant Molecular Biology*, 40(1): 37–44. <https://doi.org/10.1023/A:1026438615520>
- Mergner J, Frejno M, List M, Papacek M, Chen X, Chaudhary A, Samaras P, Richter S, Shikata H, Messerer M, Lang D, Altmann S, Cyprys P, Zolg DP, Mathieson T, Bantscheff M, Hazarika RR, Schmidt T, Dawid C, Kuster B. 2020. Mass-spectrometry-based draft of the Arabidopsis proteome. *Nature*, 579(7799): 409–414. <https://doi.org/10.1038/s41586-020-2094-2>
- Narayanan N, Beyene G, Chauhan RD, Gaitán-Solís E, Gehan J, Butts P, Siritunga D, Okwuonu I, Woll A, Jiménez-Aguilar DM, Boy E, Grusak MA, Anderson P, Taylor NJ. 2019. Biofortification of field-grown cassava by engineering expression of an iron transporter and ferritin. *Nature Biotechnology*, 37(2): 144–151. <https://doi.org/10.1038/s41587-018-0002-1>
- Narayanan N, Beyene G, Chauhan RD, Gaitán-Solís E, Grusak MA, Taylor N, Anderson P. 2015. Overexpression of Arabidopsis *VTI* increases accumulation of iron in cassava roots and stems. *Plant Science*, 240: 170–181. <https://doi.org/10.1016/j.plantsci.2015.09.007>
- Schuler M, Rellán-Álvarez R, Fink-Straube C, Abadía J, Bauera P. 2012. Nicotianamine functions in the phloem-based transport of iron to sink organs, in pollen development and pollen tube growth in Arabidopsis. *Plant Cell*, 24(6): 2380–2400. <https://doi.org/10.1105/tpc.112.099077>
- Vigani G, Zocchi G, Bashir K, Philippar K, Briat JF. 2013. Signals from chloroplasts and mitochondria for iron homeostasis regulation. *Trends in Plant Science*, 18(6): 305–311. <https://doi.org/10.1016/j.tplants.2013.01.006>