Identification of the Elusive Chloroplast Ascorbate Transporter Extends the Substrate Specificity of the PHT Family

Most of the enzymatic reactions of ascorbate biosynthesis occur in the cytosol, with the exception of the terminal step, catalyzed by L-galactono-1,4-lactone dehydrogenase, which is localized on the inner mitochondrial membrane. Given the location of L-galactono-1,4-lactone dehydrogenase, the rate of ascorbate biosynthesis is intimately associated with the rate of mitochondrial respiration (Nunes-Nesi et al., 2005). Ascorbate has legion roles in the plant cell including mitosis, cell division, expansion and cell wall hydroxylation, hormone biosynthesis, signal transduction, programmed cell death, as well as protection against environmental stresses via detoxification of reactive oxygen species and regeneration of other antioxidants. This list has recently expanded with the epigenetic role exerted as a cofactor for DNA and histone demethylases in the nucleus and ascorbate may also be implicated in iron uptake (Pick and Weber, 2014).

In photosynthetic tissues, ascorbate is highly important to control guard cell signaling and stomatal movement, as well as the expression levels of both nuclear and chloroplastic components of the photosynthetic apparatus. Moreover, it participates in the Mehler peroxidase reaction with ascorbate peroxidase to regulate the redox state of photosynthetic electron carriers and detoxification of H_2O_2. In the thylakoid lumen, ascorbate acts as a cofactor for violaxanthin de-epoxidase, an enzyme involved in xanthophyll cycle-mediated photoprotection (reviewed in Gest et al., 2013). It has also been demonstrated to donate electrons to photosystem II and as such to contribute to the plant's ability to tolerate heat stress (Tóth et al., 2011).

The major route of ascorbate biosynthesis in plants is the Smirnoff–Wheeler pathway; however, other alternative pathways have also been unraveled to some detail. From the mitochondria, ascorbate has to be transported to the various cell compartments, necessitating specific transport systems, since neither ascorbate nor its oxidized form, dehydroascorbate (DHA), can easily diffuse through lipid bilayers. The functions of ascorbate in the cell highlight the need for transporters in the membranes of basically all cell compartments as well as in the plasma membrane. The photosynthetic functions of ascorbate also largely necessitate the presence of ascorbate and possibly DHA transporters in the chloroplast envelope and the thylakoid membrane, a fact re-enforced by the observation that the chloroplastic ascorbate concentration can reach up to 20 mM (Zechmann, 2011).

However, ascorbate transport remains poorly characterized at the molecular level. Ascorbate has been shown to be transported throughout the plant via the phloem from source to sink tissues (Franceschi and Tarlyn, 2002), despite the fact that its biosynthetic pathways appear to be ubiquitously active. It has been suggested that exclusively DHA is taken up via the plasma membrane and the transporter is distinct from glucose carriers, whereas a mitochondrially localized DHA transporter is most probably similar to a glucose transporter (Szarka et al., 2013). The chloroplast membrane was suggested earlier to contain an ascorbate translocator, whereas it was suggested that the thylakoid membrane contains no ascorbate transport system and the uptake occurs via diffusion (reviewed in Szarka et al., 2013; Pick and Weber, 2014). Recently, the 12-member *Arabidopsis* nucleobase-ascorbate transporter family was identified and molecularly characterized in *Arabidopsis* (Maurino et al., 2006) and more recently in rice and tomato (reviewed in Pick and Weber, 2014), however, ascorbate transport activity has yet to be determined for any of the proteins encoded and, given that none of these proteins are localized to the chloroplast membrane, they cannot explain the full range of plant subcellular ascorbate distribution. Within this context, the recent identification that AtPHT4:4 is a chloroplast-localized ascorbate transporter and subsequent experiments (Miyaji et al., 2015) thus represent a substantial breakthrough.

Figure 1 provides a schematic representation of possible intracellular and plasma membrane ascorbate transporters required to explain the documented presence of ascorbate in different cellular compartments and cell types.

The SLC17 transporter family was initially reported as the six-member *Arabidopsis* Na$^+$ or H$^+$/phosphate co-transporter family (PHT4; Guo et al., 2008), among which AtPHT4;1 and AtPHT4;4 showed a 10-fold increased expression on light exposure; surprisingly, none of the family showed changes in expression under conditions of phosphate deficiency leading to the assumption that they have additional roles to those of phosphate transport (Guo et al., 2008). On the basis of this expression analysis, Miyaji et al. (2014) chose to express one gene of each subgroup of the *Arabidopsis* PHT4 family in *Escherichia coli*, prior to purification and incorporation into proteoliposomes. They demonstrated that those proteoliposomes containing purified AtPHT4;4 have significant ascorbate uptake activity. Ascorbate uptake showed an absolute requirement for Cl$^−$ but was insensitive to DHA, phosphate, glutamate, and ATP. Antibodies raised against AtPHT4;4 had a very similar pattern of localization to the canonical envelope membrane marker TIC40. Critically AtPHT4;4 knockout mutants exhibited reduced levels of ascorbate in the leaves and in the chloroplast

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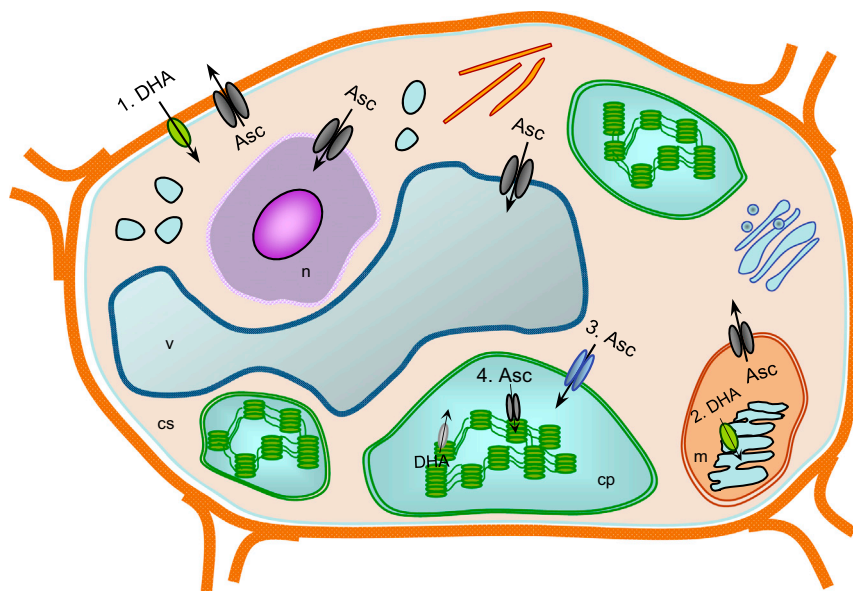
Figure 1. Identified and Putative Ascorbate Transporters in the Plant Cell.
The final step of ascorbate biosynthesis occurs in the mitochondrion from which ascorbate is transported into all the other cellular compartments and it is also transported throughout the plant via the phloem. Characterized ascorbate transporters include (1) a dehydroascorbate (DHA) transporter in the plasma membrane that is distinct from glucose carriers, (2) a DHA transporter, probably similar to glucose transporters, is located in the mitochondrion, (3) AtPHT4;4 was identified as a chloroplast-localized ascorbate transporter. Putative ascorbate and DHA transporters in the plant cell (indicated in gray) include PHT4;1, a candidate ascorbate transporter at the thylakoid membrane, ascorbate transporters responsible for the export of ascorbate out of the mitochondrion and through the plasma membrane, and transporters for ensuring the uptake of ascorbate into the vacuole and possibly into the nucleus (alternatively, ascorbate diffuses through the nuclear pores). DHA also has to be transported out of the thylakoid lumen for regeneration.
cp, chloroplast; cs, cytosol; m, mitochondrion; n, nucleus; v, vacuole.

(approximately 30% decrease), and this reduction was most pertinent under high light conditions. An important consequence of this reduced ascorbate content was an impairment of the xanthophyll cycle with the mutant exhibiting a decrease in nonphotochemical quenching. However, plant growth and photosystem II efficiency were not affected, suggesting that the decrease in ascorbate content due to the absence of PHT4;4 was not severe.

While representing a major advance for research on ascorbate, particularly with regard to the co-ordination of plant energy metabolism, this study is also of great interest from an evolutionary perspective. Indeed, the authors themselves speculate on the molecular nature of the changes that led to this member of a phosphate carrier family generating the capacity to transport ascorbate. Another important feature of the study is the finding that ascorbate transport is apparently controlled by the $\Delta\psi$. If this were the only mechanism of uptake, it would suggest that the concentration gradient between cytosol and chloroplast is unlikely to vary greatly since the $\Delta\psi$ does not change under physiological conditions; however, it is important to note that the concentration of ascorbate may be twice as high in the cytosol as in the chloroplast (Zechmann, 2011). This fact notwithstanding, the biochemical properties of the reconstituted AtPHT4;4 fit well with those of ascorbate uptake by isolated chloroplasts (reviewed in Szarka et al., 2013; Pick and Weber, 2014). However, this is not to say that AtPHT4;4 is the only plastid membrane ascorbate transporter; indeed, preliminary studies indicate that AtPHT4;1 is a candidate thylakoid membrane localized transporter and it is conceivable that there are yet further transporters localized to this organelle. While the study of Miyaji et al. (2015) provides strong evidence of the importance of ascorbate uptake by the chloroplast for its various roles, the identification of this transporter and its corresponding mutants provides the tools for a wide range of

questions to be addressed concerning the function of ascorbate within the chloroplast as well as offering hope that the proteins that transport ascorbate across other subcellular and even cellular barriers will be identified at the molecular level.

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http://dx.doi.org/10.1016/j.molp.2015.02.006

REFERENCES
Franceschi, V.R., and Tarlyn, N.M. (2002). L-Ascorbic acid is accumulated in source leaf phloem and transporter to sink tissues in plants. Plant Physiol. **130**:649–656.

Gest, N., Gautier, H., and Stevens, R. (2013). Ascorbate as seen through plant evolution: the rise of a successful molecule? J. Exp. Bot. **64**:33–35.

Guo, B., Jin, Y., Wussler, C., Blancaflor, E.B., Motes, C.M., and Versaw, W.K. (2008). Functional analysis of the *Arabidopsis* PHT4 family of intracellular phosphate tranporters. New Phytol. **177**: 889–898.

Maurino, V.G., Grube, E., Zielinski, J., Schild, A., Fischer, K., and Flügge, U.I. (2006). Identification and expression analysis of twelve members of the nucleobase-ascorbate transporter (NAT) gene family in *Arabidopsis thaliana*. Plant. Cell Physiol. **47**:1381–1393.

Miyaji, T., Kuromori, T., Takeuchi, Y., Yamaji, N., Yokosho, K., Shimazawa, A., Sugimoto, E., Omote, H., Ma, J.F., Shinozaki, K., et al. (2015). AtPHT4;4 is a chloroplast-localized ascorbate transporter in *Arabidopsis*. Nat. Commun. **6**:5928.

Nunes-Nesi, A., Carrari, F., Lytovchenko, A., Smith, A.M., Loureiro, M.E., Ratcliffe, R.G., Sweetlove, L.J., and Fernie, A.R. (2005). Enhanced photosynthetic performance and growth as a consequence of decreasing mitochondrial malate dehydrogenase activity in transgenic tomato plants. Plant Physiol. **137**:611–612.

Pick, T.R., and Weber, A.P.M. (2014). Unknown components of the plastidial permeome. Front. Plant Sci. **5**:410.

Szarka, A., Bánhegyi, G., and Asard, H. (2013). The inter-relationship of ascorbate transport, metabolism and mitochondrial, plastidic respiration. Antioxid. Redox Signal. **19**:1036–1044.

Tóth, S.Z., Nagy, V., Puthur, J.T., Kovács, L., and Garab, G. (2011). The physiological role of ascorbate as photosystem II electron donor: protection against photoinactivation in heat-stressed leaves. Plant Physiol. **156**:382–392.

Zechmann, B. (2011). Subcellular distribution of ascorbate in plants. Plant Signal. Behav. **6**:360–363.