AtDTX25, a member of the Multidrug And Toxic compound Extrusion family, is a vacuolar ascorbate transporter that controls intracellular iron cycling in Arabidopsis

Minh Thi Thanh Hoang, Minh Thi Thanh Hoang, Diego Almeida, Sandrine Chay, Carine Alcon, Claire Faillie, Catherine Curie, Stéphane Mari

To cite this version:

Minh Thi Thanh Hoang, Minh Thi Thanh Hoang, Diego Almeida, Sandrine Chay, Carine Alcon, et al.. AtDTX25, a member of the Multidrug And Toxic compound Extrusion family, is a vacuolar ascorbate transporter that controls intracellular iron cycling in Arabidopsis. New Phytologist, Wiley, 2021, 10.1111/nph.17526. hal-03258348

HAL Id: hal-03258348

https://hal.inrae.fr/hal-03258348

Submitted on 1 Sep 2022

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Distributed under a Creative Commons Attribution 4.0 International License
**TASC1**, a member of the Multidrug And Toxic compound Extrusion family, is a vacuolar ascorbate transporter that controls intracellular iron cycling in Arabidopsis

<table>
<thead>
<tr>
<th>Journal:</th>
<th><em>New Phytologist</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Manuscript ID</td>
<td>Draft</td>
</tr>
<tr>
<td>Manuscript Type</td>
<td>MS - Regular Manuscript</td>
</tr>
<tr>
<td>Date Submitted by the Author:</td>
<td>n/a</td>
</tr>
<tr>
<td>Complete List of Authors:</td>
<td>Hoang, Minh; UNIVERSITY OF SIENCE, Department of Plant Biotechnology and Biotransformation Almeida, Diego; INRAE, BPMP Chay, Sandrine; INRAE, BPMP Alcon, Carine; CNRS, BPMP Corratge-Faillie, Claire; CNRS, BPMP Curie, Catherine; Institut National de la Recherche Agronomique, Université Montpellier II, Laboratoire de Biochimie et Physiologie Moléculaire des Plantes Mari, Stéphane; INRA Montpellier, UMR5004 Biochimie et Physiologie Moléculaire des Plantes AGRO-M/ INRA 2</td>
</tr>
<tr>
<td>Key Words:</td>
<td>iron, transport, vacuole, ascorbate, MATE transporter</td>
</tr>
</tbody>
</table>
TASC1, a member of the Multidrug And Toxic compound Extrusion family, is a vacuolar ascorbate transporter that controls intracellular iron cycling in Arabidopsis

Minh Hoang*, Diego Almeida*, Sandrine Chay, Carine Alcon, Claire Faillie, Catherine Curie, Stephane Mari

BPMP, Univ Montpellier, CNRS, INRAE, Institut Agro, Montpellier, France

Author for correspondence:
Stephane Mari
stephane.mari@inrae.fr

* These authors contributed equally to this work

Abstract
- Iron (Fe) is an essential element, its transport is regulated by the cell redox balance. In seeds, Fe enters the embryo as Fe$^{2+}$ and is stored in vacuoles as Fe$^{3+}$. Through its ferric reduction activity, ascorbate plays a major role in Fe redox state and hence Fe transport within the seed.
- We have searched for ascorbate membrane transporters responsible for controlling Fe reduction through a screening in the yeast ferric reductase-deficient fre1 strain and have isolated a member of the Multidrug And Toxic compound Extrusion (MATE) family.
- TASC1 (for Transporter of ASCorbate 1) was shown to mediate ascorbate efflux when expressed in yeast and Xenopus oocytes, in a pH-dependent manner. In planta, TASC1 is highly expressed during germination and encodes a vacuolar membrane protein. Isolated vacuoles from a tasc1-1 knockout mutant contained less ascorbate and more Fe than WT and mutant seedlings were highly sensitive to Fe deficiency. Iron imaging further showed that the remobilization of Fe from vacuoles was highly impaired in mutant seedlings.
- Taken together, our results establish TASC1 as a vacuolar ascorbate transporter, required during germination to promote reduction of the pool of stored Fe$^{3+}$ and its remobilization to feed the developing seedling.

**Introduction**

Iron (Fe), the second most abundant metal of the Earth crust, is essential for life. Chronologically, iron has been the first metal used by living organisms as a cofactor for a wide range of biological processes such as molecular oxygen activation, reduction of ribonucleotides, metabolism of peroxides and electron transfer reactions. Despite its apparent abundance, Fe poses a real conundrum to all living organisms since environmental Fe is found mostly as the poorly soluble (10^{-18}M), exchange-inert, Fe$^{3+}$ form whereas the active form, Fe$^{2+}$, has a strong pro-oxidant activity. Redox cycling between Fe$^{2+}$ and Fe$^{3+}$ is thus a central process for the regulation of Fe uptake, storage and utilization. Most living organisms, including mammals, plants and fungi, possess membrane-bound metal reductases belonging to two major families, cytochrome b561 (mammals) and flavo-cytochromes (fungi, plants), that play a crucial role in generating Fe$^{2+}$ that is either readily transported by divalent metal transporters or transferred to ferroxidase/permease transport systems (reviewed in (Kosman, 2010; Jain et al. 2014)). At the intracellular level, storage and remobilization of Fe also rely on redox cycling. The incorporation of excess Fe into ferritin protein complexes requires a ferroxidation step catalyzed by ferritin itself whereas Fe release from these complexes requires a one electron reduction step that is most probably mediated by physiological reductants such as ascorbate (Watt et al., 1988; Laulhere & Briat, 1993; De Domenico et al., 2006; Melman et al., 2013). Yeast cells, that do not produce ferritins, store Fe in the vacuole as Fe$^{3+}$-(poly)-phosphate (Cockrell et al., 2011; Park et al., 2014) and the remobilization of this pool is mediated by the lumen-oriented ferric reductase FRE6 that supplies Fe$^{2+}$ either to the ferrous efflux transporter Smf3p or to the ferroxidase/permease complex encoded by Fet5p/Fthp (Singh et al., 2007). In mature embryo of the model plant *Arabidopsis thaliana* Fe is also stored as Fe$^{3+}$ in vacuoles of specific cells surrounding the provascular strands (Lott & West, 2001; Lanquar et al., 2005; Kim et al., 2006; Roschitztardtz et al., 2009). Although the overall mechanism of Fe retrieval from vacuoles remains uncharacterized, two divalent metal
transporters encoded by NRAMP3 and NRAMP4 play a key role in the efflux of Fe from vacuoles during germination (Lanquar et al., 2005), implying that, as shown in yeast, a reduction step is likely to be required to generate Fe$^{2+}$, the bona fide substrate of NRAMP proteins. Storage and remobilization of this particular Fe pool is crucial for the fate of the future seedling since mutations in either the vacuolar influx transporter VIT1 or the efflux transporters NRAMP3 and NRAMP4 severely compromises the growth of the seedling in Fe-limiting conditions (Lanquar et al., 2005; Kim et al., 2006).

In plants, we have recently demonstrated that redox cycling of Fe is also crucial for the transport between maternal tissues (seed coat) and the embryo. Indeed, isolated embryos from Arabidopsis and pea exhibit high ferric reduction activity, necessary for Fe uptake, which is not encoded by the expected membrane reductases of the FRO family. Instead, embryos efflux massive amounts of ascorbate, which reduces Fe$^{3+}$ ions that are delivered from the maternal tissues as Fe-citrate-malate complexes (Grillet et al., 2014). The association of ascorbate-assisted reduction of Fe with transmembrane uptake of Fe$^{2+}$ thus uncovered in plants is reminiscent of the Fe uptake machinery described in human brain; indeed neuronal cells take up Fe from Fe$^{3+}$-citrate complexes following a reduction step, which is catalyzed by ascorbate released from these cells (Lane et al., 2010). Taken together, these discoveries in both animal and plant systems have shed a new light on Fe homeostasis, where ascorbate efflux represents a new paradigm for ferric reduction and transport (Lane & Richardson, 2014).

Nevertheless, although ascorbate efflux activities have been reported in many instances, the identity of the corresponding transport system remains elusive. To date, several plausible candidates have been proposed, including exocytosis and/or plasma membrane anion channels (reviewed in (Wilson, 2005)) however, no clear-cut demonstration of their identity and function as ascorbate efflux transporters has been provided.

In the present work, we describe the isolation of an ascorbate efflux transporter that belongs to the Multidrug And Toxic Compound Extrusion family (MATE). This transporter, named **TASC1** (for Transporter of ASCorbatte 1) was isolated through a yeast complementation screening using the ferric reductase mutant Δfre1. We further show that **TASC1**, which encodes a protein targeted to the vacuolar membrane in plant cells, is expressed in seeds and most importantly during germination, where vacuolar Fe is crucial to sustain growth of the developing seedling. Phenotypic analysis of plants mutated in **TASC1** further allowed
establishing that this protein plays a direct role in the redox cycling of Fe and its export from the vacuole, a mechanism that is key to sustain growth of the developing seedling. Since MATE are ubiquitous efflux transporters, these findings pave the way for future discoveries linking ascorbate to Fe homeostasis in all living eukaryotic organisms.

Materials and Methods

Yeast screening conditions. The yeast mutant defective in the ferric reductase Fre1p (Δfre1, MATa, his3, leu2, met15, ura3, fre1::kanMX4) was used in this work. Transformants were grown on Yeast Nitrogen Base (YNB) medium supplemented with glucose (2% w/v), histidine, leucine and methionine (50 µg.L⁻¹). Iron deficiency was induced by the addition of 75µM bathophenanthroline disulfonate (BPDS). The screening media also contained 5mM of the ascorbate precursor L-galactono-lactone (GL). The Δfre1 cells were transformed with an Arabidopsis cDNA library (Minet & Dufour, 1992) constructed in the pFL61 shuttle vector. Clones growing in the YNB medium containing BPDS and GL were further selected for plasmid extraction and sequencing.

Measurement of ferric reduction activity. To measure ferric reduction activity of the yeast cells, the cells grown on medium with or without 10 mM L-GL overnight. Cell suspensions were centrifuged and washed 3 times with sterilized water. The cells were then incubated in the assay solution containing 5 mM MES buffer pH = 5.5, 300 µM (BPDS) and 100 µM Fe³⁺-EDTA. Fe reduction activity was monitored as the concentration of Fe²⁺-BPDS³⁻ (bathophenanthroline-disulfonate) complex formed in the assay solution by measuring absorbance at 535 nm with a Hitachi U-2800 spectrophotometer. OD535 was measured after 1 hour incubation in the dark at 22-25 °C with 250 RPM shaking.

Heterologous expression of TASC1 in Xenopus laevis oocytes and efflux activity assay. The TASC1 coding region was cloned into modified pGEM-HE vector. cRNA were synthesized from 1 µg of linearized vector, Nheli-digested, using the HiScribe™ T7 ARCA mRNA Kit with tailing (NEB, http://www.NEB.com). Oocytes obtained surgically from benzocaine-anesthetized Xenopus laevis were defolliculated using a 1 h collagenase-treatment under gentle shaking at 20°C in 25 ml of OR2 medium (NaCl 82.5mM, KCl 2mM, MgCl₂ 1 mM, HEPES-NaOH pH 7.4) supplemented with 25 mg of collagenase (type IA, Sigma). Sorted stage V and VI oocytes were then stored at 20°C in ND96 medium (NaCl 96mM, KCl 2mM, 1.8mM MgCl₂, CaCl₂ 1mM, 2.5mM Na-Pyruvate, HEPES-NaOH pH7.4) supplemented with 50 mg/ml gentamicin. Oocytes
were injected with 32 ng (50 nl) of AtTasc1 cRNA using a micropipette (10–15 µm tip diameter) and a pneumatic injector. After two days, TASC1 cRNA injected oocytes and non-injected oocytes (used as control) were transferred to fresh ND96 solution (pH 6.5) supplemented with 10 mM L-Ascorbic acid and injected with 25 nl of 100 mM [13C]-labeled L-Ascorbic acid (Sigma-Aldrich, Ref. 795097, prepared in 100 mM Tris-MES pH 7.5). After 5 min of recovery, injected oocytes were washed 5 times with ice-cold ND96 solution (pH 6.5), then placed for efflux measurements by batches (n=3) of 10 oocytes into 2 ml vials at room temperature in ND96 medium. The requirement of a proton gradient was measured by incubations in different ND96 set at different pH values (pH 5.5, 6.5 or 7.5) for 2 hrs. After incubation, 100 µl of ND96 medium from each batch were sampled in 3 replicates and dried. The [13C] abundance was analyzed from sampled ND96 medium using an IsoPrime stable isotope analyzer Mass Spectrometer (https://www.elementar.de).

**HPLC measurement of ascorbate.** For plant samples, material was collected, weighed and put in the eppendorf tubes containing a plastic bead and stored in liquid nitrogen. Samples were then ground and extracted with 5% (v/v) o-phosphoric acid. The homogenate was centrifuged at 14000 rpm for 10 min at 4°C and the supernatant was collected and filtered for the analysis of total ascorbate. Total ascorbate was determined after treatment with 2 mg/ml DTT for 25 min at room temperature in darkness. Isolated vacuoles were treated with phosphoric acid, then vortexed vigorously and ascorbate concentration was measured by HPLC. The separation was performed with isocratic elution using 0.1% TFA in water. The analyses were carried out by injecting 10µL of samples onto a Nucleodur C18 column (particle size 5µm, pore size 100A) connected to a Varian PrepStar pump using a flowrate of 0.7 mL.min⁻¹. Ascorbate was detected using a UV-Visible spectrophotometric detector at 244nm and quantified with an external standard calibration curve with pure ascorbate.

**Plant materials and growth conditions.** For soil-grown plants, seeds were stratified at 4°C for 2-3 days and sown onto the potting soil. Alternatively, 7-days old seedlings grown in vitro were transferred into the soil. The greenhouse conditions for soil-grown plants were 16 hours light, 8 hours dark at 22°C and 70% humidity. For in vitro culture, after surface sterilization with 50% ethanol, 12.5% bleach solution and 37.5% water for 10 min, seeds were rinsed three times with 100% ethanol and stratified at 4°C for 2 days. For growth test on agar plates containing different iron conditions, seeds were germinated on half strength MS. Iron sufficient medium is half strength Murashige and Skoog (MS) medium containing 0.05% MES, 1% sucrose, 0.8%
agar and 50µM Fe³⁺-citrate. The iron deficient medium is the same without Fe and a
supplement of 5 µM BPDS. For hydroponic cultures, 10-day-old plantlets were transferred
from half strength MS agar plates to Arabidopsis hydroponic solution (Hoagland solution-
appendix1). The hydroponic medium was renewed every 10 days. The growth chambers
conditions were 16 hour light/8 hour dark photoperiod at 21°C temperature and 65% the
relative humidity. The tasc1-1 homozygous mutant line was isolated from GABI-Kat (GK_912H)
using the following primers EP16 (5’-TAAAAGTGGTGCTTGTGGTATCAG-3’) and EP17 (5’-
AATAAATGAGTATTTTTATGTAG-3’). Total RNA was extracted with Trizol and first strand cDNA
was synthesized with the RevertAid reverse transcription kit (Thermo Scientific). Full-length
TASC1 cDNA was amplified by RT-PCR with TASC1-FP (5’-GGAGATAGAACCATGAGGTGGTTGAGA-3’) and TASC1-RP (5’-
TCCACCTCCGGATCCTACTTCTCTCTCTACTCTTTG-3’), AtACT2 cDNA (actin 2, AT3G18780),
used as loading control, was amplified using the following primers: 5’-
TGCTGTTGACTACGAGG-3’ and 5’-TCTGCTGGAATGTGCTGAGG-3’. The search for putative
truncated versions of the full-length cDNA was realized using seven different reverse primers
hybridizing the end of the different exons, in order to amplify cDNA fragments of increasing
size up to the full-length version. The forward primers used were, respectively: rev1: 5’-
CCATAATACCTGATGAAGC-3’; rev2: 5’-CAAAGCATGAAGCCGGAGGA-3’; rev3: 5’-
CATATGGAGAAAGCGGAAATTG-3’; rev4: 5’-CATGCTGCACCCTACACTAGCAG-3’; rev5: 5’-
CCAGATAGAATGGCCTGGATAC-3’; rev6: 5’-CTTAAATCCAAAATGAAAAAC-3’; rev7: 5’-
TCACCTTCTCTCCTTCTTTG-3’.

DNA cloning. All cloning reactions were realized using the Gateway™ system (Invitrogen). All
attB-tailed PCR products were initially cloned into pDONR207 using BP Clonase, and their
sequence verified before subcloning into various plant expression vectors (using LR Clonase)
mentioned elsewhere. The cDNAs or promoter regions were amplified by a two step-PCR using
the high-fidelity Phusion polymerase (Thermo Scientific, Waltham, MA, USA) and the following
specific primers (At5g17700 FP, 5’-GGAGATAGAACCATGAGGTGGTTGAGA-3’;
At5g17700 RP stop, 5’-TCCACCTCCGGATCTACCTTTCTTCTCTCTACTCTTTG-3’; At5g17700 RP C-
ter, 5’-TCCACCTCCGGATCCTCTTCTCTCTCTCTCTTTG-3’; promoAt5g17700 FP, 5’-
GGAGATAGAACC CAATAGTTATGAGTCTCATGCA-3’; promoAt5g17700 RP, 5’-
TCCACCTCCGGATCC TTTTCTCTCTTTGATTTTCTTTT-3’) containing universal Gateway attB1 and
universal Gateway attB2 sequences (underlined) for gateway recombination. For the TASC1-
GFP fusion, the TASC1 cDNA was amplified without its stop codon and cloned into the pDONR 207 entry vector (Gateway; Invitrogen). TASC1 cDNA was subsequently fused with GFP by recombination with the destination vector pGWB5 to generate the C-terminal GFP fusion, under the control of the CaMV 35S promoter.

Confocal microscopy. For intracellular localization, the homozygous T2 transgenic plants were imaged by confocal microscopy (Leica SP8) with laser excitation at 488 nm, emission light at 535 nm for GFP and laser excitation at 515 nm, emission light at 640-670 nm for FM4-64. Roots of 10-day-old plantlets were mounted in water for microscopy observation or stained with 0.1 µg/ml FM4-64 for 5 minutes and washed twice in water before observation under the microscope. For the fluorescent labelling of vacuoles, 10-day old seedlings were incubated with Neutral Red (4 µM) for 20 minutes and signal was acquired at 500-540 nm with an excitation at 561 nm.

Glucuronidase histochemical staining. Histochemical staining for GUS in transgenic Arabidopsis plants was performed according to standard procedures with minor modifications. Plant tissues at different development stages including germinating seeds, seedlings, rosette leaves, flower and siliques were picked using clean forceps and immersed in a X-Gluc stain solution (50 mM NaPO$_4$, 0.5 mM ferrocyanide, 0.5 mM ferricyanide, 0.05% (v/v) Triton X-100, pH 7.2, and 1 mM 5-bromo-4-chloro-3-indolyl-β -D-glucuronide) overnight at 37°C. For tissues containing chlorophyll, they were soaked in ethanol with 50%, 70%, 90% and 100% successively until the samples were clear. For GUS histochemical staining in embryos, embryos were fixed by 30 min of vacuum infiltration followed by 15 hour of soaking in fixation solution containing 2% (w/v) paraformaldehyde, 1% (v/v) glutaraldehyde and 1% (w/v) caffeine in 100 mM phosphate buffer (pH 7). Embryos were then dehydrated in successive baths of 50%, 70%, 90%, 95% and 100% ethanol, butanol/ethanol 1:1 and 100% butanol and embedded in the Technovit Resin according to the manufacturer’s instructions. Thin sections (4-6µm) were cut and observed under the microscope.

Iron staining with the Perls/DAB procedure. The staining protocol for Arabidopsis seedling and histological sections described by Roschztattardtz et al. (2009) was followed.

Vacuole isolation from leaves. Vacuoles were isolated as described by Robert et al. (2007) with some modifications. Firstly, to isolate mesophyll protoplasts, 2 g of rosette leaves from 30-day-old plants were collected and cut into small pieces. The processed leaves were floated on protoplast enzyme solution (1% w/v cellulase, 1% w/v macerozyme, 0.4 % w/v CaCl$_2$.2H$_2$O...
400 mM Mannitol and 10 mM MES pH6.0) and incubated in 4 hours with gentle shaking. The released protoplasts were collected by centrifugation for 10 min at 19g and the pellets were re-suspended in the washing buffer to remove hydrolytic enzymes. To isolate vacuoles, 12.5 ml of pre-warmed lysis buffer (0.2 M Mannitol, 10% v/v Ficoll, 10 µM EDTA pH=8, 5µM NaPO₄ pH 8.0 and 0.05% Neutral Red) were added to protoplast and re-suspended gently by pipetting 5-8 times and checking the protoplast disruption and vacuoles release under the light microscope every 2 minutes. Vacuoles were then purified by overlaying with 6 ml 4 % (v/v) Ficoll solution (mix 3 ml of lysis buffer and 4.5 ml of vacuole buffer : 450 mM mannitol, 5 µM NaPO₄ pH 7.5, 2 µM EDTA pH 8.0), and addition of 2 ml of cold vacuole buffer on the top of the gradient. After centrifugation for 50 min at 100000g at 10°C in Optima L-90K ultracentrifuge using slow acceleration and slow deceleration, the vacuoles were recovered from the interface between middle and upper layers.

**Elemental analyses.** Shoots were collected and briefly rinsed with distilled water. Roots were desorbed by washing for 10 min with 2mM CaSO4 and 10mM EDTA and for 3 min with 0.3 mM BPDS and 5.7 mM sodium dithionite and were then rinsed twice in deionized water. Samples were dried at 80°C for 2 days. For mineralization, tissues were digested completely in 70% (v/v) HNO₃ and 30% (v/v) H₂O₂ for 45 minutes at 180°C in a microwave (Berghof speedwave digestion system). Elemental analysis was performed by Microwave Plasma-Atomic Emission Spectrometer (MP-AES, Agilent) using calibration standard solutions provided by the manufacturer.

**Data analysis.** All numerical values showed are means ± standard deviation, the number of biological replicates is indicated as n. When the number of replicates was higher than 6, statistical significance was tested using the Student’s test, p values are indicated in the figure legends. For experiments realized with a number of replicates lower than 6, the statistical significance was tested with the Wilcoxon non parametric test.

**Results**

**TASC1 mediates ascorbate transport in yeast cells and Xenopus oocytes**

Yeast complementation was used as a strategy to search for ascorbate (AsA) efflux transporters from Arabidopsis. The yeast Δfre1 strain, mutated in the major ferric chelate reductase of the cell surface, is unable to acquire Fe in deficiency conditions and thus grows
poorly in Fe-limiting media (Georgatsou et al., 1997). A cDNA library from Arabidopsis thaliana was transformed in Δfre1 with the idea that expression of a transporter able to mediate AsA efflux in the medium should restore, at least in part, the ferric reduction capacity of the cell. The growth medium was supplemented with L-galactono-lactone (GL), the biosynthetic precursor that is readily converted in AsA by yeast cells that otherwise do not produce AsA naturally (Berczi et al., 2007). Among the positive clones picked for their restored growth on Fe-limiting media, a clone expressing a cDNA coding for a member of the Multidrug And Toxic Compound Extrusion family (MATE) was considered of high interest. Indeed, the hallmark of the MATE proteins characterized so far is their capacity to mediate efflux of organic anions such as organic acids and xenobiotics. According to the MATE nomenclature, this clone corresponded to AtDTX25 (At5g17700) and was named TASC1, for Transporter of ASCorbate 1. Based on the ability of TASC1 expression to restore growth of the Δfre1 mutant (Fig. 1a) and since this gene belongs to a family of bona fide efflux transporters, we then analyzed the capacity of yeast cells to reduce Fe$^{3+}$ through the efflux of AsA. The ferric reduction activity of yeast cells was measured in vitro using the Fe$^{2+}$ specific chromophore bathophenanthroline disulfonic acid (BPDS). Cells expressing TASC1 displayed a significant increase in ferric reduction, compared to empty vector controls, specifically when cells had been pre-loaded with the AsA precursor GL. This result indicated that the production of Fe$^{2+}$ was mediated by TASC1 and was ascorbate-dependent (Fig. 1b). We then analyzed the AsA efflux activity of whole cells by measuring the concentration of AsA released in the medium, after preloading cells with GL. In these conditions it was possible to show that cells expressing TASC1 were able to efflux significant amounts of AsA in the medium within 3 hours, compared to empty vector control cells (Fig. 1c). In order to further support these results with protein localization, we also expressed an TASC1-GFP fusion in the yeast Δfre1 strain. Depending on the level of expression in the different clones, TASC1-GFP could be visualized at both the plasma membrane and endomembranes including the vacuole (Fig. S1a) or only at the plasma membrane (Fig. S1b). Drop tests further showed that this TASC1-GFP fusion was functional, on the basis of the complementation of the growth defect of the Δfre1 strain (Fig. S1e). Further direct evidence of the ascorbate transport activity of TASC1 was obtained by expressing TASC1 in Xenopus oocytes. The release of $^{13}$C in the medium was monitored from oocytes previously injected with a 10mM $^{13}$C-ascorbate solution over a four-hour period (Fig. 1d). A significant efflux of ascorbate could be detected from oocytes expressing TASC1 with a
progressive increase in the efflux activity over the 4-hour period tested. Based on this result, we then tested the requirement of a proton gradient for the efflux activity of TASC1 by raising the pH to 7.5. Compared to the efflux rate measured at pH 5.5, incubating the oocytes at pH 7.5 totally abolished ascorbate efflux, which indicates that TASC1 activity does require a proton gradient. Taken together, these results strongly support the hypothesis that TASC1 is capable of mediating transmembrane efflux of ascorbate from the cytosol. The dependency on a pH gradient for the transport activity of TASC1 is an additional indication that this transport activity corresponds to an antiport system that requires a proton gradient for the secondary transport of ascorbate.

**TASC1 is a vacuolar transporter expressed during seed development and germination**

To determine the expression profile of TASC1 in planta, we examined the activity of 2 kb of its promoter fused to the *uidA* reporter gene encoding the β-glucuronidase (GUS). GUS activity was observed in the seed testa (outer and inner integuments), seed endosperm, developing embryo and in seedlings (Fig. 2a-c). TASC1 promoter was also active in flowers, throughout the developmental stages. In young flowers GUS activity could first be detected in the anthers, anther filaments and in petals. The expression of TASC1 in petals increased with the age of flowers (Fig. 2d).

The subcellular localization of TASC1 was then analyzed in planta by observing GFP fluorescence of transgenic Arabidopsis plants expressing TASC1-GFP under the control of the CaMV 35S promoter. Confocal microscopy analyses of root cells showed that the GFP fluorescence did not co-localize with the plasma membrane marker FM4-64 (Fig. 3a). Instead, the GFP signal was clearly observed on the membrane of a compartment occupying the vast majority of the cell volume and depicting an invagination that is reminiscent of the localization of the nucleus (Fig. 3a, arrowheads), these features corresponding to the vacuole. In order to further confirm the tonoplastic localization of TASC1, we incubated TASC1-GFP seedlings with Neutral Red, a dye used to stain the vacuoles of living cells. Besides being a chromophore, Neutral Red can also be used as a fluorophore, that emits a red fluorescence when the molecule is trapped in vacuoles (Dubrovsky et al., 2006). The merging of TASC1-GFP and Neutral Red signals clearly showed red fluorescent vacuoles surrounded by a green fluorescent signal of TASC1-GFP, confirming that in planta TASC1 is targeted to the tonoplast and would therefore play a role at the level of the vacuole.
TASC1 regulates vacuolar Fe export

In order to study the function of TASC1 in planta, we isolated a T-DNA mutant (tasc1-1) with an insertion that was further located by sequencing in the last exon of the TASC1 coding region (Fig. S2a). Semi-quantitative reverse transcriptase amplification of the TASC1 cDNA using seven different reverse primers located at the end of the different exons of TASC1 showed that some truncated versions of the TASC1 cDNA could be amplified, never beyond the sixth exon (Fig. S2b). These truncated forms were still missing ca 40% of the full-length cDNA and it was thus concluded that the tasc1-1 mutation corresponded most likely to a knock-out allele (Fig. S2b). To test the function of TASC1, we first focused on the growth of seedlings just after germination. Indeed, during these early stages of growth, when TASC1 is highly expressed, the Fe pools stored in the vacuoles of the embryo are actively remobilized to feed the expanding tissues, before the seedling reaches an autotrophic regime (Lanquar et al., 2005). Therefore, seeds of WT, tasc1-1 and a TASC1-GFP complemented line were sown on standard (50 µM Fe-citrate) and Fe deficient (no added Fe, supplemented with the Fe chelator BPDS) media and grown for 7 days. Whereas no difference in growth was observed in Fe-replete condition, tasc1-1 seedlings appeared somewhat stunted in –Fe, with paler cotyledons and shorter roots, compared to WT (Fig. 4a-b). This sensitivity of tasc1-1 seedlings to Fe deficiency was alleviated by the ectopic expression of a functional version of TASC1, indicating that the increased sensitivity phenotype was indeed attributable to the inactivation of TASC1 (Fig. 4a-b).

To analyze in more depth the effect of TASC1 mutation on the fate of Fe pools during germination and seedling growth, we monitored Fe remobilization from the vacuoles by histochemical staining using the Perls/DAB procedure (Roschztardtz et al., 2009). Embryos dissected from imbibed seeds of WT, tasc1-1 mutant and complemented lines displayed equivalent Fe staining (brown/black pigments) in the pro-vascular system of cotyledons and hypocotyle (Fig. 5 upper lane). After 3 and 5 days of growth without Fe, in both WT and complemented lines, Fe was completely remobilized since Fe was no more detectable in the vasculature of cotyledons and hypocotyls (Fig. 5). In contrast, tasc1-1 mutant seedlings retained staining in the vascular system, mostly in the cotyledons (Fig. 5, arrowheads). Histological analysis of Fe localization in thin sections of resin-embedded seedlings further confirmed that Fe was indeed still visible in vacuoles of the tasc1-1 mutant, whereas it had...
completely disappeared in both WT and complemented line (Fig. 5, lower row, arrows). These results indicated that in the tasc1-1 mutant, the Fe remobilization process was strongly affected, which could explain the reduced growth capacity of tasc1-1 mutants in -Fe conditions when seedlings strictly depend on this particular vacuolar Fe pool for their survival and growth.

We next investigated whether impaired Fe remobilization from vacuoles of the tasc1-1 mutant also occurred in older plants. When grown in Fe-deficient hydroponic condition during three weeks, the growth of tasc1-1 mutant plants was severely reduced, compared with WT and complemented line (Fig. 6). Like for young seedlings, Perls-DAB staining on histological sections of mature leaves showed the presence of Fe in the vacuoles of cells surrounding the vascular system in tasc1-1 (Fig. 7a, arrows), but not in WT and complemented line. This observation therefore suggested that TASC1 function is not restricted to the early development but also participates in Fe retrieval from vacuoles at different stages of plant growth.

Taken together, our results strongly suggested that the function of TASC1 is to transport ascorbate in vacuoles, in order to reduce Fe$^{3+}$ and thus remobilize the Fe pools stored in these vacuoles. To test this hypothesis, we isolated vacuoles from leaf protoplasts and quantified metals (Fe, Zn, Mn) and ascorbate (Fig. 7b and c, respectively). Vacuoles of tasc1-1 mutant protoplasts contained 3 times more Fe than WT vacuoles, consistent with the Fe imaging analyses. The concentration of other metals such as Zn and Mn remained similar between tasc1-1 and WT however. We therefore concluded that Fe is immobilized in the vacuoles of the tasc1-1 mutant. Contrary to Fe, ascorbate accumulated twice less in tasc1-1 vacuoles compared to WT (Fig. 7c), despite the fact that at the whole leaf level, both WT and tasc1-1 mutants did contain comparable amounts of ascorbate (Fig. S3). The concomitant increase of Fe and decrease of ascorbate measured in tasc1-1 vacuoles strongly suggested that remobilization of vacuolar Fe requires the presence of ascorbate within the vacuole, which itself likely relies on TASC1 ascorbate influx across the tonoplast. In this scenario, the inability of tasc1-1 cells to retrieve Fe from vacuoles would limit plant growth, reducing in fine its tolerance to Fe deficiency.

Discussion
In the model plant *Arabidopsis thaliana*, the general mechanism of ionic Fe transport across membranes, that is based on trans-membrane ferric reductases of the FRO family to generate Fe$^{2+}$, has been recently challenged by the discovery of the role of ascorbate as an iron-reducing molecule directly involved in the Fe transport process in seeds (Grillet *et al.*, 2014). This finding, reminiscent of the Fe uptake from non-transferrin bound iron (NTBI) described in human astrocyte cells (Lane *et al.*, 2010), represents a new mechanism to couple Fe reduction with transmembrane transport at the surface of the embryo. Here, we show through the characterization of the MATE transporter TASC1, that this mechanism also occurs at the intracellular level, between the vacuolar lumen and the cytosol.

Indeed, we have demonstrated that TASC1, a tonoplast-targeted protein from Arabidopsis, is competent in the transport of ascorbate when expressed in the yeast ferric reductase-defective mutant *fre1* and in Xenopus oocytes. Moreover, in Arabidopsis, TASC1 participates in the control of the efflux of Fe from the vacuole since we show that the *tasc1-1* mutant fails to release Fe from vacuoles. MATE transporters mediate the secondary transport of a wide variety of molecules such as nicotine, citrate, flavonoids, salicylic acid, protocatechuic acid and abscisic acid (Takanashi *et al.*, 2014). Given the physiological importance of these molecules, MATE transporters have emerged as important actors in some developmental processes such as root development as well as in response to environmental stresses like aluminum toxicity or iron and phosphorus translocation in deficiency conditions. Accordingly, MATE transporters are generally classified based on the family of substrates transported or by the physiological processes they are involved in. TASC1 could thus be part of the “Fe translocation” group of MATE proteins, that includes the citrate transporter FRD3 and related proteins (Rogers & Guerinot, 2002) and the protocatechuic acid transporters PEZ1 and PEZ2 (Bashir *et al.*, 2011; Ishimaru *et al.*, 2011). Citrate efflux in the apoplast is crucial for the iron mobility between cells that are symplastically disconnected, such as the root xylem parenchyma and pollen grains (Roschztardtz *et al.*, 2011). Likewise, in rice the efflux of protocatechuic acid in the xylem sap plays a central role in the Fe deficiency response by increasing the solubilization of precipitated Fe (Bashir *et al.*, 2011; Ishimaru *et al.*, 2011). Although TASC1 transport activity can be linked with “Fe translocation”, the nature of its substrate, ascorbate, represents an entire new transport function, related but potentially not limited to Fe homeostasis.

Despite the fact that ascorbate is an essential molecule for plants that plays multiple roles in many subcellular compartments, the only ascorbate transporter identified until now is
PHT4;4, a member of the PHosphate Transporter family (PHT) that mediates the influx of ascorbate in chloroplasts. The role of PHT4;4 is to fuel chloroplasts with ascorbate that is required as a co-substrate for enzymatic reactions of the non-photochemical quenching, the key metabolic responses of chloroplasts to high light stresses (Miyaji et al., 2015).

Here, we have provided biochemical, physiological and genetic evidences, which together establish that TASC1 is a new ascorbate transporter in plants. Using yeast cells and oocytes as heterologous systems, we have shown that TASC1 has the capacity to mediate the efflux of ascorbate in the medium. Moreover, the ascorbate transport activity was shown to require a proton gradient as the driving force, suggesting that TASC1 may function as an ascorbate/H+ antiporter, which is the typical transport signature of all the MATE transporters characterized so far (Takanashi et al., 2014). The targeting of TASC1 at both the plasma membrane and the tonoplast in yeast cells, revealed by transport activity assays and confocal microscopy, should be taken with caution as it likely resulted from TASC1 being produced in a heterologous system. Actually, the overexpression of plant tonoplastic proteins in Saccharomyces cerevisiae often leads to a default targeting of a fraction of the molecules to the plasma membrane, thus explaining the complementation of a plasma membrane function by tonoplastic proteins. It is the case for NRAMP3 and NRAMP4, which despite being specifically targeted at the tonoplast in plants, were shown to efficiently complement the plasma membrane uptake systems Smf1p and Fet3p/Fet4p in yeast (Thomine et al., 2000).

Our study has shed a new light on the role(s) of ascorbate in the vacuole. Compared to other organelles such as the chloroplast or the nucleus, the relative concentration of ascorbate reported in the vacuoles is unexpectedly low. Immunocytochemical studies have established that vacuoles contain no more than 3% of the total ascorbate labeling in cells (Zechmann et al., 2011). However, upon high light stresses, the highest induction of ascorbate accumulation was observed in vacuoles, suggesting a role of this intracellular ascorbate pool in stress responses (Zechmann et al., 2011). Given its high reactivity towards oxidizing molecules, the antioxidant activity of ascorbate will inevitably generate monodehydro-ascorbate (MDHA), the oxidized form of ascorbate. The classical regeneration of ascorbate from MDHA, known as the Foyer-Halliwell-Asada cycle, is not likely to take place in vacuoles since none of the enzymatic components of the cycle have been located in the vacuolar lumen. Instead, an alternative mechanism has been proposed to occur, specifically in vacuoles. This mechanism would involve transmembrane electron transfer proteins from the cytochrome b561 family.
For Peer Review

(Cyt-B) that are ubiquitous proteins, found in animal and plant phyla. The Cyt-B proteins characterized so far were shown to use ascorbate as the electron donor, on the cytosolic side, to catalyze a one-electron transfer to an acceptor that could be MDHA or ferric iron, on the extra-cytosolic side (Berczi & Zimanyi, 2014; Lu et al., 2014). Interestingly, several members of the Cyt-B family have been identified as tonoplastic ascorbate-dependent oxido-reductases in plants (Preger et al., 2005; Berczi et al., 2007).

Based on these findings and on our new results, it is possible that tonoplastic cytochrome b561 would not be directly involved in Fe redox metabolism in the vacuole but rather in the regeneration of ascorbate from MDHA that is produced as a result of Fe$^{3+}$ reduction. This mechanism would take place rapidly after germination to provide Fe to the developing plant.

Indeed, all the partners potentially required for this process, AtTASC1, AtCYB1 and AtCYB2, AtNRAMP3 and AtNRAMP4 are highly expressed in the vascular tissues after germination (Verelst et al., 2004; Lanquar et al., 2005).

However, contrary to most of the genes involved in the response to Fe deficiency, the expression of TASC1 was not induced by Fe limitation (Fig. S4). Therefore, the regulation of TASC1-mediated ascorbate-dependent Fe remobilization might occur at the level of ascorbate itself. Indeed, Fe deficiency was shown to increase ascorbate accumulation in several plant systems (Zaharieva & Abadia, 2003; Zaharieva et al., 2004; Urzica et al., 2012), through the induction of genes involved in ascorbate biosynthesis (VTC2) and recycling (MDAR1) (Urzica et al., 2012). The rapid adjustments in ascorbate concentration might thus be more efficient to cope with fluctuations of Fe status than changes in TASC1 expression. In terms of expression profile, TASC1 and the citrate effluxer FRD3 display some overlap, since both genes are expressed in the embryo and integument of the developing seed, in young seedlings and in anthers (Roschztardtz et al., 2011). This overlap in expression profile could be an indication that both transport activities, and therefore both ascorbate and citrate, are required for the movement of Fe between symplastically disconnected cells, as previously reported for FRD3 (Roschztardtz et al., 2011). Such a mechanism where citrate and ascorbate act together for Fe translocation is reminiscent of the Fe trafficking in the brain where Fe-citrate (generally referred to as non-transferrin-bound iron) is reduced by ascorbate into Fe$^{2+}$ that is then imported by transporters of the NRAMP family (Moos et al., 2007; Lane & Lawen, 2008; Lane & Richardson, 2014).
In the proposed mechanism of Fe remobilization from vacuoles, it is assumed that Fe is stored in vacuoles under its oxidized form. Although the speciation of Fe in vacuoles has never been fully addressed, several experimental evidences support this hypothesis and thus the need for a reduction step during its remobilization. First, this vacuolar Fe pool in embryos reacts with ferrocyanide, an iron dye that is specific for Fe$^{3+}$ (Roschzttardtz et al., 2009). Second, during seed maturation, the ascorbate concentration drops, reaching undetectable levels in dry seeds (Gest et al., 2013). This will generate a pro-oxidant state, in favor of the accumulation of the oxidized ferric form over the ferrous. Finally, it was shown that iron and phosphorus co-localize in specific structures in vacuoles, the globoids (Lanquar et al., 2005) that are known to accumulate phosphorus under the form of phytate (Lott & West, 2001), which is a highly efficient ligand of Fe$^{3+}$ (Veiga et al., 2015). Actually, the affinity of phytate (inositol-hexakis-phosphate, InsP6) for Fe$^{3+}$ is at least 10 orders of magnitude higher than for Fe$^{2+}$ (Veiga et al., 2015). Moreover, this preferential binding to Fe$^{3+}$ is strongly increased in acidic conditions since it has been shown, in vitro, that below pH5 the InsP6-Fe$^{2+}$ complexes totally dissociate whereas almost 100% of Fe$^{3+}$ will remain bound to InsP6 (Veiga et al., 2015). Therefore, from a biological point of view, the acidification of the vacuolar lumen that takes place during germination (Maeshima et al., 1994; Bolte et al., 2011; Wilson et al., 2016) will be necessary but not sufficient to dissociate Fe$^{3+}$ from phytate. The drop in pH has thus to be accompanied by a reduction step in order to dissociate Fe from its ligand and generate the more mobile Fe$^{2+}$ form that will be readily transported out of the vacuole by the divalent metal transporters NRAMP3 and NRAMP4 (Lanquar et al., 2005).

In conclusion, we have identified a new ascorbate transporter in the vacuolar membrane, belonging to the MATE family, that plays an important role in the process of Fe remobilization in embryos during germination. The characterization of this new transporter has shed a new light on the role of ascorbate, the main anti-oxidant molecule in plant cells, in Fe homeostasis. Our work has contributed to add a new brick to the complex mechanism by which iron, stored in vacuoles in Arabidopsis embryos, is remobilized to feed the developing seedling. In conditions of Fe deficiency, TASC1-mediated ascorbate transport appears to be as crucial as NRAMP3 and NRAMP4 for the overall remobilization process.

**Acknowledgements**
The authors wish to thank the Vietnamese Ministry of Horticulture for the funding of MH PhD fellowship and the French National Research Agency for the funding of the project (ANR-16-CE20-0019).
Figure 1. Isolation and characterization of TASC1, an ascorbate efflux transporter. (A) ∆*fre1* mutant yeast was transformed with empty vector (pDR195) or with the vector expressing TASC1 (pDR195-TASC1). Serial dilutions of an OD=1 initial culture were plated on YNB media supplemented with 5 mM GL and either Fe (+Fe) or no Fe (-Fe) obtained by the addition of 75 µM BPDS. (B) Ferric reduction activity of whole yeast cells. Transformants were grown overnight in YNB with (grey bars) or without (black bars) 10 mM GL and ferric reduction activity was measured. Data are means ± SD of n=3 independent clones, * P<0.01, Wilcoxon test. (C) Ascorbate efflux in the medium from whole cells that had been grown overnight in the presence of 10 mM GL. Ascorbate concentration in the medium was measured by HPLC. Data are means ± SD, n=4 independent clones. (D) Time course of $^{13}$C-ascorbate efflux from *Xenopus* oocytes expressing TASC1, measured at pH5.5 or pH7.5. Data are means ± SD, n=4 independent sets containing 10 oocytes per condition, ** indicates a significant difference from control using the t-test, P<0.01%.
Figure 2. TASC1 is expressed in flowers, seeds and seedlings. Histochemical staining of GUS activity in a transgenic plant expressing the TASC1 promoter-GUS fusion. (a) Seed cross section at mature green embryo stage, (b-c) seedlings, 1-2 (B) and 4 (c) days after imbibition, (d) GUS staining of a whole flower bud; oi=outer integument, ii=inner integument, end=endosperm, hyp=embryo hypocotyl, coty=embryo cotyledons. (D)
Figure 3. TASC1 is a tonoplastic protein. Confocal microscopy of roots from 1 week-old tasc1-1 seedlings expressing TASC1-GFP (green) fusion. (a) Plasma membrane was stained with FM4-64 (magenta), (b) vacuoles were stained with Neutral Red (magenta). Arrowheads indicate invaginations of the tonoplast surrounding the nucleus. bar = 10µm.
Figure 4. TASC1 is required for seedling growth in Fe deficiency. (A) tasc1-1 mutants are sensitive to Fe deficiency. Seedlings were grown for 5 days in standard (50µM Fe-citrate, +Fe) or deficiency (no Fe added, 50µM BPDS, -Fe) Fe condition. (B) primary root length of seedlings grown in –Fe. Data are means ± SD, n=10 seedlings per genotype, different letters indicate significant differences by Student’s t-test, P<0.01.
Figure 5. TASC1 participates in the remobilization of Fe during germination. Histochemical staining of Fe with the Perls/DAB procedure on embryos dissected from imbibed seeds (upper lane) 3- and 5-day old seedlings grown without Fe were stained for Fe (brown coloration in the vascular system, indicated by arrowheads), bar=0.5 mm. Lower lane, Perls/DAB staining of Fe on ultra-thin sections of cotyledons, Fe is visible as brown deposits in the vacuoles (arrows), bar=50µm.
**Figure 6. TASC1 is involved in the tolerance to Fe deficiency.** Ten day-old seedlings were transferred in hydroponic conditions and grown for 2 weeks in standard (+Fe, 10µM Fe-citrate) or Fe deficient (-Fe, no Fe added). Lower panel, biomass quantification of plants grown on –Fe. Data are means ± SD, n=12 plants per genotype, ** indicate significant differences with Student’s test, P<0.01.
**Figure 7.** Vacuolar Fe remobilization depends on TASC1-mediated ascorbate transport. (A) Perls/DAB staining of Fe on ultra-thin sections of leaves from 3-week-old plants grown in hydroponic condition. Arrows indicate Fe depositions in vacuoles of mesophyll cells (bar=20µm). (B, C) metals and ascorbate quantification, respectively, from vacuoles isolated from leaf protoplasts of plants grown in +Fe. Data are means±SE of n=3 independent preparations, * indicate statistical difference with Wilcoxon test, P<0.1.


Lane DJR, Richardson DR. 2014. The active role of vitamin C in mammalian iron metabolism: Much more than just enhanced iron absorption! *Free Radical Biology and Medicine* **75**: 69-83.

Lane DJR, Robinson SR, Czerwinska H, Bishop GM, Lawen A. 2010. Two routes of iron accumulation in astrocytes: ascorbate-dependent ferrous iron uptake via the divalent


