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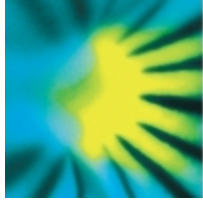
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TASC1, a member of the Multidrug And Toxic compound Extrusion family, is a vacuolar ascorbate transporter that controls intracellular iron cycling in Arabidopsis

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1 **TASC1, a member of the Multidrug And Toxic compound Extrusion family, is a vacuolar**
2 **ascorbate transporter that controls intracellular iron cycling in Arabidopsis**

3

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14

15 **Abstract**

16 - Iron (Fe) is an essential element, its transport is regulated by the cell redox balance. In
17 seeds, Fe enters the embryo as Fe²⁺ and is stored in vacuoles as Fe³⁺. Through its ferric
18 reduction activity, ascorbate plays a major role in Fe redox state and hence Fe
19 transport within the seed.

20 - We have searched for ascorbate membrane transporters responsible for controlling
21 Fe reduction through a screening in the yeast ferric reductase-deficient *fre1* strain and
22 have isolated a member of the Multidrug And Toxic compound Extrusion (MATE)
23 family.

24 - TASC1 (for Transporter of ASCorbate 1) was shown to mediate ascorbate efflux when
25 expressed in yeast and *Xenopus* oocytes, in a pH-dependent manner. *In planta*, *TASC1*
26 is highly expressed during germination and encodes a vacuolar membrane protein.
27 Isolated vacuoles from a *tasc1-1* knockout mutant contained less ascorbate and more
28 Fe than WT and mutant seedlings were highly sensitive to Fe deficiency. Iron imaging
29 further showed that the remobilization of Fe from vacuoles was highly impaired in
30 mutant seedlings.

31 - Taken together, our results establish TASC1 as a vacuolar ascorbate transporter,
32 required during germination to promote reduction of the pool of stored Fe³⁺ and its
33 remobilization to feed the developing seedling.

34
35

36

37 **Introduction**

38 Iron (Fe), the second most abundant metal of the Earth crust, is essential for life.
39 Chronologically, iron has been the first metal used by living organisms as a cofactor for a wide
40 range of biological processes such as molecular oxygen activation, reduction of
41 ribonucleotides, metabolism of peroxides and electron transfer reactions. Despite its
42 apparent abundance, Fe poses a real conundrum to all living organisms since environmental
43 Fe is found mostly as the poorly soluble (10⁻¹⁸M), exchange-inert, Fe³⁺ form whereas the active
44 form, Fe²⁺, has a strong pro-oxidant activity. Redox cycling between Fe²⁺ and Fe³⁺ is thus a
45 central process for the regulation of Fe uptake, storage and utilization. Most living organisms,
46 including mammals, plants and fungi, possess membrane-bound metal reductases belonging
47 to two major families, cytochrome b561 (mammals) and flavo-cytochromes (fungi, plants),
48 that play a crucial role in generating Fe²⁺ that is either readily transported by divalent metal
49 transporters or transferred to ferroxidase/permease transport systems (reviewed in (Kosman,
50 2010; Jain *et al.*, 2014)). At the intracellular level, storage and remobilization of Fe also rely on
51 redox cycling. The incorporation of excess Fe into ferritin protein complexes requires a
52 ferroxidation step catalyzed by ferritin itself whereas Fe release from these complexes
53 requires a one electron reduction step that is most probably mediated by physiological
54 reductants such as ascorbate (Watt *et al.*, 1988; Laulhere & Briat, 1993; De Domenico *et al.*,
55 2006; Melman *et al.*, 2013). Yeast cells, that do not produce ferritins, store Fe in the vacuole
56 as Fe³⁺-(poly)-phosphate (Cockrell *et al.*, 2011; Park *et al.*, 2014) and the remobilization of this
57 pool is mediated by the lumen-oriented ferric reductase FRE6 that supplies Fe²⁺ either to the
58 ferrous efflux transporter Smf3p or to the ferroxidase/permease complex encoded by
59 Fet5p/Fthp (Singh *et al.*, 2007). In mature embryo of the model plant *Arabidopsis thaliana* Fe
60 is also stored as Fe³⁺ in vacuoles of specific cells surrounding the provascular strands (Lott &
61 West, 2001; Lanquar *et al.*, 2005; Kim *et al.*, 2006; Roschzttardt *et al.*, 2009). Although the
62 overall mechanism of Fe retrieval from vacuoles remains uncharacterized, two divalent metal

63 transporters encoded by NRAMP3 and NRAMP4 play a key role in the efflux of Fe from
64 vacuoles during germination (Lanquar *et al.*, 2005), implying that, as shown in yeast, a
65 reduction step is likely to be required to generate Fe²⁺, the *bona fide* substrate of NRAMP
66 proteins. Storage and remobilization of this particular Fe pool is crucial for the fate of the
67 future seedling since mutations in either the vacuolar influx transporter VIT1 or the efflux
68 transporters NRAMP3 and NRAMP4 severely compromises the growth of the seedling in Fe-
69 limiting conditions (Lanquar *et al.*, 2005; Kim *et al.*, 2006).

70 In plants, we have recently demonstrated that redox cycling of Fe is also crucial for the
71 transport between maternal tissues (seed coat) and the embryo. Indeed, isolated embryos
72 from Arabidopsis and pea exhibit high ferric reduction activity, necessary for Fe uptake, which
73 is not encoded by the expected membrane reductases of the FRO family. Instead, embryos
74 efflux massive amounts of ascorbate, which reduces Fe³⁺ ions that are delivered from the
75 maternal tissues as Fe-citrate-malate complexes (Grillet *et al.*, 2014). The association of
76 ascorbate-assisted reduction of Fe with transmembrane uptake of Fe²⁺ thus uncovered in
77 plants is reminiscent of the Fe uptake machinery described in human brain; indeed neuronal
78 cells take up Fe from Fe³⁺-citrate complexes following a reduction step, which is catalyzed by
79 ascorbate released from these cells (Lane *et al.*, 2010). Taken together, these discoveries in
80 both animal and plant systems have shed a new light on Fe homeostasis, where ascorbate
81 efflux represents a new paradigm for ferric reduction and transport (Lane & Richardson,
82 2014).

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

88 In the present work, we describe the isolation of an ascorbate efflux transporter that belongs
89 to the Multidrug And Toxic Compound Extrusion family (MATE). This transporter, named
90 *TASC1* (for Transporter of ASCorbate 1) was isolated through a yeast complementation
91 screening using the ferric reductase mutant $\Delta fre1$. We further show that *TASC1*, which
92 encodes a protein targeted to the vacuolar membrane in plant cells, is expressed in seeds and
93 most importantly during germination, where vacuolar Fe is crucial to sustain growth of the
94 developing seedling. Phenotypic analysis of plants mutated in *TASC1* further allowed

95 establishing that this protein plays a direct role in the redox cycling of Fe and its export from
96 the vacuole, a mechanism that is key to sustain growth of the developing seedling. Since MATE
97 are ubiquitous efflux transporters, these findings pave the way for future discoveries linking
98 ascorbate to Fe homeostasis in all living eukaryotic organisms.

99

100 **Materials and Methods**

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

110 **Measurement of ferric reduction activity.** To measure ferric reduction activity of the yeast
111 cells, the cells grown on medium with or without 10 mM L-GL overnight. Cell suspensions were
112 centrifuged and washed 3 times with sterilized water. The cells were then incubated in the
113 assay solution containing 5 mM MES buffer pH = 5.5, 300 μM (BPDS) and 100 μM Fe^{3+} -EDTA.
114 Fe reduction activity was monitored as the concentration of Fe^{2+} -BPDS₃
115 (bathophenanthrolinedisulfonate) complex formed in the assay solution by measuring
116 absorbance at 535 nm with a Hitachi U-2800 spectrophotometer. OD535 was measured after
117 1 hour incubation in the dark at 22-25 °C with 250 RPM shaking.

118 **Heterologous expression of *TASC1* in *Xenopus laevis* oocytes and efflux activity assay.** The
119 *TASC1* coding region was cloned into modified pGEM-HE vector. cRNA were synthesized from
120 1 μg of linearized vector, *NheI*-digested, using the HiScribe™ T7 ARCA mRNA Kit with tailing
121 (NEB, <http://www.NEB.com>). Oocytes obtained surgically from benzocaine-anesthetized
122 *Xenopus laevis* were defolliculated using a 1 h collagenase-treatment under gentle shaking at
123 20°C in 25 ml of OR2 medium (NaCl 82,5mM, KCl 2mM, MgCl₂ 1 mM, HEPES-NaOH pH 7.4)
124 supplemented with 25 mg of collagenase (type IA, Sigma). Sorted stage V and VI oocytes were
125 then stored at 20°C in ND96 medium (NaCl 96mM, KCl 2mM, 1.8mM MgCl₂, CaCl₂ 1mM,
126 2.5mM Na-Pyruvate, HEPES-NaOH pH7.4) supplemented with 50 mg/ml gentamicin. Oocytes

127 were injected with 32 ng (50 nl) of *AtTasc1* cRNA using a micropipette (10–15 μ m tip diameter)
128 and a pneumatic injector. After two days, *TASC1* cRNA injected oocytes and non-injected
129 oocytes (used as control) were transferred to fresh ND96 solution (pH 6.5) supplemented with
130 10 mM L-Ascorbic acid and injected with 25 nl of 100 mM 13 C-labeled L-Ascorbic acid (Sigma-
131 Aldrich, Ref. 795097, prepared in 100 mM Tris-MES pH 7.5). After 5 min of recovery, injected
132 oocytes were washed 5 times with ice-cold ND96 solution (pH 6.5), then placed for efflux
133 measurements by batches (n=3) of 10 oocytes into 2 ml vials at room temperature in ND96
134 medium. The requirement of a proton gradient was measured by incubations in different
135 ND96 set at different pH values (pH 5.5, 6.5 or 7.5) for 2 hrs. After incubation, 100 μ l of ND96
136 medium from each batch were sampled in 3 replicates and dried. The 13 C abundance was
137 analyzed from sampled ND96 medium using an IsoPrime stable isotope analyzer Mass
138 Spectrometer (<https://www.elementar.de>).

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

151 **Plant materials and growth conditions.** For soil-grown plants, seeds were stratified at 4°C for
152 2-3 days and sown onto the potting soil. Alternatively, 7-days old seedlings grown *in vitro* were
153 transferred into the soil. The greenhouse conditions for soil-grown plants were 16 hours light,
154 8 hours dark at 22°C and 70% humidity. For *in vitro* culture, after surface sterilization with 50%
155 ethanol, 12.5% bleach solution and 37.5% water for 10 min, seeds were rinsed three times
156 with 100% ethanol and stratified at 4°C for 2 days. For growth test on agar plates containing
157 different iron conditions, seeds were germinated on half strength MS. Iron sufficient medium
158 is half strength Murashige and Skoog (MS) medium containing 0.05% MES, 1% sucrose, 0.8%

159 agar and 50µM Fe³⁺-citrate. The iron deficient medium is the same without Fe and a
 160 supplement of 5 µM BPDS. For hydroponic cultures, 10-day-old plantlets were transferred
 161 from half strength MS agar plates to *Arabidopsis* hydroponic solution (Hoagland solution-
 162 appendix1). The hydroponic medium was renewed every 10 days. The growth chambers
 163 conditions were 16 hour light/8 hour dark photoperiod at 21°C temperature and 65% the
 164 relative humidity. The *tasc1-1* homozygous mutant line was isolated from GABI-Kat (GK_912H)
 165 using the following primers EP16 (5'-TAAAAGTGGTCTTGTGGTATCAG-3') and EP17 (5'-
 166 AATAAATGAGTATTTTATTGTAG-3'). Total RNA was extracted with Trizol and first strand cDNA
 167 was synthesized with the RevertAid reverse transcription kit (Thermo Scientific). Full-length
 168 *TASC1* cDNA was amplified by RT-PCR with *TASC1-FP* (5'-
 169 GGAGATAGAACCATGAGTGGAGGTGGTGGAGA-3') and *TASC1-RP* (5'-
 170 TCCACCTCCGGATCCTCACTTTCTCTCTTCATCTCTTG-3'), *AtACT2* cDNA (actin 2, AT3G18780),
 171 used as loading control, was amplified using the following primers: 5'-
 172 TGCTGTTGACTACGAGCAGG-3' and 5'-TCTGCTGGAATGTGCTGAGG-3'. The search for putative
 173 truncated versions of the full-length cDNA was realized using seven different reverse primers
 174 hybridizing the end of the different exons, in order to amplify cDNA fragments of increasing
 175 size up to the full-length version. The forward primers used were, respectively: rev1: 5'-
 176 CCATAATACCGTAGATGAAGC-3'; rev2: 5'-CAAAGCATGAAGCCGGAGGA-3'; rev3: 5'-
 177 CATATGGAGAAAGCGGAAATTG-3'; rev4: 5'-CATGCTGCACCCATCAAGC-3'; rev5: 5'-
 178 CCAGATAGAATGGGCTGGAT-3'; rev6: 5'-CCTTAATCCCAAATTGAAAACG-3'; rev7: 5'-
 179 TCACTTTCTCTCTTCATCTCTTG-3'.

180 **DNA cloning** . All cloning reactions were realized using the Gateway™ system (Invitrogen). All
 181 *attB*-tailed PCR products were initially cloned into pDONR207 using BP Clonase, and their
 182 sequence verified before subcloning into various plant expression vectors (using LR Clonase)
 183 mentioned elsewhere. The cDNAs or promoter regions were amplified by a two step-PCR using
 184 the high-fidelity Phusion polymerase (Thermo Scientific, Waltham, MA, USA) and the following
 185 specific primers (*At5g17700* FP, 5'-GGAGATAGAACCATGAGTGGAGGTGGTGGAGA-3' ;
 186 *At5g17700* RP stop, 5'-TCCACCTCCGGATCCTCACTTTCTCTCTTCATCTCTTG-3' ; *At5g17700* RP C-
 187 ter, 5'-TCCACCTCCGGATCCCTTTCTCTCTTCATCTCTTG-3' ; *promoAt5g17700* FP, 5'-
 188 GGAGATAGAACC CAATAGTTTATGAGTTCATGCA-3' ; *promoAt5g17700* RP, 5'-
 189 TCCACCTCCGGATCC TTTTCTCTCTCTTTGATTTTC-3') containing universal Gateway *attB1* and
 190 universal Gateway *attB2* sequences (underlined) for gateway recombination. For the *TASC1*-

191 GFP fusion, The *TASC1* cDNA was amplified without its stop codon and cloned into the pDONR
192 207 entry vector (Gateway; Invitrogen). *TASC1* cDNA was subsequently fused with GFP by
193 recombination with the destination vector pGWB5 to generate the C-terminal GFP fusion,
194 under the control of the CaMV 35S promoter.

195 **Confocal microscopy.** For intracellular localization, the homozygous T2 transgenic plants were
196 imaged by confocal microscopy (Leica SP8) with laser excitation at 488 nm, emission light at
197 535 nm for GFP and laser excitation at 515 nm, emission light at 640-670 nm for FM4-64. Roots
198 of 10 day-old plantlets were mounted in water for microscopy observation or stained with 0.1
199 $\mu\text{g/ml}$ FM4-64 for 5 minutes and washed twice in water before observation under the
200 microscope. For the fluorescent labelling of vacuoles, 10-day old seedlings were incubated
201 with Neutral Red (4 μM) for 20 minutes and signal was acquired at 500-540 nm with an
202 excitation at 561 nm.

203 **Glucuronidase histochemical staining.** Histochemical staining for GUS in transgenic
204 Arabidopsis plants was performed according to standard procedures with minor
205 modifications. Plant tissues at different development stages including germinating seeds,
206 seedlings, rosette leaves, flower and siliques were picked using clean forceps and immersed
207 in a X-Gluc stain solution (50 mM NaPO_4 , 0.5 mM ferrocyanide, 0.5 mM ferricyanide, 0.05%
208 (v/v) Triton X-100, pH 7.2, and 1 mM 5-bromo-4-chloro-3-indolyl- β -D-glucuronide) overnight
209 at 37°C. For tissues containing chlorophyll, they were soaked in ethanol with 50%, 70%, 90%
210 and 100% successively until the samples were clear. For GUS histochemical staining in
211 embryos, embryos were fixed by 30 min of vacuum infiltration followed by 15 hour of soaking
212 in fixation solution containing 2% (w/v) paraformaldehyde, 1% (v/v) glutaraldehyde and 1%
213 (w/v) caffeine in 100 mM phosphate buffer (pH 7). Embryos were then dehydrated in
214 successive baths of 50%, 70%, 90%, 95% and 100% ethanol, butanol/ethanol 1:1 and 100%
215 butanol and embedded in the Technovit Resin according to the manufacturer's instructions.
216 Thin sections (4-6 μm) were cut and observed under the microscope.

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

219 **Vacuole isolation from leaves.** Vacuoles were isolated as described by Robert et al. (2007)
220 with some modifications. Firstly, to isolate mesophyll protoplasts, 2 g of rosette leaves from
221 30-day-old plants were collected and cut into small pieces. The processed leaves were floated
222 on protoplast enzyme solution (1% w/v cellulase, 1% w/v macerozyme, 0.4 % w/v $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$

223 400 mM Mannitol and 10 mM MES pH6.0) and incubated in 4 hours with gentle shaking. The
224 released protoplasts were collected by centrifugation for 10 min at 19g and the pellets were
225 re-suspended in the washing buffer to remove hydrolytic enzymes. To isolate vacuoles, 12.5
226 ml of pre-warmed lysis buffer (0.2 M Mannitol, 10% v/v Ficoll, 10 μ M EDTA pH=8, 5 μ M NaPO₄
227 pH 8.0 and 0.05% Neutral Red) were added to protoplast and re-suspended gently by pipetting
228 5-8 times and checking the protoplast disruption and vacuoles release under the light
229 microscope every 2 minutes. Vacuoles were then purified by overlaying with 6 ml 4 % (v/v)
230 Ficoll solution (mix 3 ml of lysis buffer and 4.5 ml of vacuole buffer : 450 mM mannitol, 5 μ M
231 NaPO₄ pH 7.5, 2 μ M EDTA pH 8.0), and addition of 2 ml of cold vacuole buffer on the top of
232 the gradient. After centrifugation for 50 min at 100000g at 10°C in Optima L-90K ultra-
233 centrifuge using slow acceleration and slow deceleration, the vacuoles were recovered from
234 the interface between middle and upper layers.

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

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

248

249 **Results**

250

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

252 Yeast complementation was used as a strategy to search for ascorbate (AsA) efflux
253 transporters from Arabidopsis. The yeast Δ *fre1* strain, mutated in the major ferric chelate
254 reductase of the cell surface, is unable to acquire Fe in deficiency conditions and thus grows

255 poorly in Fe-limiting media (Georgatsou *et al.*, 1997). A cDNA library from *Arabidopsis thaliana*
256 was transformed in $\Delta fre1$ with the idea that expression of a transporter able to mediate AsA
257 efflux in the medium should restore, at least in part, the ferric reduction capacity of the cell.
258 The growth medium was supplemented with L-galactono-lactone (GL), the biosynthetic
259 precursor that is readily converted in AsA by yeast cells that otherwise do not produce AsA
260 naturally (Berczi *et al.*, 2007). Among the positive clones picked for their restored growth on
261 Fe-limiting media, a clone expressing a cDNA coding for a member of the Multidrug And Toxic
262 Compound Extrusion family (MATE) was considered of high interest. Indeed, the hallmark of
263 the MATE proteins characterized so far is their capacity to mediate efflux of organic anions
264 such as organic acids and xenobiotics. According to the MATE nomenclature, this clone
265 corresponded to AtDTX25 (At5g17700) and was named TASC1, for Transporter of ASCorbate
266 1. Based on the ability of TASC1 expression to restore growth of the $\Delta fre1$ mutant (Fig. 1a) and
267 since this gene belongs to a family of *bona fide* efflux transporters, we then analyzed the
268 capacity of yeast cells to reduce Fe^{3+} through the efflux of AsA. The ferric reduction activity of
269 yeast cells was measured *in vitro* using the Fe^{2+} specific chromophore bathophenanthroline
270 disulfonic acid (BPDS). Cells expressing TASC1 displayed a significant increase in ferric
271 reduction, compared to empty vector controls, specifically when cells had been pre-loaded
272 with the AsA precursor GL. This result indicated that the production of Fe^{2+} was mediated by
273 TASC1 and was ascorbate-dependent (Fig. 1b). We then analyzed the AsA efflux activity of
274 whole cells by measuring the concentration of AsA released in the medium, after preloading
275 cells with GL. In these conditions it was possible to show that cells expressing TASC1 were able
276 to efflux significant amounts of AsA in the medium within 3 hours, compared to empty vector
277 control cells (Fig. 1c). In order to further support these results with protein localization, we
278 also expressed an TASC1-GFP fusion in the yeast $\Delta fre1$ strain. Depending on the level of
279 expression in the different clones, TASC1-GFP could be visualized at both the plasma
280 membrane and endomembranes including the vacuole (Fig. S1a) or only at the plasma
281 membrane (Fig. S1b). Drop tests further showed that this TASC1-GFP fusion was functional,
282 on the basis of the complementation of the growth defect of the $\Delta fre1$ strain (Fig. S1e).
283 Further direct evidence of the ascorbate transport activity of TASC1 was obtained by
284 expressing TASC1 in *Xenopus* oocytes. The release of ^{13}C in the medium was monitored from
285 oocytes previously injected with a 10mM ^{13}C -ascorbate solution over a four-hour period (Fig.
286 1d). A significant efflux of ascorbate could be detected from oocytes expressing TASC1 with a

287 progressive increase in the efflux activity over the 4-hour period tested. Based on this result,
288 we then tested the requirement of a proton gradient for the efflux activity of TASC1 by raising
289 the pH to 7.5. Compared to the efflux rate measured at pH5.5, incubating the oocytes at pH7.5
290 totally abolished ascorbate efflux, which indicates that TASC1 activity does require a proton
291 gradient. Taken together, these results strongly support the hypothesis that TASC1 is capable
292 of mediating transmembrane efflux of ascorbate from the cytosol. The dependency on a pH
293 gradient for the transport activity of TASC1 is an additional indication that this transport
294 activity corresponds to an antiport system that requires a proton gradient for the secondary
295 transport of ascorbate.

296

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

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

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

319

320 ***TASC1 regulates vacuolar Fe export***

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

339

340 To analyze in more depth the effect of *TASC1* mutation on the fate of Fe pools during
341 germination and seedling growth, we monitored Fe remobilization from the vacuoles by
342 histochemical staining using the Perls/DAB procedure (Roschztardt *et al.*, 2009). Embryos
343 dissected from imbibed seeds of WT, *tasc1-1* mutant and complemented lines displayed
344 equivalent Fe staining (brown/black pigments) in the pro-vascular system of cotyledons and
345 hypocotyle (Fig. 5 upper lane). After 3 and 5 days of growth without Fe, in both WT and
346 complemented lines, Fe was completely remobilized since Fe was no more detectable in the
347 vasculature of cotyledons and hypocotyls (Fig. 5). In contrast, *tasc1-1* mutant seedlings
348 retained staining in the vascular system, mostly in the cotyledons (Fig. 5, arrowheads).
349 Histological analysis of Fe localization in thin sections of resin-embedded seedlings further
350 confirmed that Fe was indeed still visible in vacuoles of the *tasc1-1* mutant, whereas it had

351 completely disappeared in both WT and complemented line (Fig. 5, lower row, arrows). These
352 results indicated that in the *tasc1-1* mutant, the Fe remobilization process was strongly
353 affected, which could explain the reduced growth capacity of *tasc1-1* mutants in -Fe
354 conditions when seedlings strictly depend on this particular vacuolar Fe pool for their survival
355 and growth.

356

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

366 Taken together, our results strongly suggested that the function of TASC1 is to transport
367 ascorbate in vacuoles, in order to reduce Fe³⁺ and thus remobilize the Fe pools stored in these
368 vacuoles. To test this hypothesis, we isolated vacuoles from leaf protoplasts and quantified
369 metals (Fe, Zn, Mn) and ascorbate (Fig. 7b and c, respectively). Vacuoles of *tasc1-1* mutant
370 protoplasts contained 3 times more Fe than WT vacuoles, consistent with the Fe imaging
371 analyses. The concentration of other metals such as Zn and Mn remained similar between
372 *tasc1-1* and WT however. We therefore concluded that Fe is immobilized in the vacuoles of
373 the *tasc1-1* mutant. Contrary to Fe, ascorbate accumulated twice less in *tasc1-1* vacuoles
374 compared to WT (Fig. 7c), despite the fact that at the whole leaf level, both WT and *tasc1-1*
375 mutants did contain comparable amounts of ascorbate (Fig. S3). The concomitant increase of
376 Fe and decrease of ascorbate measured in *tasc1-1* vacuoles strongly suggested that
377 remobilization of vacuolar Fe requires the presence of ascorbate within the vacuole, which
378 itself likely relies on TASC1 ascorbate influx across the tonoplast. In this scenario, the inability
379 of *tasc1-1* cells to retrieve Fe from vacuoles would limit plant growth, reducing *in fine* its
380 tolerance to Fe deficiency.

381

382 **Discussion**

383 In the model plant *Arabidopsis thaliana*, the general mechanism of ionic Fe transport across
384 membranes, that is based on trans-membrane ferric reductases of the FRO family to generate
385 Fe²⁺, has been recently challenged by the discovery of the role of ascorbate as an iron-reducing
386 molecule directly involved in the Fe transport process in seeds (Grillet *et al.*, 2014). This
387 finding, reminiscent of the Fe uptake from non-transferrin bound iron (NTBI) described in
388 human astrocyte cells (Lane *et al.*, 2010), represents a new mechanism to couple Fe reduction
389 with transmembrane transport at the surface of the embryo. Here, we show through the
390 characterization of the MATE transporter TASC1, that this mechanism also occurs at the
391 intracellular level, between the vacuolar lumen and the cytosol.

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

413 Despite the fact that ascorbate is an essential molecule for plants that plays multiple roles in
414 many subcellular compartments, the only ascorbate transporter identified until now is

415 PHT4;4, a member of the PHosphate Transporter family (PHT) that mediates the influx of
416 ascorbate in chloroplasts. The role of PHT4;4 is to fuel chloroplasts with ascorbate that is
417 required as a co-substrate for enzymatic reactions of the non-photochemical quenching, the
418 key metabolic responses of chloroplasts to high light stresses (Miyaji *et al.*, 2015).

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

434 Our study has shed a new light on the role(s) of ascorbate in the vacuole. Compared to other
435 organelles such as the chloroplast or the nucleus, the relative concentration of ascorbate
436 reported in the vacuoles is unexpectedly low. Immunocytochemical studies have established
437 that vacuoles contain no more than 3% of the total ascorbate labeling in cells (Zechmann *et al.*,
438 2011). However, upon high light stresses, the highest induction of ascorbate accumulation
439 was observed in vacuoles, suggesting a role of this intracellular ascorbate pool in stress
440 responses (Zechmann *et al.*, 2011). Given its high reactivity towards oxidizing molecules, the
441 antioxidant activity of ascorbate will inevitably generate monodehydro-ascorbate (MDHA),
442 the oxidized form of ascorbate. The classical regeneration of ascorbate from MDHA, known as
443 the Foyer-Halliwell-Asada cycle, is not likely to take place in vacuoles since none of the
444 enzymatic components of the cycle have been located in the vacuolar lumen. Instead, an
445 alternative mechanism has been proposed to occur, specifically in vacuoles. This mechanism
446 would involve transmembrane electron transfer proteins from the cytochrome b561 family

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

453 Based on these findings and on our new results, it is possible that tonoplasmic cytochrome
454 b561 would not be directly involved in Fe redox metabolism in the vacuole but rather in the
455 regeneration of ascorbate from MDHA that is produced as a result of Fe³⁺ reduction. This
456 mechanism would take place rapidly after germination to provide Fe to the developing plant.
457 Indeed, all the partners potentially required for this process, *AtTASC1*, *AtCYB1* and *AtCYB2*,
458 *AtNRAMP3* and *AtNRAMP4* are highly expressed in the vascular tissues after germination
459 (Verelst *et al.*, 2004; Lanquar *et al.*, 2005).

460 However, contrary to most of the genes involved in the response to Fe deficiency, the
461 expression of *TASC1* was not induced by Fe limitation (Fig. S4). Therefore, the regulation of
462 *TASC1*-mediated ascorbate-dependent Fe remobilization might occur at the level of ascorbate
463 itself. Indeed, Fe deficiency was shown to increase ascorbate accumulation in several plant
464 systems (Zaharieva & Abadia, 2003; Zaharieva *et al.*, 2004; Urzica *et al.*, 2012), through the
465 induction of genes involved in ascorbate biosynthesis (*VTC2*) and recycling (*MDAR1*) (Urzica
466 *et al.*, 2012). The rapid adjustments in ascorbate concentration might thus be more efficient
467 to cope with fluctuations of Fe status than changes in *TASC1* expression. In terms of expression
468 profile, *TASC1* and the citrate effluxer *FRD3* display some overlap, since both genes are
469 expressed in the embryo and integument of the developing seed, in young seedlings and in
470 anthers (Roschzttardtz *et al.*, 2011). This overlap in expression profile could be an indication
471 that both transport activities, and therefore both ascorbate and citrate, are required for the
472 movement of Fe between symplastically disconnected cells, as previously reported for *FRD3*
473 (Roschzttardtz *et al.*, 2011). Such a mechanism where citrate and ascorbate act together for
474 Fe translocation is reminiscent of the Fe trafficking in the brain where Fe-citrate (generally
475 referred to as non-transferrin-bound iron) is reduced by ascorbate into Fe²⁺ that is then
476 imported by transporters of the NRAMP family (Moos *et al.*, 2007; Lane & Lawen, 2008; Lane
477 & Richardson, 2014).

478 In the proposed mechanism of Fe remobilization from vacuoles, it is assumed that Fe is stored
479 in vacuoles under its oxidized form. Although the speciation of Fe in vacuoles has never been
480 fully addressed, several experimental evidences support this hypothesis and thus the need for
481 a reduction step during its remobilization. First, this vacuolar Fe pool in embryos reacts with
482 ferrocyanide, an iron dye that is specific for Fe³⁺ (Roschztardt et al., 2009). Second, during
483 seed maturation, the ascorbate concentration drops, reaching undetectable levels in dry
484 seeds (Gest et al., 2013). This will generate a pro-oxidant state, in favor of the accumulation
485 of the oxidized ferric form over the ferrous. Finally, it was shown that iron and phosphorus co-
486 localize in specific structures in vacuoles, the globoids (Lanquar et al., 2005) that are known
487 to accumulate phosphorus under the form of phytate (Lott & West, 2001), which is a highly
488 efficient ligand of Fe³⁺ (Veiga et al., 2015). Actually, the affinity of phytate (inositol-hexakis-
489 phosphate, InsP6) for Fe³⁺ is at least 10 orders of magnitude higher than for Fe²⁺ (Veiga et al.,
490 2015). Moreover, this preferential binding to Fe³⁺ is strongly increased in acidic conditions
491 since it has been shown, *in vitro*, that below pH5 the InsP6-Fe²⁺ complexes totally dissociate
492 whereas almost 100% of Fe³⁺ will remain bound to InsP6 (Veiga et al., 2015). Therefore, from
493 a biological point of view, the acidification of the vacuolar lumen that takes place during
494 germination (Maeshima et al., 1994; Bolte et al., 2011; Wilson et al., 2016) will be necessary
495 but not sufficient to dissociate Fe³⁺ from phytate. The drop in pH has thus to be accompanied
496 by a reduction step in order to dissociate Fe from its ligand and generate the more mobile Fe²⁺
497 form that will be readily transported out of the vacuole by the divalent metal transporters
498 NRAMP3 and NRAMP4 (Lanquar et al., 2005).

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

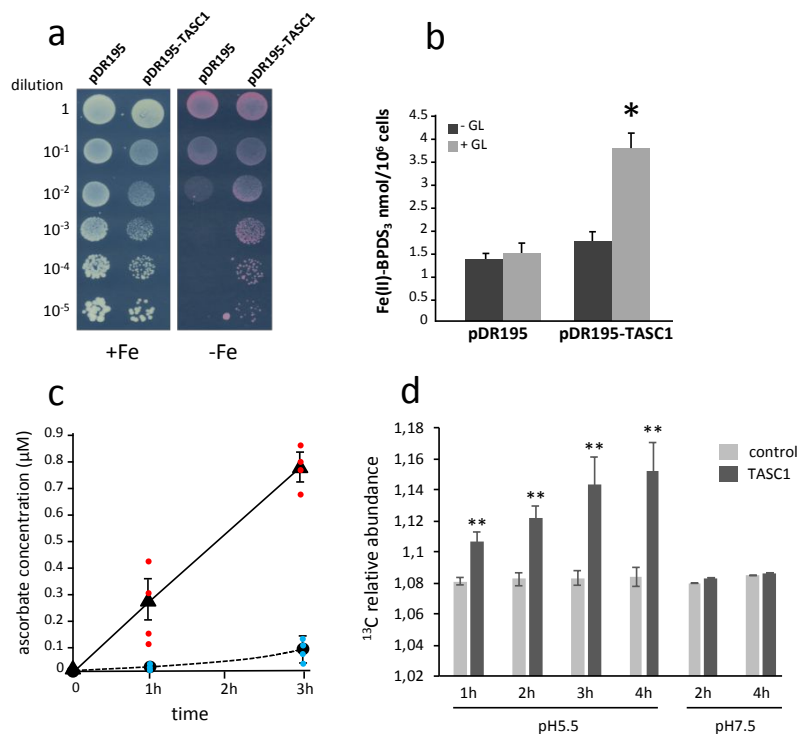
507

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For Peer Review

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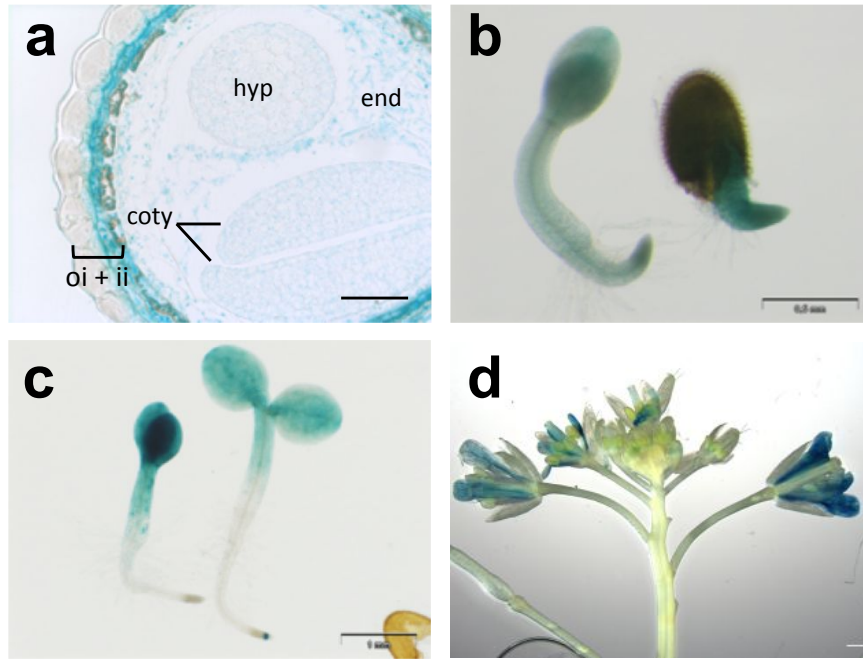


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517 **Figure 1. Isolation and characterization of TASC1, an ascorbate efflux transporter.** (A) *Δfre1*
 518 mutant yeast was transformed with empty vector (pDR195) or with the vector expressing
 519 *TASC1* (pDR195-TASC1). Serial dilutions of an OD=1 initial culture were plated on YNB media
 520 supplemented with 5 mM GL and either Fe (+Fe) or no Fe (-Fe) obtained by the addition of 75
 521 μM BPDS. (B) Ferric reduction activity of whole yeast cells. Transformants were grown
 522 overnight in YNB with (grey bars) or without (black bars) 10 mM GL and ferric reduction
 523 activity was measured. Data are means ± SD of n=3 independent clones, * P<0.01, Wilcoxon
 524 test. (C) Ascorbate efflux in the medium from whole cells that had been grown overnight in
 525 the presence of 10 mM GL. Ascorbate concentration in the medium was measured by HPLC.
 526 Data are means ± SD, n=4 independent clones. (D) Time course of ¹³C-ascorbate efflux from
 527 *Xenopus* oocytes expressing TASC1, measured at pH5.5 or pH7.5. Data are means ± SD, n=4
 528 independent sets containing 10 oocytes per condition, ** indicates a significant difference
 529 from control using the t-test, P<0.01%.



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534 **Figure 2. *TASC1* is expressed in flowers, seeds and seedlings.** Histochemical staining of GUS

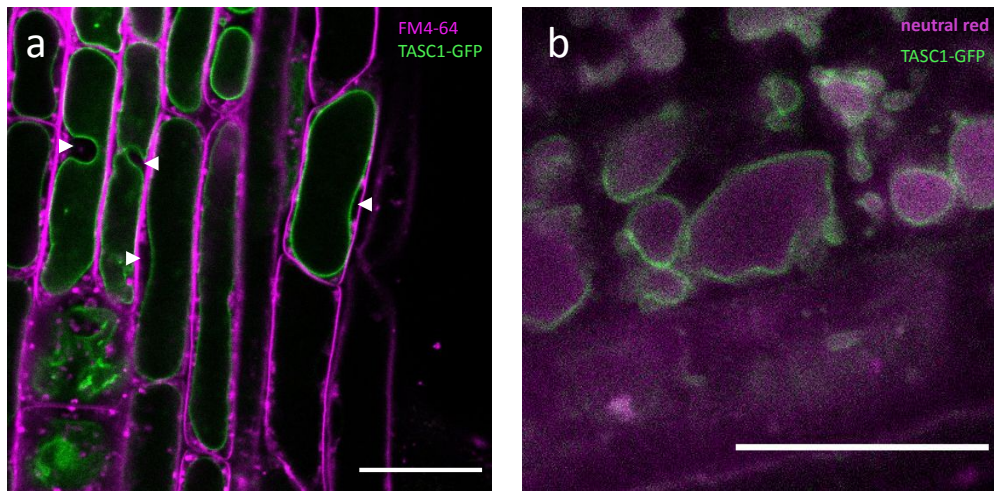
535 activity in a transgenic plant expressing the *TASC1* promoter-GUS fusion. (a) Seed cross section

536 at mature green embryo stage, (b-c) seedlings, 1-2 (B) and 4 (c) days after imbibition, (d) GUS

537 staining of a whole flower bud; oi=outer integument, ii=inner integument, end=endosperm,

538 hyp=embryo hypocotyl, coty=embryo cotyledons. (D)

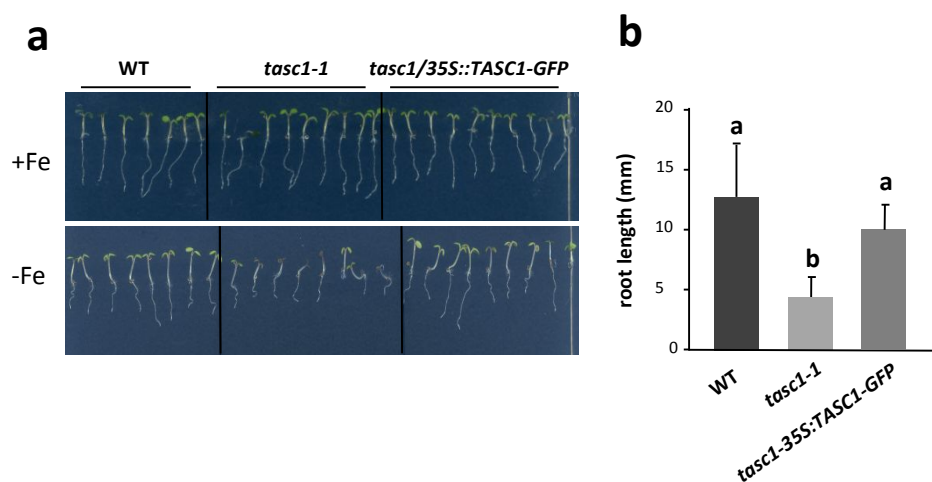
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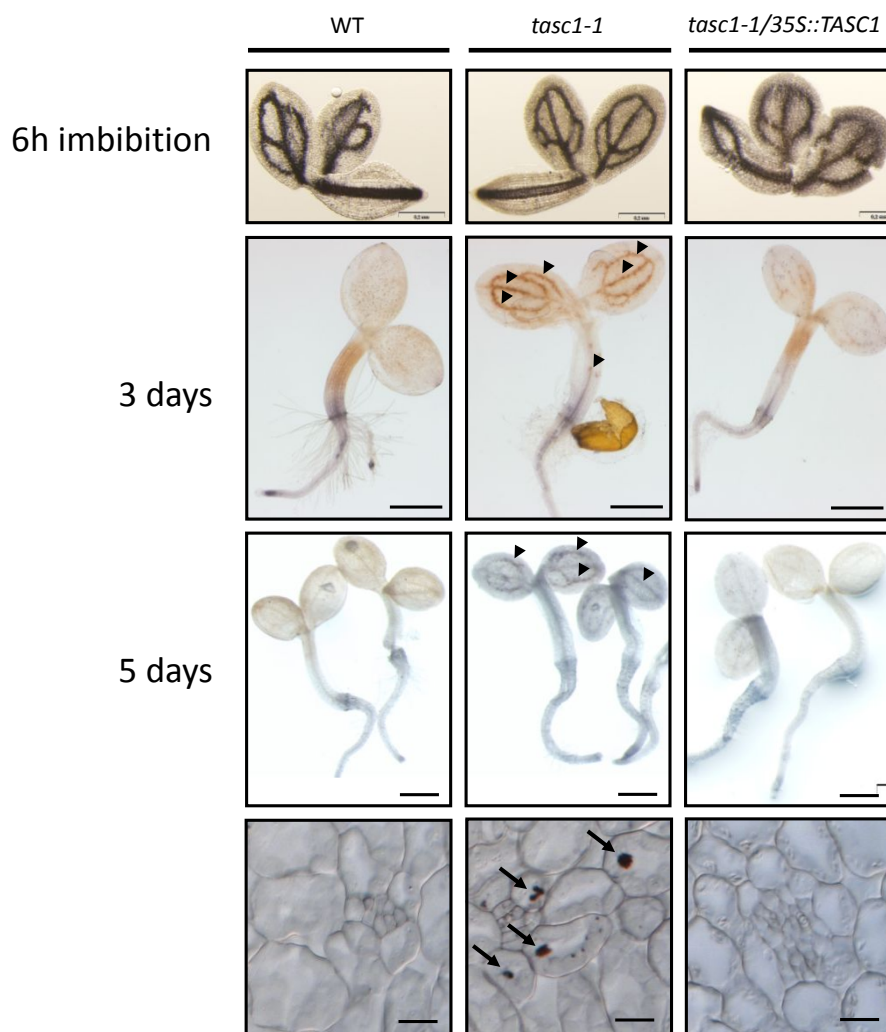
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541 **Figure 3. TASC1 is a tonoplasmic protein.** Confocal microscopy of roots from 1 week-old *tasc1-*
 542 *1* seedlings expressing TASC1-GFP (green) fusion. (a) Plasma membrane was stained with FM4-
 543 64 (magenta), (b) vacuoles were stained with Neutral Red (magenta). Arrowheads indicate
 544 invaginations of the tonoplast surrounding the nucleus. bar = 10μm.

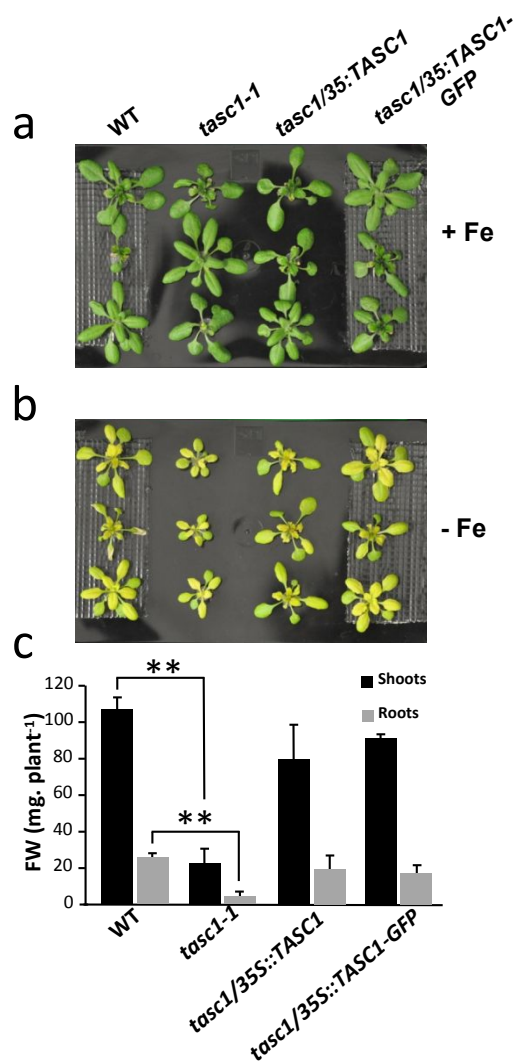
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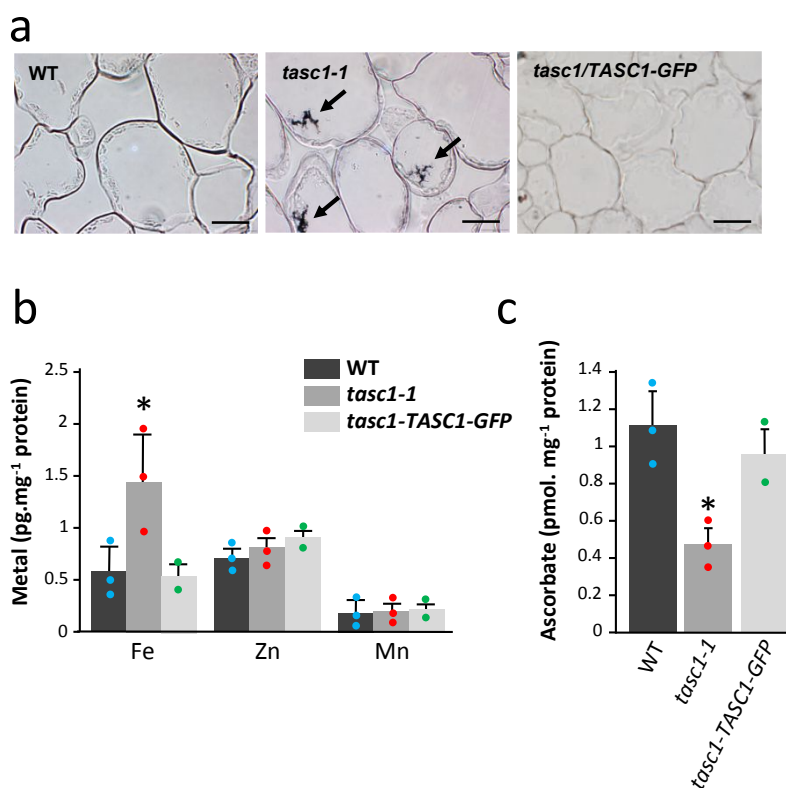
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 547 **Figure 4. TASC1 is required for seedling growth in Fe deficiency.** (A) *tasc1-1* mutants are
 548 sensitive to Fe deficiency. Seedlings were grown for 5 days in standard (50 μ M Fe-citrate, +Fe)
 549 or deficiency (no Fe added, 50 μ M BPDS, -Fe) Fe condition. (B) primary root length of seedlings
 550 grown in -Fe. Data are means \pm SD, n=10 seedlings per genotype, different letters indicate
 551 significant differences by Student's *t*-test, P<0.01.
 552



553
 554 **Figure 5. TASC1 participates in the remobilization of Fe during germination.** Histochemical
 555 staining of Fe with the Perls/DAB procedure on embryos dissected from imbibed seeds (upper
 556 lane) 3- and 5-day old seedlings grown without Fe were stained for Fe (brown coloration in
 557 the vascular system, indicated by arrowheads), bar=0.5 mm. Lower lane, Perls/DAB staining
 558 of Fe on ultra-thin sections of cotyledons, Fe is visible as brown deposits in the vacuoles
 559 (arrows), bar=50 μ m.
 560



561
 562 **Figure 6. TASC1 is involved in the tolerance to Fe deficiency.** Ten day-old seedlings were
 563 transferred in hydroponic conditions and grown for 2 weeks in standard (+Fe, 10 μ M Fe-citrate)
 564 or Fe deficient (-Fe, no Fe added). Lower panel, biomass quantification of plants grown on –
 565 Fe. Data are means \pm SD, n=12 plants per genotype, ** indicate significant differences with
 566 Student's test, P<0.01.
 567



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570 **Figure 7. Vacuolar Fe remobilization depends on TASC1-mediated ascorbate transport. (A)**571 Perls/DAB staining of Fe on ultra-thin sections of leaves from 3-week-old plants grown in
572 hydroponic condition. Arrows indicate Fe depositions in vacuoles of mesophyll cells573 (bar=20 μ m). (B, C) metals and ascorbate quantification, respectively, from vacuoles isolated574 from leaf protoplasts of plants grown in +Fe. Data are means \pm SE of n=3 independent

575 preparations, * indicate statistical difference with Wilcoxon test, P<0.1.

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