The *Arabidopsis thaliana* Gulono-1,4 γ-lactone oxidase 2 (GULLO2) facilitates iron transport from endosperm into developing embryos and affects seed coat suberization

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Abstract

Plants synthesize ascorbate (ASC) *via* the D-mannose/L-galactose pathway whereas animals produce ASC and H_2O_2 *via* the UDP-glucose pathway, with Gulono-1,4 γ-lactone oxidases (GULLO) as the last step. *A. thaliana* has seven isoforms, *GULLO1-7*; previous *in silico* analysis suggested that *GULLO2*, mostly expressed in developing seeds, might be involved in iron (Fe) nutrition. We isolated *atgullo2-1* and *atgullo2-2* mutants, quantified ASC and H_2O_2 in developing siliques, Fe(III) reduction in immature embryos and seed coats. Surfaces of mature seed coats were analyzed *via* atomic force and electron microscopies; suberin monomer and elemental compositions of mature seeds, including Fe, were profiled via chromatography and inductively coupled plasma-mass spectrometry.

Lower levels of ASC and H_2O_2 in *atgullo2* immature siliques are accompanied by an impaired Fe(III) reduction in seed coats and lower Fe content in embryos and seeds; *atgullo2* seeds displayed reduced permeability and higher levels of C18:2 and C18:3 ω-hydroxyacids, the two predominant suberin monomers in *A. thaliana* seeds. We propose that GULLO2 contributes to ASC synthesis, for Fe(III) reduction into Fe(II). This step is critical for Fe transport from endosperm into developing embryos. We also show that alterations in GULLO*2* activity affect suberin biosynthesis and accumulation in the seed coat.

Keywords: Ascorbate, embryos, Gulono-1,4 γ-lactone oxidase, iron, seeds, suberin, vitamin C.

Highlights

- Gulono-1,4 γ-lactone oxidases (GULLO) are enzymes devoted to biosynthesis of ascorbic acid (vitamin C) in animal cells
- GULLO enzymes are also present in plant cells
- We suggest that *Arabidopsis thaliana* GULLO2 isoform catalyzes the *in vivo* production of ascorbic acid
- *A. thaliana* GULLO2 contributes to Fe transport from endosperm into the developing embryo and to suberin biosynthesis in the seed coat.

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1. Introduction

Plants' growth and development are regulated by redox reactions with pro-oxidant and anti-oxidant metabolites playing pivotal roles in almost all plant developmental stages, from seed germination to plant senescence, and in cellular processes ranging from cell cycle to cell wall stiffening (Considine & Foyer, 2021). Reactive oxygen species (ROS), such as hydrogen peroxide (H_2O_2) and ion superoxide (O_2^-), are the major pro-oxidants produced during aerobic metabolism as unavoidable by-products, but they are also produced in specific pathways involved in differentiation and signaling (Noctor *et al.*, 2018; Waszczak *et al*., 2018; Sies *et al*., 2022). Plant cells are provided with several enzymatic and non-enzymatic strategies aimed at keeping ROS levels under control and avoiding and/or limiting ROS-derived oxidative stresses (Lodde *et al*., 2021 and references therein).

Ascorbate (ASC, vitamin C), together with glutathione, constitute the most important redox hub due to its anti-oxidative role (Foyer & Noctor, 2011). ASC is involved in many protective reactions, being the specific electron donor of ascorbate peroxidase that reduces H_2O_2 to water (Murgia *et al.*, 2004; Caverzan *et al*., 2012; Locato *et al.*, 2018). However, the metabolic roles of ASC go beyond redox homeostasis (Gest *et al*., 2013); as an example, ASC plays an important role in plant cell expansion and division as cofactor of prolyl hydroxylases responsible for the post-translational modification of prolyl residues of cell wall glycoproteins (De Gara *et al.*, 1991; De Tullio *et al.*, 1999; Fragkostefanakis *et al.*, 2014).

Consistently with its multifaceted roles, ASC is present in all plant cell compartments where its concentrations, ranging from less than 5 mg to over 800 mg per 100 g fresh weight, depend on the species, cultivar, and within same plant, on the tissue, developmental stage and whether such tissue is photosynthetically active or not (Bulley & Laing, 2016; Smirnoff, 2018); *Actinidia eriantha* fruits (a kiwifruit species) contains one of the highest ASC documented concentrations, reaching 1200 mg per 100 g fresh weight, during the growing stages (Bulley *et al*., 2009).

The biochemical pathways leading to ASC synthesis differ among kingdoms: the main ASC biosynthetic pathway in plants proceeds from GDP-mannose and its last biosynthetic step is the oxidation of L-galactono-γ-lactone by galactolactone dehydrogenase (GLDH); in mammals, instead, the pathway proceeds from UDP-D-glucose and its last biosynthetic step is the oxidation of L-gulono-1,4 γ-lactone by Gulono-1,4 γ-lactone oxidases (GULLO) (Wheeler *et al*., 2015, Fernie & Tohge, 2015; Smirnoff, 2018). Almost all living organisms can produce ASC, except some animals, including humans, which rely on diet for ASC intake (Wheeler *et al*., 2015; Fernie & Tohge, 2015). Humans and other primates have a pseudogene instead of a functional *GULLO* gene, as highlighted by many random nucleotide changes (Ohta & Nishikimi, 1999; Drouin *et al*., 2011), and are therefore

unable to produce ASC.

ASC biosynthetic pathways were analyzed in evolutionary terms and a possible single ancestral gene for both *GULLO* and *GLDH* has been proposed (Wheeler *et al.*, 2015). An interesting feature of ASC synthesis by GULLO is the production of hydrogen peroxide H_2O_2 as byproduct, a feature not shared with the GLDH reaction (Banhegyi *et al.*, 1997; Locato *et al.*, 2013). A selective pressure against *GULLO* genes in photosynthetic organisms has been suggested, due to the production of this oxidant species, H_2O_2, which would cause an augmented oxidative burden for organisms already exposed to photooxidative stress (Wheeler *et al.*, 2015, Fernie & Tohge, 2015). Furthermore, a mutually exclusive distribution of *GULLO* and *GLDH* genes has been observed, together with a lack of GULLO enzymes in *Arabidopsis thaliana*, *Oryza sativa* and *Zea mais* (Wheeler *et al.*, 2015); the authors highlighted how the *GULLO* gene homologs identified in *A. thaliana* (Maruta *et al.*, 2010) belong to a different clade from canonical animal *GULLO* and with an unclear role in the *de novo* ASC synthesis (Wheeler *et al.*, 2015).

Previous studies highlighted *GULLO* genes as potential key players in ASC metabolism. For example, transgenic tobacco and lettuce plants transformed with a rat (*Rattus norvegicus*) cDNA, encoding *GULLO*, showed higher ASC levels (Jain & Nessler, 2000). Correspondingly, overexpression of this gene in the ASC-deficient *A. thaliana vtc* (vitamin C defective) mutants (*vtc1-1*, *vtc2-2*, *vtc3-1* and *vtc 4-1*) rescued their ASC pools and ameliorated the stunted growth phenotype (Radzio *et al.*, 2003). Further experiments, showing the efficacy of overexpressing animal *GULLO* genes into plants with consequent elevation of ASC values, have been made (Lisko *et al.*, 2013).

A. thaliana possesses seven *GULLO* isoforms sharing 23-28% gene identity with rat *GULLO*: *GULLO1* (At1g32300), *GULLO2* (At2g46750), *GULLO3* (At5g11540), *GULLO4* (At5g56490), *GULLO5* (At2g46740), *GULLO6* (At2g46760), and *GULLO7* (At5g56470) (Maruta *et al.*, 2010; Aboobucker *et al.*, 2017). Interestingly, *GULLO1-6* share two conserved features of the *GULLO* family, the N-terminus flavin adenine dinucleotide (FAD)-binding and an arabinono-lactone oxidase catalytic domain (Aboobucker *et al.*, 2017). Transgenic Tobacco BY-2 Cells overexpressing *GULLO2*, *3* and *5*, show increased ASC values, when fed with the substrate gulono-lactone (Maruta *et al.*, 2010). However, up to date, no clear evidence of a genuine *in vivo* role of any plant GULLO enzymes has been reported.

Previously we functionally characterized the circadian-regulated *A. thaliana* P450 gene CYP82C4 involved in the early Fe deficiency response (Murgia *et al.*, 2011). CYP82C4 is expressed in roots and it catalyzes the conversion of fraxetin into the novel coumarin molecule, named sideretin (Rajniak *et al.*, 2018). Notably, among the list of genes that highly co-express with CYP82C4, besides those with established roles in Fe nutrition, we found At2g46750 containing an RY element and IDE1-like

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motifs within its 1500-bp promoter region, that was annotated at the time of publication as a FAD-containing protein (Murgia *et al.*, 2011). This gene corresponds to the *A. thaliana GULLO2*. Indeed, we recently proposed a possible link between GULLO2 activity and plant Fe nutrition (Murgia *et al.*, 2022). According to *in silico* bioinformatics data (*Arabidopsis* eEF browser developmental map; Winter *et al*., 2007), the expression of *GULLO2* appears the highest in developing seeds; however, it is also expressed in roots (Schmid *et al*., 2005).

The iron loading into seeds has been explored in recent years (Murgia *et al.*, 2022). Fe(III) is reduced by ASC into Fe(II), for its transport from endosperm into the developing embryo, in both *A. thaliana* and pea with a mechanism different from the classical membrane-bound ferric reductase (Grillet *et al*., 2014).

Given these premises, in the current study we sought to investigate the possible *in vivo* roles of GULLO2 in Fe nutrition and in seed development. To this end, we isolated and characterized two *A. thaliana* independent insertional mutants *atgullo2-1* and *atgullo2-2*. Here we found that such mutants have lower content, in their developing siliques, of the two enzymatic products of animal GULLO, i.e. ASC and H_2O_2. This result indicates that *A. thaliana* GULLO2 acts, *in vivo*, as a fully functioning GULLO enzyme, similarly to its animal counterpart. This finding is utterly relevant as the endogenous GULLO role in plants has been debated for years; until now the D-mannose/L-galactose pathway of ASC biosynthesis, with L-galactone 1,4 lactone dehydrogenase GLDH as the last enzymatic step, was indeed considered as the only pathway, *in vivo*, for ASC synthesis in plants (Wheeler *et al.* 2015; Fernie & Tohge, 2015; Smirnoff, 2018).The diminished ASC pool in *atgullo2* developing seeds has an impact on their Fe(II) reduction capacity and hence, in the amount of Fe transported into *atgullo2* embryos, which is indeed lower in such mutant embryos and whole seeds.

Moreover, GULLO2 influences the composition of suberin in mature seed coats: *atgullo2* seeds have higher levels of C18:2 and C18:3 ω-hydroxyacids, the two predominant suberin monomers in *A. thaliana* seeds; consequently, permeability of their seed coats is reduced. Taken together, these findings open the way to further studies on the GULLO gene family and its role in Fe nutrition and suberization, in the various plant organs and tissues.

2. Materials and Methods

2.1 A. thaliana growth in soil and in vitro

A. thaliana wt Col and *atgullo2-1* and *atgullo2-2* mutants were grown in control soil as described previously (Murgia *et al*., 2015), or in ½ MS medium at various pHs (achieved by adding HCl).

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2.2 Isolation of atgullo2-1 and atgullo2-2 mutants

A. thaliana mutant lines GABI KAT_241A05 and SM_3_30489 lines (Nottingham Arabidopsis Stock Center NASC) carry, respectively, a T-DNA and a transposon insertion in the first and in the second exon of *GULLO2* (At2g46750) gene. To isolate homozygous lines, progeny plants were tested by PCR with following primers:

GKF: 5'-ACTTATGCACACGGCTGTCT-3'; GKR: 5'-TTGACCGAACCAGGACTAAC-3', pLB 08409: 5'-ATATTGACCATCATACTCATTGC-3'; SMF 5'-AGACTACGACTCCTAACGAG-3'; SMR: 3'-GGAGTCTGGATCGGATAATG-5';

dSPM32:5'TACGAATAAGAGCGTCCATTTTAGAGTGA)-3'.

PCR conditions: 2 min 94 °C; 34 cycles: 20 s 94 °C, 1 min 63 °C, 1 min 72 °C; 5 min at 72 °C.

Line GABI KAT_241A05 homozygous for the insertion in *GULLO2* gene was named *atgullo2-1*, whereas line SM_3_30489 homozygous for insertion in *GULLO2* was named *atgullo2-2*.

Insertion junctions in the *atgullo1-1* and *atgullo1-2* mutants were sequenced by using pLB 08409 and dSPM32primers, respectively (BMR Genomics, s.r.l., Italy).

2.3 Seed staining procedures

Perls staining of mature embryos for Fe: mature seeds were immersed for 3 h in distilled water, then embryos were gently separated from seed coats under a stereomicroscope and stained with Perls reagent as indicated in Roschzttardtz *et al.* (2009). Pictures were taken with Stereomicroscope Leica MZ6 and Zeiss Axio imager d1.

Staining of seeds mucilage with ruthenium red: seeds were imbibed 5 min in distilled water, then sonicated 40 s and stained with a 0.01% ruthenium red solution for 90 min; seeds were then thoroughly rinsed in distilled water; control seeds (not sonicated) were stained after imbibition.

2.4 Fe(III) reduction activity in seed coats and embryos

The protocol has been adapted from Grillet *et al*. (2014). Siliques from *A. thaliana* plant grown in control soil were collected when still immature, with embryos at torpedo/bent cotyledon stage with gelatinous/sticky endosperm; such siliques roughly correspond to the fourth or fifth silique above the

first senescing (yellowing) one. The sampled siliques were placed onto a microscope slide, and their extremities were fixed with sellotape and the siliques were opened with a sharp surgical blade under the stereomicroscope; extracted seeds were gently pressed with a blade tip, to extrude their intact embryos and the endosperm. The seed coats deprived of embryos and of part of the endosperm extruded together with the embryos were collected into Eppendorf tubes containing 1 ml 5 mM MES at pH 5.5. Each sample, consisting of forty emptied seeds, was prepared in the following way: emptied seeds were gently washed twice in 5 mM MES with finger flicking (without centrifugation) and then suspended in 1 ml 5 mM MES containing 300 μM Bathophenanthroline (BPS) and 100 μM Fe(III) EDTA (complete samples). Control samples, i.e. without BPS, were also prepared, in the same way as described above. After 2 h incubation in the dark, absorbance $A_{535\ nm}$ of each sample was measured at the spectrophotometer, and the final absorbance of the Fe(II)EDTA complex was calculated as $A_{535\ nm}$ (complete sample)- $A_{535\ nm}$ (sample without BPS), by using 5 mM MES as blank. Fe(III) reduction activity of seed coats was calculated by using ε_λ Fe (II)-BPS: 22.14 $mM^{-1}\ cm^{-1}$ (Waters *et al*., 2002; Vasconcelos *et al*., 2014). The same procedure described above for emptied seeds, was also applied for measuring Fe(III) reduction activity of embryos isolated from mature seeds, with 35 embryos for each sample.

2.5 Seed germination assays

Treatment with commercial bleach: mature seeds were fully immersed for either 1 min, 5 min or 10 min in Eppendorf tubes containing commercial bleach (Ace, Procter & Gamble), they were then thoroughly washed six times in distilled water and plated onto moist filter paper (with distilled water), in petri dishes and germinated at 22-25 °C, 14 h light/10 h dark, 100 μE $m^{-2}\ s^{-1}$.

Treatment with abscisic acid (ABA): sterile filter paper circles Whatman 42 (7.0 cm) were placed onto sterile petri dishes, in sterile conditions; the same volume (1.5 ml) of 0, 0.5, 1, 5 or 10 μM ABA solution (Sigma, A-1012) was added to each plate; the seeds, previously sterilized as described in Murgia *et al*. (2015) were then laid on the various plates and germinated as described above.

2.6 ASC and H_2O_2 quantification

Immature siliques from 6 weeks old plants grown in control soil were sampled; for that, siliques from no.8 to no.15 (no.1 being the youngest developing silique on the stem) were collected. Siliques were frozen in liquid nitrogen and then freeze-dried overnight at 4 °C.

For ASC and dehydroascorbate (DHA) determination, freeze-dried *A. thaliana* silique were collected, ground in liquid nitrogen and homogenized with 8 volumes of cold 5% meta- phosphoric acid at 4 °C. The homogenate was centrifuged at 20000 g for 15 min at 4 °C, and the supernatant used for the

analysis as previously described in de Pinto *et al.* (1999). The redox state was calculated as ratio ASC/(ASC+DHA). H_2O_2 determination was performed according to Sabetta *et al.* (2019). Briefly, freeze-dried *A. thaliana* siliques were quickly ground in liquid nitrogen and homogenized with 6 volumes of cold 40 mM Tris-HCl pH 7.0 in presence of 20 μM of 2′,7′-dihydrodichlorofluorescein diacetate (H2DCF-DA). The samples were incubated for 1 h in the dark. The H_2O_2 levels were measured using a spectrofluorometer (excitation λ = 495 nm; emission λ = 520 nm) and normalized for mg protein. Results are expressed as unit of fluorescence relative to wt Col samples. Siliques from no.16 or older ones were also collected, cleared as described in Vazzola *et al*. (2007) and observed at the stereomicroscope.

2.7 Microscopical analyses

Atomic force microscopy (AFM): mature dry seeds were imbibed in deionized water for 5 min and then deprived of their mucilage by 40 s sonication; the seeds were then air dried. Dry seeds were immobilized on glass with glue Power Epoxy 5min Loctite. To obtain the mechanical properties of the outer cell wall only, the maximum indentation force was set to 10 μN to achieve a maximum indentation of no more than 80 nm. The cantilever used was "Nano World" (Nanosensors Headquarters, Neuchâtel, Switzerland) SD-R150-T3L450B tips with a spring constant of 10 N m^{-1} (the one used around 25 N m^{-1}) with silicon point probe tips of a 150-nm radius. All force microscopy experiments were performed as previously described (Feng *et al*., 2018; Peaucelle, 2014; Peaucelle *et al*., 2015). Briefly, stiffness of samples was determined as follows: an AFM cantilever loaded with a spherical tip was used to indent the sample over a 60 × 60 μm square area, within the area 60 × 60 measurements were made resulting in 3600 force indentation experiments; each force-indentation experiment was treated with a Hertzian indentation model to extract the apparent Young's modulus (E_A); each pixel in a stiffness map represents the apparent Young's modulus from one force-indentation point. The E_A was calculated using the JPK Data Processing software (ver. Spm - 4.0.23, JPK Instruments AG, Germany), which allows a more standardized analysis than the estimation of the E_A using a standard Hertzian contact model (Peaucelle, 2014; Peaucelle *et al*., 2015). Only the retraction curve was used in our analyses as is typically the case in nano-indentation experiments. A Poisson ratio of 0.5 was assumed for the material.

Scanning Electron Microscopy (SEM): seeds samples were fixed on aluminium stubs (ø 12.5 mm; 3.2 x 8 mm pin), coated with double-sided adhesive disks, and the seeds were covered with gold metallization with Scancoat Six Sputter Coater (Edwards). Samples were then analysed at SEM microscope SEM-EDS JSM-IT500 LV (JEOL). Pictures were taken at high vacuum (HV), by using 20 kV acceleration voltage, 2.53 μA load current and a 30 μA electron beam.

2.8 Elemental composition analysis via ICP-MS

Samples of mature seeds (30-40 mg) collected from wt Col, *atgullo2-1* and *atgullo2-2* senescing plants were dried in an oven at 100 °C; 500 µl 65% nitric acid (v/v) was added in each sample and seeds were mineralized at 130 °C. Each white pellet was resuspended in 400 µl 65% nitric acid (v/v), and 200 µl of such resuspension was diluted 1:40 with water (8 ml final volume). The concentrations of various elements were then measured by inductively coupled plasma mass spectrometry (ICP-MS) as described in Murgia *et al*. (2020).

2.9 Suberin polyester analysis via GC-MS

Suberin monomers were extracted from mature seeds of the wt Col and *atgullo2* mutant according to the protocols previously described (Cohen *et al*., 2019, 2020; Arya *et al.*, 2022). A sample volume of 1 µL was injected in splitless mode on a GC-MS system (Agilent 7693A Liquid Auto injector, 8860 gas chromatograph and 5977B mass spectrometer). GC was performed (HP-5MS UI column; 30 m length, 0.250 mm diameter, and 0.25 µm film thickness; Agilent J&W GC Columns) with injection temperature of 270 °C, interface set to 250 °C, and the ion source to 200 °C. Helium was used as the carrier gas at a constant flow rate of 1.2 mL min^{-1}. The temperature program was 0.5 min isothermal at 70 °C, followed by a 30 °C min^{-1} oven temperature ramp to 210 °C and a 5 °C min^{-1} ramp to 330 °C, then kept constant during 21 min. Mass spectra were recorded with an *m/z* 40 to 850 scanning range. Chromatograms and mass spectra were evaluated using the MassHunter Quantitation software (Agilent). Integrated peaks of mass fragments were normalized for sample dry weight and the respective C_{32}-alkane internal standard signal. For identification, the corresponding mass spectra and retention time indices were compared with the NIST20 library as well as in-house spectral libraries.

2.10 Statistical analysis

ASC and H_2O_2 concentrations were analyzed by two-way or one-way ANOVA respectively, followed by a Tukey test; concentrations of suberin monomers were analysed by two-tailed t-test and seed content of various elements, Fe(III) reduction activity, germination after bleaching or after exposure to ABA, chlorophyll content in seedlings at various pH, were analysed by one-tailed t-test.

2.11 In silico co-expression analyses

Transcript correlation analysis, including the starting dataset present in the Arabidopsis ATH1 Genome Array of Affymetrix, were performed as described in Menges *et al.* (2008) and Murgia *et al*. (2020).

3. Results

3.1 A. thaliana GULLO2 encodes an enzyme acting in vivo as Gulono-1,4 γ-lactone oxidase, in developing seeds

A. thaliana possesses seven *GULLO* isoforms, similar to rat (*Rattus norvegicus*) Gulono-1,4 γ-lactone oxidase, namely *Gullo1* to *Gullo7* (Maruta *et al.*, 2010; Aboobucker *et al*., 2017). Among these isoforms, *GULLO2* (At2g46750) is one of the top correlators of CYP82C4 (Murgia *et al*., 2011; Murgia *et al*., 2022), with peak expression in developing seeds, particularly in seed coats (Table 1), according to the eFP browser (Winter *et al*., 2007) based on the Developmental map and Seed microarray series, respectively (Schmid *et al*., 2005; Le *et al*., 2010).

AGI code	Gene name	Gene expression peak	References
AT1G32300	*GULLO1*	flower stage 12	Schmid *et al*., 2005
AT2G46750	*GULLO2*	seed stage 8-10	Schmid *et al*., 2005; Maruta *et al*., 2010;
		maturation green	Le *et al*., 2010
AT5G11540	*GULLO3*	flower stage 15	Schmid *et al*., 2005; Maruta *et al*., 2010; Aboobucker *et al*., 2017
AT5G56490	*GULLO4*	flower stage 15	Schmid *et al*., 2005
AT2G46740	*GULLO5*	root	Schmid *et al*., 2005; Maruta *et al*., 2010; Aboobucker *et al*., 2017
AT2G46760	*GULLO6*	first node	Schmid *et al*., 2005
AT5G56470	*GULLO7*	mature pollen	Schmid *et al*., 2005

Table 1 *A. thaliana* L-gulono-1,4 γ-lactone oxidase gene isoforms *GULLO1-7*. For each gene, the AGI code, name, the tissue/organs in which its maximal expression is observed, according to *Arabidopsis* eEF browser developmental map, and references of published studies, are reported.

The evidence assembled suggests a potential involvement of GULLO2 in plant Fe nutrition, possibly during seed development. To test this hypothesis, two independent T-DNA insertional mutants were isolated, namely *atgullo2-1* and *atgullo2-2* (Fig. **S1**). These mutants show no altered phenotype (no chlorosis or impaired growth) when grown in control soil, with respect to wt Col (Fig. **1a**). When grown on mildly alkaline soil (pH 7.7) causing Fe deficiency, their stunted growth and chlorosis was like that observed for wt (Fig. **1b**).

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Eliminato: Alteration in the plant Fe nutritional status and homeostasis can affect progeny seeds and their ability to germinate and thrive; the transgenerational analysis can be a valid tool to uncover potential phenotypes in the progeny, which remain hidden in the mother plant (Murgia *et al.*, 2015). Germination rates and phenotype of *atgullo2-1* and *atgullo2-2* mutants and of their respective pH 7.7 s1 progenies were therefore evaluated on control or pH 7.7 soil. Germination of wt and mutant seeds was similar in control soil, and all the seedlings thrived (Fig. S2a). When grown at pH 7.7, both wt and mutant seeds showed similar symptoms of Fe deficiency, i.e. smaller rosettes and chlorotic leaves (Fig. S2b), however their germination rates were, after 4 d, above 80% in all tested lines (Fig. S2c). The few progeny seeds produced from the plants grown on alkaline soil, i.e. wt pH 7.7 s1, *atgullo2-1* pH 7.7 s1 and *atgullo2-2* pH 7.7 s1 were collected and tested back on pH 7.7 soil; they all showed lower germination percentage, than their control counterparts, when observed in 24 d time span (Fig. S2c); moreover germination of *atgullo2-1* and *atgullo2-2* pH 7.7 s1 seeds is even lower than their wt counterpart (Fig. S2c). Some of the already germinated *atgullo2* pH 7.7 s1 seedlings died, as shown in the declinining germination values in the 7-24 d time span (Fig.S2c). After 32 d, 18 wt pH 7.7 s1 seedlings, 0 *gullo2-1* pH 7.7 s1 and 5 *gullo2-2* pH 7.7 s1 seedlings survived and grew, out of 30 total seeds per line (Fig. **S2c, d**).

atgullo2-1 and *atgullo2-2* mutants were also germinated *in vitro* in acidic growth medium at various pH: 6.0, 5.2, 4.7 and 4.0; chlorophyll concentration, in 4 d old *atgullo2* seedlings, was lower than wt at all pH tested (Fig. **S2a**), but seedlings weight was similar in all tested lines (Fig. **S2c**). After 19 d, a diminished chlorophyll concentration in *atgullo2* mutants was observed at three pH values (5.2 , 4.7, 4.0) (Fig. **S2b**) without, again, consistent differences in seedlings weight between wt and mutants (Fig. **S2d**).

Next, to explore if ASC synthesis was truly affected in *atgullo2* mutants, the two products of a canonical animal GULLO activity, i.e. ASC and H_2O_2 were measured in developing siliques of wt Col, *atgullo2-1* and *atgullo2-2* plants grown in control soil. For that, siliques with seeds in the developing window of *GULLO2* expression peak (as reported above, see also Materials and Methods section) were sampled. Around 50% less ASC, both in its reduced form as well as total, calculated as ASC plus its oxidized form dehydroascorbate DHA (i.e. ASC+DHA), was measured in *atgullo2-1* and *atgullo2-2* siliques, in comparison with wt (Fig. **2a**). Moreover, H_2O_2 concentration was also significantly lower in *atgullo2-1* and *atgullo2-2* siliques, with respect to wt (Fig. **2b**).

The diminished level of both ASC and H_2O_2 in *atgullo2* developing siliques was not accompanied by an altered cellular redox state: the ratio between reduced ASC and total ascorbate content [ASC/(ASC+DHA)], which is an indicator of the cellular redox state (de Pinto *et al*., 1999) was indeed statistically similar in all tested lines, being 0.73 ± 0.06 for Col siliques, 0.66 ± 0.09 for *atgullo2-1* siliques and 0.65 ± 0,07 for *atgullo2-2* ones (one-way ANOVA).

3.2 atgullo2 seeds have lower Fe content and impaired Fe(III) reduction activity

An ASC-mediated reduction step of Fe(III) into Fe(II) is required before its transport from the endosperm into the developing embryo (Grillet *et al*., 2014; Curie & Mari, 2017). To test GULLO2 contribution to the ASC pool necessary for such a reduction step, wt Col, *atgullo2-1* and *atgullo2-2* embryos from mature seeds were stained with Perls reagent which detects Fe by forming a blue color (Grillet *et al*., 2014). As expected, the blue staining formed by Perls reagent was localized in the provascular tissues in all embryos; both *atgullo2* embryos, however showed weaker staining, when compared to wt counterpart (Fig. **3a-i**) suggesting lower Fe content in such embryos with respect to wt ones. Fe content, measured by ICP-MS in whole mature seeds, is also lower in both *atgullo2* mutants when compared to wt values (Fig. **3j**).

To test an association of the lower Fe content, as observed in *atgullo2* embryos and mature seeds, with an altered ASC-mediated Fe(III) reducing activity in developing seeds, immature seeds with embryos at late torpedo/bent cotyledon stage were sampled from siliques of wt Col, *atgullo2-1* and *atgullo2-2* plants. Seeds were deprived, under a stereomicroscope, of their embryos; the emptied

seeds were tested for their ability to reduce Fe(III) into Fe(II). Both *atgullo2* emptied seeds show a diminished Fe(III) reduction activity, with respect to wt Col, being Fe(III) reduction in *atgullo2* mutants around 50% of the wt (Fig. **3k**). The embryos isolated from siliques of both *atgullo2* mutants, at the same developmental stage described for the emptied seeds, showed similar Fe(III) reduction activity to the wt ones (Fig. **S3**).

The ionome profile of mutants revealed that both *atgullo2* mutant seeds have lower content of Zinc (Zn), Manganese (Mn), Calcium (Ca) and Sodium (Na), whereas Molybdenum (Mo), Copper (Cu) and Magnesium (Mg) levels are similar to wt (Fig. **4**).

3.3 Gullo2 expression correlates with genes involved in suberin biosynthesis

An *in silico* co-expression analysis was performed, to identify genes highly similar to *GULLO2* in expression pattern. Markedly, several key genes involved in suberin metabolism exhibited high correlation coefficients with *GULLO2*, both in logarithmic (Table 2) and linear analysis (Table S1), suggesting a possible link between its activity and suberin biosynthesis.

The suberin biosynthetic pathways with known and hypothetical enzymes involved in the various steps were recently proposed (Vishwanath *et al*., 2015; Lashbrooke *et al*., 2016; Shukla & Barberon, 2021; Nomberg et al., 2022; Woolfson *et al*., 2022). *A. thaliana* alcohol-forming fatty acyl-coenzyme A reductases FAR1, FAR4 and FAR5 catalyse the synthesis of the C22:0, C20:0 and C18:0 fatty alcohols present in suberin of various plant tissues, respectively; *far* double and triple mutants have reduced fatty alcohol composition and altered seed coat permeability (Vishwanath *et al*., 2013). FAR4 is one of the *GULLO2* top correlators, in both log and linear analysis, with 0.819 and 0.847 Pearson coefficients, respectively (Table **2**, Table **S1**).

Cytochrome P450 CYP86A1 encodes a fatty acid omega-hydroxylase involved in suberin monomer biosynthesis (Höfer *et al*., 2008; Lashbrooke *et al*., 2016) whereas CYP82B1 is required for long chain hydroxyacid and dicarboxylic acid synthesis, in root and seed suberin polyesters (Molina *et al*., 2009). CYP82A1 is among the top correlators, both in log (Table **2**) and in linear analysis (Table **S1**), with 0.805 and 0.761 Pearson coefficients, respectively.

AGI code	**Gene name**	**Pearson coeff. (log)**	**Pearson coeff. (lin)**	**Subcellular localization**
AT2G46750	GULLO2	1	1	extracellular
AT3G09220	LAC7	0.851	0.837	extracellular

AT2G44010	unknown function	0.850	0.795	nucleus
AT5G22890	STOP2	0.846	0.830	nucleus
AT3G58550	LPTG22	0.845	0.783	extracellular
AT1G78340	GSTU22	0.840	0.747	cytosol
AT2G18370	LTP8	0.835	0.820	extracellular
AT3G25830; AT3G25820	TPS27; TPS23	0.830	0.770	plastid; plastid
AT5G63560	**FACT**	0.829	0.801	cytosol
AT5G59520	ZIP2 (IRT-LIKE 2)	0.828	0.782	PM
AT1G56320	unknown function	0.828	0.722	extracellular
AT4G17215	(Ole e 1) allergen type	0.826	0.725	extracellular
AT5G66690	UGT72E2	0.824	0.728	PM
AT3G44540	**FAR4**	0.819	0.847	PM
AT2G18980	PRX16	0.818	0.695	extracellular
AT5G41040	**ASFT**	0.82	0.595	cytosol
AT5G06300	LOG7	0.815	0.738	cytosol
AT3G06390	CASPL1D2	0.812	0.665	PM
AT2G30210	LAC3	0.811	0.595	extracellular
AT2G23030	SNRK2-9	0.810	0.698	nucleus
AT3G54040	PAR1	0.810	0.745	extracellular
AT1G68850	PRX11	0.809	0.596	extracellular
AT3G01420	DOX1	0.809	0.864	cytosol
AT3G21510	AHP1	0.805	0.674	nucleus
AT5G58860	**CYP86A1**	0.805	0.554	secret. pathway
AT3G01190	PER27	0.805	0.554	extracellular
AT2G29330	TRI	0.804	0.668	PM
AT1G47600;AT1G51470	BGLU34; BGLU22	0.804	0.614	extracel; extracel
AT3G22620	LTPG20	0.803	0.667	extracellular
AT5G13900	LTPG30	0.802	0.660	extracellular
AT1G18980	RmlC-like cupins fam	0.802	0.823	extracellular

Table 2. Top correlators of *A. thaliana GULLO2* gene expression, in logarithmic analysis. From left: the AGI codes, gene names, Pearson correlation coefficients in logarithmic and linear analysis and the subcellular localization of the thirty *GULLO2* top correlators are reported (PM: plasma membrane). The genes taking part in suberin biosynthesis are indicated in bold. For each gene, the

subcellular localization, according to Subcellular Localization Database for Arabidopsis Proteins SUBA5 (at https://suba.live/), is reported.

The Feruloyl Transferase Fatty Alcohol: Caffeoyl-CoA Caffeoyl Transferase (FACT) (Compagnon *et al*., 2009) is also a key enzyme in suberin biosynthesis (Nomberg *et al*., 2022) and among the top *GULLO2* correlators, with 0.829 and 0.801 Pearson coefficients, in log and linear analysis, respectively (Table **2**, Table **S1**).

Lastly, the Feruloyl transferase ASFT, also required for suberin biosynthesis (Nomberg *et al*., 2022) is among *GULLO2* top correlators in the log analysis, with 0.82 Pearson coefficient (Table **2**).

To uncover the extent of correlations between the expression of *GULLO2* and other suberin biosynthetic genes not listed among the top correlators described above (Table 2, Table S1), Pearson coefficients between *GULLO2* and 14 genes recently identified as relevant for suberin biosynthesis (Lashbrooke *et al*., 2016; Shukla & Barberon, 2021; Shukla *et al*., 2021; Nomberg *et al*., 2022; Woolfson *et al*., 2022) were also extracted (Table S2). Among these, Embryo Sac Development Arrest 4 EDA4, SGNH hydrolase-type esterase SGNH, Enhanced-Suberin 1 ESB1, ATP-binding cassette (ABC) transporter ABCG6, MYB93, Lipid Transfer Protein LTPG15, MYB36, MYB92, NAC domain containing protein 58 NAC058, Schengen 3SGN3, Suberin synthase SUS1, FAR5, MYB92, Peroxidase 64 (PER64) and CYP82B1 all turned out to be good *GULLO2* correlators, with coefficients >0.7, in the log analysis. Notably, SUS1 is a network hub for suberin biosynthesis, according to Lashbrooke *et al*. (2016); SUS is a GDSL-type Esterase-Lipase protein, more recently annotated as GELP51 by other authors (Ursache *et al*., 2021; Nomberg *et al*., 2022). The complete list is reported in Table S3.

3.4 atgullo2 seed coats have altered suberin composition and permeability

The *GULLO2* gene displayed remarkably high correlation coefficients with a subset of key biosynthetic, transport and regulatory genes previously shown to facilitate suberin deposition in the *A. thaliana* seed coat. To test whether the content and composition of the suberin in the seed coats of *atgullo2* mutants might be altered, the polyester content and composition was extracted and profiled in wt Col and *atgullo2* mature seeds via gas chromatography-mass spectrometry (GC-MS)-based protocols (Cohen *et al*., 2019, 2020; Arya *et al*., 2022). 23 archetypal suberin monomers in both wt Col and *atgullo2* mutant seeds belonging to the biochemical class of aromatic components, fatty acids, fatty alcohols, ω-hydroxyacids, and α, ω-diacids were identified (Fig. **5**). Interestingly, among these monomers only 3 exhibited different levels between wt and *atgullo2* mutant seeds, being

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significantly higher in both *atgullo2* mutant seeds. These include C20 fatty acid, and the two most predominant suberin monomers in *A. thaliana* seeds, C18:2 and C18:3 ω-hydroxyacids (Fig. **5**). These increases, in turn, impacted the total suberin content which was significantly elevated in both *atgullo2* mutant seeds, compared to wt (Fig. **5**).

The suberin content in the seed coat was previously directly associated with seed permeability capacities. The chemical changes detected in the seed coat of both *atgullo2* mutant seeds might therefore impact their permeability. To test this hypothesis, wt Col *atgullo2-1* and *atgullo2-2* seeds were bleached, for up to 10 min (Fig. **S4a**). Bleaching of the brown colour of the seed coats was more pronounced in wt Col seeds than in *atgullo2* mutants; after 10 min, patches of seed coat discoloration were observed on wt Col seeds only. The lower permeability of the *atgullo2* seed tegument was also confirmed by the different effect of bleach treatment on seed germination, since it affected the germination rate of wt Col more than both *atgullo2-1* and *atgullo2-2* mutants: a 10 min treatment reduced the mean germination value to 52% in wt seeds whereas germination values were higher, i.e. 68% and 75%, in *atgullo2-1* and *atgullo2-2* seeds (Fig. **S4b**).

To further test seed coat alterations in permeability, wt Col and *atgullo2* mutants were germinated, *in vitro*, in increasing abscisic acid (ABA) concentrations (0, 0.5, 1 and 10 μM ABA), given the established role of ABA as repressor of germination in mature seeds (Garciarrubio *et al*., 1997; Kang *et al*., 2015) and germination rates were scored for four days (1d- 4d). A significant difference in germination, between wt and *atgullo2* mutant seeds, could already be observed at 2d, when exposed to either 1 or 10 μM ABA. Differences between wt and *atgullo2* germination were observed also at 3d and 4d; in particular, at 4d, wt Col showed a residual germination rate < 30%, which was instead around 50-55% in *atgullo2-1* and *atgullo2-2* mutants, at the highest ABA concentration (Fig. **6**).

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3.5 atgullo2 seeds show features of altered seed coat morphology

The morphological and mechanical properties of wt Col, *atgullo2-1* and *atgullo2-2* mature seeds were also explored, with Scanning Electron Microscopy (SEM) and Atomic Force Microscopy (AFM). As expected (Windsor *et al*., 2000; Ezquer *et al*., 2016), the surface of mature wt dry seeds show the typical reticulate pattern of hexagonal cells with thickened radial cell wall (RCW), and the central volcano-shaped columella (Fig. **7a, d**). Such a reticular pattern was maintained in both *atgullo2-1* (Fig. **7b, e**) and *atgullo2-2* (Fig. **7c, f**). However, occasionally the pattern was slightly altered in both mutants with cell shapes of round appearance due to an altered disposition of cell walls and consequent distortion of the characteristic radial pattern and with columellas of variable shape, from flattened to elongated, as indicated by arrows (Fig. **7e, f**). SEM analyses were repeated with two more independent seed batches, collected from plants grown during different rounds of cultivation; results

show that phenotypic expression of the disruption of the regular reticulate pattern is variable in *atgullo2* mutants, and in one seed batch such disruption appeared more severe (Figs. **S5-S7**).

The apparent Young's modulus (elastic modulus) of wt Col, *atgullo2-1* and *atgullo2-2* dry seeds was also evaluated, by Atomic Force Microscopy (AFM). To avoid distortions due to the seeds' mucilage, which could affect measurements, seeds were first hydrated, their adherent mucilage was removed by 40 s sonication (Zhao *et al.*, 2017) and the seeds were dried again; ruthenium red staining of imbibed wt Col, *atgullo2-1* and a*tgullo2-2* seeds (Fig. **S8**) and staining after sonication (Fig. **S9**), shows that such treatment is effective in completely removing the mucilage without damaging the seed coat.

The AFM analysis does not highlight any differences in the tested lines (Fig. S10), suggesting that the altered suberin composition in both *atgullo2* seeds did not impact the mechanical properties of their coats.

4. Discussion

Given the importance of ASC in plant responses to changes in ROS concentrations during exposure to stresses, a major effort has been put into the elucidation of the biosynthetic pathway of ASC in plants (Smirnoff & Wheeler, 2000; Wheeler *et al*., 2015; Smirnoff, 2018).

Various *A. thaliana vtc* mutants, characterized by reduced ASC levels, have been isolated and analysed; they are mainly defective in the enzymes involved in the Smirnoff-Wheeler biosynthetic pathway (Conklin *et al*., 2000): *vtc1* in the GDP-Mannose pyrophosphorylase (Conklin *et al*., 1999), *vtc2* and *vtc5* in either of the two isoforms of GDP-L-galactose phosphorylase (Linster et al, 2007; Gao *et al*., 2011; Lim *et al*., 2016), whereas *vtc4* in L-Galactose-1-P Phosphatase (Conklin *et al*., 2006). Decreased growth and altered patterns of photosynthesis (Talla *et al*., 2011) accelerated senescence, and altered response to pathogens (Barth *et al*., 2004) are traits associated, in most cases, with diminished ASC levels in such *vtc* mutants. A study proposed that the altered growth observed in the *vtc2-1* mutant is unrelated to the decreased ASC levels (Lim *et al*., 2016). Analysis of all these *vtc* mutants allowed the physiological role of ASC in plants to be better defined.

Besides the well-known ASC role as antioxidant hub and in various housekeeping functions, ASC is also involved in the transport of Fe into developing seeds; mature seeds need to be filled, during their maturation stage, with an amount of Fe in the proper chemical form, able to support early growth stages after their germination. Such seed Fe loading is therefore finely tuned and it involves, in *A. thaliana* and in *Pisum sativum*, the reduction of Fe(III) to Fe(II) in the endosperm, for subsequent Fe(II) transport into the developing embryos (Grillet *et al*., 2014; Murgia *et al*., 2022).

The co-production of ASC and H_2O_2 by plant GULLO enzymes, similarly to what is demonstrated for animal GULLOs, still remains an open question (Aboobucker and Lorence, 2016). Nonetheless, our data suggest that, in developing *A. thaliana* seeds, Gulono-1,4 γ-lactone oxidase 2 GULLO2 genuinely catalyses an oxidase reaction generating both ASC and H_2O_2. Indeed, developing siliques of both *atgullo2* mutants show diminished levels of both ASC and H_2O_2. A more in-depth study on the catalytic properties of plant GULLO enzymes is however needed, to confirm this similarity with the animal counterpart.

Due to the necessity to collect enough biological material, such measurements have been performed on whole siliques and not simply on their developing seeds. This unavoidable experimental constraint might therefore have caused an underestimation of the differences in ASC and H_2O_2 values, between wt and *atgullo2* seeds; the sampled material also included the valves and the replum, where differences in ASC and H_2O_2 levels are not expected between wt and *atgullo2* lines. Our work also suggests that the ASC produced by GULLO2 contributes to the ASC pool required to reduce endospermic Fe(III) into Fe(II) for its transport into the developing embryos. The mutant seeds,

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devoid of their embryos, showed a diminished Fe(III) reduction activity, which is hence attributable to the lack of GULLO2 in their coat/endosperm. Consistently, *atgullo2* embryos showed reduced Fe content. These results represent, to our knowledge, the first documented evidence of an *in vivo* role of a GULLO enzyme, in plants.

These results are in agreement with an extracellular localization of GULLO2, as predicted by the Subcellular Localization Database for Arabidopsis Proteins SUBA (https://suba.live/suba-app/factsheet.html?id=AT2G46750.1) which uses 22 topological predictors (Hooper *et al.*, 2017); GULLO2 is localized in the apoplast of the innermost layers of the seed coats, as suggested by previous data (Aboobucker and Lorence, 2016).

The reduced Fe concentration in *atgullo2* mutants was observed not only in embryos but also in whole seeds. This suggests a feedback control of the Fe transport into seeds and involving the Fe nutritional status of the endosperm; the Fe transported from the stem into the endosperm might be modulated according to the amount of Fe transported from endosperm itself into the embryos, possibly to avoid unnecessary Fe accumulation in the endosperm and consequent toxicity. Without this feedback control, the lower extent of Fe(III) reduction caused by diminished ASC availability in *atgullo2* seeds, should have caused Fe accumulation in the tissues surrounding the embryos, without affecting the total Fe content in whole seeds, contrary to what is observed in *atgullo2* mutants. The elucidation of the metabolic players involved in this putative feedback control of Fe transport into developing seed and involving both the endosperm and the embryos, could represent potential regulators of seed Fe loading which are sought after (Roschzttardtz *et al.*, 2020). Also, these regulators might represent novel candidates for plant Fe biofortification approaches (Connorton & Balk, 2019). Some of these regulators of Fe loading has been recently identified in *A.thaliana*; the transcription factor ERF95 contributes to seed Fe loading (Sun *et al.*, 2020). Moreover, the YABBY transcription factor INNER NO OUTER (INO) binds to a specific region of NRAMP1 promoter, thus inhibiting its expression; in this way, INO prevents excess Fe accumulation in seeds and prevents Fe-dependent oxidative damage (Sun et al., 2021).

GULLO2 also impacts seed coat suberization. Suberin is a complex hetero-polymer consisting of polyaliphatic and polyphenolic domains (Philippe *et al.*, 2020; Shukla & Barberon, 2021; Nomberg *et al.*, 2022). The deposition of suberin in plants occurs in various specialized tissues including tree bark, potato tuber skin, russeted and reticulated skin of several fleshy fruits, root endodermis as well as seed coats (Pollard *et al.*, 2008; Schreiber, 2010; Cohen *et al.*, 2017). Fe deficiency was documented to affect the content of both aliphatic and aromatic components of suberin in bean roots; such effect was attributed to a lower activity of suberin-specific peroxidases (Sijmons *et al.*, 1985).

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The reduction in suberin accumulation is the root endodermis, has been also described in roots of Fe-deficient *A. thaliana* plants, as well as its dependence on ethylene signaling (Barberon *et al*., 2016); the lower suberization of root cells would favour nutrient transport by increasing cell wall permeability (Ogden et al, 2018).

The connection between seed Fe nutritional status and suberin content and composition, in the seed coats, is unexplored. Our results show that the knocking out of *GULLO2* affects the deposition patterns of suberin in the *A. thaliana* seed coat, leading to an increase of total suberin due to higher level of specific monomers, in particular C18:2 and C18:3 ω-hydroxyacids. The future profiling of the suberin content in seed coats of *A. thaliana* mutants impaired in Fe accumulation as well as in *vtc* mutants, which cause an alteration in ASC content in different tissues and cellular compartments, will help entangle the relationship between GULLO2 activity and suberin biosynthesis. In particular, it will help to clarify whether the altered suberin content in *atgullo2* mutants shown in the present work, can be directly attributed to the metabolites produced by GULLO2 activity or, indirectly, to the altered Fe content in *atgullo2* seeds. Notably, GULLO2 is expressed also in roots, though at much lower extent with respect to expression in developing seeds, according to *Arabidopsis* eEF browser developmental map (Schmid *et al*., 2005; Winter *et al*., 2007). The deposition of suberin in *atgullo2* roots might therefore be also altered; this, in turn, might alter nutrient uptake (Barberon *et al*., 2016; Ogden *et al*., 2018). On the other hand, the lack of GULLO2 activity truly affects not only Fe content but also the content of other nutrients in *atgullo2* seeds; the lower levels of Zn and Mn in seeds of both mutant genotypes indeed paralleled the observed reduction in Fe levels. The relationship between the possible alterations in suberification process in *atgullo2* roots and the observed altered levels of some nutrients in *atgullo2* seeds will be therefore investigated, in the future, by using *in situ* suberin visualization techniques (Serra and Geldner, 2022) and a thorough analysis of *atgullo2* roots, stems and leaves ionomes. A direct involvement of this gene in the synthesis of the affected suberin monomers cannot be ascertained, yet. Indeed, it is known that H_2O_2 can inhibit P450 enzymes, and thus, this might directly up-regulate the activity of CYP86A1, which participates in the oxidation of C18:2 and C18:3 suberin monomers (Hofer et al., 2008; Nomberg *et al.*, 2022). The reduced levels of H_2O_2 detected in *atgullo*2 developing siliques might lead to higher CYP86A1 activity, which in turn, might cause an over-accumulation of C18:2 and C18:3 ω-hydroxyacids. This hypothesis, however, implies that H_2O_2 produced by GULLO2 in the apoplastic space of the developing seed coat would affect the CYP86A1-driven C18:2 and C18:3 biosynthesis occurring in the endoplasmic reticulum. H_2O_2 is the most diffusible ROS, since its diffusion across membranes is known to be facilitated by aquaporins (Bienert & Chaumont, 2014). Alternatively, both H_2O_2 and ASC might be involved in polymerization of the phenolics, with involvement of superoxide SOD and peroxidases

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(Woolfson *et al*., 2022) which, in turn, might impact the alteration in the monomeric composition of suberin, shown in the *atgullo2* mutants. Notably, in *A. thaliana* the expression of *GULLO5*, encoded by At2g46740 gene, increases in Fe deficient roots with respect to Fe sufficient counterpart and such regulation is lost in *fit1-1* mutant altered in the basic helix-loop-helix FIT1, which is one of the regulators of the Fe-deficiency response in *A. thaliana* (Colangelo and Guerinot, 2004). The reduction of suberization in Fe-deficient roots, as described in (Barberon *et al*., 2016) suggests that the increase in GULLO5 activity in *A. thaliana* Fe-deficient root, is associated with a reduction in suberin. This hypothesis should be tested in *atgullo5* mutants and it would be consistent with the fact that, inversely, an impaired GULLO2 activity increases suberin content, in *atgullo2* mutants.

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The isolation and characterization of *atgullo2* mutants in vtc background could be relevant to clarify the relationship between GULLO2 physiological activity and the plant canonical pathway of ASC biosynthesis.

STOP2 is a homolog of STOP1, a zinc-finger protein regulating transcription of genes involved in tolerance to Aluminium (Al) or to low pH (Kobayashi *et al*., 2014); these authors list some of the co-expressed genes with STOP2 which overlap between the stress from Al and stress from low pH: with the exception of zigzag suppressor 2 gene (ZIP2, At1g08190). GULLO2 is highly positively correlated with all these genes, i.e. Al-activated malate transporter 1 (ALMT1), a Leu-rich kinase gene (At1g51830), Rapid Alkalinization Factor-Like 27 (RALFL27), a cysteine-rich receptor-like protein kinase (CRK25), as their Pearson coefficients for GULLO 2 are 0.7014, 0.7207, 0.720, 0.775, respectively (in the log analysis). STOP2 is expressed in roots, hydathodes, vascular bundles, flowers, and siliques' extremities whereas GULLO2 is expressed in stems and leaves, although at much lower extent than in seeds. It will be interesting to investigate, in the future, if the reduced chlorophyll concentration observed in both *atgullo2* seedlings at very early stage, when grown at low pH, is due to altered STOP2-mediated stress responses, in *atgullo2* mutants.

The present work uncovered an *in vivo* physiological role of one of the seven GULLO isoforms in *A. thaliana* in Fe nutrition and seed development; further investigations will be necessary to clarify the link with suberin formation and to uncover possible involvements of other *A. thaliana* GULLO isoforms in Fe nutrition and suberization processes in various plant organs and tissues.

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author Contribution

IM and PM conceived the work and the experimental plan. IM, AM, SC, LDG, PM, EM, HC, AP performed the experiments. IM, AM, SC, LDG, PM, EM, HC, AP analysed the data. IM wrote the manuscript, with PM, SC, LDG, HC contributions.

References

Aboobucker SI, Lorence A. 2016. Recent progress on the characterization of aldonolactone oxidoreductases. *Plant Physiol Biochem* 98:171-85. doi: 10.1016/j.plaphy.2015.11.017

Aboobucker SI, Suza WP, Argelia Lorence A. 2017. Characterization of two Arabidopsis L-Gulono-1,4-lactone Oxidases, AtGulLO3 and AtGulLO5, involved in ascorbate biosynthesis. *Reactive Oxygen Species* **4**: 389-417.

Arya GC, Dong Y, Heinig U, Shahaf N, Kazachkova Y, Aviv-Sharon E, Nomberg G, Marinov O, Manasherova E, Aharoni A, Cohen, H. 2022. The metabolic and proteomic repertoires of periderm tissue in skin of the reticulated Sikkim cucumber fruit. *Hortic. Res.* **9**: uhac092.

Banhegyi G, Braun L, Csala M, Puskas F, Mandl J. 1997. Ascorbate metabolism and its regulation in animals. *Free Radic. Biol. Med.* **23**: 793–803.

Barberon M, Vermeer JEM, De Bellis D, Wang P, Naseer S, Andersen TG, Humbel BM, Nawrath C, Takano J, Salt DE, Geldner N. 2016. Adaptation of Root Function by Nutrient-Induced Plasticity of Endodermal Differentiation. *Cell* **164**: 447-459.

Barth C, Moeder W, Klessig DF, Conklin PL. 2004. The timing of senescence and response to pathogens is altered in the ascorbate-deficient *Arabidopsis* mutant vitamin c-1. *Plant Physiol.* **134**: 1784-1792.

Bienert GP, Chaumont F. 2014. Aquaporin-facilitated transmembrane diffusion of hydrogen peroxide. *Biochim. Biophys. Acta* **1840**,5: 1596-1604. https://doi.org/10.1016/j.bbagen.2013.09.017.

Bulley SM, Rassam M, Hoser D, Otto W, Schünemann N, Wright M, MacRae E, Gleave A, Laing W. 2009. Gene expression studies in kiwifruit and gene over-expression in *Arabidopsis* indicates that GDP-L-galactose guanyltransferase is a major control point of vitamin C biosynthesis, *Journal of Experimental Botany*, **60**: 765–778.

Bulley S, Laing W. 2016. The regulation of ascorbate biosynthesis. *Current Opinion in Plant Biology* **33**: 15-22.

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Caverzan A, Passaia G, Rosa SB, Ribeiro CW, Lazzarotto F, Margis-Pinheiro M. 2012. Plant responses to stresses: role of ascorbate peroxidase in the antioxidant protection. *Genetics and Molecular Biology* **35**: 1011-1019.

Cohen H, Dong Y, Szymanski J, Lashbrooke J, Meir S, Almekias-Siegl E, Zeisler-Diehl VV, Schreiber L, Aharoni A. 2019. A multilevel study of melon fruit reticulation provides insight into skin ligno-suberization hallmarks. *Plant Phys.* **179**: 1486-1501.

Cohen H, Fedyuk V, Wang C, Wu S, Aharoni A. 2020. SUBERMAN regulates developmental suberization of the Arabidopsis root endodermis. *Plant Journal* **102**: 431-447.

Compagnon V, Diehl P, Benveniste I, Meyer D, Schaller H, Schreiber L, Franke R, Pinot F. 2009. CYP86B1 is required for very long chain omega-hydroxyacid and alpha, omega-dicarboxylic acid synthesis in root and seed suberin polyester. *Plant Physiol.* **150**: 1831-1843.

Conklin PL, Norris SR, Wheeler GL, Williams EH, Smirnoff N, Last RL. 1999. Genetic evidence for the role of GDP-mannose in plant ascorbic acid (vitamin C) biosynthesis. *Proc. Natl. Acad. Sci. USA* **96**: 4198-4203.

Conklin PL, Saracco SA, Norris SR, Last RL. 2000. Identification of ascorbic acid-deficient Arabidopsis thaliana mutants. *Genetics* **154**: 847-856.

Conklin PL, Gatzek S, Wheeler GL, Dowdle J, Raymond MJ, Rolinski S, Isupov M, Littlechild JA, Smirnoff N. 2006. Arabidopsis thaliana VTC4 encodes L-Galactose-1-P Phosphatase, a plant ascorbic acid biosynthetic enzyme. *Journal of Biological Chemistry* **281**: 15662-15670.

Connorton JM, Balk J. 2019. Iron biofortification of staple crops: lessons and challenges in plant genetics. *Plant Cell Physiol.* **60**: 1447-1456.

Considine MJ, Foyer CH. 2021. Oxygen and reactive oxygen species-dependent regulation of plant growth and development. *Plant Physiology* **186:** 79-92.

Curie C, Mari S. 2017. New routes for plant iron mining. *New Phytologist* **214**: 521-525.

De Gara L, Tommasi F, Liso R, Arrigoni O. 1991. Ascorbic acid utilization by prolyl hydroxylase *in vivo*. *Phytochemistry* **30:** 1397-1399.

de Pinto MC, Francis D, De Gara L. 1999. The redox state of the ascorbate-dehydroascorbate pair as a specific sensor of cell division in tobacco BY-2 cells. *Protoplasma* **209:** 90-97.

De Tullio MC, Paciolla C, Della Vecchia F, Rascio N, D'Emerico S, De Gara L, Liso R, Arrigoni O. 1999. Changes in onion root development induced by the inhibition of peptidyl prolyl hydroxylase and influence of the ascorbate system on cell division and elongation. *Planta* **209:** 424-434.

Drouin G, Godin JR, Pagé B. 2011. The Genetics of Vitamin C Loss in Vertebrates. *Current Genomics* **12**: 371-378.

Ezquer I, Mizzotti C, Nguema-Ona E, Gottè M, Beauzamy L, Ebeling Viana V, Dubrulle N, Costa de Oliviera A, Caporali E, Koroney AS et al. 2016. The developmental regulator SEEDSTICK controls structural and mechanical properties of the *Arabidopsis* seed coat. *Plant Cell* **28**: 2478-2492.

Feng W, Kita D, Peaucelle A, Cartwright HN, Doan V, Duan Q, Liu M, Maman J, Steinhorst L, Schmitz I, Yvon R, Kudla J, Wu H, Cheung A, Dinneny J. 2018. The FERONIA receptor kinase maintains cell-wall integrity during salt stress through Ca2+ signalling. *Curr. Biol.* **28**: 666-675.

Fernie AR, Tohge T. 2015. A cross-kingdom history. *eLife* 4:e07527.

Foyer CH, Noctor G. 2011. Ascorbate and glutathione: the heart of the redox hub. *Plant Physiology* **155**: 2-18.

Fragkostefanakis S, Sedeek KEM, Raad M, Zaki MS, Kalaitzis P. 2014. Virus induced gene silencing of three putative prolyl 4-hydroxylases enhances plant growth in tomato (*Solanum lycopersicum*). *Plant Molecular Biology* **85**: 459-471.

Gao Y, Badejo AA, Shibata H, Sawa Y, Maruta T, Shigeoka S, Page M, Smirnoff N, Ishikawa T. 2011. Expression analysis of the VTC2 and VTC5 genes encoding GDP-L-galactose

Eliminato: ¶
Dubeaux G, Neveu J, Zelazny E, Vert G. 2018. Metal Sensing by the IRT1 transporter-receptor orchestrates its own degradation and plant metal nutrition. *Mol. Cell.* **69**: 953-964.e5.¶

phosphorylase, an enzyme involved in ascorbate biosynthesis, in Arabidopsis thaliana. *Biosci. Biotechnol. Biochem.* **75**: 1783-1788.

Garciarrubio A, Legaria JP, Covarrubias AA. 1997. Abscisic acid inhibits germination of mature Arabidopsis seeds by limiting the availability of energy and nutrients. *Planta* **203**: 182-187.

Gest N, Gautier H, Stevens R. 2013. Ascorbate as seen through plant evolution: the rise of a successful molecule? *Journal of Experimental Botany* **64**: 33-53.

Grillet L, Ouerdane L, Flis P, Hoang MTT, Isaure MP, Lobinski R, Curie C, Mari S. 2014. Ascorbate efflux as a new strategy for iron reduction and transport in plants. *J. Biol. Chem.* **289**: 2515-2525.

Höfer R, Briesen I, Beck M, Pinot F, Schreiber L, Franke R. 2008. The *Arabidopsis* cytochrome P450 CYP86A1 encodes a fatty acid omega-hydroxylase involved in suberin monomer biosynthesis. *J. Exp. Bot.* **59**: 2347-2360.

Hooper CM, Castleden I, Tanz SK, Aryamanesh N, Millar AH. 2017. SUBA4: the interactive data analysis centre for Arabidopsis subcellular protein locations. *Nucleic Acids Res.* **45**: D1064-D1074.

Jain AK, Nessler CL. 2000. Metabolic engineering of an alternative pathway for ascorbic acid biosynthesis in plants. *Mol. Breed.* **6**: 73-78.

Kang J, Yim S, Choi H, Kim A, Lee KP, Lopez-Molina L, Martinoia E, Lee Y. 2015. Abscisic acid transporters cooperate to control seed germination. *Nat. Commun***. 6**: 8113.

Kobayashi Y, Ohyama Y, Kobayashi Y, Ito H, Iuchi S, Fujita M, Zhao CR, Tanveer T, Ganesan M, Kobayashi M, Koyama H. 2014. STOP2 activates transcription of several genes for Al- and low pH-tolerance that are regulated by STOP1 in Arabidopsis. *Mol. Plant.* **7**: 311-22.

Lashbrooke J, Cohen H, Levy-Samocha D, Tzfadia O, Panizel I, Zeisler V, Massalha H, Stern A, Trainotti L, Schreiber L, Costa F et al. 2016. MYB107 and MYB9 homologs regulate suberin deposition in Angiosperms. *The Plant Cell* **28**: 2097-2116.

Le BH, Cheng C, Bui AQ, Wagmaister JA, Henry KF, Pelletier J, Kwong L, Belmonte M, Kirkbride R, Horvath S, Drews GN, Fischer RL, Okamuro JK, Harada JJ, Goldberg RB. 2010. Global analysis of gene activity during Arabidopsis seed development and identification of seed-specific transcription factors. *Proc. Natl. Acad. Sci. U S A.* **107**: 8063-8070.

Lim B, Smirnoff N, Cobbett CS, Golz JF. 2016. Ascorbate-deficient vtc2 mutants in Arabidopsis do not exhibit decreased growth. *Front Plant Sci.***7**: 1025.

Linster CL, Gomez TA, Christensen KC, Adler LN, Young BD, Brenner C, Clarke SG. 2007. Arabidopsis VTC2 encodes a GDP-L-galactose phosphorylase, the last unknown enzyme in the Smirnoff-Wheeler pathway to ascorbic acid in plants. *J. Biol. Chem.* **282**: 18879-18885.

Lisko KA, Torres R, Harris RS, Belisle M, Vaughan MM, Jullian B, Chevone BI, Mendes P, Nessler CL, Lorence A. 2013. Elevating vitamin C content via overexpression of myo-inositol oxygenase and L-gulono-1,4-lactone oxidase in *Arabidopsis* leads to enhanced biomass and tolerance to abiotic stresses. *In Vitro Cell Dev. Biol. Plant.* **49**: 643-655.

Locato V, Cimini S, De Gara L. 2013. Strategies to increase Vitamin C in plants: from plant defense perspective to food biofortification. *Front. Plant Sci.* **4**:152.

Locato V, Cimini S, De Gara L. 2018. ROS and redox balance as multifaceted players of cross-tolerance: Epigenetic and retrograde control of gene expression. *Journal of Experimental Botany* **69:** 3373-3391.

Lodde V, Morandini P, Costa A, Murgia I and Ezquer I. 2021. cROStalk for life: Uncovering ROS signalling in plants and animal systems, from gametogenesis to early embryonic development. *Genes* **12**. 10.3390/genes12040525

Maruta T, Ichikawa Y, Mieda T, Takeda T, Tamoi M, Yabuta Y, Ishikawa T, Shigeoka S. 2010. The contribution of *Arabidopsis* homologs of L-Gulono-1,4-lactone Oxidase to the biosynthesis of ascorbic acid. *Biosci. Biotechnol. Biochem.* **74**: 1494-1497.

Menges M, Dóczi R, Ökrész L, Morandini P, Mizzi L, Soloviev M, Murray JAH, Bögre L. 2008. Comprehensive gene expression atlas for the Arabidopsis MAP kinase signalling pathways. *New Phytol*. **179**: 643-662.

Molina I, Li-Beisson Y, Beisson F, Ohlrogge JB, Pollard M. 2009. Identification of an *Arabidopsis* feruloyl-coenzyme A transferase required for suberin synthesis. *Plant Physiology* **151**: 1317-1328.

Murgia I, Tarantino D, Vannini C, Bracale M, Carravieri S, Soave C. 2004. Arabidopsis thaliana plants overexpressing thylakoidal ascorbate peroxidase show increased resistance to Paraquat-induced photooxidative stress and to nitric oxide-induced cell death. *The Plant Journal* **38**: 940-953.

Murgia I, Tarantino D, Soave C, Morandini P. 2011. The Arabidopsis CYP82C4 expression is dependent on Fe availability and the circadian rhythm and it correlates with genes involved in the early Fe-deficiency response. *Journal of Plant Physiology* **168**: 894-902.

Murgia I, Giacometti S, Balestrazzi A, Paparella S, Pagliano C, Morandini P. 2015. Analysis of the transgenerational iron deficiency stress memory in *Arabidopsis thaliana* plants. *Front. Plant Sci.* **6**: 745.

Murgia I, Vigani G, Di Silvestre D, Mauri P, Rossi R, Bergamaschi A, Frisella M, Morandini P. 2020. Formate dehydrogenase takes part in molybdenum and iron homeostasis and affects dark-induced senescence in plants. *Journal of Plant Interactions* **15**: 386-397.

Murgia I, Marzorati F, Vigani G and Morandini P. 2022. Plant iron nutrition in the long road from soil to seeds. *Journal of Experimental Botany* **73**: 1809-1824.

Noctor G, Reichheld JP, and Foyer CH. 2018. ROS-related redox regulation and signaling in plants. *Semin. Cell Dev. Biol*. **80**: 3–12.

Nomberg G, Marinov O, Arya GC, Ekaterina Manasherova E, Cohen H. 2022. The key enzymes in the suberin biosynthetic pathway in plants: an update. *Plants* **11**: 392.

Ogden M, Hoefgen R, Roessner U, Persson S, Khan GA. 2018. Feeding the walls: how does nutrient availability regulate cell wall composition? *International Journal of Molecular Sciences*. **19**:2691. https://doi.org/10.3390/ijms19092691

Ohta Y, Nishikimi M. 1999. Random nucleotide substitutions in primate non-functional gene for L-gulono-g-lactone oxidase, the missing enzyme in L-ascorbic acid biosynthesis. *Biochimica Biophysica Acta* **1472**: 408-411.

Peaucelle A. 2014. AFM-based mapping of the elastic properties of cell walls: at tissue, cellular, and subcellular resolutions. *J. Vis. Exp.* **89** : e51317.

Peaucelle A, Wightman R, Höfte H. 2015. The control of growth symmetry breaking in the Arabidopsis hypocotyl. *Current Biol.* **25:** 1746-1752.

Philippe G, Sørensen I, Jiao C, Sun X, Fei Z, Domozych DS, Rose JK. 2020. Cutin and suberin: assembly and origins of specialized lipidic cell wall scaffolds. *Curr. Opin. Plant. Biol.* **55**: 11-20.

Pollard M, Beisson F, Li Y, Ohlrogge JB. 2008. Building lipid barriers: biosynthesis of cutin and suberin. *Trends Plant. Sci.* **13**: 236-246.

Radzio JA, Lorence A, Chevone BI, Nessler CL. 2003. l-Gulono-1,4-lactone oxidase expression rescues vitamin C-deficient Arabidopsis (vtc) mutants. *Plant Molec. Biol.* **53**: 837-844.

Rajniak J, Giehl RFH, Chang E, Murgia I, von Wirén N, Sattely ES. 2018. Biosynthesis of redox-active metabolites in response to iron deficiency in plants. *Nature Chemical Biology* **14**: 442-450.

Roschzttardtz H, Conéjéro G, Curie C, Mari S. 2009. Identification of the Endodermal Vacuole as the Iron Storage Compartment in the Arabidopsis Embryo. *Plant Physiology* **151**: 1329-1338.

Roschzttardtz H, Gaymard F, Dubos C. 2020. Transcriptional Regulation of iron distribution in seeds: a perspective. *Frontiers in Plant Science* **11**: 725.

Sabetta W, Vandelle E, Locato V, Costa A, Cimini S, Bittencourt Moura A, Luoni L, Graf A, Viggiano L, De Gara L, Bellin D, Blanco E, de Pinto MC. 2019. Genetic buffering of cyclic AMP

in *Arabidopsis thaliana* compromises the plant immune response triggered by an avirulent strain of *Pseudomonas syringae* pv. Tomato. *The Plant Journal* **98:** 590-606.

Schmid M, Davison TS, Henz SR, Pape UJ, Demar M, Vingron M, Schölkopf B, Weigel D, Lohmann JU. 2005. A gene expression map of Arabidopsis thaliana development. *Nat. Genet.* **37**: 501-506.

Schreiber L. 2010. Transport barriers made of cutin, suberin and associated waxes. *Trends Plant Sci.* **15**: 546-553.

Serra O, Geldner N. 2022. The making of suberin. *New Phytologist* **235**: 848–866.

Shukla V, Barberon M. 2021. Building and breaking of a barrier: Suberin plasticity and function in the endodermis. *Current Opinion in Plant Biology* **64**: 102153.

Shukla V, Han JP, Cléard F, Lefebvre-Legendre L, Gully K, Flis P, Berhin A, Andersen TG, Salt DE, Nawrath C, Barberon M. 2021. Suberin plasticity to developmental and exogenous cues is regulated by a set of MYB transcription factors. *Proceedings of the National Academy of Sciences USA* **118**: 39.

Sies H, Belousov VV, Chandel NS, Davies MJ, Jones DP, Mann GE, Murphy MP, Yamamoto MY, Winterburn C. 2022. Defining roles of specific reactive oxygen species (ROS) in cell biology and physiology. *Nat. Rev. Mol. Cell Bio.l* **23**: 499-515.

Sijmons PC, Kolattukudy PE, Bienfait HF. 1985. Iron Deficiency Decreases Suberization in Bean Roots through a Decrease in Suberin-Specific Peroxidase Activity. *Plant Physiol*. 78:115-20.

Smirnoff N, Wheeler GL. 2000. Ascorbic acid in plants: biosynthesis and function. *Critical Reviews in Biochemistry and Molecular Biology* **35**: 291-314.

Smirnoff N. 2018. Ascorbic acid metabolism and functions: A comparison of plants and mammals. *Free Radic. Biol. Med.* **122**: 116-129.

Sun Y, Li JQ, Yan JY, Yuan JJ, Li GX, Wu YR, Xu JM, Huang RF, Harberd NP, Ding ZJ, Zheng SJ. 2020 Ethylene promotes seed iron storage during *Arabidopsis* seed maturation via ERF95 transcription factor. *J. Integr. Plant Biol.* **62**:1193-1212.

Sun L, Wei YQ, Wu KH, Yan JY, Xu JN, Wu YR, Li GX, Xu JM, Harberd NP, Ding ZJ, Zheng SJ. 2021. Restriction of iron loading into developing seeds by a YABBY transcription factor safeguards successful reproduction in *Arabidopsis*. *Mol. Plant* **14**:1624-1639.

Talla S, Riazunnisa K, Padmavathi L, Sunil B, Rajsheel P, Raghavendra AS. 2011. Ascorbic acid is a key participant during the interactions between chloroplasts and mitochondria to optimize photosynthesis and protect against photoinhibition. *J. Biosci.* **36**: 163-173.

Ursache R, De Jesus Vieira Teixeira C, Dénervaud Tendon V. *et al.* 2021. GDSL-domain proteins have key roles in suberin polymerization and degradation. *Nat. Plants* **7**: 353-364.

Vasconcelos MW, Clemente TE and Grusak MA. 2014. Evaluation of constitutive iron reductase (AtFRO2) expression on mineral accumulation and distribution in soybean (Glycine max. L). *Front. Plant Sci.* **5**: 112.

Vazzola V, Losa A, Soave C, Murgia I. 2007. Knockout of frataxin causes embryo lethality in Arabidopsis. *FEBS Letters* **581**: 667-672.

Vishwanath SJ, Kosma DK, Pulsifer IP, Scandola S, Pascal S, Joubes J, Dittrich-Domergue F, Lessire R, Rowland O, Domergue F. 2013. Suberin-associated fatty alcohols in *Arabidopsis*: distribution in roots and contributions to seed coat barrier properties. *Plant Physiology* **163**: 1118-1132.

Vishwanath SJ, Delude C, Domergue F, Rowland O. 2015. Suberin: biosynthesis, regulation, and polymer assembly of a protective extracellular barrier. *Plant Cell Reports* **34**: 573-586.

Waszczak C, Carmody M, and Kangasjärvi J. 2018. Reactive Oxygen Species in Plant Signaling. *Annual Review of Plant Biology* **69**: 209-236.

Waters BM, Blevins DG, Eide DJ. 2002. Characterization of FRO1, a Pea Ferric-Chelate Reductase Involved in Root Iron Acquisition. *Plant Physiology* **129**: 85-94.

Wheeler G, Ishikawa T, Pornsakit V, Smirnoff N. 2015. Evolution of alternative biosynthetic pathways for vitamin C following plastid acquisition in photosynthetic eukaryotes. *eLife* 4:e06369.

Windsor JB, Symonds VV, Mendenhall J, Lloyd AM. 2000. *Arabidopsis* seed coat development: morphological differentiation of the outer integument. *Plant J.* **22**: 483-493.

Winter D, Vinegar B, Nahal H, Ammar R, Wilson GV, Provart NJ. 2007. An "Electronic Fluorescent Pictograph" Browser for Exploring and Analyzing Large-Scale Biological Data Sets. *PLOS ONE* **2(8):** e718.

Woolfson KN, Esfandiari M, Bernards MA. 2022. Suberin Biosynthesis, Assembly, and Regulation. *Plants* **11**: 555.

Zhao X, Qiao L, Wu AM. 2017. Effective extraction of Arabidopsis adherent seed mucilage by ultrasonic treatment. *Scientific Reports* **7**: 40672.

Figure Legends

Figure 1

Phenotype of at*gullo2* mutants. wt Col, *atgullo2-1* and *atgullo2-2* plants grown four weeks in **(a)** control soil or **(b)** alkaline soil, at pH 7.7.

Figure 2

ASC and H_2O_2 contents in *atgullo2* developing siliques **(a)** Alterations in the ASC levels in wt Col, *atgullo2-1* and *atgullo2-2* developing siliques were monitored as variations in the content of total ascorbate (ASC plus its oxidized form dehydroascorbate DHA, ASC + DHA) and of ASC. **(b)** Changes in H_2O_2 content in wt Col, *atgullo2-1* and *atgullo2-2* developing siliques. Results are expressed as fluorescence relative to wt Col values and normalized for mg of proteins. Bars represent the mean values from three independent samples, each consisting of siliques sampled from at least four plants, ± standard deviation. Statistical significance was determined by two-way ANOVA (a) and One-way ANOVA (b) followed by a Tukey test ($P < 0.01$). Different letters indicate significant differences.

Figure 3

Fe content and Fe(III) reducing activity in *atgullo2* seeds. Embryos extracted from mature **(a,b)** wt Col, **(c,d)** *atgullo2-1* and **(e,f)** *atgullo2-2* seeds stained for Fe with Perls reagent. For each line, two embryos are shown; bar corresponds to 100 µm. Details of mature embryos extracted from **(g)** wt Col, **(h)** *atgullo2-1* and **(i)** *atgullo2-2* seeds stained for Fe with Perls reagent; bar corresponds to 100 µm. **(j)** Fe content, expressed as µg Fe g dry weight $^{-1}$, in mature wt Col, *atgullo2-1* and *atgullo2-2* seeds quantified by ICP-MS; each bar corresponds to mean value of three independent samples of 40 mg seeds each, ± SE. **(k)** Fe(III) reducing activity of emptied seed (without embryos) isolated from wt Col, *atgullo2-1* and *atgullo2-2* developing seeds at late torpedo/bent cotyledon stage, measured as pmoles of the complex Fe(II)-BPS produced per single seed coat. Each bar corresponds to the mean value ± SE, from 4 independent samples, each containing 40 seed coats. Significant difference between wt Col and *atgullo2* values, according to one-tailed T-test, are indicated with (*) ($P < 0.1$ in **(j)** and $P < 0.05$ in **(k))**.

Eliminato: (a)

Eliminato: (b)

Figura 4

Ionome profile of mature *atgullo2* seeds. Zn, Mn, Ca, Na, Mo, Cu, K, Mg content, expressed as mg g^{-1} dry weight (DW), in mature wt Col, *atgullo2-1* and *atgullo2-2* seeds quantified by ICP-MS; each

bar corresponds to mean value of three independent samples of 40 mg seeds each ± SE. Significant differences in concentration between wt Col and *atgullo2* values, are indicated with (*), according to one-tailed T-test (P <0.1).

Figure 5

Suberin polyester analysis in mature *atgullo2* seeds. Suberin profiles were measured by Gas Chromatography-Mass Spectrometry. The y-axis represents the relative peak areas following normalization to a C32-alkane internal standard. Data in bars represent mean ± SE of three biological replicates where asterisks indicate statistically significant differences (P value < 0.05) compared with wt Col, determined by two-tailed t-test.

Permeability of *atgullo2* seeds. Germination percentage of wt Col and *atgullo2-1* and *atgullo2-2* seeds, when exposed to 0.5, 1, 5, 10 µM ABA, after 1, 2, 3 or 4 days. Each bar the mean germination percentage ± SE of 4 plates, each containing 100 seeds. Significant differences between wt Col and *atgullo2* values, according to one-tailed T-test, are indicated with (*) (P<0.05).

Figure 7

SEM images of *atgullo2* seeds. **(a)** wt Col, **(b)** *atgullo2-1*, **(c)** *atgullo2-2* whole mature seeds. Details of **(d)** wt Col, **(e)** *atgullo2-1*, **(f)** *atgullo2- 2* seeds. Bars in (**a, b, c**) correspond to 100 µm bars; bars in (**d, e, f**) correspond to 10 µm.

Eliminato: Figure 6¶
Permeability of *atgullo2* seeds. **(a)** wt Col, *atgullo2-1* and *atgullo2-2* seeds were treated, either 5 or 10 min, with commercial bleach or simply immersed onto distilled water (control); they were then thoroughly rinsed six times with distilled water and soon after inspected at the stereomicroscope. **(b)** wt Col and *atgullo2-1* and *atgullo2-2* seeds, after treatment described in **(a),** were germinated on moist paper in petri dishes and germination percentage was scored after 4 d. Each bar represents the mean germination percentage ± SE of 3 plates, each containing at least 60-150 seeds each.

Eliminato: (c)

Eliminato: P <0.1 in (b) and

Eliminato: in (c)