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PRX2 and PRX25, peroxidases regulated by COG1, are involved in seed longevity in Arabidopsis

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PRX2 and *PRX25*, peroxidases regulated by COG1, are involved in seed longevity in Arabidopsis

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Abstract

Permeability is a crucial trait that affects seed longevity and is regulated by different polymers including proanthocyanidins, suberin, cutin and lignin located in the seed coat. By testing mutants in suberin transport and biosynthesis, we demonstrate the importance of this biopolymer to cope with seed deterioration. Transcriptomic analysis of *cog1-2D*, a gain-of-function mutant with increased seed longevity, revealed the upregulation of several peroxidase genes. Reverse genetics analysing seed longevity uncovered redundancy within the seed coat peroxidase gene family, however after controlled deterioration treatment, seeds from the *prx2 prx25* double and *prx2 prx25 prx71* triple mutant plants presented lower germination than wild-type plants.. Transmission electron microscopy (TEM) analysis of the seed coat of these mutants showed a thinner palisade layer, but no changes were observed in proanthocyanidin accumulation or in the cuticle layer. Spectrophotometric quantification of acetyl bromide-soluble lignin components indicated changes in the amount of total polyphenolics derived from suberin and/or lignin in the mutant seeds. Finally, the increased seed coat permeability to tetrazolium salts observed in the *prx2 prx25* and *prx2 prx25 prx71* mutant lines suggested that the lower permeability of the seed coats caused by altered polyphenolics is likely to be the main reason explaining their reduced seed longevity.

Keywords **A**ccelerating aging · seed longevity · tetrazolium salts · TEM · Sudan · · RNA-Seq.

Abbreviations TF Transcription factor · SDT seed deterioration treatment · CDT controlled deterioration treatment · WT wild type · ROS reactive oxygen species · ABA abscisic acid · DEGs Differentially expressed genes · ChIP-seq Chromatin Immuno-Precipitation sequencing · %ABSL acetyl bromide soluble lignin.

Introduction

The progressive oxidation of nucleic acids, proteins and lipids of the embryo by external environmental sources accelerates seed deterioration (Bailly et al., 2008). One of the most important mechanisms in plants to prevent the impact of ROS on embryo integrity is the formation of the seed coat, a structure that functions as a barrier between the embryo and its external environment, thus conferring chemical and physical protection (Sano et al., 2016).

In Arabidopsis, the outermost epidermal cells of seed coat secrete mucilage, a pectic polysaccharide, that has high water-retention properties, but does not appear to play a role in seed deterioration (Haughn and Chaudhury, 2005). Below the epidermis is the palisade layer followed by the seed coat endothelium, which is the innermost layer and the site of proanthocyanidin synthesis. These flavonoid compounds are antioxidants that act as ROS scavengers. On the other hand, the structure and biosynthesis of lignin have received little attention with regard to seed coat location. Some testa cells develop lignified secondary cell walls that are thought to reinforce the cell and make the seed coat impermeable to water and gas (Tobimatsu et al., 2013, Liang et al., 2006).

In addition, cell walls of the palisade layer of Arabidopsis seed coats contain suberin and the cell walls of the inner integument contain cutin (Molina et al., 2008), These polymers constitute a lipophilic cell wall barrier that controls the flux of gases, water and solutes. In particular, suberin is a complex biopolymer consisting of a polyaliphatic domain (polyester) and a polyphenolic domain (Bernards et al., 2002). Although a role for suberin has been described in relation to different biotic and abiotic stresses (Franke et al., 2005; Franke and Schreiber 2007; Vishwanath et al., 2015), no studies have linked this complex molecule with seed longevity. Since suberin in the seed coat could limit the diffusion of atmospheric oxygen into the seed, it might play an important role in preventing oxidation and associated deteriorative processes that contribute to seed aging.

The order of the reactions in suberin biosynthesis has yet to be definitively elucidated, but several enzyme families are known to be involved, including β-ketoacyl-CoA synthases (KCS), fatty acid oxidases of the cytochrome P450 monooxygenases (CYP), fatty acyl reductases (FAR), glycerol-3-phosphate acyl-transferases (GPAT), and HXXXD-motif family hydroxycinnamoyl-CoA transferases (ASFT or FHT) (Pollard et al., 2008; Li-Beisson et al., 2013; Vishwanath et al., 2015). *GPAT5* acquires relevance, since it encodes a glycerol-3-phosphate acyltransferase specifically involved in the synthesis of acylglycerol precursors of the suberin polymer. Seed coat lipid polyester analysis of the *gpat5* mutant revealed a strong reduction in 22:0/24:0 fatty acids and their derivatives, in agreement with a reduction in seed coat suberin content observed after Sudan Red staining (Beisson et al., 2007). Several of these suberin biosynthesis genes are positively regulated in seeds by MYB107, a transcription factor (TF) that directly binds directly to their promoters (Gou et al., 2017). On the other hand, suberin transport to the cell wall may occur through the secretory pathway and, in part, also mediated by ATP-binding cassette (ABC) transporters and/or lipid transfer proteins (LTPs). In *Arabidopsis*, an *abcg2 abcg6 abcg20* triple mutant was shown to have alterations in the structure, composition and properties of root and seed coat suberin

(Yadav et al. 2014). In addition, an important role for peroxidases in the macromolecular assembly of the suberin polyphenolic domain at the cell wall has also been postulated (Bernards et al., 2004). Peroxidases are heme-containing proteins that catalyze the reduction of H_2O_2 by transferring electrons from various donor molecules and, in the case of plant peroxidases (class III), these acceptor molecules can be very different, for example lignin precursors or the growth hormone auxin (Passardi et al., 2004). This type of peroxidase gene family appeared with the colonization of land by plants and consists of a large (73 genes in *Arabidopsis thaliana*) and varied multigene family in land plants (Duroux and Welinder, 2003). Class III peroxidases participate in a broad range of physiological processes such as lignin formation, synthesis of phytoalexins or metabolism of ROS (Almagro et al., 2009). Our results suggest a new function for these peroxidases in the maintenance of seed viability likely due to their role in the polymerization of suberin polyphenolics and lignin in the seed coat.

Results

Suberin accumulation in the seed coat subepidermal layer is a key factor for tolerance to seed deterioration

Seed storability can be measured by many different approaches, however the International Seed Testing Association (ISTA) has defined very specific protocols for seed deterioration treatment (SDT), which is thought to reflect seed vigour, and for controlled deterioration treatment (CDT), the standard method to measure seed longevity (ISTA, 2018). COG1 (At1g29160) is a TF that positively regulates the tolerance of *Arabidopsis* seeds to deterioration. The most likely mechanism of the gain of function mutant *cog1-2D* is the high accumulation of suberin in the palisade layer of the seed coat (Bueso et al. 2016). To confirm this hypothesis, we carried out a deterioration test of full germination capacity of seeds from mutants affected in suberin content. We used *cog1-2D* seeds as the resistant mutant control in a seed deterioration test (SDT). As expected, we observed an increase in germination for this mutant, as compared to WT plants (80 versus 50 %, respectively; Fig. 1). In addition, *gpat5, abcg2 abcg6 abcg20* and *myb107* mutants, all showing altered seed coat aliphatic suberin composition, were also tested. All three mutants with defects in suberin composition presented 100% germination in non-aged seeds, but were highly sensitive to SDT with less than 25% of the seeds able to germinate one week after sowing (Fig. 1). These results suggest that suberin accumulation in the palisade layer of the outer integument is important for tolerance to seed deterioration during aging.

COG1 regulates peroxidase activity

It is unclear how the COG1 TF could regulate suberin accumulation in the palisade layer of the seed coat, however the participation of growth and stress hormones is expected (Bueso et al., 2016). To shed light on the global effect of the overexpression of this TF in the whole plant, we sequenced the mRNA in both WT and COG1-overexpressing seedlings. The analysis of this data (FDR < 0.05) revealed 3124 differentially expressed genes (DEGs) in *cog1-2D*. Of these, 1360 genes were upregulated and 1764 genes were downregulated (Fig. S1 and Table S1). In order to

determine the processes affected in this gain of function mutant, we searched for over-represented gene ontology (GO) terms within differentially expressed genes, and performed Singular Enrichment Analysis (SEA) as implemented in the agriGO website. Regarding "biological process", we detected 90 GO terms significantly over-represented in the sets of DEGs in *cog1-2D* using a significance level of 0.01 (Table S2). Interestingly, some enriched GO terms could be related to suberin biosynthesis. For instance, related to the polyaliphatic fraction, we identified the "lipid metabolic process" (GO:0006629), and "carboxylic acid biosynthetic process" (GO:0046394) categories. In addition, our analysis revealed 71 DEGs classified as "phenylpropanoid biosynthetic process" (GO:0009699), which are genes that could be directly involved in suberin and lignin synthesis (Table S3). Phenylpropanoids are a diverse family of organic compounds, which are all initially synthesized via the shikimate pathway, with 4-coumaryl CoA as a central metabolite that provides the basis for all subsequent branches and resulting compounds (Vogt, 2010). In addition, our analysis identified a glycerol-3-phosphate acyltransferase, two cinnamyl alcohol dehydrogenases, several fatty acyl-CoA reductases, and putative and described laccases as DEGs. Although class III family peroxidases are not included in GO:0009699, these enzymes catalyse the oxidation of phenylpropanoids to their phenoxyl radicals, and the subsequent non-enzymatic coupling controls the pattern and extent of polymerization (Russell et al., 2006). For instance, using the KEGG PATHWAY, peroxidases are included in the "phenylpropanoid biosynthesis" group (ath00940) and we observed that both *PRX33* and *PRX39* were induced at least two-fold (Table S4). In accordance with this observation and regarding the "molecular function" the most significant group was the GO:0016491: "oxidoreductase activity", where 13 class III peroxidases were identified as DEGs (Fig. 2). This result points to peroxidases as a COG1-regulated gene family.

prx2 prx25 and *prx2 prx25 prx71* are sensitive to accelerated aging treatments

The RNA sequencing analysis from *cog1-2D* seedlings displayed differentially expressed peroxidases genes that are highly expressed in this vegetative stage, but not in seeds. Thus, these isoforms are unlikely to play an important role in the seed coat formation. However, we hypothesized that peroxidases expressed in the seed coat may have a physiological role in determining seed longevity. Therefore, we performed a reverse screening of loss-of-function mutants of peroxidases with high expression in seed coat based on data provided by Belmonte et al. (2013) and represented in eFP browser (http://bar.utoronto.ca/efp/cgi-bin/efpWeb.cgi). We isolated homozygous mutant lines of peroxidases 3, 12, 22, 23, 25, 36, 43, 50, 55 and 64 and sensitivity to deterioration was checked using an SDT treatment. The viability of the seeds of all these single mutants was the same as the wild type (Fig. S2).

We next conducted experiments to establish which of these seed coat peroxidases are regulated by COG1 during seed formation. According to databases, COG1 is highly expressed in the seed coat at the end of embryogenesis. To determine with more precision the spatial-temporal expression of COG1 during seed development, we generated transgenic plants expressing green fluorescent protein (GFP) under the control of the COG1 promoter. On the third day, COG1 expression starts to be detected

in seeds and at the end of embryo differentiation the expression of this TF is higher in the columella and in the radial walls of cells that form the seed epidermis (Fig. 3a). Thus, 6-8 days after pollination (DAP), wild type and *cog1-2D* siliques were collected and seed coat peroxidase expression was analysed by qRT-PCR. The COG1 TF positively regulated seed coat peroxidases *PRX12*, *PRX22*, *PRX25* and *PRX43* (Fig. 3b).

On the other hand, overexpression of COG1 increases gibberellin levels during seed maturation (Bueso et al., 2016) and some peroxidases are induced by exogenous application of GA3 (Zieslin and Ben-Zaken 1992; Wang et al., 2017). To ascertain whether COG1-regulated seed coat peroxidases are induced by this growth hormone, siliques from GA3-treated plants were collected and peroxidase gene expression was analysed. All these peroxidases were induced after this treatment (Fig. 3c).

PRX25 was the most expressed in *cog1-2D*, but seeds from this loss-of-function mutant were not affected in seed viability (Fig. S2). Shigeto et al., 2013 reported that *PRX25* and two homologues genes, *PRX2* and *PRX71,* are involved in stem lignification. *PRX2* was also induced in *cog1-2D* and in GA3-treated siliques more than 3-fold, while *PRX71* was clearly repressed in this activation-tagging mutant (Fig. 3b and Fig. 3c).

In order to find out whether these peroxidases are involved in seed viability, single loss-of-function mutants *prx2*, *prx25, prx71,* double mutants *prx2 prx25* and *prx25 prx71* and the *prx2 prx25 prx71* triple mutant were subjected to both accelerated aging treatments (SDT and CDT). All mutants presented 100% germination in non-aged seeds. As mentioned above, the SDT treatment, used to evaluate seed vigour, revealed that the double mutant *prx2 prx25* and the *prx2 prx25 prx71* triple mutant were sensitive to the deterioration treatment to the same extent, indicating that the loss-of-function of *PRX71* was not additive (Fig. 4). In addition, these mutants were subjected to a controlled deterioration treatment, an alternative test to check seed longevity where seeds are exposed to high humidity instead of being imbibed in water. Both the *prx2 prx25* and the *prx2 prx25 prx71* triple mutant exhibited same sensitivity after 14 days of accelerated aging, with less than 50% of the sowed seeds germinating (Fig. 4).

***prx2 prx25* and *prx2 prx25 prx71* show seed coat alterations**

To characterize potential defects in the seed coat of these peroxidase double and triple mutants, we performed histological sections of dry seeds and observed them using transmission electron microscopy (TEM). Analysing the different layers starting from the inside of the seed (Fig. 5), the cutin layer (CL) was located in the inner integument and associated to the cell wall of the endosperm. The *prx2 prx25* and *prx2 prx25 prx71* mutant lines presented a structure comparable to the wild type. Tannins are deposited in the endothelium (EN), which is observed as a broad brown pigment layer (Gou et al., 2017). This layer was also very similar between the wild type and mutants. The next identified layer is called the palisade (PL), where suberin is deposited adjacent to cell walls. The thickness of this subepidermal layer was highly reduced in both mutants. Finally, no change was detected in the mucilage (MU) extrusion in any of the samples analyzed. This result suggests the specific participation of these peroxidases in the

formation of the palisade layer in the seed coat. To completely discard the role of these peroxidases in both mucilage and proanthocyanidins synthesis, ruthenium red and vanillin stainings were performed respectively. As shown in Fig. S3, no differences were observed between the wild type and mutant lines. Finally, the fact that the double and triple mutants do not display a *transparent testa* phenotype also discards a role for these peroxidases in tannin accumulation (Debeaujon et al., 2000).

***PRX2* and *PRX25* regulate cell wall polyphenolics in seed coat**

Suberin is a complex heteropolymer, characterized by both polyphenolic and polyaliphatic domains (Bernards et al., 2002). There is no single method by which both fractions can be effectively isolated and studied together (Vishwanath et al., 2015), and there is no method to chemically characterize suberin polyphenolics and lignin separately. Although the exact structure of the suberin-associated polyaromatics remains an open question, it is known that polyaromatics are a substantial component of suberized cells (Graça et al., 2015). The analysis of the insoluble suberin-associated polyaromatics includes degradative lignin techniques like the acetyl bromide protocol, based on the formation of acetyl derivatives in non-substituted OH groups and bromide replacement of the α-carbon OH groups to produce a complete solubilisation under acidic conditions (Rains et al., 2018). In order to confirm whether PRX2, PRX25 and PRX71 have an important role in the macromolecular assembly of lignin and/or the suberin polyphenolic domain in seed coats, we performed a cell wall preparation from seeds that was solubilized with acetyl bromide. Spectrophotometry measurements indicated that the *prx2 prx25* mutant had a 35% reduction of polyphenolic compounds, and the triple mutant seed preparations showed similar values (Fig. 6). Aliphatic components of suberin were also measured by GC-MS in these mutants. Total suberin amounts were comparable to WT in the in *prx2 prx25* and in *prx2 prx25 prx71* mutants (Fig. S4).

***prx2 prx25* and *prx2 prx25 prx71* seeds are more permeable.**

The correlation between seed permeability and the lack of some components, such as proanthocyanidins, suberin and cutin, in the seed coat has been widely studied (Debaujon et al., 2000; Vishwanath et al., 2013; Beisson et al., 2007 and Yadav et al., 2014). A common method to assess seed permeability consists of incubating seeds in an aqueous solution of colourless tetrazolium salts. When seeds are permeable, tetrazolium is metabolized by NADH-dependent reductases present in embryo cells, producing red-colored insoluble precipitates made up of formazans (Debeaujon et al., 2000). Our results indicate that the mutants sensitive to accelerated aging, *prx2 prx25* and *prx2 prx25 prx71*, have significant compositional alterations in seeds and, as explained above, these seeds should present higher permeability. To test this, we incubated seeds from both mutants in a tetrazolium salt solution and after 48 h we observed the amount of red staining (Molina et al., 2008). All seeds from the mutant peroxidase lines displayed increased accumulation of formazan precipitates compared to the WT, as

determined by microscopy (Fig. 7a). To quantify seed permeability, we extracted the red-coloured formazans after 72 h of incubation and measured the absorbance of the solution at 485 nm. Extracts from mutant seeds presented higher absorbance than WT (Fig. 7b) confirming our microscopical observations.

Discussion

The seed coat is a maternal tissue developed from the integuments of the ovule whose main function is to protect the embryo from external factors. Class III peroxidases are enzymes with a role in the polymerization of different seed coat components, as well as their hydrolysis (Francoz et al., 2015). For instance, PEROXIDASE36 (PER36) is required for seed coat mucilage extrusion (Kunieda et al., 2013). Proanthocyanidins are oligomeric and polymeric end products of the flavonoid biosynthetic pathway and peroxidases could be involved in the polymerization of these phenolic compounds, although no gene encoding a protein with this function has been reported so far (Dixon et al., 2004). The accumulation of these flavonoids provides a barrier, as demonstrated by two main studies. Debeaujon and collaborators compared mutants affected in testa pigmentation, *transparent testa (tt)* and *transparent testa glabra (ttg)* for dormancy, tetrazolium uptake and storability and they established an inverse correlation between permeability and the presence of catechins and proanthocyanidins (Debeaujon et al., 2000). In addition, Clerkx et al., 2004 showed that condensed tannins are crucial to maintain seed viability because of their antioxidant properties. Finally, lipid polyesters are compounds commonly found in seed coats of different species. It was described that both suberin and cutin are found in *Arabidopsis* seed coats (Molina et al., 2008). In addition, peroxidases also have a role in assembling monolignols during lignin synthesis. Although seed coat lignin has been less explored than vascular lignin, there is evidence of its accumulation in cell walls of seed coat integuments, possibly making seeds less permeable to water and gases (Tobimatsu et al., 2013; Liang et al., 2006). Finally, the biosynthesis of the suberin poly(phenolic) domain is hypothesized to follow a peroxidase-mediated oxidative coupling process (Bernards et al., 2004). This apoplastic lipid barrier is located on the palisade cell walls of the outer integument of the seed coat (Molina et al., 2008). It has been postulated to have a function in dormancy (Fedi et al., 2017) and in abiotic stress protection during germination (Gou et al., 2017). Our work demonstrates that suberin deposition in the palisade layer of testa also increases seed tolerance to deterioration and it constitutes the first barrier protecting the embryo from this stress. Mutants sensitive to SDT such as *gpat5, myb107* and *abcg2 abcg6 abcg20* display an important reduction in the suberin layer in seeds that generates very permeable seeds (Beisson et al., 2007, Gou et al., 2017 and Yadav et al., 2014).

On the other hand, class III peroxidases are a gene family with a large number of members involved in numerous processes, suggesting a particular specialization for each isoform. Genetically, this hypothesis is also supported by the nucleotide variation observed in the peroxidase promoters. Another feature of these oxidoreductases is the

high redundancy, which substantially complicates the observation of a specific phenotype upon mutation of a single peroxidase gene (Cosio and Dunand, 2009). Our data reinforce this concept, as no single mutant line in seed coat peroxidases showed lower germination after SDT, as compared to control seeds. The most studied function of peroxidases is in cell wall stiffening mostly through lignin polymerization. The first and most well-studied is the ATP A2 peroxidase, named PRX53 using the accepted peroxidase classification nomenclature. A mutant with high levels of this peroxidase presents an increase in lignin accumulation. Moreover, the substrate specificity for p-coumaryl and coniferyl alcohols was determined by X-ray crystallography (Ostergaard et al., 2000). In *Arabidopsis*, the role of PRX64 in the Casparian Strip formation has also been reported (Lee et al., 2013), as well as the importance of PRX2, PRX25 and PRX71 in controlling the content of lignin in stem (Shigeto et al., 2015). Pedreira et al., 2010 described the function of PRX37 as a peroxidase associated with vascular bundles functioning mainly in mature leaves and the flower stem. Finally, the *prx72* mutant was reported to present a low content in syringyl units and a decrease in the amount of lignin (Herrero et al., 2013).

Suberin is a biopolymer involved in cell wall permeability and stiffening and the polyphenolic domain shares some similarities with lignin, because they are partly formed by monolignols, such as coumaryl, coniferyl and sinapyl alcohols. In addition, peroxidases are proposed to participate in the oxidative coupling process of this domain (Bernards et al., 2004). This role of peroxidases has been confirmed in the case of *TPX1*, a tomato peroxidase gene whose expression was detected in the endodermis and exodermis where suberin accumulates (Quiroga et al., 2000). Our study shows the important function of PRX2 and PRX25 in the normal development of the palisade layer, where suberin is accumulated in the cell wall. In addition, *prx2 prx25* double mutant seeds present a marked reduction in the % of ABSL that measures the amount of suberin and lignin polyphenolic compounds (Rains et al., 2018). Thus, our results suggest that the high permeability of these mutants is because of a deficient phenolic-based polymer. However, our analyses cannot establish if the affected polymer represents a part of suberin or lignin or both.

During the last years, we have been trying to define the molecular mechanism underpinning COG1 and ATHB25-mediated resistance to seed aging. Overexpression of these TFs produces seeds with an increase in the accumulation of suberin in the palisade layer of the seed coat, likely reducing the embryo oxidation and extending seed viability (Bueso et al., 2014; Bueso et al., 2016). The specific target genes and processes involved in mediating this reinforcement of the seed coat is the next issue to clarify. *cog1-2D* and *athb25-1D* accumulate more ABA. Interestingly, suberization in potato tuber by exogenous ABA application has been known for a long time (Soliday et al., 1978, Cottle and Kolattukudy, 1982). More recently, MYB41 has been described as an ABA-inducible TF that activates steps necessary for aliphatic suberin synthesis and deposition (Kosma et al., 2014). Another explanation is that COG1 and ATHB25 could directly regulate suberin biosynthetic genes, including glycerol-3-phosphate acyltransferases, cinnamyl alcohol dehydrogenases, fatty acyl-CoA reductases and laccases. At this time, we do not favour a mechanism in which the peroxidase genes are

directly regulated by these TFs because we are unable to detect binding of this transcription factor to the *PRX25* promoter in chromatin immunoprecipitation experiments. Thus, the most plausible mechanism is that the COG1 TF regulates the expression of GA-inducible seed coat peroxidases *PRX2* and *PRX25* through direct regulation of gibberellic oxidase biosynthesis genes.

Material and methods

Plant material and growth conditions

Arabidopsis thaliana seeds were surface sterilized by soaking in 70% ethanol 0.1% Triton X-100 for 15 min, followed by incubation in commercial bleach (2.5%) 0.1% Triton X-100 for 10 min and rinsing three times with sterile water. Seed stratification was performed for 3 days at 4 ºC. Seeds were then germinated on plates containing Murashige and Skoog salts (MS) with 1% (w/v) sucrose, 10 mM 2-(N-morpholino)ethanesulfonic acid and 1% (w/v) agar, pH adjusted to 5.7 with Tris base. *Arabidopsis* plants were grown under greenhouse conditions (16 h light/8h dark, at 23±2 ºC and 70 ± 5% relative humidity) in pots containing a 1:2 vermiculite:soil mixture. Plants treated with GA3 were sprayed twice per week with a 50 µM solution. We used the following *Arabidopsis* homozygous mutant lines N816472 (loss-of-function of AT1G05250 "PRX2") N640204 (loss-of-function of AT1G05260 "PRX3"), N543965 (loss of function of AT1G71695 "PRX12"), N644487 (loss of function of AT2G38380 "PRX22"), N561249 (loss of function of AT2G38390 "PRX23"), N468724 (loss of function of AT2G41480 "PRX25"), N873555 (loss of function of AT3G50990 "PRX36"), N863633 (loss of function of AT4G25980 "PRX43"), N563662 (loss of function of AT4G37520 "PRX50"), N602284 (loss of function of At5g14130 "PRX55"), N691891 (loss of function of AT5G42180 "PRX64"), N623643 (loss of function of AT5G64120 "PRX71") and N811227 (loss of function of AT3G02940 "MYB107"). The *prx2 prx25*, *prx25 prx71* double mutant and the *prx2 prx25 prx71* triple mutant were provided by Dr Shigeto (Shigheto et al., 2015). The *gpat5* mutant and *abcg2 abcg6 abcg20* triple mutant were obtained from Dr. I. Molina (Beisson et al., 2007) and Dr. JW Reed (Yadav et al., 2014) respectively. The *cog1-2D* is an activation mutant isolated in our laboratory (Bueso et al., 2016)

Seed deterioration tests

Seeds used for testing were harvested at the same time, dried, and stored under the same conditions for at least 2 weeks prior to the experiment. The seeds were imbibed in tubes and incubated at 41 ºC for 24h. After treatment, the seeds were cooled to room temperature and plated on MS medium. All germination analyses were performed with 4 replicates using around 100 seeds per biological replicate. Controlled deterioration test was performed as is described in Châtelain et al 2013.

Molecular techniques

Genomic DNA was extracted from seedlings and young leaves, as described (Weigel and Glazebrook, 2002). Primers for genotyping heterozygous lines are listed in Supplementary Material. For GFP gene promoter-reporter fusion, a 2 Kb fragment corresponding to the native COG1 (-1957 to 0 relative to COG1 translation start) promoter were placed before the 5´-GUS-GFP-3´gene fusion in pCAMBIA1303 (Muñoz-Bertomeu et al., 2009) between the sites for restriction enzymes XbaI and NcoI. The primers used to amplify the COG1 promoter were 5´-TGCTCTAGATCATCCAATCATGAAAAAGGTAC-3´ and 5´-CTTGGGTCCATGGTAAAATGAGAGAGAGATAGAG-3´. All PCR-derived constructs were verified by DNA sequencing. Arabidopsis plants were transformed with the constructs by the floral dipping method with Agrobacterium tumefaciens carrying pSOUP .

Total RNA was extracted from one-week-old siliques in wild type, activation tagging mutant *cog1-2D*, and GA3 treated wild type following the protocol of Oñate-Sánchez and Vicente-Carbajosa (2008). RNA was then purified using NucleoSpin RNA II (Macherey-Nagel). 3 µg RNA was reverse transcribed using the Maxima first-strand cDNA synthesis kit for RT-qPCR (Thermo Fisher Scientific) according to the manufacturer´s instructions. PCR amplifications were performed on first strand cDNA corresponding to 150 ng of total RNA and each reaction was performed in triplicate in a total volume of 20 µl. Quantitative (real-time) PCR (qRT-PCR) was performed using an Applied Biosystems 7500 Real-Time PCR System (Thermo Fisher Scientific) with the 5 PyroTaq EvaGreen qPCR Mix Plus (ROX) (Cultek S.L.U., Madrid, Spain) according to the manufacturer's protocol. Data are the mean of three biological samples. PCR amplification specificity was confirmed with a heat-dissociation curve (from 60 to 95 ºC). The efficiency of the PCR was calculated and different internal standards (PP2AA3 or At5g55840) were selected depending on the efficiency of the primers (Czechowski et al., 2005). Relative mRNA abundance was calculated using the comparative ΔCt method (Pfaffl, 2001). The list of primers for qRT-PCR utilized is provided in Supplementary Material.

RNA-Seq and bioinformatics analysis

Total RNA from 7 day-old-seedling was isolated using NucleoSpin RNA II (Macherey-Nagel). Library construction was performed using the TruSeq Stranded mRNA Library Preparation Kit (Illumina) and the resulting fragments were sequenced in the Illumina Hiseq 2000 system at Sistemas Genómicos (Paterna, Spain). The sequence alignment and the quantification of gene expression levels were performed as previously described in Mandel et al., 2016 with some modifications. The reads (an average of 25 M per sample at 101 nucleotides long) were quality filtered and trimmed using Trimmomatic version 0.36 (Bolger et al., 2014). The resulting reads were then aligned to the TAIR10 version of the *Arabidopsis thaliana* genome sequence (https://www.arabidopsis.org/) using Hisat2 version 2.1.0 (Kim et al., 2015). The resulting read alignments (in BAM format) were used for transcript quantification with cuffdiff program of the Cufflinks version 2.2.1 package (Trapnell et al., 2013). Three biological replicates were used for each genotype. The resulting read alignments were visualized and explored using Tablet

software (Milne et al., 2013) and CummeRbund R package version 2.23.0 (Goff et al., 2014). Differentially expressed genes (DEGs) were subjected to Singular Enrichment Analysis (SEA) for the identification of overrepresented Gene Ontology (GO) terms using agriGO (http://bioinfo.cau.edu.cn/agriGO/) (Berardini et al., 2004) with the default options (statistical test: hypergeometric, multi-test adjustment method: Yekutieli, significance level: 0.01). Using the criteria of two-fold up- or down-regulation, DEGs were also mapped to metabolic pathways using the KEGG PATHWAY online tool (http://www.genome.jp/kegg/pathway.html) (Aoki-Kinoshita et al., 2007). The RNA seq data from this article have been submitted to Gene Expression Omnibus (GEO) (https://www.ncbi.nlm.nih.gov/geo/) and assigned the identifier GSE128953.

Acetyl bromide method

Acetyl bromide method was used to measure polyphenolic compounds from suberin and lignin in seed (Moreiro-Vilar et al 2015). % ABSL was calculated according Rains et al., 2018.

Seed coat permeability test

Tetrazolium red assays were used for seed coat permeability tests. Dried *Arabidopsis* seeds were incubated in the dark in an aqueous solution of 1% (w/v) tetrazolium red (2,3,5-triphenyltetrazolium chloride, Sigma-Aldrich) at 30°C for 48 h. The seeds were observed for change in color and imaged using a macroscope Leica DMS1000. The quantitative formazan assay was carried out as described Vishwanath et al., (2013). If the tetrazolium salts penetrate into the seed, they are reduced by the embryo to a red precipitate called formazans. 50 mg of seeds were incubated in 500 µl of 1% (w/v) aqueous solution of tetrazolium red at 30 ºC for 72 in darkness. After incubation, the samples were washed twice with water, resuspended in 1 mL 95% (v/v) ethanol, and finely ground with a mortar and pestle to extract formazans. The final volume was adjusted to 2 mL with 95% (v/v) ethanol and immediately centrifuged for 3 min at 15,000 *g*, and the supernatant was recovered. Each sample was assayed in triplicate.

Lipid polyester analysis

Seed coat lipid polyester monomers were identified and quantified following the protocol described in Molina et al. (2006).

TEM analyses

Samples were hydrated and chemically fixed with Karnovsky's solution. Seeds were then washed in phosphate buffer and postfixed in 2% osmium tetroxide. Samples were dehydrated through a graded ethanol series and embedded in LR resin for 24h in 55 ºC. All the samples were sectioned (60-90 nm) using an Ultracut Leica UC6 with a diamond knives (Diatome). Sections for each sample were examined and imaged with a

JEM-1010 transmission electron microscope (JEOL-1010).

Staining of the mucilage and proanthocyanidins

Staining of the mucilage with Ruthenium red was performed as in Bueso et al., 2014 and vanillin staining to detect proanthocyanidin accumulation followed indications from Debaujon et al., 2000.

Author contribution
IMA, RN, JR and GB performed the mutant genotyping and gene expression analysis.
AS and IM performed seed lipid polyester analysis
JMC conducted the RNA-seq analysis.
JMB and IF generated transgenic lines.
JS and YT generated double and triple peroxidase mutants.
JG, RS, and EB designed experiments and coordinated the work.
JG, RS, IM, and EB wrote the manuscript.

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LEGENDS TO THE FIGURES

Fig. 1 Mutants with less suberin in the palisade layer of seed coat are sensitive to seed deterioration treatment. Seeds from wild type (WT), activation tagging *cog1-2D*, glycerol-3-phosphate acyltransferase (*gpat5*), ABCG half-transporters (*abcg2 abcg6 abcg20*) and MYB107 transcription factor (*myb107*) mutants were subjected to seed deterioration treatment for 24 h at 41 ºC and sown on MS plates. The percentage of germination was recorded after 1 week. The results are the average of four experiments with 100 seeds per line, and bars indicate standard errors. Not-aged seeds from all lines germinated more than 99% after 3 days. *Significantly differing from controls (wild type) at $P < 0.05$ (Student´s t-test).

Fig. 2 COG1 regulates peroxidase activity in seedlings. Molecular function chart resulted from mRNA sequencing comparison analysis using agriGO between seedlings wild type and *cog1-2D* mutant. Inserted chart represents 13 peroxidases differentially expressed between both genotypes.

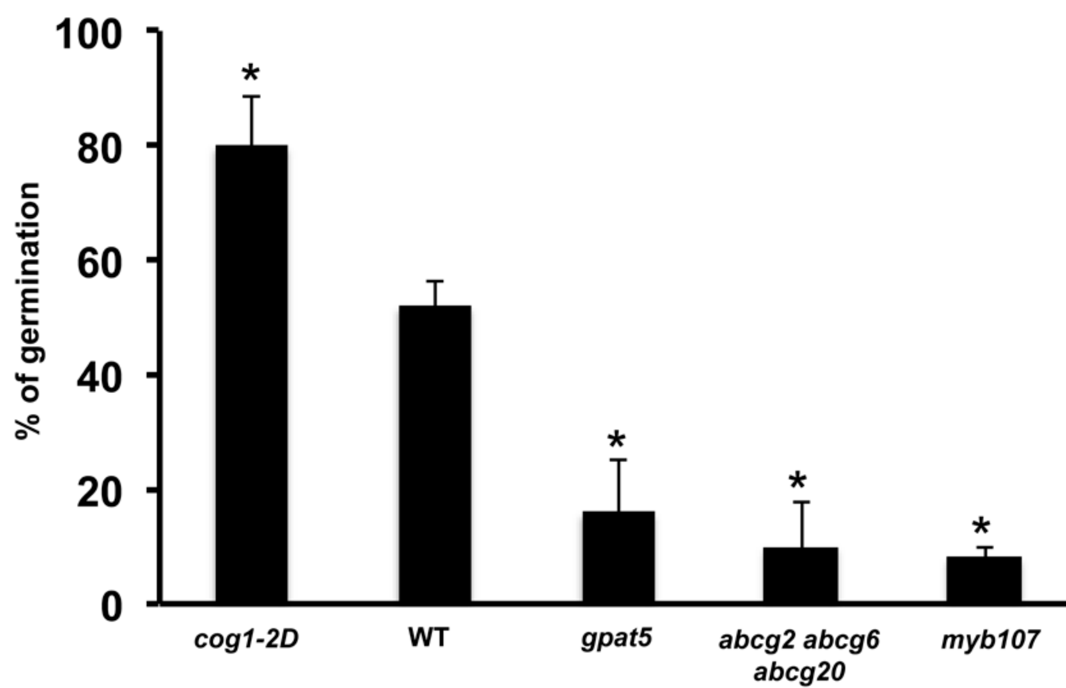
Fig. 3 Gene expression analysis of COG1 in developing seeds and seed coat peroxidases in *cog1-2D* and GA3 treated plants. **a** Fluorescence images of 3, 6 and 8 DAP Arabidopsis seeds transformed with Pro_{COG1}*::GUS:GFP* using confocal laser scanning microscopy (CLSM). **b** qRT-PCR analysis of expression of *PRX2* (2), *PRX3* (3), *PRX12* (12), *PRX22* (22), *PRX23* (23), *PRX25* (25), *PRX36* (36), *PRX43* (43), *PRX50* (50), *PRX55* (55), *PRX64* (64) and *PRX71* (71) from *cog1-2D* mutant one-week-old siliques. **c** qRT-PCR analysis of expression of *PRX2* (2) *PRX12* (12), *PRX22* (22), *PRX25* (25) and *PRX43* (43) from GA3 treated wild type one-week-old siliques. Expression values are relative to housekeeping genes ("see Methods") and the resulting ratios are normalized to wild type, taken as 1. Results are the average of three determinations with bars corresponding to standard errors.

Fig. 4 Double mutant *prx2 prx25* and triple mutant *prx2 prx25 prx71* are sensitive to accelerated aging treatments. Seeds from wild type (WT), *prx2 (2)* , *prx25 (25)*, *prx71 (71)*, *prx2 prx25 (2-25)*, *prx25 prx71 (25-71)* and *prx2 prx25 prx71 (2-25-71)* mutants were subjected to seed deterioration treatment for 24 h at 41 ºC (black bars) and to controlled deterioration treatment (grey bars) for 14 days and sown on MS plates. The percentage of germination was recorded after 1 week. The results are the average of four experiments with 100 seeds per line, and bars indicate standard errors. Not-aged seeds from all lines germinated more than 99% after 3 days. *Significantly differing from controls (wild type) at $P < 0.05$ (Student´s t-test).

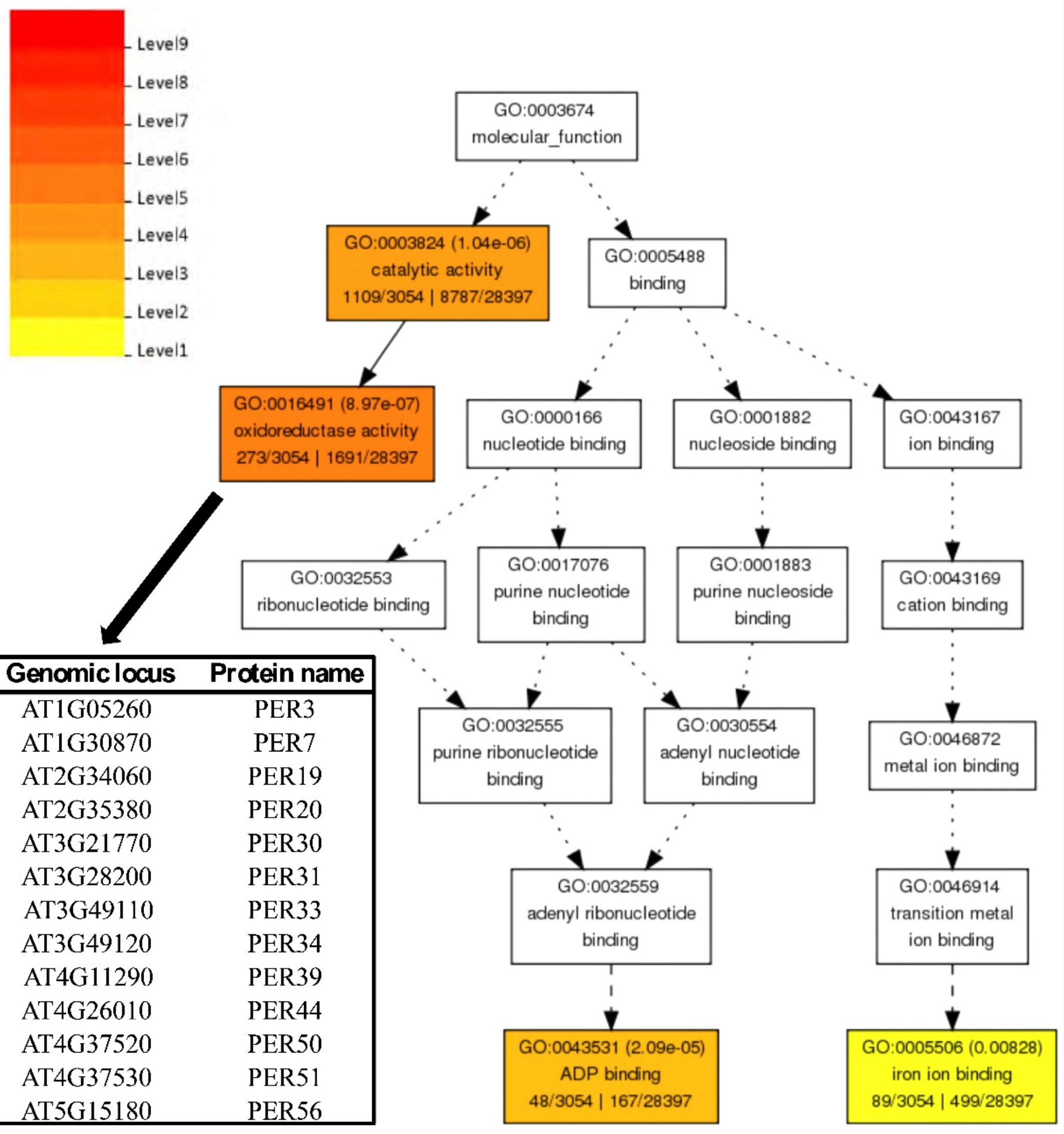
Fig. 5 Double mutant *prx2 prx25* and triple mutant *prx2 prx25 prx71* are altered in the formation of palisade layer of seed coat. TEM observation of representative seed coat of the wild type, *prx2 prx25* and *prx2 prx25 prx71*. Mature seeds were ultrathin sectioned and image was focus to show seed coat and. MU, mucilage; PL, palisade layer; EN, endothelium; CL, cutin layer. The pictures are representative of 20 different seeds. Arrows indicate the thickness of palisade layer.

Fig. 6 PRX2 and PRX25 regulate cell wall polyphenolics in seed coat**.** Total acetyl bromide (AcBr) soluble polyphenolic from suberin and lignin from wild type, *prx2 prx25* and *prx2 prx25 prx71* seeds. The results are the average of 10 samples. *Significantly differing from controls (wild type) at $P < 0.05$ (Student´s t-test). %ABSL: acetyl bromide soluble lignin percentage.

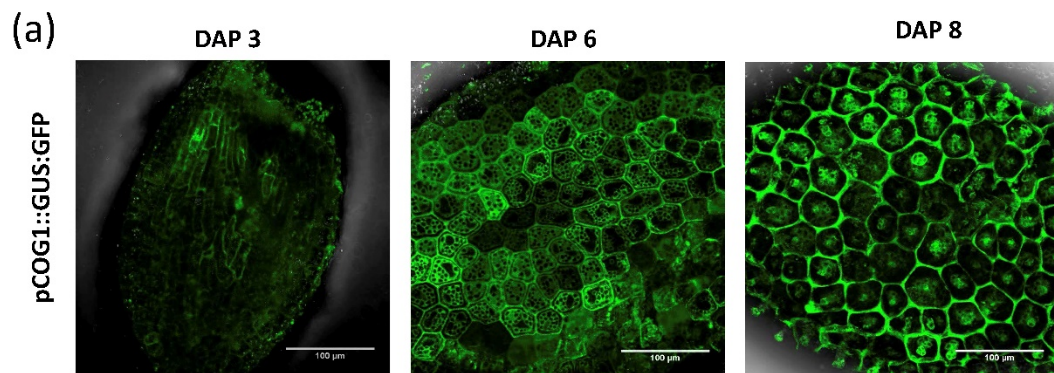
Fig. 7 Seed permeability is increased in SDT-sensitive seed coat peroxidases mutants. **a** Staining pattern in seeds of wild type (WT) and mutants incubated in 1% tetrazolium red at 30°C and imaged at the end of 48 h. The red colour reflects formazan accumulation after penetration of tetrazolium red into the embryo and metabolic reduction. **b** Quantification of formazan extracted from seeds of different genotypes incubated in 1% tetrazolium red at 30°C at the end of 72 h.. The data (absorbance at 485 nm) are mean values of three replicates and bars indicate standard errors. *Significantly differing from controls (wild type) at $P < 0.05$ (Student´s t-test).

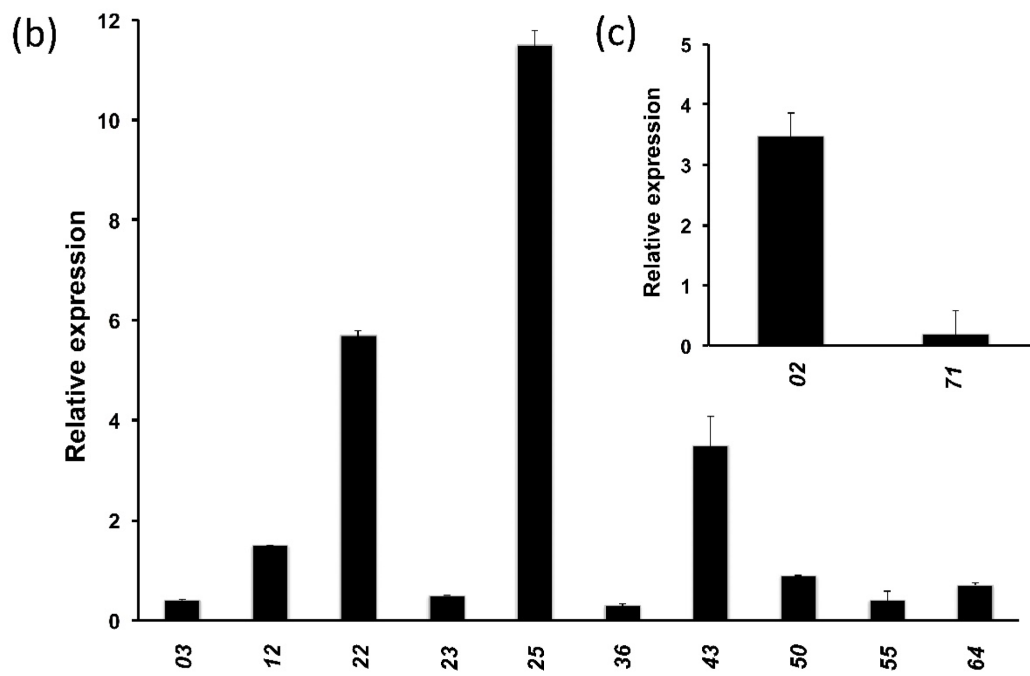


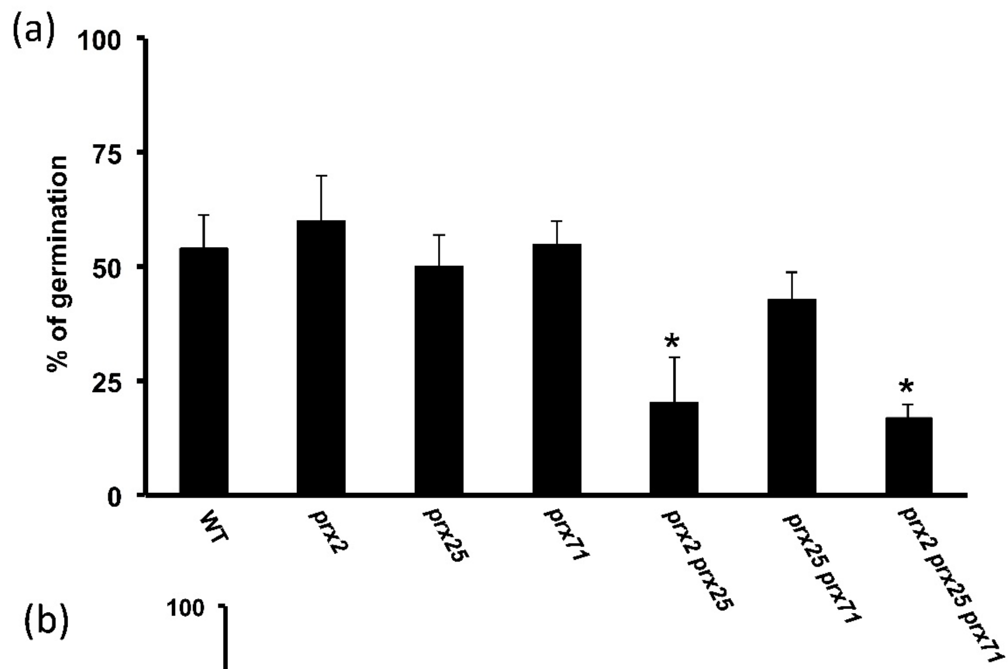
100
80
60
40
20
0
% of germination
*
*
*
*
cog1-2D
WT
gpat5
abcg2 abcg6
abcg20
myb107

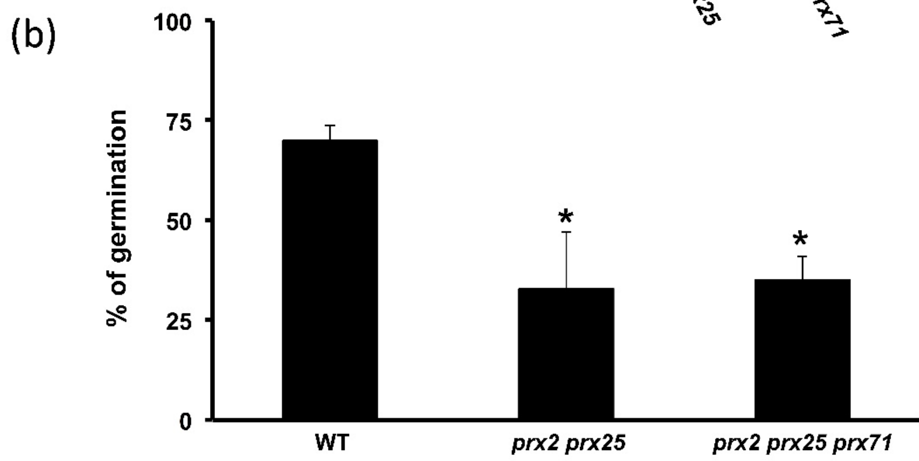


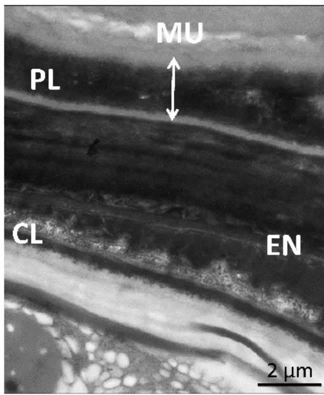
Genomic locus	Protein name
AT1G05260	PER3
AT1G30870	PER7
AT2G34060	PER19
AT2G35380	PER20
AT3G21770	PER30
AT3G28200	PER31
AT3G49110	PER33
AT3G49120	PER34
AT4G11290	PER39
AT4G26010	PER44
AT4G37520	PER50
AT4G37530	PER51
AT5G15180	PER56

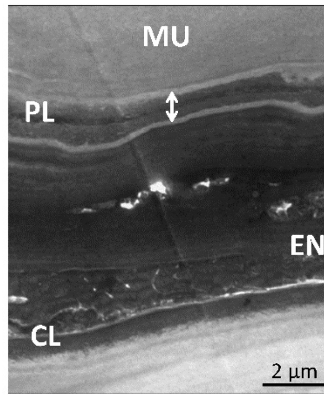

(a)
DAP 3
DAP 6
DAP 8
pCOG1::GUS:GFP

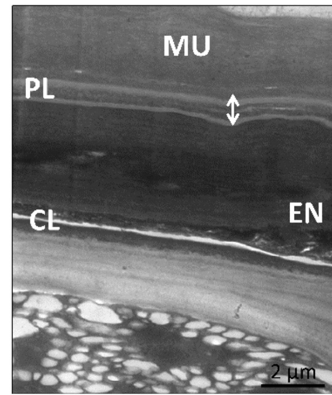

(b)
Relative expression
0
2
4
6
8
10
12
03
12
22
23
25
36
43
50
55
64
(c)
Relative expression
0
1
2
3
4
5
02
71


(a)
100
75
50
25
0
% of germination
*
*
WT
prx2
prx25
prx71
prx2 prx25
prx25 prx71
prx2 prx25 prx71

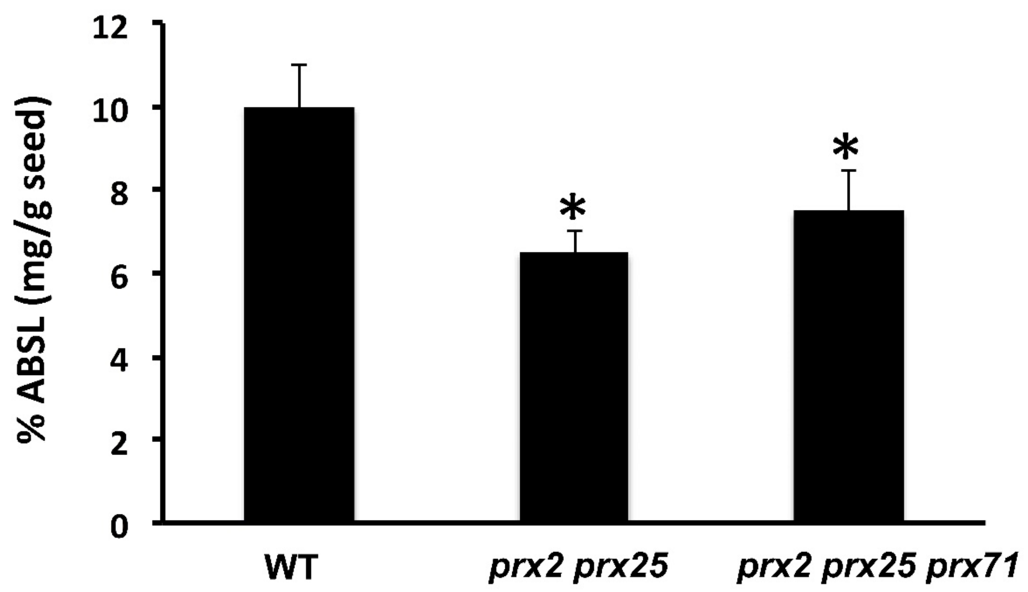

(b)
100
75
50
25
0
% of germination
*
*
WT
prx2 prx25
prx2 prx25 prx71



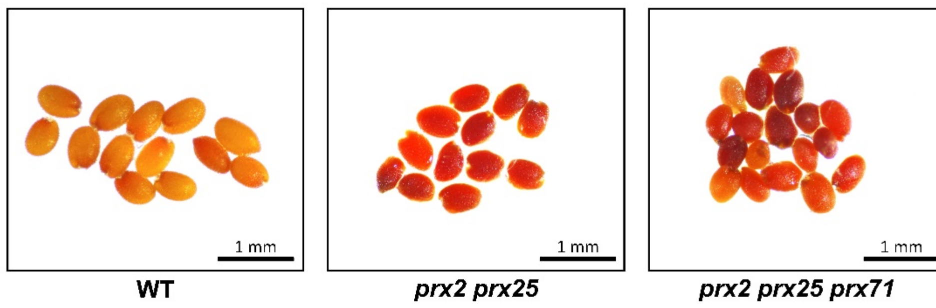




WT ***prx2 prx25*** ***prx2 prx25 prx71***


% ABSL (mg/g seed)
12
10
8
6
4
2
0
*
*
WT
prx2 prx25
prx2 prx25 prx71

(a)



(b)