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Coordination of five class III peroxidase encoding genes for early germination events of *Arabidopsis thaliana*.

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Coordination of five class III peroxidase encoding genes for early germination events of *Arabidopsis thaliana*.

Five Class III peroxidases have specific spatio-temporal expression pattern and antagonistic effects to control the endosperm cell wall rupture during *Arabidopsis* seed germination events

Abstract (236 words)

The Class III peroxidases (CIII Prxs) belong to a plant-specific multigene family. Thanks to their double catalytic cycle they can oxidize compounds or release reactive oxygen species (ROS). They are either involved in different cell wall stiffening processes such as lignification and suberization, in cell wall loosening or defense mechanisms. Germination is an important developmental stage requiring specific peroxidase activity. However, little is known about which isoforms are involved. Five CIII Prx encoding genes: *AtPrx04*, *AtPrx16*, *AtPrx62*, *AtPrx69*, and *AtPrx71* were identified from published microarray data mining. Delayed or induced testa and endosperm rupture were observed for the corresponding CIII Prx mutant lines indicating either a gene-specific inducing or repressing role during germination, respectively. Via *in situ* hybridization *AtPrx16*, *AtPrx62*, *AtPrx69* and *AtPrx71* transcripts were exclusively localized to the micropylar endosperm facing the radicle, and transcriptomic data analysis enabled positioning the five CIII Prxs in a co-expression network enriched in germination, cell wall, cell wall proteins and xyloglucan hits. Evidence were produced showing that the five CIII Prxs were cell wall-targeted proteins and that the micropylar endosperm displayed complex cell wall domain topochemistry. Finally, we drew a spatio-temporal model highlighting the fine sequential gene expression and the possible involvement of micropylar endosperm, cell wall domains to explain the non-redundant cell wall stiffening and loosening functions of the CIII Prxs in a single cell type. We also highlighted the necessity of a peroxidase homeostasis to accurately control the micropylar endosperm cell wall dynamics during *Arabidopsis* germination events.

Key words: *Arabidopsis*, Class III peroxidase, testa rupture, micropylar endosperm rupture, germination, cell wall domain remodeling,

Abbreviations: Class III peroxidase (CIII Prx), micropylar endosperm (MEN), reactive oxygen species (ROS).

1 **Introduction**

2

3 Seeds are important resistant structures because they ensure the transition between two
4 successive generations. *Arabidopsis thaliana* mature seed formation takes about twenty days and
5 seeds are composed of three main tissues: (i) the testa or seed coat, (ii) the endosperm which is
6 divided into 3 parts (the peripheral, the chalazal and the micropylar endosperm, the latter being
7 adjacent to the radicle) and (iii) the embryo which is enclosed in the first two compartments
8 (Dekkers et al., 2013).

9 Germination is the focus of numerous studies since it represents a crucial process by which
10 the embryo will start to grow in order to develop a seedling. It starts after imbibition and resuming
11 of the metabolic activity. Germination is considered as completed when root, hypocotyl, and
12 cotyledons can be observed. *Sensu stricto* germination ended with the embryo emergence (usually
13 the radicle first) and consists in two observable events: the testa rupture (TR) due to the embryo and
14 endosperm expansion and the (micropylar) endosperm rupture (ER) {Dekkers, 2013; Lariguet,
15 2013; Nonogaki, 2007}. Several aspects of this agro-economically relevant developmental stage
16 have been previously analyzed in details. Beside the well-known hormonal balance control of
17 germination by the antagonist ABA and GA₃ phytohormones (Ali-Rachedi et al., 2004; Penfield
18 and Hall, 2009), we started to have a knowledge about micropylar endosperm cell wall proteins
19 influencing this process by facilitating the TR and the ER. As examples, genes encoding endo-β-
20 mannanase isoforms (MAN) (Iglesias-Fernández et al., 2011) or a pectin methylesterase inhibitor
21 (PMEI5/At2g31430) {Müller, 2013} have been characterized.

22 Reactive oxygen species (ROS) initially described as cell-damaging by-products, can also be
23 produced in a controlled manner for a specific purpose. In particular, embryo releases ROS through
24 the seed coat to protect the emerging embryo against pathogen attacks {Schopfer, 2001}. They have
25 also a function during seed germination and dormancy break {Ogawa, 2001; Müller, 2009, Barba-
26 espin, 2010, Leymarie, 2012}. Among the battery of proteins able to regulate ROS homeostasis,
27 NADPH oxidases, class III peroxidases (CIII Prx), and thioredoxins have a role during germination
28 {Müller, 2009; Ortiz-Espín, 2017; Lariguet, 2013; Linkies, 2010}.

29 Peroxidase enzymes catalyze the oxidation of substrate to the detriment of hydrogen
30 peroxide (Francoz et al., 2014). CIII Prxs are exclusive of the green lineage and have a broad range
31 of functions like for example, auxin catabolism, lignin polymerization, cell wall domain loosening
32 or defense responses (Cosio and Dunand, 2009; Francoz et al., 2014; 2019a; Hiner et al., 2002;
33 Passardi et al., 2005). They constitute a multigenic family in plants and are mainly considered as
34 secreted proteins: 73 members have been identified in *A. thaliana* (Tognolli *et al.*, 2002), and

35 thanks to their dual peroxidative and hydroxylic cycle, they contribute to the cell wall stiffening by
36 the radical coupling of aromatic compounds and to cell wall loosening by the non-enzymatic
37 polysaccharide breaking thank to ROS production, respectively (Passardi et al., 2005). Due to the
38 high sequence conservation of CIII Prxs within species, putative functional redundancy is expected
39 which explains the difficulty to attribute a specific function to each member of this large multigenic
40 family (Cosio and Dunand, 2009). Several recent studies illustrated both this possible functional
41 redundancy and conversely the means by which individual CIII Prxs may reach their specificity.
42 The elegant demonstration of the involvement of a lignin molecular brace controlling the abscission
43 of sepals and petals from the receptacle showed that 23 out of the 73 *CIII Prxs* were differentially
44 expressed between the receptacle and the secession cells, impairing the identification of individual
45 *CIII Prx* mutants with abscission phenotype (Lee *et al.*, 2018). Similarly, in search of the CIII Prx
46 involved in Casparian strip lignification in the root endodermis, the quadruple mutant
47 *atprx3/9/39/72* for four co-expressed *AtPrxs* did not display a Casparian strip lignification
48 phenotype arguing for possible functional redundancy (Lee *et al.*, 2013). However, in the same
49 study, *AtPrx64*, a fifth co-expressed CIII Prx was demonstrated to be anchored by the help of the
50 trans-membrane CASP1 protein in the Casparian strip cell wall domain, enabling the lignification of
51 the Casparian strip in the root endodermis (Hosmani et al., 2013; Lee et al., 2013). Moreover,
52 *AtPrx36* which is specifically produced in the outermost integument cells (*oi2*) during the seed
53 development at torpedo stage and localized at the junction of radial/outer periclinal cell wall
54 microdomain of the seed coat, is involved in the mucilage extrusion by controlling the polarized *oi2*
55 cell wall domain loosening (Kunieda et al., 2013; Francoz et al., 2019a). Therefore, CIII Prxs
56 commonly appear as functionally redundant and rare recent examples start making a link between
57 the specific activity of individual members because of spatio-temporal positioning to remodel cell
58 wall domain zones. The molecular mechanisms enabling such accurate positioning of individual
59 CIII Prxs in cell wall domains start to be uncovered (Hosmani et al., 2013; Lee et al., 2013; Francoz
60 et al., 2019a). However, to date there are no studies showing within a single cell type, the
61 localization of individual CIII Prxs in multiple cell wall domains and/or individual cell wall domain
62 microphenotype of the corresponding mutants.

63 In addition, CIII Prxs are also able to regulate ROS homeostasis (Cosio and Dunand, 2009)
64 to protect the embryo emergence against pathogens (Schopfer et al., 2001) or to control cell wall
65 loosening or stiffening thus leading to a probable TR an ER boosting or delay, respectively. A
66 *Lepidium sativum* transcriptomic search led to the demonstration of a germination acceleration
67 phenotype in *atprx16* mutant from *A. thaliana* (Linkies *et al.*, 2010). Here, using recently published
68 tissue-specific transcriptomics focused on *A. thaliana* germination (Dekkers *et al.*, 2013), we

69 identified a micropylar and chalazal endosperm-specific co-expression cluster encompassing five
70 *CIII Prxs* including *AtPrx16*. We showed that the expression was restricted to the micropylar
71 endosperm and that the corresponding proteins were localized to the cell wall. We also showed
72 either a delayed or accelerated germination phenotype for each corresponding individual mutant
73 ruling out the possibility of functional redundancy. In depth analysis of these expression cluster
74 enabled us identifying enrichment in cell wall, cell wall protein and xyloglucan hits. We also
75 produced a comprehensive topochemical characterization of micropylar endosperm cell walls
76 showing a complex multi-cell wall domain organization. Finally, we discuss how sequential
77 temporal gene expression and cell wall domains could account for the implication of CW
78 remodeling enzymes from multigenic families, including *CIII Prxs*, to control micropylar
79 endosperm cell wall dynamics during seed germination.

80

81 **Materials and methods**

82

83 **Microarray data analysis and expression clustering of *Class III peroxidases***

84 All *CIII Prx* expression values were retrieved in a Microsoft Excel file from the microarray
85 data deposited by (Dekkers *et al.*, 2013) in the national center for biotechnology information Gene
86 expression Omnibus with accession number GSE 41212 (www.ncbi.nlm.nih.gov/geo/). The raw data
87 was first annotated and edited by sorting the genes according to their maximal log₂ expression
88 value and by identifying the genes whose expression values were above the cutoff log₂ value of
89 “5” previously defined (Dekkers *et al.*, 2013). The generated Microsoft Excel file was then
90 analyzed by different means. (i) *AtPrx62* which was the earliest *CIII Prx* to be highly expressed in
91 the Micropylar and chalazal endosperm (MCE) pool, was chosen as a bait to calculate the Pearson
92 Correlation Coefficient (PCC) along with all the genes of the data array. The genes were sorted and
93 ranked following decreasing PCC values. Only the *CIII Prxs* were selected and their relative
94 ranking and PCC were displayed allowing to select five MCE-sequentially expressed *CIII Prx*
95 candidates. The expression profiles were highlighted with a red (maximal value)-to-yellow (cutoff
96 5)-to grey (values below the cutoff) heat map. (ii) The co-expression networks of the five selected
97 *CIII Prxs* were similarly built and the four other *CIII Prxs* were positioned in each of the network to
98 identify the *AtPrx69* network as those displaying the best co-expression of the five *CIII Prxs*. (iii)
99 The *AtPrx69* network was analyzed in depth using 12 lists of genes constituting so-called “tool
100 boxes” related to subcellular compartments, cell wall polymers and physiological processes. The

103 the search tool from TAIR (<https://www.arabidopsis.org/index.jsp>). Each tool box was used to
104 search and filter the corresponding hits within the *AtPrx69* network. For each hit, the relative rank
105 (%) was calculated by dividing the “tool box rank” by the “absolute rank. Results were summarized
106 by plotting the relative rank (y axis) vs. the PCC (x axis) for each of the cross-analysis.

107

108 **Plant material and seed germination assays**

109 Wild type Col-0 *A. thaliana* (Col) and T-DNA insertion lines in Col background (*atprx04-1*
110 [SALK_110617], *atprx04-3* [FLAG_242H09], *atprx16-1* [SAIL_317_H04], *atprx16-2*
111 [SALK_118245], *atprx62-1* [GK_287E07], *atprx62-2* [SALK_151762], *atprx69-1*
112 [SAIL_691_G12], *atprx69-2* [SALK_137991], *atprx71-1* [SALK_123643] and *atprx71-2*
113 [SALK_121202]) were obtained from the European Arabidopsis Stock Centre
114 (<http://arabidopsis.info/>) and the INRA Versailles Stock Centre (<http://publiclines.versailles.inra.fr>).
115 Using standard procedures, homozygous mutant plants were identified by PCR genotyping with
116 respective primers (Table S16). T-DNA insertion sites were confirmed by sequencing using the
117 same primers.

118 Seeds of the Col and mutant lines were simultaneously grown in Jiffy peat pellets under the
119 same conditions (continuous light, 120 $\mu\text{mol photons.m}^{-1}.\text{s}^{-1}$, 22°C, 67 % relative humidity) in order
120 to obtain homogeneous seed batches. For *in vitro* experiments, seeds were surface-sterilized with a
121 solution containing 30 % bleach (v/v), 0.1 % triton X-100 (v/v), Seeds were put by spots of 50 to
122 100 seeds in Petri dishes containing $\frac{1}{2}$ MS medium including sucrose (10 g.L^{-1}) with or without 200
123 mM NaCl for stress conditions. Petri dishes were stored at 4°C for cold stratification during 24-48
124 hours, and incubated in a germination cabinet with continuous light (77 $\mu\text{mol photons.m}^{-1}.\text{s}^{-1}$, 24°C)
125 for the germination time course.

126 At different points of the germination kinetics (18, 24, 30, 32, 36, 38, 44, 50 and 77 hours
127 after sowing (HAS) for Col, *atprx04-1* and *atprx16-1* and 24 and 36 HAS for at least 4 repeats with
128 all the lines from 200 seed per batches), the Petri dishes were observed using a Zeiss Axiozoom
129 V16 stereomicroscope. The seed pictures were analyzed using ImageJ to categorize and count the
130 seeds at different germination stages: 1- intact testa and endosperm, 2- the testa rupture (TR) and 3-
131 the endosperm rupture (ER).

132

133 ***In situ* hybridization**

134 *in situ* RNA hybridization was realized essentially as previously described (Francoz et al.,

137 [http://www.brc.riken.jp/lab/epd/catalog/cdnaclone.html; (Seki et al., 1998, Seki et al.,
138 2002)] as templates : *AtPrx16* (pda18344), *AtPrx62* (pda13156), *AtPrx69* (pda07505), *AtPrx71*
139 (pda06662). *AtPrx04* cDNA was directly cloned in pGEMT-easy using the primers prx04_S-rt and
140 prx04CodSequ-SmaI/AS listed in Table S16. Hundreds of Col seeds at various developmental
141 stages were fixed in FAA, infiltrated in paraplast and allowed to sediment in 50 ml falcon tubes
142 used as primary embedding molds. Following paraplast solidification at 4°C, each samples of the
143 kinetics were unmolded, and the resulting paraplast blocks were trimmed and assembled in a final
144 metallic embedding mold constituting a tissue microarray encompassing hundreds of seeds from
145 each developmental stage. Serial sections were deparaffinized and used for the previously described
146 *in situ* hybridization protocol (Francoz et al., 2016a; Francoz et al., 2019b). Slides were then
147 scanned using the Nanozoomer 2.0HT (Hamamatsu Photonics, Tokyo, Japan). After scanning the
148 image analysis was performed using the Hamamatsu photonics, Image viewer (NanoZoomer Digital
149 Pathology, NDP) for “Virtual Microscopy”. To insure fair comparison, the same individual seeds
150 hybridized with the 10 riboprobes were retrieved on the synchronized scans from the serial sections
151 and used for Fig. mounting and semi-quantitative analysis of signal intensities.

152

153 **Transient expression in *Nicotiana benthamiana* leaves**

154 *AtPrx04*, *AtPrx16*, *AtPrx62*, *AtPrx69* and *AtPrx71* coding sequences were amplified by PCR
155 using cDNAs from *A. thaliana* 10-day old plantlets with the primers listed in Table S16. The PCR
156 products were digested with the appropriate restriction enzymes and cloned into TagRFP-AS-N
157 vector (Evrogen).

158 *AtPrx04*, *AtPrx16*, *AtPrx62*, *AtPrx69* and *AtPrx71-TagRFP* fusions were subcloned (LR
159 reaction, Gateway Technology, Invitrogen) into pEAQ-HT-DEST1 vector (Sainsbury *et al.*, 2009).
160 Every construct was checked by restriction analysis and sequencing, and transferred into
161 *Agrobacterium tumefaciens* (strain GV3101). The aquaporin PIP2A-YFP fusion protein pm-yb
162 CD3-1006 (Nelson *et al.*, 2007) was used as a plasma membrane marker. Alternatively, the
163 endoplasmic reticulum sp-YFP-HDEL (En Re) marker CD3-958 (Nelson *et al.*, 2007) was also
164 used.

165 *A. tumefaciens* strains grown at an optical density of 0.5 at 600 nm and containing each Prx-
166 TagRFP and PIP2A-YFP or sp-YFP-HDEL constructs were co-infiltrated using a ratio of $\frac{2}{3}$ AtPrx-
167 TagRFP – $\frac{1}{3}$ into *N. benthamiana* 30-day old lower side leaves. 48 hours after infiltration, the
168 leaves were detached, counterstained with Calcofluor and infiltrated or not in 30% glycerol for 5-10

171 sequential mode: Sequence 1 for Calcofluor (excitation: 405 nm ; emission 416-468 nm), sequence
172 2 for TagRFP (excitation: 561 nm; emission: 556-605 nm or excitation: 543 nm; emission: 555-636
173 nm); sequence 3 for YFP (excitation: 488 nm; emission: 498-559 nm). 1024x1024 images
174 corresponded to single confocal sections merged and false colored using ImageJ
175 (<http://imagej.nih.gov/ij/>).

176

177 **RNA isolation and residual gene transcript analysis by end-point RT-PCR**

178 Total RNA was prepared from 10-day old *in vitro*-grown Col and mutant plantlets using the
179 Tri-reagent solution (Sigma-Aldrich). After quantification by spectrophotometry and verification by
180 electrophoresis, RNA was treated with the RQ1 RNase-free DNase I (Promega) and quantified
181 again. One microgram of total RNA was reverse transcribed using the oligo(dT)15 primer and the
182 MMLV-RT (Promega) according to the manufacturer's instructions. PCR was carried out with the
183 gene-specific intron-spanning primers (Table S16). The cycling conditions were 94°C for 3 min,
184 followed by 94°C for 30 s, 60°C for 30 s and 72°C for 1 min, for 20 to 40 cycles. The PCR was
185 ended with one final step at 72°C for 5 min, and analyzed by gel electrophoresis and ethidium
186 bromide staining. The signal from the actin-encoding *ACT2* gene (Fulton and Cobbett, 2003) was
187 used as a control for cDNA quantity and quality. Three independent experiments were performed
188 with consistent results.

189

190 **Peroxidase activity.**

191 Soluble proteins were extracted from seeds 24 HAS grinded in 20mM HEPES, pH 7.0,
192 containing 1 mM EGTA, 10mM ascorbic acid, and PVP PolyclarAT (100mg g⁻¹ fresh material;
193 Sigma). The extract was centrifuged twice for 10 min at 10,000 g. Each extract was assayed for
194 protein levels with the Bio-Rad assay (Bio-Rad) and for total peroxidase activity following the
195 oxidation of 8 mM guaiacol (Fluka) at 470 nm in the presence of 2 mM H₂O₂ (Carlo Erba) in a 200
196 mM phosphate buffer pH 6.0. Enzyme activity was expressed in nkatal/mg protein. Values are the
197 mean of three replicates ± SD. P-value of one-way anova followed by Tukey's significance test,
198 (***) P < 0.001, (**) P < 0.0001.

199

200 **Immunofluorescence screening**

201 Dry *A. thaliana* Col seeds were embedded in LR White resin using an optimized protocol
202 adapted for this dry material (Francoz et al., 2019b). Semi-thin serial sections (1 µm) were disposed
203 on silane coated microscopy slides and either stained with toluidine blue (TBO) or Calcofluor for

205 of 38 cell wall probes (<http://www.plantprobes.net>; Ralet et al., 2010) listed in Table S17: The
206 secondary antibody was anti-Rat IgG-Alexa488, anti-mouse IgG-Alexa488 or anti-His-Alexa488
207 (Invitrogen) as detailed in Table S17. Attempts to perform a calcofluor counterstaining resulted in
208 irregular efficiency of staining. The slides were then mounted in ProLong® Gold Antifade medium
209 and imaged using confocal microscopy. HCX APO CS 63.0 x 1.40 oil immersion objective under
210 sequential mode: Sequence 1 for Alexa488 (excitation: 488 nm; emission: 499-578 nm), sequence 2
211 for Calcofluor/autofluorescence (excitation: 405 nm ; emission 426-468 nm). Images corresponded
212 to single confocal sections merged and false colored using ImageJ (<http://imagej.nih.gov/ij/>).

213
214

215 **Phylogenetic analysis and taxonomic distribution**

216 The 10 CIII Prx protein sequences including AtPrx69, AtPrx71, AtPrx16, AtPrx62,
217 AtPrx04, their paralogs and AtPrx42 as outgroup sequence, were aligned using MAFFT. The tree
218 was constructed using the Neighbor-Joining method (Saitou and Nei, 1987) and evolutionary
219 analyses were conducted in MEGA7 with 100 bootstraps (Kumar *et al.*, 2016).

220 Taxonomic distribution of the four clusters along the green lineage was performed with
221 OrthoMCL clustering. The numbers corresponded to the copy number found in each cluster.
222 All protein sequences used for phylogenetic analysis and taxonomic distribution are available from
223 the PeroxiBase (<http://peroxibase.toulouse.inra.fr>, (Fawal *et al.*, 2013)).

224

225 **Results**

226

227 **Microarray analysis of CIII Prx expression profiles leads to a clusterized micropylar and** 228 **chalazal endosperm specific pattern**

229

230 In order to explore the role of CIII Prxs during seed germination, we extensively analysed
231 published microarray dataset based on laser capture microdissection of different seed sections
232 during the germination time course of *A. thaliana* (Dekkers *et al.*, 2013) (data are deposited in the
233 NCBI gene expression omnibus, GSE 41212, and for complete list refer to Tables S1). Among the
234 highly expressed CIII Prxs in the micropylar and chalazal endosperm (MCE) pool, *AtPrx62*, which
235 was the earliest expressed CIII Prx during the studied kinetics, has been used as a bait to build a co-
236 expression network ranked by decreasing individual Pearson correlation coefficient (PCC) among
237 all this microarray dataset. Other CIII Prx-encoding genes were then selected based on their rank
238 and PCC value (Fig. 1A; Tables S2). A MCE-specific CIII Prx cluster could be identified since four

239 additional *CIII Prxs* (*AtPrx69*, *AtPrx71*, *AtPrx16* and *AtPrx04*) were found within the 1,000 top
240 genes belonging to the *AtPrx62* network with PCC values ranging from 0.888 to 0.739 and
241 relatively high expression values (Fig. 1A; Tables S2). It should be noted that although these *CIII*
242 *Prxs* are strongly expressed during the early germination steps with particular expression patterns,
243 they have also been detected in other organs such as roots, stems and leaves {Shigeto, 2013;
244 Arnaud, 2018}.

245 The expression of *AtPrx62* and *AtPrx69* sequentially started before the testa rupture (TR)
246 and persisted until the completion of the endosperm rupture (ER). *AtPrx71*, *AtPrx16* and *AtPrx04*
247 had a similar expression profile starting after the TR, slightly delayed as compared to *AtPrx62* and
248 *AtPrx69*. Gene expression analysis using more global transcriptomic data overall confirmed the
249 clusterization but with a lower level of resolution (Fig. 1B). *AtPrx62* was the earliest expressed
250 gene among the five selected genes (expression from 3 h, Fig. 1A), and at 24 h and 48 h, the five
251 selected genes were co-expressed. This co-expression was specific as compared to non-selected
252 corresponding paralog genes for *AtPrx69*, *AtPrx16* and *AtPrx04* (*AtPrx70*, *AtPrx25* and *AtPrx05*,
253 respectively). However, the low spatial resolution of global transcriptomic data (Fig. 1B), could
254 lead to wrongly select *AtPrx46* as candidate while its expression is only restricted to cotyledons and
255 radicle during germination but not to MCE (Fig. 1A). These results indicate the advantage of our
256 approach in order to specifically select *CIII Prxs* involved in «seed germination». Considering the
257 importance of both TR and ER events during the germination time course, and the availability of at
258 least two independent T-DNA mutants for the five selected *CIII Prxs* (*AtPrx62*, *69*, *71*, *16*, and *04*),
259 we decided to perform cellular and sub-cellular localizations and phenotyping analysis.

260

261 ***AtPrx62*, *AtPrx69*, *AtPrx71* and *AtPrx16* expression is restricted to the micropylar endosperm**

262

263 The tissue-specific transcriptomic study used here for the selection of the candidates was
264 valuable since sampling was restricted to a limited number of tissues (Dekkers et al., 2013).
265 However, the sampling of chalazal and micropylar endosperm pool could not discriminate whether
266 the candidate genes were expressed within the micropylar endosperm (ME), the chalazal endosperm
267 (CE), or both tissues. This piece of information was crucial to relate these genes to germination
268 since the *CIII Prx* activity involved in seed germination is concentrated in the ME together with
269 H₂O₂ (Lariguet *et al.*, 2013). As the complementarity between *in situ* hybridization (ISH) and
270 transcriptomics has been demonstrated (Francoz *et al.*, 2016a), a systematic ISH experiment was
271 engaged using digoxigenin-labelled riboprobes specific for each of the five *CIII Prx* candidate
272 genes. Serial sections from the paraffin-embedded tissue array containing kinetics of hundreds of

273 germinating seeds around the time of TR and ER were used. Transcripts from *AtPrx62*, *AtPrx69*,
274 *AtPrx71* and, to a lower extend, *AtPrx16* were specifically localized in the ME, and neither in the
275 CE nor in the peripheral endosperm (PE), at 34 hour after sowing (HAS) (Fig. 2; Fig. S1 and S2).
276 We carefully analyzed serial sections from individual seeds from the tissue microarray between 24
277 and 38 HAS with each riboprobe and summarized the semi-quantitative results (Fig. 3). The time
278 window analyzed by ISH was compared to the transcriptomics kinetics (Fig. 3A, B). A relative
279 coherence could be obtained between the transcriptomic expression levels and the ISH signal
280 intensities, since *AtPrx69* displayed the best ISH signal while *AtPrx04* expression level was just
281 below the ISH detection limit. Similarly, the correlation between transcriptomics and ISH was
282 relatively good while looking at the kinetics since *AtPrx62* and *AtPrx69* were more readily detected
283 than *AtPrx71* and *AtPrx16* at 26 HAS (Fig. 3A, B). Finally, this analysis at the individual-seed level
284 through ISH performed on serial sections allowed monitoring the number of genes among the five
285 *CIII Prxs* for which transcripts were simultaneously detected in the ME of a same seed (Fig. 3C).
286 For example, at 34 HAS, transcripts from four out of the five *CIII Prxs* could be detected in the
287 same seed for 23 % of the analyzed seeds. Beyond the MCE co-expression observed by
288 transcriptomics, this semi-quantitative ISH results showed the actual co-expression restricted to the
289 ME at the seed level of up to five *CIII Prxs*, raising the question of whether or not these genes had a
290 redundant function in this tissue during germination.

291

292 **AtPrx62, AtPrx69, AtPrx71 and AtPrx04 are cell wall-targeted class III peroxidases**

293

294 In *A. thaliana*, 64 *CIII Prxs* are predicted or localized to the CW and 9 are targeted to the
295 vacuole in relation with a C-terminal extension acting as a demonstrated or putative vacuolar
296 targeting sequence (Carter *et al.*, 2004; Francoz *et al.*, 2015). Heterologous expression experiments
297 were performed in *Nicotiana benthamiana* to assess whether these five *CIII Prxs* belonging to ME-
298 specific expression cluster co-localized to the same subcellular compartment. Using constructs
299 encoding for *CIII Prx*-TagRFP fusion proteins under the control of CaMV 35S promoter, transient
300 expression experiments were conducted in *N. benthamiana* leaves with PIP2A-YFP used as a
301 plasma membrane marker (Nelson *et al.*, 2007) and Calcofluor as a CW marker. For three
302 constructs (*AtPrx62*-TagRFP, *AtPrx69*-TagRFP and *AtPrx71*-TagRFP), we were able to detect a
303 co-localization for the RFP, the YFP signals and the calcofluor CW counterstaining (Fig. 4). Under
304 water mounting, the calcofluor signal (green false colored) co-localized with the RFP signal
305 (magenta false colored) for the three constructs leading to a white signal when the two channels
306 were merged, predicting a CW localization for *AtPrx62*, *AtPrx69* and *AtPrx71* (Fig. 4A-C). As

307 shown in Fig. 4D-L when the cells were plasmolyzed, the RFP signal was still localized to the cell
308 wall while the YFP signal (green false colored) was essentially found in the plasma membrane,
309 demonstrating that AtPrx62, AtPrx69 and AtPrx71 were targeted to the CW. This was in agreement
310 with the fact that most of the CIII Prxs (including AtPrx62, AtPrx69 and AtPrx71) had a secretory
311 pathway signal peptide. In addition, several studies have shown that CIII Prxs could have a function
312 in the CW dynamics thanks to their CW loosening or stiffening roles (Francoz et al., 2015;. Wilson
313 et al., 2015). AtPrx16-TagRFP fluorescence signal totally co-localized with signal peptide-YFP-
314 HDEL used as an endoplasmic reticulum marker, respectively (Fig. S3A-C) while AtPrx04-TagRFP
315 showed a cell wall localization with some residual retention to the endoplasmic reticulum (Fig.
316 S3D-F). Therefore, although AtPrx04 and AtPrx16 both contained predicted secreted signal peptide
317 and no C-terminal HDEL/KDEL endoplasmic reticulum retention signal, they seemed to be at least
318 partially retained to the endoplasmic reticulum regardless of the time of observation following
319 agroinfiltration. This surprising result was counterbalanced by the analysis of cell wall proteomics
320 data (Fig. S3G) showing the detection of AtPrx04 and AtPrx16 in CW-enriched root fractions.
321 AtPrx69, AtPrx71 and *Brassica olearacea* AtPrx62 ortholog were also detected in CW proteome in
322 agreement with our confocal observations. Comparative analysis of subcellular proteomes
323 confirmed the presence of these five CIII Prxs in CW-enriched fractions as compared to other sub-
324 cellular compartment-enriched fractions, and did not show any detection of AtPrx04 and AtPrx16 in
325 endoplasmic reticulum-enriched fractions (Fig. S3H). Altogether, AtPrx62, AtPrx69 and AtPrx71
326 are CW localized proteins and AtPrx04 and AtPrx16 are probably CW proteins as well. Therefore,
327 these five CIII Prxs are good candidates to specifically or redundantly play a role in CW remodeling
328 of the ME cells, thus accelerating or delaying the germination of *A. thaliana* seeds.

329

330 **Class III peroxidases have antagonist effects on early germination steps**

331

332 Two independent T-DNA mutant lines were isolated for each of the five genes (Fig. S4A).
333 As demonstrated by RT-PCR, they were all knockout mutants except for *atprx04-1*, *atprx62-2* and
334 *atprx71-2*, which presented 40-50 % residual transcriptional activity (Fig. S4A). Accordingly,
335 significant reduction of the total peroxidase activity was observed in all mutant lines suggesting a
336 relation between CIII Prx transcript and protein levels (Fig. S4C). Germination kinetics were
337 performed and the three following stages were quantified: (i) intact testa and endosperm, (ii) testa
338 rupture (TR) and (iii) endosperm rupture (ER) (Fig. 5A). Complete kinetics of germination were
339 performed for wild-type seeds (Col) and two mutants that show opposite phenotypes (*atprx04-1* and
340 *atprx16-1*) in order to determine the optimal time to well discriminate the two rupture events (Fig.

341 5B). Then, TR and ER rates were extensively assessed 24 hours after sowing (HAS) and 36 HAS,
342 respectively. At 24 HAS, the TR was high enough and the ER more or less equal to zero to allow
343 the observation of differences between all the different lines. In addition, at 36 HAS, the ER was
344 also high enough and the TR close to 100 % to discriminate between the mutants.

345 At 24 HAS, *atprx04-1* and *atprx04-3* exhibited a severe TR delay (-75% and -52 %
346 respectively) and *atprx16-1*, *atprx16-2*, *atprx69-1*, *atprx69-2*, *atprx71-1* and *atprx71-2* seeds
347 showed a clear increase of TR (between 30% and 82%) when compared to the wild type (Fig. 5C).
348 No significant effect was observed in *atprx62-1* and *atprx62-2* regarding the % of TR at 24 HAS.
349 At 36 HAS, WT and mutants seeds had more than 80% of TR, which no longer allowed observing a
350 significant TR phenotype (Fig. 5B). However, this second observation time revealed ER
351 phenotypes. Indeed, *atprx04-1* and *atprx04-3* presented an ER delay (-44% and -63 % respectively,
352 Fig. 5C) and the eight other mutants including the two *atprx62* lines presented a large increase of
353 ER (between 58 to 206 %) as compared to the wild type seeds (Fig. 5D). Strikingly, despite the
354 observed strong gene co-expression, mutants from all the five genes displayed a clear germination
355 phenotype in disagreement with anticipated putative functional redundancy. To summarize, two
356 opposite germination phenotypes were observed in the *CIII Prx* mutant lines: (i) TR and/or ER
357 induction for *atprx16*, *atprx62*, *atprx69* and *atprx71* mutants suggesting a CW stiffening action of
358 AtPrx16, AtPrx62, AtPrx69 and AtPrx71 that negatively regulates germination. (ii) TR and ER
359 repression for the two *atprx04* allelic mutants suggesting that AtPrx04 had a promoting CW
360 loosening role on both testa and ME rupture. These phenotypes were observed for both KO and KD
361 mutants suggesting that a 50-60% reduction of gene expression was sufficient to lose the function
362 (Fig. 5C, D; Fig. S4B).

363 The process of germination is delayed in presence of salt concentration (Lee and Zhu, 2010)
364 and public data indicate that the expression of *AtPrx04*, *AtPrx16*, *AtPrx62* and *AtPrx69* are
365 regulated by salt stress at the seedling root level (Fig. S5A, {Killian, 2007}). The kinetics of TR and
366 ER in presence of 200 mM NaCl were performed with Col, and two mutants with contrasted
367 phenotypes (*atpr04-1* and *atprx16-1*, Fig. 5) and allowed determining the optimal reading frame of
368 early germination steps (Fig. S5B). As expected, the TR and ER were both delayed and the
369 germination rate was also reduced when Col seeds were grown with salt. In order to determine the
370 putative relationship between salt and *CIII Prx* action, the TR was quantified 77 HAS for 5 mutants
371 and compared with the results obtained without NaCl. For *atprx04-1*, the strong TR delay observed
372 at 24 HAS without NaCl was no longer observed and converted to a slight TR induction at 77 HAS.
373 For *atprx62-1* and *atprx16-1*, and to a lesser extent, for *atprx71-2*, seeds at 77 HAS in presence of
374 NaCl showed a higher TR induction when compared with the germination performed without NaCl

375 (Fig. S5C). *atprx69-1* TR induction was slightly decreased in presence of NaCl. Therefore, mutants
376 from *AtPrx04* and *AtPrx62* that were the most induced by salt treatment (Fig. S5A), were those
377 displaying the most dramatical phenotype changes in presence of NaCl (attenuated and amplified
378 phenotypes for *atprx04-1* and *atprx62-1*, respectively). These results suggested that among the five
379 CIII Prxs, *AtPrx62* seemed to be the most implicated in salt tolerance during germination. A link
380 between salt stress tolerance during germination and CW remodeling has been recently proposed
381 for a pectin methylesterase inhibitor (PME113/AT5G62360) and a pectin methylesterase
382 (PME31/[AT3G29090](#)) since the germination phenotype of the corresponding mutants
383 (overexpression and T-DNA respectively) were only revealed under salt stress conditions {Yan,
384 2018; Chen, 2018}.

385

386 **The CIII Prx co-expression network is enriched in genes related to “germination”, “cell wall”**
387 **“cell wall proteins” and “xyloglucans”.**

388

389 In order to better understand the implication of the five CIII Prxs during early germination
390 steps, each of the CIII Prx was used as a bait to generate five respective co-expression networks
391 using the seed germination tissue-specific transcriptomics data (Dekkers et al. 2013). We compared
392 the five networks in order to identify which one displayed the strongest co-expression of the five
393 CIII Prxs (Tables S3A, B). *AtPrx69* co-expression network appeared as the most interesting to
394 simultaneously analyze commonly co-expressed genes with the five CIII Prxs. We assembled
395 twelve “tool box” lists of genes related to various sub-cellular compartments, cell wall components
396 and physiological processes (Table S4B-15B), and used each of these tool boxes for a cross-
397 comparison with *AtPrx69* co-expression network (Table S4A-15A). For each of these cross-
398 analyses, we calculated the Pearson correlation coefficient and the relative rank corresponding to
399 the ratio of the tool box rank vs. the initial absolute rank (see Methods for details). The results were
400 summarized in 12 plots representing the relative rank (%) vs. the PCC for each of the tool box genes
401 found in *AtPrx69* co-expression network, using the same x and y scales for fair comparison. It
402 appeared that the *AtPrx69* co-expression network was clearly enriched in genes from the “cell wall”
403 as compared to the other sub-cellular compartment tool boxes (Fig. 6A-D). The “cell wall genes”
404 enrichment further supports the ME endosperm CW remodeling hypothesis. Interestingly, the
405 comparison of tool boxes from different CW polymers revealed that *AtPrx69* co-expression network
406 was enriched in “cell wall proteins” and in “xyloglucan” hits rather than in “cellulose” and “pectin”
407 hits (Fig. 6E-H). This observation gave more insight on how the five CIII Prxs might regulate the
408 ME CW dynamics through possible functional connections with these types of CW polymers.

409 Finally, while performing similar search using tool boxes related to different physiological
410 processes, we observed the enrichment of *AtPrx69* co-expression network in hits from “seed
411 germination” and not from “seed storage”, “photosynthesis” and “cell division” used as controls
412 (Fig. 6I-L). Altogether, these cross-analyses of transcriptomic data reinforced our experimental
413 results indicating the role of the five CIII Prxs in ME CW dynamics controlling seed germination.
414 These enrichments in categories of genes did not give direct evidence as to the role of the five CIII
415 Prxs, but argued in favor of a potential ME CW modification function during seed germination in
416 relation to xyloglucan. Bibliographic search within the “seed germination” hits from *AtPrx69* co-
417 expression network showed that the best hits were the four other *CIII Prxs* coming from this study,
418 as well as STEROL CARRIER PROTEIN 2, a unrelated peroxisomal protein involved in β -
419 oxidation already described for its role during seed germination (Zheng *et al.*, 2008) (Table S12A).
420 The other “germination hits” were found farther along the co-expression network indicating that the
421 *CIII Prx* co-expression was somehow separated from the previously characterized genes for their
422 role in germination suggesting that potential new candidates could be found between the position
423 270 and 2010 of the absolute rank.

424

425 **Dry seed ME CW displayed topochemical specificities suggesting the occurrence of CW**
426 **microdomains.**

427

428 A previous immunofluorescence study illustrated the CW topochemistry of ME as compared
429 to other seed tissues in *A. thaliana* imbibed seeds (Lee *et al.*, 2012). It appeared that ME CW was
430 labeled with LM25 and to a lesser extent LM15 (xyloglucans), LM19 (unmethylesterified
431 homogalacturonans), LM6, LM13 and LM16 (arabinans). Of particular interest was the different
432 labeling observed with the arabinan-specific antibodies: LM13 labeled the inner and outer CW and
433 not the radial CW while LM6 and LM16 labeled all the ME CW, illustrating the complexity and
434 diversity of ME CW topochemistry. With the aim to better understand this complexity, we
435 performed an additional systematic immunofluorescence screening focusing on serial longitudinal
436 sections from dry seeds that are in the “starting blocks” preceding the action of the five studied CIII
437 Prxs (Fig. 7; Fig. S17). We obtained ME-specific signals with 13 CW probes (Fig. 7). Interestingly,
438 only LM25 and LM16 displayed an homogeneous labeling all around the ME CW (Fig. 7E, L). The
439 remaining eleven probes all gave patchy and/or polarized and/or differentially intense labeling
440 around ME CW (Fig. 7C, D, G, I-K, M-P, R). While Calcofluor cytochemical labeling of β -glucans
441 including cellulose showed a regular labeling of ME walls, crystalline cellulose-specific labeling
442 with CBM3a was restricted to the ME inner wall/radial wall corners (Fig. 7B, C). The LM15

443 labeling on ME inner wall and radial and outer wall patches contrasted with the LM25 more regular
444 labeling (Fig.7D, E). LM15 recognizes somehow less substituted and shorter xyloglucan (XG)
445 models than LM25, though the differences of specificity of these antibodies are subtle and complex
446 (Pedersen et al., 2012; Ruprecht et al., 2017). The only homogalacturonan (HG) probe showing ME
447 CW labeling was LM19 restricted to the inner wall (Fig. 7G). Four rhamnogalacturonan I (RGI)
448 probes displayed labeling with a tendency of more intense labeling on the ME outer wall than on
449 the inner wall and a patchy labeling pattern of RU2 (Fig. 7I-L). Contrarily, three extensin (EXT)
450 antibodies show the somehow opposite pattern with regular labeling on the ME inner wall and less
451 intense, absent or patchy labeling of the radial and/or outer wall (Fig. 7M-O). Finally, two arabino
452 galactan protein (AGP) antibodies showed a patchy and/or polarized labeling (Fig. 7P-R). These
453 results complemented those published on imbibed seeds (Lee *et al.*, 2012) since LM25 and LM16
454 gave similar ME CW labeling patterns in both studies while LM19, LM6, LM13 and JIM12 ME
455 CW labeling patterns were more polarized in our study. The other antibodies were not presented in
456 Lee et al (2012). This antibody panel and the previous study on imbibed seeds illustrated the
457 complexity and diversity in the distribution of ME CW polymers since different topochemistries
458 could be observed on the inner, radial and outer CW with some patchy labeling constituting
459 multiple cell wall domains altogether suggesting the necessity for a correlated complexity/diversity
460 in CW remodeling enzymes, in agreement with our results showing the involvement of five non
461 redundant CIII Prxs in a single cell type.

462 In *A. thaliana*, AtPrx04, AtPrx16, AtPrx62, AtPrx69 and AtPrx71 belong to the same
463 evolutionary cluster containing four other members (AtPrx05, AtPrx25, AtPrx45, AtPrx70). The
464 five CIII Prxs selected in this study present from 34 to 74% identity (Fig. S6) All the genes member
465 of this cluster were found to be expressed in roots and only the five selected genes presented a
466 specific seed expression (Fig. S7A). The cluster resulted from four recent duplications in *A.*
467 *thaliana* and also in other Brassicaceae (Fig. S7A and B, blue circles). Older duplications can be
468 detected (Fig. S7A, orange circles) since no AtPrx04 and AtPrx69 orthologs has been detected
469 before mono-/dicotyledon separation (Fig. S7B). From an evolutionary point of view, this raises
470 questions on the putative neofunctionalization of some ortholog genes in Angiosperms, and on their
471 possible conserved function in controlling germination.

472

473

474 **Discussion**

475

476 Germination is a critical stage during *A. thaliana* and other seed plants lifespan since it
477 represents the transition between two successive generations (Salomé et al., 2008). This stage
478 consists in two crucial and sequential events: the testa rupture and endosperm rupture necessitating
479 CW remodeling, particularly in the ME facing the protruding radicle. Here, we exploited an elegant
480 published microarray data (Dekkers et al., 2013) to identify a MCE co-expression cluster
481 comprising five *CIII Prx* candidates (*AtPrx69*, *AtPrx71*, *AtPrx16*, *AtPrx62*, *AtPrx04*). We further
482 demonstrated their expression in the ME rather than in the CE. This location is similar to that of the
483 H₂O₂ that strongly accumulated in the distended endosperm prior its rupture (Lariguet *et al.*, 2013).
484 We also provided evidence and discussion indicating that the five genes encoded CW proteins all
485 displaying peroxidase activity. Interestingly, we observed a germination phenotype for the ten
486 corresponding mutants in disagreement with the anticipated putative functional redundancy. Our
487 results indicated that the ME remodeling was finely controlled by the coordinated action of four
488 *CIII Prxs* (*AtPrx62*, 69, 71, 16) having a repressive role on TR and ER and one *CIII Prx* (*AtPrx04*)
489 having the opposite activating role on TR and ER. The repressive role could be related with cell
490 wall stiffening as suggested for *AtPrx71* whose corresponding mutants display increased growth,
491 enhanced leaf epidermal cell size (Raggi *et al.*, 2015) and lignin structure modification {Shigeto,
492 2013}. The activating role could be associated with cell wall loosening due to ROS spatio-temporal
493 production as well as cell wall stiffening on the border of the rupture area. *AtPrx04*, which has been
494 described to play a role in stem lignification {Fernandez, 2015}, could reinforce the cells around
495 rupture region.

496 This raised the question on the means by which this coordination was achieved. Our ISH
497 results demonstrated that at certain points of the kinetics, individual seeds actually co-expressed at
498 least four of these genes. However, transcriptomics and ISH also demonstrated that the expression
499 of these genes sequentially started at different time points. The four *CIII Prx* genes that were
500 sequentially expressed at first were those encoding *AtPrx62*, 69, 71, 16 (Fig. 8A) suggesting a
501 cumulative ME stiffening action of these enzymes that could counter-balance the increasing
502 pressure of the growing radicle to avoid a premature germination (Fig. 8B). In turn, *AtPrx04* that
503 encoded an enzyme that could account for the opposite ME CW loosening action was the last
504 expressed among the five *CIII Prxs*, and its expression correlated with the ER (Fig. 8). The means
505 by which these *CIII Prxs* either stiffened or loosened the ME CW is unknown but in depth cross-
506 analysis of their co-expression network argued for a link with other CW proteins and possibly
507 xyloglucans. Among the ME-expressed genes encoding CW remodeling or putative CW integrity
508 sensors, members of three multigenic families were previously characterized. These corresponded
509 to three mannanases (Iglesias-Fernandez *et al.*, 2011), three Lectin-receptor kinases (Xin *et al.*,

510 2009) and two xyloglucan xylosyltransferases (Lee *et al.*, 2012). On the one hand, single mutants
511 of the three mananases displayed a germination delay phenotype similarly to the situation observed
512 for the five CIII Prx mutants. On the other hand, clear germination phenotypes were observed with
513 multiple mutants for the LecRKs and XXTs and never in single mutants. By comparing the ME-
514 specific expression profiles of all these genes, it appeared that the non-redundant functions of the
515 three MANs and the five CIII Prxs during ME CW remodeling was associated with a subtle
516 sequential expression while the redundant functions of the three LecRKs and the two XXTs could
517 be associated to more pronounced co-expression (Fig. 8A). It also appeared that the sequential
518 expression of the three *MANs* preceded the sequential expression of the four *CIII Prxs* that could
519 account for stiffening action, together preceding the final expression of *AtPrx04* with putative
520 loosening function (Fig. 8B). These combined results showed the complexity of ME CW
521 remodeling allowing to finely tune the precise time of radicle protrusion. The particularity of CIII
522 Prxs also relied on their involvement in both H₂O₂ cellular regulation and ROS generation which
523 could have a role in CW dynamics through non enzymatic polysaccharide breaking or aromatic
524 compounds covalent linking (Cosio and Dunand, 2009; Hiraga *et al.*, 2001). However, the actual
525 mode of action used by the five CIII Prxs to stiffen or loosen the ME CW still needs to be
526 investigated. The precise localization of each of these enzymes within ME CW might be crucial for
527 the developmental output as reported for two CIII Prxs localized in polarized CW microdomains in
528 *Arabidopsis* seed coat or root endodermis (Kunieda *et al.*, 2013; Lee *et al.*, 2013; Francoz *et al.*,
529 2019a). So in addition to the subtle sequential temporal expression of the five *CIII Prxs*, we cannot
530 exclude that putative localization of the corresponding proteins to ME CW domains could also
531 account for the observed non redundant functions. This last hypothesis would fit with the observed
532 ME CW topochemical heterogeneity.

533 Altogether, these results show that multiple CIII Prxs may have non redundant CW
534 remodeling function in a given cell type in agreement with the emerging description of the
535 localization of this type of enzyme to remote CW domains necessitating specific remodeling.

536

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Figure legends

Fig. 1. Class III Peroxidase expression data mining allows identifying a micropylar and chalazal endosperm (MCE)-specific CIII Prx co-expression cluster. (A) The original gene chip dataset (tissue-specific gene expression of 21,313 protein-coding genes in laser-capture microdissected tissues along *A. thaliana* seed germination kinetics, Dekkers et al., 2013) was retrieved as series matrix files (GSE41212) at <https://www.ncbi.nlm.nih.gov/geo/> and edited (Table S1). *AtPrx62* (At5g64100) which was the earliest highly expressed *CIII Prx* along the micropylar and chalazal endosperm (MCE) kinetics was used as a bait to calculate the Pearson correlation

coefficient (PCC) for all genes. The genes were subsequently ranked following a decreasing PCC value order. The 23 expressed *CIII Prx* genes present on the microarray are displayed here clearly illustrating a MCE-specific *CIII Prx* co-expression cluster highlighted in blue. A red (max value)-to-yellow (cutoff 5)-to-grey (min value below cutoff) heatmap was drawn for log₂ expression values. RAD, radicle; COT, cotyledons; MCE, micropylar and chalazal endosperm; PE, peripheral endosperm; TR, testa rupture; ER, endosperm rupture. The complete data including 37 *AtPrxs* below the cutoff is shown in **Table S2**. **(B)** Seed germination expression profile of the five candidates (blue text) and their paralogs (red text) showing the germination-specific expression of the candidates. Note that the paralog of *AtPrx62* is also a candidate (*AtPrx71*). Data was retrieved from Genevestigator (Zimmermann *et al.*, 2004) and corresponded to Narsai *et al.* (2011) (Narsai *et al.*, 2011).

Fig. 2. *AtPrx62*, *AtPrx69*, *AtPrx71* and *AtPrx16* transcripts are restricted to the micropylar endosperm of germinating seeds by *in situ* hybridization. Longitudinal sections of 34 hour after sowing (HAS) *A. thaliana* Col germinating seeds were hybridized with antisense (AS, first column) or sense (S, second column) digoxigenin-labeled riboprobes specific for the five *CIII Prxs* selected by co-expression network analysis. The hybridization signals (arrows: purple labeling present on the AS images on the left column and absent in the corresponding S images on the right column) were restricted to the micropylar endosperm and not to the chalazal endosperm for *AtPrx62*, *AtPrx69*, *AtPrx71* and *AtPrx16*. No signal could be detected for *AtPrx04*, the least expressed of these five *CIII Prxs* according to transcriptomics (**Table S2**). T: testa (non-specific seed coat natural brown color both in AS and S images); C: cotyledons; R: radicle; PE: peripheral endosperm; CZE: chalazal endosperm; ME: micropylar endosperm; Scale bars: 100 μ m. Whole seed views are shown in **Fig. S1**.

Fig. 3. Semi-quantitative comparison of tissue-specific transcriptomics and *in situ* hybridization results. **(A)** The transcriptomics kinetics data from the micropylar and chalazal endosperm (MCE) was extracted from **Fig. S2** for the five *CIII Prx* candidates showing the sequential initiation of the expression of the five genes. **(B)** Semi-quantitative analysis of *in situ* hybridization (ISH) performed on tissue microarrays at 26, 34, 36 and 38 HAS. For each stage, 10 tissue array serial sections individually hybridized with antisense or sense probes for the five genes were analyzed for expression in micropylar endosperm (ME). Each column represents individual seed serial-section (n = 30 per developmental stage). Color-coding: Red, strong ISH signal in ME; yellow, low ISH signal in ME; white, no ISH signal in ME; black, missing sample. The arrows at

the bottom show seed serial-sections with at least 3 out of the 5 *CIII Prxs* co-expressed. (C) Distribution (%) of seed serial-sections displaying ISH signal in ME for 0-to-5 genes along developmental stages. (D) The wide view of the tissue array serial-section hybridized with *AtPrx69* AS probe is shown as an example of the method. Individual seed within the white frames have been carefully analyzed at $\times 20$ magnification and color-coded as described above using NDP-view (Hamamatsu).

Fig. 4. AtPrx62, AtPrx69 and AtPrx71 are cell wall localized. Pieces of *Nicotiana benthamiana* leaves transformed with constructs expressing AtPrx62-TagRFP (A, D-F), AtPrx69-TagRFP (B, G-I) or AtPrx71-TagRFP (C, J-L) either alone (A-C) or with constructs expressing PIP2A-YFP used as a plasma membrane (PM) marker (D-L) were cut 48 h post-agroinfiltration, and either counterstained with aqueous Calcofluor to label cellulose (A-C) or subjected to 30 % glycerol mediated plasmolysis to separate the plasma membrane from the cell wall (D-L). The first line directly shows the merge of the TagRFP fusion proteins as indicated (magenta false color) and calcofluor (green false color) signals. The white signals illustrate the colocalization of AtPrx62-TagRFP (A), AtPrx69-TagRFP (B) and AtPrx71-TagRFP (C) with the cell wall marker. Under plasmolysis, the fluorescence signal (magenta false color) of AtPrx62-TagRFP (D-F), AtPrx69-TagRFP (G-I) and AtPrx71-TagRFP (J-L) is excluded from the fluorescence of PIP2A-YFP localized to the plasmolysed plasma membrane (green false color) confirming the cell wall localization of the three TagRFP fusion proteins. CW: cell wall, PM: plasma membrane, Scale bars: 20 μ m.

Fig. 5. Testa and endosperm ruptures of *Arabidopsis thaliana* seeds are dependent on antagonistic action of several CIII Prxs. (A) Pictures of Col *A. thaliana* seed early germination steps. (B) The kinetics of testa rupture (TR, full curves) and endosperm rupture (ER, dashed curves) were monitored for Col, *atprx04-1* and *atprx16-1*. The rates of TR and ER are expressed in % of total number of seeds (\pm SD) for each time point and for each line. The dashed line corresponds to 50% TR or ER. The time to get 50% of rupture is provided for each line. (C) The % of induction or repression of TR at 24 HAS versus Col (\pm SD) for the ten *CIII Prx* mutants. (D) The % of induction or repression of ER at 36 HAS versus Col (\pm SD) for the ten *CIII Prx* mutants. . The values are the mean of 200 seeds per experiment and they are representative of at least four independent batches of seeds.

Fig. 6. The *AtPrx69* co-expression network is enriched in genes from the “cell wall”, “cell wall protein”, “xyloglucan” and “germination” tool boxes. Among the co-expression networks of the five *CIII Prx* candidates, *AtPrx69* co-expression network was chosen for this tool box cross-analysis since it showed the most co-expressed pattern of the five *CIII Prxs* (**Table 3**). Twelve tool boxes gathering lists of genes related to various sub-cellular compartments (**A-D**), cell wall polymers (**E-H**) and physiological processes (**I-L**) as labeled in the top right corner of each panel, were searched within *AtPrx69* co-expression network built using Dekkers et al (2013) dataset. The x axis corresponds to the Pearson correlation coefficients (PCC) values and the y axis represents the relative rank (%) corresponding for each gene to the ratio of the “tool box rank” hits among *AtPrx69* expression network vs the absolute rank within the whole *AtPrx69* expression network. The x axis crosses the y axis at specific thresholds values corresponding to the proportion (%) of the number of expressed genes of each tool box among the 14,317 expressed genes on the microarray (maximum expression value above the “5” cutoff expression value of the transcriptomics). For more details, see **Tables S4 to S15**.

Fig. 7. Complex topochemical diversity of the micropylar endosperm cell wall domains in dry seeds. *A. thaliana* Col dry seed serial longitudinal sections were stained with Toluidine blue (TBO) (**A**) or Calcofluor (**B**) to visualize cell wall morphology and β -glucans, respectively. Serial sections were also immunolabeled with cell wall-specific antibodies and Alexa fluor 488-labeled secondary antibodies. The position of micropylar endosperm (men) is shown with * marks (**C-R**). Negative controls without primary antibodies were also performed (**S-T**). The antibodies are noted on the top right corner of the images and classified using background color codes. The green lines/spots around the simplified drawing displayed on the right of each image summarize the observed immunofluorescence patterns within a men cell. The absence of green line with some antibodies corresponds to the absence of labeling as compared to the negative controls (See Table S17 and methods for a complete list of the antibodies tested). The non specific autofluorescence allowing to visualize the morphology is displayed in magenta false color. The Alexa-488 specific signal is visualized in bright green. Note that the confocal microscopy settings were adjusted to visualize either intense and fainter A488 signals depending on the antibodies. The greenish autofluorescence labels correspond to fainter signals. The negative control without primary antibodies is displayed using both settings. pd, radicle protodermis; men (*), micropylar endosperm; ii1, inner integument 1 cell layer; oi2, outer integument 2 cell layer. Bars: 25 μ m.

Fig. 8. Integrated model of spatiotemporal coordinated roles of functionally redundant and non-redundant ME CW-remodeling or ME CW integrity-sensing proteins from four multigenic families. (A) The tissue-specific germination transcriptomics data (Dekkers *et al.*, 2013) was used to assemble MCE-specific expression values for the five *CIII Prxs* and to integrate those with corresponding values from previously characterized additional ME CW-remodeling enzymes: 3 Mananases (MAN) (Iglesias-Fernandez *et al.*, 2011) and 2 xyloglucan xylosyltransferases (XXT) (Lee *et al.*, 2012), and putative ME CW integrity sensors: 3 Lectin Receptor kinases (LecRK) (Xin *et al.*, 2009). Comparison of phenotypes and expression profiles indicated that the genes having slightly sequential expression had non redundant functions since simple mutant displayed germination phenotypes (3 MANs and 5 *CIII-Prxs*) while those having more highly co-expression profiles had redundant functions requiring multiple mutants to see the germination phenotypes (3 LecRKs and 2 XXTs). The ME CW loosening (green label) or stiffening (red lab) roles were deducted from the delayed and accelerated germination phenotypes, respectively. (B) Spatio-temporal integrated model of ME CW fine remodeling necessary to control germination. The drawings show a zoom on the interface between the growing radicle (Rad), the micropylar endosperm (ME) and the testa at various times expressed as hours after sowing. The timing of apparition of individual ME CW remodeling proteins follows transcriptomics. The growing-radicle increasing pressure (large green arrow) is first accompanied by the ME CW sequential loosening role of 3 MANS (green label), which in turn is counterbalanced by the ME CW sequential stiffening role of 4 *CIII Prxs* (red label) and finally accompanied by the ME CW loosening role of AtPrx04 (green label). The additional ME CW redundant loosening role of the two constitutive XXTs and the 3 late expressed LecRKs was not displayed for clarity. Altogether, the phenotypes observed in each *man* and *CIII prx* mutants, indicating their non-redundant roles, could be explained by their subtle sequential apparition and/or by putative yet-to-be demonstrated ME microdomain localization.

Fig. 2

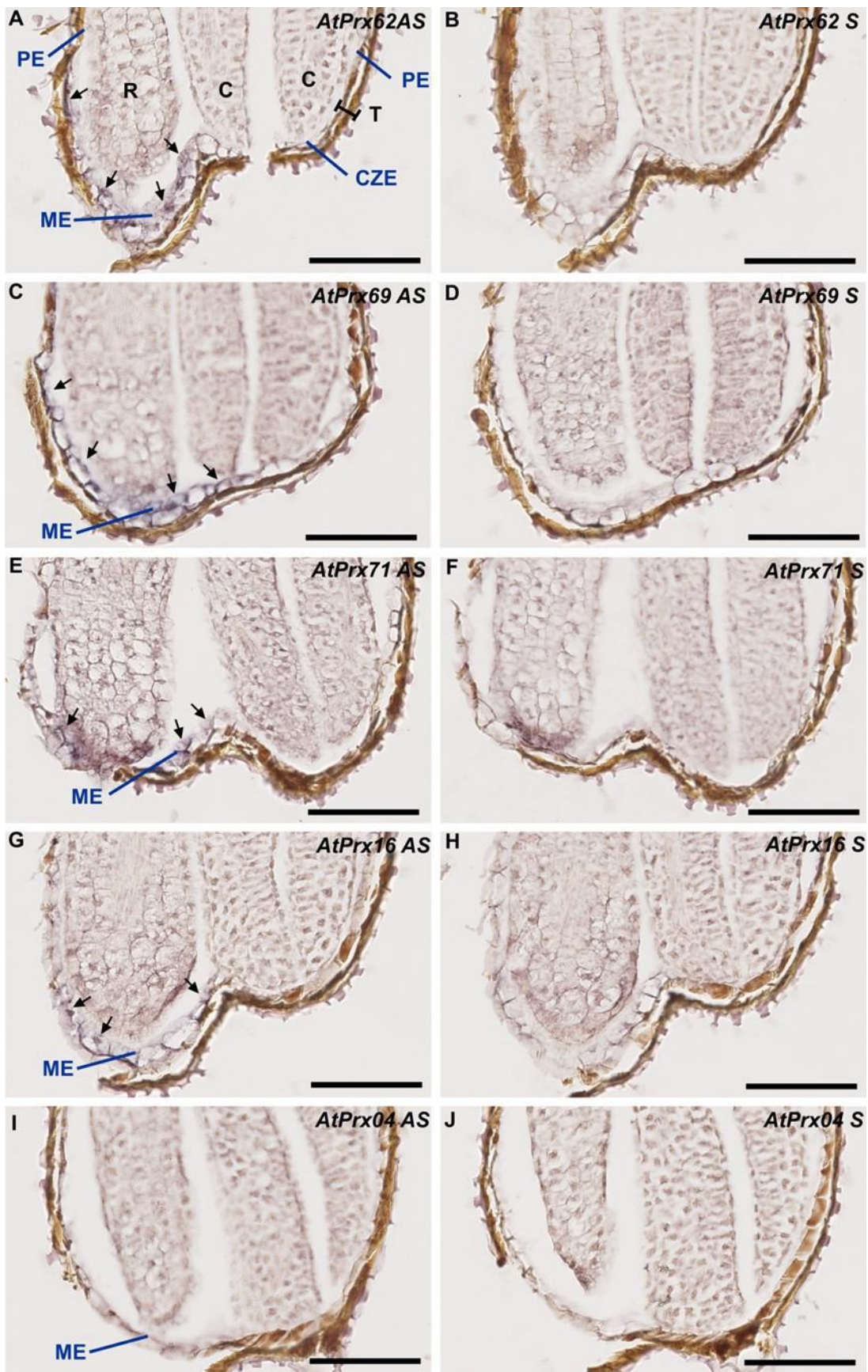


Fig. 3

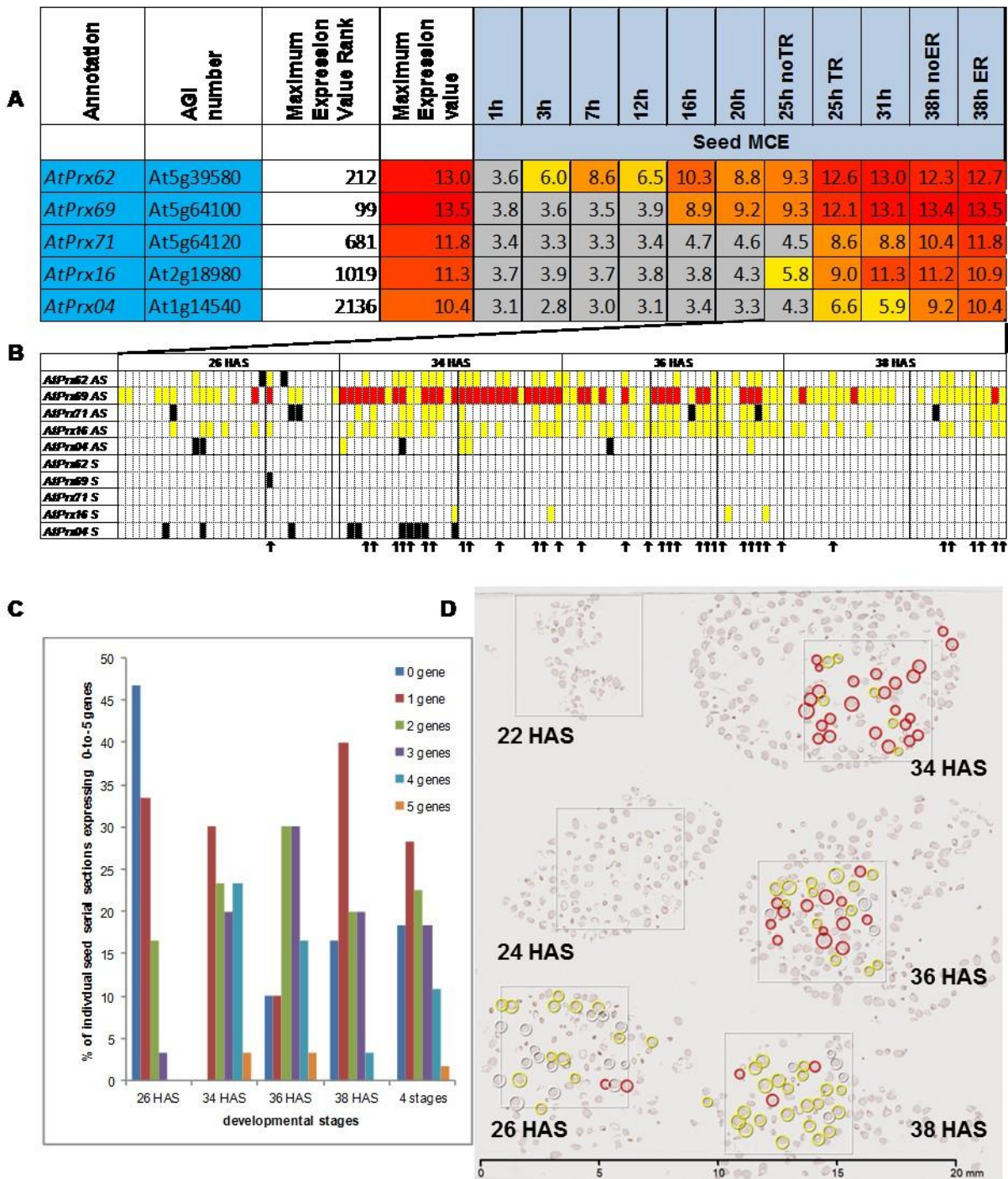


Fig. 4

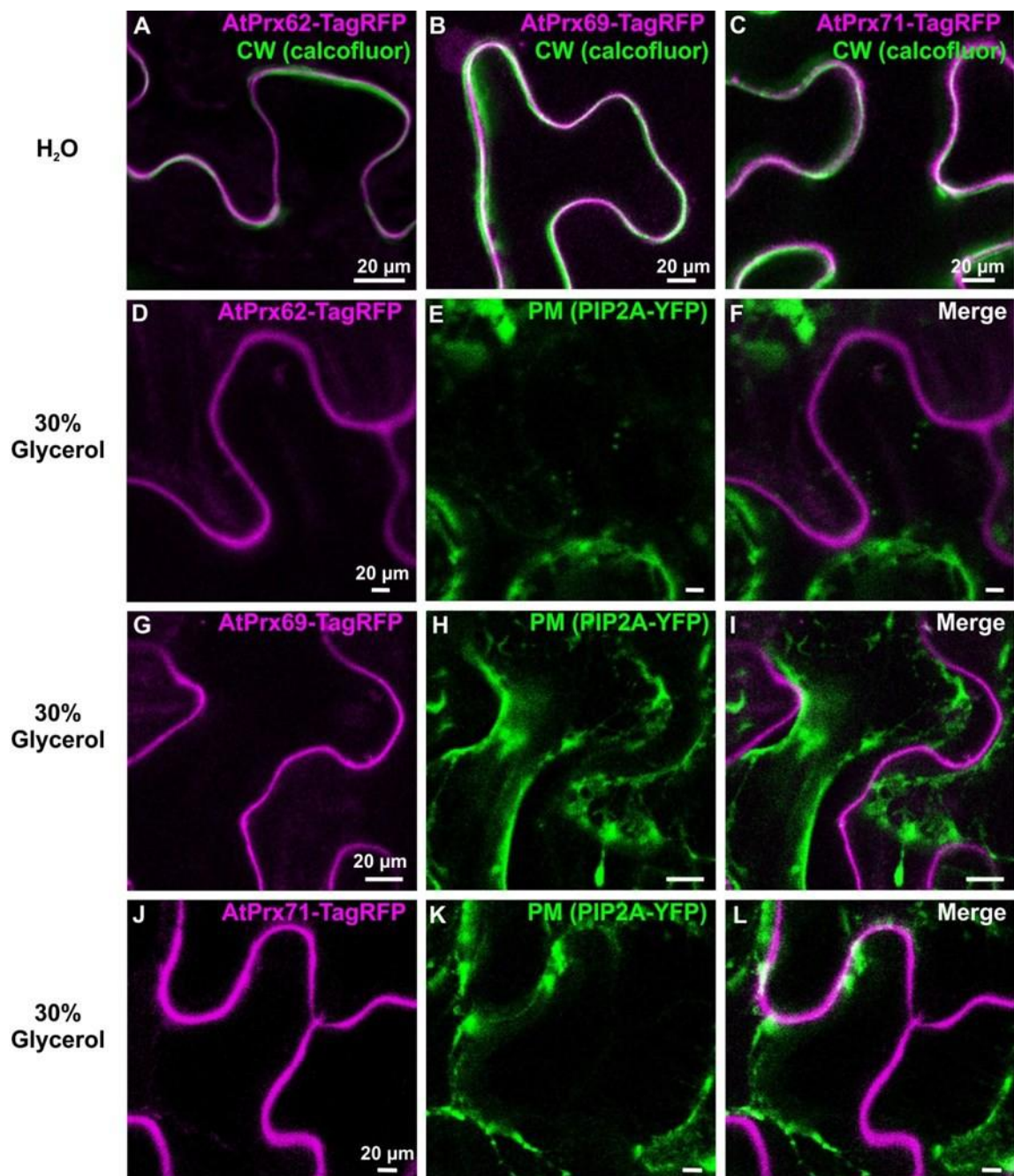


Fig. 5

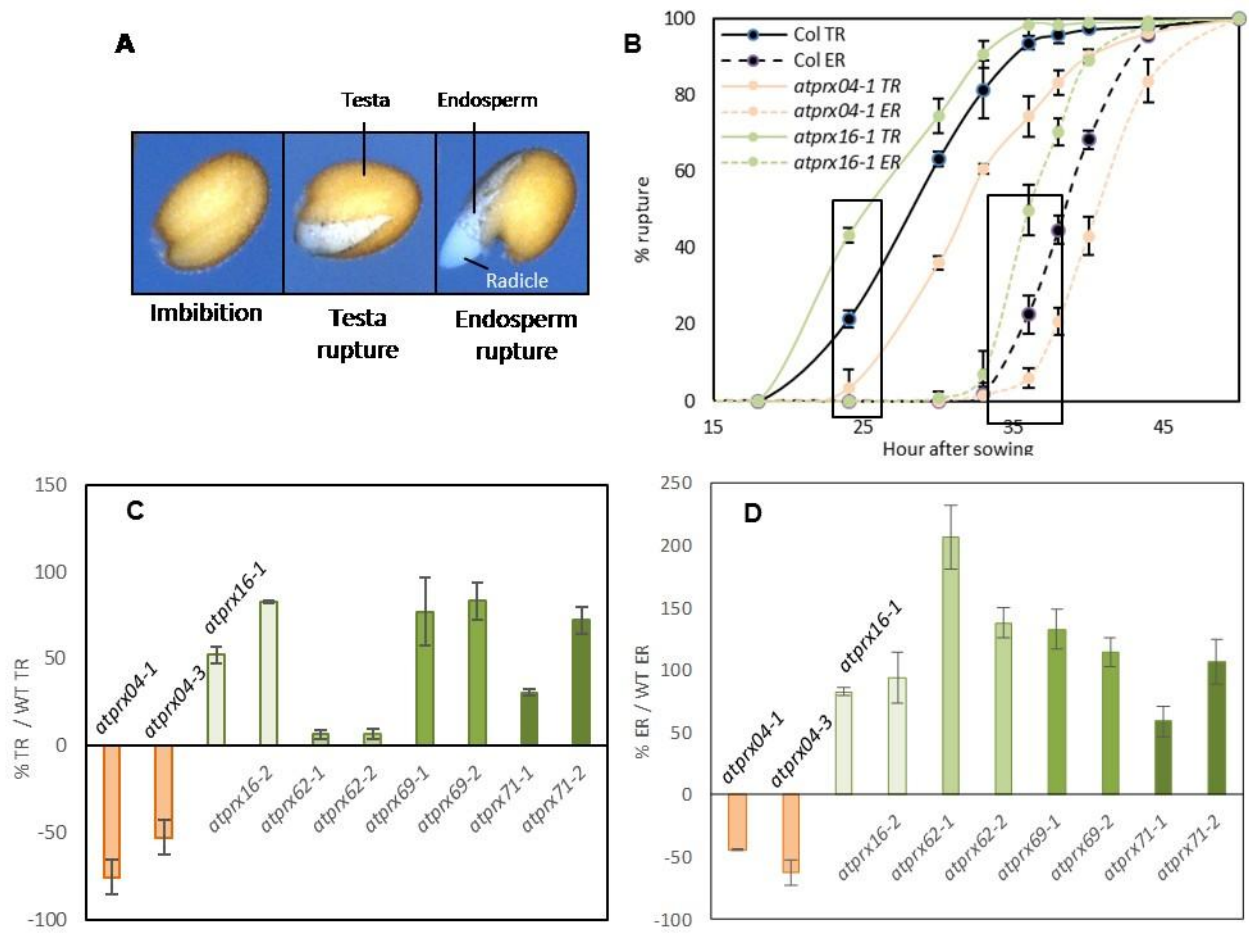


Fig. 6

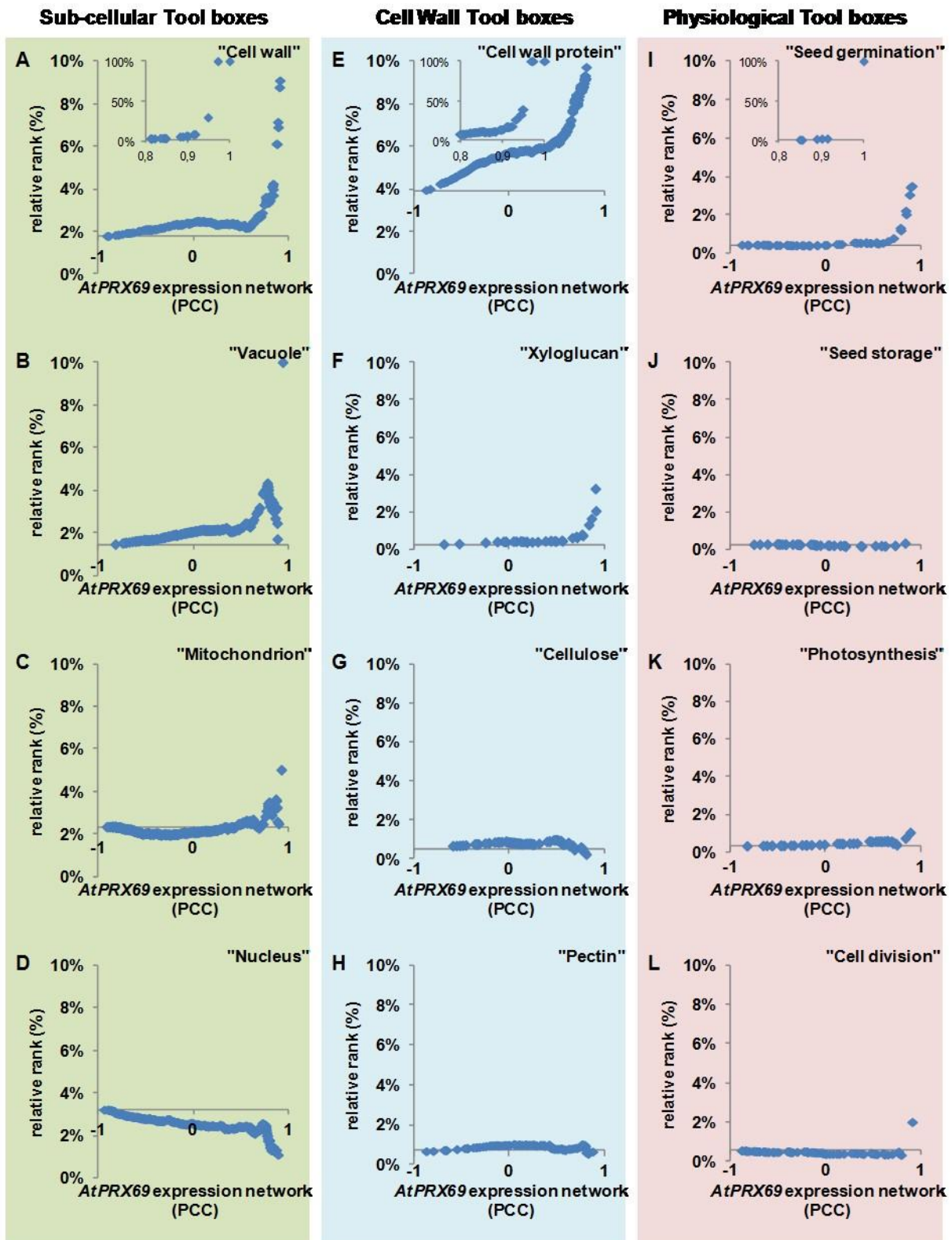
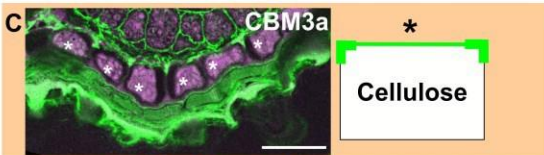
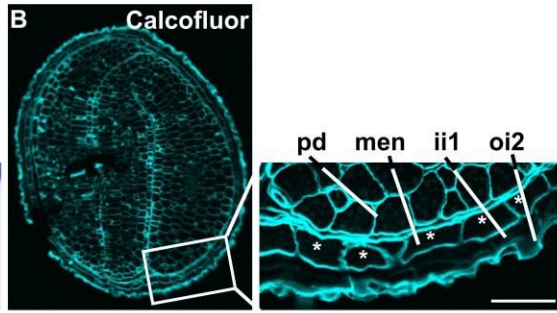
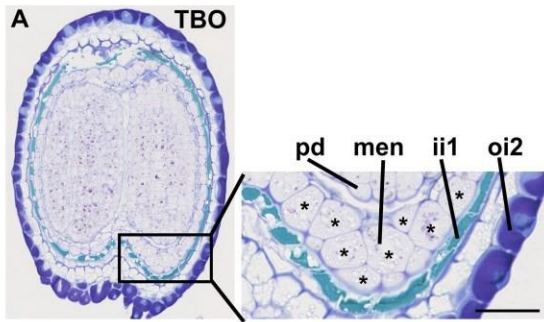
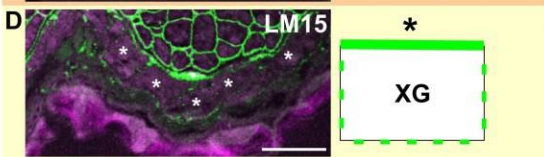


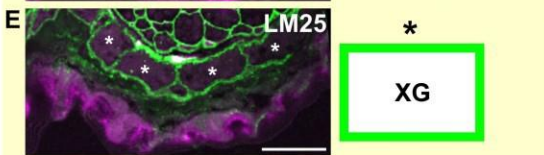
Fig. 7



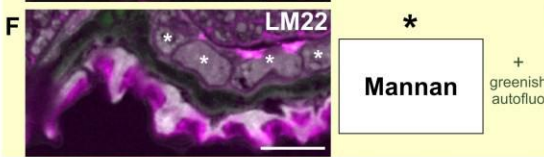
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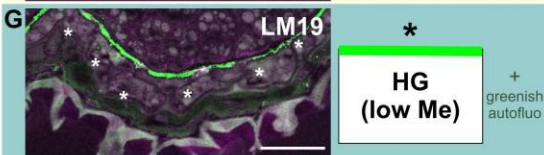
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XG



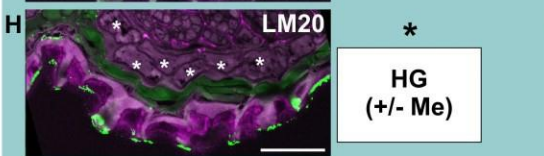
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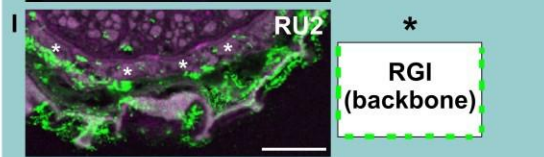
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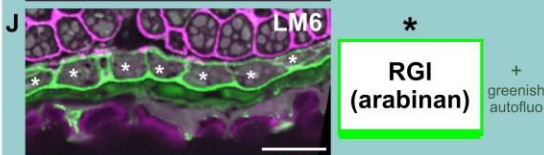
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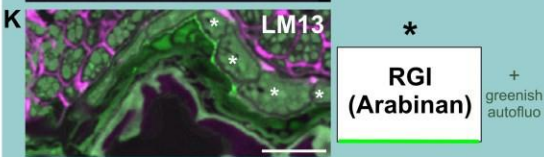
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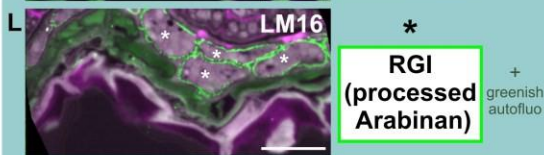
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RGI (backbone)



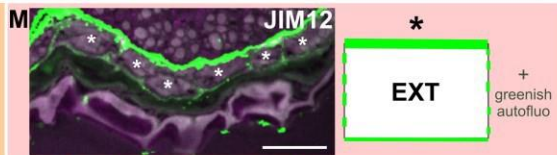
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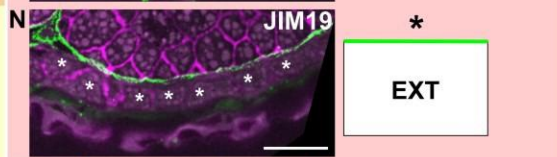
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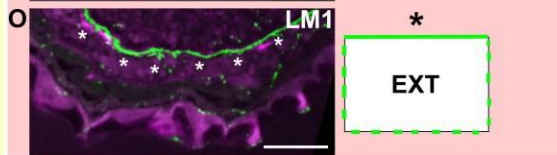
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RGI (processed Arabinan) + greenish autofluo



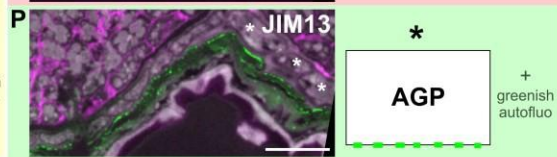
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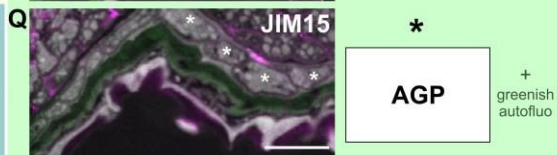
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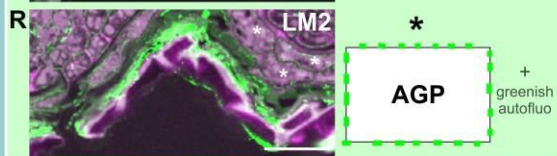
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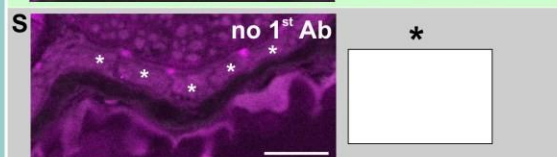
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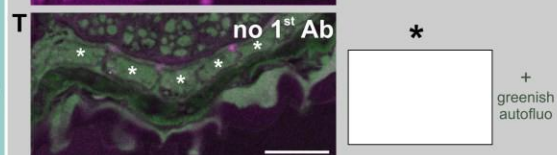
*
AGP + greenish autofluo



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AGP + greenish autofluo



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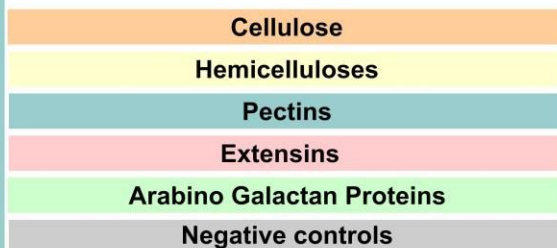


Fig. 8

A

Functional redundancy	AGI number	Annotation	1h	3h	7h	12h	16h	20h	25h noT R	25h TR	31h	38h noE R	38h ER	Germination phenotype	Deducted ME CW remodeling role
Seed MCE															
Non Redundant	AT4g28320	<i>AtMAN5</i>	5.0	5.6	4.6	3.9	3.8	3.6	3.7	3.6	3.6	3.5	3.0	Delayed	Loosening
Non Redundant	AT5g01930	<i>AtMAN6</i>	5.7	6.8	5.7	4.5	4.3	4.1	4.0	4.0	3.9	3.9	4.0	Delayed	Loosening
Non Redundant	AT5g66460	<i>AtMAN7</i>	6.3	5.8	6.5	10.3	11.4	11.6	11.6	10.9	9.3	6.9	5.4	Delayed	Loosening
Non Redundant	AT5g39580	<i>AtPrx62</i>	3.6	6.0	8.6	6.5	10.3	8.8	9.3	12.6	13.0	12.3	12.7	Accelerated	Stiffening
Non Redundant	AT5g64100	<i>AtPrx69</i>	3.8	3.6	3.5	3.9	8.9	9.2	9.3	12.1	13.1	13.4	13.5	Accelerated	Stiffening
Non Redundant	AT2g18980	<i>AtPrx16</i>	3.7	3.9	3.7	3.8	3.8	4.3	5.8	9.0	11.3	11.2	10.9	Accelerated	Stiffening
Non Redundant	AT5g64120	<i>AtPrx71</i>	3.4	3.3	3.3	3.4	4.7	4.6	4.5	8.6	8.8	10.4	11.8	Accelerated	Stiffening
Non Redundant	AT1g14540	<i>AtPrx04</i>	3.1	2.8	3.0	3.1	3.4	3.3	4.3	6.6	5.9	9.2	10.4	Delayed	Loosening
Redundant	AT5g01550	<i>AtLecRKA4.2</i>	3.8	3.6	3.5	3.5	3.5	3.3	3.4	3.6	3.8	5.2	6.8	Delayed	Loosening
	AT5g01560	<i>AtLecRKA4.3</i>	3.1	3.3	3.1	3.2	3.1	3.1	3.1	3.1	3.1	4.2	5.3		
	AT5g01540	<i>AtLecRKA4.1</i>	3.2	3.3	3.4	3.5	3.4	3.5	3.5	3.8	3.7	4.9	6.1		
Redundant	AT3g62720	<i>AtXXT1</i>	6.9	8.5	7.8	7.9	9.8	8.8	8.9	9.9	9.0	8.9	8.8	Delayed	Loosening
	AT4g02500	<i>AtXXT2</i>	8.5	8.2	7.5	6.5	6.7	7.6	7.9	8.4	8.1	7.4	7.3		

B

