

1 NADP-dependent malic enzyme 1 participates in the abscisic acid response in *Arabidopsis*
2 *thaliana*

3 Running title: NADP-ME1 participates in the abscisic acid response

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25 **Abstract**

26 *Arabidopsis thaliana* possesses three cytosolic (NADP-ME1-3) and one plastidic
27 (NADP-ME4) NADP-dependent malic enzymes. NADP-ME2 and -ME4 show constitutive
28 expression, in contrast to NADP-ME1 and -ME3, which are restricted to particular tissues.
29 Here, we show that NADP-ME1 transcript and protein were almost undetectable during
30 normal vegetative growth, but gradually increased and reached levels higher than those of
31 the other isoforms in the latest stages of seed development. Accordingly, in knockout *nadp-*
32 *me1* mature seeds the total NADP-ME activity was significantly lower than in wild type
33 mature seeds. The phenotypic analysis of *nadp-me1* plants indicated alterations of seed
34 viability and germination. Besides, the treatment with abscisic acid (ABA), NaCl and
35 mannitol specifically induced the accumulation of NADP-ME1 in seedlings. In line with
36 this, *nadp-me1* plants show a weaker response of primary and lateral root length and
37 stomatal opening to the presence of ABA.

38 The results suggest that NADP-ME1 plays a specialized role, linked to ABA
39 signalling during the seed development as well as in the response to saline and osmotic
40 stress.

41
42 Key words: osmotic stress, phytohormone, plant metabolism, root, seed development, water
43 deficit.

44

45 **Introduction**

46 NADP-dependent malic enzyme (NADP-ME; EC 1.1.1.40) catalyzes the oxidative
47 decarboxylation of malate to generate pyruvate, CO₂ and NADPH. In plants, the NADP-
48 ME family is represented by several members, localized to cytosol and plastids. One of the
49 better-established roles of this enzyme is the participation as malate decarboxylase in C4
50 and CAM photosynthesis (Drincovich *et al.*, 2011; Saigo *et al.*, 2013). Other functions are
51 suggested based on the importance of malate balance for pH regulation, stomatal opening
52 or lipogenesis (Laporte *et al.*, 2002; Hurth *et al.*, 2005; Gerrard Wheeler *et al.*, 2016).
53 *Arabidopsis* possesses three cytosolic (NADP-ME1-3) and one plastidic NADP-ME
54 isoforms (NADP-ME4; AT1G79750) (Gerrard Wheeler *et al.* 2005). NADP-ME2
55 (AT5G11670) is responsible for most of the NADP-ME activity measured in mature organs
56 and has been involved in sugar metabolism in veins (Brown *et al.*, 2010) and in the
57 oxidative burst triggered by hemibiotrophic fungal pathogen infection (Voll *et al.*, 2012).
58 NADP-ME3 (AT5G25880) is only found in trichomes and pollen (Gerrard Wheeler *et al.*
59 2005).

60 Regarding NADP-ME1 (AT2G19900), its expression is very low in seedlings and
61 adult plants, but significantly higher levels were found in maturing seeds and roots (Gerrard
62 Wheeler *et al.*, 2005). This enzyme belongs to a particular phylogenetic group composed by
63 NADP-MEs from different plant species (Gerrard Wheeler *et al.* 2005). In *Zea mays*, a
64 similar NADP-ME that is also specific of embryo roots, has shown similar expression
65 pattern and kinetic characteristics such as lower catalytic efficiency and activation by
66 succinate (Detarsio *et al.*, 2008; Alvarez *et al.*, 2013). Thus, the delimited localization and
67 particular kinetic properties of NADP-ME1 probably reflect a particular role yet unknown.
68 In a recent work based on transcriptome analysis and reverse genetics aimed at identifying
69 differentially expressed genes during the imbibition and after-ripened seeds, *NADP-ME1*
70 was found up-regulated in dormant *Arabidopsis* genotypes (Yazdanpanah *et al.*, 2017). The

71 knock-out mutant *nadp-me1* showed disturbed seed traits compared to Col-0 plants
72 (Yazdanpanah *et al.*, 2017).

73 Here, to disclose the biological role of *A. thaliana* NADP-ME1, we performed a
74 deep analysis of its expression pattern measuring transcript accumulation, enzymatic
75 activity and by using reporter genes. Besides, we evaluated the phenotypic parameters and
76 physiological processes throughout Arabidopsis plant life affected by the absence of
77 NADP-ME1. Overall, these findings indicate that there is a tight link of NADP-ME1 with
78 processes related to abscisic acid (ABA) responses in seeds, roots and leaves.

79

80 **Materials and methods**

81

82 **Plant lines, growing conditions and sampling**

83 *Arabidopsis thaliana* Columbia-0 lines analyzed in this work include homozygous
84 knockout mutants with T-DNA inserted into the genes encoding NADP-ME1 (*nadp-me1*;
85 SALK_036898) and NADP-ME2 (*nadp-me2*; SALK_020607) and a triple mutant *nadp-*
86 *me2x3x4* obtained by crosses (Gerrard Wheeler *et al.*, 2005). The position of the T-DNA
87 insertion into each *NADP-ME* gene was verified by amplifying and sequencing the T-DNA
88 flanking genomic DNA.

89 Arabidopsis transgenic lines were obtained by transforming wild type plants
90 (Columbia-0, WT) with a construct carrying the complete coding sequence of NADP-ME1
91 fused to YFP (yellow fluorescent protein) gene, under the control of the *NADP-ME1*
92 (referred as NADP-ME1::YFP) or the double 35SCaMV promoter. The construction that
93 holds *NADP-ME1* promoter contains the 2,000 bp long sequence upstream transcription +1
94 site and the first intron of the gene. The binary vector ER-yb (Nelson *et al.*, 2007) was
95 used. Inflorescences were incubated with cultures of *Agrobacterium tumefaciens* strain
96 GV3101 using the protocol described in (Clough and Bent, 1998.) The transformed plants
97 were selected with the herbicide BASTA. Four homozygous T3 lines for each construction
98 were analyzed.

99 Seeds were sterilized with 0.5% (v/v) Triton X-100 and 50% (v/v) ethanol for 3
100 min, washed with 95% (v/v) ethanol and dried on filter paper. Seeds were stratified for 72 h
101 at 277 K in the dark to synchronize germination, unless otherwise is stated. Plants were
102 grown in 1X MS plates (Murashige and Skoog, 1962) or in soil in a culture room at days of
103 16 h of light with a flux density of 100 $\mu\text{E m}^{-2} \text{s}^{-1}$ at 296-298 K. Seeds were collected at
104 different stages, including 7, 12 and 18 days after pollination (DAP), mature (28 DAP), and
105 1 and 2 days after imbibition (DAI). Seedlings of 8 days grown in MS plates were
106 transferred to plates supplemented with 100 mM NaCl, 225 mM mannitol or 0.5-10 μM
107 ABA and collected at different times. All samples were frozen in liquid N₂ and stored at
108 193 K.

109

110 **Real time polymerase chain reaction (qPCR) assays**

111 Total RNA was extracted using a method developed for seed samples of
112 Arabidopsis (Oñate-Sánchez and Vicente-Carbajosa, 2008) or a phenol-based one
113 (Chomczynski and Sacchi, 1987) and plant RNA purification columns (PureLink, Amicon)
114 for the rest of plant tissues, and then treated with RQ1DNase (Promega). The quantity and
115 quality were evaluated by spectrophotometric measurements and electrophoresis in agarose
116 gels. cDNAs were synthesized using MMLV (Promega) and random primers
117 (Biodynamics). Relative expression was determined using specific primers (NADP-

118 ME1left: 5'-CAAGGCAATAAAAACCGACTG-3', NADP-ME1right: 5'-
119 CATTTTTGGCTAGTGGAAGCC-3', NADP-ME2left: 5'-
120 ACGATGGCAAACCTACTTG-3', NADP-ME2right: 5'-
121 ATTGGCGTAATGCTCTTCTG-3', NADP-ME3left: 5'-
122 GGCACCAATCAGACTCAGATCT-3', NADP-ME3right: 5'-
123 AGCAAGTCCTTTATTGTAACGT-3', NADP-ME4left: 5'-
124 CTTTCGAACCCAACCTTCTCA-3', NADP-ME4right: 5'-
125 CATTATTAGCCCCGAGTCCAA-3', YFPleft: 5'-ACGTAAACGGCCACAAGTTC-3',
126 YFPright: 5'-AAGTCGTGCTGCTTCATGTG-3') and polyubiquitin 10 gene
127 (AT4G05320; Czechowski *et al.*, 2005) as normalizer. The amplifications were performed
128 on a Stratagene Mx3000P cyler, using the SYBR Green I dye (Invitrogen) as a fluorescent
129 reporter. PCR controls were made to ensure that the RNA samples were free of DNA
130 contamination. The PCR specificity was verified by melting curve and gel electrophoresis
131 analysis of the products. The relative expression was calculated using a modified version of
132 the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001), the efficiencies and the propagation of errors determined
133 according to (Liu and Saint, 2002) and (Hellemans *et al.*, 2007).

134

135 **Microscopy analysis**

136 Detection of YFP was achieved using a YFP filter (excitation, 488 nm; emission,
137 505-550 nm) and a Karl Zeiss Lsm880 or a Nikon Eclipse TE-2000 Model-E2 confocal
138 microscope. Roots were mounted in propidium iodide dye (Invitrogen) and the imaging
139 settings were 488 nm excitation and >585 nm emission.

140

141 **NADP-ME activity measurements in extracts and western blot analysis**

142 Samples were homogenized in mortars according to (Badia *et al.*, 2015) and the
143 extracts were desalted through Sephadex G-50 spin columns. Protein concentration was
144 determined by the BioRad protein assay using total serum protein as standard. NADP-ME
145 activity was assayed at 303 K in a Jasco spectrophotometer following the appearance of
146 NADPH at 340 nm ($\epsilon_{340nm}=6.22 \text{ mM}^{-1} \text{ cm}^{-1}$) using 50 mM MOPS-KOH pH 6.8, 10 mM
147 MgCl_2 , 0.5 mM NADP and 10 mM malate. One unit (U) is defined as the amount of
148 enzyme that catalyzes the formation of 1 μmol of NADPH min^{-1} under the specified
149 conditions.

150 SDS-PAGE was performed in 10% (w/v) polyacrylamide gels according to
151 Laemmli, 1970. Proteins were then electroblotted onto a nitrocellulose membrane.
152 Antibodies against green fluorescent protein (Abcam), which also immunodetect YFP
153 fusion proteins, were used. Bound antibodies were visualized by linking to alkaline
154 phosphatase conjugated goat anti-rabbit IgG according to the instructions of the
155 manufacturer (Sigma). Alkaline phosphatase activity was detected colorimetrically.

156

157 **Determination of phenotypic parameters**

158 The analyzed lines were grown simultaneously with a randomized physical
159 arrangement and frequently rotated. Germination was evaluated by counting the number of
160 seeds with visible radicles, in plate growth assays without previous stratification. Seeds
161 freshly collected or stored at room temperature for 1-11 years were used. For primary and
162 lateral root length measurement, 5-day-old seedlings were transplanted to MS plates
163 without or with 10 μM ABA and monitored for 6 days.

164

165 **Controlled deterioration test**

166 The controlled deterioration test was performed as described previously (Mao and
167 Sun, 2015) with minor modifications. Briefly, freshly harvested seeds were dried in a
168 desiccator containing silica gel and then equilibrated for 3 days at 288 K and 85-90%
169 relative humidity (RH) in a hygostat of KCl. Then, the hygostat was transferred to 313 K
170 which resulted in 80-85% RH. After 1-7 days at high temperature, the seeds were stored at
171 293 K and 33% RH for 3 days in a hygostat of MgCl₂ and dried again in a desiccator with
172 silica gel (6% RH). The RH and temperature were monitored in all steps with a datalogger.
173 The germination was assayed in replicates of 100 seeds in agar plates and recorded after 7
174 days.

175 **Stomatal opening assays**

176 WT and *nadp-me1* plants were grown in soil pots for 2-3 weeks. The stomatal
177 response was evaluated in detached leaves measuring the stomatal aperture after treatment
178 with 30 μM ABA according to (Wohlbach *et al.*, 2008).
179

180 **In silico phylogenetic analysis**

181 Protein sequences were retrieved from Phytozome 9.1 database
182 (www.phytozome.net), using *A. thaliana* NADP-ME2 as query. The evolutionary history
183 was inferred using the Neighbor-Joining method (Saitou and Nei, 1987). The evolutionary
184 distances were computed using the Poisson correction method (Zuckermandl and Pauling,
185 1965). The analysis involved 40 amino acid sequences. All positions containing gaps and
186 missing data were eliminated. There were a total of 488 positions in the final dataset.
187 Evolutionary analyses were conducted in MEGA7 (Kumar *et al.*, 2016). The detection of
188 ABA related elements was performed with a phylogenetic footprinting tool with a set of
189 characterized motifs (Table S1, cis-analyzer, Gismondi unpublished).
190

191 **Statistical analysis**

192 Significance was determined by the ANOVA or Student's t test using the SigmaPlot
193 software. For the case of qualitative variables, such as germination analysis, the data were
194 analyzed using comparisons of proportions through the Z statistic. The sample size is
195 indicated in each figure or table.
196

197 **Results**

198 ***NADP-ME1* increases during seed maturation in Arabidopsis**

199 The expression profile of NADP-ME1 was analyzed along Arabidopsis seed
200 maturation and germination. The transcript level of *NADP-ME1* sharply increases during
201 seed maturation from 7 DAP until complete maturation, reaching a value 136-fold higher at
202 18 DAP (Fig. 1A). By contrast, the transcript levels of *NADP-ME2*, *NADP-ME3* and
203 *NADP-ME4* remain fairly constant along seed maturation (Fig. 1A). In germinating seeds
204 the transcript level of *NADP-ME1* drastically decreases to the low levels detected at 7 DAP
205 (Fig. 1A). A similar pattern of *NADP-ME1* expression was observed using the YFP reporter
206 gene under the control of *NADP-ME1* promoter. In embryos, YFP fluorescence is
207 undetectable up to 9 DAP; increase from 11 to 18 DAP; and then decrease in germinating
208 embryos (Fig. 1D).
209
210

211 NADP-ME activity in Arabidopsis siliques increases from 7 DAP to 18 DAP (Fig.
212 1B), following a similar profile as that observed for *NADP-ME1* transcript level (Fig. 1A).
213 The increase of NADP-ME activity in *nadp-me2x3x4* triple mutant, in which NADP-ME1
214 is the only NADP-ME found, matches the increase of activity found in WT. Thus, the
215 NADP-ME activity profile in Arabidopsis WT may be endorsed to NADP-ME1 increase
216 (Fig. 1B). When comparing NADP-ME activity in mature seeds of WT and *nadp-me*
217 mutant lines, the lack of NADP-ME2 alone or in combination with NADP-ME3 and
218 NADP-ME4 does not affect the total NADP-ME activity. In contrast, a drastic decrease of
219 NADP-ME activity is observed in *nadp-me1* knockout mutant (Fig. 1C). Overall, it is clear
220 that NADP-ME1 is the isoform that contributes the most to NADP-ME activity at mature
221 seed stage (Fig. 1C).

222

223 ***NADP-ME1* is up-regulated by NaCl, mannitol and ABA in Arabidopsis seedlings and** 224 **roots**

225 *NADP-ME1-4* transcript levels were assayed in Arabidopsis rosettes of 8 days (Fig.
226 2A, Supplementary fig. 1A). As previously shown (Gerrard Wheeler et al 2005), the
227 transcripts of *NADP-ME1* and -3 display very low levels in relation to *NADP-ME2* and -4
228 in control conditions (MS, Supplementary fig. 1A). However, when 8-day-old seedlings are
229 treated with 100 mM NaCl; 225 mM mannitol or 10 μ M ABA, a strong induction *NADP-*
230 *ME1* transcript is observed (53, 79 and 55 times, respectively). Neither *NADP-ME2* nor
231 *NADP-ME3* or *NADP-ME4* shows such a significant response as *NADP-ME1* (Fig. 2A,
232 Supplementary fig. 1A). We further analyzed the response of 8-day-old seedlings to
233 different ABA concentrations (0.5, 1, 5 and 10 μ M of ABA) and exposure times. The level
234 of *NADP-ME1* transcript increases 5-6 folds in 0.5, 1 and 5 μ M ABA and 10 folds in 10
235 μ M ABA, reaching a level which is almost twice as high as that of *NADP-ME2*
236 (Supplementary fig. 1B). When the length of the ABA treatment was tested, we found that
237 *NADP-ME1* increases 6 and 12 folds at 6 and 12 h, respectively and *NADP-ME2* levels did
238 not varied significantly (Supplementary fig. 1C).

239 To test if NADP-ME1 protein was affected by the presence of ABA, we used the
240 transgenic *NADP-ME1::YFP* lines to immunodetect the fusion protein. Consistent with the
241 previous observations, a strong NADP-ME1 induction by ABA at the level of transcript
242 and protein was observed in the lines expressing *NADP-ME1::YFP* under the control of
243 *NADP-ME1* promoter (Fig. 2 B and C).

244 To further analyze the expression pattern of NADP-ME1 under water stress and
245 ABA treatments we observed the YFP fluorescence in roots of 8-day-old seedlings of the
246 transgenic lines expressing *NADP-ME1::YFP* under the control of *NADP-ME1* promoter.
247 ABA, NaCl and mannitol produced similar induction of the NADP-ME1, especially in the
248 root apical meristem, the differentiation zone and in lateral roots (Fig. 2D).

249

250 **Seeds of *nadp-me1* mutant are less sensitive to the ABA repression of germination and** 251 **loss viability more rapidly than WT**

252 The number of *nadp-me1* and WT germinated seeds with visible radicles was
253 counted at different times after seeding. Almost 100% of *nadp-me1* seeds germinate
254 approximately 50 hours after seeding in MS medium but WT seeds reach that value almost
255 48 hours later (Fig. 3A). In the presence of exogenous ABA, the percentages of germinated
256 seeds are lower for both lines but *nadp-me1* seeds reach 2.5-3 folds higher germination
257 percentages than WT at 62 and 86 hours (Fig. 3B). Then, these results show that under both

258 conditions the germination rate of *nadp-me1* is faster than WT and a lower sensitivity of
259 *nadp-me1* seeds to ABA repression of germination.

260 Seed viability was also analyzed for the *nadp-me1* mutant. The viability of recently
261 harvested *nadp-me1* and WT seeds is 100%. However, when we tested seeds stored for
262 long times, we found that *nadp-me1* seeds loss viability earlier than WT (Fig. 3C).

263 To examine whether the longevity is affected in seeds lacking NADP-ME1, we
264 performed a controlled deterioration test based on the exposure of seeds to high
265 temperature (40°C) and relative humidity (80-85%) for several days. Although the viability
266 is the same as WT without treatment and after complete treatment (7 days), *nadp-me1* seed
267 decay is faster than WT (Fig. 3D).

268

269 **Stomata and roots in knockout *nadp-me1* are less sensitive to ABA**

270 Accordingly to the very low levels of *NADP-ME1* expression observed in leaves,
271 *nadp-me1* mutant plants do not show differences in stomatal aperture compared to WT
272 under normal conditions. However, in the presence of 30 μ M ABA the stomata pore size is
273 larger in plants lacking NADP-ME1 than in WT (Fig. 4A).

274 Root growth responses to ABA, mannitol and NaCl are also different in *nadp-me1*
275 and WT. When 5-day-old seedlings are transferred to MS medium supplemented with 10
276 μ M ABA, increased primary root (PR) elongation rate and total length of lateral roots (LR)
277 are found in *nadp-me1* mutants with respect to WT (Fig. 3B and C). When the seedlings are
278 transferred to MS supplemented with 100 mM NaCl, the rate of growth of the PR is not
279 significantly different for *nadp-me1* mutants compared to WT, but the average total length
280 of LR is higher for the mutant plants (Fig. 3B and C). On contrary, mannitol treatment
281 inhibits the PR growth to a lesser extent in *nadp-me1* plants compared to WT and do not
282 show a significant effect on the total length of LR (Fig. 3B and C).

283

284 **Discussion**

285

286 **NADP-ME1 expression is up-regulated by ABA and its absence weakens the ABA** 287 **response in different Arabidopsis organs**

288

289 Despite *A. thaliana* having three cytosolic NADP-ME isoforms, NADP-ME1
290 displays a distinctive and specific expression pattern. Our results indicate that under normal
291 conditions, *NADP-ME1* transcript is detected almost exclusively in maturing seeds (Fig. 1),
292 while it accumulates in the rosettes and roots under saline or osmotic stresses (Fig. 2).
293 Besides, we found that ABA increases *NADP-ME1* transcript, which is correlated with an
294 increase in NADP-ME1 protein (Fig. 2A B and C). This up-regulation is exclusively
295 exerted over *NADP-ME1*, since *NADP-ME2*, *NADP-ME 3* and *NADP-ME 4* do not show
296 this response in the conditions assayed here.

297 Considering that ABA mediates part of the response to saline and osmotic stress and
298 the regulation of NADP-ME1 expression by ABA treatment, the sharp increase in the
299 expression of *NADP-ME1* in maturing seeds could be caused by the accumulation of ABA
300 (Fig. 1A). Thus, it seems that this phytohormone acts as one of the major signals
301 controlling *NADP-ME1* expression.

302 Besides the control of NADP-ME1 expression exerted by ABA, we show that the
303 absence of NADP-ME1 affects ABA response in different Arabidopsis organs. Particularly,
304 we found that the lack of NADP-ME1 affects not only the longevity of the seeds but also

305 the control of the germination (Fig. 3). *nadp-me1* mutants exhibit less tolerance to
306 prolonged storage and are less sensitive to the inhibition of the germination exerted by
307 ABA (Fig. 3). Plants lacking NADP-ME1 also exhibit less sensitivity than the WT to
308 stomatal closure and root growth inhibition induced by ABA, NaCl and mannitol (Fig. 4A
309 and B).

310 It is well-known that ABA regulates vital processes associated to normal late seed
311 development such as synthesis of reserve compounds, tolerance to desiccation, dormancy,
312 longevity, and germination (North *et al.*, 2010). In addition, this phytohormone
313 accumulates in large quantities during saline, osmotic or drought stress conditions and
314 controls root growth and transpiration through stomatal closure (Priest *et al.*, 2006;
315 Christmann *et al.*, 2007). ABA target genes belong to functional categories such as seed
316 maturation (oleosins, dehydrins, or late embryogenesis abundant proteins), protein stability
317 (proteases), cellular structure (expansins and wall synthesis enzymes), signalling (kinases
318 and phosphatases), response to stress (heat shock proteins) and metabolism (Reeves *et al.*,
319 2011). Here, we found that there is a link between NADP-ME1 expression and ABA in
320 *Arabidopsis thaliana*. Moreover, our results suggest that NADP-ME1 would be important
321 to cope with conditions of water deficit, where the plant responses are mainly mediated by
322 ABA.

323

324 **NADP-ME1: an old and conserved NADP-ME with particular physiological roles** 325 **linked to ABA response**

326 The sequence analysis of the complete set of NADP-ME isoforms from the dicot
327 species *A. thaliana*, *Glycine max*, *Medicago truncatula*, *Phaseolus vulgaris*, *Ricinus*
328 *communis* and the monocot species *Oryza sativa*, *Setaria italica* and *Zea mays* showed that
329 monocot cyt2, cyt3 and plastidic isoforms belong to a monophyletic cluster independent
330 from dicot cyt2 and plastidic isoforms that also cluster together (Fig. 5A). On the other
331 hand, monocot cyt1 and dicot cyt1 groups show origins different from the rest of the family
332 members and are more similar to the ancestral NADP-ME present in the origin of
333 angiosperms (Fig. 5A). In different studies it has been shown that many of these *NADP-ME*
334 cyt1 genes have expression patterns similar to *NADP-ME1*. In *G. max* and *R. communis* the
335 transcripts encoding the isoforms homologous to NADP-ME1 also showed an induction
336 along the seed maturation (Gerrard Wheeler *et al.*, 2016). The study of the *Z. mays* NADP-
337 ME family showed that one cytosolic isoform also accumulated along the grain maturation
338 and increased after ABA treatment in roots (Detarsio *et al.*, 2008; Alvarez *et al.*, 2013).
339 Furthermore, Chi and collaborators (2004) observed that a cytosolic isoform of *O. sativa* is
340 specifically induced by mannitol and NaCl and confers salt tolerance in transgenic over-
341 expressing Arabidopsis (Chen and Long, 2007). These results suggest a conserved role for
342 cyt1 NADP-ME proteins both in monocot and dicot species.

343 *In silico* analysis of the promoter and 5'UTR region of monocot cyt1 and dicot cyt1
344 genes showed that they conserve elements that are linked to the ABA response (Fig. 5B,
345 Tables S1 and S2). In this sense, we observed an increment of the *NADP-ME1* transcript
346 level in response to dose and time of ABA treatment (Supplementary Fig. 1B and C). It is
347 also important to mention that these regulatory elements are also present in the bryophyte
348 *Physcomitrella patens*, suggesting an ancestral and strongly conserved regulation of *NADP-*
349 *ME* genes (Fig. 5 and Table S3). In spite of the evolutionary distance of this bryophyte with
350 respect to angiosperms, ABA is also involved in tolerance to stress by balancing the water
351 level in its tissues (Takezawa *et al.*, 2011). This could indicate that the participation of

352 NADP-ME in the response to ABA existed in plants even before the appearance of stomata,
353 vascular system and seeds.

354 Based on the phylogenetic and promoter analysis and in the phenotypic analysis
355 carried out here, we propose that NADP-ME1 is an isoform that plays a very specialized
356 role compared to the rest of the NADP-ME family members, possibly linked to the
357 signaling initiated by ABA, as an intermediary or final effector (Fig. 6). This enzyme could
358 fulfill its role by consuming malate and/or generating pyruvate and NADPH in the cytosol.
359 Malate is a metabolic intermediate that is being recognized for its regulatory functions
360 (Finkemeyer *et al.*, 2013). For example, the decrease of ion entrance through a vacuolar
361 channel that promotes the stomata closure is regulated by the decrease of the cytosolic
362 malate concentration (De Angeli *et al.*, 2013). Besides, the growth and the response of the
363 roots to the availability of water also depend on the hydric potential generated by the
364 movement of osmolytes through cellular compartments (Robbins and Dinneny, 2015). On
365 the other hand, the NADPH generated could be used for the biosynthesis of compounds that
366 accompany the stress response or to control reactive oxygen species generation. Although
367 future experiments are necessary to elucidate the mechanism by which NADP-ME1
368 participates in the ABA signaling pathway, the results presented here clearly show the
369 participation of this particular isoenzyme from the NADP-ME family in the response to a
370 hormone which is a key in different physiological responses in plants. NADP-ME1 could
371 work as an intermediate or end effector, possibly regulating the concentration of the
372 reaction substrates and/or products to feed metabolic pathways and regulatory functions
373 triggered by the water stress (Fig. 6). The findings shown here contribute to the
374 understanding of the maturation and germination seed processes and the response of plants
375 to water stress, and may help us to establish innovative strategies to generate crops with a
376 better use of natural resources.

377

378 Abbreviations: ABA, abscisic acid; DAI, days after imbibition; DAP, days after
379 pollination; LR, lateral roots; ME, malic enzyme; MS, Murashige and Skoog medium; PR,
380 primary root; qPCR, real time polymerase chain reaction assay; RH, relative humidity; U,
381 unit; WT, wild type; YFP, yellow fluorescent protein.

382

383

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391

392 **Author Contributions Statement**

393

394 CLA, TP, GT, MBB, MG, MCGW and MS performed the experiments. VGM contributed
395 to the plant lines. CLA, CSA, MFD, MCGW and MS planned the experiments, analyzed
396 the data, and wrote the paper.

397

398 **Conflict of Interest Statement**

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400
401
402

The authors certify that they have no conflict of interest with any organization or entity.

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Figure legends

Fig. 1: NADP-ME1 expression in Arabidopsis seeds. (A) Relative level of the transcripts of *NADP-ME* genes in seeds collected at 7, 12 and 18 days after pollination (DAP), mature stage (M), and at 1 and 2 days after imbibition (DAI). The polyubiquitin 10 (UBQ) gene was used as reference. NADP-ME activity was assayed in siliques throughout maturation (B) and in mature seeds (C) for WT and simple or triple mutant lines. The values are the average of at least two independent experiments \pm SD. Values marked with an asterisk (*) indicate that are significant different ($p < 0.05$). (D) Expression of the fusion protein NADP-ME1::YFP during seed development and germination. The reporter contains the complete coding sequence of NADP-ME1 fused to YFP gene, under the control of the *NADP-ME1* promoter. YFP fluorescence in representative embryos at 7; 9; 11; 12 and 18 DAP and at 1, 1-2 and 2 DAI are shown. Scale bars are indicated in each panel.

Fig. 2: NADP-ME1 expression in response to NaCl, mannitol and ABA. (A) Relative levels of the transcripts of *NADP-ME* genes in rosettes in control conditions (MS) or after 6 hs treatments of seedling with 100 mM NaCl, 225 mM mannitol or 10 μ M ABA. The polyubiquitin 10 gene (UBQ) was used as reference. The values are the average of at least two independent experiments \pm SD. (B) Response of *NADP-ME1* promoter in transgenic lines. ME1-YFP3 and 4 are two independent transgenic lines expressing NADP-ME1::YFP under the control of *NADP-ME1* promoter. YFP denotes a line expressing the YFP coding sequence under the control of the double 35SCaMV promoter. The level of *NADP-ME1* was compared in control conditions (MS) and after treatment with 10 μ M ABA during 6 h was applied. (C) Western blot of the seedling protein extract (30 μ g) from the transgenic lines. Molecular mass markers were run in parallel and stained with Coomassie Blue to localize the position of fusion and YFP proteins. (D) Expression of the fusion protein NADP-ME1::YFP in roots in response to ABA, NaCl and mannitol. YFP fluorescence in different parts of roots of 8-day-old seedlings incubated with 10 μ M ABA, 100 mM NaCl or 225 mM mannitol for 6 h. Scale bars are indicated in each panel. Right panels show the fluorescence distribution of the control YFP line.

Fig. 3: Germination and seed viability assay in *nadp-me1* mutant. Germination was evaluated by counting the number of seeds with visible radicles in MS plates (A) or MS supplemented with 10 μ M ABA (B). The proportion of germinated seeds after evaluating 300 seeds per line is shown. Germination was also evaluated using long time stored seeds (C) or after exposing them to high temperature (313 K) for 1-7 days (D). The asterisk denotes significant differences ($p < 0.05$) between WT and *nadp-me1* lines.

Fig. 4: Stomatal opening and root length assays in *nadp-me1* mutant. (A) Stomata pore size was determined in the light and in the presence or absence of 30 μ M ABA. Between 70-100 stomata aperture were measured. Primary (PR, B) and lateral (LR, C) root length were assayed in seedling transferred to MS plates supplemented with 10 μ M ABA, 100 mM NaCl or 225 mM mannitol for 6 days. All values are presented and the statistical descriptions of each set of data are shown as box plots. The asterisk denotes significant differences ($p < 0.05$) between WT and *nadp-me1* line.

Fig. 5: ABA and drought response elements in the promoters of *NADP-ME1* and related genes. (A) The evolutionary history of the complete set of NADP-ME isoforms from the dicot species *Arabidopsis* (At), soybean (*Glyma*), *Medicago truncatula* (Medtr), *Phaseolus vulgaris* (Phvul), *Ricinus communis* (Rco) and the monocot species rice (Os), *Setaria italica* (Si) and maize (GRMZM) was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length=2.44632768 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances are in the units of the number of amino acid substitutions per site. (B) The occurrence of ABA responsive elements was analyzed in the promoters of monocot cyt 1 and dicot cyt1 groups.

Fig. 6: Schematic scheme showing the participation of NADP-ME1 in ABA response in *Arabidopsis*. ABA induces NADP-ME1 expression in particular organs and cell types producing a modification of malate, pyruvate and NADPH levels, which impact on seed longevity and germination, stomata opening and root architecture.

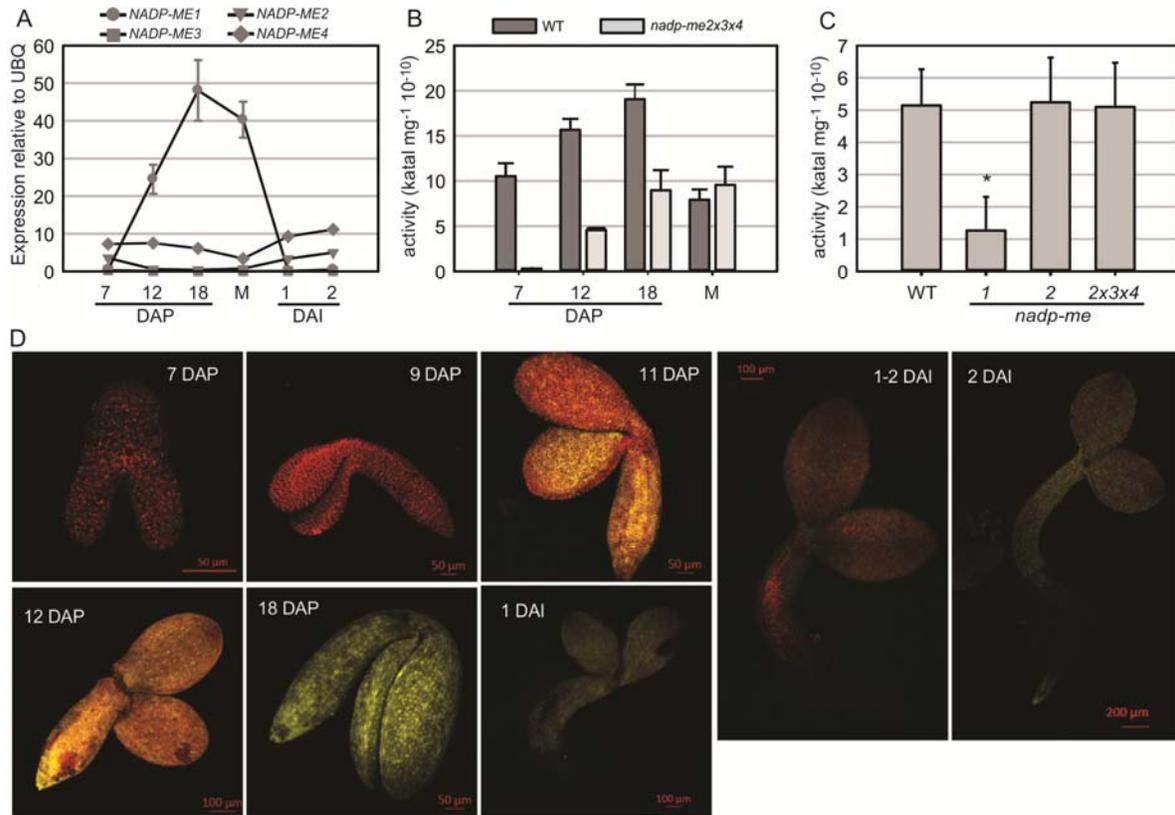
Supplementary Figure 1: NADP-ME1 expression in response to NaCl, mannitol and ABA. (A) Relative levels of the transcripts of NADP-ME genes in rosettes in control conditions (MS) or after 6 hs treatments of seedling with 100 mM NaCl, 225 mM mannitol or 10 μ M ABA. The expression levels of NADP-ME genes are normalized to expression of the reference gene polyubiquitin 10 (UBQ). The values are the average of at least two independent experiments \pm SD. In (B) and (C) the levels of *NADP-ME1* and *NADP-ME2* transcripts after 12 h of ABA treatment or 10 μ M ABA, respectively, are shown.

Table S1: Motifs selected for the analysis of occurrence in promoters and 5'UTR.

Table S2: Location of motifs in the promoters and 5'UTR of the genes selected.

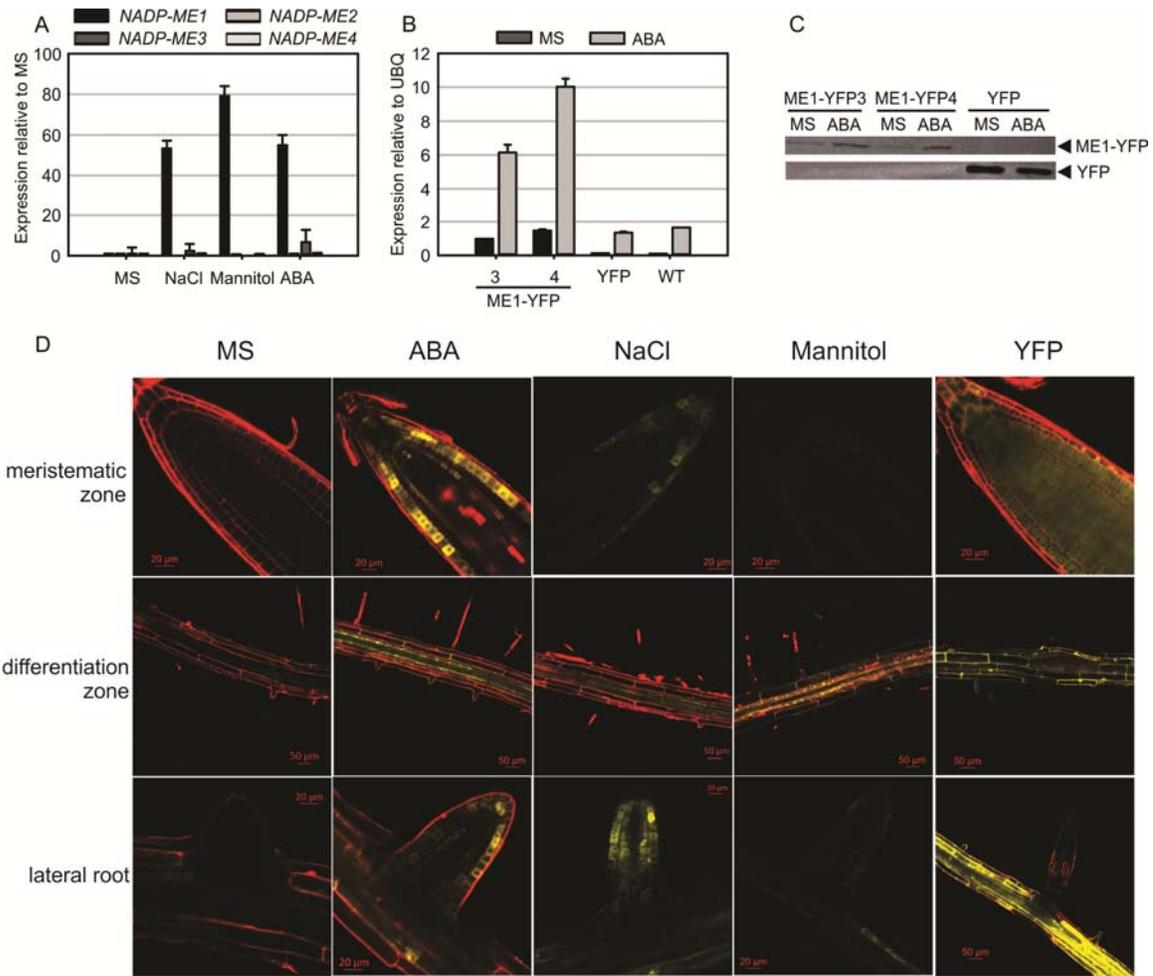
Table S3: Location of motifs in the promoters and 5'UTR of NADP-ME genes from *P. patens*.

Figure 1



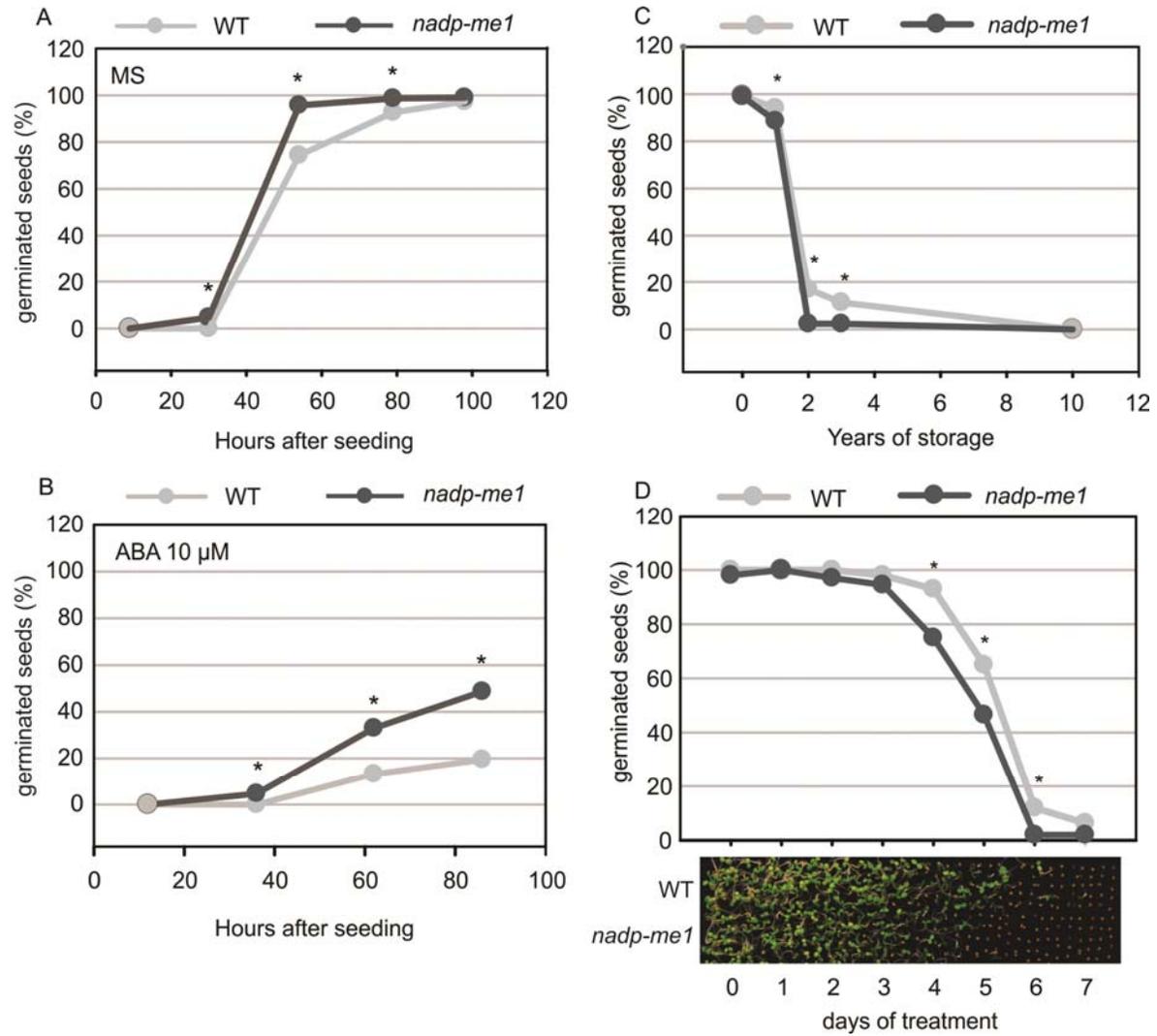
403

Figure 2



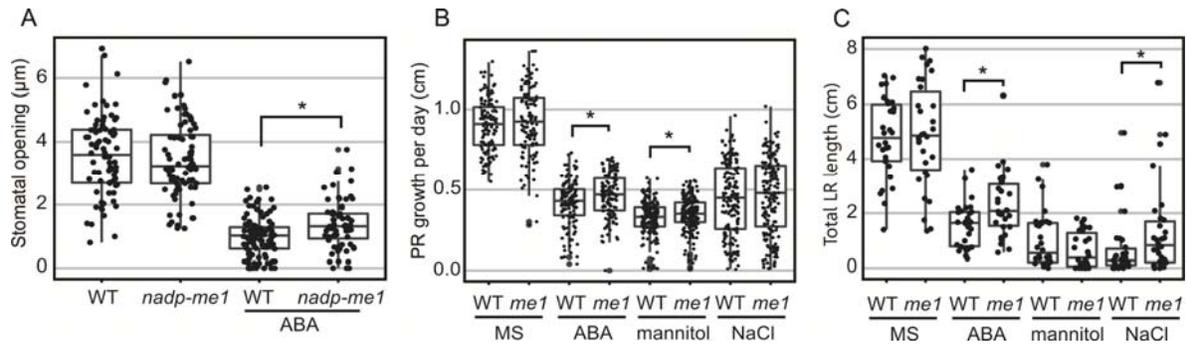
404

Figure 3



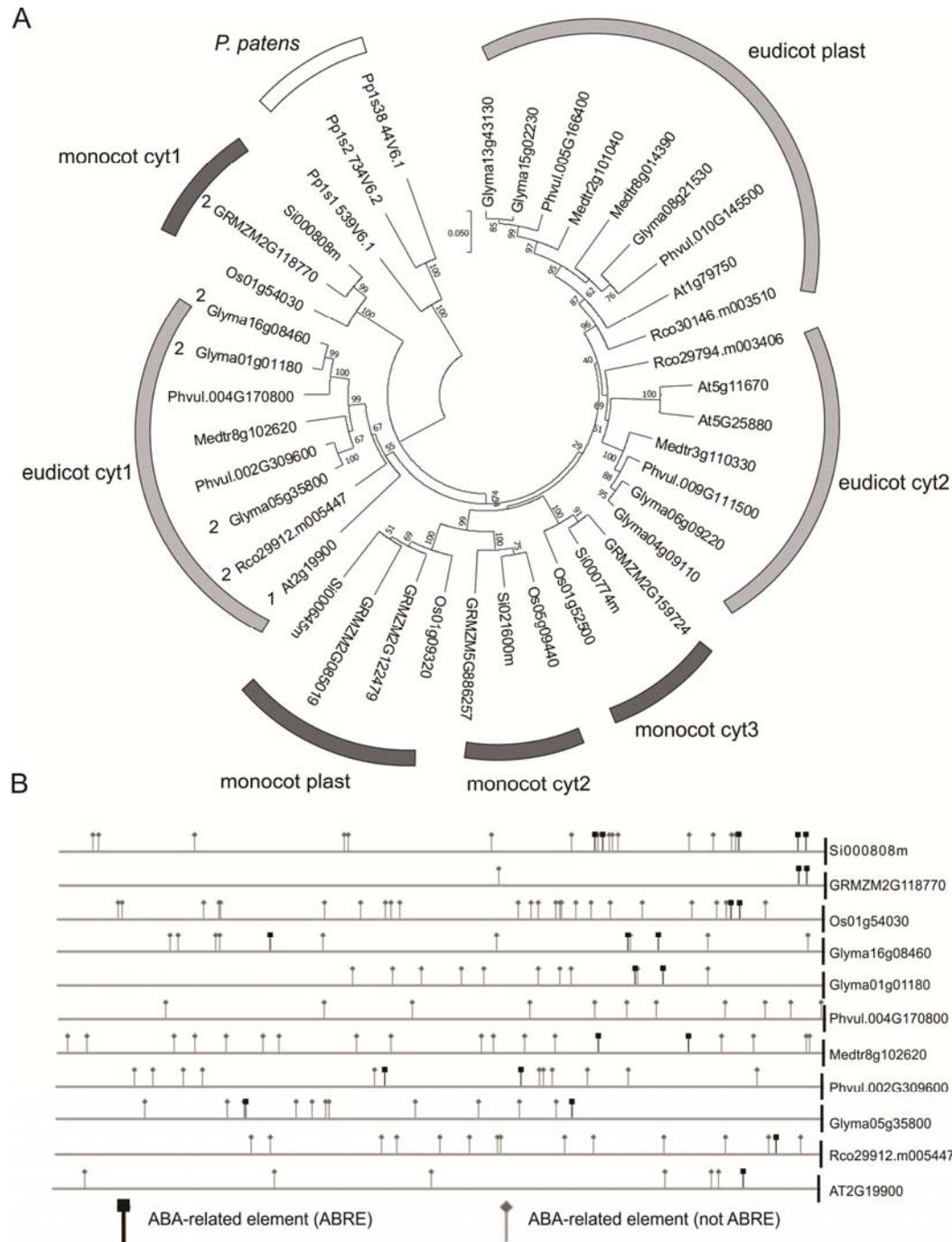
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Figure 4



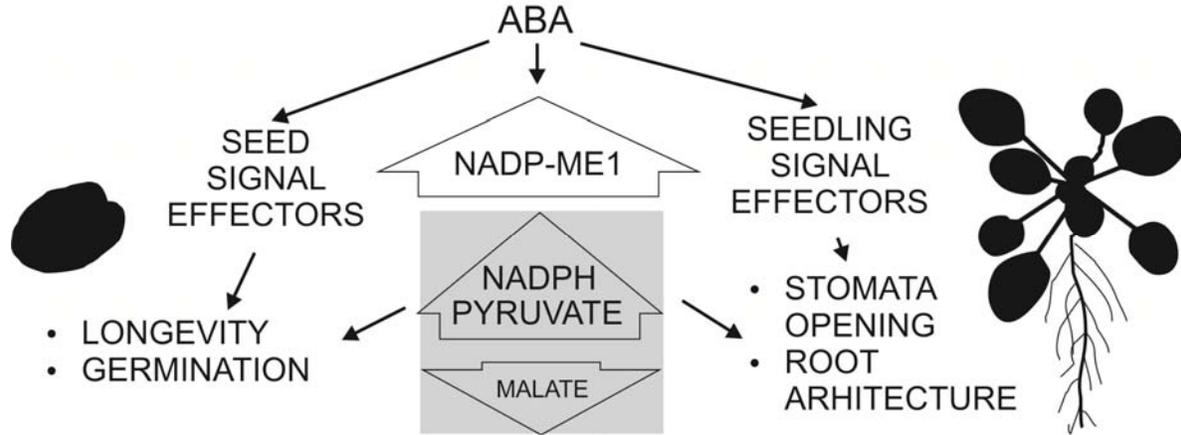
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Figure 5



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Figure 6



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Supplementary figure 1

