Short Title: Regulation of ABI4 activity by phosphorylation

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Title: Phosphorylation of Serine114 of the transcription factor ABSCISIC ACID INSENSITIVE 4 is essential for activity

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One sentence summary:

Stress and abscisic acid repressed growth and gene expression requires phosphorylation of a single amino acid in the transcription factor ABSCISIC ACID INSENSITIVE 4.

List of author contributions:

N.E. performed most of the experiments and participated in writing the draft of the manuscript; T.M. performed some experiments; E.C.S. cloned, expressed and purified the recombinant MAPKs; Da.B.Z. and S.B. analyzed the RNA seq data and participate in writing the manuscript; R.F. contributed to experiment planning, data analysis and writing the manuscript; Du.B.Z. conceived the project idea, designed and analyzed the experiments, wrote part of the manuscript.

Funding information:

This work was supported by a grant No. 2011097 from the US Israel Binational Science Foundation (BSF) (to R.F. and Du.B.Z.)

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ABSTRACT

The transcription factor ABA-INSENSITIVE(ABI)4 has diverse roles in regulating plant growth, including inhibiting germination and reserve mobilization in response to ABA and high salinity, inhibiting seedling growth in response to high sugars, inhibiting lateral root growth, and repressing light-induced gene expression. ABI4 activity is regulated at multiple levels, including gene expression, protein stability, and activation by phosphorylation. Although ABI4 can be phosphorylated at multiple residues by MAPKs, we found that S114 is the preferred site of MPK3. To examine the possible biological role of S114 phosphorylation, we transformed *abi4-1* mutant plants with *ABI4pro::ABI4* constructs encoding wild type (114S), phosphorylation-null (S114A) or phosphomimetic (S114E) forms of ABI4. Phosphorylation of S114 is both necessary and sufficient for the response to ABA, glucose, salt stress, and lateral root development, where the *abi4* phenotype could be complemented by expressing ABI4(114S) or ABI4(S114E) but not ABI4(S114A). Comparison of root transcriptomes in ABA-treated roots of *abi4-1* mutant plants transformed with constructs encoding the different phosphorylation-forms of S114 of ABI4 revealed that 85% of the ABI4-regulated genes whose expression pattern could be restored by expressing ABI4(114S) are down-regulated by ABI4. Over half of the ABI4-modulated genes were independent of the phosphorylation state of ABI4; these are enriched for stress responses. Phosphorylation of S114 was required for regulation of 35% of repressed genes, but only 17% of induced genes. The genes whose repression requires the phosphorylation of S114 are mainly involved in embryo and seedling development, growth and differentiation, and regulation of gene expression.

59

60

61 **INTRODUCTION**

62

63 The transcription factor ABA-INSENSITIVE(ABI)4 was initially identified on the basis
64 of ABA-resistant germination in loss of function mutants (Finkelstein, 1994; Finkelstein et al.,
65 1998). Additional mutants were found in screens for resistance to stress responses due to high
66 salinity (Quesada et al., 2000), high sugar concentrations (Arenas-Huertero et al., 2000; Huijser
67 et al., 2000; Laby et al., 2000) or inhibited sugar-induced gene expression (Rook et al., 2001).
68 Further characterization of these mutants revealed roles in lipid reserve mobilization, lateral root
69 growth, regulation of light-regulated gene expression, retrograde signaling from mitochondria,
70 redox signaling, and some pathogen responses (reviewed in Wind et al., 2013). ABI4 has also
71 been implicated in retrograde signaling from plastids (Koussevitzky et al., 2007), but this is
72 controversial (Kacprzak et al., 2019).
73 Although *ABI4* is most strongly expressed in embryos, imbibing seeds and maturing
74 pollen (Soderman et al., 2000; Nakabayashi et al., 2005; Penfield et al., 2006), its diversity of
75 functional roles reflects expression in vegetative tissues. In addition to developmental contexts,
76 *ABI4* promoter activity is regulated by environmental stresses and interactions with signaling via
77 other hormones including cytokinin and auxin (Arroyo et al., 2003; Shkolnik-Inbar and Bar-Zvi,
78 2010; Huang et al., 2017). ABI4 protein accumulation is further regulated by proteasomal
79 degradation, but is stabilized in the dark or by exposure to high salt or sugar concentrations
80 (Finkelstein et al., 2011; Gregorio et al., 2014; Xu et al., 2016).
81 Like many cellular proteins, ABI4 protein activity may also be subject to regulation by
82 post-translational modifications such as phosphorylation. The primary amino acid sequence of
83 ABI4 is highly enriched in the phosphorylatable residues Ser and Thr: 47 and 20 residues,
84 respectively, out of the 328 amino acid residues, corresponding to 20% of the total amino acid
85 residues of ABI4. An *in vitro* phosphorylation study of the Arabidopsis proteome by MAPKs
86 showed that ABI4 could be phosphorylated by 3 out of the 10 tested MAPKs (Popescu et al.,
87 2009). The phosphorylating MAPKs (MPK3, MPK4, and MPK6) regulate numerous signaling
88 pathways, including stress response, in Arabidopsis (Colcombet and Hirt, 2008; de Zelicourt et
89 al., 2016) and their activities were shown to be activated by ABA treatment, suggesting that this

90 could be a mechanism for stress-regulation of ABI4 activity. MAPK signaling may also regulate
91 ABI4 activity at the level of expression via effects on as-yet unidentified transcription factor(s)
92 (Li et al., 2017).
93 Previous studies of ABI4 phosphorylation focused on its role in retrograde signaling
94 controlling light-regulated gene expression (Guo et al., 2016) and inhibiting adventitious root
95 formation (Bai et al., 2020). Guo et al. demonstrated Ca^{2+}-dependent scaffolding of MAPK
96 cascades resulting in activation of MPK3 and MPK6, which in turn activated ABI4 to repress the
97 photosynthesis-associated nuclear gene LHCB. In the present study, we tested the importance of
98 ABI4 phosphorylation by MAPKs in regulating ABA and stress responses in seeds and
99 seedlings.
100
101
102 **RESULTS**
103
104 **Phosphorylation of ABI4 by MAPKs**
105 We first set out to check the interaction between ABI4 and MAPKs in our system. Using
106 bimolecular fluorescence complementation we confirmed interactions between ABI4 and both
107 MPK3 and MPK6 in nuclei of *N. benthamiana* leaves transiently expressing these proteins
108 (Supplemental Figure S1). We then used purified recombinant proteins to confirm the *in vitro*
109 phosphorylation of ABI4 by MPK3, MPK4, and MPK6. We chose to use the basal activity of the
110 protein kinases, rather than the enhanced activity following their phosphorylation by MAPKKs,
111 because the basal activity may phosphorylate sites with higher affinity, that cannot be
112 distinguished from lower affinity sites that may be phosphorylated by the enhanced kinase
113 activities attained by the phosphorylation of a MAPK by a MAPKK. Although our data
114 confirmed the reported finding that ABI4 could be phosphorylated by MPK3, MPK4, and
115 MPK6, these kinases varied in their potency to phosphorylate ABI4: all three kinases showed
116 similar degrees of autophosphorylation, but MPK3 was more effective than MPK4 and MPK6
117 for phosphorylating ABI4 (Figure 1). Thus, we chose to focus on MPK3.
118

119 **ABI4 Phosphorylation sites**

120 MAPKs phosphorylate serine and threonine residues in their substrate proteins (Mishra et
121 al., 2006). MAPKs are well studied in eukaryotes, and thus many of their phosphorylation sites
122 are characterized. Two consensus sequences, P-X-S/T-P and S/T-P were found to be highly
123 abundant in protein substrates of MAPKs (Seger and Krebs, 1995). Searching the primary amino
124 acid sequence of ABI4 for these motifs, we found that ABI4 has one of the 4-amino acid long
125 MAPK phosphorylation consensus motifs, with S114 as the suggested phosphorylated residue,
126 and two shorter 2-amino acid motifs at residues S140 and T111 (Supplemental Figure S3). Since
127 the short T111 motif overlaps with the longer S114 motif, we first tested whether MPK3 could
128 phosphorylate Ser residues 114 and 130 of ABI4 by mutating the respective codons to encode
129 Ala, a residue that cannot be phosphorylated. Single and double mutations were constructed and
130 verified by DNA sequencing. Wild-type and mutated His-tagged recombinant ABI4 proteins
131 were expressed in *E. coli*, purified by affinity chromatography, and assayed for phosphorylation
132 by MPK3. Replacing S114 of ABI4 with Ala greatly diminished MPK3's ability to
133 phosphorylate ABI4, whereas mutating S130 did not affect the extent of the observed
134 phosphorylation of ABI4 by MPK3 (Figure 2). The lack of phosphorylation of the S114A/S130A
135 double mutant of ABI4 by MPK3 also suggested that T111 is not a preferred phosphorylation
136 site of this protein kinase.

137

138 **Construction of transgenic Arabidopsis plants expressing ABI4 harboring mutations in**
139 **codons of S114 and S130**

140 To evaluate if the observed *in vitro* phosphorylation of ABI4 by MPK3 (Figure 2) has
141 any biological significance, we tested the potency of ABI4 proteins mutated in S114 and S130 to
142 complement the phenotype of *abi4* mutants. Since overexpression of ABI4 using a strong
143 promoter severely inhibits growth (Shkolnik-Inbar and Bar-Zvi, 2010; Finkelstein et al., 2011),
144 we chose to use the 2 kb ABI4 promoter that was sufficient for the regulated expression of this
145 gene (Shkolnik-Inbar and Bar-Zvi, 2010). Codons of ABI4 encoding S114 and S130 were
146 mutated to codons encoding Ala, resulting in phosphorylation-null forms of the protein. A
147 phosphomimetic ABI4 mutant was prepared by replacing the respective Ser residues with Glu,
148 which resembles the negatively charged phosphorylated Ser residue. *Arabidopsis thaliana abi4-1*
149 mutant plants were transformed by *Agrobacteria* carrying pCAMBIA plasmids encoding wild-

150 type ABI4(114S/130S) or the mutated proteins ABI4(S114A), ABI4(S130A), ABI4(S114E), and
151 ABI4(S130E). Homozygous T3 plants resulting from the integration of single T-DNAs were
152 selected by germination and growth in the presence of kanamycin. The phenotype of the
153 resulting transgenic plants was indistinguishable from that of wild-type or *abi4* plants when
154 assayed for germination on plates containing solid MS medium with 0.5% sucrose and 0.55%
155 plant agar, or growth in pots (Supplemental Figure S4).
156
157 **Germination in the presence of ABA**
158 A pronounced phenotype of *abi4* mutants is their ability to germinate in the presence of
159 inhibitory concentrations of ABA (Finkelstein, 1994). To test the functional significance of the
160 MAPK phosphorylation sites, seeds of transgenic and control lines were sown on 0.5x MS plates
161 containing 10 µM ABA, and radicle emergence was scored daily for 7 days (Figure 3). All tested
162 genotypes fully germinated with similar kinetics when sown on agar plates containing 0.5x MS
163 medium supplemented with 0.5% sucrose. In agreement with the known phenotype of *abi4,*
164 seeds of the mutant line were less sensitive to ABA inhibition than wild-type plants. For
165 example, 7 days after sowing on plates containing 10 µM ABA, the *abi4* seeds fully germinated
166 whereas only half of the wild type seeds showed radicle emergence. Transformation of *abi4*
167 mutant plants with a construct encoding wild-type ABI4 whose expression was driven by the
168 *ABI4* promoter complemented the germination phenotype (Figure 3, green). Although expression
169 of the ABI4(S114A) phosphorylation-null protein did not complement the germination
170 phenotype, transformants with a construct encoding phosphomimetic ABI4(S114E) yielded
171 seeds with even higher ABA sensitivity than those expressing the wild-type protein. In contrast,
172 the transformation of the *abi4* mutant plants with constructs expressing wild-type, S130A or
173 S130E forms of ABI4 all completely restored the ABA sensitivity. These results clearly suggest
174 that the phosphorylation of S114 but not S130 is necessary and sufficient for the activity of
175 ABI4.
176 Following radicle emergence, we also scored the development of cotyledon expansion
177 and greening 10 days after sowing on media supplemented with ABA, in which 9.8% of the
178 wild-type seeds developed green expanded cotyledons (Figure 3C). In contrast, 81.8 % of the
179 *abi4* seeds developed green expanded cotyledons under these conditions. Cotyledon
180 development in the plants expressing ABI4(S114A) was similar to that of the parental *abi4*

181 plants, whereas those expressing ABI4(114S) and ABI4(S114E) resembled those of the wild-
182 type plants, indicating that these constructs express biologically active forms of the ABI4
183 protein.
184
185 **Seedling sensitivity to high glucose**
186 High glucose and sucrose concentrations are known to inhibit seed germination and
187 seedling development, and multiple *abi4* mutants have been selected as resistant to these sugars
188 (Arenas-Huertero et al., 2000; Huijser et al., 2000; Laby et al., 2000). We thus tested seedling
189 development in the presence of 7% glucose. The plants were grown at 22 ºC with 16 h light and
190 8 h dark conditions. Although no differences were obtained in the seed germination rate, the
191 tested genotypes showed differences in cotyledon development rate at 4 days after sowing.
192 Under these conditions, very few seeds of the wild-type plants had expanded cotyledons (Figure
193 4). In contrast, 89% of the seeds of the *abi4* mutants developed open cotyledons 4 days after
194 plating. The wild-type phenotype could be restored by complementation of the *abi4* mutants with
195 constructs expressing wild-type ABI4(114S) or the phosphomimetic ABI4(S114E) mutant, but
196 not by the phosphorylation-null S114A form of ABI4. Preliminary experiments showed that
197 mutating S130 to Ala or Glu did not affect the protein potency to complement the mutated
198 phenotype, similar to their lack of effects on ABA sensitivity.
199
200 **Response to salt**
201 The salt-tolerant *salobreño*(*sañ*) mutants were selected for germination of Arabidopsis
202 seeds in the presence of 250 mM NaCl (Quesada et al., 2000). One of the isolated mutants, *sañ5,*
203 also termed *abi4-2* resulted from a frameshift mutation in codon 94 of the *ABI4* gene. We thus
204 tested the seeds of *abi4* mutants transformed with constructs expressing the different
205 phosphorylation forms of ABI4 for their potency to germinate in the presence of 0.2 M NaCl.
206 Whereas 92% of the *abi4* seeds germinated 3 days after sowing, only 29% of the wild-type seeds
207 germinated under the same conditions (Figure 5A). Transformation of the *abi4* plants with
208 constructs expressing the wild-type (114S) or phosphomimetic (S114E) form of ABI4 restored
209 sensitivity to salt for inhibition of germination, whereas transformation with the phosphorylation-
210 null S114A form was ineffective, reaching 89% germination at 3 days.

211 We previously showed that *abi4* mutants display higher salt tolerance when exposed to
212 NaCl at the rosette stage (Shkolnik-Inbar et al., 2013). To test the relevance of ABI4
213 phosphorylation for this response, one-month-old pot grown rosettes were irrigated with 0.25 M
214 NaCl and 16 mM $CaCl_2$, and plant survival was scored 17d later. In agreement with earlier
215 studies, *abi4* mutants displayed enhanced salt tolerance compared with wild-type plants (Figure
216 5). This tolerance could be reversed by complementation with wild-type ABI4(114S) and
217 ABI4(S114E) phosphomimetic mutant proteins, but not by a transgene encoding the
218 phosphorylation-null ABI4(S114A) mutant.
219
220 **Lateral root development**
221 The *A. thaliana abi4* mutants produce more lateral roots than wild-type plants, whereas
222 ABI4 expression inhibits lateral root development (De Smet et al., 2003; Shkolnik-Inbar and
223 Bar-Zvi, 2010). Seeds were sown in Petri dishes containing 0.5x MS, 0.5% sucrose and 0.5%
224 plant agar, and plates were incubated for 12 days in a growth room in 12-hour light/dark
225 conditions. The plants were harvested, and the lengths of the primary roots (PR) and first lateral
226 roots (LR) were measured. The lengths of the PR did not differ among genotypes, whereas the
227 length of the LR and the calculated ratios of the LR/PR lengths of the *abi4* mutant, and plants
228 expressing ABI4(S114A) were significantly higher than those of the wild-type, and plants
229 expressing ABI4(114S) and ABI4(S114E) (Figure 6).
230
231 **DNA binding activity**
232 DNA binding potency of the different phosphorylated states of S14 of ABI4 was studied
233 *in vitro* by using Electrophoresis Mobility Shift Assays (EMSAs) with the respective
234 recombinant ABI4 proteins and radiolabeled short DNA fragments containing two sequences
235 from the Arabidopsis *HKT1;1* gene previously shown to be bound by ABI4 (Shkolnik-Inbar et
236 al., 2013). The wild-type ABI4 and the phosphomimetic protein ABI4(S114E) bound the DNA
237 sequences, whereas the phosphorylation-null ABI4(E114A) form did not bind the probes
238 (Supplemental Figure S5).
239

240 **Phosphorylation of Serine 114 of ABI4 is essential for modulating gene expression *in***
241 ***planta*.**
242 ABI4 is known to act as both an activator and a repressor of transcription. To determine
243 whether the phosphorylation state of the S114 residue affects transcriptional control by ABI4, we
244 analyzed the root transcriptomes of 1-week-old seedlings of the 5 genotypes following a 4 hr
245 treatment with 50 μM ABA. Principal component analyses show that wild-type and *abi4*
246 transcriptomes display the greatest difference in this set, with all 3 transgenic lines intermediate
247 with respect to PCA1 (Supplemental Figure S6).
248 We first compared the transcriptomes of ABA-treated roots of wild-type and *abi4*
249 seedlings to identify the complete set of ABI4-regulated genes (see Methods). This analysis
250 identified 823 genes, differentially expressed between the two genotypes, where the expression
251 levels of 592 and 231 genes were higher and lower, respectively, in ABA-treated roots of the
252 *abi4* mutant (Supplemental Figure S7, and Supplemental Table S1). Our results also showed that
253 transformation of *abi4* mutants with a construct expressing wild-type ABI4(114S) protein,
254 partially restored the transcription pattern of the wild-type plants. Nevertheless, as expressing
255 ABI4(114S) in *abi4* mutants was sufficient for complementation of the lateral root development
256 phenotype (Figure 6), as well as ABA and stress responses at germination and seedling growth of
257 the *abi4* mutant (Figures 3-5), we focused our analysis on 420 genes whose expression is
258 significantly different between roots of *abi4* mutants and both wild-type and *abi4* plants
259 expressing wild type ABI4(114S) (Figure 7A and Supplemental Table S2). Of these, the
260 expression levels of 355 genes (84.5%) were higher in *abi4* mutant roots, suggesting that ABI4
261 acts mostly as a repressor.
262 To determine whether phosphorylation state affected ABI4 activity *in planta*, we
263 compared the expression levels of these genes in the transgenic plants expressing ABI4(114S),
264 ABI4(S114A) and ABI4(S114E) relative to the respective levels in the *abi4* plants to determine
265 whether phosphorylation state affected ABI4 activity *in planta* (Figure 7A and Supplemental
266 Figure S8). Heat map and Venn diagrams show that the gene expression profile in roots of ABA-
267 treated plants expressing the phosphomimetic ABI4(S114E) protein more closely resembles that
268 of the wild-type ABI4(114S) profile than that of plants expressing the phosphorylation-null
269 ABI4(S114A) (Figure 7A & B). Expression levels of 52% and 68% of the genes whose transcript
270 levels were higher or lower, respectively, in *abi4* mutants than in wild-type and *abi4* expressing

271 ABI4(114S) plants, were reversed by either ABI4(S114A) or ABI4(S114E) proteins (Figure 7B
272 & C, Supplemental Table S3, marked as WSAE), suggesting that modulation of the expression
273 of these genes by ABI4 is independent of the phosphorylation state of S114. In contrast, the
274 phosphorylated state of S114 is required for modulation of the expression of 35% and 17% of the
275 ABI4-regulated genes whose levels in *abi4* were higher or lower, respectively, than in *abi4*
276 mutants expressing ABI4(S114E), but not ABI4(S114A) (Figure 7B & C, Supplemental Table
277 S4, marked as WSE no A). Even fewer ABI4-modulated genes require the non-phosphorylated
278 form of S114 for regulation: only 5% and 14% for repression or activation, respectively (Figure
279 7B and C, Supplemental Table S5, marked as WSA no E).
280 The genes that require phosphorylation of S114 for repression are enriched for those
281 affecting development (embryo, post-embryonic and reproductive), morphogenesis, cell growth,
282 differentiation, and regulation of gene expression. Those that are repressed regardless of
283 phosphorylation state are enriched for responses to stress, abiotic and endogenous stimuli (Figure
284 7D). The few ABI4-induced genes that require phosphorylation of S114 include several that are
285 involved in defense response and/or are induced by salt treatment of roots (Kilian et al., 2007).
286 In terms of molecular function, the largest classes of genes whose expression is
287 modulated by the phosphorylation of S114 encode nucleic acid binding proteins, transporters,
288 and enzymes, especially hydrolases and transferases including those involved in cell wall
289 synthesis (Supplemental Figure S9). Consistent with the ABA-resistant growth of the *abi4*
290 mutants, the ABI4-repressed genes that were most highly enriched relative to their abundance in
291 the genome code for factors needed for growth including nucleic acid binding proteins mediating
292 transcription, RNA splicing, chromatin modification; translation initiation and elongation
293 factors; and ion channels.
294 Screening the upstream sequences of the differentially expressed genes for 6 DNA
295 sequences previously reported to be bound by ABI4 identified an average of 2.55 putative ABI4
296 binding sites per gene. The most abundant are the CE1-like element (CACCK), ABRE-like
297 sequence (BACGTGKM), and DRE core motif (RCCGAC) (Supplemental Figure S10). The
298 differences between the numbers of the different binding sites in genes whose expression levels
299 is affected by the different phosphorylation states of S114 of ABI4 maybe too small to be
300 significant. Moreover, these sites are present in similar abundance in all the root expressed genes
301 (Supplemental Figure S10), suggesting that the occurrence of these sites in the promoter DNA

302 sequence is not enough to be regulated by ABI4. Furthermore, some of these genes may be
303 indirectly regulated by ABI4 so would not contain ABI4 binding sites in their promoters.
304
305
306 **DISCUSSION**
307
308 ABI4 activity has been shown to be regulated at the level of gene expression, protein
309 stability, and most recently by phosphorylation. Previous studies of ABI4 phosphorylation by
310 MAP kinases identified 3 MAPKs that could phosphorylate ABI4 *in vitro* (Popescu et al., 2009)
311 and focused on how MAPKs activated by MAPKKs affected ABI4 function in chloroplast
312 retrograde signaling (Guo et al., 2016). Guo et al. identified 3 residues phosphorylated by MPK3
313 and MPK6: T111, S114, and S130. Although the relevance of each phosphosite was not tested
314 individually, a mutant form of ABI4 with all 3 residues converted to Ala was unable to bind an
315 *LHCB* promoter *in vitro* or repress *LHCB* expression in transgenic plants. More recently,
316 phosphorylation of ABI4 by MPK3 and MPK6 was found to repress production of adventitious
317 roots at the root-hypocotyl junction, in part by inhibiting accumulation of reactive oxygen
318 species and programmed cell death in overlying cell layers (Bai et al., 2020). Furthermore, these
319 MPKs phosphorylate PP2C12, which dephosphorylates ABI4, thereby inactivating PP2C12 and
320 stabilizing ABI4 in an activated state. As for *LHCB* regulation, this study did not distinguish
321 functional significance among the 3 potential phosphosites.
322 In the current study, we focused on activation of ABI4 by MPK3, the kinase with the
323 highest intrinsic affinity for ABI4 without prior activation by a MAPKK. Under these conditions,
324 substitution of both S114 and S130 with Ala was necessary and sufficient to block
325 phosphorylation of ABI4, suggesting that T111 is not phosphorylated by MPK3 displaying its
326 basal activity (Figure 2). Furthermore, the extent of phosphorylation suggests that the
327 phosphorylation of S114 is preferred over S130 (Figure 2). Although both S114 and S130 were
328 phosphorylated *in vitro*, functional tests of the individual phosphosites showed that
329 phosphorylation of S114 was necessary and sufficient for ABI4 activity in promoting inhibition
330 of growth by ABA, high sugar or salinity, including inhibition of lateral root growth. This
331 modification could enhance ABI4 activity by increasing stability and/or binding to target
332 promoters. *In vitro* assays showed that S114 phosphorylation or substitution with the

333 phosphomimic S114E was required for binding to at least one class of ABI4-regulated promoter
334 elements. Consistent with this, the root transcriptome of the ABI4(S114A) plant is most similar
335 to that of the *abi4* mutant, whereas the transcriptomes of ABI4(S114) and ABI4(S114E) are
336 closer to that of wild type plants. It is not clear why transgenic expression of the wild type ABI4
337 protein restored regulation of only half of the genes mis-regulated in the *abi4* mutant, but this
338 might reflect different threshold levels of ABI4 needed for proper expression of its targets. If so,
339 even small differences in expression or accumulation of ABI4 could account for this incomplete
340 complementation, and would be consistent with the transcriptome variability observed among
341 biological replicates of any given genotype.

342 ABI4-regulated individual genes and transcriptomes have been reported in a variety of
343 contexts, including seed development, dormancy and germination (Nakabayashi et al., 2005;
344 Penfield et al., 2006; Shu et al., 2013), oxidative stress response (Kerchev et al., 2011), ABA-
345 treatment of ectopic expression lines (Reeves et al., 2011), salt stress response (Li et al., 2016),
346 and retrograde signaling from organelles (Koussevitzky et al., 2007; Giraud et al., 2009; Leon et
347 al., 2012). In dry seeds, expression of over 2200 genes was reduced at least two-fold in *abi4*
348 mutants, while over 2800 genes were expressed at least two-fold higher in this background
349 (Nakabayashi et al., 2005), reflecting primarily repressive action of ABI4. Approximately 50-
350 70% of genes activated by ABA treatment of ABI4 overexpression lines were also activated in
351 wild-type plants exposed to ABA, high salt or osmotic stress at different ages (Reeves et al.,
352 2011). In contrast, consistent with the diversity of physiological and developmental contexts
353 described above, there is very little overlap between the ABI4 target genes identified in these
354 studies. A further complication for making comparisons is that many ABA or stress responses
355 proceed through phases of early vs. late gene expression, so different studies may simply be
356 examining different phases or transient responses (Finkelstein, 2013). However, all of these
357 studies show that ABI4 can function as either an activator or repressor of distinct subsets of
358 genes.

359 The present study analyzed ABI4-regulated genes in seedling roots following a brief
360 exposure to ABA. Comparison of wild-type and *abi4* mutants identified at least 2.5-fold more
361 genes that are repressed by ABI4 under these conditions than genes that are activated
362 (Supplemental Figure S7). Filtering out those genes whose expression was not restored to wild-
363 type levels by the complementing transgene removed about half of these genes, but preferentially

364 removed ABI4-induced genes, resulting in a 4-fold difference between repressed and induced
365 genes. Further narrowing the focus to genes whose regulation was restored in roots of plants
366 expressing ABI4(S114E) but not in ABI4(S114A) seedling roots, the ratio of repressed to
367 induced genes was greater than 10-fold, suggesting that phosphorylation of S114 of ABI4 might
368 be more important for action as a repressor. Whereas the genes requiring phosphorylation of
369 ABI4 for repression were enriched in functions associated with reproductive development and
370 growth, most of the few transcripts that were higher in the ABA-treated roots of wild-type and
371 ABI4(S114E) than *abi4* were associated with abiotic and/or biotic stress responses.
372 Many transcription factors are known to require phosphorylation for activity. For
373 example, in the core ABA signaling pathway, stability and/or activity of the ABA and stress-
374 responsive ABI5/ABF/AREB clade of bZIPs depend on phosphorylation by SnRK2 family
375 protein kinases (Cutler et al., 2010), possibly mediated by interactions with 14-3-3 proteins
376 (Sirichandra et al., 2010). However, the effects of phosphorylation of these transcription factors
377 on accumulation of specific transcripts has not been systematically analyzed. Our results show
378 that phosphorylation of a single residue of ABI4 affects its ability to regulate some, but not all,
379 ABI4 target genes. Similar results were described for the neurogenic transcription factor,
380 SOX11, in transfected cells (Balta et al., 2018). In this case, transactivation assays testing wild-
381 type, phosphomimetic and phosphorylation-null mutants for effects on three different promoter-
382 LUC fusions produced three distinct results: no difference between wild type and mutant
383 SOX11, and both phosphomutants more active than wild type but differing in whether
384 phosphomimetic or phosphorylation-null was more effective. These results suggest that the
385 balance between phosphorylation states may be another important aspect regulating activity
386 relative to specific target genes.
387 Surprisingly, some of the genes that are repressed in ABA-treated roots by ABI4,
388 regardless of its phosphorylation state, show reduced expression in *abi4* mutant seeds (e.g.,
389 AT3G53040, encoding a LEA protein) (Nakabayashi et al., 2005), indicating that ABI4 may be
390 either an activator or repressor of a given gene in different developmental contexts. This may
391 reflect interactions with a variety of other transcription factors. The putative ABI4 binding sites
392 have diverse sequences and previous EMSA studies indicate relatively low affinity and
393 specificity of binding to several of these (Reeves et al., 2011; Shkolnik-Inbar et al., 2013),
394 suggesting that *in vivo* binding may be modulated by associations with other factors. Two recent

395 reports document formation of complexes containing ABI4 and the histone deacetylase HDA9,
396 leading to repression of a subset of ABI4-regulated genes by chromatin modification during
397 drought stress (Baek et al., 2020; Khan et al., 2020). Furthermore, a recent report indicates that
398 intrinsically disordered domains in transcription factors can influence *in vivo* binding site
399 specificity (Brodsky et al., 2020), and more than half of ABI4 is predicted to be disordered.
400 In summary, our results confirm previously reported MAPK phosphorylation sites, but
401 show that phosphorylation of only one of these is actually necessary and sufficient for ABI4
402 activity in responding to ABA, glucose and salt stress. Furthermore, the transcriptome
403 experiment identified a novel subset of ABI4-regulated genes, approximately one-third of which
404 are dependent on phosphorylation of ABI4 for proper expression in roots, and this regulation is
405 primarily repressive.
406
407
408 **MATERIALS AND METHODS**
409
410 **Expression and purification of recombinant proteins**
411 cDNA sequences of Arabidopsis genes encoding ABI4, MPK3, MPK4, and MPK6 were
412 amplified by a polymerase chain reaction (PCR) using gene-specific primers. Resulting
413 sequences were subcloned into the *Sal*I and *Xho*I sites of pGST-parallel1 bacterial expression
414 vector. The resulting plasmids were verified by DNA sequencing and introduced into *E. coli* BL-
415 21 cells. The respective GST-MAPK proteins were expressed and affinity-purified on GST-
416 agarose column.
417 The coding sequence of ABI4 was cloned into the *Xho*I and *Sal*I sites of pRSET A,
418 downstream to a sequence encoding hexahistidine (Shkolnik-Inbar et al., 2013). Site-directed
419 mutagenesis was carried out according to the QuikChange protocol (Agilent Technologies). The
420 primers used for mutagenesis are shown in Supplemental Table S6 using the pRSET A-ABI4
421 plasmid as a template. The introduced respective mutations were confirmed by DNA sequencing.
422 Plasmids were introduced into *E. coli* BL-21 cells and cultures were grown at 16 ºC in LB
423 medium containing in addition 100 mg/ml ampicillin, to OD600 nm=0.6. The expression of the
424 recombinant protein was induced by the addition of 1 mM IPTG. The culture was incubated

425 overnight in the shaker at 16 ºC. Bacteria were then harvested, and cells were osmotically
426 shocked to reduce the abundance of cation chelators within the bacterial intermembrane space,
427 which was shown to reduce the binding capacity of low-expressed recombinant His-tagged
428 proteins to matrices containing immobilized nickel ions (Magnusdottir et al., 2009).
429 Hypertonically shocked bacteria were suspended in a lysis buffer containing 50 mM Tris-HCl
430 pH 8.0, 0.5 M NaCl, and 10 mM imidazole, using a ratio of 1.5 buffer ml/gr bacteria and
431 sonicated on ice by 60 seconds of 70Ω bursts using a tip sonicator (Sonics Vibra-Cell). The
432 resulting homogenate was centrifuged for 45 min at 150,000 × g and 4 ºC in an ultracentrifuge,
433 and the resulting supernatant was loaded on a 1ml HiTrap IMAC FF nickel-agarose column (GE)
434 attached to an AKTA start chromatography apparatus. The column was thoroughly washed with
435 the lysis buffer and then eluted with a 20–300 mM imidazole gradient in a buffer containing 50
436 mM Tris-HCl pH 8.0 and 0.5 M NaCl. Eluted fractions were analyzed by SDS-PAGE; fractions
437 containing ABI4 were pooled, and their buffer was replaced by 50 mM Tris-HCl pH 8.0 and 0.5
438 M NaCl by using a 10 kDa cutoff Amicon Ultra 0.5ml centrifugal filter (Merck).
439
440 **Protein phosphorylation assay**
441 Approximately 0.5 µg of affinity-purified His-tagged ABI4 proteins (WT and proteins
442 containing the respective amino acid changes) were incubated in a phosphorylation reaction mix
443 containing 20 mM HEPES-NaOH pH 7.4, 15 mM $MgCl_2$, 5 mM EDTA, 1 mM DTT, 3 µl of
444 carrier-free [γ-^{32}P]ATP (3000 Ci/mmol; 5 mCi/ml), and 0.1µg of each of recombinant GST-
445 MAPK3, GST-MAPK4, or GST-MAPK6 proteins (in phosphate-buffered saline, PBS). Reaction
446 mixtures were incubated for 1 hour at 30 ºC, followed by adding 10 µl of 5 × SDS-PAGE sample
447 buffer. Proteins were denatured by incubation for 5 min at 95 ºC, and samples were loaded on
448 SDS-PAGE (Laemmli, 1970). Following the electrophoresis run, the gel was stained with
449 Coomassie blue, vacuum dried for 2 hours at 80 ºC, and exposed to a phosphorimager screen.
450 Signals were detected by a Typhoon phosphorimager (GE Life Sciences).
451
452 **Plant material and growth**
453 All the studies used *Arabidopsis thaliana*– Columbia ecotype. Seeds of *abi4-1* mutants
454 were obtained from an Arabidopsis stock center in Columbus, Ohio. The plants were grown at
455 22–25 ºC in day/night periods of 12 hours each and 50% humidity. For seed collection, plants

456 were grown in long daylight regimes (16/8 hours, day/night) to accelerate flowering time. Seeds
457 were surface sterilized by incubation for 20 min with constant rotation in 20% commercial
458 bleach containing 1% Triton X-100, followed by eight washes with sterile deionized water.
459 Seeds were resuspended in soft agar and incubated for 3–5 days at 4 ºC, to enhance germination
460 rate and uniformity. Cold stratified seeds were sown in Petri dishes containing solidified 0.5 ×
461 Murashige and Skoog (MS, (Murashige and Skoog, 1962)), 0.5% sucrose and 0.55% plants agar,
462 containing, also, the added materials as indicated. Plants were also sown in 6 × 6 × 7 cm black
463 pots using a potting mix containing 20 % tuff, 30% peat, 30% vermiculite, 20% perlite, and
464 slow-release fertilizer. Pot-flats were placed in trays and were irrigated by filling the trays with
465 deionized water.
466
467 **Preparation of constructs and transformation of plants**
468 DNA sequence comprised of ABI4 encoding sequence and 2 kbp of upstream promoter
469 sequence was cloned by PCR using gene-specific primers (Supplemental Table S6) and template
470 comprised of genomic DNA prepared form WT Arabidopsis plants. The resulting PCR product
471 was verified by DNA sequencing and subcloned into the *Kpn*I and *Xba*I sites of the plant
472 transformation vector pCAMBIA 2300. Constructs expressing ABI4(S114A), ABI4(S114E),
473 ABI4(S130A) or ABI4(S130E) mutated proteins were constructed by replacing the *Bam*HI-*Blp*I
474 restriction fragment of the wild-type ABI4 in pCAMBIA 2300 by the respective fragments of
475 constructs in pRSET encoding the respective mutation. The resulting plasmids were verified by
476 DNA sequencing and were introduced into *Agrobacterium tumefaciens* (strain GV3101). The
477 transformed bacteria were used to transform *abi4* mutant Arabidopsis plants (Clough and Bent,
478 1998). Transgenic plants were selected by germination in Kanamycin containing plates.
479 Homozygous seeds from the T2 and T3 generations, resulting from a single T-DNA insertion
480 event, were chosen for this study.
481
482 **Plant phenotyping**
483
484 **Plate grown seedlings**
485 Cold-treated, surface-sterilized Arabidopsis seeds were plated on Petri dishes containing
486 0.5xMS, 0.5% sucrose, and 0.55% plant agar, supplemented with the indicated concentrations of

487 ABA, glucose or NaCl. Plates were incubated in the growth room in 12 h of dark/light conditions
488 at 22ºC, and radicle emergence was assayed daily for one week. Cotyledon development was
489 assayed by measuring seedlings with spread cotyledons. Lateral root development was assayed
490 in 12 days old seedlings grown on Petri dishes containing 0.5 × MS, 0.5% sucrose, and 0.5%
491 plant agar. Seedlings were harvested, placed on a transparent sheet, and roots were arranged
492 before taking their images by a scanner. The lengths of primary and lateral roots were measured
493 from the resulting images. All assays were carried out at least 3 times with more than 50
494 seedlings per line in each assay. Statistical significance of the data was analyzed using Tukey's
495 HSD post-hoc test.
496
497 **Salt treatment of pot-grown rosettes**
498 Seeds of plants of the indicated genotypes were sowed in 6 × 6 × 7 cm pots. The pots were
499 placed in the growth room under conditions of 12h of dark/light at 22 ºC for approximately 1
500 month. Plants were then irrigated with water or with a solution containing 0.25 M NaCl and 16
501 mM $CaCl_2$. Rosettes were followed daily for 3 weeks. Rosettes having even a small amount of
502 green tissue were scored as live plants, where all yellow-brown rosettes were scored as dead
503 plants. All assays were carried out at least 3 times with more than 15 seedlings per line in each
504 assay. Statistical significance of the data was analyzed using Tukey's HSD post-hoc test.
505
506 **Electrophoresis mobility shift assay (EMSA)**
507 Recombinant His-tagged ABI4 WT, or ABI4 containing the indicated mutations in
508 positions 114 or 130, were expressed in *E. coli* and purified on nickel-agarose columns as
509 described above. Double stranded 5' ^{32}P-radiolabelled oligonucleotides corresponding to two
510 sequences from HKT1;1 promoter, termed A6-1 and A6-5, each containing an ABE motif
511 previously shown to bind ABI4 were prepared as described (Shkolnik-Inbar et al., 2013). DNA
512 binding assays were performed as previously described (Shkolnik-Inbar et al., 2013). Following
513 PAGE, gels were dried, and a radiogram was obtained by using a Typhoon phosphorimager.
514
515 **RNA Sequencing and expression analysis**
516 Arabidopsis seeds were plated on top of a sterilized silk print mesh placed on growth
517 medium (0.5 x MS, 0.5% sucrose and 0.5% agar) and grown for 1 week as described above. Nets

518 with the germinated seedlings were transferred to fresh Petri dishes containing Whatman No 1
519 filter paper soaked with 0.5 x MS, 0.5% sucrose and 50 μM of the plant hormone ABA. Plates
520 were incubated in the light at room temperature for 4 h, roots were harvested, and total RNA was
521 extracted by using Quick-RNA Plant Kit (Zymo Research). The RNA concentrations were
522 determined by using NanoDrop (DeNovix DS-11 FX). Four biological repeats, each containing
523 all tested genotypes, were performed on different days.

524 Sequencing libraries were prepared and sequenced by the Israel National Center for
525 Personalized Medicine (INCPM) at the Weizmann Institute. Briefly, total RNA was fragmented,
526 followed by reverse transcription and second-strand cDNA synthesis. The double-strand cDNA
527 was subjected to end repair, A base addition, adapter ligation, and PCR amplification to create
528 libraries. Libraries were evaluated by Qubit and TapeStation. Sequencing libraries were
529 constructed with barcodes to allow multiplexing of the samples on two lanes of Illumina HiSeq
530 2500 V4 instrument, resulting in ~25 million single-end 60-bp reads per sample. Poly-A/T
531 stretches and Illumina adapters were trimmed from the reads using cutadapt (Martin, 2011);
532 resulting reads shorter than 30 bp were discarded. Reads were mapped to the *A. thaliana*
533 reference genome TAIR10 using STAR (Dobin et al., 2012), supplied with gene annotations
534 downloaded from Ensembl (and with EndToEnd option and outFilterMismatchNoverLmax was
535 set to 0.04). Expression levels for each gene were quantified using htseq-count (Anders et al.,
536 2014), using the annotation of the reference genome above.

537 The total reads of each sample were normalized to 20 million, and $\log_2$ transformed.
538 Values below 2 ($\log_2$) were removed, and only genes where at least two out of the 18 samples
539 had a threshold value of 6 ($\log_2$) were included in the analysis. Data were then normalized to
540 remove the day bias: for each gene, the median value of all samples from the same day was
541 subtracted from the value of each sample. Three outlier samples were not included in the
542 analysis.

543 In order to find the genes regulated by the WT, we first compared it to *abi4*. In each
544 comparison, genes were assigned to 30 bins according to the sum of expression of the two
545 genotypes (values used for binning are $\log_2$ values prior to day normalization, for indication of
546 absolute expression levels). Z-score of the delta $\log_2$ expression between the two genotypes was
547 calculated for each bin, using the values normalized by experiment day. A cutoff of $|\text{Z-score}| \geq 2$

548 was implemented in order to find genes that are differentially expressed. The same was done in
549 order to find genes that are differentially expressed between the *abi4* mutant and the transgenic
550 lines expressing ABI4(114S), ABI4(S114A) or ABI4(S114E). Since expressing wild-type
551 ABI4(114S) in the *abi4* genetic background complemented the wild-type phenotype (Figures 3-
552 6), we focused our analysis on genes whose expression could be complemented by ABI4(114S).
553 In order to find the genes that are affected by the specific phosphorylation in S114, we checked
554 which of those genes could also be complemented by expressing the phosphorylation-null
555 ABI4(S114A) or the phosphomimetic ABI4(S114E) in *abi4*.

556 ABI4 DNA binding sites were searched by using the Patmatch tool at TAIR. DNA sites
557 searched were: S box (CACYKSCA), CE-1 like (CACCK), ABRE (YACGTGGC), ABRE-like
558 (BACGTGKM), DRE core motif (RCCGAC) (Reeves et al., 2011), and ABE motif (GCSCTTT)
559 (Shkolnik-Inbar et al., 2013). The resulting list was searched for genes whose expression in *abi4*
560 mutants compare with wild-type plants, could be complemented in the *abi4* plants expressing
561 ABI4(114S), ABI4(S114A) or ABI4(S114E). As a control we used the list of genes whose
562 expression was detected by RNA-seq.

563 Gene Ontology (GO) was performed using the agriGO v2.0
564 (http://systemsbiology.cau.edu.cn/agriGOv2/) (Tian et al., 2017) using the Fisher statistical test
565 method, Yekutieli (FDR dependency) at significant level of 0.05 and the Plant GO slim option.
566
567

568 **Accession Numbers**

569 ABI4, At2G40220; MPK3, At3G45640; MPK4, At4G01370; MPK5, At2G43790; HKT1;1,
570 At4G10310.

571

572 **Large Datasets**

573 The SRA accession number for the RNA-seq data reported in this paper is
574 RJNA655114.

575

576 ## Supplemental Data

577

578 **Supplemental Figure S1.** BiFC interactions between ABI4 and MPK3 and MPK6.

579

580 **Supplemental Figure S2.** Coomassie blue-stained gel of ABI4 phosphorylation by MAPKs.

581

582 **Supplemental Figure S3**. Putative MAPK phosphorylation sites in ABI4.

583

584 **Supplemental Figure S4.** Phenotype of the studies genotype grown in standard conditions.

585

586 **Supplemental Figure S5.** Effect of ABI4 S114 residue on its interaction with DNA.

587

588 **Supplemental Figure S6**. Principal component (PCA) analysis of gene expression levels in
589 roots of ABA-treated plants.

590

591 **Supplemental Figure S7.** Expression of ABI4-regulated genes in roots of ABA-treated plants.

592

593 **Supplemental Figure S8.** Heatmap of genes that are differentially expressed both in the wild-
594 type and abi4 complemented by ABI4(114S) genotypes versus abi4.

595

596 **Supplemental Figure S9.** Molecular function of the differential expressed genes.

597

598 **Supplemental Figure S10.** Analysis of putative ABI4-binding sites in the differentially
599 expressed genes.

600

601 **Supplemental Table S1.** List of genes with different root expression between ABA-treated wild-
602 type and *abi4* mutant plants.

603 **Supplemental Table S2.** List of genes with different root expression in both wild type and 114S
604 compared to *abi4*.

605

606 **Supplemental Table S3.** List of genes with different root expression in both wild-type and 114S
607 compared to abi4, that are also differentially expressed in both 114A and 114E.
608
609 **Supplemental Table S4.** List of genes with different root expression in both wild-type and 114S
610 compared to abi4, that are also differentially expressed in 114E but not in 114A.
611
612 **Supplemental Table S5.** List of genes with different root expression in both wild-type and 114S
613 compared to abi4, that are also differentially expressed in 114A but not in 114E.
614
615 **Supplemental Table S6.** List of primers used in this study.

616

617 **ACKNOWLEDGMENTS**

618 Du.B.Z. is the incumbent of The Israel and Bernard Nichunsky Chair in Desert Agriculture, Ben-
619 Gurion University of the Negev.

620

621 **FIGURE LEGENDS**

622 **Figure 1.** ABI4 phosphorylation by MAPKs. Recombinant ABI4 was incubated with GST-
623 tagged MPK3, MPK4, and MPK6 in the presence of [γ–^{32}P]ATP. Samples were resolved on 10%
624 SDS-PAGE, fixed by staining with coomassie-blue, dried, and exposed to a phosphorimager
625 screen. The radiolabeled bands of MAPK and ABI4 are marked. Autophosphorylation of MAPK
626 serves as a control for the kinase activity. The image of the coomassie blue-stained gel is
627 presented in Supplemental Figure S2.
628
629 **Figure 2.** Test of phosphorylation of S114A and S130E mutants of ABI4 by MPK3. The
630 indicated His-tagged ABI4 proteins (WT and the respective Ser to Ala mutants) were expressed
631 in *E. coli*, affinity purified and assayed for phosphorylation by MPK3 in the presence of
632 [γ–^{32}P]ATP. Proteins were resolved by SDS-PAGE, gels were stained with coomassie blue,

633 dried, and exposed to a phosphorimager screen. A: Autoradiogram showing phosphorylation of
634 ABI4. B Coomassie staining of the different ABI4 proteins.

635 **Figure 3.** Effect of ABA on seed germination. Surface sterilized cold treated seeds of the
636 indicated genotypes were sown on plates containing agar solidified growth medium without
637 (dashed lines) or supplemented with 10 µM (solid lines) ABA. A&B, Radicle emergence was
638 scored daily. Data represent mean ± SE. A. Mutants of S114. B. Mutants of S130. Dashed gray –
639 germination of all tested genotypes in the absence of ABA. Black, WT plants; Red, *abi4*
640 mutants; Green, Orange and Blue, *abi-4* plants transformed with constructs expressing ABI4 in
641 which amino acid residues 114 (A) or 130 (B) are Ser, Ala or Glu, respectively. C. Expanded
642 cotyledons were scored 10 days after sowing on medium without (shaded) or with (black) 10 µM
643 ABA. The phosphomimetic ABI4(S114E) mutant but not the phosphorylation null ABI4(S114A)
644 mutant could complement the *abi4* phenotype. Data represent mean ± SE. Bars with different
645 letters represent statistically different values using Tukey's HSD post-hoc test (P ≤0.05).
646

647 **Figure 4.** ABI4(S114E), but not ABI4(S114A) mutations, complement *abi4* resistance to
648 glucose effects on cotyledon development. Seeds of plants from the indicated genotypes were
649 sown on plates containing 0.5 x MS salts and 0.7% agar (grey bars) or the same medium
650 supplemented with 7% glucose. Expanded cotyledons were scored 4 days later. Grey and black
651 bars represent data obtained in without or with glucose, respectively. Data represent mean ± SE.
652 Bars with different letters represent statistically different values using Tukey's HSD post-hoc test
653 (P ≤0.05).
654

655 **Figure 5.** ABI4(S114E), but not ABI4(S114A) mutations, complement *abi4* resistance to NaCl
656 effects on seed germination and viability of rosettes. A. Seeds from the indicated genotypes were
657 sown on plates containing 0.5 x MS salts, 0.5% sucrose and 0.7% agar, containing in addition 0
658 (shaded) or 0.2 M NaCl (black). Radicle emergence was scored 3 days later. Data represent mean
659 ± SE. Bars with different letters represent statistically different values using Tukey's HSD post-
660 hoc test (P≤0.01).
661 B. Seeds of the indicated genotypes were sown in a pot containing potting mix and irrigated with
662 water for one month. Rosettes were than irrigated twice a week with water (shaded) or 0.25 M

663 NaCl and 16 mM $CaCl_2$. Plant survival was scored 17 d later. Data represent mean ± SE. Bars
664 with different letters represent statistically different values using Tukey's HSD post-hoc test
665 (P≤0.01).
666
667 **Figure 6.** ABI4(S114E), but not ABI4(S114A) mutations, complement over-production of
668 lateral roots by *abi4* mutants. Length of primary (blue bars) and 1st lateral roots (orange bars) in
669 12 d old seedlings were measured. (●) The ratio of lateral root/primary root lengths. Data
670 represent mean ± SE. Bars with different letters represent statistically different values using
671 Tukey's HSD post-hoc test (P ≤0.01).

672
673 **Figure 7.** Expression of ABI4-regulated genes that are affected by the phosphorylation status of
674 S114.
675 A. Heatmap of genes that are differentially expressed both in the wild-type and *abi4*
676 complemented by ABI4(114S) genotypes versus *abi4.* Color indicate the Z-score values of the
677 differential expression of the indicated genotypes compared to *abi4* (see Methods).
678 B. Venn diagram presenting ABI4-regulated genes shown in A. WS, genes that are differentially
679 expressed both in wild type and ABI4(114S); WSA, genes that are differentially expressed in
680 WS and ABI4(S114A); WSE, WS and ABI4(S114E).
681 C. Expression patterns of gene clusters whose expression is independent of the phosphorylation
682 state of ABI4-S114 (WSAE), or dependent on the non-phosphorylated (WSA no E) or
683 phosphorylated (WSE no A) states of ABI4 S114, corresponding to the Figure 7B. Plots show
684 log_2 values of medians of each gene in the group normalized to the expression of the respective
685 gene in the wild-type plant, where plots of individual genes are shown by colored lines, and the
686 average values in thick black lines.
687 D. Biological processes of the proteins encoded by the ABI4-downregulated genes in (B).
688 Biological processes were analyzed by AgriGo ver2. Blue bars represent ABI4-down regulated
689 genes whose expression is not affected by the phosphorylation state of ABI4 (WSAE) ; orange
690 bars represent genes in group WSE, that are down-regulated by the phosphorylated state of S114
691 of ABI4 (WSE no A). The WSA no E group was too small for the analysis.
692

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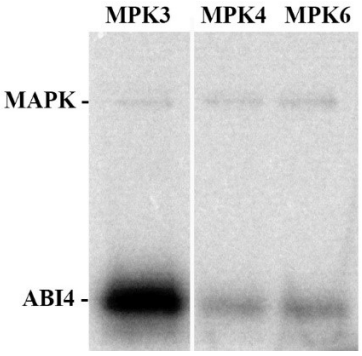

Figure 1. ABI4 phosphorylation by MAPKs. Recombinant ABI4 was incubated with GST-tagged MPK3, MPK4, and MPK6 in the presence of [γ–^{32}P]ATP. Samples were resolved on 10% SDS-PAGE, fixed by staining with coomassie-blue, dried, and exposed to a phosphorimager screen. The radiolabeled bands of MAPK and ABI4 are marked. Autophosphorylation of MAPK serves as a control for the kinase activity. The image of the coomassie blue-stained gel is presented in Supplemental Figure S2.

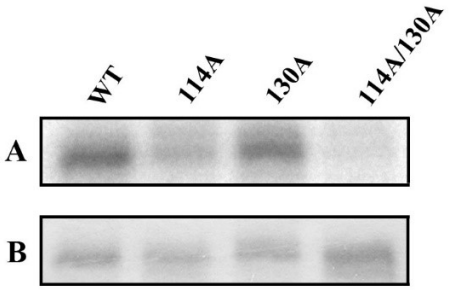

Figure 2. Test of phosphorylation of S114A and S130E mutants of ABI4 by MPK3. The indicated His-tagged ABI4 proteins (WT and the respective Ser to Ala mutants) were expressed in *E. coli*, affinity purified and assayed for phosphorylation by MPK3 in the presence of [γ–^{32}P]ATP. Proteins were resolved by SDS-PAGE, gels were stained with coomassie blue, dried, and exposed to a phosphorimager screen. A: Autoradiogram showing phosphorylation of ABI4. B Coomassie staining of the different ABI4 proteins.

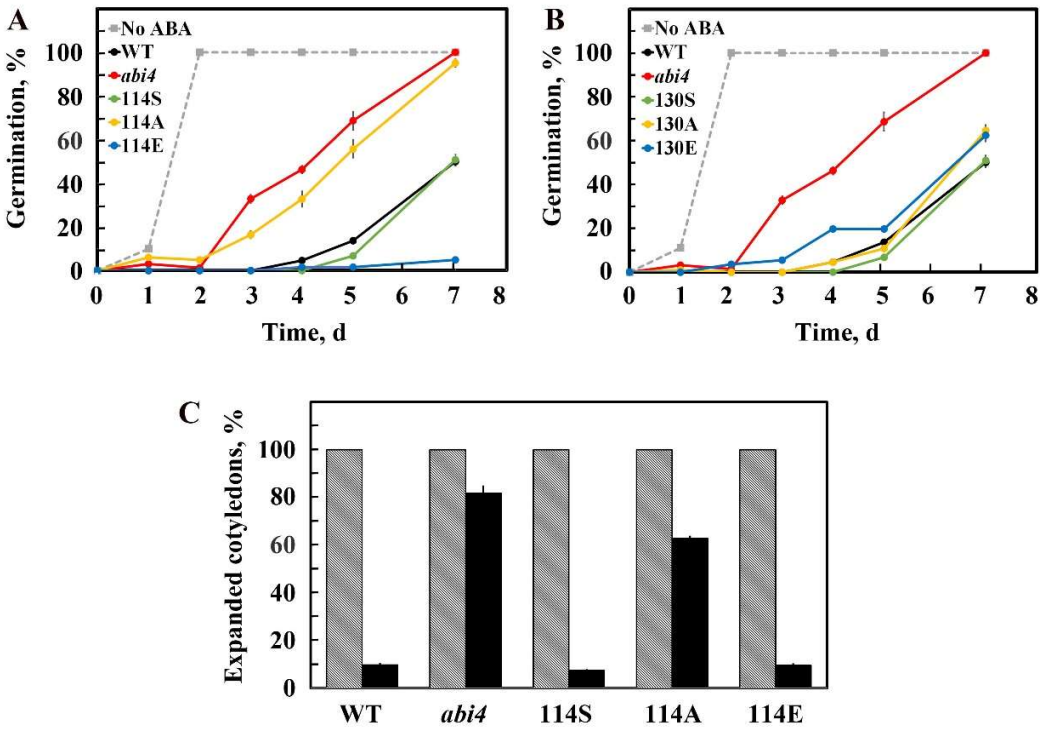

Figure 3. Effect of ABA on seed germination. Surface sterilized cold treated seeds of the indicated genotypes were sown on plates containing agar solidified growth medium without (dashed lines) or supplemented with 10 µM (solid lines) ABA.

A&B, Radicle emergence was scored daily. Data represent mean ± SE

A. Mutants of S114. B. Mutants of S130. Dashed gray – germination of all tested genotypes in the absence of ABA. Black, WT plants; Red, *abi4* mutants; Green, Orange and Blue, *abi-4* plants transformed with constructs expressing ABI4 in which amino acid residues 114 (A) or 130 (B) is Ser, Ala or Glu, respectively.

C. Expanded cotyledons were scored 10 days after sowing on medium without (shaded) or with (black) 10 µM ABA. The phosphomimetic ABI4(S114E) mutant but not the phosphorylation null ABI4(S114A) mutant could complement the *abi4* phenotype. Data represent mean ± SE. Bars with different letters represent statistically different values using Tukey's HSD post-hoc test ($P \leq 0.05$).

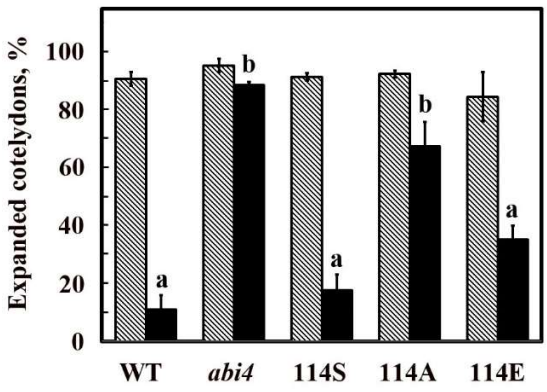

Figure 4. ABI4(S114E), but not ABI4(S114A) mutations, complement *abi4* resistance to glucose effects on cotyledon development. Seeds of plants from the indicated genotypes were sown on plates containing 0.5 x MS salts and 0.7% agar (grey bars) or the same medium supplemented with 7% glucose. Expanded cotyledons were scored 4 days later. Grey and black bars represent data obtained in without or with glucose, respectively. Data represent mean ± SE. Bars with different letters represent statistically different values using Tukey's HSD post-hoc test (P ≤0.05).

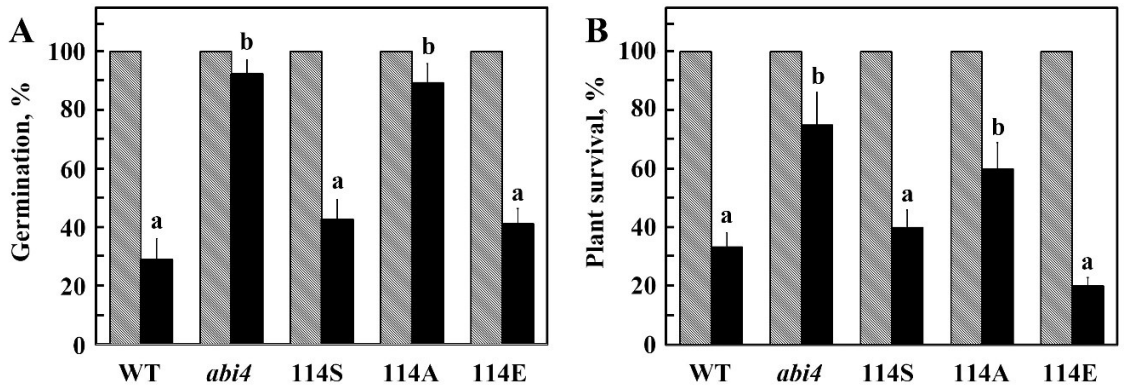

Figure 5. ABI4(S114E), but not ABI4(S114A) mutations, complement *abi4* resistance to NaCl effects on seed germination and viability of rosettes.
A. Seeds from the indicated genotypes were sown on plates containing 0.5 x MS salts, 0.5% sucrose and 0.7% agar, containing in addition 0 (shaded) or 0.2 M NaCl (black). Radicle emergence was scored 3 days later. Data represent mean ± SE. Bars with different letters represent statistically different values using Tukey's HSD post-hoc test (P≤0.01).
B. Seeds of the indicated genotypes were sown in a pot containing potting mix and irrigated with water for one month. Rosettes were than irrigated twice a week with water (shaded) or 0.25 M NaCl and 16 mM $CaCl_2$. Plant survival was scored 17 d later. Data represent mean ± SE. Bars with different letters represent statistically different values using Tukey's HSD post-hoc test (P≤0.01).

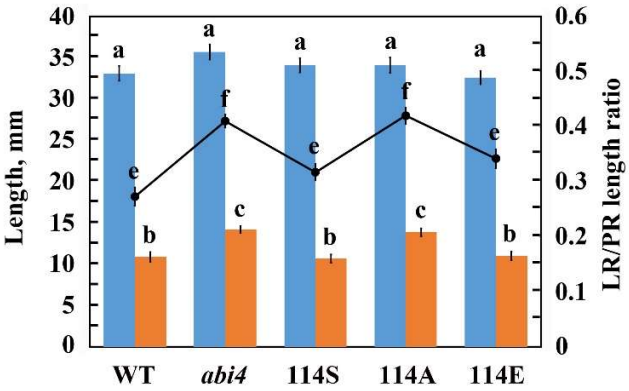

Figure 6. ABI4(S114E), but not ABI4(S114A) mutations, complement over-production of lateral roots by *abi4* mutants. Length of primary (blue bars) and 1st lateral roots (orange bars) in 12 d old seedlings were measured. (●) The ratio of lateral root/primary root lengths. Data represent mean ± SE. Bars with different letters represent statistically different values using Tukey's HSD post-hoc test (P ≤0.01).

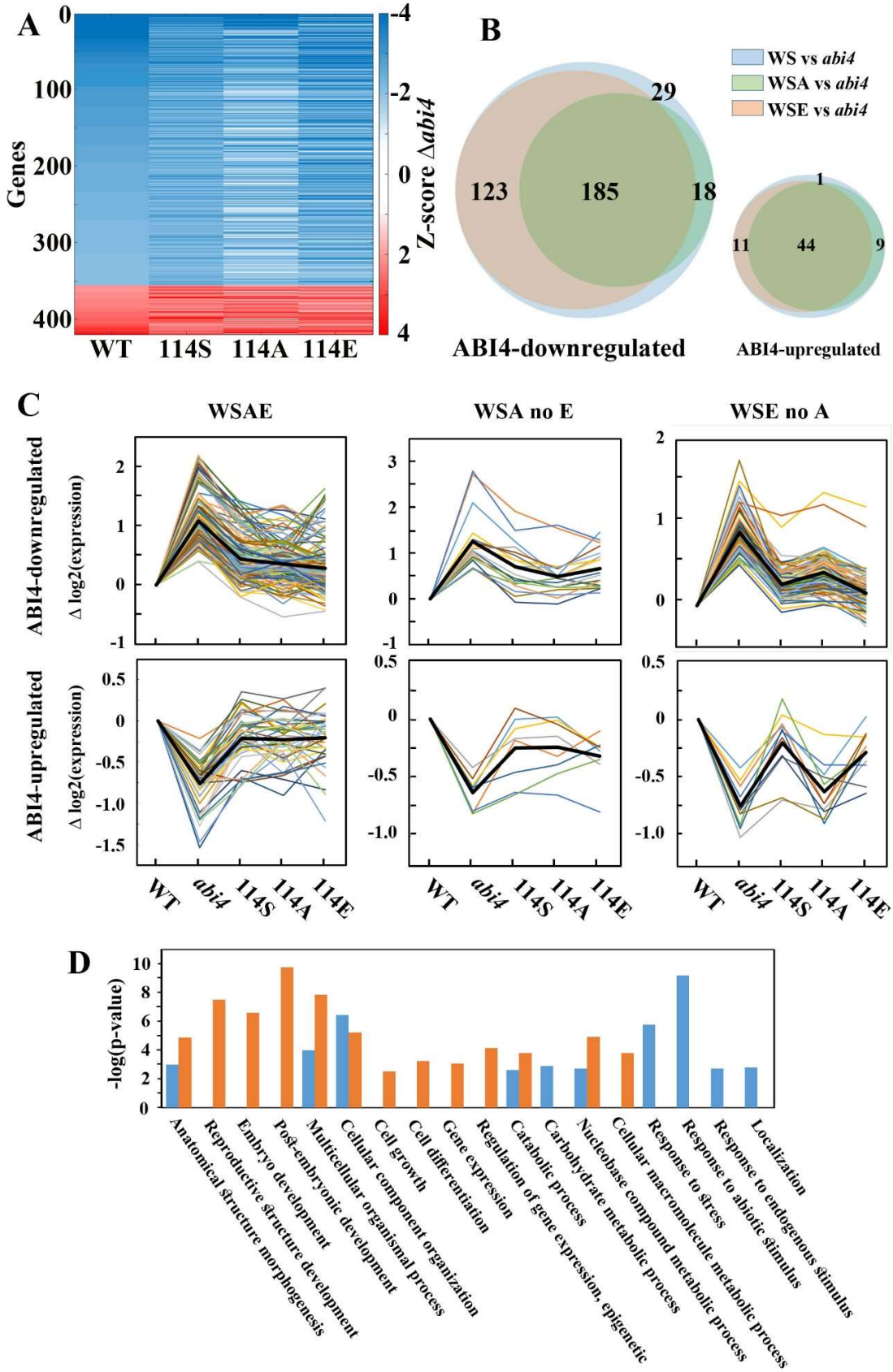
A
Genes
0
100
200
300
400
WT
114S
114A
114E
-4
-2
0
2
4
Z-score Δabi4
B
WS vs abi4
WSA vs abi4
WSE vs abi4
29
123
185
18
1
11
44
9
ABI4-downregulated
ABI4-upregulated
C
WSAE
WSA no E
WSE no A
ABI4-downregulated
ABI4-upregulated
Δ log2(expression)
WT
abi4
114S
114A
114E
D
-log(p-value)
Anatomical structure morphogenesis
Reproductive structure development
Embryo development
Post-embryonic development
Multicellular organismal process
Cellular component organization
Cell growth
Cell differentiation
Gene expression
Regulation of gene expression, epigenetic
Catabolic process
Carbohydrate metabolic process
Nucleobase compound metabolic process
Cellular macromolecule metabolic process
Response to stress
Response to abiotic stimulus
Response to endogenous stimulus
Localization

Figure 7. Expression of ABI4-regulated genes that are affected by the phosphorylation status of S114.

A. Heatmaps of genes that are differentially expressed both in the wild-type and *abi4* complemented by ABI4(114S) genotypes versus *abi4.* Color indicate the Z-score values of the differential expression of the indicated genotypes compared to *abi4* (see Methods).

B. Venn diagram presenting ABI4-regulated genes shown in A. WS, genes that are differentially expressed both in wild type and ABI4(114S); WSA, genes that are differentially expressed in WS and ABI4(S114A); WSE, WS and ABI4(S114E).

C. Expression patterns of gene clusters whose expression is independent of the phosphorylation state of ABI4-S114 (WSAE), or dependent on the non-phosphorylated (WSA no E) or phosphorylated (WSE no A) states of ABI4 S114, corresponding to the Figure 7B. Plots show $\log_2$ values of medians of each gene in the group normalized to the expression of the respective gene in the wild-type plant, where plots of individual genes are shown by colored lines, and the average values in thick black lines.

D. Biological processes of the proteins encoded by the ABI4-downregulated genes in (B). Biological processes were analyzed by AgriGo ver2. Blue bars represent ABI4-down regulated genes whose expression is not affected by the phosphorylation state of ABI4 (WSAE) ; orange bars represent genes in group WSE, that are down-regulated by the phosphorylated state of S114 of ABI4 (WSE no A). The WSA no E group was too small for the analysis.