Short title: PSKRs maintain growth under mannitol stress

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Title:

PSK signaling controls ABA homeostasis and promotes shoot growth under mannitol stress

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One-sentence summary

PSK receptor signaling is required to delay cotyledon greening in response to mannitol and ABA nad it promotes shoot growth under mannitol stress by controlling ABA levels and ABA responsiveness.

Footnotes

Author contributions: MS conceived the project. MS, MDS and KR designed the experiments. BH performed ABA measurements and data analysis. MDS and KR performed experiments. MDS and KR analyzed data. MS wrote the manuscript with contributions from MDS and BH.

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ABSTRACT

We hypothesized that the growth-promoting activity of the peptide hormone phytosulfokine (PSK), may be important to maintain growth under abiotic stress. To test this hypothesis, we employed mannitol as a stressor and analyzed the involvement of abscisic acid (ABA) that mediates a subset of mannitol responses. Inhibition of seed germination by ABA or mannitol was independent of PSK receptor signaling whereas repression of cotyledon greening was partially dependent on PSKR signaling with receptor null *pskr1-3 pskr2-1* seedlings showing enhanced greening. Mannitol led to dose-dependent shoot growth inhibition that was alleviated by PSKR signaling. With mannitol, *pskr1-3 pskr2-1* seedlings had strongly reduced shoot fresh and dry weights incompared to wild type. Analysis of *pskr1-3* and *pskr2-1* single receptor loss-of-function lines revealed that signaling via *PSKR1* is crucial for shoot growth promotion under mannitol stress. Mannitol and ABA induced expression of *PSK3* and *PSKR1* and ABA promoted expression of *PSK2* and *PSK4*. In turn, PSKR signaling was required for mannitol-induced accumulation of ABA in the shoot revealing a regulatory feedback loop. PSKRs repressed expression of *UGT71B6* encoding an ABA UDP-glucosyltransferase and maintained expression of *BG1*, an ABA glucosidase which may lead to reduced ABA-glucose conjugation and enhanced ABA remobilization. Marker gene expression analyses supported the conclusion that PSKR and mannitol signaling pathways crosstalk, a conclusion that was supported by the observation that PSK receptor signaling enhanced sensitivity to ABA in mannitol-stressed shoots. In conclusion, PSKR signaling maintains shoot growth under mannitol stress in part by interfering with the ABA pathway.

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64 **INTRODUCTION**

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67 The non-ionic osmolyte mannitol is a metabolite produced by plants and microorganisms
68 that is frequently used in osmotic stress and plant growth research (Nikonorova et al.,
69 2018; Kalve et al., 2020). Plants respond to mannitol by reduced growth and by stoma
70 closure that reduces water loss (Munemasa et al., 2015). The plant hormone abscisic
71 acid (ABA) accumulates in plants exposed to osmotic stress and controls core plant
72 responses to counteract water deficit (Bartels and Sunkar, 2005; Yamaguchi-Shinozaki
73 and Shinozaki, 2006; Tuteja, 2007 Finkelstein, 2013). Active ABA levels are determined
74 by synthesis, degradation, inactivation and remobilization. ABA is synthesized from
75 xanthophylls with 9-*cis*-epoxycarotenoid dioxygenase (NCED) catalyzing a key regulated
76 step. Indeed, elevation of ABA levels during drought conditions goes along with elevated
77 NCED activity (Finkelstein, 2013; Dejonghe et al., 2018). Degradation of ABA is initiated
78 by ABA 8'-hydroxylase, an enzyme that is encoded by four members of the cytochrome
79 P450 CYP707A family in Arabidopsis (Kushiro et al., 2004; Saito et al., 2004). ABA
80 hydroxylation has been identified as the key step in ABA catabolism (Dejonghe et al.,
81 2018). However, inactivation of ABA also occurs through conjugation to glucose by ABA
82 UDP-glucosyltransferase (UGT) encoded by *UGT71B6-B8*, in Arabidopsis (Dong and
83 Hwang 2004; Dong et al., 2014). ABA-glucose ester is a storage form of ABA that can
84 be remobilized by ABA glucosidases BG1 and BG2 (Lee et al., 2006). Thus, ABA
85 homeostasis depends on synthesis and inactivation pathways whereby many of the
86 genes involved are regulated in response to dehydration and other stresses (Xu et al.,
87 2013).

88 Shoot growth is inhibited by mannitol in a dose-dependent manner with inhibition
89 observed at low concentrations (25-50 mM), and high levels of ABA (Saab et al., 1990;
90 Skirycz et al., 2010; Skirycz and Inzé, 2010; Verelst et al., 2013). Growth reduction as a
91 response to abiotic stresses is thought to save resources that can be used to provide
92 stress resistance and ensure plant survival and reproduction in an adverse environment.

93 The mechanisms that balance growth and stress responses are however not much
94 explored.

95 PSK is a secreted signaling peptide that is perceived by a receptor at the plasma
96 membrane that belongs to the leucine-rich repeat receptor-like kinase family. The
97 Arabidopsis genome encodes two genes, *PSKR1* and *PSKR2* (Matsubayashi et al.,
98 2006; Sauter, 2015). Knockout of both receptor genes results in reduced root elongation
99 and shoot growth whereas exposure of wild type seedlings to PSK promotes growth by
100 enhancing cell expansion (Kutschmar et al., 2009; Stührwohldt *et al*., 2011). At the
101 molecular level, PSKR1 interacts with the co-receptor BRASSINOSTEROID-
102 INSENSITIVE 1 (BAK1) and the H^+-ATPases AHA1 and AHA2, and indirectly through
103 BAK1 and AHAs with the CYCLIC NUCLEOTIDE-GATED CHANNEL 17 (CNGC17).
104 These proteins assemble in a nanocluster at the plasma membrane and were proposed
105 to form a functional unit that drives cell expansion (Ladwig et al., 2015). PSKR signaling
106 was shown to cause protoplast expansion in a CNGC17-dependent manner and was
107 proposed to lead to cell wall acidification, water uptake, accompanied by osmotic
108 adjustment, and consequently to expansion of cells. Based on the growth-promoting
109 activity of PSKRs, we hypothesized that PSK signaling may also contribute to maintain
110 growth under mannitol stress thereby providing a molecular mechanism that integrates
111 growth and mannitol stress responses. To test this hypothesis, we employed the *pskr1-3*
112 *pskr2-1* double knockout mutant that is deficient in PSKR signaling (Hartmann *et al*.,
113 2013) and compared shoot growth under mannitol, that is well suited to study growth
114 regulation under mild stress conditions (Skirycz *et al.*, 2011*c*; Van den Broeck *et al.*,
115 2017), to that of wild type.Our results show that PSK signaling maintains shoot growth
116 under moderate mannitol stress. In the shoot, PSKR, mannitol and ABA interact at the
117 level of gene expression resulting in PSKR-dependent induction of ABA under mild
118 mannitol stress. We further show that PSKR signaling is required for the delay of
119 cotyledon greening induced by mannitol and ABA. Together, these findings reveal that
120 PSKR signaling interacts with ABA and mannitol pathways to control early seedling
121 development and shoot growth. under abiotic stress.
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125 # RESULTS

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127 **PSK receptors mediate inhibition of cotyledon greening in response to ABA and**
128 **mannitol**

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130 To answer the question if PSK receptor signaling controlled early seedling development
131 under stress conditions we used the uncharged osmolyte mannitol and the stress
132 hormone abscisic acid (ABA) which have unique but also overlapping signaling
133 pathways (Yoshida et al., 2014). We first studied seed germination under mannitol
134 stress. Seeds of wild type, the PSK receptor null line *pskr1-3 pskr2-1*, and PSKR1
135 overexpressors were exposed to 1 µM PSK, to 350 mM mannitol or a combination of
136 both and seed germination was scored for up to 21 days (Supplemental Fig. S1). No
137 significant differences in germination rate were detected between genotypes and
138 treatments. ABA inhibited seed germination, but treatment with 1 µM PSK in the
139 presence or absence of 1 µM ABA did not alter seed germination (Supplemental Fig.
140 S2). Likewise, knockout or overexpression of PSK receptors had no influence on seed
141 germination at any given treatment indicating that seed germination was not controlled
142 by PSK signaling.
143 We next studied the role of PSKR signaling in cotyledon greening. In the absence
144 of an effector, cotyledon greening did not differ between wild type, *pskr1-3 pskr2-1*, and
145 *PSKR1* overexpressors (Fig. 1A; Supplemental Fig. S3). Seedling development was
146 delayed by 1 µM ABA while 1 µM PSK did not have an effect (Fig. 1B; Supplemental
147 Fig. S3). However, wild type seedlings that were treated with PSK in addition to ABA
148 showed enhanced inhibition of cotyledon greening indicative of a synergistic effect
149 between ABA and PSK signaling. This conclusion was supported by the finding that the
150 degree of inhibition by ABA was genotype-dependent (Fig. 1B; Supplemental Fig. S3). In
151 wild type, ABA inhibited cotyledon greening by 65% compared to control conditions
152 whereas greening was reduced by only 18% in the PSK receptor null line *pskr1-3 pskr2-*
153 *1* and by 81-96% in lines overexpressing PSKR1 indicating that the ABA-induced delay
154 in seedling development was enhanced by PSKR signaling. The response to 350 mM
155 mannitol was similar to that of ABA. A delay in seedling development was observed that
156 was enhanced by PSK (Fig. 1C). Furthermore, *pskr1-3 pskr2-1* seedlings were less
157 responsive to mannitol whereas PSKR1 overexpressors showed a hypersensitive
158 response. Taken together, these findings revealed crosstalk between PSKR and
159 mannitol and between PSKR and ABA signaling pathways in cotyledon greening.
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162 **PSKR1 promotes shoot growth under mannitol stress**

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164 To study seedling growth regulation by PSKR signaling in response to mannitol,
165 seedlings were pre-grown for 4 days without mannitol and subsequently transferred to
166 plates containing 0, 25 mM, 50 mM, 100 mM or 200 mM mannitol (Fig. 2). By chosing
167 this experimental setup, we circumvented the effects of mannitol on cotyledon greening
168 described above. During pre-growth under control conditions, early seedling
169 development was completed after 4 days in all genotypes (Supplemental Figs. S1, S2
170 and S3). Wild type seedlings that were transferred to mannitol displayed a dose-
171 dependent inhibition of shoot size, shoot fresh weight and shoot dry weight that was
172 exacerbated in *pskr1-3 pskr2-1* seedlings (Fig. 2A, B, and C). The strongest growth
173 inhibition compared to wild type was observed at low concentrations of 25 mM and 50
174 mM mannitol suggesting that PSKR signaling maintains shoot growth during mild
175 mannitol stress. Growth reduction was brought about mainly by reduced leaf size, and
176 reduced leaf number (Fig. 2D; Supplemental Fig. S4). Exacerbated growth inhibition in
177 *pskr1-3 pskr2-1* seedlings was also seen with sorbitol (Supplemental Fig. S5). Unlike
178 shoot growth, root elongation was unaffected by mannitol up to 100 mM in wild type and
179 *pskr1-3 pskr2-1*. Root growth was inhibited by At 200 mM mannitol in both genotypes
180 with a significantly stronger inhibition in *pskr1-3 pskr2-1* (Fig. 2E).

181 To pinpoint which of the two PSK receptors mediated resistance to mannitol-
182 induced growth inhibition of the shoot, we used single PSK receptor gene knock out
183 lines (Kutschmar *et al*., 2009) (Fig. 3). After 2 and 3 weeks, growth inhibition by 50 mM
184 mannitol was comparable in *pskr1-3* and *pskr1-3 pskr2-1* whereas *pskr2-1* plants
185 showed wild-type growth indicating that PSKR1 promotes growth of plant shoots
186 exposed to mannitol (Fig. 3A, B).

187 To test whether the PSKR-dependent maintenance of shoot growth was a
188 response to low water potential, we exposed wild type and *pskr1-3 pskr2-1* seedlings to
189 PEG6000 at concentrations between 0 and 10% (Supplemental Fig. S6). While shoot
190 fresh and dry weights were reduced in a PEG6000 dose-dependent manner, the growth
191 reduction was not significantly different between wild type and *pskr1-3 pskr2-1* indicating
192 that the response to mannitol was due to a specific chemical property of mannitol rather
193 than a water-stress condition.

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196 **Mannitol and ABA promote expression of *PSKR* and *PSK* genes**
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198 To investigate whether expression of *PSK* and *PSKR* genes was regulated by mannitol
199 and ABA in leaves, we employed *promoter:GUS* lines that were described previously
200 (Kutschmar *et al*., 2009; Stührwohldt *et al*., 2011) to obtain spatial resolution. Of the five
201 PSK precursor genes, *PSK2*, *PSK3*, *PSK4* and *PSK5* were expressed in the shoot in
202 accord with the previous observation that *PSK1* is root-specific (Kutschmar *et al*., 2009)
203 (Fig. 4A). *PSK3* promoter activity was particularly high in young, expanding leaves.
204 Regulation of PSK precursor gene expression by 200 mM mannitol or 5 µM ABA was
205 studied by qPCR (Fig. 4B-F). In wild type, *PSK3* was significantly induced by mannitol
206 while *PSK2*, *PSK3* and *PSK4* were induced by ABA revealing, in part, differential
207 responses to the two signals. Knockout of PSK signaling in *pskr1-3 pskr2-1* resulted in
208 elevated *PSK2* and *PSK4* expression at control conditions suggestive of a feedback
209 inhibitory loop between PSK signaling and precursor gene expression. Interestingly,
210 *PSK1*, *PSK3* and *PSK5* were hyperinduced in the receptor-deficient background
211 compared to wild type revealing that *PSK1*, *PSK3* and *PSK5* are negatively controlled by
212 PSKR signaling during mannitol stress. Induction of *PSK3* mRNA levels by ABA was
213 abolished in *pskr1-3 pskr2-1* indicating that *PSK3* is upregulated by ABA in a PSKR-
214 dependent manner pointing to cross-talk between these signaling pathways.
215 *PSKR1:GUS* activity was detected in cotyledons, true leaves and in the root (Fig.
216 5A-F) while *PSKR2:GUS* activity was overall low in the shoot (Fig. 5G). *PSKR1:GUS*
217 seedlings exposed to 50 mM mannitol displayed increased GUS activity specifically in
218 young true leaves (Fig. 5D), a finding that was confirmed by qPCR analysis (Fig. 5H).
219 *PSKR1* transcripts increased in response to mannitol or ABA at low and high
220 concentrations (Fig. 5H). By contrast, *PSKR2* mRNA levels increased to a minor degree
221 at high mannitol or ABA levels (Fig. 5I) in accord with the observation that PSKR1 is
222 crucial for maintenance of growth (Fig. 3).
223 The data revealed highly differential PSK gene regulation in young leaves by a
224 network of ABA-dependent and ABA-independent mannitol stress signaling that
225 crosstalk with the PSK signaling pathway. As a consequence, mannitol and ABA induce,

226 both, *PSKR1* and *PSK* precursor genes which promote shoot growth during mannitol
227 stress.
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230 **PSKR signaling controls ABA levels and ABA responsiveness**
231
232 Hyperinduction of *PSK1*, *PSK3* and *PSK5* by mannitol in the PSK receptor null
233 background (Fig. 4B-D) revealed crosstalk between PSK and mannitol signaling.
234 Induction of *PSK3* by mannitol and ABA suggested that ABA may participate in PSK-
235 dependent growth promotion under mannitol stress. To learn more about this regulatory
236 network, we next asked whether ABA levels were regulated by PSKR signaling. Analysis
237 of ABA levels by mass spectrometry revealed a dose-dependent increase in ABA in
238 shoots and roots in response to mannitol in wild type (Fig. 6A, B). By contrast, ABA
239 levels did not increase in *pskr1-3 pskr2-1* shoots in response to mannitol suggesting that
240 PSKR signaling is required for ABA accumulation under mannitol stress. In roots, ABA
241 levels increased in *pskr1-3 pskr2-1* seedlings at 200 mM mannitol albeit overall levels
242 were lower than in wild type. Genotypic differences in ABA accumulation in response to
243 mannitol were confirmed using the ABA sensor ABAleon2.1 (Waadt et al. 2014) that was
244 introduced in wild type and *pskr1-3 pskr2-1*. Photobleaching-based FRET analysis
245 (Supplemental Fig. S7) revealed a decreased FRET efficiency, reporting elevated ABA,
246 in wild type but not in *pskr1-3 pskr2-1* in response to mannitol (Fig. 6C).The difference in
247 absolute FRET efficiency between genotypes may be owed to the fact that the lines
248 were transformed rather than introgressed.
249 We next explored whether PSKR signaling affected the sensitivity to ABA in
250 mannnitol-exposed seedlings. A dose-response analysis showed that ABA at 0.1 µM
251 and higher inhibited shoot growth in wild type under control conditions in a concentration
252 dependent manner whereas *pskr1-3 pskr2-1* seedling growth was inhibited at 0.3 µM
253 ABA and higher (Fig. 6D, E). In the presence of mannitol, shoot growth of wild type was
254 promoted at 0.01 µM ABA and inhibited at 0.3 µM ABA and higher while growth of
255 *pskr1-3 pskr2-1* seedlings increased between 0.1 and 1 µM ABA and decreased at
256 higher ABA concentrations indicating that PSKR signaling altered the sensitivity to ABA
257 under mild mannitol stress. The reduced leaf number in mannitol exposed *pskr1-3*

258 *pskr2-1* seedlings was restored to wild type level at 0.3-1 μM ABA (Supplemental Fig.
259 S4). In summary, the responsiveness of seedlings that lack PSKR receptors is shifted to
260 higher ABA concentrations compared to wild type. The need for higher ABA required to
261 promote shoot growth may be explained by a lower endogenous ABA content fostering
262 the idea that PSKR promotes growth in part by increasing ABA in response to mannitol.
263 It should be noted, however, that growth inhibition by mannitol was only partially
264 alleviated by ABA in *pskr1-3 pskr2-1* suggesting that other levels of regulation exist. To
265 gain more insight into the network that connects ABA with mannitol and PSKR
266 pathways, we analyzed expression of genes involved in ABA homeostasis (Fig. 6F).

267 To identify the molecular targets of PSKR signaling in ABA metabolism, we
268 analyzed key ABA biosynthetic *NCED* genes, *CYP707A1-4* genes coding for ABA 8'-
269 hydroxylases that degrade ABA to phaseic acid (Kushiro et al., 2004; Saito *et al*., 2004;
270 Okamoto et al., 2006), *UGT71B6-8* genes coding for ABA uridine diphosphate
271 glucosyltransferases that catalyze ABA inactivation through glycosylation (Dong et al.,
272 2014), and *BG1* and *BG2* genes encoding glucosidases that mobilize ABA from ABA-
273 glucose ester (Lee et al., 2006; Xu et al., 2012) (Fig. 6F). *NCED3* and *5* transcripts were
274 more abundant in young leaves than *NCED9* transcripts (Fig. 7A). *NCED3* was
275 upregulated by mannitol whereas *NCED5* was induced by ABA (Fig. 7B-D). However,
276 PSKR signaling did not significantly alter *NCED* expression.

277 *CYP707A2* and *A3* were expressed at higher levels than *CYP707A1* and
278 *CYP707A4* confirming a previous report (Fig. 8A) (Kushiro *et al*., 2004). A more detailed
279 analysis revealed induction of *CYP707A1*, *A2* and *A4* by 1 μM and 5 μM ABA in wild
280 type indicating increased ABA degradation when plants encounter high ABA (Fig. 8B-E;
281 Supplemental Fig. S8). Mannitol did not alter mRNA abundance of *CYP707A* genes. In
282 *pskr1-3 pskr2-1*, *CYP707A1* transcripts were elevated over wild type revealing
283 repression of this gene by PSKR signaling (Fig. 8B). By contrast, *CYP707A2* and *A4*
284 transcripts decreased in PSKR-deficient plants indicative of gene activation by PSKR
285 signaling (Fig. 8C) while transcript levels of *CYP707A3* were not significantly different
286 between mutant and wild type (Fig. 8D; Supplemental Fig. S8). Taken together,
287 *CYP707A1*, *A2* and *A4* are targets of PSK receptor signaling. Since *CYP707A1* was
288 upregulated and *CYP707A2* and *CYP707A4* were downregulated in mannitol stressed

289 *pskr1-3 pskr2-1* plants, it is difficult to assess the contribution of ABA degradation to
290 overall ABA levels.
291 Among the three ABA UDP-glucose transferase genes *UGT71B6-B8* (Fig. 9),
292 *UGT71B6* transcripts were elevated in wild type and hyperinduced in *pskr1-3 pskr2-1*
293 plants exposed to mannitol (Fig. 9B). *UGT71B7* was upregulated by mannitol and ABA
294 in both genetic backgrounds (Fig. 9C), while *UGT71B8* was not regulated. These data
295 suggested that ABA conjugation is enhanced under mannitol stress in PSKR-deficient
296 plants compared to wild type.
297 Genes coding for the ABA glucosidases BG1 and BG2 were upregulated by ABA
298 in wild type and *pskr1-3 pskr2-1* while mannitol reduced *BG1* expression in *pskr1-3*
299 *pskr2-1* compared to wild type (Fig. 9E-G; Supplemental Fig. S9) suggesting that PSKR
300 signaling increases ABA levels by releasing it from the ABA-glucose pool. In summary,
301 repression of *UGT71B6* and enhanced expression of *BG1* by PSKR signaling may be
302 responsible for ABA accumulation in response to mannitol.
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304
305 **PSKR-dependent marker gene expression in response to ABA and mannitol**
306
307 Since ABA responsiveness was controlled by PSKR signaling (Fig. 1A, B, Fig. 6D, E,
308 Supplemental Fig. S3), we studied expression of ABA- and mannitol-responsive genes
309 in wild type and PSKR-deficient seedlings (Fig. 10A-C). The dehydration-responsive
310 element/C-repeat (DRE/CRT)-binding protein 2 (DREB2) transcription factor plays a key
311 role in ABA-independent gene expression in response to osmotic stress such as induced
312 by mannitol while also interacting with ABA-dependent gene expression (Yoshida et al.,
313 2014). *DREB2A* transcripts were elevated in *pskr1-3 pskr2-1* but not in wild-type
314 seedlings in response to mannitol but not ABA (Fig. 10A). Similarly, the ABA receptor
315 gene *PYL6* was induced by mannitol in the receptor null mutant compared to wild type
316 (Fig. 10B) as was the basic helix-loop-helix transcriptional repressor gene *bHLH129*
317 (Tian et al., 2015) (Fig. 10C) providing evidence that these genes are repressed by
318 PSKRs during mannitol stress signaling possibly to dampen the stress response and to
319 allow for seedling growth. Analysis of knockout lines for these and other genes related to
320 ABA synthesis (*ABA2, NCED3, NCED9*) and signaling (*PYL6*, *OST1*, *SnRK2.9*, *ABI1*,

321 *ABI3*, *bHLH129*, *DREB2A*) revealed that their response to mannitol with regard to shoot
322 growth was indistinguishable from wild type and did hence not phenocopy *pskr1-3*
323 *pskr2-1* seedlings (Fig. 10D) leading us to conclude that these genes are not required
324 for or act redundantly in PSKR-mediated growth control during mannitol stress.
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328 # DISCUSSION

329

330 **Inhibition of cotyledon greening by ABA or mannitol is dependent on PSK**
331 **receptor signaling**

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333 Seed germination and seedling establishment are stringently controlled by
334 environmental conditions to ensure seedling survival. In particular, water availability is a
335 requirement for seedling establishment. Under water-limited conditions, ABA acts as a
336 hormonal signal that prevents delays germination and early seedling development. Here
337 we showed that ABA and mannitol delayed seed germination independent of PSKR
338 signaling whereas inhibition of cotyledon greening by ABA or mannitol was PSKR-
339 dependent. *pskr1-3 pskr2-1* seedlings displayed accelerated cotyledon greening and
340 *PSKR1* overexpressors were hypersensitive to ABA and mannitol with delayed
341 cotyledon greening suggesting that PSKR signaling altered the responsiveness to ABA
342 and mannitol pointing to crosstalk between these pathways.

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345 **PSK ligand and receptor are induced by mannitol and ABA to promote shoot**
346 **growth**

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348 Growth of plant shoots was supported by PSKR signaling particularly during mannitol
349 stress. Plants exposed to mannitol displayed elevated transcript levels of *PSKR1* and
350 *PSK3,* that were also induced by ABA suggesting that the PSK signal pathway is
351 promoted by mannitol signaling through ABA to actively maintain shoot growth. A crucial
352 role of *PSKR1* in promoting mannitol resistance was revealed in single receptor gene
353 knockout lines. *pskr1-3* seedlings displayed the same growth retardation as seedlings of
354 the double receptor knock out line *pskr1-3 pskr2-1* whereas *pskr2-1* seedlings had a wild
355 type phenotype. Mannitol and ABA exerted common but also differential control of PSK
356 signaling genes pointing to a fine-tuned regulatory network rather than a linear pathway
357 of growth control. *PSK2* and *PSK4* were induced by ABA but not by mannitol revealing
358 ABA regulation of PSK signaling in a pathway that is unrelated to mannitol. Intricate
359 regulatory loops were revealed by hyperinduction of *PSK1*, *PSK3* and *PSK5* by mannitol

360 in the PSK receptor null background compared to wild type which suggested that PSK
361 signaling feedback-inhibits expression of ligand precursor genes under mannitol stress
362 possibly to balance growth (Fig. 11). The regulated signaling genes *PSK3* and *PSKR1*
363 were expressed in young expanding leaves in accord with their role in cell expansion
364 (Hartmann et al., 2013; Ladwig et al., 2015). It was suggested that plant cells
365 communicate via extracellular signals and plasma membrane-bound receptors despite
366 of a continuous network of plasmodesmata that enable cell-cell communication because
367 apoplastic signals allow for signal integration and output coordination (Chivasa and
368 Goodman, 2020). Induction of PSK signaling may allow for coordinated leaf growth in
369 particular when leaves encounter local mannitol accumulation e.g. from mannitol-
370 producing pathogens (Jennings et al., 2002; Mosher et al., 2013; Meena et al., 2015).
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372
373 **ABA homeostasis under mannitol stress is controlled through the PSKR pathway**
374
375 Exposure to mannitol increased ABA levels in a PSKR-dependent manner raising the
376 question how PSK signaling controls ABA homeostasis at the molecular level. The
377 steady-state level of active ABA is determined by ABA synthesis, catabolism,
378 inactivation and remobilization (Cutler and Krochko, 1999; Nambara and Marion-Poll,
379 2005). Nine-*cis*-epoxycarotenoid dioxygenases (NCEDs) are key enzymes of ABA
380 synthesis that cleave 9-*cis*-xanthophylls to xanthoxin, a precursor of ABA. Arabidopsis
381 NCED3 was previously described as the major stress-induced *NCED* gene in leaves
382 with *NCED5* and *NCED9* being induced to a minor degree in response to water-deficit
383 (Tan et al., 2003). In this study, *NCED3* was induced by mannitol in wild type and
384 hyperinduced in the PSKR null mutant pointing to elevated ABA synthesis in PSKR-
385 deficient shoots. On the other hand, *CYP707A1* encoding an ABA degrading ABA 8'-
386 hydroxylase (Kushiro et al., 2004) was likewise hyperinduced in the PSK receptor null
387 mutant compared to wild type in the presence of mannitol while *CYP707A2* transcript
388 levels went down. Elevated synthesis and elevated degradation rates in *pskr1-3 pskr2-1*
389 seedlings compared to wild type could result in increased flux in the ABA
390 synthesis/degradation pathway but do not explain why ABA levels did not increase
391 under mannitol stress. The *CYP707A3* gene was previously shown to be repressed in

392 the shoot by the bHLH transcription factor bHLH122 under drought stress conditions
393 resulting in up-regulated ABA levels (Liu et al., 2014). In this study, *CYP707A3* was not
394 significantly regulated by mannitol or ABA in young leaves.

395 Aside from synthesis and degradation, ABA is subject to inactivation through
396 conjugation to glucose by UDP-glucose transferases UGT71B6, UGT71B7 and
397 UGT71B8 (Dong et al., 2014) and by remobilization of ABA from ABA-glucose stores by
398 ABA-glucose ß-glucosidases BG1 and BG2 (Lee et al., 2006; Xu et al., 2012).
399 Regulation of reversible inactivation is one level to control ABA homeostasis (Ma et al.,
400 2018). BGs were previously described as key regulators of osmotic stress resistance
401 (Xu et al., 2012). *BG1* is regulated by miR165/166. Downregulation of miR165/166
402 resulted in elevated *BG1* expression and in elevated ABA levels (Yan et al., 2016). A
403 comparison between wild type and *pskr1-3 pskr2-1* seedlings exposed to mannitol
404 revealed that PSKR signaling repressed induction of *UGT71B6* and promoted
405 expression of *BG1* possibly shifting the equilibrium between conjugation and
406 remobilization toward more free ABA. Vice versa, increased conjugation and reduced
407 ABA release from ABA-glucose ester in *pskr1-3 pskr2-1* could prevent accumulation of
408 free ABA in response to mannitol in this genotype.

409 A molecular link between ABA and PSKR pathways was established by marker
410 gene expression. The ABA receptor gene *PYL6* (Ma et al., 2009; Aleman et al., 2016)
411 was upregulated in PSKR null plants and hyperinduced by mannitol suggesting that
412 PSKR signaling dampens PYL6-induced ABA responses at the level of receptor
413 abundance. Plants respond to osmotic stress through ABA-dependent and ABA-
414 independent pathways (Yoshida et al., 2014). The dehydration-responsive element/C-
415 repeat (DRE/CRT), and DRE-/CRT-binding protein 2A (DREB2A) integrates ABA-
416 dependent and ABA-independent drought stress signaling. DREB2A promotes stress-
417 response genes and at the same time inhibits growth (Qin et al., 2008; Kim et al., 2011).
418 However, the PSKR-dependent pathway that confers resistance to growth inhibition by
419 mannitol was not observed when plants were dehydrated by PEG suggesting that
420 PSKRs do not mediate a drought response mimicked by PEG6000. Mannitol has
421 chemical properties beyond being an osmolyte that may be recognized in the growth-
422 related pathway that links it to PSKR signaling. *DREB2A* was induced in the PSKR
423 knockout line suggesting that repression of *DREB2A* may contribute to growth

maintenance by PSKRs under mannitol stress. However, even though *DREB2A* was reported to have adverse effects on plant growth (Yoshida et al., 2014), the loss-of-function mutant *dreb2* showed wild-type shoot growth in the presence of mannitol. A wild-type growth phenotype was also observed for other ABA signaling and synthesis mutants exposed to mannitol possibly indicating redundant activities from other members of the gene families that encode the ABA synthesis and signaling components (Nambara and Marion-Poll, 2005; Joshi-Saha, 2011).

CONCLUSION

PSKR signaling mediates the delay in cotyledon greening in response to ABA and mannitol and supports shoot growth in plants exposed to mannitol. ABA, mannitol and PSKR signaling pathways are intricately linked as evident from the regulation of *PSK* and *PSKR* genes by mannitol and ABA and by the regulation of ABA levels by PSKR signaling under mannitol stress. Regulation of ABA levels could result from PSKR-dependent repression of *UGT71B6* responsible for ABA conjugation and upregulation of *BG1* responsible for release of ABA from ABA-glucose. Our study reveals a role of PSKR signaling in balancing growth during mannitol stress through interaction with ABA homeostasis.

448 # METHODS

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450

451 **Plant material and growth conditions**

452

453 All experiments were performed with *Arabidopsis thaliana* (L.) Heynh. ecotype Columbia
454 (Col-0) and mutants in the Col-0 background. The following lines were described
455 previously: *pskr1-3* (Kutschmar *et al*., 2009; Stührwohldt *et al*., 2011), *pskr2-1* (Amano *et*
456 *al*., 2007; Stührwohldt *et al*., 2011), *pskr1-2 pskr2-1* (Stührwohldt *et al*., 2011; Hartmann
457 *et al*., 2013), *PSKR1ox2* and *PSKR1ox12* (Hartmann *et al*., 2013), *35S:PSKR1-GFP*
458 (Hartmann *et al*., 2015). For GUS analyses of PSK receptors the lines *PSKR1:GUS-4*
459 (Kutschmar *et al*., 2009; Stührwohldt *et al*., 2011) and *PSKR2:GUS-3* were used. For
460 growth on plates, seeds were surface-sterilized for 25 min with 2% (w/v) sodium
461 hypochlorite (NaOCl) followed by four washing steps with autoclaved water and placed
462 on square plates containing half-strength MS medium (Murashige & Skoog, 1962; basal
463 salt mixture, Duchefa Biochemie) and 1% (w/v) sucrose except for PEG6000
464 experiments with 0.5% (w/v) sucrose (Supplemental Fig. S6), solidified with 0.4% (w/v)
465 Gelrite (Duchefa Biochemie). After two days of stratification at 4°C in the dark, plates
466 were transferred to long day with a 16 h light (70 µM photons$\cdot m^{-2} \cdot s^{-1}$) and 8 h dark at
467 22°C and 60% humidity.

468 For germination and greening assays, wild type and mutant parental plants were
469 grown and harvested at the same time to ensure equal seed quality. Seeds were placed
470 on medium supplemented with or without 1 µM (+)-*cis*, *trans*-abscisic acid (ABA)
471 (Duchefa Biochemie), 350 µM mannitol and/or 1 µM PSK (Pepscan). PSK and ABA
472 were always added freshly. ABA was diluted right before use. The penetration of the
473 endosperm or testa by the embryo radicle was counted as successful germination event.
474 Three independent experiments were performed with 100 seeds each per genotype per
475 treatment.

476 For shoot growth analyses under stress conditions, seedlings were pregrown
477 vertically under sterile conditions for 4 days to complete germination and cotyledon
478 greening (Supplemental Fig. S3) and subsequently transferred to new plates
479 supplemented with mannitol, sorbitol, polyethylene glycol 6000 (PEG6000), and/or ABA

480 as indicated and grown for the times indicated. Growth was quantified with a fine scale
481 as shoot fresh weight or shoot dry weight. Plant pictures are shown on a black
482 background for better visualization. Primary root lengths were determined 7 days after
483 transfer of 4-day-old seedlings to mannitol because the roots reached the bottom of the
484 plate after longer times making it impossible to measure root lengths lateron.
485
486
487 **Histochemical GUS analysis**
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489 To analyze spatial distribution of *PSKR* and *PSK* expression *β*-glucuronidase (GUS)
490 assays were performed as described (Weigel & Glazebrook, 2002) with minor changes.
491 Seedlings were collected in 90% (v/v) isopropanol, incubated for 10 minutes and
492 washed with 50 mM sodium phosphate buffer (pH 7.2). Staining was performed for 15 h
493 and stopped by transferring the seedlings to 70% (v/v) ethanol. Tissues were cleared
494 with a chloral hydrate:deionized water:glycerol 6:2:1 (g/ml/ml) mixture that was added on
495 microscopy slides instead of water. Seedlings were visualized under bright-field
496 illumination with a Nikon SMZ18 binocular (Nikon) and photographed with a DIGITAL
497 SIGHT-Ri1 camera (Olympus).
498
499
500 **RNA isolation, RT-PCR and quantitative real time PCR**
501
502 Total RNA was isolated from true leaves using TRI Reagent (Merck) following
503 manufacturer's protocol. cDNA was synthesized from 1 µg DNase I-treated (Thermo
504 Fisher Scientific) total RNA by oligo(dT)-primed reverse transcription using RevertAid
505 Reverse Transcriptase (Thermo Fisher Scientific). To avoid gDNA contamination and to
506 ensure an equal amount of RNA input, cDNA was tested by PCR for *ACTIN2*
507 (At3g18780) transcripts prior to qPCR analysis with the primers ACT2for
508 5'-CAAAGACCAGCTCTTCCATCG-3' and ACT2rev 5'-
509 CTGTGAACGATTCCTGGACCT-3'. qPCR was used to examine the expression of
510 *PSKR1*, *PSKR2*, *PSK1*, *PSK2*, *PSK3*, *PSK4*, *PSK5*, *NCED3*, *NCED5*, *NCED9*,
511 *CYP707A1*, *CYP707A2*, *CYP77A3*, *CYP707A4*, *UGT71B6*, *UGT71B7*, *UGT71B8*, *BG1*,

512 *BG2*, *DREB2A*, *PYL6* and *bHLH129* with primers listed in Supplemental Table 1 using
513 the Rotor Gene SYBR Green PCR Kit (Qiagen) according to manufacturers' protocol in
514 a Rotor gene Q cycler (Qiagen). Ten ng cDNA per sample were applied in a total volume
515 of 15 µl. Estimation of raw data was done with the Rotor-Gene Q 2.3.1.49 (Qiagen)
516 program.
517 The relative transcript abundance was calculated based on the ΔΔCP method
518 including primer efficiency to normalize the data with two reference genes (Pfaffl, 2001;
519 Van Desompele *et al*., 2007). In relation to each reference gene (*ACT2* and
520 *GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE 1* (*GAPC1*)) (Supplemental
521 Table 1) values were averaged from three independent biological replicates with two
522 technical replicates each. The expression of wild type under control conditions was set
523 to 1 and all other values were calculated as fold change of that.
524
525
526 **ABA measurements**
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528 Four-day-old seedlings were transferred to medium containing mannitol as indicated and
529 grown for additional 7 days. Roots and shoots were harvested separated and
530 immediately frozen in liquid nitrogen. About 50 mg of homogenized, frozen material was
531 extracted with 500 µl of methanol containing isotope-labelled internal standard $^{2}H_{6}$-ABA
532 (0.1 ng μl^{-1}) followed by centrifugation. The supernatant was diluted with 4.5 ml water
533 and subjected to solid-phase extraction on HR-XC (Chromabond, Macherey-Nagel,
534 Düren, Germany) column. Fractions containing ABA were eluted with acetonitrile and
535 separated using the ACQUITY UPLC System (Waters, Eschborn, Germany) (Balcke et
536 al. 2012). Detection of ABA and $^{2}H_{6}$-ABA was done by ESI-tandem mass spectrometry
537 (MS/MS) using a 3200 Q TRAP® LC/MS/MS mass spectrometer (Waters) (Balcke et al.,
538 2012). ABA content per sample was calculated using the ratio of ABA and $^{2}H_{6}$-ABA peak
539 heights. Data were obtained from 5 biological replicates each.
540 To an alternative method to measure changes in ABA levels in response to
541 mannitol, we obtained the *ABAleon2.1* line and the plasmid *barII-UT-ABAleon2.1* from
542 Rainer Waadt (Waadt et al. 2014) that we used to generate a homozygous *pskr1-3*
543 *pskr2-1 ABAleon2.1* line. Plants were transformed with *Agrobacterium tumefaciens*

544 *EHA105,* containing the *barII-UT-ABAleon2.1* construct with the floral dip method
545 (Clough and Bent, 1998). The progeny were selected with BASTA®. In the T2 generation
546 the seedlings were additionally screened with the FastGene® Blue/Green LED Flashlight
547 (Nippon Genetics Europe GmbH) with an orange/blue filter to exclude silencing of the
548 transgene. Acceptor photobleaching Förster resonance energy transfer (FRET) was
549 used to measure relative differences in ABA levels in each genotype using a Leica SP5
550 CLSM. Cells were excited sequentially at 458 and 514 nm and emission recorded with
551 adequate filter sets. Post-bleach images were captured at 458 nm excitation
552 (Supplemental Fig. S7). FRET is visualized as an increase in mTurquoise fluorescence
553 following cpVenus173 photobleaching (Supplemental Figure S7). FRET efficiency was
554 calculated according to the formula $FRET_{eff} = (D_{post} - D_{pre})/D_{post}$ with D_{post}=fluorescence
555 intensity of the donor after acceptor photobleaching and D_{pre}=fluorescence intensity of
556 the donor before acceptor photobleaching.
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561 **Statistical analysis**
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563 Statistical analyses were done using Minitab® 16.1. Data that were not ditributed
564 normally were evaluated using a Kruskal-Wallis or Mann-Whitney test for pairwise
565 comparison. Normally distributed data were tested for equal variance. In the case of
566 equal variance, ANOVA, otherwise a *t*-test for pairwise comparison was chosen.
567
568
569 **Accession numbers**
570
571 *ACT2* - At3g18780, *GAPC1* - At3g04120, *PSKR1* - At2g02220, *PSKR2* - At5g53890,
572 *PSK1* - At1g13590, *PSK2* - At2g22860, *PSK3* - At3g44735, *PSK4* - At3g49780, *PSK5* -
573 At5g65870, *NCED3* - At3g14440, *NCED5* - At1g30100, *NCED9* - At1g78390,
574 *CYP707A1* - At4g19230, *CYP707A2* - At2g29090, *CYP707A3* - At5g45340, *CYP707A4*

575 - At3g19270, *UGT71B6* - At3g21780, *UGT71B7* - At3g21790, *UGT71B8* - At3g21800,
576 *BG1* - At1g52400, *BG2* - At2g32860, *DREB2A* - At5g05410, *PYL6* - At2g40330,
577 *bHLH129* - At2g43140.
578
579
580
581 **ACKNOWLEDGEMENTS**
582
583 Hagen Stellmach (IPB Halle) is acknowledged for helping in ABA measurements. We
584 are grateful to Rainer Waadt for providing the *ABAleon2.1* construct and line and to
585 Lena Carstens for generating the *pskr1-3 pskr2-1 ABAleon2.1* reporter line.
586
587

588

589 # FIGURE LEGENDS

590

591

592 **Figure 1. Repression of early seedling development by ABA or mannitol is**
593 **mediated by PSK receptor signaling.**
594 **A** Cotyledon greening of wild type, *pskr1-3 pskr2-1*, and *PSKR1* overexpressing
595 seedlings exposed to 1 µM ABA or 350 mM mannitol or no treatment for the times
596 indicated. The scheme on the lower left side indicates the genotypes grown on each
597 plate
598 **B** Cotyledon greening of 8-day-old wild type seedlings, *PSKR* knockout and of three
599 independent *PSKR1* overexpressing lines treated with 1 µM PSK, 1 µM ABA, or both.
600 Data are means ±SE of 3 independent experiments each evaluating 100 seeds per
601 genotype. Different letters indicate significantly different values (one-way ANOVA,
602 Tukey's test, $p<0.05$).
603 **C** Cotyledon greening of 8-day-old wild type seedlings, *PSKR* knockout and of three
604 independent *PSKR1* overexpressing lines treated with 1 µM PSK, 350 mM mannitol, or
605 both. Data are means ±SE of 3 independent experiments each evaluating 100 seeds per
606 genotype. Different letters indicate significantly different values (one-way ANOVA,
607 Tukey's test, $p<0.05$).

608

609

610 **Figure 2. PSK receptor signaling promotes shoot growth under mannitol stress.**
611 Four-day-old wild type and *pskr1-3 pskr2-1* seedlings were transferred to media
612 containing mannitol as indicated.
613 **A** Shoot phenotypes of wild type (wt) and *pskr1-3 pskr2-1* plants grown for 2 weeks.
614 Scale bars = 10 mm.
615 **B** Shoot fresh weights of wt and *pskr1-3 pskr2-1* plants grown as in A.
616 **C** Shoot dry weights of wt and *pskr1-3 pskr2-1* plants analyzed in B.
617 **D** Leaf numbers, sizes and morphology of wild type and *pskr1-3 pskr2-1* plants grown on
618 mannitol-free medium or on 25 mM mannitol for 3 weeks. Scale bar = 10 mm.

619 **E** Primary root lengths of seedlings grown for 7 days after transfer. (B, C, E) Significantly
620 different values within a genotype are denoted by different letters (Kruskal-Wallis,
621 Tukey‘s ,test, $p<0.05$, n≥36, 3 independent experiments). Asterisk indicates significant
622 differences between genotypes (Mann-Whitney test, $P<0.05$).
623
624
625 **Figure 3. *PSKR1* promotes growth under mild mannitol stress.**
626 Four-day-old wild type, *pskr1-3*, *pskr2-1,* and *pskr1-3 pskr2-1* seedlings were transferred
627 to media with or without 50 mM mannitol.
628 **A** Shoot phenotypes after 2 weeks; scale bar = 10 mm.
629 **B** Mean (±SE) shoot fresh weights of seedlings after 3 weeks. Different letters indicate
630 significant differences (Kruskal-Wallis, Tukey's test, $p<0.05$, n≥36, 3 independent
631 experiments).
632
633
634 **Figure 4. *PSK* genes are differentially regulated by mannitol, ABA and *PSKR***
635 **signaling.**
636 **A** GUS staining of *PSK1:GUS-3*, *PSK2:GUS-2*, *PSK3:GUS-3*, *PSK4:GUS-4* and
637 *PSK5:GUS-1* seedlings; scale bar = 2 mm.
638 **(B-F)** Seven-day-old seedlings were exposed to 200 mM mannitol or 5 µM ABA for 1 day
639 for qPCR analyses in first true leaves. Results (±SE) are averages from 3 biological
640 replicates with two technical repeats each. Different letters indicate significant
641 differences.
642 **B** Transcript levels of PSK genes relative to *PSK3* that was set to 1.
643 **C-F** Relative transcript levels of *PSK1-5* in wild type and in *pskr1-3 pskr2-1* seedlings
644 treated with mannitol or ABA as indicated (B, F: Kruskal-Wallis, Tukey's test, $p<0.05$; C-
645 E: one-way ANOVA, Tukey‘s test, $p<0.05$).
646
647
648 **Figure 5. *PSKR1* expression is induced by mannitol and ABA.**
649 **A-F** *PSKR1:GUS-4* activity in 7-day-old seedlings treated with 50 mM mannitol for 1 day
650 or left untreated as a control. (A-C) Expression in shoot, and root under control

conditions. (D-F) Expression in shoot, and root under mannitol. Arrowheads point at true leaves; scale bar = 0.5 mm (A, D), = 0.1 mm (B, E), = 2 mm (C, F).

G *PSKR2:GUS-3* activity in the shoot; scale bar = 0.5 mm.

H, I Seven-day-old seedlings exposed to 50 mM, 200 mM mannitol, 1 µM or 5 µM ABA for 1 day or left untreated as a control. Relative transcript levels of (H) *PSKR1* and (I) *PSKR2* were analyzed by RT-qPCR in true leaves. Values are means (±SE); different letters indicate significant differences (one-way ANOVA, Tukey‘s test, $p<0.05$, 3 biological replicates).

Figure 6. PSKR and ABA pathways interact.

A Four-day-old wild type and *pskr1-3 pskr2-1* seedlings were transferred to 0 mM, 50 mM or 200 mM mannitol and ABA levels in the shoot were determined after 7 days. Different letters indicate statistically significant differences (Kruskal-Wallis, Tukey‘s test, $p<0.05$, 5 biological replicates).

B ABA levels in roots of seedlings treated as in A (one-way ANOVA, Tukey‘s test, $p<0.05$, 5 biological replicates).

C ABA levels in the first true leaf were compared by photobleaching-based FRET analysis in wild type and *pskr1-3 pskr2-1* seedlings treated as in A. Decreased FRET efficiency indicates elevated ABA levels. Different capital and minor letters indicate significantly different values between treatments (Mann-Whitney test, $p<0.05$, $n\geq142$, 3 independent experiments).

D Shoot fresh weights of wild type seedlings that were transferred after 4 days to plates containing ABA and mannitol at the concentrations indicated were determined after 2 weeks.

E Shoot fresh weight of *pskr1-3 pskr2-1* seedlings grown as described in (D). (D, E) Different capital and minor letters indicate significantly different values within a given treatment (Kruskal-Wallis, Tukey‘s test, $p<0.05$; n=36).

F Overview of genes encoding for enzymes of ABA synthesis (*NCED*s), degradation (*CYP707A*s), inactivation (*UGT71B*s) and mobilization (*BG*s). PA=phaseic acid.

Figure 7. *NCED* genes are differentially regulated by mannitol and ABA.

Seven-day-old wild type and *pskr1-3 pskr2-1* seedlings were exposed to 200 mM mannitol or 5 µM ABA for 1 day to analyze relative *NCED3, 5* and *9* transcript levels in true leaves.

A Relative *NCED* mRNA levels in wild type under control conditions (Kruskal-Wallis, Tukey‘s test, $p<0.05$; 3 biological replicates with two technical replicates each).

B-D Changes in *NCED3*, *NCED5* and *NCED9* transcript levels in response to mannitol, ABA and genotype. Values are means (±SE) with different letters indicating significant differences (Kruskal-Wallis, Tukey‘s test, $p<0.05$; n=6, 3 biological replicates).

Figure 8. Regulation of *CYP707A* ABA-8‘-hydroxylase transcripts by mannitol, ABA and PSKR signaling.

Seven-day-old wild type and *pskr1-3 pskr2-1* seedlings were exposed to 200 mM mannitol or 5 µM ABA for 1 day to analyze gene expression in true leaves.

A Relative *CYP707A* transcript levels in wild type under control conditions. Values are means (±SE) with different letters indicating significant differences (Kruskal-Wallis, Tukey‘s test, $p<0.05$, n=6; 3 biological replicates).

B-E Relative *CYP707A1-4* transcript levels in response to genotype and treatment (Kruskal-Wallis, Tukey‘s test, $p<0.05$, n=6, 3 biological replicates).

Figure 9. Regulation of *UGT71B* and *BG* transcripts by mannitol, ABA and PSKR signaling.

Seven-day-old wild type and *pskr1-3 pskr2-1* seedlings were exposed to 200 mM mannitol or 5 µM ABA for 24 h for qPCR analysis in true leaves. Values are means (± SE) of 3 biological replicates with different letters indicating significant differences

A Relative *UGT71B6-8* transcript abundance in wild type under control conditions.

B-D Relative mRNA levels of *UGT71B6-8* in response to genotype and treatment (Kruskal-Wallis, Tukey‘s test, $p<0.05$).

E Relative *BG* transcript abundance in wild type under control conditions.

F, G Relative mRNA levels of *BG1 and 2* in response to genotype and treatment (one-way ANOVA, Tukey‘s test, $p<0.05$).

715 **Figure 10. Regulation of ABA and mannitol-responsive genes and their impact on**
716 **growth under low mannitol stress.**
717 Seven-day-old wild type and *pskr1-3 pskr2-1* seedlings were exposed to 200 mM
718 mannitol or 5 µM ABA for 24 h to analyze gene expression in true leaves. Values are
719 means (±SE) of 3 biological replicates with different letters indicating significant
720 differences.
721 **A** Relative transcript levels of the osmotic stress response gene *DREB2A* (Kruskal-
722 Wallis, Tukey‘s test, $p<0.05$).
723 **B** Relative transcript level of the ABA receptor gene *PYL6* (one-way ANOVA, Tukey test,
724 $p<0.05$).
725 **C** Relative transcript level of the transcriptional repressor *bHLH129* (Kruskal-Wallis,
726 Tukey‘s test, $p<0.05$).
727 **D** Four-day-old wild type and knockout seedlings were exposed to 50 mM mannitol for
728 21 days. Capital letters indicate significant differences between lines under control
729 conditions. Percentages show values of treated compared to untreated seedlings within
730 each genotype. Minorcase letters indicate significant differences between lines exposed
731 to mannitol (one-way ANOVA, Tukey‘s test, $p<0.05$; $n\geq18$, 2 biological experiments).
732
733
734
735 **Figure 11. Model of shoot growth control during mannitol stress by PSKR1 and**
736 **ABA.**
737 Mannitol leads to elevated ABA that promotes PSK and PSKR gene expression. PSKR
738 signaling, in a feedback regulatory loop, targets ABA metabolic genes that may control
739 ABA accumulation. PSKR signaling maintains shoot growth in the presence of low levels
740 of mannitol likely by interacting with ABA synthesis and signaling.
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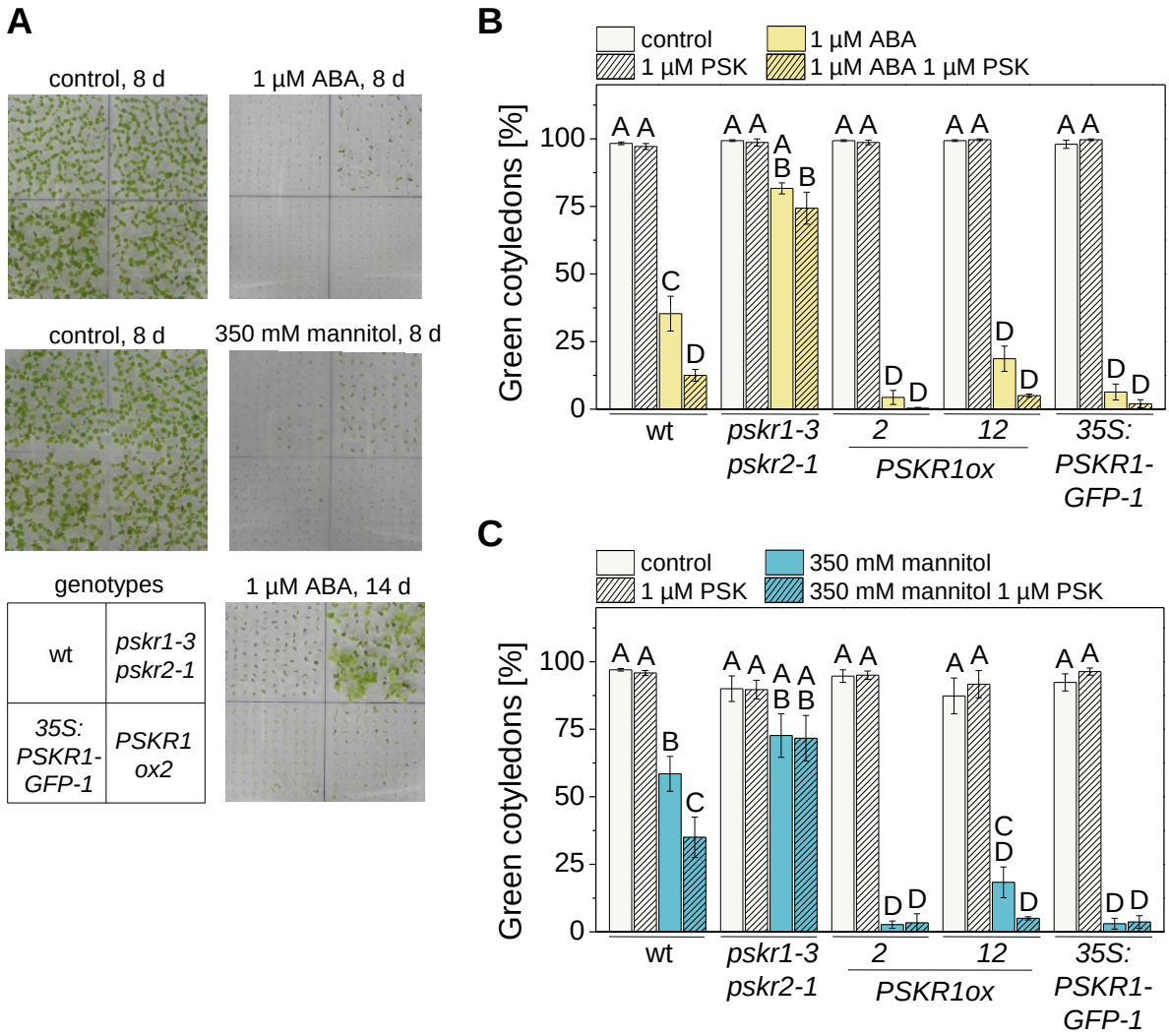

Figure 1. Repression of early seedling development by ABA or mannitol is mediated by PSK receptor signaling.

A Cotyledon greening of wild type, *pskr1-3 pskr2-1*, and *PSKR1* overexpressing seedlings exposed to 1 µM ABA or 350 mM mannitol or no treatment for the times indicated. The scheme on the lower left side indicates the genotypes grown on each plate

B Cotyledon greening of 8-day-old wild type seedlings, *PSKR* knockout and of three independent *PSKR1* overexpressing lines treated with 1 µM PSK, 1 µM ABA, or both. Data are means ±SE of 3 independent experiments each evaluating 100 seeds per genotype. Different letters indicate significantly different values (one-way ANOVA, Tukey's test, $p<0.05$).

C Cotyledon greening of 8-day-old wild type seedlings, *PSKR* knockout and of three independent *PSKR1* overexpressing lines treated with 1 µM PSK, 350 mM mannitol, or both. Data are means ±SE of 3 independent experiments each evaluating 100 seeds per genotype. Different letters indicate significantly different values (one-way ANOVA, Tukey's test, $p<0.05$).

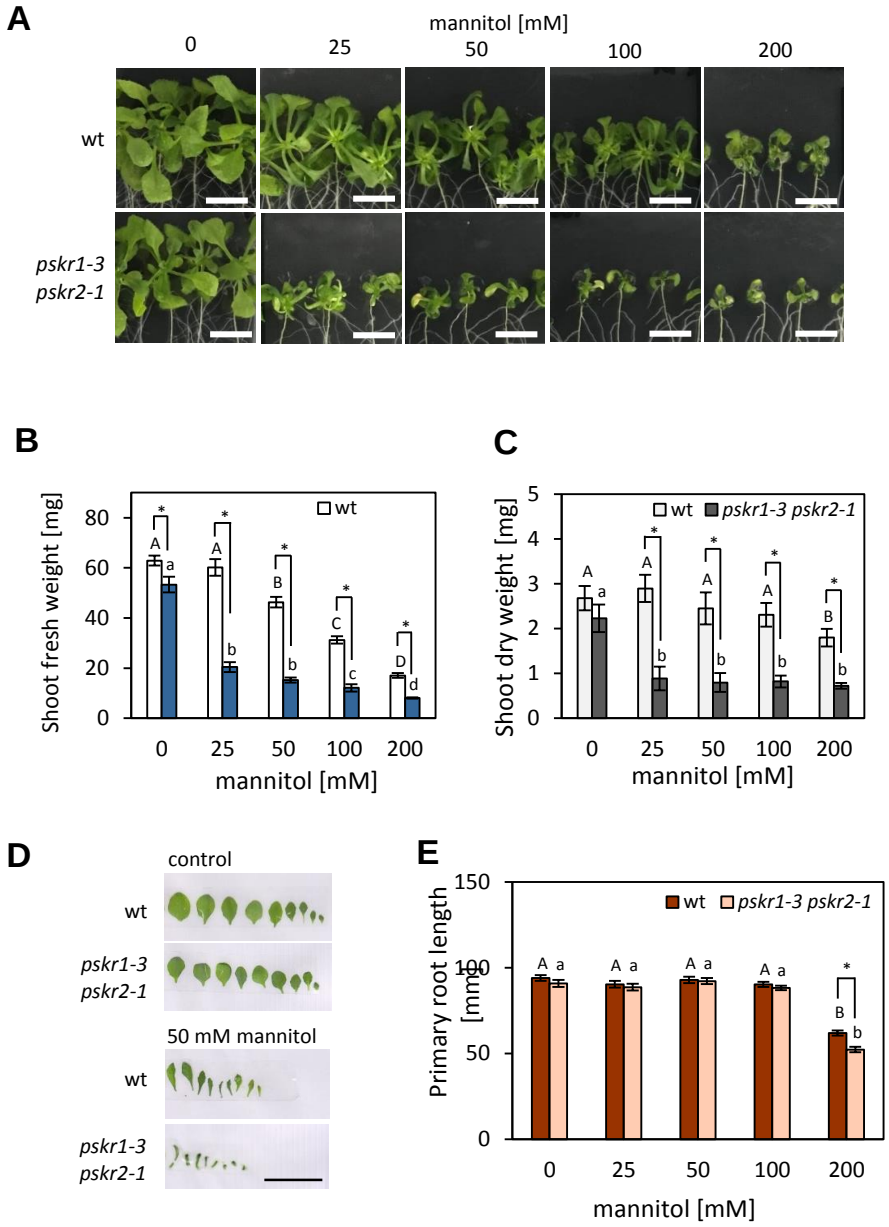

Figure 2. PSK receptor signaling promotes shoot growth under mannitol stress.
Four-day-old wild type and *pskr1-3 pskr2-1* seedlings were transferred to media containing mannitol as indicated.
A Shoot phenotypes of wild type (wt) and *pskr1-3 pskr2-1* plants grown for 2 weeks. Scale bars = 10 mm.
B Shoot fresh weights of wt and *pskr1-3 pskr2-1* plants grown as in A.
C Shoot dry weights of wt and *pskr1-3 pskr2-1* plants analyzed in B.
D Leaf numbers, sizes and morphology of wild type and *pskr1-3 pskr2-1* plants grown on mannitol-free medium or on 25 mM mannitol for 3 weeks. Scale bar = 10 mm.
E Primary root lengths of seedlings grown for 7 days after transfer. (B, C, E) Significantly different values within a genotype are denoted by different letters (Kruskal-Wallis, Tukey‘s ,test, $p<0.05$, $n\geq36$, 3 independent experiments). Asterisk indicates significant differences between genotypes (Mann-Whitney test, $p<0.05$).

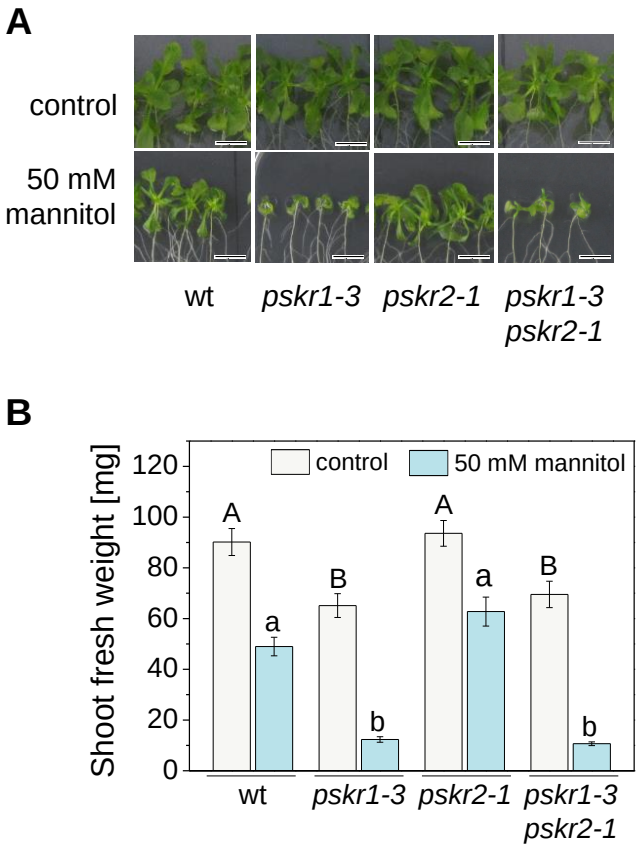

Figure 3. *PSKR1* promotes growth under mild mannitol stress.
Four-day-old wild type, *pskr1-3*, *pskr2-1,* and *pskr1-3 pskr2-1* seedlings were transferred to media with or without 50 mM mannitol.
A Shoot phenotypes after 2 weeks; scale bar = 10 mm.
B Mean (±SE) shoot fresh weights of seedlings after 3 weeks. Different letters indicate significant differences (Kruskal-Wallis, Tukey's test, $p<0.05$, n≥36, 3 independent experiments).

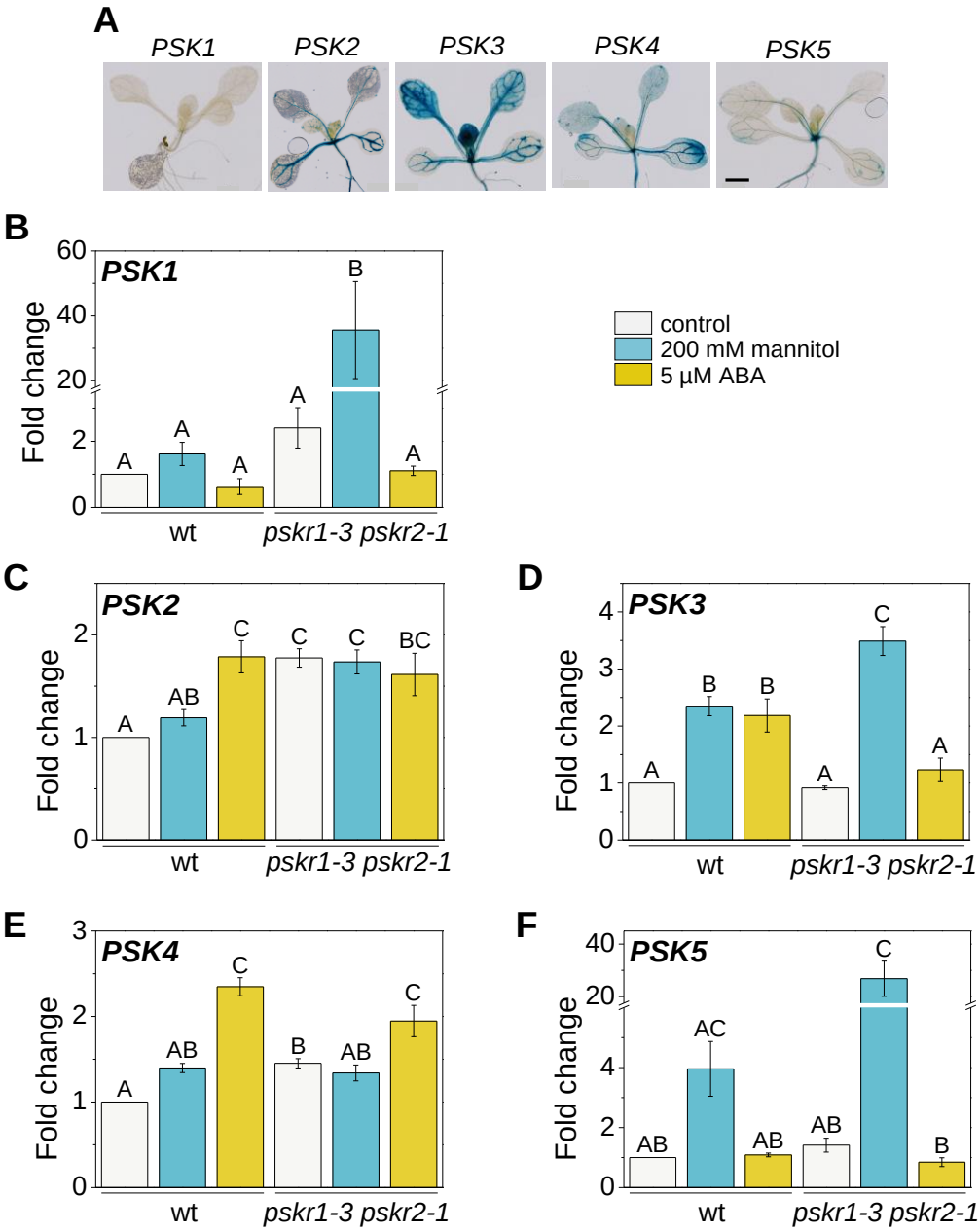

Figure 4. *PSK* genes are differentially regulated by mannitol, ABA and *PSKR* signaling.

A GUS staining of *PSK1:GUS-3*, *PSK2:GUS-2*, *PSK3:GUS-3*, *PSK4:GUS-4* and *PSK5:GUS-1* seedlings; scale bar = 2 mm.

(B-F) Seven-day-old seedlings were exposed to 200 mM mannitol or 5 µM ABA for 1 day for qPCR analyses in first true leaves. Results (±SE) are averages from 3 biological replicates with two technical repeats each. Different letters indicate significant differences.

B Transcript levels of PSK genes relative to *PSK3* that was set to 1.

C-F Relative transcript levels of *PSK1-5* in wild type and in *pskr1-3 pskr2-1* seedlings treated with mannitol or ABA as indicated (B, F: Kruskal-Wallis, Tukey's test, $p<0.05$; C-E: one-way ANOVA, Tukey's test, $p<0.05$).

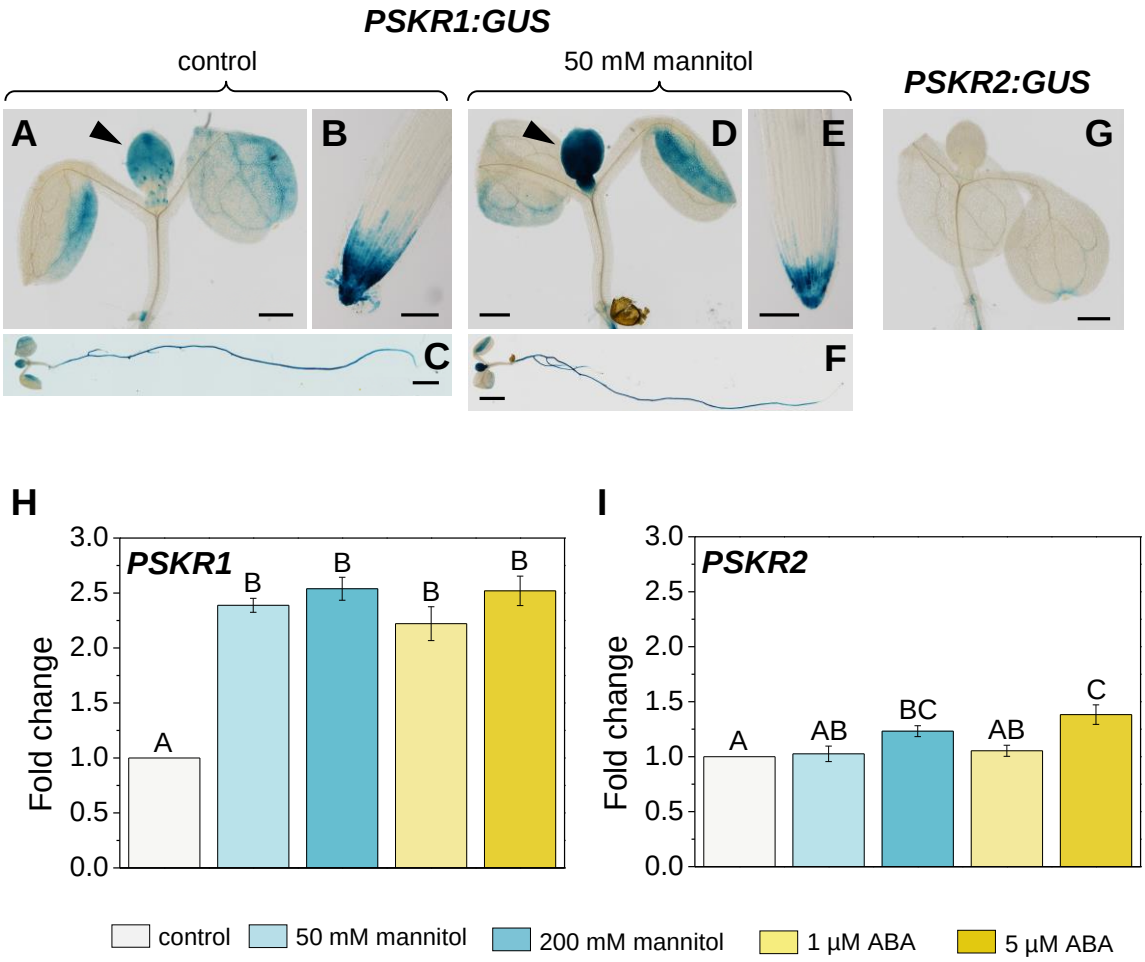

Figure 5. *PSKR1* expression is induced by mannitol and ABA.
A-F *PSKR1:GUS-4* activity in 7-day-old seedlings treated with 50 mM mannitol for 1 day or left untreated as a control. (A-C) Expression in shoot, and root under control conditions. (D-F) Expression in shoot, and root under mannitol. Arrowheads point at true leaves; scale bar = 0.5 mm (A, D), = 0.1 mm (B, E), = 2 mm (C, F).
G *PSKR2:GUS-3* activity in the shoot; scale bar = 0.5 mm.
H, I Seven-day-old seedlings exposed to 50 mM, 200 mM mannitol, 1 µM or 5 µM ABA for 1 day or left untreated as a control. Relative transcript levels of (H) *PSKR1* and (I) *PSKR2* were analyzed by RT-qPCR in true leaves. Values are means (±SE); different letters indicate significant differences (one-way ANOVA, Tukey‘s test, $p<0.05$, 3 biological replicates).

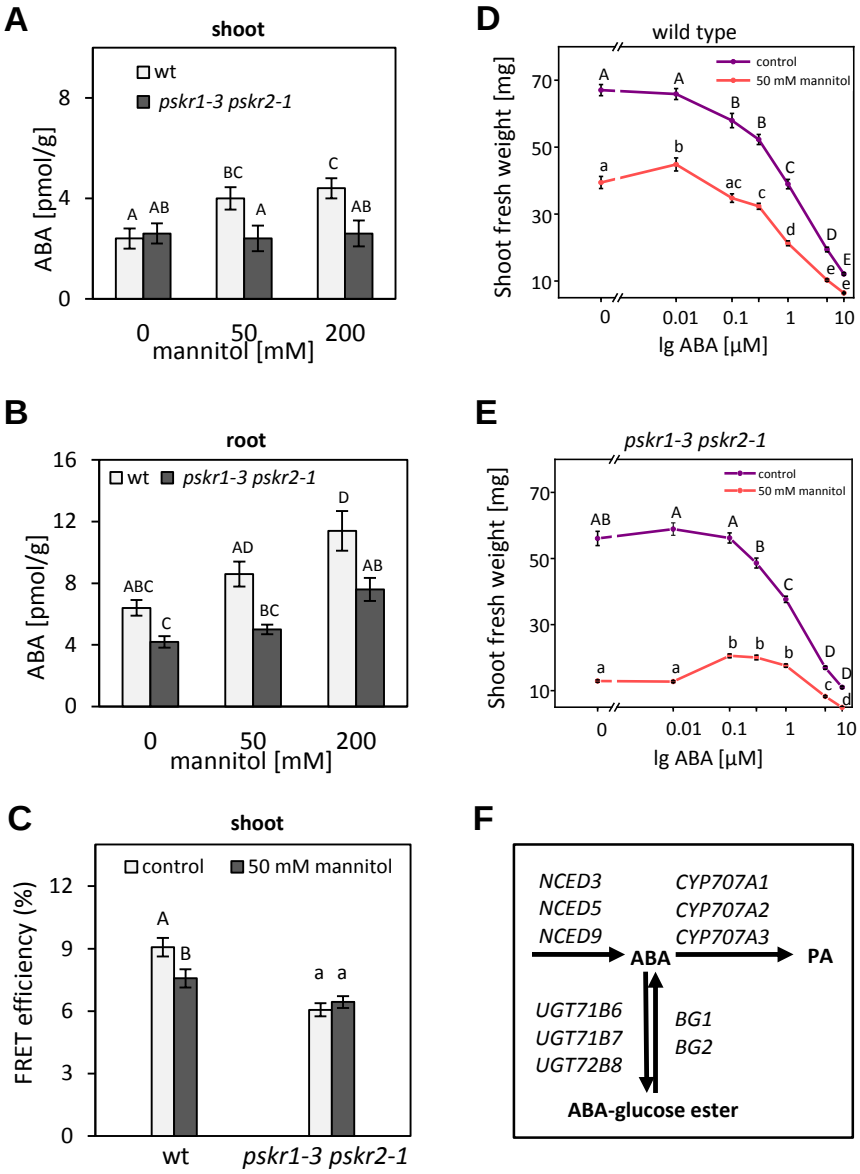
A
shoot
wt
pskr1-3 pskr2-1
ABA [pmol/g]
0
4
8
A
AB
BC
A
C
AB
0
50
200
mannitol [mM]
B
root
wt
pskr1-3 pskr2-1
ABA [pmol/g]
0
4
8
12
16
ABC
C
AD
BC
D
AB
0
50
200
mannitol [mM]
C
shoot
control
50 mM mannitol
FRET efficiency (%)
0
3
6
9
12
A
B
a
a
wt
pskr1-3 pskr2-1
D
wild type
control
50 mM mannitol
Shoot fresh weight [mg]
10
30
50
70
0
0.01
0.1
1
10
lg ABA [µM]
E
pskr1-3 pskr2-1
control
50 mM mannitol
Shoot fresh weight [mg]
10
30
50
70
0
0.01
0.1
1
10
lg ABA [µM]
F
NCED3
NCED5
NCED9
CYP707A1
CYP707A2
CYP707A3
ABA
PA
UGT71B6
UGT71B7
UGT72B8
BG1
BG2
ABA-glucose ester

Figure 6. PSKR and ABA pathways interact.

A Four-day-old wild type and *pskr1-3 pskr2-1* seedlings were transferred to 0 mM, 50 mM or 200 mM mannitol and ABA levels in the shoot were determined after 7 days. Different letters indicate statistically significant differences (Kruskal-Wallis, Tukey‘s test, $p<0.05$, 5 biological replicates).

B ABA levels in roots of seedlings treated as in A (one-way ANOVA, Tukey‘s test, $p<0.05$, 5 biological replicates).

C ABA levels in the first true leaf were compared by photobleaching-based FRET analysis in wild type and *pskr1-3 pskr2-1* seedlings treated as in A. Decreased FRET efficiency indicates elevated ABA levels. Different capital and minor letters indicate significantly different values between treatments (Mann-Whitney test, $p<0.05$, $n\geq142$, 3 independent experiments).

D Shoot fresh weights of wild type seedlings that were transferred after 4 days to plates containing ABA and mannitol at the concentrations indicated were determined after 2 weeks.

E Shoot fresh weight of *pskr1-3 pskr2-1* seedlings grown as described in (D). (D, E) Different capital and minor letters indicate significantly different values within a given treatment (Kruskal-Wallis, Tukey‘s test, $p<0.05$; n=36).

F Overview of genes encoding for enzymes of ABA synthesis (*NCED*s), degradation (*CYP707A*s), inactivation (*UGT71B*s) and mobilization (*BG*s). PA=phaseic acid.

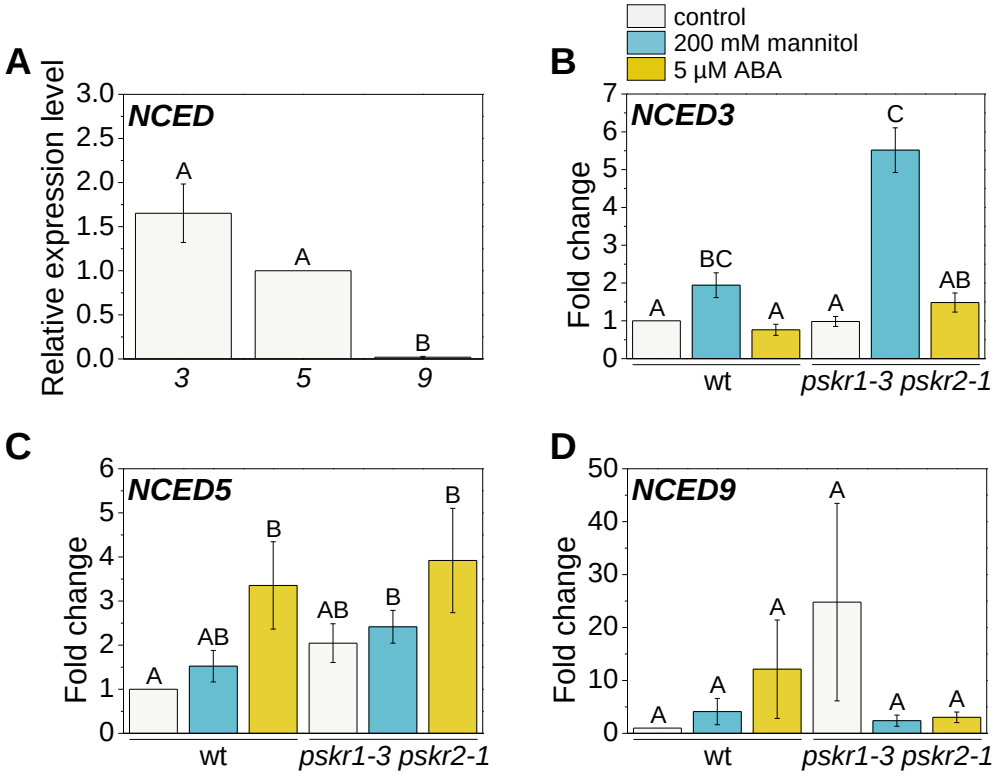

Figure 7. *NCED* genes are differentially regulated by mannitol and ABA.
Seven-day-old wild type and *pskr1-3 pskr2-1* seedlings were exposed to 200 mM mannitol or 5 µM ABA for 1 day to analyze relative *NCED3, 5* and *9* transcript levels in true leaves.
A Relative *NCED* mRNA levels in wild type under control conditions (Kruskal-Wallis, Tukey‘s test, $p<0.05$; 3 biological replicates with two technical replicates each).
B-D Changes in *NCED3*, *NCED5* and *NCED9* transcript levels in response to mannitol, ABA and genotype. Values are means (±SE) with different letters indicating significant differences (Kruskal-Wallis, Tukey‘s test, $p<0.05$; n=6, 3 biological replicates).

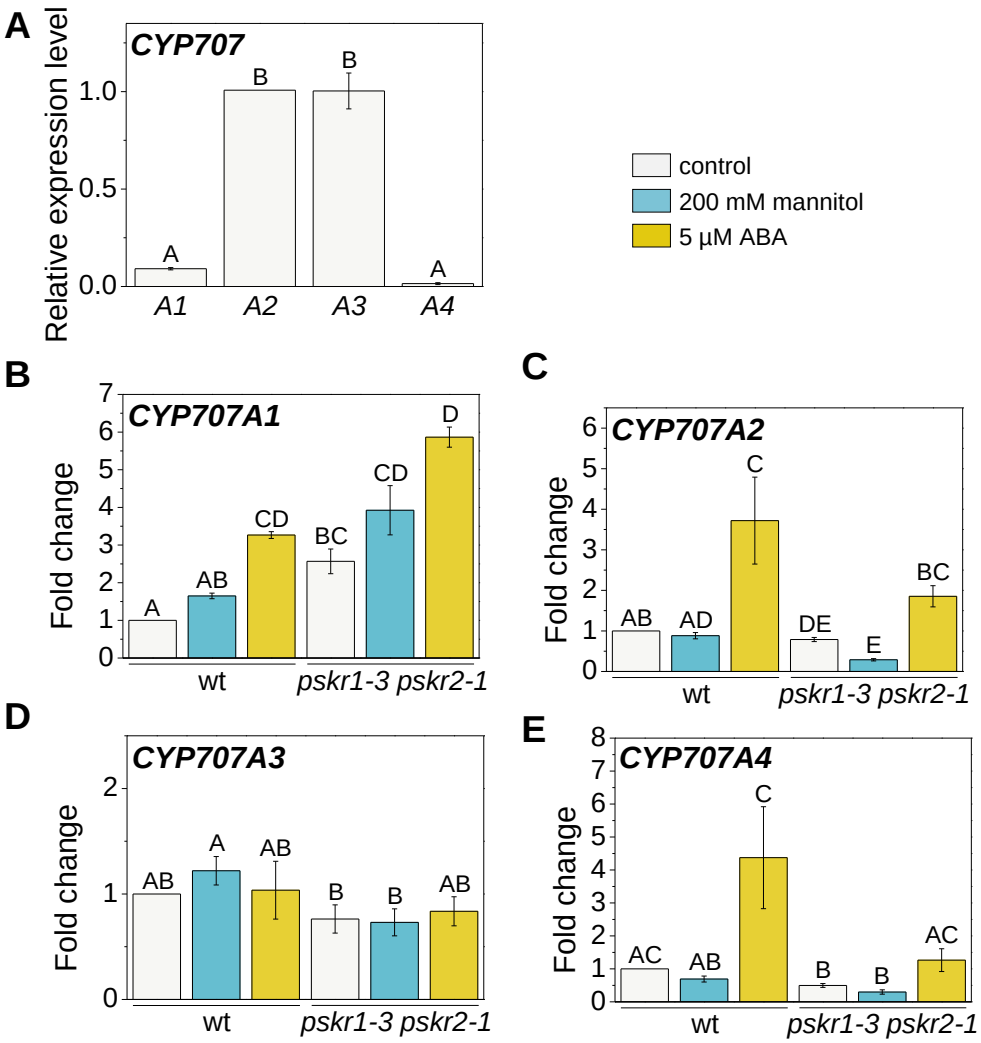

Figure 8. Regulation of *CYP707A* ABA-8'-hydroxylase transcripts by mannitol, ABA and PSKR signaling.

Seven-day-old wild type and *pskr1-3 pskr2-1* seedlings were exposed to 200 mM mannitol or 5 µM ABA for 1 day to analyze gene expression in true leaves.

A Relative *CYP707A* transcript levels in wild type under control conditions. Values are means (±SE) with different letters indicating significant differences (Kruskal-Wallis, Tukey's test, $p<0.05$, n=6; 3 biological replicates).

B-E Relative *CYP707A1-4* transcript levels in response to genotype and treatment (Kruskal-Wallis, Tukey's test, $p<0.05$, n=6, 3 biological replicates).

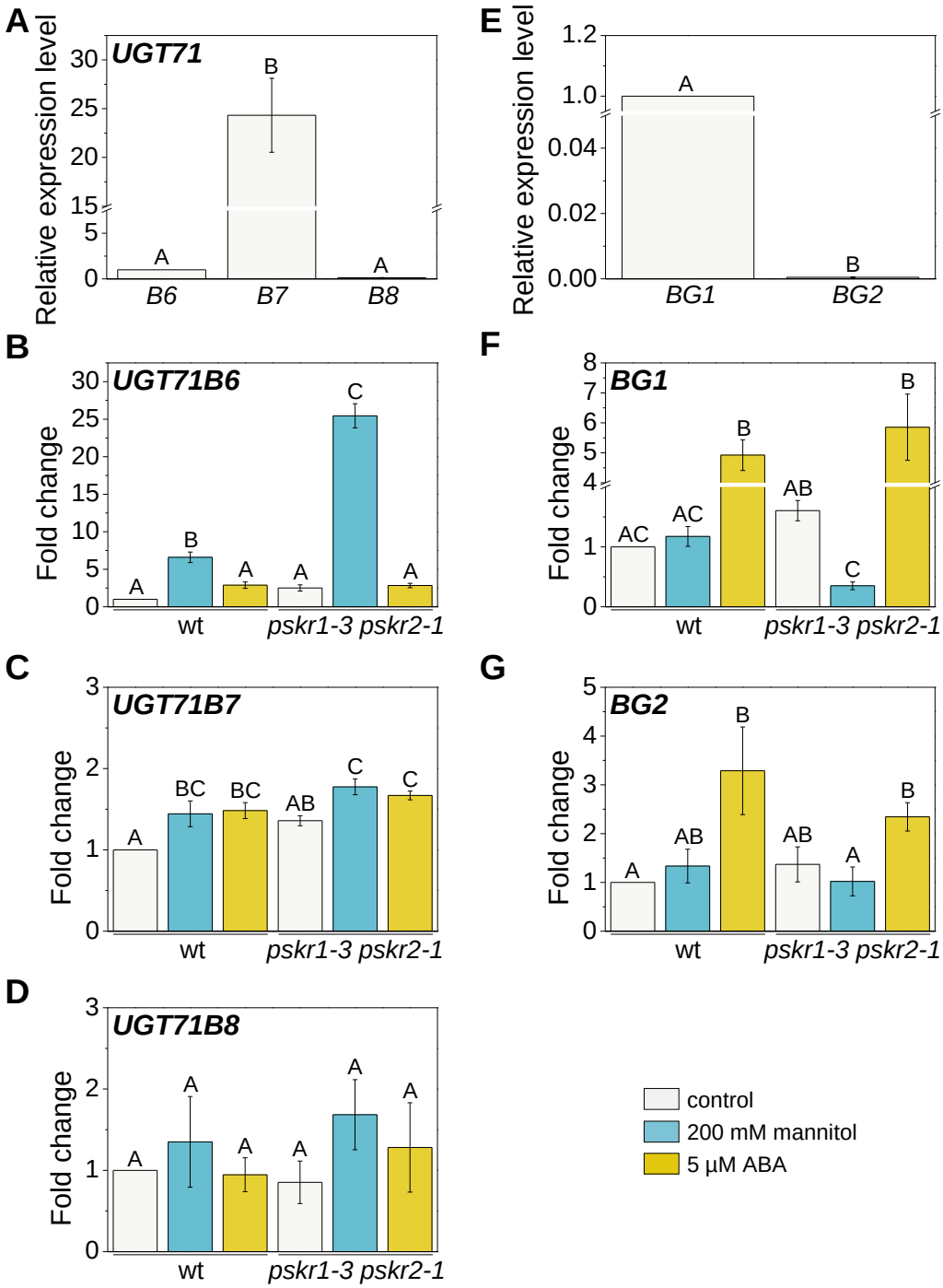

Figure 9. Regulation of *UGT71B* and *BG* transcripts by mannitol, ABA and PSKR signaling.

Seven-day-old wild type and *pskr1-3 pskr2-1* seedlings were exposed to 200 mM mannitol or 5 µM ABA for 24 h for qPCR analysis in true leaves. Values are means (± SE) of 3 biological replicates with different letters indicating significant differences

A Relative *UGT71B6-8* transcript abundance in wild type under control conditions.

B-D Relative mRNA levels of *UGT71B6-8* in response to genotype and treatment (Kruskal-Wallis, Tukey's test, $p<0.05$).

E Relative *BG* transcript abundance in wild type under control conditions.

F, G Relative mRNA levels of *BG1 and 2* in response to genotype and treatment (one-way ANOVA, Tukey's test, $p<0.05$).

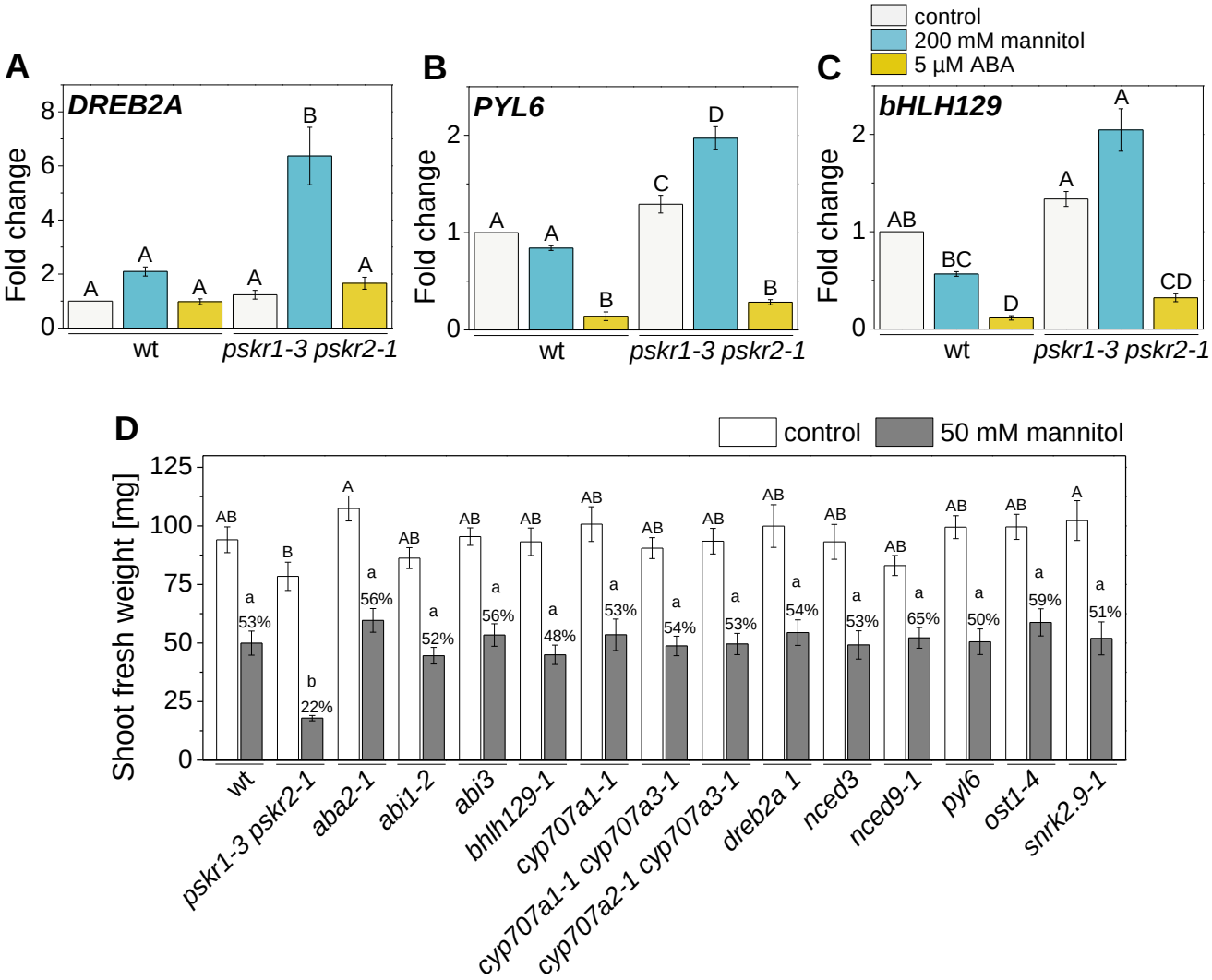

Figure 10. Regulation of ABA and mannitol-responsive genes and their impact on growth under low mannitol stress.

Seven-day-old wild type and *pskr1-3 pskr2-1* seedlings were exposed to 200 mM mannitol or 5 µM ABA for 24 h to analyze gene expression in true leaves. Values are means (±SE) of 3 biological replicates with different letters indicating significant differences.

A Relative transcript levels of the osmotic stress response gene *DREB2A* (Kruskal-Wallis, Tukey‘s test, $p<0.05$).

B Relative transcript level of the ABA receptor gene *PYL6* (one-way ANOVA, Tukey test, $p<0.05$).

C Relative transcript level of the transcriptional repressor *bHLH129* (Kruskal-Wallis, Tukey‘s test, $p<0.05$).

D Four-day-old wild type and knockout seedlings were exposed to 50 mM mannitol for 21 days. Capital letters indicate significant differences between lines under control conditions. Percentages show values of treated compared to untreated seedlings within each genotype. Minorcase letters indicate significant differences between lines exposed to mannitol (one-way ANOVA, Tukey‘s test, $p<0.05$; n≥18, 2 biological experiments).

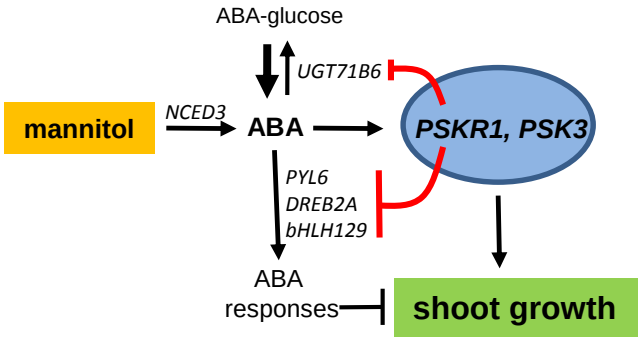

Figure 11. Model of shoot growth control during mannitol stress by PSKR1 and ABA.
Mannitol leads to elevated ABA that promotes PSK and PSKR gene expression. PSKR signaling, in a feedback regulatory loop, targets ABA metabolic genes that may control ABA accumulation. PSKR signaling maintains shoot growth in the presence of low levels of mannitol likely by interacting with ABA synthesis and signaling.

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