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► **To cite this version:**

Masahiko Otani, Ryo Tojo, Sarah Regnard, Lipeng Zheng, Takumi Hoshi, et al.. The MKK3 MAPK cascade integrates temperature and after-ripening signals to modulate seed germination. 2024. hal-04433687

HAL Id: hal-04433687

<https://hal.inrae.fr/hal-04433687>

Preprint submitted on 2 Feb 2024

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1 **Title**

2 The MKK3 MAPK cascade integrates temperature and after-ripening signals to
3 modulate seed germination

4
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24 **Abstract**

25 Temperature is a major environmental cue for seed germination. The permissive
26 temperature range for germination is narrow in dormant seeds and expands
27 during after-ripening. Quantitative trait loci analyses of pre-harvest sprouting in
28 cereals have revealed that MKK3, a mitogen-activated protein kinase (MAPK)
29 cascade protein, is a negative regulator of grain dormancy. Here we show that
30 the MAPKKK19/20-MKK3-MPK1/2/7/14 cascade modulates germination
31 temperature range in Arabidopsis seeds by elevating germinability of the seeds
32 at sub- and supra-optimal temperatures. The expression of *MAPKKK19* and
33 *MAPKKK20* is regulated by an unidentified temperature sensing and signaling
34 mechanism the sensitivity of which is modulated during after-ripening of the
35 seeds, and MPK7 is activated at the permissive temperature for germination
36 regulated by expression levels of *MAPKKK19/20*. Activation of the MKK3
37 cascade represses abscisic acid (ABA) biosynthesis enzyme gene expression,
38 and induces expression of ABA catabolic enzyme and gibberellic acid
39 biosynthesis enzyme genes, resulting in expansion of the germinable
40 temperature range. Our data demonstrate that the MKK3 cascade integrates
41 temperature and after-ripening signals to germination processes including
42 phytohormone metabolism.

43 **Introduction**

44 Temperature and seed dormancy are two important factors controlling seed
45 germination. Temperature is a major environmental factor and seed dormancy is
46 an adaptive trait that enables the seeds to germinate in optimal season for
47 vegetative and reproductive growth. It has been shown that seed
48 responsiveness to temperature is closely related to the dormancy level in
49 soil-buried seeds of winter and summer annuals (Baskin and Baskin, 2014).
50 Primary dormancy of freshly harvested seeds gradually decreases with
51 after-ripening, due to an expansion in the range of permissive germination
52 temperatures (Baskin and Baskin 2014). In winter annual species such as
53 *Arabidopsis* (*Arabidopsis thaliana* L. Heynh.), the seeds are dispersed from
54 mother plants in spring, but at that time they do not germinate under any
55 temperature conditions. From spring to autumn, the maximal permissive
56 temperature for germination rises gradually during after-ripening, but
57 germination is still suppressed by because temperatures are still higher than the
58 upper limit for germination. So, the seeds do not germinate until the autumn
59 when the temperature falls below the upper limit (Baskin and Baskin 2014).
60 Therefore, the temperature sensing and signaling mechanism is modulated
61 during after-ripening, and this allows the seeds to germinate in the appropriate
62 season for their growth.

63 Abscisic acid (ABA) and gibberellic acid (GA) are the main phytohormones
64 that antagonistically regulate seed germination. Studies have shown that
65 supra-optimal high temperatures inhibit the germination of imbibed *Arabidopsis*
66 and lettuce seeds by inducing expression of the ABA biosynthesis enzyme gene

67 *NCED*, and repressing expression of the GA biosynthesis enzyme gene *GA3ox*,
68 which increases ABA levels and decreases GA levels (Gonai et al. 2004, Toh et
69 al. 2008, Argyris et al. 2008).

70 Pre-harvest sprouting (PHS) of maturing seeds severely reduces yield and
71 quality of grain crops such as rice, wheat and barley (Singh et al. 2021). PHS
72 tolerance has been shown to be closely linked with seed dormancy and
73 regulated by quantitative trait loci (QTL). Several genes have been identified
74 from major QTLs in rice (Sugimoto et al., 2010), wheat (Nakamura et al. 2011;
75 Barrero et al. 2015) and barley (Nakamura et al. 2011; Barrero et al. 2015; Sato
76 et al. 2016). Mitogen-activated protein kinase (MAPK) cascades are a common
77 mechanism for transducing external and internal signals to cellular responses in
78 eukaryotes. The MAPK cascades consist of at least three protein kinases, MAPK
79 kinase kinase (MAPKKK), MAPK kinase (MKK), and MAPK (MPK), and are
80 activated by consecutive phosphorylation (Ichimura *et al.*, 2002). In plants, it has
81 been reported that MAPK cascades are involved in various cellular processes
82 such as biotic/abiotic stress responses, phytohormone responses, embryo
83 development and plant growth (Xu and Zhang, 2015). The Arabidopsis genome
84 codes for 80 MAPKKKs, 10 MAPKKs and 20 MPKs, and specific members of the
85 families are involved in the specific signaling pathways (Ichimura et al. 2002,
86 Jonak et al. 2002). MAPKKK activity is thought to be regulated by
87 phosphorylation, for example in the case of immunity (Bi et al. 2018).
88 Nevertheless, transcriptional regulation of clade III *MAPKKKs* seems to be the
89 determinant of MKK3 module activation, explaining the delayed activation
90 kinetics of its downstream group C MPKs (Colcombet et al. 2016). Recently,

91 PHS QTL analyses of wheat and barley identified MKK3 as a negative regulator
92 of seed dormancy (Nakamura et al. 2016; Torada et al. 2016). MKK3 has been
93 reported to have multiple-functions in stress responses in both plantlets and
94 adult plants (Colcombet et al. 2016). In the current study, we identified MKK3
95 containing MAPK cascade components which are involved in temperature
96 signalling, and revealed their regulation mechanism and role in germination
97 temperature range regulation in both freshly harvested and after-ripened
98 Arabidopsis seeds.

99

100

101 **Results**

102 **Arabidopsis MKK3 regulates germination response to temperature in** 103 **freshly harvested and after-ripened seeds**

104 We first analyzed the function of MKK3 on Arabidopsis seed dormancy and
105 germination by using loss-of-function mutant alleles, *mkk3-1* and *mkk3-2*
106 (Supplementary Fig. 1a; Takahashi et al. 2007, Sözen et al. 2020). Freshly
107 harvested seeds of *mkk3-1* and *mkk3-2* showed slower speeds and lower
108 percentages of germination than wild type (WT; Col-0) when imbibed at 22 °C
109 (Supplementary Fig. 1b, c). The seeds of *mkk3-1* had a prolonged after-ripening
110 period for coming out from dormancy (Fig. 1a), and their germination was
111 stimulated by cold stratification (Fig. 1b). These observations indicate that *MKK3*
112 works as a negative regulator of primary dormancy in Arabidopsis, as has been
113 reported in wheat and barley (Torada et al. 2016, Nakamura et al. 2016).

114 We next analyzed germination at various temperatures by using freshly

115 harvested (FH) and after-ripened (AR) seeds. The maximum germination ability,
116 represented by germination index (GI), in FH seeds of WT was 15 °C, whereas
117 in AR seeds the optimal germination temperature increased to around 26 °C, as
118 commonly observed in winter-annual species (Fig. 1c, Baskin & Baskin 1983).
119 The seeds of *mkk3-1* showed higher sensitivity to supra-optimal temperatures,
120 with germination of FH and AR seeds respectively requiring ca. 6 °C and 2 °C
121 lower temperatures, than WT. Also, at suboptimal temperatures, the germination
122 speed of *mkk3-1* FH and AR seeds was clearly slower than WT (Fig. 1c for GI,
123 Supplemental Fig. 1d, e for germination time course). These results suggest that
124 MKK3 is a positive regulator of germination at both sub- and supra-optimal
125 temperatures, and enables seeds to germinate over a range of temperatures.

126

127 **Expression of *MAPKKK19* and *MAPKKK20* is regulated by temperature** 128 **during seed imbibition**

129 It has been reported that transcriptional up-regulation of clade-III *MEKK-like*
130 *MAPKKKs* by ABA is responsible for the activation of downstream kinases
131 (Matsuoka et al. 2015, Danquah et al. 2015, Colcombet et al. 2016). In the same
132 way, MKK3-MPK2 activation by wounding has been shown to depend on the
133 transcriptional up-regulation of several clade-III *MAPKKKs* (Sözen 2020). In the
134 current study, our transcriptome analysis (GSE229182) revealed that of all the
135 clade-III *MAPKKKs*, only *MAPKKK19* and *MAPKKK20* were induced in
136 germinating seeds (Supplementary Fig. 2). Expression of *MAPKKK19* was
137 relatively high in dry seeds, but decreased to low levels after imbibition at
138 supra-optimal temperatures in both FH (26 °C) and AR (34 °C) seeds (Fig. 2a

139 and b). In germinating AR seeds imbibed at 26 °C, the expression levels were
140 initially reduced during the first 6 h, but increased to high levels after 24 h.
141 Expression of *MAPKKK20* was repressed in freshly harvested seeds at 26 °C,
142 but temporarily induced in after-ripened seeds and peaked at 3h after the start of
143 imbibition irrespective of temperature and germination (Fig. 2a and b). Also,
144 *MAPKKK20* expression was re-induced in germinating AR seeds imbibed at the
145 optimal temperature, peaking at 24 h after the start of imbibition, but was
146 repressed in non-germinating FH and AR seeds imbibed at supra-optimal
147 temperatures.

148 In concert with germination ability (GI), the maximum expression of
149 *MAPKKK19* in 24 h imbibed AR seeds was at around 26 °C and reduced at
150 supra- and sub-optimal temperatures (Fig. 2c and d). Furthermore, the
151 expression of *MAPKKK20* was also temperature-dependent, with maximum
152 expression at 5 °C but steadily decreasing as the temperature rose (Fig. 2d).

153

154 ***MAPKKK19* and *MAPKKK20* are involved in the regulation of seed**
155 **germination in response to temperature**

156 In order to understand the role of *MAPKKK19* and *MAPKKK20* on seed
157 germination response to temperature, we isolated DNA insertion mutants. We
158 also isolated DNA insertion mutant of *MAPKKK21* which is a closest paralog of
159 *MAPKKK19* and *MAPKKK20* to elucidate the possibility of redundant function of
160 the genes (Supplementary Fig. 2a). Expression of *MAPKKK21* was very low in
161 the imbibed FH and AR seeds at any temperature conditions (Fig. 2b, d), but the
162 expression was evident during seed development (Supplementary Fig. 4).

163 *mapkkk19-1* and *mapkkk21-1* were transposon insertion lines of the Nossen
164 ecotype, and were backcrossed with Col-0 (Supplementary Fig. 3). *mapkkk20-3*
165 was a T-DNA insertion line of Col-0. The FH seeds of the single mutants showed
166 almost the same germination as wild type at 22 °C, but the seeds of the double
167 mutants showed lower germination percentage than WT (Fig. 3a). Furthermore,
168 the FH seeds of *mapkkk19/20/21* triple mutant showed a lower germination
169 percentage than WT and the double mutants (Fig. 3a). *mapkkk19/20/21* seeds
170 had a prolonged after-ripening period, and their germination was stimulated by
171 cold stratification as observed in *mkk3-1* seeds (Supplementary Fig. 5a and b).
172 These results suggest that all the three *MAPKKKs* are involved in the regulation
173 of seed dormancy.

174 We next analyzed the function of the *MAPKKKs* on germination response to
175 temperature with AR seeds. At supra-optimal temperature (32 °C), the seeds of
176 *mapkkk19/20* showed significantly lower germination percentage than WT (Fig.
177 3b). In contrast to the FH seeds, AR seeds of *mapkkk19/20/21* showed similar
178 germination to *mapkkk19/20* (Fig. 3b). At sub-optimal temperature (5 °C), the
179 germination speed of *mapkkk19/20* seeds was slower than WT, and the delayed
180 germination phenotype was not enhanced in *mapkkk19/20/21* seeds (Fig. 3c,
181 Supplementary Fig. 5c). These results suggest that *MAPKKK19* and
182 *MAPKKK20* are responsible for the germination response to temperature in both
183 FR and AR seeds, but *MAPKKK21* is no longer effective in AR seeds.

184 We also analyzed the contribution of other clade III *MAPKKKs* on
185 germination of FH and AR seeds. *MAPKKK13* and *MAPKKK14* are known to
186 have putative transmembrane motifs at C-terminal domains (Schwacke et al.

187 2003, Sözen et al 2020). The gain-of-function mutant, *mapkkk14-1*, produces a
188 mutant protein that lacks a C-terminal transmembrane domain, and has been
189 reported to show higher MPK2 activation ability than WT in response to
190 wounding (Sözen et al. 2020). However, in our study the seeds of *mapkkk14-1*
191 showed no germination phenotypes (Supplementary Fig. 6a to c). We also
192 isolated the *mapkkk13-1* allele which has T-DNA insertion between kinase and
193 the transmembrane domains, and produced a *mapkkk13-1/14-1* double mutant.
194 However, the FH and AR seeds also showed very similar germination to WT
195 (Supplementary Fig. 6c). We next used the gene editing loss-of-function alleles
196 of *MAPKKK13* and *MAPKKK14* (Sözen et al. 2020; Regnard et al. 2024), but
197 again the germination response to temperature of the FH and AR seeds of
198 *map3k13CR/14CR* double mutant was very similar to WT (Supplementary Fig.
199 6d). Furthermore, the seeds of *mapkkk15/16* and *mapkkk17/18* also showed
200 similar germination response to temperature as WT (Supplementary Fig. 7, 8).
201 Therefore, these results suggest that *MAPKKK13* to *MAPKKK18* have almost no
202 function in seed germination response to temperature.

203 To evaluate the contribution of transcriptional regulation of *MAPKKKs* on
204 seed germination response to temperature, we analyzed *MAPKKK20*
205 overexpression (*MAPKKK20^{OX}*) lines (Supplementary Fig. 9a). The two
206 independent lines accumulated ca. 36-fold more *MAPKKK20* transcripts in dry
207 seeds than the non-transformant wild type (Supplementary Fig. 9b). The FH and
208 AR seeds of *MAPKKK20^{OX}* lines showed significantly higher percentage
209 germination than WT at supra-optimal temperatures (Fig. 4a and b). These
210 results suggest that transcriptional regulation of *MAPKKK20* plays an important

211 role in germination response to temperature. On the other hand, *MAPKKK20*
212 overexpression had almost no effect on germination at 5 oC (Supplementary Fig.
213 9c). The low temperature induced expression nature of the native *MAPKKK20*
214 may mask the effect of the trans gene (Fig. 2d).

215 We analyzed the genetic interaction between *MAPKKK20* and *MKK3* by
216 creating a *MAPKKK20^{OX} mkk3-1* double mutant. In contrast to *MAPKKK20^{OX}*
217 seeds, the double mutant seeds showed lower percentage germination than WT,
218 having almost the same germination rate as *mkk3-1* (Fig. 4b). This epistatic
219 nature of *mkk3-1* suggests that *MAPKKK20* works upstream of *MKK3* for
220 germination.

221

222 **Group C MPKs are involved in the regulation of seed germination response** 223 **to temperature**

224 It has been reported that *MKK3* activates group C MPKs (i.e. *MPK1*, *MPK2*,
225 *MPK7* and *MPK14*) by direct binding (Dóczy et al. 2007, Lee et al. 2008,
226 Matsuoka et al. 2015, Danquah et al. 2015). To identify MPKs which are involved
227 in the regulation of seed germination response to temperature, we isolated and
228 analyzed multiple knockout mutants of the group C *MPKs* (Supplementary Fig.
229 10). Among the four single mutants, only *mpk7-1* FH seeds showed lower
230 percentage germination than WT at 22 °C (Fig. 5a). The seeds of *mpk1/2/14*
231 triple mutant showed almost the same germination as WT, but multiple mutant
232 seeds containing *mpk7-1* showed reduced germination (Fig. 5a). Among these
233 multiple mutants, the seeds of *mpk1/2/7*, *mpk1/7/14* and *mpk1/2/7/14* showed
234 greatly reduced germination phenotype (Fig. 5a). Dormancy of the *mpk1/7/14*

235 and *mpk1/2/7/14* seeds was alleviated by after-ripening and cold-stratification
236 treatment, similar to *mkk3* (Supplementary Fig. 11). These results indicate that
237 *MPK7* has a prominent role, but that other group C MPKs redundantly work on
238 germination of FH seeds, i.e. dormancy.

239 The AR seeds of *mpk1/7/14* and *mpk1/2/7/14* were more sensitive to
240 supra-optimal temperature than WT (Fig. 5b). At sub-optimal temperature (5 °C),
241 the seeds of *mpk7-1* and *mpk1/7/14* showed a 1-day delay in germination, and
242 the *mpk1/2/7/14* seeds showed a 3-day delay when compared with WT (Fig. 5c,
243 Supplementary Fig. 11c). These results indicate that *MPK7* and other group C
244 MPKs redundantly work on promoting germination at supra-optimal
245 temperatures. At sub-optimal low temperatures, *MPK2* may have a major role,
246 while other group C MPKs have redundant function on germination.

247

248 **MPK7 activity is regulated by MAPKKK19/20-MKK3 module in response to** 249 **temperature**

250 To understand the relationship between *MAPKKK19/20* expression, MPK
251 activation and seed germination, we first analyzed *MPK7* activity in germinating
252 and non-germinating seeds since *MPK7* was shown to have a major role in the
253 regulation of germination (Fig. 5a, b). Germinating WT seeds showed a peak in
254 *MPK7* activity after 12 to 24 h of imbibition, but this increased activity was not
255 observed in non-germinating FH and AR seeds imbibed at supra-optimal
256 temperatures (Fig. 6a, Supplementary Fig. 12a). A peak in *MPK7* activity was
257 also observed in after-ripened seeds imbibed for 3h, irrespective of the
258 imbibition temperature, but not detected in dormant seeds. This *MPK7* activity

259 was synchronized with the expression of *MAPKKK20* (Fig. 2b). However, this
260 activation at 3 h was not detected in *mapkkk19/20* and *mapkkk19/20/21* seeds
261 (Fig. 6b and c, Supplementary Fig. 12b and c). Therefore, the early activation of
262 MPK7 appears to be induced by the expression of *MAPKKK20*, but it may not be
263 enough for the completion of germination. These results suggest that activation
264 of group C MPK after 12 to 24 h of imbibition is responsible for the germination
265 response to temperature. We detected almost no MPK7 activity in germinating
266 *mkk3-1* and *mapkkk19/20* seeds throughout the imbibition period (Fig. 6b).
267 Unexpectedly, some MPK7 activity was detected after 1 h of imbibition in the
268 *mapkkk19/20/21* seeds, but the activity diminished during subsequent imbibition
269 (Fig. 6b and c), suggesting that the recorded activity might be the result of the
270 complementation effect of other clade III *MAPKKK* genes expressed during
271 development of the triple mutant seeds. These results suggest that MKK3,
272 *MAPKKK19* and *MAPKKK20* are responsible for the activation of group C MPKs
273 during imbibition, but *MAPKKK21* is not. The contribution of *MAPKKK21* was
274 clearly observed for germination of freshly harvested seeds but not for
275 germination of after-ripened seeds (Fig. 3), suggesting that *MAPKKK21* may be
276 activated only during seed development, and it work on germination of freshly
277 matured seeds.

278

279 **Effect of ABA and GA in *MAPKKK19/20* expression**

280 It has been reported that high temperature inhibits seed germination by
281 inducing ABA biosynthesis and suppressing GA biosynthesis in Arabidopsis and
282 lettuce seeds (Toh et al. 2008, Argyris et al. 2008). So, we analyzed the effect of

283 endogenous and exogenously applied ABA and GA on the expression of
284 *MAPKKK19/20*. FH seeds of the ABA deficient *aba2-2*, showed almost no
285 dormancy and germinated well at 28 °C, but this germination was inhibited by
286 the application of ABA (Fig. 7a). Expression of *MAPKKK19* and *MAPKKK20* was
287 repressed in the imbibed WT dormant seeds, but was de-repressed in *aba2-2*
288 seeds (Fig. 7b). The expression of *MAPKKK19* and *MAPKKK20* in *aba2-2* seeds
289 was moderately suppressed by the exogenously applied ABA. However, the
290 expression of both *MAPKKK19* and *MAPKKK20* was not apparently affected in
291 the seeds treated with the ABA biosynthesis inhibitor, fluridone, and ABA (Fig. 7c
292 and d). These results suggest that the expression of *MAPKKK19* and
293 *MAPKKK20* is not directly regulated by ABA, but the expression is controlled by
294 temperature and physiological status of the seeds.

295 After-ripened GA deficient, *ga3ox1-3 ga3ox2-1* double mutant seeds
296 imbibed at 30 °C could not germinate like similarly imbibed WT seeds, but
297 application of exogenous GA₃ enabled the double mutant seeds to germinate
298 (Fig. 7e). Expression of *MAPKKK19* was repressed in the GA deficient mutant
299 seeds, and this repression was not reversed by the application of exogenous GA
300 (Fig. 7f). In addition, we could not detect any significant effect of the GA
301 deficiency or exogenous GA application on the expression of *MAPKKK20* (Fig.
302 7f). These results suggest that the expression of *MAPKKK19* and *MAPKKK20* is
303 not regulated by GA.

304

305 **MKK3-MAPK cascade modulates ABA and GA metabolism**

306 To understand the molecular mechanism of germination regulation by the

307 MKK3-MAPK cascade, we analyzed the expression of ABA and GA metabolism
308 enzyme genes in the seeds of *MAPKKK20* over-expression lines. In this
309 experiment, *MAPKKK20^{OX}* seeds showed higher percentage germination than
310 WT at 34 °C (Fig. 8a). It has been reported that the key ABA biosynthesis
311 enzyme genes, *NCED2*, *NCED5* and *NCED9* are induced under supra-optimal
312 temperature conditions (Toh et al. 2008, Fig. 8b). The expression of all the three
313 *NCEDs* was reduced in *MAPKKK20^{OX}* seeds imbibed at supra-optimal
314 temperature, 34 °C, when compared with WT (Fig. 8b). The ABA catabolism
315 enzyme genes, *CYP707A1*, *CYP707A2* and *CYP707A3*, have been reported to
316 be involved in germination, and *CYP707A2* has a major role in the rapid
317 decrease in ABA content right after imbibition (Kushiro et al. 2004, Okamoto et al.
318 2006). At the permissive 26 °C temperature, all three ABA catabolism enzyme
319 genes showed significantly higher expression levels in *MAPKKK20^{OX}* seeds than
320 in WT (Fig. 8c). Expression levels of *CYP707A2* and *CYP707A3* were also
321 up-regulated at supra-optimal 34 °C temperature in *MAPKKK20^{OX}* seeds (Fig.
322 8c). These results suggest that the MKK3-MAPK module stimulates seed
323 germination by reducing ABA levels through repression of ABA biosynthesis
324 genes and inducing ABA catabolism genes.

325 GA3ox1 and GA3ox2 are the key enzymes of active GA biosynthesis,
326 and the expression of the genes are regulated by the germination stimulating
327 signals, light and temperature (Toyomasu et al. 1998, Yamaguchi et al. 1998,
328 Yamauchi et al.2004, Toh et al. 2008). Under both in the permissive and
329 non-permissive supra-optimal temperature conditions, *GA3ox1* and *GA3ox2*
330 showed significantly higher expression levels in *MAPKKK20^{OX}* seeds than in WT

331 seeds (Fig. 8d). These results suggest the MKK3-MAPK cascade stimulates the
332 expression of GA biosynthesis enzyme genes.

333

334

335 **Discussion**

336 We identified the MAPKKK19/20-MKK3-MPK1/2/7/14 cascade module (MKK3
337 module) as a mediator of the temperature signal to control Arabidopsis seed
338 germination. The MKK3 module is not required for germination itself since the
339 loss of function alleles of MKK3 could germinate at the optimal temperature
340 condition (Fig. 1c). However, the MKK3 module had a critical role in the
341 modulation of germination temperature range of the seeds, and positively
342 regulated germination at both supra- and sub-optimal temperatures (Fig. 1c,
343 Supplementary Fig. 1f, Fig. 3, Supplementary Fig. 5c, Fig. 5, Supplementary Fig.
344 8c). The germination temperature range is closely related with dormancy, and
345 expands during after-ripening of winter and summer annual seeds (Baskin &
346 Baskin 2014, Fig. 1c). *MKK3* has been considered as a negative regulator of
347 dormancy in cereals (Torada et al. 2016, Nakamura et al. 2016), but our data
348 suggest that its primary function is as a modulator of germination response to
349 temperature. In addition, the MKK3 module is not essential for after-ripening
350 since the germination temperature range was expanded even in the
351 loss-of-function mutant seeds during dry storage of the seeds (Fig. 1c).

352 In this study, the MKK3 module activity was regulated by internal and
353 external signals, namely after-ripening of the seeds and environmental
354 temperature (Fig. 9). The MAP kinase cascade can be rapidly activated

355 post-translationally, but relatively slow activation of clade III *MAPKKKs* at the
356 transcriptional level has been reported for ABA, nitrate, and various stress
357 responses (Colcombet et al. 2016). Our data suggest that transcriptional
358 regulation of the two clade III *MAPKKKs*, *MAPKKK19* and *MAPKKK20*, by
359 temperature is key for activation of the MKK3 module and seed germination (Fig.
360 2, Fig. 6, Fig. 9). This temperature dependent gene expression is modulated by
361 after-ripening, as demonstrated by the fact that repression of *MAPKKK19/20*
362 expression in FH seeds at 26 °C was relieved in AR seeds at the same
363 temperature (Fig. 1a, Fig. 2, Fig. 6, Fig. 9). It has been shown that the
364 germination regulator gene *SOMNUS* is regulated by temperature as well as by
365 light (Lim et al. 2013), but the temperature sensing and signaling mechanism in
366 seeds still needs to be clarified. Thus, elucidation of the temperature signaling
367 and its modulation process during after-ripening may be important for better
368 understanding of seed dormancy and germination. Rice is a summer-annual
369 species, and the germination temperature range expands to downwards during
370 after-ripening, which is in contrast to the winter-annual wheat, barley and
371 *Arabidopsis*. Recently, it has been suggested that rice MEKK family genes,
372 *OsMAPKKK62* and *OsMAPKKK63* are negative regulators of seed dormancy
373 (Mao et al. 2019, Na et al. 2019). Therefore, it would be interesting to compare
374 the temperature signaling systems between summer and winter annual plants.

375 Our data indicate that supra-optimal high temperature represses *MAPKKK19*
376 and *MAPKKK20* expression in the imbibed seeds, and that the MKK3 module
377 regulates seed germination by modulating ABA and GA metabolism (Fig. 7, Fig.
378 8, Fig. 9). However, in the nitrate induced germination system, the expression of

379 the hormone metabolism genes was not regulated by the MKK3 module
380 (Regnard et al. 2024). In the temperature induced germination system, we could
381 not find any contribution of *MAPKKK13* or *MAPKKK14* (Supplementary Fig. 6),
382 but expression of these genes was induced by nitrate, and they were partially
383 responsible for the activation of MPK7 in response to nitrate (Regnard et al.
384 2024). These observations suggest that while different environmental signals
385 can activate the MKK3 cascade, there is a signal specific transduction
386 mechanism that controls transcription of specific *MAPKKKs* in the cascade
387 activation.

388 Our results suggest that the expression of *MAPKKK19/20* is not controlled
389 by ABA, but *MAPKKK17* and *MAPKKK18* have been shown to be induced by
390 ABA in Arabidopsis seedlings, and the *MAPKKK17/18*-MKK3-MPK1/2/7/14
391 module has been shown to be involved in the regulation of leaf senescence
392 (Matsuoka et al 2015, Danquah et al 2015). However, in the current study we
393 could not detect any expression of *MAPKKK17/18* in the imbibed seeds, and the
394 seeds of *mapkkk17/18* double mutant showed no germination phenotype
395 (Supplementary Fig. 2, Supplementary Fig. 8). Therefore, tissue, stage and
396 signal specific regulation of different clade III *MAPKKKs* may support the diverse
397 functions of the MKK3 containing MAPK cascade to control plant growth and
398 development.

399 In addition to *MAPKKK19/20*, *MAPKKK21* was also revealed to be involved
400 in the regulation of FH seed germination. The limit of *MAPKKK21*, to only having
401 a role in FH seed germination may be explained by its expression only occurring
402 during the seed developmental stage (Fig. 2, Supplemental Fig. 4). *MAPKKK21*

403 may activate downstream MKK3 and group C MPKs during seed development,
404 which can modulate the temperature response of freshly matured seeds (Fig.
405 6c).

406 In the MKK3 module, among the group C MPKs, MPK7 was shown to have a
407 major role in seed germination, especially at supra-optimal temperatures (Fig. 5,
408 Supplementary Fig. 8). We also found that MPK2 had a major contribution to
409 germination at sub-optimal low temperatures (Fig. 5c, Supplemental Fig. 11c).
410 Therefore, different group C MPKs may have different substrate specificity, and
411 different systems may regulate seed germination at supra- and sub-optimal
412 temperature conditions.

413 MPK7 activity showed dual peaks at 3 h and from 12 to 24 h after the start
414 of imbibition during germination (Fig. 6). The first activity peak may not be
415 sufficient for the completion of germination since this initial activity was also
416 detected in non-germinating after-ripened seeds imbibed at supra-optimal
417 temperature. The second MPK7 activity peak was not observed at
418 non-permissive supra-optimal temperatures in either FH or AR seeds,
419 suggesting that it has a critical role for germination (Fig. 2, Fig. 6, Fig. 9). During
420 the germination process, seed water uptake is divided in to three phases,
421 passive and rapid water uptake (phase I), stationary (phase II) and seedling
422 growth associated water uptake (phase III) (Bewley 1997). In Arabidopsis, phase
423 I is completed by 1 to 3 h after the start of imbibition, and radicle protrusion is
424 observed after around 30 h (Preston et al. 2009). Therefore, the MPK7 activity
425 peaks observed in the current study correspond to the early and late stages of
426 phase II. In the early stage of phase II, the MKK3 module may target pre-existing

427 and newly synthesized proteins which stimulate the initiation of the germination
428 process in AR seeds, including respiration, macromolecule repair, transcription
429 and translation (Bewley et al. 2013, Preston et al. 2009). In the late stage of
430 phase II, the MKK3 module may phosphorylate proteins which stimulate the
431 completion of the germination process including ABA and GA metabolism.

432 Collectively, the MKK3 module integrates internal after-ripening and
433 external temperature signals into the germination regulation process, and
434 modulates the germination temperature range which is critical to establish the
435 dormancy levels and germination timing of the seeds.

436

437

438 **Materials and Methods**

439

440 **Plant materials and growth conditions**

441 T-DNA insertion lines of Arabidopsis (*Arabidopsis thaliana* L. Heynh.)
442 Columbia-0 (Col-0) accession were obtained from the Arabidopsis Biological
443 Resource Center [*mkk3-1* (SALK_051970; Takahashi *et al.*, 2007), *mkk3-2*
444 (SALK_208528C; Sözen *et al.*, 2020), *mpk1-1* (SALK_063847C; Enders *et al.*,
445 2017), *mpk2-2* (SALK_047422C; Lv *et al.*, 2021), *mpk7-1* (SALK_113631),
446 *mpk14-1* (SALK_022928C; Lv *et al.*, 2021), *mapkkk13-1* (GK-277E09),
447 *map3k14-1* (GK-653B01; Sözen *et al.*, 2020), *mapkkk15-1* (SALK_084817),
448 *map3k16* (Choi *et al.*, 2017), *mapkkk17-2* (SALK_080309C; Romero-Hernandez
449 and Martinez, 2022), *mkkk18-2* (GK-676E02; Mitula *et al.*, 2015) and
450 *mapkkk20-3* (GK-458D07)]. Ds-transposon insertion lines of Arabidopsis

451 Nossen accession were obtained from RIKEN BioResource Research Center
452 [*mapkkk19-1* (pst14411) and *mapkkk21-3* (psh20310)]. Homozygous insertion
453 lines were selected by PCR with the specific primer sets (Supplementary Table 1
454 and 2). Genome editing lines of Arabidopsis Col-0 accession *mapkkk13/14CR*
455 were created by using the CRISPR-Cas9 system (Sözen et al. 2020, Regnard et
456 al. 2024). *MAPKKK20^{ox}* lines were created by transformation of Col-8 accession
457 using the pGWB20 destination vector (Nakagawa et al. 2007). *aba2-2* (Nambara
458 et al., 1998) was kindly provided by Dr. E. Nambara (Toronto University, Toronto),
459 and *ga3ox1-3* (SALK_004521) and *ga3ox1-3 ga3ox2-1* were kindly provided by
460 Dr. E. Nambara (Toronto University, Toronto) and Dr. S. Yamaguchi (Kyoto
461 University, Kyoto), respectively (Nambara et al. 1998, Mitchum et al. 2006).

462 To generate multiple group C *MPK* mutants, we first isolated *mpk1-1*
463 *mpk2-2* and *mpk7-1 mpk14-1* double mutants. Then, we crossed the double
464 mutants, and isolated quadruple, triple, double, single mutants and the wild type
465 siblings from the segregants by PCR, as described above.

466 *mapkkk19-1* and *mapkkk21-3* in Nossen background were backcrossed
467 four times with Col-0, and the introgression lines *mapkkk19-1C* and
468 *mapkkk21-3C* were selected from BC₄F₂ and BC₃F₂ siblings, respectively, by
469 PCR. To obtain multiple *mapkkk* mutants, we first crossed *mapkkk19-1C* and
470 *mapkkk21-3C*, and then crossed the F₁ plant with *mapkkk20-3*. Then, multiple
471 mutants were isolated from F₂ and F₃ plants. Genotyping was done by PCR with
472 the gene-specific primers and either T-DNA left-border or Ds-transposon H-edge
473 primers (Supplementary Table 2). We crossed *MAPKKK20^{ox}* (#125) and *mkk3-1*
474 and selected double mutants from the F₂ plants by PCR using specific primer

475 sets for the overexpression vector pGWB20 and T-DNA insertion in *MKK3*
476 (Supplementary Table 2).

477 Seeds were stratified for 4 days at 4 °C and then directly sown and grown in
478 soil (Co-op N-150; Katakura & Co-op Agri, Co., Tokyo, Japan) in a growth
479 chamber (continuous illumination at 22 °C). Then, after the plants reached
480 physiological maturity, when about one-half of the fruits on a plant turned to
481 yellow, their seeds were harvested and dried for 2 days in a desiccator. These
482 were used as freshly harvested (FH) seeds. After-ripened (AR) seeds were
483 obtained by storing FH seeds in a desiccator at room temperature for at least 2
484 months. To maintain dormancy, some of the FH seeds were stored at –80 °C
485 with silica gel.

486

487 **Germination assay**

488 Thirty seeds were imbibed with 300 µL of ultra-pure water in the well of a
489 24-well plate at constant temperature in continuous light for 5 to 14 days without
490 cold stratification, unless otherwise stated. Germination was scored as radicle
491 protrusion. The germination ability of the seeds was evaluated as the final
492 germination percentage or as the Germination Index (GI). The GI was calculated
493 with maximum weight given to the seeds that germinated early and less weight
494 given to those that germinated late as follows: $GI = \sum_{n=1}^N G_n(N + 1 - n)/N$,
495 where G_n is the percentage of germinated seeds on day n , but not a cumulative
496 value. Germination tests were done with at least three independent seed
497 batches with three replicates in each, unless otherwise stated. Each batch
498 contained the seeds harvested from at least 4 plants. A typical result was

499 presented because there was some variation in germination percentages
500 between batches, but the relative differences between treatments in any batch
501 were very similar for all batches.

502 For the chemical treatments, 30 seeds were imbibed with 250 μ L of
503 hormone solutions in the well of a 24-well plate at constant temperature in
504 continuous light for 5 to 7 days without cold stratification. \pm ABA (SIGMA, A1049),
505 GA₃ (SIGMA, G7645) and fluridone (Daw Elanco, recrystallized) were first
506 dissolved in dimethylsulfoxide (DMSO) and then diluted to the final
507 concentrations with ultra-pure water. The final concentration of DMSO was 0.1%,
508 and 0.1% DMSO solution was used as the control.

509

510 **RNA isolation and qRT-PCR analysis**

511 Dry or imbibed seeds (15-20 mg dry weight), seedlings and siliques were
512 frozen with a ϕ 5mm stainless bead in LN₂ immediately after sampling, and
513 stored at -80 °C until use. The seeds in developmental stages were collected as
514 described previously (Zheng et al. 2022). In brief, we marked open flowers at
515 anthesis (day zero) by thread, and collected the fruits at 3, 6, 9, 12, 15 and 18
516 days post-anthesis (DPA). Total RNAs were isolated from 20 siliques with seeds
517 (DPA 3 and 6) and seeds collected by opening the 20 siliques (DPA 9 to 18). The
518 frozen tissues were squashed in a 2 ml tube by a bead mill type homogenizer
519 (Biomedical Science, Tokyo). Total RNA was extracted using the
520 hexadecyltrimethylammonium bromide method as described previously (Zheng et
521 al. 2022). RNA was reverse-transcribed to cDNA by using DNA removal and a
522 cDNA synthesis kit (PrimeScript™ RT reagent Kit with gDNA Eraser; TaKaRa,

523 Kusatsu, Japan) with a mixture of oligo-dT and random primers. Quantification of
524 transcript was done by qRT-PCR with fluorescent-labelled nucleotide substrate
525 (TB Green™ Premix Ex Taq™ II, Takara or PowerUp SYBR Green Master Mix,
526 Thermo Scientific) as described previously (Shigeyama et al. 2016, Zheng et al.
527 2022). Forward and reverse primer sequences for semi-quantitative RT-PCR
528 and qRT-PCR are listed in Supplementary Table 2. Reactions were done using
529 the 7500 Fast system (Applied Biosystems, ABI), and the data were analyzed
530 using ABI Prism 7700 SDS software (ABI). For each sample, the mean value
531 from triplicate qRT-PCRs was used to calculate the transcript abundance.
532 At2g20000 was used as a reference genes for transcript normalization (Graeber
533 et al. 2011). For each sample, the mean value from triplicate reactions was used
534 to calculate transcript abundance, with the mean values being plotted along with
535 the standard deviations. To confirm biological reproducibility, experiments were
536 performed at least three times using samples harvested in different batches;
537 similar results were obtained. Typical results are presented unless otherwise
538 stated.

539

540 **Microarray analysis**

541 Freshly harvested Col-0 seeds were imbibed at 26 oC, and after-ripened seeds
542 were imbibed at either 26 °C or 34 °C under continuous illumination. Total RNA
543 was extracted from 20 mg seeds (dry weight) using the RNAqueous™ Small
544 scale phenol-free total RNA isolation kit (Ambion: catalog #1912) according to
545 the manufacturer's protocol. Three seed batches harvested from independently
546 grown plants were used for biological replications. Cyanine 3-labelled cRNA was

547 synthesized from 150 ng RNA using the Low Input Quick Amp Labeling Kit
548 (Agilent) and predicated using RNeasy Mini Kit (QIAGEN) according to the
549 manufacturer's protocol. The labeled cRNA was fragmented and hybridized to
550 Agilent Arabidopsis 4 Oligo Microarrays (G2519F) for 17 h at 65 °C. After
551 hybridization on 4 x 44K array slide, the arrays were washed and scanned by
552 Agilent DNA Microarray Scanner (G2505B) according to one-color methods.
553 Signal intensities were measured by Feature Extraction Software 11.5.1.1
554 (Agilent) and data analysis were performed by Gene Spring (Agilent) and R
555 software. Normalization was conducted using Modified Histogram Matching
556 Normalization (MHMN) method (Astola and Molenaar, 2014) . Araport11 (TAIR)
557 was set for gene annotation. Raw data is available at GEO database (Clough
558 and Barrett 2016); accession #GSE229182.

559

560 **Kinase assay of MPK7 in seeds**

561 FH and AR seeds (10 mg) were imbibed at permissive or supra-optimal
562 temperatures for germination under continuous illumination. The seeds were
563 frozen in LN₂ immediately after sampling, and stored at -80 °C until use. Protein
564 extraction, kinase assay and western blotting were performed as described
565 (Sözen et al. 2020, Regnard et al. 2024). In brief, frozen seeds were ground, and
566 then soluble proteins were extracted using a non-denaturing buffer,
567 supplemented with phosphatase inhibitors. After normalization on total protein
568 amount, MPK7 was immunoprecipitated using a specific antibody, and its activity
569 was assayed as the ability to phosphorylate the substrate MBP. Phosphorylated
570 MBP was revealed on a SDS-PAGE gel. Western blots were performed using

571 indicated antibodies (Sözen et al. 2020, Regnard et al. 2024).

572

573

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769

770

771 **Acknowledgments**

772 We thank Dr. Atsushi Torada at Hokuren and Dr. Taishi Umezawa at Tokyo
773 University of Agriculture and Technology greatly for valuable discussion. We
774 thank Dr. Tsuyoshi Nakagawa in Interdisciplinary Center for Science Research,
775 Shimane University for providing pGWB vector plasmids. We also thank Dr. Eiji
776 Nambara at Toronto University and Dr. Shinjiro Yamaguchi at Kyoto University
777 for providing ABA deficient (*aba2-2*) and GA deficient (*ga3ox1* and *ga3ox2*)
778 mutant seeds, respectively. We thank RIKEN BioResource Research Center and
779 ABRC for providing transposon and T-DNA insertion mutant seeds, respectively.
780 We thank Kohei Yokota at Faculty of Agriculture, Kagawa University, Yuichi
781 Kashiwakura and Ryoshun Nishioka at the Department of Life Sciences, Meiji
782 University, for their technical assistance. We thank Dr. Iain McTaggart at School
783 of Agriculture, Meiji University for critical reading and English editing of the
784 manuscript. This work was supported partly by the Program for the Strategic
785 Research Foundation at Private Universities, 2014–2018 of MEXT, Japan.

786

787 **Author contributions**

788 M.O. and N.K. conceived the project; M.O. and N.K. conducted experiments and
789 data analysis; M.O., R.T., L.Z., T.H. and S.O. performed mutant isolation and
790 characterization; S.R. performed kinase assays; K.I. constructed *MAPKKK20^{ox}*
791 lines; T.S. performed microarray experiment; N.T. and S.K. performed the
792 bioinformatics; J.C. and K.I. contributed to discussion; M.O. wrote the initial
793 manuscript; All authors edited the manuscript.

794

795 **Competing interests**

796 The authors declare that they have no competing interests.

797

798 **Data availability**

799 The microarray data generated from this study have been deposited in the Gene
800 Expression Omnibus under accession code GSE229182. The unique biological
801 materials are available upon appropriate requests. Source data are provided
802 with
803 this paper.

804

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807 **Figure legends**

808

809 **Fig. 1 MKK3 is a modulator of germination response to temperature in**
810 **both freshly harvested and after-ripened seeds.** Asterisks indicate statistical
811 differences between wild type (Col-0) and *mkk3-1* ($p < 0.05$, Student's t test). **a**
812 Enhanced dormancy phenotype of *mkk3-1* seeds. Freshly harvested seeds were
813 stored in a desiccator at room temperature for up to 3 weeks. Seeds were
814 imbibed at 22 °C for 7 days without cold stratification. Values are means of three
815 technical replicates with SDs. We obtained similar results from triplicate
816 experiments, and typical data are presented. **b** Effect of cold stratification on
817 germination of the freshly harvested seeds. The seeds at DAH 2 were imbibed at
818 22 °C for 5 days with (+) or without (-) cold stratification at 4 °C for 4 days.
819 Results from three independent seed batches are shown with averages and SDs.
820 **c** MKK3 is a modulator of germination temperature range. Germination index of
821 freshly harvested (FH, DAH 2, open symbols) and after-ripened (AR, DAH 150,
822 closed symbols) seeds imbibed at 5 to 35 °C for 14 days are shown. Results
823 from three independent seed batches are shown with averages and SDs.

824

825 **Fig. 2 Temperature dependent expression *MAPKKK19* and *MAPKKK20* in**
826 **FH and AR seeds.** **a** Final percentage germination of FH (DAH 2) and AR
827 (DAH 49) Col-0 seeds used for gene expression analysis in **b**. The FH seeds
828 were imbibed at 26 °C, and the AR seeds were imbibed at either 26 °C or 34 °C
829 for 7 days. Results from three technical replicates are shown with averages and
830 SDs. **b** Effect of imbibition temperature on the expression time course of

831 *MAPKKK19, 20* and *21* in FH and AR seeds. FH seeds imbibed at 26 °C (orange
832 circles), and AR seeds imbibed at 26 °C (blue triangles) or 34 °C (red diamonds)
833 were used for RNA extraction. **c** GI of AR (DAH 330) Col-0 seeds used for gene
834 expression analysis in **d**. The seeds were imbibed at various temperatures for 14
835 days. Values are means of three technical replicates with SDs. **d** Effect of
836 imbibition temperature on the expression of *MAPKKK19, 20* and *21*. Total RNA
837 was prepared from 24 h imbibed seeds. Dashed red lines indicate the
838 expression levels in dry seeds. **b** and **d** Transcript levels were quantified by
839 qRT-PCR. We obtained similar results from three biological replicates, and
840 typical data is presented.

841

842 **Fig. 3** *MAPKKK19* and *20* are responsible for germination of FH and AR
843 seeds, and *MAPKKK21* contributes to germination of FH seeds. Significant
844 differences between samples are indicated by different letters (Tukey HSD tests,
845 $P < 0.05$). **a** Germination of FH seeds of single and multiple mutants at 22 °C.
846 Freshly harvested (DAH 2) seeds were imbibed for 7 days. Results from three
847 biological replicates are shown with averages and SDs. **b** Effect of supra-optimal
848 temperature on germination of AR seeds. The seeds were imbibed at 22 °C and
849 32 °C for 5 days. We obtained similar results from three biological replicates, and
850 a typical result is shown. **c** Effect of sub-optimal temperature on germination of
851 AR seeds. The seeds were imbibed at 5 °C for 14 days. GI values were
852 presented as mean (\pm SD) of three biological replicates.

853

854 **Fig. 4** *MAPKKK20* over-expression stimulates germination of FH and AR
855 seeds at supra-optimal temperatures in the MKK3 pathway. The values are
856 the means (\pm SD) of three biological replicates. Significant differences between
857 lines are indicated by different letters (Tukey HSD tests, $P < 0.05$).
858 **a** FH (DAH 2) and AR (DAH 64) *35S::MAPKKK20-10xMYC* (*MAPKKK20^{OX}*)
859 seed germination response to imbibition temperature. The seeds were imbibed
860 for 5 days. **b** *mkk3-1* is epistatic to *MAPKKK20^{OX}*. Freshly harvested (DAH 2)
861 seeds were imbibed at 22 and 26 °C for 7 days without stratification.

862

863 **Fig. 5** Group C MPKs are responsible for germination of FH and AR seeds.
864 The values are the means (\pm SD) of three biological replicates. Significant
865 differences between lines are indicated by different letters (Tukey HSD tests,
866 $P < 0.05$). **a** Germination of FH (DAH 2) seeds of single and multiple mutants
867 at 22 °C. The seeds were imbibed for 7 days without cold stratification. **b** Effect
868 of supra-optimal temperature on germination of AR seeds. The seeds were
869 imbibed at either 22 °C or 32 °C for 5 days. **c** Effect of sub-optimal temperature
870 on germination of AR seeds. Germination index of after-ripened seeds imbibed
871 at 5 °C for 14 days.

872

873 **Fig. 6** MPK7 activity in germinating and non-germinating seeds. MBP was
874 used as a phosphorylation substrate of MPK7 which was immunoprecipitated
875 from imbibed seeds with an anti-MPK7 antibody. Amount of MPK7 protein was
876 monitored by immunoblot using an anti-MPK7 antibody. Equal loading was
877 indicated by Coomassie staining of the LEA protein (LEAp) on the membrane. **a**

878 Effect of imbibition temperature and seed dormancy status (after-ripening) on
879 MPK7 activity. **b** MPK7 activity in after-ripened *mkk3-1* and *mapkkk19/20/21*
880 seeds imbibed at permissive temperature for germination (28 °C). **c** MPK7
881 activity in after-ripened *mapkkk19/20* and *mapkkk19/20/21* seeds imbibed at
882 permissive temperature for germination (28 °C).

883

884 **Fig. 7 Effect of ABA and GA on the expression of *MAPKKK19* and**
885 ***MAPKKK20*.** Seed germination and gene expression data are the means (\pm SD)
886 of three biological replicates. Total RNA was prepared from FH (**a**; DAH2) or AR
887 (**c**; DAH 80, **e**; DAH 50) seeds imbibed for 24 h or 48 h. Transcript levels were
888 quantified by qRT-PCR with At2g20000 as an internal standard. Significant
889 differences in the gene expression levels are indicated by different letters (Tukey
890 HSD tests, $P \leq 0.05$). **a-d** Effect of endogenous and exogenous ABA on seed
891 germination (**a**, **c**) and gene expression (**b**, **d**). The endogenous ABA levels in
892 FH seeds imbibed at 28 °C were reduced by *aba2-2* mutation (**a**, **b**) or by the
893 ABA biosynthesis inhibitor, fluridon in Col-0 AR seeds imbibed at 32 °C (**c** and **d**).
894 Total RNA was prepared from seeds imbibed for 24 h. **e**, **f** Effect of endogenous
895 and exogenous GA on seed germination (**e**) and gene expression (**f**). AR seeds
896 were imbibed at 30 °C. Total RNA was prepared from AR seeds imbibed for 24h.

897

898 **Fig. 8 *MAPKKK20* can induce expression of ABA catabolism and GA**
899 **biosynthesis genes and reduce expression of ABA biosynthesis genes.**

900 Values are means of three technical replicates with SDs. We obtained similar
901 results from triplicate experiments, and typical data are presented. Transcript

902 levels were quantified by qRT-PCR with At2g20000 as an internal control for
903 normalization. **a** Germination of wild type (Col-8) AR (DAH 554) seeds used for
904 the gene expression analysis. The seeds were imbibed for 7 days. **b** Expression
905 of ABA biosynthesis enzyme genes, *NCEDs*, in seeds imbibed for 24 h. **c**
906 Expression of ABA catabolism enzyme genes. The expression of *CYP707A1*
907 and *CYP707A3* were analyzed at 24h after the start of imbibition, and
908 *CYP707A2* was analyzed at 3h after the start of imbibition. **d** Expression of GA
909 biosynthesis enzyme genes, *GA3ox1* and *GA3ox2* in seeds imbibed for 12 h.
910

911 **Fig. 9 Modulation of germination temperature range by**

912 **MAPKKK19/20-MKK3-MPK1/2/7/14 module. A model.** The MKK3 module is
913 regulated by temperature through the regulation of *MAPKKK19/20* expression,
914 and it regulates seed germination at sub- and supra-optimal temperature
915 conditions through modulation of ABA and GA metabolism. Expression of
916 *MAPKKK19/20* is regulated by temperature and unknown signal of after-ripening.
917 Accumulation of MAPKKK19 and MAPKKK20 proteins induce activation of the
918 MKK3 module, and the kinase cascade signaling decreases ABA accumulation
919 and increases GA production. During after-ripening of the seeds, an unidentified
920 mechanism modulates *MAPKKK19/20* expression, and expands the permissive
921 temperature range of *MAPKKK19/20* induction by suppressing ABA production.

922 **Legends to Supplementary Figures**

923

924 **Supplementary Fig. 1 Germination of *MKK3* loss-of-function mutant**
925 **seeds.**

926 **(a)** Gene model of *MKK3* and the position of T-DNA insertion. **(b, c)** Germination
927 time course of WT (Col-0), *mkk3-1* and *mkk3-2* FH (DAH 2) seeds. The seeds
928 were imbibed at 22 °C without stratification. Typical germination time course data
929 from three (b) or two (a) biological replicates are shown. The values are the
930 means (\pm SD) of three technical replicates, and we had similar results in different
931 replicates. **(d, e)** Germination time course of FH and AR seeds imbibed at 5 °C
932 **(d)** and at 10 °C **(e)**. The values are the means (\pm SD) of three biological
933 replicates.

934

935 **Supplementary Fig. 2 Expression of clade-III *MEKKs* during imbibition of**
936 **FH and AR seeds.**

937 **(a)** Phylogenetic tree of MEKK-like MAPKKK in Arabidopsis. Phylogenetic
938 analysis with amino acid sequences was done by neighbor-joining method using
939 MEGA X (Kumar et al. 2018). Percentages of clustering reproducibility
940 (bootstrap test with 1000 replicates) are shown at the branch points. The
941 evolutionary distances were calculated using the Poisson correction method. **(b)**
942 Expression of clade-III MEKK-like *MAPKKK* during imbibition of dry mature
943 seeds (GEO accession: GSE229182). Values shown are means (\pm SD) of three
944 biological replicates of microarray analysis with Arabidopsis 4 Oligo Microarray
945 (Agilent).

946

947 **Supplementary Fig. 3 T-DNA insertion alleles of *MAPKKK19*, *20* and *21*.**

948 **(a)** Position of T-DNA (white triangles) and transposon (black triangles) insertion
949 in Col-0 (*mapkkk20-3*) and Nossen (*mapkkk19-1* and *mapkkk21-3*) accessions,
950 respectively. Positions of primers used for genotyping (white arrows) and
951 expression (black arrows) analyses are indicated. **(b)** Semi-quantitative RT-PCR
952 for the mutant allele expression analysis. Total RNA was extracted from 24 h
953 imbibed seeds for *MAPKKK19* and *MAPKKK20*, and from dry seeds for
954 *MAPKKK21*. 18s rRNA was used as an internal control. PCR cycle numbers
955 were 27 for *MAPKKK19* and *MAPKKK20*, 30 for *MAPKKK21*, 21 for 18S rRNA.

956

957 **Supplementary Fig. 4 Expression of *MAPKKK19/20/21* during seed**
958 **development.**

959 Total RNA was prepared from seeds with siliques at 3 and 6 days after flowering
960 (DAF) and from seeds without siliques at 9 to 21 DAF. Transcript levels were
961 quantified by quantitative RT-PCR using At2g20000 as an internal control. The
962 quantification was done with three independent plant populations, and typical
963 data are presented. We obtained similar results from the different experiments.

964

965 **Supplementary Fig. 5 Seed dormancy and germination of**
966 ***mapkkk19/20/21* at sub-optimal temperature.**

967 Asterisks indicate statistical differences between wild type (Col-0) and
968 *mapkkk19/20/21* ($p < 0.05$, Student's t test). **(a)** Enhanced dormancy phenotype
969 of *mapkkk19/20/21* seeds. Freshly harvested seeds were stored in a desiccator

970 for up to 3 weeks at room temperature. Seeds were imbibed at 22 °C for 7 days
971 without cold stratification. Values are means (\pm SD) of three technical replicates.
972 We obtained similar results from triplicate experiments, and typical data are
973 presented. **(b)** Effect of cold stratification on germination of the freshly harvested
974 seeds. The seeds at DAH 2 were imbibed at 22 °C for 5 days with (+) or without
975 (-) preceding cold stratification at 4 °C for 4 days. **(c)** Germination time course of
976 AR seeds imbibed at 5 °C. **(b, c)** Results from three independent seed batches
977 are shown with averages and SDs.

978

979 **Supplementary Fig. 6 Germination of gain- and loss-of-function mutant**
980 **seeds of *MAPKKK13* and *MAPKKK14*.**

981 **(a)** Gene model of *MAPKKK13* and *MAPKKK14*, and the positions of T-DNA
982 insertion (*mapkkk13-1*, *mapkkk14-1*) and gene editing (*mapkkk13CR*,
983 *mapkkk14CR*) positions. Asterisk indicates stop codon created by single
984 nucleotide insertion by CRISPR-Cas9. **(b)** Semi-quantitative RT-PCR for
985 *mapkkk13-1* expression analysis. Total RNA was extracted from dry seeds. 18s
986 rRNA was used as an internal control. PCR cycle numbers were described
987 above the gel image. **(c)** Germination of the gain-of-function mutant seeds. FH
988 (DAH 2) and AR (DAH 111-113) seeds were imbibed for 7 days. We obtained
989 similar results from triplicate experiments, and typical data are presented. The
990 values were presented as mean (\pm SD) of three technical replicate. **(d)**
991 Germination of loss-of-function *mapkkk13/14-cr1* and *mapkkk13/14-cr2* double
992 mutant seeds. FH (DAH 2) were imbibed for 7 days, and AR (DAH 390) seeds
993 were imbibed for 5 days. Results from three independent seed batches are

994 shown with averages and SDs.

995

996 **Supplementary Fig. 7 Germination of loss-of-function mutant seeds of**
997 ***MAPKKK15* and *MAPKKK16*.**

998 (a) Gene model of *MAPKKK15* and *MAPKKK16*, and the positions of T-DNA
999 insertion positions. (b) Semi-quantitative RT-PCR for the mutant gene
1000 expression analysis. Total RNA was extracted from 7-days-old seedlings treated
1001 with 10 μ M ABA for 1 h. 18s rRNA was used as an internal control. PCR cycle
1002 numbers were 30 for *MAPKKK15* and *MAPKKK16*, and 21 for 18S rRNA. (c)
1003 Germination of FH (DAH 2) and AR seeds (DAH 111-113). The seeds were
1004 imbibed for 7 days. The experiments were performed in three independent seed
1005 batches with three replicates in each. We obtained similar results from the three
1006 experiments, and typical data are presented. The values were presented as
1007 mean (\pm SD) of three technical replicates ($n = 8$). We could not find significant
1008 differences between WT and the mutants (Tukey HSD tests, $P \leq 0.05$).

1009

1010 **Supplementary Fig. 8 Germination of loss-of-function mutant seeds of**
1011 ***MAPKKK17* and *MAPKKK18*.**

1012 (a) Gene model of *MAPKKK17* and *MAPKKK18*, and the positions of T-DNA
1013 insertion positions. Positions of primers used for expression analysis are
1014 indicated by arrows. (b) Semi-quantitative RT-PCR for the mutant gene
1015 expression analysis. Total RNA was extracted from 7-day-old seedlings treated
1016 with 10 μ M ABA for 3 h. 18s rRNA was used as an internal control. PCR cycle
1017 numbers were 30 for *MAPKKK17* and *MAPKKK18*, and 21 for 18S rRNA. (c) FH

1018 (DAH 2) and AR (DAH 80) seeds were imbibed for 7 and 5 days, respectively.
1019 We obtained similar results from triplicate experiments, and typical data are
1020 presented. We could not find significant differences between WT and the
1021 mutants (Tukey HSD tests, $P < 0.05$).

1022

1023 **Supplementary Fig. 9 Over-expression of *MAPKKK20* and the effect on**
1024 **germination at sub-optimal temperature.**

1025 (a) Schematic representation of *MAPKKK20*^{OX} construct. Positions of primers
1026 used for expression analysis are indicated by arrows. (b) Quantification of
1027 *MAPKKK20* transcripts by qRT-PCR with At2g20000 as an internal control.
1028 Relative expression to WT (Col-8) is indicated by the fold change expression in
1029 *MAPKKK20*^{OX} dry seeds. The values are the means (\pm SD) of three technical
1030 replicates. (c) Germination time course of AR (DAH 167) seeds imbibed at 5 °C.
1031 The values are the mean (\pm SD) of three biological replicates.

1032

1033 **Supplementary Fig. 10 T-DNA insertion alleles of group C MPKs.**

1034 (a) Schematic representation of the genes and position of T-DNA (white
1035 triangles) insertion in Col-0. Positions of primers used for genotyping (white
1036 arrows) and expression (black arrows) analyses are indicated. (b-d)
1037 Semi-quantitative RT-PCR for the mutant allele expression analysis. Total RNA
1038 was extracted from 24 h imbibed seeds. 18S rRNA was used as an internal
1039 control. PCR cycle numbers were 30 for *MPKs* and 21 for 18S rRNA.

1040

1041 **Supplementary Fig. 11 Seed dormancy and germination of group C MPK**

1042 **multiple mutants at sub-optimal temperature.**

1043 (a) Enhanced dormancy phenotype of *mpk1/7/14* and *mpk1/2/7/14* seeds.

1044 Freshly harvested seeds were stored in a desiccator at room temperature.

1045 Seeds were imbibed at 22 °C for 7 days without cold stratification. Values are

1046 means of three technical replicates with SDs. We obtained similar results from

1047 triplicate experiments, and typical data are presented. (b) Effect of cold

1048 stratification on germination of the freshly harvested seeds. The seeds at DAH 2

1049 were imbibed at 22 °C for 5 days with (+) or without (–) preceding cold

1050 stratification at 4 °C for 4 days. (c) Germination time course of AR seeds imbibed

1051 at 5 °C. (a, b) Significant differences between samples are indicated by different

1052 letters (Tukey HSD tests, $P < 0.05$). (b, c) Results from three independent

1053 seed batches are shown with averages and SDs.

1054

1055 **Supplementary Fig. 12 MPK7 activity in germinating and non-germinating**

1056 **seeds (Biological replication of Fig. 6).** In panel c, electrophoresis and

1057 staining of the proteins in the kinase assay mixture has not been done.

1058

1059

1060 **Supplementary Data**

1061

1062 **Supplementary Table 1** Genes and its mutant alleles used in this study

1063

1064 **Supplementary Table 2** Primers used in this study

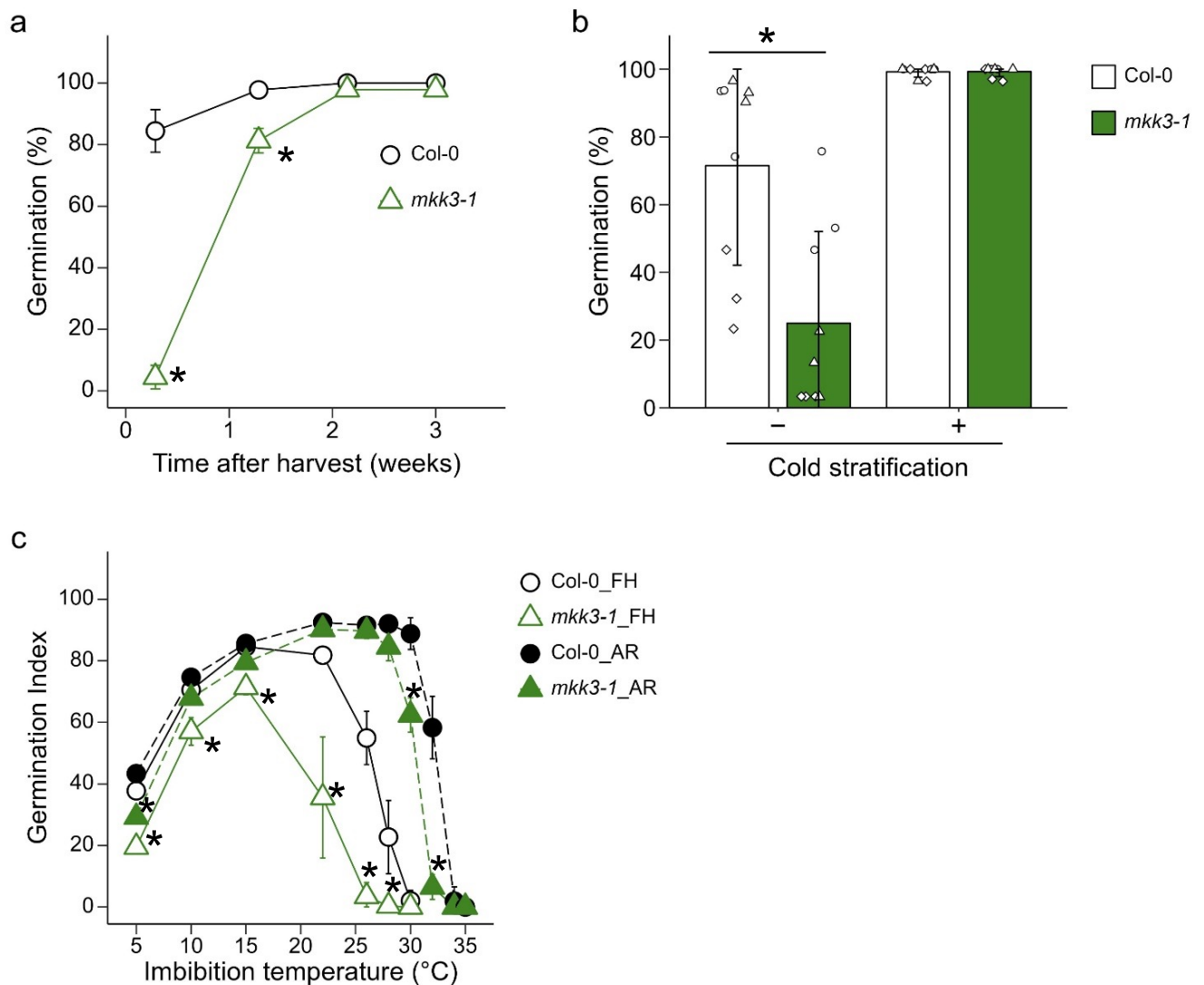


Fig. 1 MKK3 is a modulator of germination response to temperature in both freshly harvested and after-ripened seeds. Asterisks indicate statistical differences between wild type (Col-0) and *mkk3-1* ($p < 0.05$, Student's t test). **a** Enhanced dormancy phenotype of *mkk3-1* seeds. Freshly harvested seeds were stored in a desiccator at room temperature for up to 3 weeks. Seeds were imbibed at 22 °C for 7 days without cold stratification. Values are means of three technical replicates with SDs. We obtained similar results from triplicate experiments, and typical data are presented. **b** Effect of cold stratification on germination of the freshly harvested seeds. The seeds at DAH 2 were imbibed at 22 °C for 5 days with (+) or without (-) cold stratification at 4 °C for 4 days. Results from three independent seed batches are shown with averages and SDs. **c** MKK3 is a modulator of germination temperature range. Germination index of freshly harvested (FH, DAH 2, open symbols) and after-ripened (AR, DAH 150, closed symbols) seeds imbibed at 5 to 35 °C for 14 days are shown. Results from three independent seed batches are shown with averages and SDs.

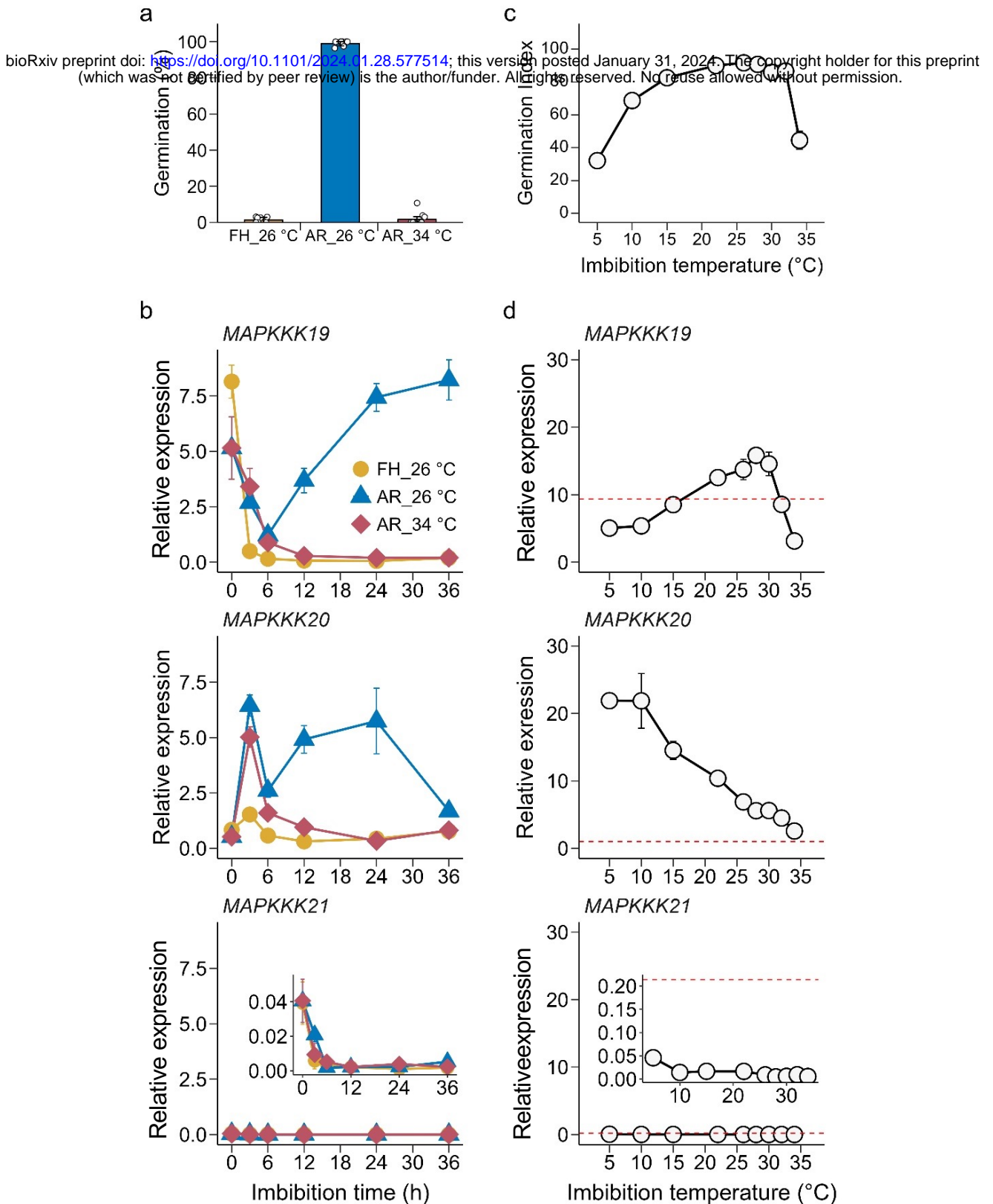


Fig. 2 Temperature dependent expression *MAPKKK19* and *MAPKKK20* in FH and AR seeds. a Final percentage germination of FH (DAH 2) and AR (DAH 49) Col-0 seeds used for gene expression analysis in **b**. The FH seeds were imbibed at 26 °C, and the AR seeds were imbibed at either 26 °C or 34 °C for 7 days. Results from three technical replicates are shown with averages and SDs. **b** Effect of imbibition temperature on the expression time course of *MAPKKK19*, *20* and *21* in FH and AR seeds. FH seeds imbibed at 26 °C (orange circles), and AR seeds imbibed at 26 °C (blue triangles) or 34 °C (red diamonds) were used for RNA extraction. **c** GI of AR (DAH 330) Col-0 seeds used for gene expression analysis in **d**. The seeds were imbibed at various temperatures for 14 days. Values are means of three technical replicates with SDs. **d** Effect of imbibition temperature on the expression of *MAPKKK19*, *20* and *21*. Total RNA was prepared from 24 h imbibed seeds. Dashed red lines indicate the expression levels in dry seeds. **b** and **d** Transcript levels were quantified by qRT-PCR. We obtained similar results from three biological replicates, and typical data is presented.

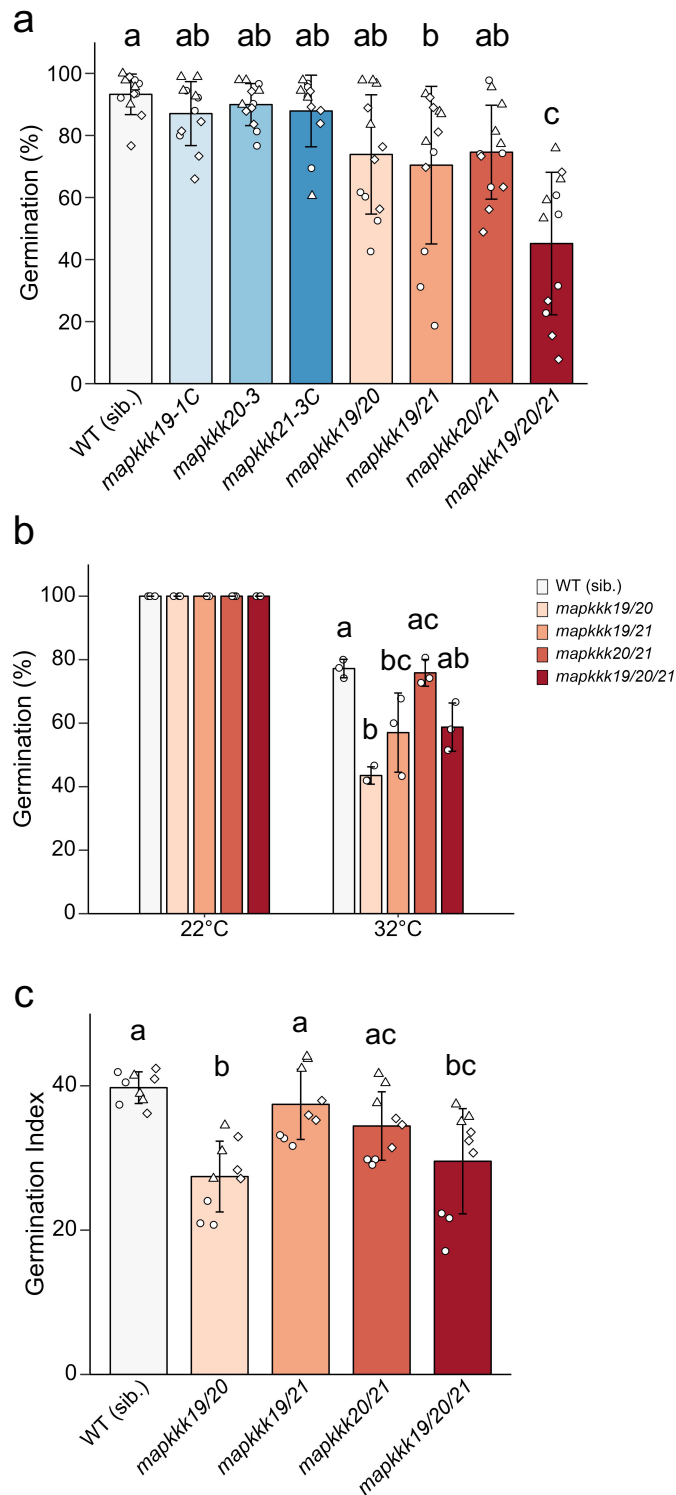


Fig. 3 *MAPK19* and *20* are responsible for germination of FH and AR seeds, and *MAPK21* contributes to germination of FH seeds. Significant differences between samples are indicated by different letters (Tukey HSD tests, $P < 0.05$). **a** Germination of FH seeds of single and multiple mutants at 22 °C. Freshly harvested (DAH 2) seeds were imbibed for 7 days. Results from three biological replicates are shown with averages and SDs. **b** Effect of supra-optimal temperature on germination of AR seeds. The seeds were imbibed at 22 °C and 32 °C for 5 days. We obtained similar results from three biological replicates, and a typical result is shown. **c** Effect of sub-optimal temperature on germination of AR seeds. The seeds were imbibed at 5 °C for 14 days. GI values were presented as mean (\pm SD) of three biological replicates.

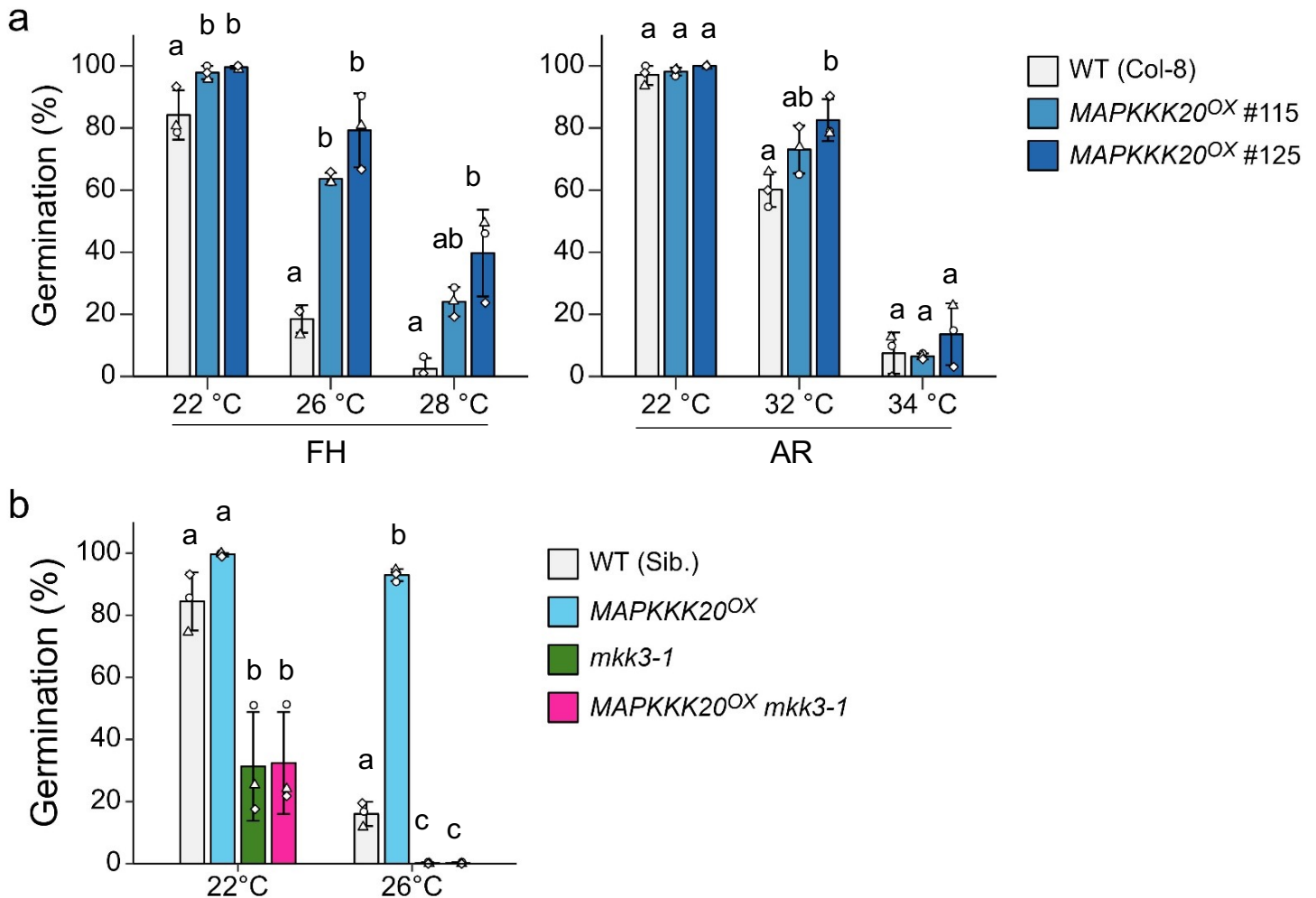


Fig. 4 *MAPKKK20* over-expression stimulates germination of FH and AR seeds at supra-optimal temperatures in the MKK3 pathway. The values are the means (\pm SD) of three biological replicates. Significant differences between lines are indicated by different letters (Tukey HSD tests, $P < 0.05$). **a** FH (DAH 2) and AR (DAH 64) 35S::*MAPKKK20-10xMYC* (*MAPKKK20^{OX}*) seed germination response to imbibition temperature. The seeds were imbibed for 5 days. **b** *mkk3-1* is epistatic to *MAPKKK20^{OX}*. Freshly harvested (DAH 2) seeds were imbibed at 22 and 26 °C for 7 days without stratification.

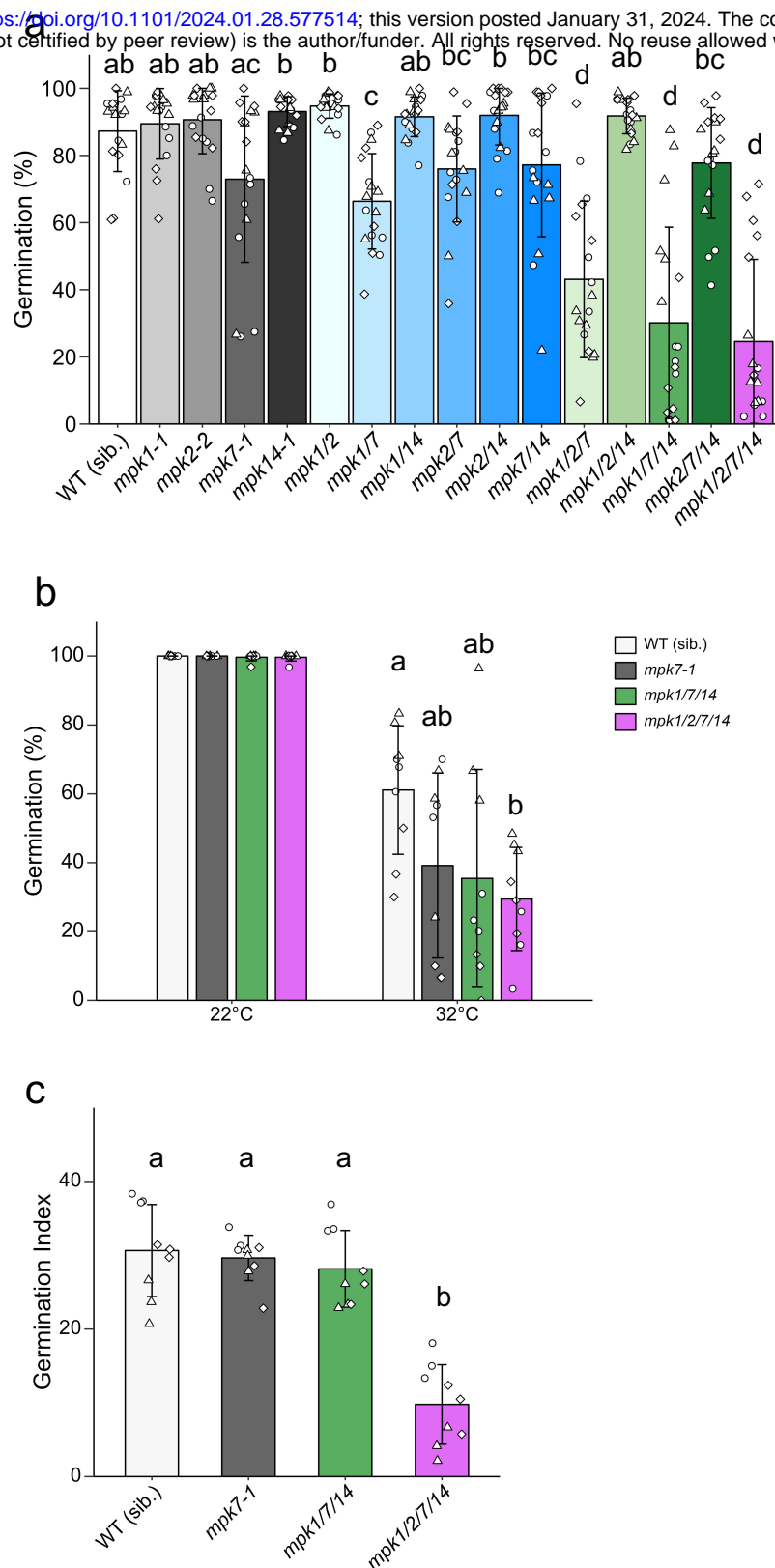


Fig. 5 Group C MPKs are responsible for germination of FH and AR seeds. The values are the means (\pm SD) of three biological replicates. Significant differences between lines are indicated by different letters (Tukey HSD tests, $P < 0.05$). **a** Germination of FH (DAH 2) seeds of single and multiple mutants at 22 °C. The seeds were imbibed for 7 days without cold stratification. **b** Effect of supra-optimal temperature on germination of AR seeds. The seeds were imbibed at either 22 °C or 32 °C for 5 days. **c** Effect of sub-optimal temperature on germination of AR seeds. Germination index of after-ripened seeds imbibed at 5 °C for 14 days.

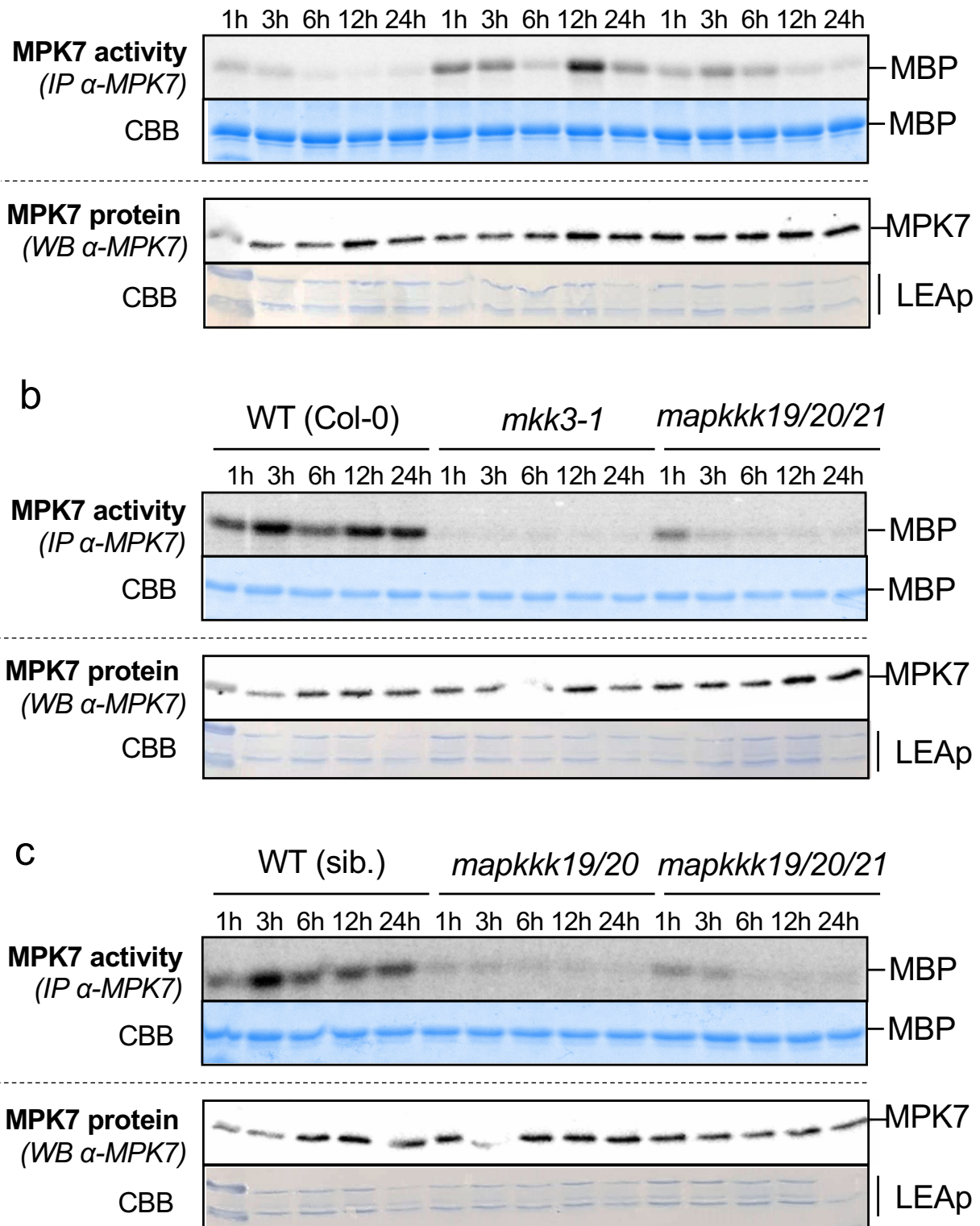


Fig. 6 MPK7 activity in germinating and non-germinating seeds. MBP was used as a phosphorylation substrate of MPK7 which was immunoprecipitated from imbibed seeds with an anti-MPK7 antibody. Amount of MPK7 protein was monitored by immunoblot using an anti-MPK7 antibody. Equal loading was indicated by Coomassie staining of the LEA protein (LEAp) on the membrane. **a** Effect of imbibition temperature and seed dormancy status (after-ripening) on MPK7 activity. **b** MPK7 activity in after-ripened *mkk3-1* and *mapkkk19/20/21* seeds imbibed at permissive temperature for germination (28 °C). **c** MPK7 activity in after-ripened *mapkkk19/20* and *mapkkk19/20/21* seeds imbibed at permissive temperature for germination (28 °C).

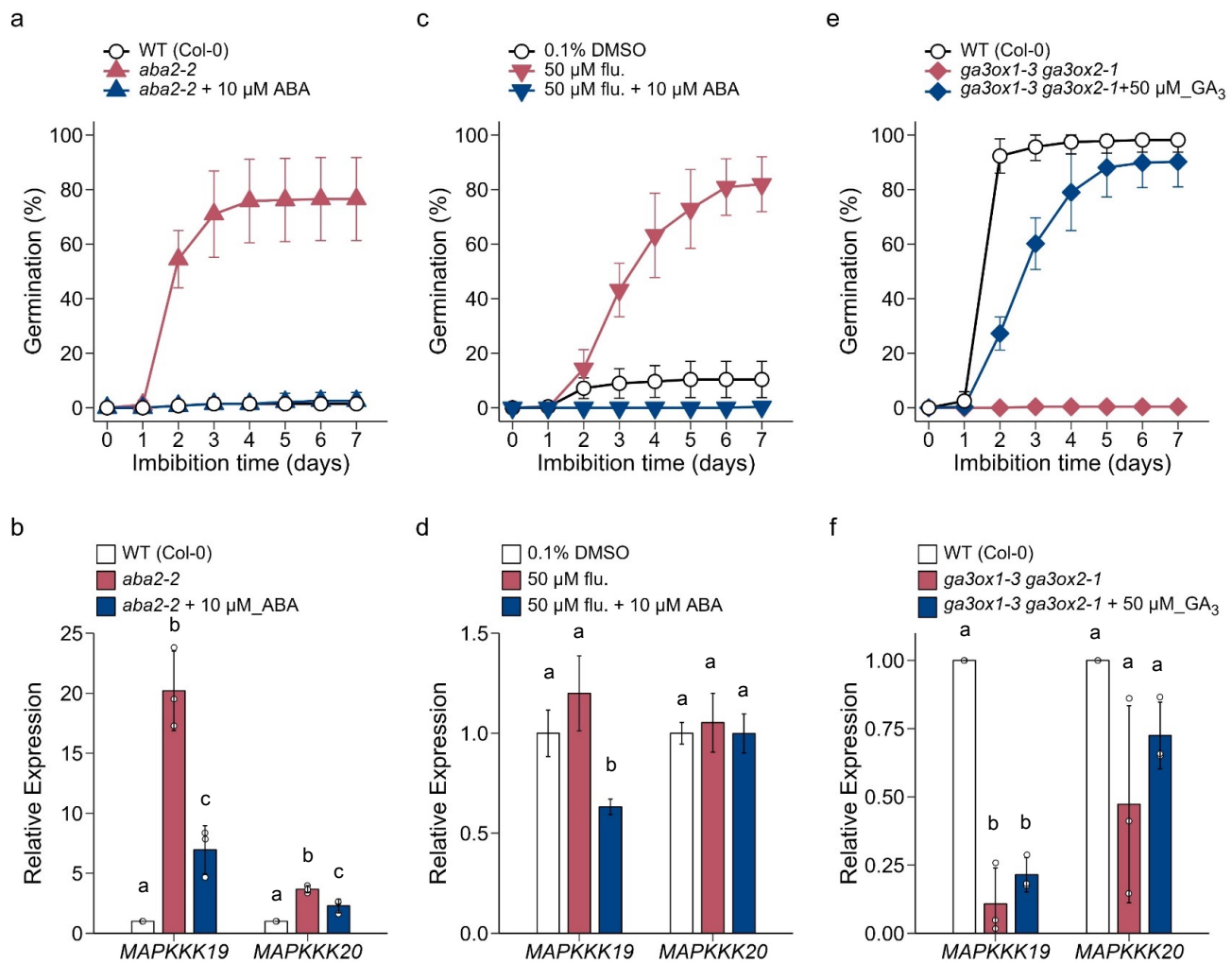


Fig. 7 Effect of ABA and GA on the expression of *MAPKKK19* and *MAPKKK20*. Seed germination and gene expression data are the means (\pm SD) of three biological replicates. Total RNA was prepared from FH (a; DAH2) or AR (c; DAH 80, e; DAH 50) seeds imbibed for 24 h or 48 h. Transcript levels were quantified by qRT-PCR with At2g20000 as an internal standard. Significant differences in the gene expression levels are indicated by different letters (Tukey HSD tests, $P < 0.05$). **a-d** Effect of endogenous and exogenous ABA on seed germination (a, c) and gene expression (b, d). The endogenous ABA levels in FH seeds imbibed at 28 $^{\circ}$ C were reduced by *aba2-2* mutation (a, b) or by the ABA biosynthesis inhibitor, fluridion in Col-0 AR seeds imbibed at 32 $^{\circ}$ C (c and d). Total RNA was prepared from seeds imbibed for 24 h. **e, f** Effect of endogenous and exogenous GA on seed germination (e) and gene expression (f). AR seeds were imbibed at 30 $^{\circ}$ C. Total RNA was prepared from AR seeds imbibed for 24h.

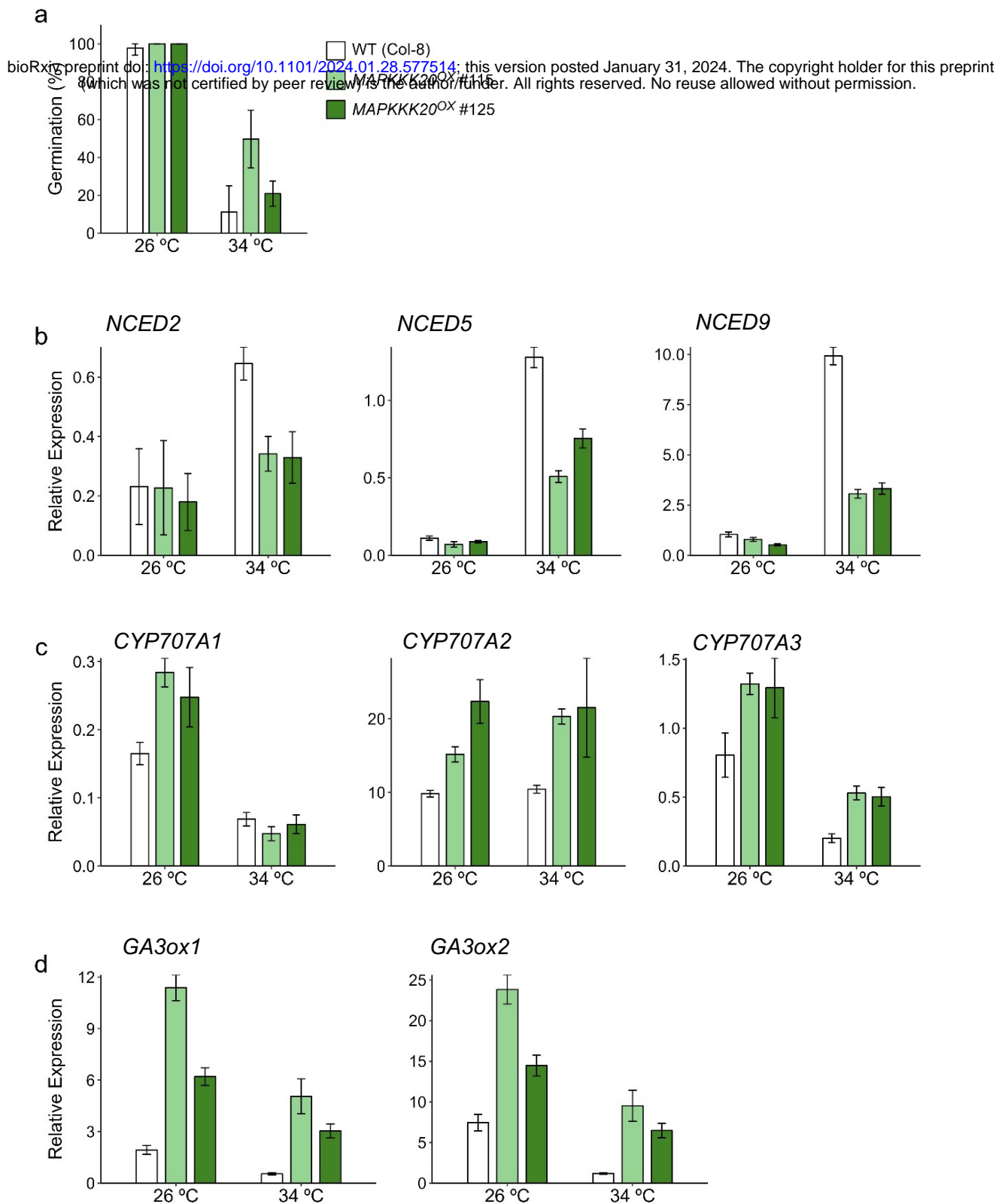


Fig. 8 *MAPKKK20* can induce expression of ABA catabolism and GA biosynthesis genes and reduce expression of ABA biosynthesis genes. Values are means of three technical replicates with SDs. We obtained similar results from triplicate experiments, and typical data are presented. Transcript levels were quantified by qRT-PCR with *At2g20000* as an internal control for normalization. **a** Germination of wild type (Col-8) AR (DAH 554) seeds used for the gene expression analysis. The seeds were imbibed for 7 days. **b** Expression of ABA biosynthesis enzyme genes, *NCEDs*, in seeds imbibed for 24 h. **c** Expression of ABA catabolism enzyme genes. The expression of *CYP707A1* and *CYP707A3* were analyzed at 24h after the start of imbibition, and *CYP707A2* was analyzed at 3h after the start of imbibition. **d** Expression of GA biosynthesis enzyme genes, *GA3ox1* and *GA3ox2* in seeds imbibed for 12 h.

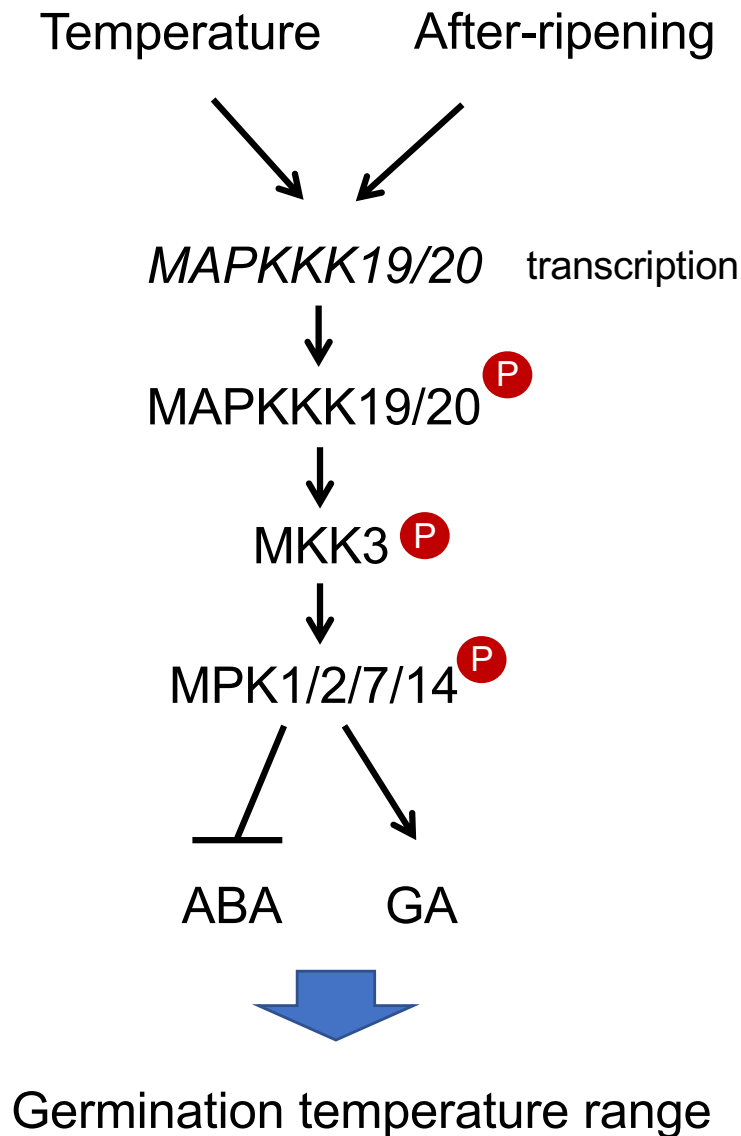


Fig. 9 Modulation of germination temperature range by MAPKKK19/20-MKK3-MPK1/2/7/14 module. A model. The MKK3 module is regulated by temperature through the regulation of *MAPKKK19/20* expression, and it regulates seed germination at sub- and supra-optimal temperature conditions through modulation of ABA and GA metabolism. Expression of *MAPKKK19/20* is regulated by temperature and unknown signal of after-ripening. Accumulation of MAPKKK19 and MAPKKK20 proteins induce activation of the MKK3 module, and the kinase cascade signaling decreases ABA accumulation and increases GA production. During after-ripening of the seeds, an unidentified mechanism modulates *MAPKKK19/20* expression, and expands the permissive temperature range of *MAPKKK19/20* induction by suppressing ABA production.