



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

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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           Absciscic 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,

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

## Results

### Arabidopsis MKK3 regulates germination response to temperature in freshly harvested and after-ripened seeds

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

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.

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

To evaluate the contribution of transcriptional regulation of *MAPKKKs* on seed germination response to temperature, we analyzed *MAPKKK20* overexpression (*MAPKKK20<sup>OX</sup>*) lines (Supplementary Fig. 9a). The two independent lines accumulated ca. 36-fold more *MAPKKK20* transcripts in dry seeds than the non-transformant wild type (Supplementary Fig. 9b). The FH and AR seeds of *MAPKKK20<sup>OX</sup>* lines showed significantly higher percentage germination than WT at supra-optimal temperatures (Fig. 4a and b). These 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<sup>OX</sup> mkk3-1* double mutant. In contrast to *MAPKKK20<sup>OX</sup>*  
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

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

278

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

It has been reported that high temperature inhibits seed germination by inducing ABA biosynthesis and suppressing GA biosynthesis in Arabidopsis and 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 GA<sub>3</sub> 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<sup>OX</sup>* 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<sup>OX</sup>* 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<sup>OX</sup>* 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<sup>OX</sup>* 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<sup>OX</sup>* 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

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

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

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

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

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

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

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

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

## Materials and Methods

### Plant materials and growth conditions

T-DNA insertion lines of Arabidopsis (*Arabidopsis thaliana* L. Heynh.) Columbia-0 (Col-0) accession were obtained from the Arabidopsis Biological Resource Center [*mkk3-1* (SALK\_051970; Takahashi et al., 2007), *mkk3-2* (SALK\_208528C; Sözen et al., 2020), *mpk1-1* (SALK\_063847C; Enders et al., 2017), *mpk2-2* (SALK\_047422C; Lv et al., 2021), *mpk7-1* (SALK\_113631), *mpk14-1* (SALK\_022928C; Lv et al., 2021), *mapkkk13-1* (GK-277E09), *map3k14-1* (GK-653B01; Sözen et al., 2020), *mapkkk15-1* (SALK\_084817), *map3k16* (Choi et al., 2017), *mapkkk17-2* (SALK\_080309C; Romero-Hernandez and Martinez, 2022), *mkkk18-2* (GK-676E02; Mitula et al., 2015) and *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<sup>ox</sup>* 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 *mapkkkk21-3* in Nossen background were backcrossed  
 467 four times with Col-0, and the introgression lines *mapkkk19-1C* and  
 468 *mapkkk21-3C* were selected from BC<sub>4</sub>F<sub>2</sub> and BC<sub>3</sub>F<sub>2</sub> siblings, respectively, by  
 469 PCR. To obtain multiple *mapkkk* mutants, we first crossed *mapkkk19-1C* and  
 470 *mapkkk21-3C*, and then crossed the F<sub>1</sub> plant with *mapkkk20-3*. Then, multiple  
 471 mutants were isolated from F<sub>2</sub> and F<sub>3</sub> 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<sup>ox</sup>* (#125) and *mkk3-1*  
 474 and selected double mutants from the F<sub>2</sub> 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

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

For the chemical treatments, 30 seeds were imbibed with 250  $\mu$ L of hormone solutions in the well of a 24-well plate at constant temperature in continuous light for 5 to 7 days without cold stratification.  $\pm$ ABA (SIGMA, A1049), GA<sub>3</sub> (SIGMA, G7645) and fluridone (Daw Elanco, recrystallized) were first dissolved in dimethylsulfoxide (DMSO) and then diluted to the final concentrations with ultra-pure water. The final concentration of DMSO was 0.1%, and 0.1% DMSO solution was used as the control.

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

Dry or imbibed seeds (15-20 mg dry weight), seedlings and siliques were frozen with a  $\phi$  5mm stainless bead in LN<sub>2</sub> immediately after sampling, and stored at -80 °C until use. The seeds in developmental stages were collected as described previously (Zheng et al. 2022). In brief, we marked open flowers at anthesis (day zero) by thread, and collected the fruits at 3, 6, 9, 12, 15 and 18 days post-anthesis (DPA). Total RNAs were isolated from 20 siliques with seeds (DPA 3 and 6) and seeds collected by opening the 20 siliques (DPA 9 to 18). The frozen tissues were squashed in a 2 ml tube by a bead mill type homogenizer (Biomedical Science, Tokyo). Total RNA was extracted using the hexadecyltrimethylammonium bromide method as described previously (Zheng et al. 2022). RNA was reverse-transcribed to cDNA by using DNA removal and a 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 °C, 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 LN<sub>2</sub> 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

## 574 **References**

575

576 **Argyris J, Dahal P, Hayashi E, Still DW, Bradford KJ.** (2008) Genetic variation  
577 for lettuce seed thermoinhibition is associated with temperature-sensitive  
578 expression of abscisic acid, gibberellin, and ethylene biosynthesis,  
579 metabolism, and response genes. *Plant Physiology* **148**, 926–947.

580 **Astola L, Molenaar J.** 2014. A new modified histogram matching normalization  
581 for time series microarray analysis. *Microarrays* **3**, 203–211

582 **Barrero JM, Cavanagh C, Verbyla KL, et al.** 2015. Transcriptomic analysis of  
583 wheat near-isogenic lines identifies PM19-A1 and A2 as candidates for a  
584 major dormancy QTL. *Genome Biology* **16**, 93.

585 **Baskin JM, Baskin CC.** 1983. Seasonal Changes in the Germination  
586 Responses of Buried Seeds of *Arabidopsis thaliana* and Ecological  
587 Interpretation. *Botanical Gazette* **144**, 540–543.

588 **Baskin CC, Baskin JM.** 2014. Germination Ecology of Seeds with Nondeep  
589 Physiological Dormancy. In *Seeds*. Elsevier, 79–117.

590 **Bewley JD.** 1997. Seed germination and dormancy. *Plant Cell* **9**, 1055–1066.

591 **Bewley JD, Bradford KJ, Hilhorst HWM and Nonogaki H.** 2013. *Seeds*  
592 *-Physiology of Development, Germination and Dormancy*, 3rd Edition.  
593 Springer.

594 **Bi, G., Zhou, Z., Wang, W., Li, L., Rao, S., Wu, Y., Zhang, X., Menke, F.L.H.,**

595       **Chen, S., and Zhou, J.M.** 2018. Receptor-like cytoplasmic kinases directly  
596       link diverse pattern recognition receptors to the activation of  
597       mitogen-activated protein kinase cascades in arabidopsis. *Plant Cell* **30**:  
598       1543–1561.

599       **Choi S-W, Lee S-B, Na Y-J, Jeung S-G, Kim SY.** 2017. Arabidopsis MAP3K16  
600       and other salt-inducible MAP3Ks regulate ABA response redundantly.  
601       *Molecules and Cells* **40**, 230–242.

602       **Clough E, Barrett T.** 2016. The gene expression omnibus database. *Methods*  
603       *Mol Biol* 1418, 93-110.

604       **Colcombet J, Sozen C and Hirt H.** 2016. Convergence of multiple MAP3Ks on  
605       MKK3 identifies a set of novel stress MAPK modules. *Front Plant Sci* **7**,  
606       1941.

607       **Danquah A, de Zélicourt A, Boudsocq M, et al.** 2015. Identification and  
608       characterization of an ABA-activated MAP kinase cascade in *Arabidopsis*  
609       *thaliana*. *Plant Journal* **82**, 232–244.

610       **Dóczi R, Brader G, Pettkó-Szandtner A, Rajh I, Djamei A, Pitzschke A, Teige**  
611       **M, Hirt H.** 2007. The Arabidopsis Mitogen-Activated Protein Kinase Kinase  
612       MKK3 Is Upstream of Group C Mitogen-Activated Protein Kinases and  
613       Participates in Pathogen Signaling. *Plant Cell* **19**, 3266–3279.

614       **Enders TA, Frick EM, Strader LC, Drive B, Louis S.** 2017. An Arabidopsis  
615       kinase cascade influences auxin-responsive cell expansion. *Plant Journal*  
616       **92**, 68–81.

617       **Farooq M, Basra SMA, Ahmad N, Hafeez K.** 2005. Thermal hardening: A new  
618       seed vigor enhancement tool in rice. *Journal of Integrative Plant Biology* **47**,

619 187–193.

620 **Fujino K, Sekiguchi H, Sato T, Kiuchi H, Nonoue Y, Takeuchi Y, Ando T, Lin**  
621 **SY, Yano M.** 2004. Mapping of quantitative trait loci controlling  
622 low-temperature germinability in rice (*Oryza sativa* L.). Theoretical and  
623 Applied Genetics. **108**, 794–799.

624 **Gonai T, Kawahara S, Tougou M, Satoh S, Hashiba T, Hirai N, Kawaide H,**  
625 **Kamiya Y, Yoshioka T.** 2004. Absciscic acid in the thermoinhibition of lettuce  
626 seed germination and enhancement of its catabolism by gibberellin. J Exp  
627 Bot 55: 111–118

628 **Graeber K, Linkies A, Wood ATA, Leubner-Metzger G.** 2011. A guideline to  
629 family-wide comparative state-of-the-art quantitative RT-PCR analysis  
630 exemplified with a Brassicaceae cross-species seed germination case study.  
631 Plant Cell **23**, 2045–2063.

632 **Ichimura K, Shinozaki K, Tena G, et al.** 2002. Mitogen-activated protein kinase  
633 cascades in plants: a new nomenclature. Trends in Plant Science **7**,  
634 301–308.

635 **Jaakola L, Pirttilä AM, Halonen M, Hohtola A.** 2001. Isolation of high quality  
636 RNA from Bilberry ( *Vaccinium myrtillus* L .) Fruit. **19**, 201–203.

637 **Jonak C, Ökrész L, Bögre L, Hirt H.** 2002. Complexity, cross talk and  
638 integration of plant MAP kinase signalling. Current Opinion in Plant Biology **5**,  
639 415–424.

640 **Kumar S, Stecher G, Li M, Knyaz C, Tamura K.** 2018. MEGA X: Molecular  
641 evolutionary genetics analysis across computing platforms. Molecular  
642 Biology and Evolution **35**, 1547–1549.

643 **Kushiro T, Okamoto M, Nakabayashi K, Yamagishi K, Kitamura S, Asami T,**  
644 **Hirai N, Koshiba T, Kamiya Y, Nambara E.** 2004. The *Arabidopsis*  
645 cytochrome P450 CYP707A encodes ABA 8'-hydroxylases: Key enzymes in  
646 ABA catabolism. *EMBO Journal* **23**, 1647–1656.

647 **Lee JS, Huh KW, Bhargava A, Ellis BE.** 2008. Comprehensive analysis of  
648 protein-protein interactions between *Arabidopsis* MAPKs and MAPK kinases  
649 helps define potential MAPK signalling modules. *Plant Signaling & Behavior*  
650 **3**, 1037–1041.

651 **Lim S, Park J, Lee N, Jeon J, Toh S, Watanabe A, Kim J, Kang H, Kim DH,**  
652 **Kawakami N and Choi G.** 2013. ABA-INSENSITIVE3, ABA-INSENSITIVE5,  
653 and DELLAs interact to activate the expression of *SOMNUS* and other  
654 high-temperature-inducible genes in imbibed seeds in *Arabidopsis*. *Plant*  
655 *Cell* **25**, 4863-4878.

656 **Lv B, Yu Q, Liu J, Wen X, Yan Z, Hu K, Li H, Kong X, Li C, Tian H, Smet ID,**  
657 **Zhang X-S, Ding Z.** 2020. Non-canonical AUX/IAA protein IAA33 competes  
658 with canonical AUX/IAA repressor IAA5 to negatively regulate auxin  
659 signaling. *EMBO Journal* **39**, e101515.

660 **Mao X, Zhang J, Liu W, Yan S, Jiu Q, Fu H, Zhao J, Huang W, Dong J,**  
661 **Zhang S, Yang T, Yang W, Liu B, Wng F.** 2019. The  
662 MKKK62-MKK3-MAPK7/14 module negatively regulates seed dormancy in  
663 rice. *Rice* **12**, <https://doi.org/10.1186/s12284-018-0260-z>

664 **Matsuoka D, Yasufuku T, Furuya T, Nanmori T.** 2015. An abscisic acid  
665 inducible *Arabidopsis* MAPKKK, MAPKKK18 regulates leaf senescence via  
666 its kinase activity. *Plant Molecular Biology* **87**, 565–575.

667 **Mitchum MG, Yamaguchi S, Hanada A, Kuwahara A, Yoshioka Y, Kato T,**  
668 **Tabata S, Kamiya Y, Sun TP.** 2006. Distinct and overlapping roles of two  
669 gibberellin 3-oxidases in Arabidopsis development. *Plant Journal* **45**,  
670 804–818.

671 **Mitula F, Tajdel M, Cieřla A, Kasproicz-Maluřki A, Kulik A,**  
672 **Babula-Skowrońska D, Michalak M, Dobrowolska G, Sadowski J,**  
673 **Ludwików A.** 2015. Arabidopsis ABA-activated kinase MAPKKK18 is  
674 regulated by protein phosphatase 2C ABI1 and the ubiquitin–proteasome  
675 pathway. *Plant & Cell Physiology* **56**, 2351-2367.

676 **Na Y, Choi H, Park MY, Choi S, Vo KTX, Jeon J-S, Kim SY.** 2019.  
677 OsMAPKKK63 is involved in salt stress response and seed dormancy  
678 control. *Plant Signal Behav* 14, e1578633

679 **Nakagawa T, Kurose T, Hino T, Tanaka K, Kawamukai M, Niwa Y, Toyooka K,**  
680 **Matsuoka K, Jinbo T, Kimura T.** 2007. Development of series of gateway  
681 binary vectors, pGWBs, for realizing efficient construction of fusion genes for  
682 plant transformation. *Journal of Bioscience and Bioengineering* **104**, 34–41.

683 **Nakamura S, Abe F, Kawahigashi H, et al.** 2011. A wheat homolog of  
684 MOTHER OF FT AND TFL1 acts in the regulation of germination. *Plant Cell*  
685 **23**, 3215–29.

686 **Nakamura S, Pourkheirandish M, Morishige H, et al.** 2016. Mitogen-Activated  
687 Protein Kinase Kinase 3 Regulates Seed Dormancy in Barley. *Current*  
688 *Biology*, **26**, 775–781.

689 **Nambara E, Kawaide H, Kamiya Y, Naito S.** 1998. Characterization of an  
690 Arabidopsis thaliana mutant that has a defect in ABA accumulation:

691 ABA-dependent and ABA-independent accumulation of free amino acids  
692 during dehydration. *Plant & Cell Physiology* **39**, 853–858.

693 **Oh E, Yamaguchi S, Hu J, Yusuke J, Jung B, Paik I, Lee H-S, Sun T-P,**  
694 **Kamiya Y, Choi G.** 2007. PIL5, a phytochrome-interacting bHLH protein,  
695 regulates gibberellin responsiveness by binding directly to the GAI and RGA  
696 promoters in *Arabidopsis* seeds. *Plant Cell* **19**, 1192–1208.

697 **Okamoto M, Kuwahara A, Seo M, Kushiro T, Asami T, Hirai N, Kamiya Y,**  
698 **Koshiba T, Nambara E.** 2006. CYP707A1 and CYP707A2, which encode  
699 abscisic acid 8'-hydroxylases, are indispensable for proper control of seed  
700 dormancy and germination in *Arabidopsis*. *Plant Physiology* **141**, 97–107.

701 **Preston J, Tatematsu K, Kanno Y, Hobo T, Kimura M, Jikumaru Y, Yano R,**  
702 **Kamiya Y, Nambara E.** 2009. Temporal expression patterns of hormone  
703 metabolism genes during imbibition of *Arabidopsis thaliana* seeds: a  
704 comparative study on dormant and non-dormant accessions. *Plant & Cell*  
705 *Physiology* **50**, 1786–1800.

706 **Regnard S, Otani M, Keruzore M, Teinturier A, Blondel M, Kawakami N,**  
707 **Krapp A, Colcombet J.** 2024. The MKK3 module integrates nitrate and light  
708 signals to modulate secondary dormancy in *Arabidopsis thaliana*. *bioRxiv*

709 **Romero-Hernandez G, Martinez M.** 2022. Opposite roles of MAPKKK17 and  
710 MAPKKK21 against *Tetranychus urticae* in *Arabidopsis*. *Frontiers in Plant*  
711 *Science* **13**, 1–15.

712 **Sato K, Yamane M, Yamaji N, Kanamori H, Tagiri A, Schwerdt JG, Fincher**  
713 **GB, Matsumoto T, Takeda K, Komatsuda T.** 2016. Alanine  
714 aminotransferase controls seed dormancy in barley. *Nature*

715 Communications **7**, 11625.

716 **Schwacke R, Schneider A, van der Graaff E, Fischer K, Catoni E, Desimone**  
717 **M, Frommer WB, Flügge U-I, Kunze R.** 2003. ARAMEMNON, a novel  
718 database for Arabidopsis integral membrane proteins. *Plant Physiology* **131**,  
719 16–26.

720 **Shigeyama T, Watanabe A, Tokuchi K, Toh S, Sakurai N, Shibuya N,**  
721 **Kawakami N.** 2016.  $\alpha$ -Xylosidase plays essential roles in xyloglucan  
722 remodelling, maintenance of cell wall integrity, and seed germination in  
723 *Arabidopsis thaliana*. *Journal of Experimental Botany* **67**, 5615–5629.

724 **Singh C, Kamble UR, Gupta V, Singh G, Sheoran S, Gupta A, Tyagi BS,**  
725 **Kumar P, Mishra CN, Gopalareddy K, Bishnoi SK, Sharma AK, Kumar S,**  
726 **Singh GP** (2021) Pre-harvest sprouting in wheat: current status and future  
727 prospects. *Journal of Cereal Research* **13** (Spl-1) :1–22.

728 **Sößen C, Schenk ST, Boudsocq M, Chardin C, Almeida-Trapp M, Krapp A,**  
729 **Hirt H, Mithöfer A, Colcombet J.** 2020. Wounding and Insect Feeding  
730 Trigger Two Independent MAPK Pathways with Distinct Regulation and  
731 Kinetics. *Plant Cell* **32**, 1988–2003.

732 **Sugimoto K, Takeuchi Y, Ebana K, et al.** 2010. Molecular cloning of Sdr4, a  
733 regulator involved in seed dormancy and domestication of rice. *PNAS, USA*  
734 **107**, 5792–5797.

735 **Takahashi F, Yoshida R, Ichimura K, Mizoguchi T, Seo S, Yonezawa M,**  
736 **Maruyama K, Yamaguchi-Shinozaki K, Shinozaki K.** 2007. The  
737 mitogen-activated protein kinase cascade MKK3-MPK6 is an important part  
738 of the jasmonate signal transduction pathway in *Arabidopsis*. *Plant Cell* **19**,



739        805–818.

740        **Takeuchi Y, Lin SY, Sasaki T, Yano M.** 2003. Fine linkage mapping enables

741        dissection of closely linked quantitative trait loci for seed dormancy and

742        heading in rice. *TAG. Theoretical and Applied Genetics* **107**, 1174–1180.

743        **Toh S, Imamura A, Watanabe A, et al.** 2008. High Temperature-Induced

744        Absciscic Acid Biosynthesis and Its Role in the Inhibition of Gibberellin Action

745        in Arabidopsis Seeds. *Plant Physiology* **146**, 1368–1385.

746        **Torada A, Koike M, Ogawa T, Takenouchi Y, Tadamura K, Wu J, Matsumoto**

747        **T, Kawaura K, Ogihara Y.** 2016. A Causal Gene for Seed Dormancy on

748        Wheat Chromosome 4A Encodes a MAP Kinase Kinase. *Current Biology*, **26**,

749        782–787.

750        **Toyomasu T, Kawaide H, Mitsuhashi W, Inoue Y, Kamiya Y.** 1998.

751        Phytochrome regulates gibberellin biosynthesis during germination of

752        photoblastic lettuce seeds. *Plant Physiology* **118**, 1517-1523.

753        **Xu J, Zhang S.** 2015. Mitogen-activated protein kinase cascades in signaling

754        plant growth and development. *Trends in Plant Science* **20**, 56–64.

755        **Yamaguchi S, Smith MW, Brown RGS, Kamiya Y, Sun TP.** 1998.

756        Phytochrome regulation and differential expression of gibberellin 3

757        beta-hydroxylase genes in germinating Arabidopsis seeds. *Plant Cell* **10**,

758        2115-2126.

759        **Yamauchi Y, Ogawa M, Kuwahara A, Hanada A, Kamiya Y, Yamaguchi S.**

760        2004. Activation of Gibberellin Biosynthesis and Response Pathways by

761        Low Temperature during Imbibition of Arabidopsis thaliana Seeds. *Plant Cell*

762        **16**, 367–378.

763 **Zhang M, Zhang S.** 2022. Mitogen-activated protein kinase cascades in plant  
764 signaling. *Journal of Integrative Plant Biology* **64**, 301–341.

765 **Zheng L, Otani M, Kanno Y, Seo M, Yoshitake Y, Yoshimoto K, Sugimoto K,**  
766 **Kawakami N.** 2022. Seed dormancy 4 like1 of Arabidopsis is a key regulator  
767 of phase transition from embryo to vegetative development. *Plant Journal*  
768 **112**, 460–475.

769

770

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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<sup>ox</sup>*  
 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  $P \leq 0.05$ ). **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

**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,  $P \leq 0.05$ ). **a** FH (DAH 2) and AR (DAH 64) *35S::MAPKKK20-10xMYC* (*MAPKKK20<sup>OX</sup>*) seed germination response to imbibition temperature. The seeds were imbibed for 5 days. **b** *mkk3-1* is epistatic to *MAPKKK20<sup>OX</sup>*. 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,  $P \leq 0.05$ ). **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**

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  $\mu$ 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  $\mu$ 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

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

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

(a) Schematic representation of *MAPKKK20<sup>OX</sup>* construct. Positions of primers used for expression analysis are indicated by arrows. (b) Quantification of *MAPKKK20* transcripts by qRT-PCR with At2g20000 as an internal control. Relative expression to WT (Col-8) is indicated by the fold change expression in *MAPKKK20<sup>OX</sup>* dry seeds. The values are the means ( $\pm$ SD) of three technical replicates. (c) Germination time course of AR (DAH 167) seeds imbibed at 5 oC. The values are the mean ( $\pm$ SD) of three biological replicates.

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

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

**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,  $P \leq 0.05$ ). (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

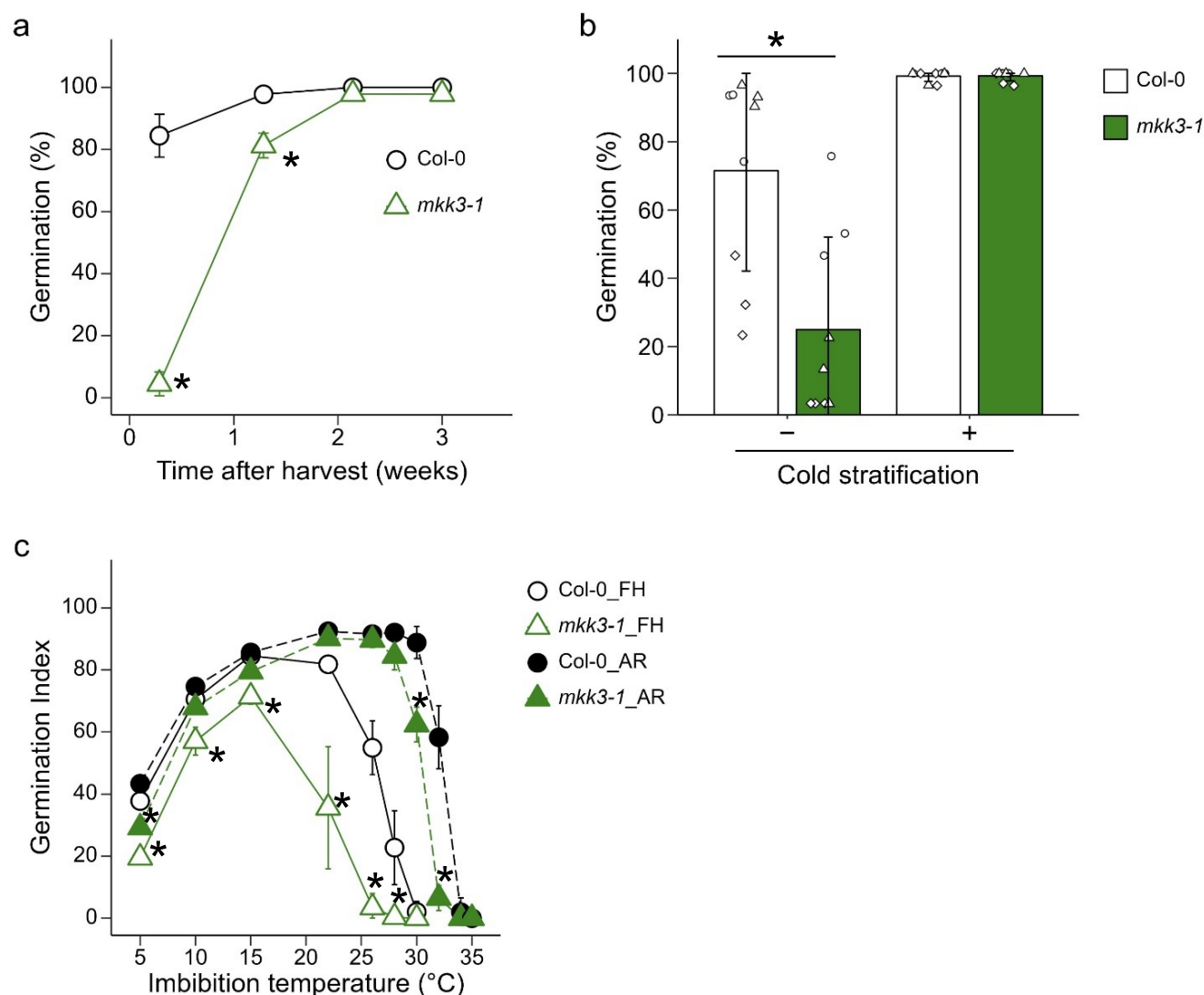
1060 **Supplementary Data**

1061

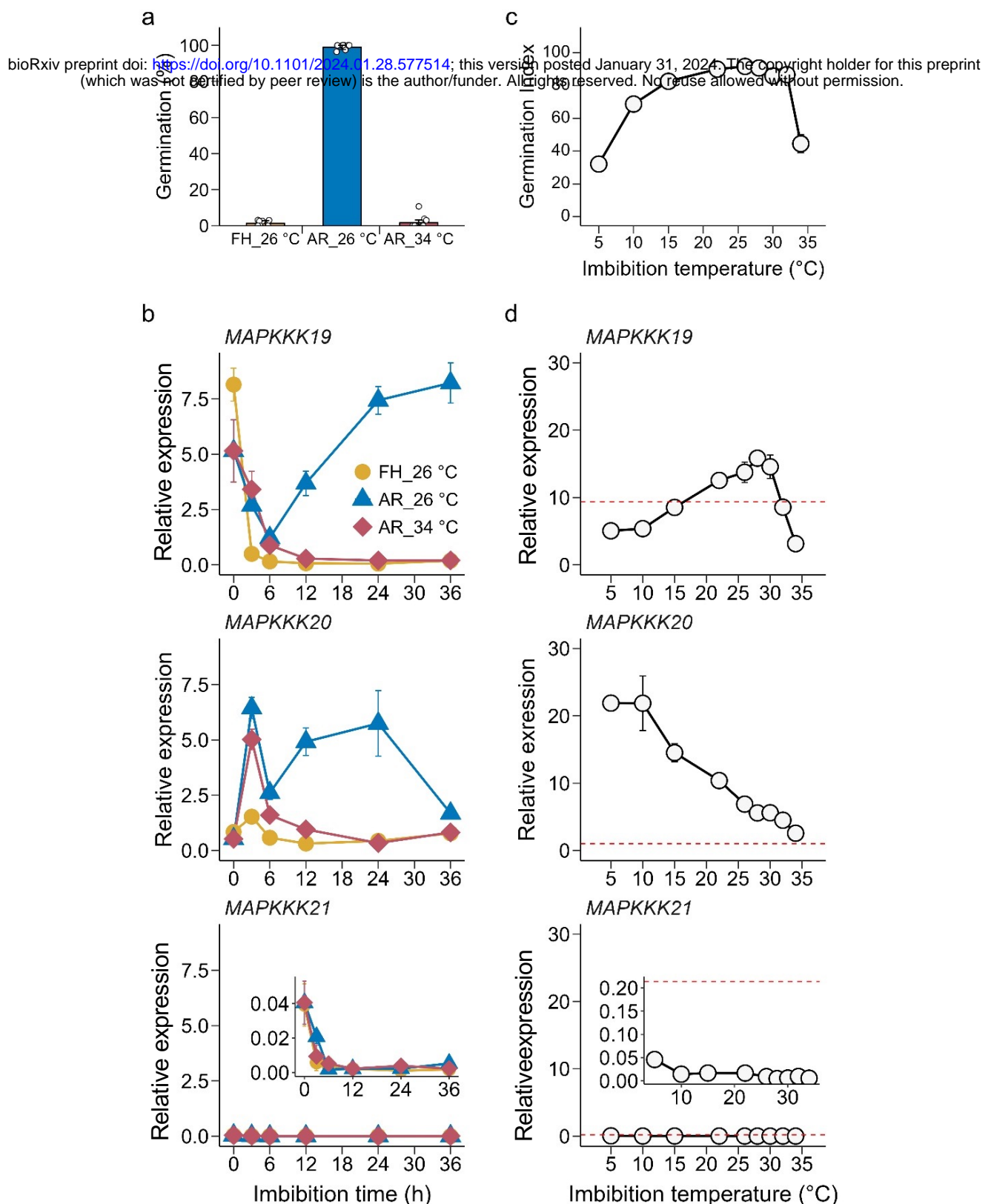
1062 **Supplementary Table 1** Genes and its mutant alleles used in this study

1063

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

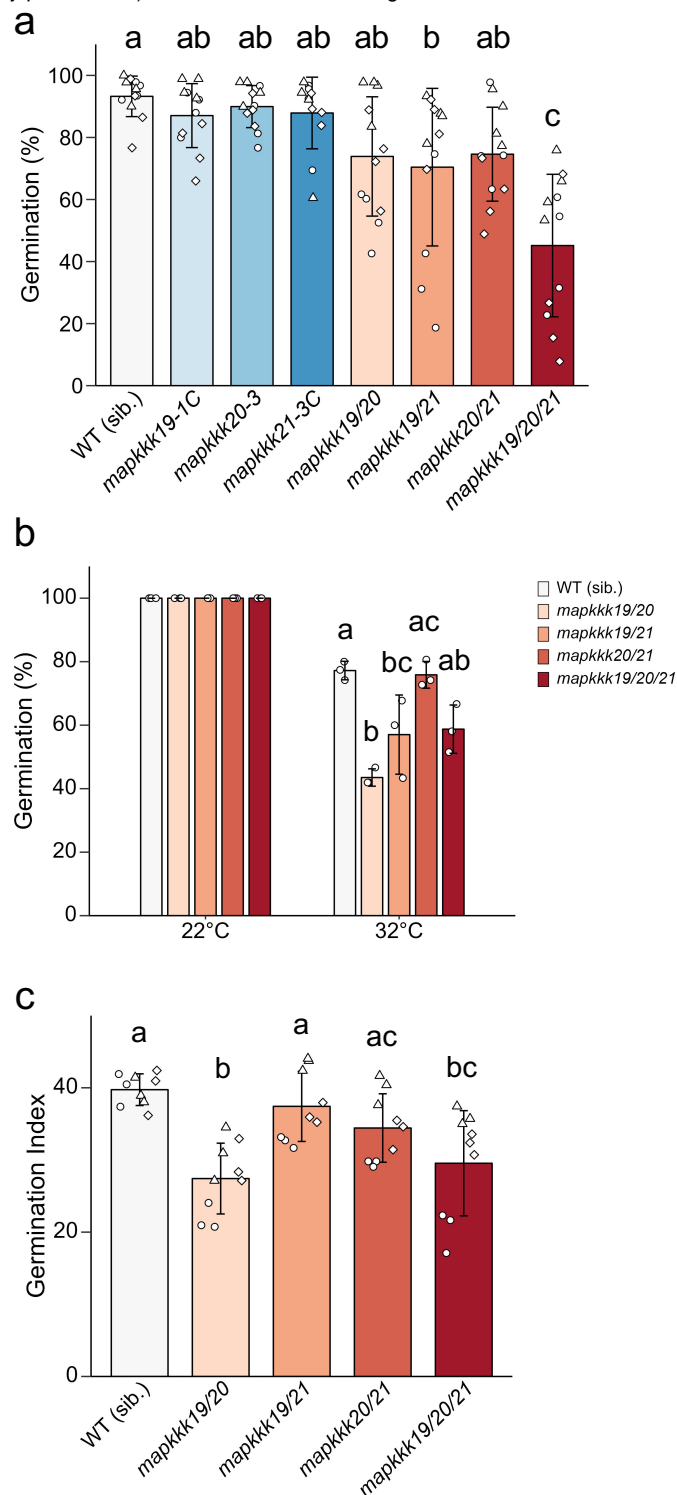


**Fig. 1 MKK3 is a modulator of germination response to temperature in both freshly harvested and after-ripened seeds.** Asterisks indicate statistical differences between wild type (Col-0) and *mkk3-1* ( $p < 0.05$ , Student's t test). **a** Enhanced dormancy phenotype of *mkk3-1* seeds. Freshly harvested 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.

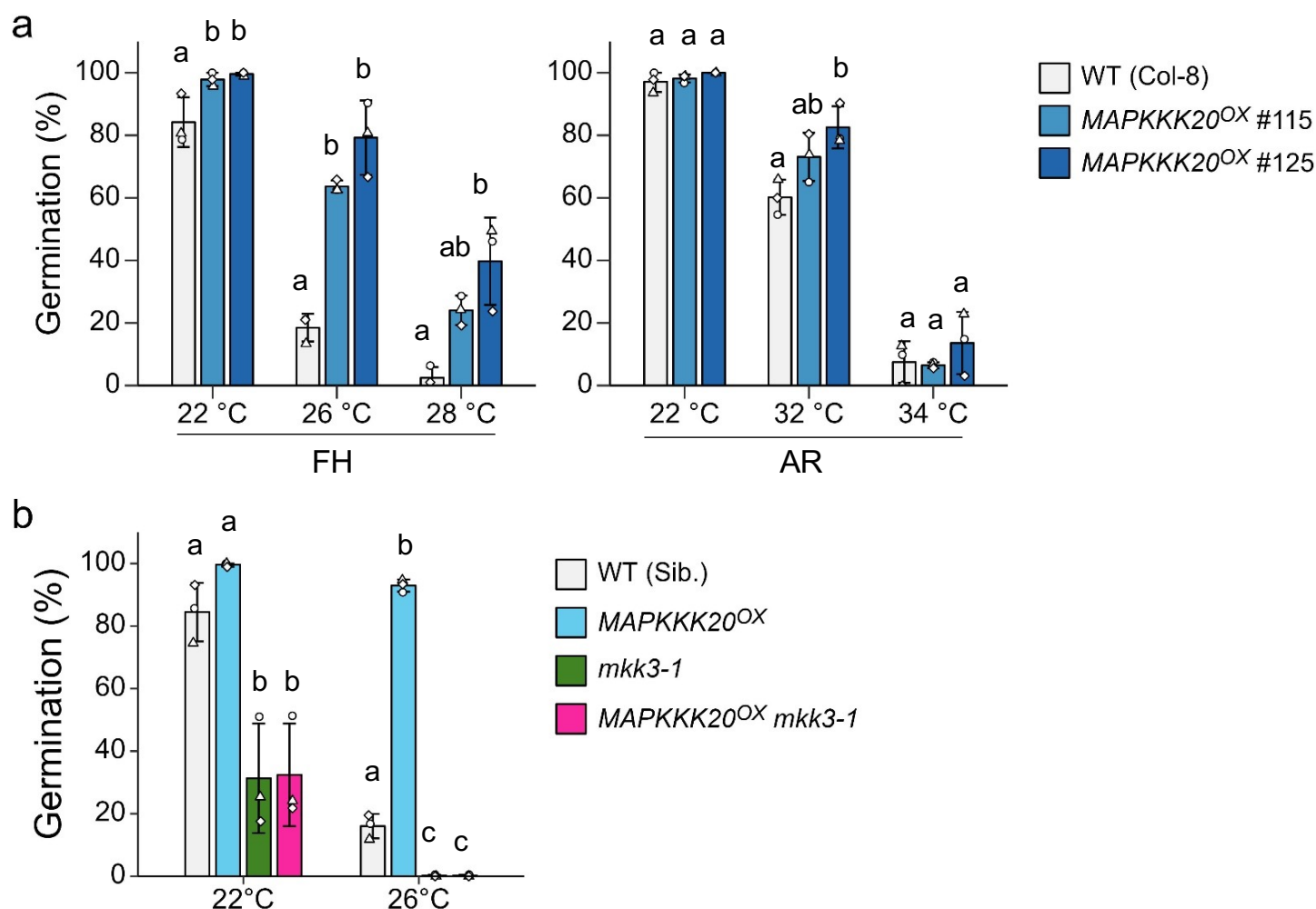


**Fig. 2 Temperature dependent expression *MAPKKK19* and *MAPKKK20* in FH and AR seeds.** **a** Final percentage germination of FH (DAH 2) and AR (DAH 49) Col-0 seeds used for gene expression analysis in **b**. The FH seeds were imbibed at 26 °C, and the AR seeds were imbibed at either 26 °C or 34 °C 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.

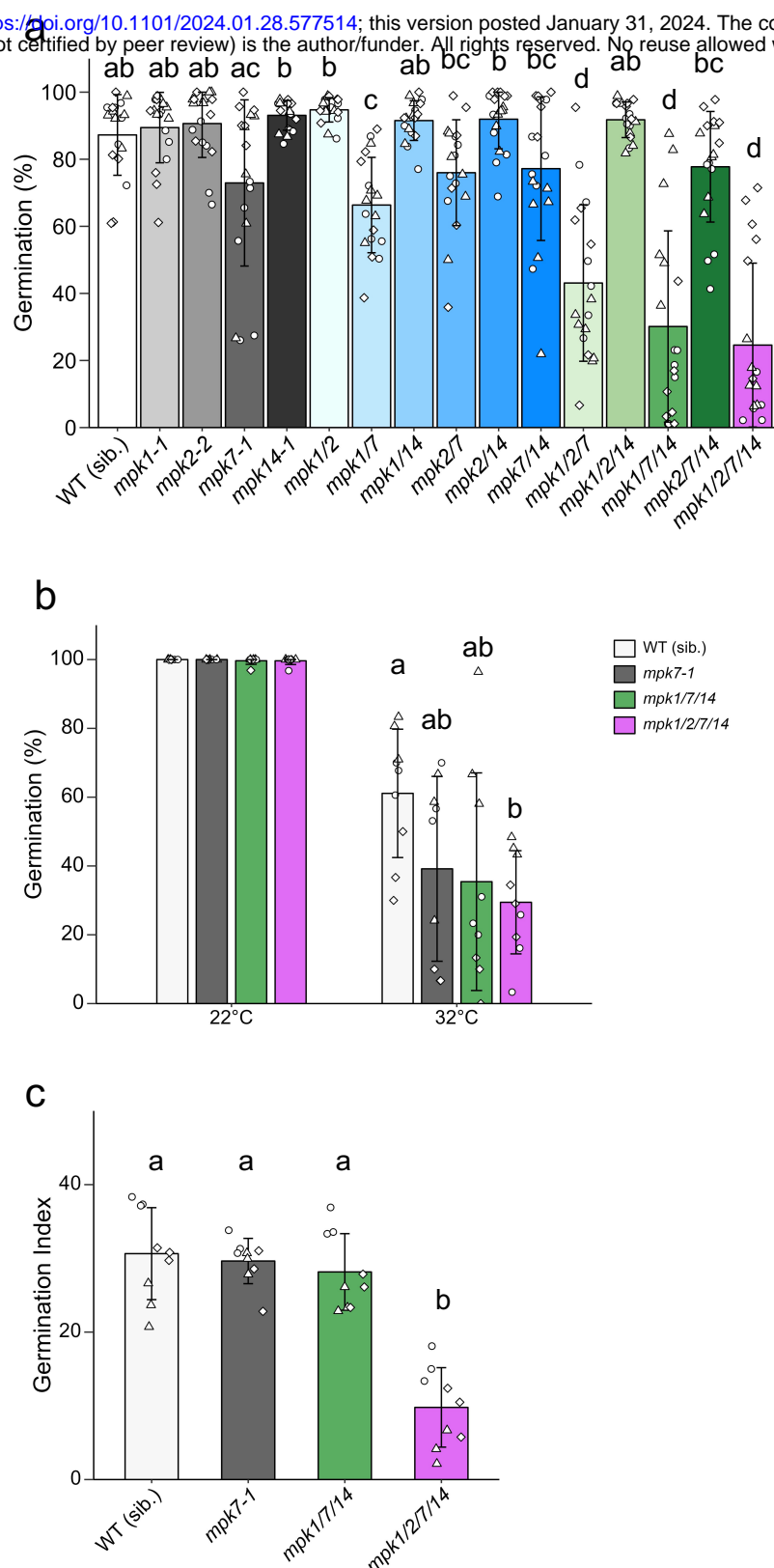




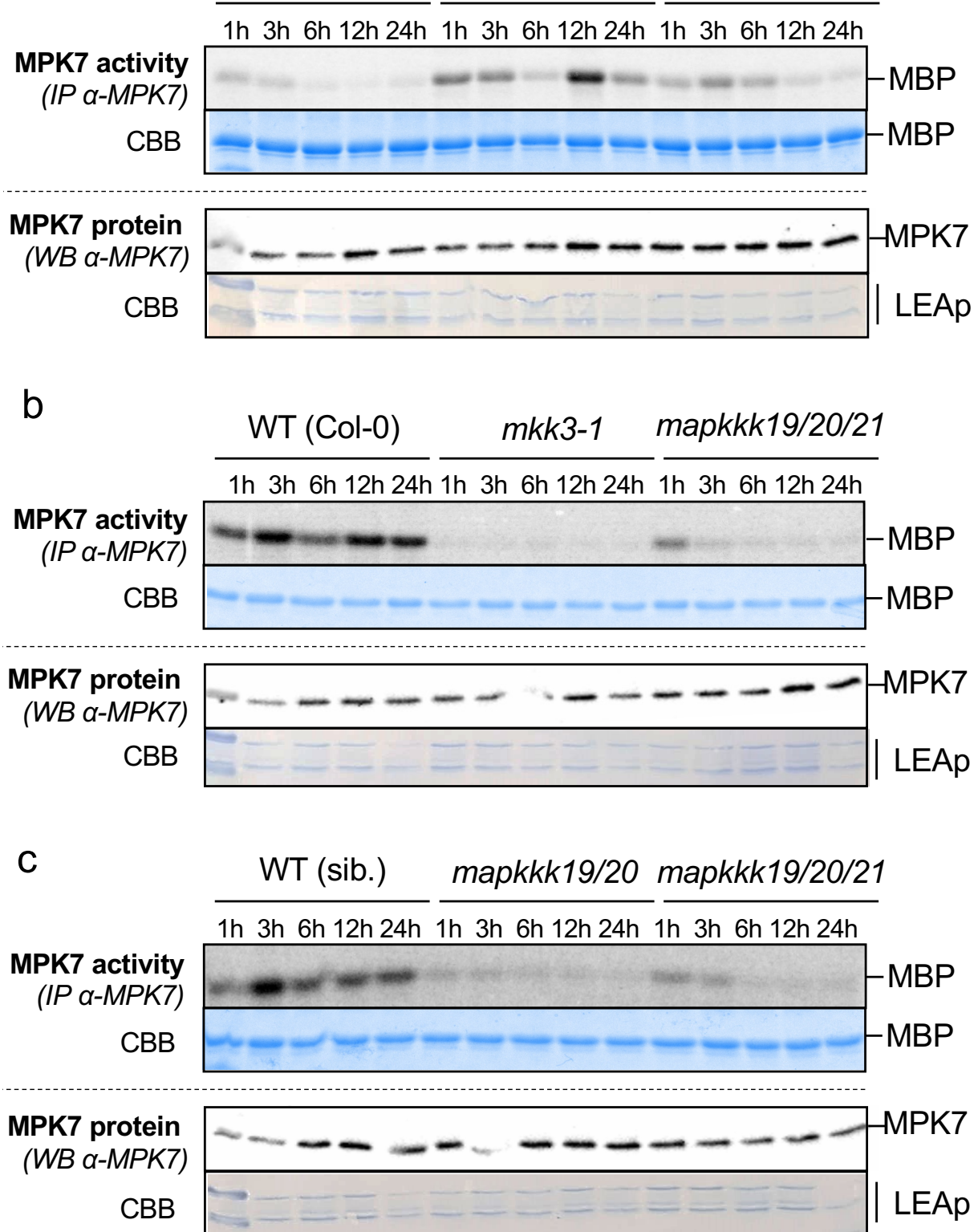
**Fig. 3** *MAPKKK19* and *20* are responsible for germination of FH and AR seeds, and *MAPKKK21* contributes to germination of FH seeds. Significant differences between samples are indicated by different letters (Tukey HSD tests,  $P < 0.05$ ). **a** Germination of FH seeds of single and multiple mutants 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.



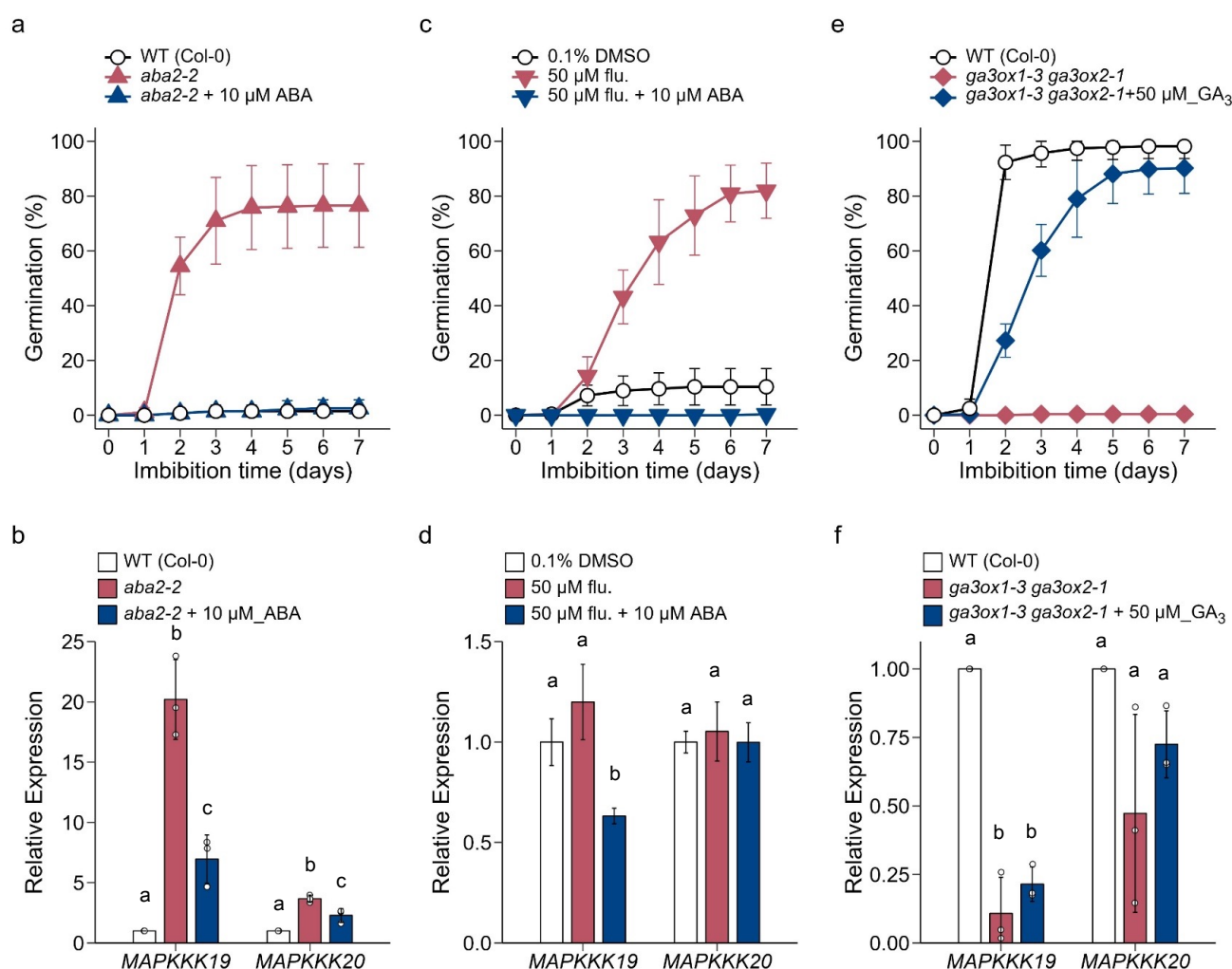
**Fig. 4 *MAPKKK20* over-expression stimulates germination of FH and AR seeds at supra-optimal temperatures in the MKK3 pathway.** The values are the means ( $\pm$ SD) of three biological replicates. Significant differences between lines are indicated by different letters (Tukey HSD tests,  $P < 0.05$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



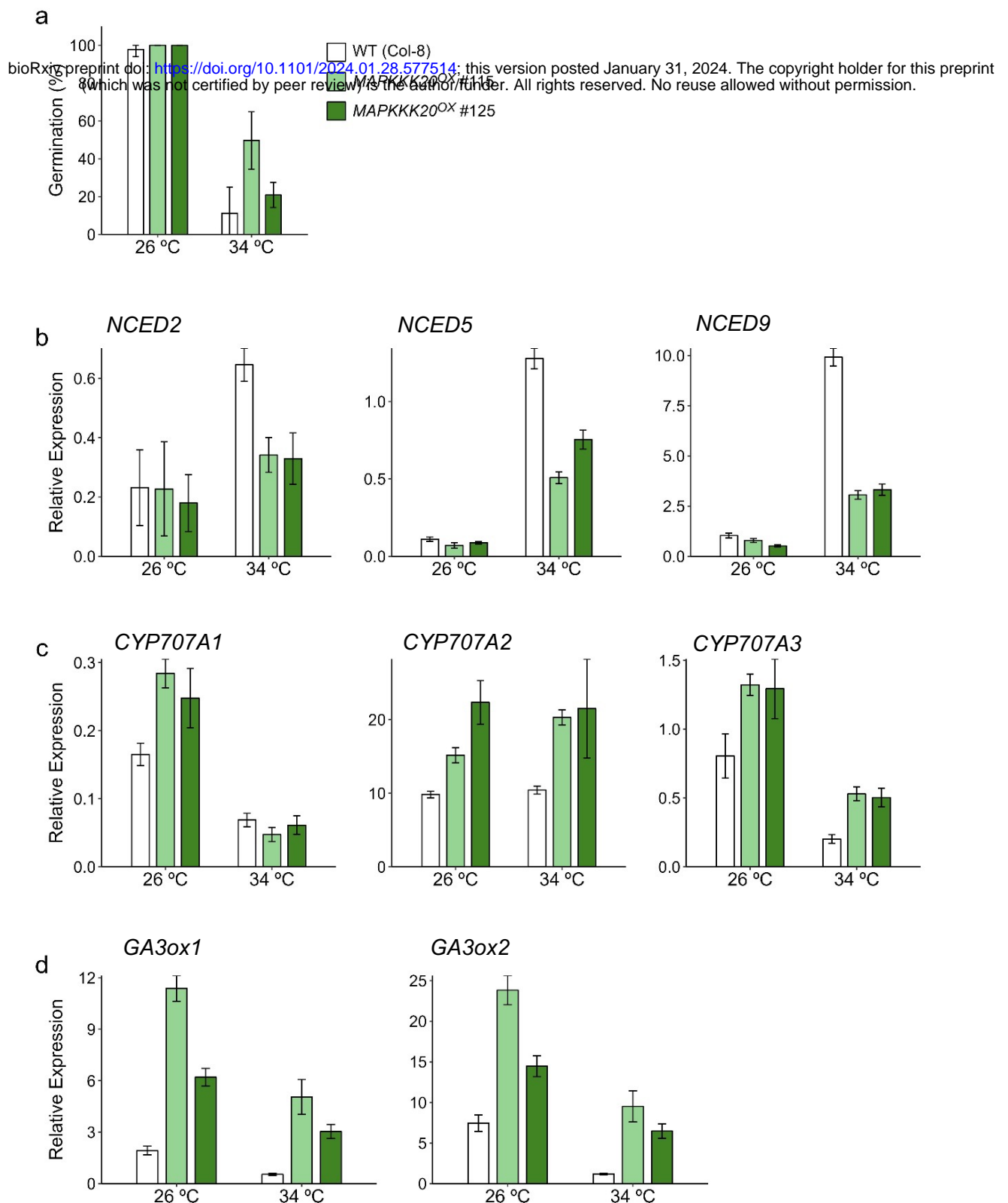
**Fig. 5 Group C MPKs are responsible for germination of FH and AR seeds.** The values are the means ( $\pm$ SD) of three biological replicates. Significant differences between lines are indicated by different letters (Tukey HSD tests,  $P < 0.05$ ). **a** Germination of FH (DAH 2) seeds of single and 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.



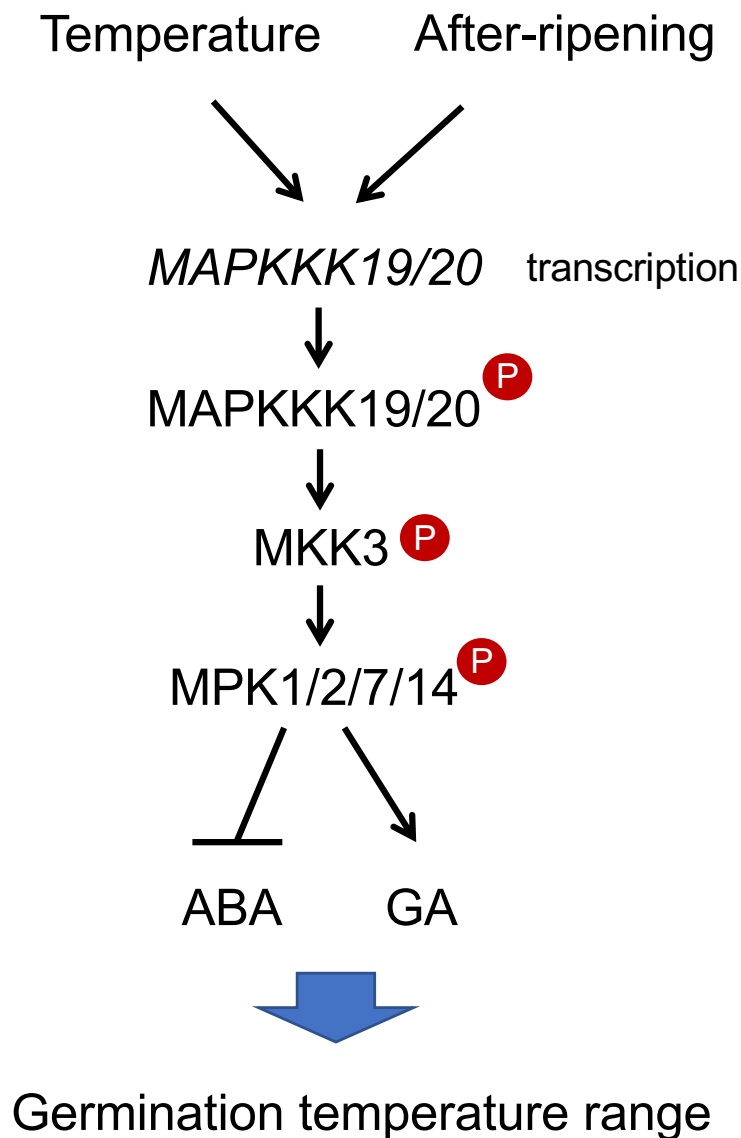
**Fig. 6 MPK7 activity in germinating and non-germinating seeds.** MBP was used as a phosphorylation substrate of MPK7 which was immunoprecipitated from imbibed seeds with an anti-MPK7 antibody. Amount of MPK7 protein was monitored by immunoblot using an anti-MPK7 antibody. Equal loading was indicated 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 °C were reduced by *aba2-2* mutation (a, b) or by the ABA biosynthesis inhibitor, fluridion in Col-0 AR seeds imbibed at 32 °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 °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.