The MKK3 module integrates nitrate and light signals to modulate secondary dormancy in *Arabidopsis thaliana*

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Abstract

Seed dormancy corresponds to a reversible blockage of germination. Primary dormancy is established during seed maturation while secondary dormancy is set up on the dispersed seed, following an exposure to unfavourable factors. Both dormancies are relieved in response to environmental factors, such as light, nitrate and coldness. QTL analyses for preharvest sprouting identified MKK3 kinase in cereals as a player in dormancy control. Here, we showed that MKK3 also plays a role in secondary dormancy in Arabidopsis within a signalling module composed of MAP3K13/14/19/20, MKK3 and clade-C MAPKs. Seeds impaired in this module acquired heat-induced secondary dormancy more rapidly than WT seeds and this dormancy is less sensitive to nitrate, a signal able to release dormancy. We also demonstrated that MPK7 was strongly activated in the seed during dormancy release, especially in response to light and nitrate. This activation was greatly reduced in *map3k13/14/19/20* and *mkk3* mutants. Finally, we showed that the module was not regulated, and apparently did not regulate, the genes controlling ABA/GA hormone balance, one of the crucial mechanisms of seed dormancy control. Overall, our work identified a whole new MAPK module controlling seed germination and enlarged the panel of functions of the MKK3-related modules in plants.

Introduction

A seed is a dispersive organ produced by plants after fertilisation. The plantlet embryo, embedded in the seed, can remain functional—although asleep—for long periods, even in harsh environmental conditions. Its genetic programme, shaped by the species' evolution in interaction with its environment, allows its germination at the appropriate time to maximise the probability of producing progeny. A critical physiological determinant of seed behaviour is its dormancy (i.e., its ability to block germination even when the environmental conditions are compatible with germination and plant development) and how it reacts to environmental inputs. Therefore, dormancy can be considered as a seed mechanism integrating environmental conditions that will drive the decision to germinate or not. Primary dormancy is acquired during the seed maturation on the mother plant (Baskin and Baskin, 2004). It is the highest when seeds have just been released and gradually decreases with time. Once they reach the ground, and depending on the species' lifestyle, seeds face a succession of favourable and unfavourable germination periods throughout the year, which requires the repetitive establishment of *de novo* dormancy and its breaking, this process being referred to as 'dormancy cycling' (Footitt et al., 2011). This secondary dormancy is acquired by dispersed seeds, which cannot germinate when the conditions are unfavourable. All aspects of seed dormancy, including its acquisition, maintenance, and breaking, are tightly modulated by environmental cues.

Dormancy is thought to be primarily controlled by a dynamic hormonal balance (Shu et al., 2016; Tuan et al., 2018). Abscisic acid (ABA) is a major inducer of seed dormancy, whereas gibberellins (GA) promote germination. Plants impaired in ABA/GA biogenesis or signalling pathways are affected in their ability to control dormancy and germination. After seed dispersal or harvest, the ABA catabolism in imbibed seeds contributes to the release of dormancy and allows the promotion of germination by GA. Many external cues modulate this balance. Seeds perceive and respond to changes in environmental conditions, such as temperature, light, storage periods (after-ripening), moisture content, and nitrate (NO_3^-). External NO_3^- promotes seed germination at low concentrations in many plant species, acting as both a nutrient and signal (Alboresi et al., 2005). Furthermore, exogenous NO_3^- breaks the primary dormancy efficiently and promotes the completion of seed germination by enhancing ABA catabolism and inhibiting ABA synthesis. In Arabidopsis, the NIN-Like

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71   Protein 8 (NLP8) transcription factor is a major regulator of NO_3^--regulated dormancy,

72   promoting NO_3^--dependent germination by upregulating ABA catabolism genes that include

73   *CYP707A1/2* (Yan et al., 2016).

74   In recent years, several reports pointed to the MKK3 kinase as an essential actor

75   promoting seed germination. Notably, MKK3 orthologous genes have been identified in

76   wheat, barley, and rice as the underlying molecular traits for QTLs controlling pre-harvest

77   sprouting (PHS) (Mao et al., 2020; Nakamura et al., 2016; Torada et al., 2016). Arabidopsis

78   seeds impaired in *MKK3* also displayed defects in germination and ABA sensitivity (Danquah

79   et al., 2015). MKK3 belongs to the MAP2K (MAPK Kinase) subfamily which is found in plants

80   and other eukaryotes. Together with MAP3K (MAP2K Kinase) and MAPK (Mitogen-Activated

81   Protein Kinase) subfamilies, it forms intracellular phosphorylation cascades, referred to as

82   MAPK modules (Jagodzik et al., 2018; Colcombet and Hirt, 2008). Our work and others have

83   suggested that the Arabidopsis MKK3 is robustly activated by clade-III MAP3Ks

84   (MAP3K13/14/15/16/17/18/20/21) and, in turn, activates clade-C MAPKs (MPK1/2/7/14).

85   Such modules were largely involved in abiotic stress signalling. Drought, through the ABA

86   core signalling module, activated a MAP3K17/18-MKK3-MPK7 module in plantlets, and

87   plants impaired in these kinases display reduced tolerance to drought (Danquah et al., 2014;

88   Mitula et al., 2015; Li et al., 2017b; Matsuoka et al., 2015; Zhou et al., 2021). Unexpectedly,

89   the module's activation was tightly controlled by the transcription-dependent MAP3K17/18

90   production, which, without stimulation, did not occur (Danquah et al., 2015; Boudsocq et al.,

91   2015). More recently, the MKK3 module was found to be activated in response to insect

92   feeding, wounding, and jasmonic acid (Sözen et al., 2020). Again, the module's activation

93   depended on the strong upregulation of several clade-III *MAP3K* genes. Last, we have

94   recently showed that NO_3^- (nitrate) activates an MKK3 module through the NIN-Like Protein

95   7 (NLP7)-dependent upregulation of *MAP3K13/14* genes (Sözen et al 2020; Schenk, Chardin,

96   Krapp, and Colcombet, unpublished). In the present work, we further investigate the

97   functioning of the MKK3 module in the control of dormancy using the model plant

98   Arabidopsis.

99   ## Results

100   ### *Seeds impaired in* MKK3 *establish a faster secondary dormancy*

101   To test whether the MKK3 module plays a role in the acquisition of secondary
102   dormancy, Col-0 and *mkk3-1* seeds produced in low NO_3^- conditions were sown on agar
103   plates containing 5 mM KCl and incubated at 30 °C in the dark. Even after 15 days, thermo-
104   inhibition prevented seeds from germinating ($n > 10$). Seeds were then transferred into
105   permissive conditions (16 h photoperiod; 22 °C) for seven days to measure their germination
106   ability. Upon this treatment, Col-0 seeds progressively acquired dormancy, with a 50%
107   decrease in germination after 7–10 days. *mkk3-1* seeds acquired dormancy more rapidly, in
108   less than two days, suggesting that the MKK3 module prevents dormancy acquisition (Figure
109   1A). Next, we wondered whether the module could function in the release of secondary
110   dormancy. Because NO_3^- was shown to be able to promote germination, we tested whether
111   it could break the heat-induced secondary dormancy. Seeds were first incubated on agar
112   plates containing 5 mM KCl at 30 °C to induce secondary dormancy and then transferred on
113   new plates supplemented with various NO_3^- concentrations and incubated for seven days in
114   permissive conditions (16 h; photoperiod 22 °C). NO_3^- was provided in the form of KNO_3, and
115   KCl was added to reach a KCl + KNO_3 concentration of 5 mM, keeping potassium and total
116   anion concentrations constant. As previously shown, seeds were largely dormant when
117   transferred on agar supplemented with only KCl (Figure 1B). *mkk3-1* seeds displayed lower
118   germination on low NO_3^- concentrations (0.05 and 0.5 mM) than Col-0. 5 mM KNO_3 was able
119   to promote about 100% germination in both genotypes. We found a similar result for *mkk3-*
120   *2*, and the *mkk3-1* complemented line presented a WT behaviour (Figures S1C and S1D). This
121   result indicates that *mkk3* seeds are less sensitive to NO_3^- although not insensitive,
122   suggesting that an MKK3-containing MAPK module could mediate a part of the NO_3^-
123   signalling to promote dormancy breaking.

124   ### MPK7 *is expressed in seeds and activated by* NO_3^- *in an MKK3-dependent way*

125   MKK3 was shown to activate MAPKs of the clade-C, namely MPK1/2/7/14 (Colcombet
126   et al., 2016; Danquah et al., 2015; Sözen et al., 2020). We evaluated by RT-qPCR analysis
127   which of these clade-C MAPKs were expressed in seeds during the first hours of germination
128   after transfer on 5 mM KCl or 5 mM KNO_3 (Figure 2). *MPK1* and *MPK7* displayed a higher

129 expression than *MPK2* and *MPK14*. None of the four clade-C MAPK genes seemed to be

130 more sensitive to NO_3^- than to Cl^-, and all four displayed a decrease in their expression after

131 transfer to germination conditions. HA-tagged MPK1/2/7-locus lines available in the

132 laboratory (Sözen et al., 2020) confirmed that MPK7 was detectable in dry seeds but

133 suggested that MPK1 and MPK2 were not (Figure S2). Seeds impaired in the four clade-C

134 MAPKs displayed an *mkk3*-like phenotype in nitrate-triggered dormancy release, whereas

135 seeds impaired in only *MPK7* (single mutant) had an intermediate phenotype (Figure 3).

136 These genetic data are in good agreement with clade-C MAPKs working downstream of

137 MKK3, suggests a functional redundancy in the modulation of secondary dormancy, and

138 indicate that MPK7 can be used as a proxy to monitor the module's activation.

139 To assess the module activation, MPK7 was immunoprecipitated from seeds using a

140 specific antibody, and its activity was assayed as the ability to phosphorylate the

141 heterologous substrate MYELIN BASIC PROTEIN (MBP) (Figures 4 and S3). MPK7 activity was

142 detectable in dry seeds but not in seeds under heat-induced dormancy. When seeds were

143 transferred to germination conditions (22 °C under light), the activity increased rapidly with

144 an apparent maximum at 8 hours. This increase of MPK7 activity was 3-4 times higher when

145 seeds were transferred on agar supplemented with 5 mM KNO_3 than agar supplemented

146 with 5 mM KCl (figure S3). In *mkk3-1* seeds, MPK7 activity was not detectable, confirming

147 that MPK7 functions downstream of MKK3. In the *mpk7* background, MPK7 activity was not

148 detectable, confirming the specificity of the antibody raised against MPK7 used in kinase

149 assays and western blots (Figure S4). To consolidate these results, an HA-antibody was used

150 to immunoprecipitate MPK7-HA from plants transformed with an HA-tagged MPK7-locus.

151 MPK7-HA displayed a similar pattern of activity, which was abolished when the *mkk3-1*

152 mutation was introgressed in the line (Figure S5). Overall, these results indicate that MPK7 in

153 seeds is activated by NO_3^- in an MKK3-dependent way. Other activators may be responsible

154 for the high activity background observed when seeds were transferred on KCl.

MAP3K13/14/19/20 are necessary for the MKK3-dependent module's activation

156 We reported that the activation of MKK3 relies on the transcriptional upregulation of

157 clade-III MAP3Ks (Colcombet et al., 2016; Danquah et al., 2015; Sözen et al., 2020). Using RT-

158 qPCR, we measured the expression of clade-III *MAP3K* genes in Col-0 seeds during dormancy

159 breaking (Figures 5 and S6). Four MAP3K genes were repeatedly upregulated in at least one

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160   of the samples. *MAP3K13* and *MAP3K14* were rapidly induced in seeds by NO_3^-, with a peak

161   at 45 minutes (Figures 5A and 5B), whereas *MAP3K19* and *MAP3K20* displayed a delayed

162   activation, typically at 3 and 8 hours, which was stronger in the presence of 5 mM KNO3

163   than 5 mM KCl (Figures 5C and 5D). Additionally, *MAP3K13* and *MAP3K19* displayed high

164   expression levels in dry seeds but were not expressed anymore in seeds under heat-induced

165   dormancy (Figures 5A et 5C). These results fit the timing of MPK7 activity in seeds (Figure 4)

166   and suggest that those four MAP3Ks may play a role in the context of seed dormancy

167   regulation. Nevertheless, the patterns of MPK7 activation and *MAP3K* transcriptional

168   regulation suggest a prominent function for MAP3K19/20, whereas the *MAP3K13/14*

169   transcription peak does not coincide with a high MPK7 activation level.

170   To test the role of these MAP3Ks in seed, we first immunoprecipitated MPK7 from

171   *map3k19-1/20-3* single and double mutants from dormant seeds transferred on KCl or

172   KNO3. MPK7 activity was weakly impaired in *map3k20-3* and strongly in *map3k19-1/20-3*,

173   notably at 8 hours (Figures 6A and S7A). Interestingly, a residual NO_3^--dependent MPK7

174   activity was observed in *map3k19-1/20-3* at 45 minutes. Since this residual kinetics fitted the

175   *MAP3K13/14* transcriptional response (figures 5A and 5B), we generated by cross the

176   quadruple mutant *map3k13cr/14cr/19-1/20-3* and showed that the MPK7 activity was

177   impaired throughout the time-course (Figure 6B and S7B). Consistently, *map3k13cr/14cr/19-*

178   *1/20-3* seeds displayed a stronger NO_3^- insensitivity than *map3k13cr/14cr* and *map3k19-*

179   *1/20-3* seeds (Figure 7). All together, these data suggested that MAP3K13/14/19/20 are the

180   only upstream activators of the nitrate-activated MKK3-MPK1/2/7/14 module involved in

181   the secondary dormancy breaking. To test, whether the MAP3K expression is sufficient to

182   reduce dormancy, we generated two lines constitutively expressing a MYC-tagged *MAP3K19*.

183   They both displayed a higher MPK7 activity (Figures 8A and S8) and did not acquire any

184   secondary dormancy (Figure 8B).

185   ***MPK7 activation by nitrate depends of NLP transcription factors***

186   Nitrate has been shown to trigger *MAP3K13/14* expression through NLP transcription

187   factors (Marchive et al., 2013; Yan et al., 2016). To test whether NLPs are also involved in the

188   MKK3   module   activation   during   the   breaking   of   secondary   dormancy,   we

189   immunoprecipitated MPK7 from dormant seeds impaired in one or several *NLP* genes.

190   Surprisingly, MPK7 activity was not reproducibly reduced in *nlp8* seed sets produced

191 independently (figure S9), whereas NLP8 has been shown to be a master regulator of the
192 nitrate regulation of primary dormancy (Yan et al., 2016). This suggest that other NLPs could
193 act redundantly of NLP8, depending of seed sets. In line, when mutations in other NLPs
194 where combined, MPK7 activity decreased robustly, indicating that NLPs play a role in the
195 *MAP3K* transcriptional regulation by nitrate and therefore in the activation of the module
196 (figures 9 and S10).

Light activates MPK7 in an MKK3-dependent way and primes MAP3K19/20 expression

198 Once treated with heat, seeds were transferred in light conditions, so we wondered
199 whether light might be an activator of the MKK3 module and responsible for the high MPK7
200 activity background in KCl conditions (see for example, figure 4). We repeated the
201 experiment in dark conditions, manipulating filters carrying dormant seeds under green
202 illumination. In these conditions, we barely detected a background MPK7 activity (Figures
203 10A and S11). Surprisingly, we did not observe a strong NO_3^--induced MPK7 activation either,
204 suggesting that the NO_3^- ability to activate MPK7 depends on light. Consistently, RT-qPCR
205 analysis revealed that the transcriptional regulation of *MAP3K19* and *MAP3K20* was strongly
206 reduced in the dark, no matter the anionic condition (Figure 11). On the contrary, the NO_3^--
207 induced *MAP3K13* and *MAP3K14* expressions were unaffected by light conditions. Other
208 clade-III *MAP3K* genes were not upregulated in these conditions (Figure S12). These results
209 suggest that the *MAP3K19/20* transcriptional regulation acts as a conditional switch for light
210 and NO_3^- in the activation of MPK7 and that the MKK3 module might contribute to the well-
211 known light-triggered germination. Coherently, MPK7 was far less activated by NO_3^- or light
212 in *phyA/B* seeds, impaired in the corresponding phytochrome receptors involved in the
213 red/far-red regulation of germination (Figure 10B).

The MKK3 module activation does not depend on the ABA/GA balance

215 The ABA/GA hormonal balance is one of the main physiological mechanisms
216 controlling the seed's decision to germinate. Since the MKK3 module promotes germination,
217 we first wondered whether the NO_3^-- and light-triggered activation of the MKK3 module
218 depended on GA synthesis or signalling. To test this possibility, we transferred dormant
219 seeds on agar media containing either KCl or KNO3, combined or not with 10 μM
220 paclobutrazol (PCZ), a potent blocker of GA biosynthesis. In these conditions, we did not

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221 measure any variation in MPK7 activity (Figures 12A and S13A). We also confirmed that an

222 active GA, GA$_3$, could not directly activate the module (Figures 12B and S13B).

223 We then tested whether ABA could modulate MPK7 activity in seeds. Dormant seeds

224 were transferred on agar containing 5 mM KCl or KNO3, with or without ABA, for 8 hours,

225 and MPK7 activity was assayed. ABA did not affect the NO$_3$-induced MPK7 activation (Figure

226 13). This result was rather surprising since we previously reported that ABA could activate

227 the module in plantlets through the transcriptional regulation of *MAP3K17/18* (Danquah et

228 al., 2015). When ABA was added to the media, no matter the anionic conditions, seeds did

229 not express *MAP3K17/18* (Figure S14). At the same time, *MAP3K13/14/19/20* displayed

230 unchanged transcriptional regulations (Figure S14). This result indicates that the ABA-

231 dependent activation of the MKK3 module is tissue-specific and that the ABA-dependent

232 regulation of seed germination does not require the MKK3 module in our conditions.

233 ***The MKK3 module does not modulate genes regulating ABA and GA contents***

234 Last, we wondered whether the MKK3 module could modulate the ABA/GA balance.

235 Therefore, we performed an RT-qPCR analysis of genes coding for ABA/GA biosynthetic and

236 catabolic enzymes. These results are presented in figures S15 and S16. As expected, the

237 expression of ABA synthesis genes (*NCEDs*, *ABA1*, *ABA2*, and *AAO3*) decreased after seed

238 transfer to germination conditions, independently of NO$_3$. Genes involved in ABA catabolism

239 had various patterns: *CYP707A1* and *CYP707A3* expression behaved like ABA biosynthetic

240 genes; *CYP707A2* expression was expressed at least until 24 hours and, consistently with the

241 literature (Yan et al., 2016), was strongly induced by NO$_3$; and *CYP707A4* expression was

242 barely detectable in our conditions. We did not find any dramatic effect of the *mkk3*

243 mutation on the expression of these ABA-related genes. GA biosynthetic genes *Ga20ox1/2/3*

244 were neither NO$_3$- nor MKK3-dependent, whereas *Ga3ox* seemed to be promoted by NO$_3$

245 and *Ga3ox2* seems to present a delayed activation in *mkk3* (Figure S13).

246 **Conclusion and perspectives**

247 Our work demonstrated that nitrate and light are activators of MKK3 module through

248 the transcriptional regulation of two different pairs of MAP3K (figure 14). MAP3K13/14

249 function specifically in the regulation by nitrate whereas MAP3K19/20 seem rather to

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250   integrate both nitrate and light signals. We also showed that classical actors of dormancy

251   regulation, such as NLP transcription factors and PHY photoreceptors, are involved in this

252   activation. Last, we bring evidence that, in the context of secondary dormancy and with the

253   assays used for this work, ABA and GA homeostasis are not the primary targets of the MKK3

254   pathway.

255   *Clade-III MAP3Ks, MKK3 and clade-C MAPKs emerge as a conserved transcription-*

256   *dependent signalling module activated by a large range of signals*

257   This work comforts the hypothesis that MKK3, together with clade-III MAP3Ks and

258   clade-C MAPKs, defines robust signalling modules in plants (Colcombet et al., 2016). This

259   specificity in kinase interaction is supported by yeast-2-hybrid and Split-YFP as well as

260   functional reconstruction of modules in Arabidopsis protoplast expression system (Sözen et

261   al., 2020; Danquah et al., 2015). Interestingly, the combined expression of MAP3K19, MKK3

262   and MPK7 in yeast strongly impaired cell growth, this impairment being suppressed if

263   MAP3K19 or MPK7 is omitted or if MKK3 carries a mutation blocking its kinase activity

264   (figure S17). This suggests that these three kinase clades are building a functional module in

265   yeast and that MAP3K19 does not require further activation by a plant specific mechanism.

266   The most striking demonstration that these kinases define functional modules comes from

267   genetics and notably the observation that clade-C MAPK activities are impaired in mutants of

268   upstream kinases. This impairment was complete in *mkk3* backgrounds whatever the

269   activating signal used (Dóczi et al., 2007; Danquah et al., 2015; Sözen et al., 2020). In

270   response to ABA and wound, clade-C MAPKs activities were also strongly reduced in mutants

271   of clade-III MAP3Ks, *map3k17/18* for the first and *map3k14* for the second (Sözen et al.,

272   2020; Danquah et al., 2015). In response to nitrate (in preparation) and nitrate/light (this

273   study), the knocking out of all the transcriptionally-regulated MAP3Ks resulted in a total

274   suppression of MAPK activation. This suggests once again that, at least for these signals,

275   MAPK activation totally relies on clade-III MAP3KsOf course, these findings do not exclude

276   the possibility MKK3 and clade-III MAP3Ks could also activate MAP2Ks/MAPKs of other sub-

277   clades, as it has been suggested previously by other groups (Sethi et al., 2014; Benhamman

278   et al., 2017; Lee, 2015; Bai and Matton, 2018; Takahashi et al., 2007; Schikora et al., 2008; Li

279   et al., 2017a; Ojha et al., 2023; Takahashi et al., 2011). Notably MKK3 has been repeatedly to

280   function upstream of MPK6 in response to light and pathogens (Takahashi et al., 2007;

281 Schikora et al., 2008; Bai and Matton, 2018; Sethi et al., 2014; Lee, 2015) although co-
282 expression in Arabidopsis protoplasts did not confirmed this connection (Danquah et al.,
283 2015).

284 Our present work also shows that the module activation is systematically under the
285 tight control of the signal-dependent transcriptional up-regulation of upstream MAP3K
286 genes. In response to wound, systematically expression analysis identified 5 clade-III
287 MAP3Ks, but when only a single one, *MAP3K14*, was knocked down, the mutant showed a
288 MPK2 activity reduction (Sözen et al., 2020). We recently showed that nitrate
289 transcriptionally regulates specifically *MAP3K13/14* genes in plantlets and that MPK7
290 activation was strongly impaired in the double mutant (Schenk, Chardin, Krapp, and
291 Colcombet, unpublished). In the present work we generated a mutant in which each of the
292 four *MAP3K* genes which are regulated in seeds transferred in germination-permissive
293 conditions were mutated. In this quadruple mutant, the MPK7 activity is totally abolished in
294 the same conditions. It is likely that the large number of clade-III MAP3Ks found in plant
295 genomes (whereas there is usually a single MKK3 gene (Colcombet et al., 2016)) is
296 evolutionarily constrained by the dual necessity of a large number of module activators and
297 the importance of transcriptional regulation. Therefore, the MAP3K specificity should appear
298 more at the promoter sequence level than at the protein sequence. Here we have identified
299 light as a new signal able to activate the module. We showed that ABA, JA, nitrate and light
300 are able to modulate the cascade but they are very likely other signals to be characterized.
301 As a matter of fact, many more signals are able to regulate clade-III *MAP3K* expression as
302 shown in Genvestigator expression database (Zimmermann et al., 2004; Colcombet et al.,
303 2016) and some of the clade-III MAP3Ks have not described function yet.

304 ***MKK3 module is a major dormancy regulator***

305 The choice to germinate or not is crucial for seed survival. Understanding how plants
306 make the decision, in interaction with their environment, has strong academic and
307 agronomic interests. In this article, we report the dissection of a molecular signalling
308 pathway integrating environmental cues to modulate secondary dormancy.

309 Our choice to work on secondary dormancy was methodological, but several
310 arguments suggest that the MKK3 module may also function to release primary dormancy.

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311   (i) some clade-III *MAP3Ks* are strongly transcriptionally regulated during imbibition (Narsai et

312   al., 2011), (ii) lines overexpressing some clade-III *MAP3Ks* and loss-of-function mutants

313   display a germination phenotype (Choi et al., 2017; Mao et al., 2020; Danquah et al., 2015),

314   and (iii) mutations in *MKK3* homologues have been identified in QTLs for wheat and barley

315   vivipary, suggesting a direct mutation effect on primary dormancy (Torada et al., 2016;

316   Nakamura et al., 2016). More directly, two recent articles support this idea. First, a whole

317   MKK3 module plays an important role in the context of the temperature control of primary

318   dormancy (Otani et al., 2024). Moreover a recent work in Arabidopsis reported that a

319   functional MKK3-MPK7 module phosphorylate, in response to dormancy breaking

320   conditions, the Ethylene Responsive Factor4 (ERF4) to target it to the proteasome. This

321   allows the expression of EXPA genes necessary for the radicle emergence and seed

322   germination. It is possible that the same players act downstream of our module described in

323   the context of secondary dormancy (Chen et al., 2023).

324   Our work and others were led on the Brassicaceae specie *Arabidopsis thaliana*. It

325   completes previous genetic investigations identifying *MKK3* mutation under important QTLs

326   for barley and wheat pre-harvest sprouting (PHS) (Torada et al., 2016; Nakamura et al.,

327   2016). These results on a dicotyledon suggest that the MKK3-dependent signalling pathway

328   is shared among angiosperms and likely existed in the common ancestor of monocotyledons

329   and dicotyledons.

330   Previously, we showed that ABA/drought and JA/wounding, through the production of

331   MAP3K17/18 and MAP3K14, respectively, were activators of MKK3 and clade-C MAPKs

332   (Colcombet et al., 2016; Sözen et al., 2020; Boudsocq et al., 2015). Our results also suggested

333   that nitrate activates the module by upregulating *MAP3K13/14* (Schenk, Chardin, Krapp, and

334   Colcombet, unpublished). Here, we unveiled that light can also activate the module and

335   proposed that MAP3K19 and MAP3K20 could be the main entry points for this activation. A

336   partial module composed of MKK3-MPK6 was shown to be activated by blue light to

337   modulate MYC2-dependent photomorphogenesis (Sethi et al., 2014). Another study

338   suggested, in the context of red light, that MKK3 rather restricts MPK6 activity in dark-light

339   period transition (Lee, 2015). In the context of seeds, we also proposed that PHYA/B were

340   the upstream light sensors, suggesting that the main compounds of the light effect are red

341   and far-red wavelengths.

342   Besides clade-C MAPKs and MPK6, MKK3 has been proposed to work upstream of a
343   clade-D MAPK, MPK8, in the context of wound-triggered Reactive Oxygen Species (ROS)
344   homeostasis (Takahashi et al., 2011). Interestingly, a recent study also highlighted the role of
345   MPK8 in regulating seed dormancy (Zhang et al., 2019). In this work, the authors show that
346   freshly harvested *mpk8* seeds display a strong dormancy phenotype, arduously released by
347   gibberellins and after-ripening. MPK8 can interact with and phosphorylate the transcription
348   factor TEOSINTE BRANCHED1/CYCLOIDEA/PROLIFERATING CELL FACTOR (TCP14) in the
349   nucleus, enhancing the activity of the latter in seeds. Transcriptomes of WT, *mpk8*, and
350   *tcp14* seeds are very similar at the dry stage but diverge after a 24 h imbibition.
351   Nevertheless, *mpk8* and *tcp14* mutants displayed a strong overlap in their misregulated
352   genes, confirming that the two proteins belong to a common signalling pathway. The
353   connection between MKK3 and MPK8 has not been confirmed yet, since MKK3 does not
354   reproducibly activate MPK8 in protoplasts (Danquah et al., 2015). Nonetheless, identifying
355   how MPK8 and TCP14 are regulated in our conditions would be interesting

356   Surprisingly, ABA is not an activator of the module in seeds, whereas it has been
357   repeatedly reported to be in plantlets and adult plants (Mitula et al., 2015; Matsuoka et al.,
358   2015; Danquah et al., 2015). We observed neither an upregulation of *MAP3K17/18* or other
359   clade-III *MAP3Ks*, nor the increase of MPK7 activity in dormant seeds transferred to
360   germination conditions supplemented with ABA. This result suggests that MAP3K promoters
361   are specifically recognised by ABA-responsive transcription factors, which are not recruited
362   by ABA in seeds or not expressed. Because ABA and nitrate/light act in opposite ways to
363   promote seed germination, it makes sense that both signals cannot activate the same
364   module. We also tested the activation by GA or its importance in MPK7 activation by
365   light/nitrate but did not observe any effects of GA or paclobutrazol. This suggests that the
366   module integrates environmental signals to modulate cellular mechanisms controlling
367   germination. One possibility is that it modulates the ABA/GA balance by regulating ABA/GA
368   biosynthetic genes.

369

13

370 **Material and methods**

371 *Biological material*

372   *mkk3-1* (SALK_051970), *mkk3-2* (SALK_208528), *mpk7-1* (SALK_113631) and lines
373 expressing HA-tagged *MPK1/2/7* loci were published previously (Dóczi et al., 2007; Sözen et
374 al., 2020). *mpk1-1/2-2/7-1/14-1* and *map3k19-1/20-3* were obtained by crossing in the Pr
375 Kawakami's laboratory from the following single mutant lines *mpk1-1* (SALK_063847C),
376 *mpk2-2* (SALK_047422C), *mpk7-1* (SALK_113631), *mpk14-1* (SALK_022928C), *map3k19*
377 (Transposon pst14411), *map3k20-3* (CS443915/GK-458D07) (Otani et al., 2024).
378 *map3k13CR/14CR* are a double crisper mutants described previously (Sözen et al 2020;
379 Schenk, Chardin, Krapp, and Colcombet, unpublished). *map3K13CR/14CR/19-1/20-3* were
380 obtained by crossings. *nlp8* (SALK_16341), *nlp7/nlp8* (SALK_026134/SALK_16341) and
381 nlp8/nlp9 (SALK_140298/SALK_025839) were published previously (Yan et al., 2016).
382 *nlp7/nlp8/nlp9* (SALK_026134/SALK_031064/SALK_025839) was created by crossing.

383   To produce *35S::MAP3K19-MYC* lines, the ORF was PCR amplified from Arabidopsis
384 thaliana (ecotype Columbia-0) cDNA using iProof DNA polymerase (Bio-Rad), specific primers
385 (ORF-MAP3K19-F: gga gat aga acc ATG GAG TGG ATT CGA GGA GAA A; ORF-MAP3K19-R tcc
386 acc tcc gga tcm CCG TAC GGT GAC CCA GCT) and a two-step amplification protocol as
387 described previously (Colcombet et al., 2013). PCR products were recombined into
388 pDONR207 (Invitrogen) using Gateway® BP Clonase® II Enzyme mix (Invitrogen). LR
389 recombination reactions were performed using Gateway® LR Clonase® Enzyme Mix
390 (Invitrogen) in order to transfer ORF sequences from Entry vectors to the pC2N1 allowing C-
391 terminal translational fusion with the 10xMyc tag under the control of the 35S promoter
392 (Bigeard et al., 2014; Berriri et al., 2012). The resulting construct, *pC2N1-MAP3K19,* was
393 introduced into the *Agrobacterium tumefaciens* strain C58C1 and used to transform
394 *Arabidopsis thaliana* Col-0 plants by the floral-dipping method (Clough and Bent, 1998).
395 Using kanamycin segregation analysis, we selected two independent transgenic lines
396 carrying a single insertion at the homozygous state. *MAP3K19-MYC* expression was assessed
397 by western blot.

398   ***Growth production***

399   Seeds were produced in 'low-nitrogen' conditions (Alboresi et al., 2005). They were

400   germinated on agar media on ½ MS plates in a growth chamber in long-day conditions for 7

401   days, and plantlets were transferred onto 'Spezialsubstrat' (Stender, ref: 19002774-A204

402   sans NPK) containing low nitrate. Plants were further grown in an Aralab® growth cabinet

403   maintained in long-day conditions (16 h of 80–100 µE m^{-2} s^{-1} light at 20°C and 8 h of

404   darkness at 18°C) with a 60% hygrometry. Pots were watered three times weekly (Monday,

405   Wednesday, and Friday), using a low-nitrogen solution (250 µM KH_2PO_4; 250 µM $MgSO_4$; 750

406   µM KNO_3; 125 µM $Ca(NO_3)_2$; 125 µM $CaCl_2$; 10 mG L^{-1} Sequestrene138FE 100SG (Syngenta);

407   0,4 µM $(NH_4)_6Mo_7O_{24}$; 243 µM H_3BO_3; 118 µM $MnSO_4$; 34,8 µM $ZnSO_4$; 10 µM $CuSO_4$)

408   containing 1 mM NO_3^- (Loudet et al., 2003) and filling the plateau with 2 cm of solution. After

409   1–2 hours, the remaining liquid was removed by draining. In these growth conditions,

410   plantlets exhibit smaller rosettes and complete their lifecycle after 3–4 months. When about

411   two-thirds of the siliques started turning yellow, watering was stopped, and plants dried.

412   The inflorescence was cut, enclosed in paper bags, and further dried for two weeks. Seeds

413   were then harvested and stored at room temperature in Eppendorf tubes.

414   ***Dormancy induction and germination tests***

415   Appropriate amounts of seeds were sterilised (15' in [50% EtOH, 0.5% Triton 100X], 2x

416   5' in 96% EtOH). Seeds were dried on sterile Whatman paper (ref: GE Healthcare Life

417   Science, ME 25/31 ST, Whatman). 5-cm round plates were filled with 8 mL minimal medium

418   (MES hydrate [M8250, Sigma Aldrich] 0.58%, Agar [HP696, Kalys 0,7%], pH adjusted to 5,75

419   with NaOH). Depending on the type of experiment performed, the minimal medium was

420   supplemented with KNO_3, KCl, 50 µM ABA (Sigma Aldrich, ref: A1049, in ethanol), 50 µM GA_3

421   (Sigma Aldrich, ref: G7645), or 10 µM Paclobutrazol (Sigma Aldrich, ref: 43900) at indicated

422   concentrations. A round filter (GE Healthcare Life Science ME 25/31 ST) was carefully placed

423   on the medium surface for experiments requiring seed transfer. Typically, 50–150 seeds

424   were sown on each plate. Plates were sealed with micropore surgical tape.

425   To induce secondary dormancy, plates were wrapped in aluminium foil and incubated

426   at 30°C in a Memmert cabinet for 1 to 15 days. To evaluate dormancy through the ability of

427   seeds to germinate, plates were shifted in a growth room in long-day conditions (16 h light

428　[80–100μE m^{-2}s^{-1}] at 22°C and 8 h dark at 18°C). To evaluate the ability of anions or

429　hormones to release dormancy, dormant seeds were delicately transferred onto new media

430　with appropriate supplementation. The germination rate was expressed as the percentage

431　of seeds with a radicle protrusion after seven days.

432　**Gene expression**

433　For gene expression analysis, seeds were collected, frozen in liquid nitrogen, and

434　ground using a plastic pestle. RNA was extracted using the NucleoSpin® RNA Plant kit

435　(Macherey-Nagel) according to the manufacturer's instructions and quantified with a

436　Nanodrop spectrophotometer. Typically, 2–5 µg of total RNA was used to perform RT, using

437　the Transcriptase inverse SuperScript™ II (Thermofisher) and following the manufacturer's

438　instructions. 10 ng of cDNA was used for qPCR with the CFX384 Touch real-time PCR

439　detection system (Bio-Rad) and ONEGreen® Fast qPCR Premix (Ozyme), following the

440　manufacturer's standard instructions. *ACT2 (*AT3G18780) was used as an internal reference

441　gene to calculate relative expression. RT-qPCR primers are listed in Supplemental Table 1.

442　**Kinase assay and western blot**

443　For biochemistry, 50–150 seeds were collected, frozen in liquid nitrogen, and ground

444　using a plastic pestle. A detailed kinase assay protocol for plant samples has been provided

445　in a previous publication (Sözen et al., 2020). The notable modification necessary for protein

446　extraction from seeds is that triton was adjusted at 1% in the extraction buffer. A new batch

447　of rabbit α-MPK7 antibodies was also prepared for this study, raised against the

448　LYYHPEAEISNA epitope. For kinase assays, immunoprecipitated kinases were resuspended in

449　15 µl of kinase buffer containing 0.1 mM ATP, 1 mg ml^{-1} MBP, and 2 µCi ATP [γ-33P]. After 30

450　min of reaction at room temperature, retain was stopped with 15µL Laemmli buffer 2x and

451　boiled for 5 minutes at 95°C. Samples were then loaded and run on a 15% SDS-PAGE gel.

452　MBP was stained with Coomassie Blue. Then, the gels were dried and revealed on a STORM

453　scanner (GE Healthcare).

454　Protein levels were monitored by immunoblotting following Bio-Rad

455　recommendations. Proteins were separated in 10% (w/v) SDS-PAGE gels and transferred

456　onto polyvinylidene fluoride membranes (Bio-Rad). Membranes were blocked in 5% (w/v)

457　nonfat dry milk. The following primary and secondary antibodies were used: α-HA (Roche

458 11867431001; 1: 10,000 dilution), α-Myc (Sigma-Aldrich C3956; 1:10,000 dilution), α -rat

459 (Sigma-Aldrich A9037, 1:10,000), α-rabbit (Sigma-Aldrich A6154, 1/20,000), and α -mouse

460 (Sigma-Aldrich A5906, 1:10,000). Horseradish peroxidase activity was detected with a Clarity

461 western ECL substrate reaction kit (Bio-Rad) and a ChemiDoc Imagers (Bio-Rad). Blots were

462 stained with Coomassie Blue for protein visualisation.

463 *Functional expression of kinase in yeast*

464 *MAP3K19*, *MKK3* and *MPK7* ORFs were amplified using couple of primers and cloned

465 into p425GPD, p426GPD and p423GPD (Mumberg et al., 1995)respectively. To generate

466 MKK3 mutants, site-directed mutagenesis was carried out using the QuickChange Lightning

467 kit from Agilent. The two targeted mutation sites were K112M and D207A. Combination of

468 plasmids were co-transformed in B4741 *Y00000* (*MAT a; his3D1; leu2D0; met15D0; ura3D0*)

469 strain derived from the S288C isogenic yeast strain. Transformed yeasts were selected and

470 grown on selective medium (Yeast Nitrogen Base, 2 % glucose, and the addition of amino

471 acids except histidine, uracil and leucine to maintain selection for the plasmids).

472 **Acknowledgements**

482

483

484 **Supplemental Tables**

485 *Supplemental Table 1*

Gene	Locus	Forward:F/ Reverse: R	Sequence (5' > 3')
MPK1	AT1G10210	F	TGGTCACTTATCACCGAGGG
		R	GCTCCACGACCAATAGGCTT
MPK2	AT1G59580	F	GGGAGGTAGAAGAATGGCGA
		R	CCGAAGAGCAAACCACACCG
MPK7	AT2G18170	F	CTCTGTAACCGATGCGCTCT
		R	AGCTTCAGGGTGGTAATAAAGCA
MPK14	AT4G36450	F	GGAACCGGAATGTAACCCGT
		R	CTCGGGGGAGGTAATGAAGC
MAP3K13	AR1G07150	F	CGCGGCGAAGGCAATAATTT
		R	CACCCAACCATCTGACTCCC
MAP3K14	AT2G30040	F	ACCAGCTTGGGAAGATCACG
		R	GAGTTCCGATAACCCCACCG
MAP3K15	AT5G55090	F	ATCGTCGATTTGGGTTGTGC
		R	CTTCACCACGTGCTACCTCC
MAP3K16	AT4G26890	F	TTGAAAATCGCCGACATGGG
		R	TCACCACGAGCAACTTCTGG
MAP3K17	AT2G32510	F	TACTCGGAGAGGATCGGACG
		R	TGTTCCTTCACACCTCGCTC
MAP3K18	AT1G05100	F	TTCACCGGTCGGAGTTCTTG
		R	TGTGGAAGGGCTCTCTCGTA
MAP3K19	AT5G67080	F	GACGGTCGAAAACGGTGAAG
		R	CACGGTGGATTCCGGTACAC
MAP3K20	AT3G50310	F	TTCAACGGTGGAGAACGGAG
		R	ACGTAACCCTCGAAGCACTG
MAP3K21	AT4G36950	F	GGGTTAGCGAAACGGAGGAG
		R	TCGCCGTGATTCACAGACTC
ABA1/ZEP	AT5G67030	F	TTGTTTGGCCGTAGTGAAGCT
		R	AGACTCGATATCCGCTGGTATAAAA
ABA2	AT1G52340	F	GGAGGAGCCACAGGGATAGG
		R	GCAGATCAACAATGCAGACTTTG
ABA3	AT1G16540	F	TCCTGAAGATTACAGTTGCTTATTCAC
		R	TGGGTCCACGGAAAAGTCTCT
AAO1	AT5G20960	F	GACGGGCTCGGCAACAG

18

		R	CATGAAAACCGGCGATACG
AAO2	AT3G43600	F	TGTCATGAAAAACGCGTACTCTCT
		R	CGCAGTGCACCGAAGCT
AAO3	AT2G27150	F	GAAGGTCTTGGAAACACGAAGAA
		R	GAAATACACATCCCTGGTGTACAAAAC
AAO4	AT1G04580	F	CAATGTTTTGGATCAGACGAGGTA
		R	CTCTATCTTTGCCAGGGTTGGTT
NCED2	AT4G18350	F	GCGGCTGAGCGTGCATTAA
		R	GGGAATAATTCCCGGCAATCT
NCED3	AT3G14440	F	GCTGCGGTTTCTGGGAGAT
		R	GTCGGAGCTTTGAGAAGACGAT
NCED5	AT1G30100	F	CCTCCGTTAGTTTCACCAACACT
		R	GGTGTGTCGGAGACGGAGTT
NCED6	AT3G24220	F	CGTTATTCCTATGGAGCAGAATCG
		R	GGAGCGAAGTTACCTGATAATTGAA
NCED8	AT1G78390	F	GGAAAACGCCATGATCTCACA
		R	AGGATCCGCCGTTTTAGGAT
CYP707A1	AT4G19230	F	CTCACTCTCTTCGCCGGAAG
		R	GGAGGGAGTGGGAGTTTGGAA
CYP707A2	AT2G29090	F	CGTCTCTCACATCGAGCTCCTT
		R	GAGGGTGTTGATGGACTTTTGG
CYP707A3	AT5G45340	F	CATGCCTTTTGGTAGTGGGATTCAT
		R	CGGCCCATACTGAATTCCATCG
CYP707A4	AT3G19270	F	CCTGAAACCATCCGTAAACTCAT
		R	TTGGCCCAAGATTGTAAGGAA
Ga20ox1	AT4G25420	F	GCCTGTAAGAAGCACGGTTTCT
		R	CTCGTGTATTCATGAGCGTCTGA
Ga20ox2	AT5G51810	F	CCCAAGGCTTTCGTTGTCAA
		R	CCGCTCTATGCAAACAGCTCT
Ga20ox3	AT5G07200	F	TCGTGGACAACAAATGGCA
		R	TGAAGGTGTCGCCTATGTTCAC
Ga3ox1	AT1G15550	F	CTTGGGGTGCCTTCCAAATC
		R	AACCTTCGGACCACATTTGC
Ga3ox2	AT1G80340	F	GTTTCACCGTTATTGGCTCTCC
		R	TCACAGTATTTGAGGTGGTGGC
Ga2ox2	AT1G30040	F	CCTAAAACCTCCGCCGTTTT
		R	CCTTCATGTACTCCTCCACCGA

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488 **Figure legends**

489 **Figure 1 -** *mkk3-1* **seeds have a faster secondary dormancy acquisition and a reduced**
490 **nitrate-triggered dormancy release**
491 A. Col-0 and *mkk3-1* seeds were imbibed at 30°C in the dark for the indicated time to induce
492 secondary dormancy. Germination ability was then assessed after 7 days in long day
493 conditions. Values are mean ± SE of seven to ten biological replicates from seed batches
494 produced independently. Values for each replicate are also shown. On the top, based on
495 Mann-Whitney test, yellow and red sticks show differences with 1% < α < 5% and α < 1%,
496 respectively, whereas gray sticks show no differences.
497 B. Col-0 and *mkk3-1* seeds were imbibed at 30°C in the dark for 10 days to induce secondary
498 dormancy and transferred on media containing indicated NO3- concentrations. Germination
499 ability was then assessed after 7 days in long day conditions. Values are mean ± SE of height
500 biological replicates from seed batches largely produced independently. Values for each
501 replicate are also shown. On the top, based on Mann-Whitney test, yellow and red sticks
502 show differences with 1% < α < 5% and α < 1%, respectively, whereas gray sticks show no
503 differences.
504
505 **Figure 2 - Clade-C** *MAPK* **genes are expressed in seeds and during secondary dormancy**
506 **release**
507 RT-qPCR analysis of clade-C MAP3K genes. Transcript levels are expressed relative to ACTIN2
508 as reference gene. Values are mean ± SE of two to six biological replicates from seed batches
509 produced independently. Values for each replicate are also shown.
510
511 **Figure 3 - Seeds impaired in C-clade MAPKs have a faster secondary dormancy acquisition**
512 **and a reduced nitrate-triggered dormancy release**
513 Col-0, *mpk7* and *mpk1/2/7/14* seeds were imbibed at 30°C in the dark for 10 days to induce
514 secondary dormancy and transferred on media containing indicated NO3- concentrations.
515 Germination ability was then assessed after 7 days in long day conditions. Values are mean ±
516 SE of three biological replicates from seed batches largely produced independently. Values
517 for each replicate are also shown. On the top, based on Mann-Whitney test, yellow sticks
518 show differences with α < 5% whereas gray sticks show no differences.
519
520 **Figure 4 - MPK7 activity in dormant seeds transferred on nitrate depends on MKK3.**
521 Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0
522 and mkk3-1 seeds, either dry (DS), after acquisition of secondary dormancy (0') and after
523 transfer on either 5mM KCl (-) or KNO3. MPK7 amount was monitored by immunoblot using
524 an anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the
525 membrane. LEAp, Late Embryogenesis Abundant proteins. Results were repeated two to six
526 times depending on the time points, the quantification of these replicates being gathered in
527 figure S3.

21

528

529 **Figure 5 - *MAP3K13, MAP3K14, MAP3K19* and *MAP3K20* are expressed in seeds and during**
530 **secondary dormancy release**
531 RT-qPCR analysis of *MAP3K13, MAP3K14, MAP3K19* and *MAP3K20* genes expression.
532 Transcript levels are expressed relative to ACTIN2 as reference gene. Values are mean ± SE of
533 two to 10 biological replicates from seed batches produced independently. Values for each
534 replicate are also shown. ND not determined.

535

536 **Figure 6 - MPK7 activity in dormant seeds transferred on nitrate depends on**
537 **MAP3K13/14/19/20**
538 Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from
539 indicated background, either dry (DS), after acquisition of secondary dormancy (0') and after
540 transfer on either 5mM KCl or KNO3. MPK7 amount was monitored by immunoblot using an
541 anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the membrane.
542 LEAp, Late Embryogenesis Abundant proteins. Results were repeated 2-6 times depending of
543 the time point and genotype for A and 3 times for B, the quantification of these replicates
544 being gathered in figures S7A and B.

545

546 **Figure 7 - *map3k13/14/19/20* seeds have a reduced nitrate-triggered dormancy release**
547 Col-0, *map3k13CR/14CR, map3k19-1/20-3* and *map3k13CR/14CR/19-1/20-3* seeds were
548 imbibed at 30°C in the dark for 10 days to induce secondary dormancy and transferred on
549 medium containing indicated NO3 concentration. Germination ability was assessed after 7
550 days in long day conditions. Values are mean ± SE of three biological replicates from seed
551 batches produced independently. Values for each replicate are also shown. On the top,
552 based on Mann-Whitney test, yellow sticks show differences with α < 5% whereas gray sticks
553 show no differences.

554

555 **Figure 8 – Constitutive expression of *MAP3K19* triggers a strong MKK3-dependent MPK7**
556 **activation and reduces the acquisition of secondary dormancy**
557 A. Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from
558 indicated background, either dry (DS), after acquisition of secondary dormancy (0') and after
559 transfer on either 5mM KCl (-) or KNO3 (+). MPK7 amount was monitored by immunoblot
560 using an anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the
561 membrane. LEAp, Late Embryogenesis Abundant proteins. Results were repeated two to
562 three times depending on the time points, the quantification of these replicates being
563 gathered in figure S8.
564 B. Seeds from indicated background were imbibed at 30°C in the dark for the indicated time
565 to induce secondary dormancy. Germination ability was assessed after 7 days in long day
566 conditions. Values are mean ± SE of 3-4 biological replicates from seed batches produced
567 independently. Values for each replicate are also shown.

568

22

569 **Figure 9 - MPK7 activity in dormant seeds transferred on nitrate depends on NLPs**
570 Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from
571 indicated background, either dry (DS), after acquisition of secondary dormancy (0') and after
572 transfer on either 5mM KCl or KNO3. MPK7 amount was monitored by immunoblot using an
573 anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the membrane.
574 LEAp, Late Embryogenesis Abundant proteins. Results were repeated 3 times, the
575 quantification of these replicates being gathered in figure S10.
576
577 **Figure 10 - MPK7 activity in dormant seeds is also triggered by light**
578 Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0 (A
579 and B) or *phya/b* (B) seeds, either dry (DS), after acquisition of secondary dormancy (0') and
580 after transfer on either 5mM KCl or KNO3 with and without whit light. MPK7 amount was
581 monitored by immunoblot using an anti-MPK7 antibody. Equal loading was controlled by
582 Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results
583 from A were repeated two to three times depending on the time points, the quantification
584 of these replicates being gathered in figure S7. Results from A
585
586 **Figure 11 - *MAP3K19* and *MAP3K20*, but not *MAP3K13* and *MAP3K14*, are differentially**
587 **regulated by light**
588 RT-qPCR analysis of *MAP3K13*, *MAP3K14*, *MAP3K19* and *MAP3K20* genes expression.
589 Transcript levels are expressed relative to ACTIN2 as reference gene. Values are mean ± SE of
590 three biological replicates from seed batches produced independently. Values for each
591 replicate are also shown. ND, not determined.
592
593 **Figure 12 - MPK7 activity in dormant seeds is not modulated by GAs**
594 A and B. Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody
595 from Col-0 seeds, either dry (DS), after acquisition of secondary dormancy (0') and after
596 transfer on either 5mM KCl or KNO3 with and without white light with and without
597 Paclobutrazol 10µM (A) or with and without GA3 50µM (B). MPK7 amount was monitored by
598 immunoblot using an anti-MPK7 antibody. Equal loading was controlled by Coomassie
599 staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results were
600 repeated two to three times depending on the time points, the quantification of these
601 replicates being gathered in figure S13.
602
603 **Figure 13 - MPK7 activity in dormant seeds is not modulated by ABA**
604 Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0
605 seeds, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on
606 either 5mM KCl or KNO3 with and without ABA 50µM. MPK7 amount was monitored by
607 immunoblot using an anti-MPK7 antibody. Equal loading was controlled by Coomassie
608 staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results were
609 repeated two times depending on the time points.

610

Figure 13 – Working model of MKK3 module-dependent regulation of secondary seed dormancy by nitrate and light

613

Figure S1 - Seeds impaired in MKK3 have a reduced nitrate-triggered dormancy release

A and B. Seeds from indicated genetic background were imbibed at 30°C in the dark for 10 days to induce secondary dormancy and transferred on medium containing indicated NO3 concentration. Germination ability was assessed after 7 days in long day conditions. Values are mean ± SE of two biological replicates from seed batches produced independently. Values for each replicate are also shown. On the top, based on Mann-Whitney test, yellow sticks show differences with $\alpha < 5\%$ whereas gray sticks show no differences.

621

Figure S2 - MPK7 is expressed in dry seeds

Western-Blot detection in dry seeds and 7-days old plantlets of lines expressing indicated HA tagged clade-C MAPK from the native locus. Equal loading was controlled by Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. RbcL, RubisCo large subunit. Results were repeated twice.

627

Figure S3 - Quantification of MPK7 activity in dormant Col-0 and _mkk3-1_ seeds transferred on nitrate.

MPK7 activities of replicates were quantified and normalized to the one of Col-0 8h NO3 (*). Values are mean ± SE of two to six biological replicates from seed batches produced independently. Values for each replicate are also shown.

633

Figure S4 - kinase activity immunoprecipitated with the anti-MPK7 antibody dependent of the expression of MPK7.

Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0 and _mpk7-1_ seeds, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl or KNO3. Seeds were produced in the green house with non limiting nitrogen fertilizer, probably explaining why there is no NO3 effect.

640

Figure S5 - MPK7-HA activity in dormant seeds expressing an HA-tagged MPK7 depends on MKK3.

Kinase activity of MPK7 after immunoprecipitation with an anti-HA antibody from _MPK7locus-HA_ and _mkk3-1 MPK7locus-HA_ seeds, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl or KNO3. MPK7-HA amount was monitored by immunoblot using an anti-HA antibody. Equal loading was controlled by Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins.

648

Figure S6 - All clade-III MAP3Ks are not expressed in seeds or during secondary dormancy release

24

651 RT-qPCR analysis of *MAP3K15, MAP3K16, MAP3K17, MAP3K18* and *MAP3K21* genes
652 expression. Transcript levels are expressed relative to ACTIN2 as reference gene. Values are
653 mean ± SE of two to 10 biological replicates from seed batches produced independently.
654 Values for each replicate are also shown.
655
656 **Figure S7 - Quantification of MPK7 activity in dormant Col-0 and map3k mutant seeds**
657 **transferred on nitrate.**
658 MPK7 activities of replicates were quantified and normalized to the one of Col-0 8h NO3 (*).
659 Values are mean ± SE of 2-6 (A) or 3 (B) biological replicates from seed batches mainly
660 produced independently. Values for each replicate are also shown.
661
662 **Figure S8 - Quantification of MPK7 activity in Col-0 and *35S::MAP3K19-MYC* seeds**
663 **transferred on nitrate.**
664 MPK7 activities of replicates were quantified and normalized to the one of Col-0 8h NO3 (*).
665 Values are mean ± SE of one to three biological replicates from seed batches produced
666 independently. Values for each replicate are also shown.
667
668 **Figure S9 – Quantification of MPK7 activity in dormant Col-0 and *nlp8* seeds transferred on**
669 **nitrate.**
670 MPK7 activities of replicates were quantified and normalized to the one of Col-0 8h NO3 (*).
671 Values are mean ± SE of two to five biological replicates from seed batches produced
672 independently. Values for each replicate are also shown.
673
674 **Figure S10 - Quantification of MPK7 activity in Col-0 and *nlp* seeds transferred on nitrate.**
675 MPK7 activities of replicates were quantified and normalized to the one of Col-0 8h NO3 (*).
676 Values are mean ± SE of 3 biological replicates from seed batches produced independently.
677 Values for each replicate are also shown.
678
679 **Figure S11 - Quantification of MPK7 activity in Col-0 transferred on nitrate and dark.**
680 MPK7 activities of replicates were quantified and normalized to the one of Col-0 8h NO3 (*).
681 Values are mean ± SE of three to five biological replicates from seed batches produced
682 independently. Values for each replicate are also shown.
683
684 **Figure S12 – Complement of clade-III *MAP3K* expression in seeds or during secondary**
685 **dormancy release by nitrate and/or light**
686 RT-qPCR analysis of *MAP3K15/16/17/18/21* genes expression. Transcript levels are
687 expressed relative to ACTIN2 as reference gene. Values are mean ± SE of three biological
688 replicates from seed batches produced independently. Values for each replicate are also
689 shown. ND, not determined.
690

691   **Figure S13 - Quantification of MPK7 activity in Col-0 seeds after transfer on Paclobutrazol**
692   **(PCZ) and GA3.**
693   A and B. MPK7 activities of replicates were quantified and normalized to the one of Col-0 8h
694   NO3 (*). Values are mean ± SE of two biological replicates from seed batches produced
695   independently. Values for each replicate are also shown.
696
697   **Figure S14 – Expression of *MAP3K13, MAP3K14, MAP3K17, MAP3K18, MAP3K19* and**
698   ***MAP3K20* is not affected by ABA during secondary dormancy release**
699   RT-qPCR analysis of *MAP3K13, MAP3K14, MAP3K17, MAP3K18, MAP3K19* and *MAP3K20*
700   genes expression. Transcript levels are expressed relative to ACTIN2 as reference gene.
701   Values are mean ± SE of two biological replicates from seed batches produced
702   independently. Values for each replicate are also shown.
703
704   **Figure S15 – Expression of genes involved in ABA biosynthesis (left) and catabolism (right)**
705   RT-qPCR analysis of indicated genes expression in Col0 and *mkk3-1*. Transcript levels are
706   expressed relative to ACTIN2 as reference gene. Values are mean ± SE of two to three
707   biological replicates from seed batches produced independently. Values for each replicate
708   are also shown.
709
710   **Figure S16 – Expression of genes involved in ABA metabolism**
711   RT-qPCR analysis of indicated genes expression in Col0 and *mkk3-1*. Transcript levels are
712   expressed relative to ACTIN2 as reference gene. Values are mean ± SE of two to three
713   biological replicates from seed batches produced independently. Values for each replicate
714   are also shown.
715
716   **Figure S17 – reconstruction of a functional MKK3 pathway in budding yeast strongly affects**
717   **its growth.**
718   Serial dilutions of the various transformed strains were spotted onto agar-based solid
719   medium. Note that the complete MKK3 module leads to growth inhibition. However, the
720   growth is restored if MAP3K19 or MPK7 is omitted, or if MKK3 carries a kinase-dead
721   mutation affecting its kinase activity.

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724 **References**

725 **Alboresi, A., Gestin, C., Leydecker, M.T., Bedu, M., Meyer, C., and Truong, H.N.** (2005).
726   Nitrate, a signal relieving seed dormancy in Arabidopsis. Plant, Cell Environ. **28**: 500–
727   512.

728 **Bai, F. and Matton, D.P.** (2018). The Arabidopsis Mitogen-Activated Protein Kinase Kinase
729   Kinase 20 (MKKK20) C-terminal domain interacts with MKK3 and harbors a typical DEF
730   mammalian MAP kinase docking site. Plant Signal. Behav. **13**: e1503498.

731 **Baskin, J.M. and Baskin, C.C.** (2004). A classification system for seed dormancy. Seed Sci.
732   Res. **14**: 1–16.

733 **Benhamman, R., Bai, F., Drory, S.B., Loubert-Hudon, A., Ellis, B., and Matton, D.P.** (2017).
734   The Arabidopsis Mitogen-Activated Protein Kinase Kinase Kinase 20 (MKKK20) Acts
735   Upstream of MKK3 and MPK18 in Two Separate Signaling Pathways Involved in Root
736   Microtubule Functions. Front. Plant Sci. **8**: 1–15.

737 **Berriri, S., Garcia, a. V., Frei dit Frey, N., Rozhon, W., Pateyron, S., Leonhardt, N.,**
738   **Montillet, J.J.-L., Leung, J., Hirt, H., and Colcombet, J.** (2012). Constitutively Active
739   Mitogen-Activated Protein Kinase Versions Reveal Functions of Arabidopsis MPK4 in
740   Pathogen Defense Signaling. Plant Cell **24**: 4281–4293.

741 **Bigeard, J., Pfl, D., Colcombet, J., Gérard, L., Mireau, H., and Hirt, H.** (2014). Protein
742   Complexes Characterization in Arabidopsis thaliana by Tandem Affi nity Purifi cation
743   Coupled to Mass Spectrometry Analysis. Methods Mol. Biol. **1171**: 237–250.

744 **Boudsocq, M., Danquah, A., de Zélicourt, A., Hirt, H., and Colcombet, J.** (2015). Plant MAPK
745   cascades: Just rapid signaling modules? Plant Signal. Behav. **10**: e1062197.

746 **Chen, X. et al.** (2023). The MKK3–MPK7 cascade phosphorylates ERF4 and promotes its rapid
747   degradation to release seed dormancy in Arabidopsis. Mol. Plant **16**: 1743–1758.

748 **Choi, S.-W., Lee, S.-B., Na, Y.-J., Jeung, S.-G., and Kim, S.Y.** (2017). Arabidopsis MAP3K16
749   and Other Salt-Inducible MAP3Ks Regulate ABA Response Redundantly. Mol. Cells **40**:
750   230—242.

751 **Clough, S.J. and Bent, A.F.** (1998). Floral dip: a simplified method for Agrobacterium-
752   mediated transformation of Arabidopsis thaliana. Plant J. **16**: 735–43.

753 **Colcombet, J. and Hirt, H.** (2008). Arabidopsis MAPKs: a complex signalling network involved
754   in multiple biological processes. Biochem. J. **413**: 217–226.

755 **Colcombet, J., Lopez-Obando, M., Heurtevin, L., Bernard, C., Martin, K., Berthomé, R., and**
756   **Lurin, C.** (2013). Systematic study of subcellular localization of Arabidopsis PPR proteins
757   confirms a massive targeting to organelles. RNA Biol. **10**: 1557–1575.

758 **Colcombet, J., Sözen, C., and Hirt, H.** (2016). Convergence of Multiple MAP3Ks on MKK3
759   Identifies a Set of Novel Stress MAPK Modules. Front. Plant Sci. **07**: 1941.

760 **Danquah, A. et al.** (2015). Identification and characterization of an ABA-activated MAP
761   kinase cascade in Arabidopsis thaliana. Plant J. **82**: 232–244.

762 **Danquah, A., de Zelicourt, A., Colcombet, J., and Hirt, H.** (2014). The role of ABA and MAPK
763   signaling pathways in plant abiotic stress responses. Biotechnol. Adv. **32**: 40–52.

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

768 **Footitt, S., Douterelo-Soler, I., Clay, H., and Finch-Savage, W.E.** (2011). Dormancy cycling in
769   Arabidopsis seeds is controlled by seasonally distinct hormone-signaling pathways.
770   Proc. Natl. Acad. Sci. U. S. A. **108**: 20236–20241.

27

771   Jagodzik, P., Tajdel-Zielinska, M., Ciesla, A., Marczak, M., and Ludwikow, A. (2018).
772   Mitogen-Activated Protein Kinase Cascades in Plant Hormone Signaling. Front. Plant Sci.
773   **9**: 100–136.
774   Lee, H. (2015). Mitogen-Activated Protein Kinase Kinase 3 Is Required for Regulation during
775   Dark-Light Transition. Mol. Cells **38**: 651–656.
776   Li, K., Yang, F., Zhang, G., Song, S., Li, Y., Ren, D., Miao, Y., and Song, C.P. (2017a). AIK1, a
777   mitogen-activated protein Kinase, modulates abscisic acid responses through the
778   MKK5-MPK6 kinase cascade. Plant Physiol. **173**: 1391–1408.
779   Li, Y., Cai, H., Liu, P., Wang, C., Gao, H., Wu, C., Yan, K., Zhang, S., Huang, J., and Zheng, C.
780   (2017b). Arabidopsis MAPKKK18 positively regulates drought stress resistance via
781   downstream MAPKK3. Biochem. Biophys. Res. Commun. **484**: 292–297.
782   Loudet, O., Chaillou, S., Merigout, P., Talbotec, J., and Daniel-Vedele, F. (2003).
783   Quantitative trait loci analysis of nitrogen use efficiency in Arabidopsis. Plant Physiol.
784   **131**: 345–358.
785   Mao, X. et al. (2020). The MKKK62-MKK3-MAPK7 / 14 module negatively regulates seed
786   dormancy in rice.
787   Marchive, C., Roudier, F., Castaings, L., Bréhaut, V., Blondet, E., Colot, V., Meyer, C., and
788   Krapp, A. (2013). Nuclear retention of the transcription factor NLP7 orchestrates the
789   early response to nitrate in plants. Nat. Commun. **4**: 1–9.
790   Matsuoka, D., Yasufuku, T., Furuya, T., and Nanmori, T. (2015). An abscisic acid inducible
791   Arabidopsis MAPKKK, MAPKKK18 regulates leaf senescence via its kinase activity. Plant
792   Mol. Biol.: 565–575.
793   Mitula, F., Tajdel, M., Ciešla, A., Kasprowicz-Maluški, A., Kulik, A., Babula-Skowrońska, D.,
794   Michalak, M., Dobrowolska, G., Sadowski, J., and Ludwików, A. (2015). Arabidopsis
795   ABA-Activated Kinase MAPKKK18 is Regulated by Protein Phosphatase 2C ABI1 and the
796   Ubiquitin-Proteasome Pathway. Plant Cell Physiol. **56**: 2351–2367.
797   Mumberg, D., Müller, R., and Funk, M. (1995). Yeast vectors for the controlled expression of
798   heterologous proteins in different genetic backgrounds. Gene **156**: 119–122.
799   Nakamura, S. et al. (2016). Mitogen-activated protein kinase kinase 3 regulates seed
800   dormancy in barley. Curr. Biol. **26**: 775–781.
801   Narsai, R., Law, S.R., Carrie, C., Xu, L., and Whelan, J. (2011). In-depth temporal
802   transcriptome profiling reveals a crucial developmental switch with roles for RNA
803   processing and organelle metabolism that are essential for germination in Arabidopsis.
804   Plant Physiol. **157**: 1342–62.
805   Ojha, M., Verma, D., Chakraborty, N., Pal, A., Bhagat, P.K., Singh, A., Verma, N., Sinha, A.K.,
806   and Chattopadhyay, S. (2023). MKKK20 works as an upstream triple-kinase of MKK3-
807   MPK6-MYC2 module in Arabidopsis seedling development. iScience **26**: 106049.
808   Otani, M., Tojo, R., Regnard, S., Zheng, L., Hishi, T., Ohmori, S., Tachibana, N., Sano, T.,
809   Koshimizu, S., Ichimura, K., Colcombet, J., and Kawakami, N. (2024). The MKK3 MAPK
810   cascade integrates temperature and after-ripening signals to modulate seed
811   germination. BioRxiv.
812   Schikora, A., Carreri, A., Charpentier, E., and Hirt, H. (2008). The dark side of the salad:
813   Salmonella typhimurium overcomes the innate immune response of arabidopsis
814   thaliana and shows an endopathogenic lifestyle. PLoS One **3**.
815   Sethi, V., Raghuram, B., Sinha, A.K., and Chattopadhyay, S. (2014). A mitogen-activated
816   protein kinase cascade module, MKK3-MPK6 and MYC2, is involved in blue light-
817   mediated seedling development in Arabidopsis. Plant Cell **26**: 3343–57.

818   **Shu, K., Liu, X.D., Xie, Q., and He, Z.H.** (2016). Two Faces of One Seed: Hormonal Regulation
819   of Dormancy and Germination. Mol. Plant **9**: 34–45.
820   **Sözen, C., Schenk, S.T., Boudsocq, M., Chardin, C., Almeida-Trapp, M., Krapp, A., Hirt, H.,**
821   **Mithöfer, A., and Colcombet, J.** (2020). Wounding and Insect Feeding Trigger two
822   Independent MAPK Pathways with Distinct Regulation and Kinetics. Plant Cell **32**: 1988–
823   2003.
824   **Takahashi, F., Mizoguchi, T., Yoshida, R., Ichimura, K., and Shinozaki, K.** (2011). Calmodulin-
825   Dependent Activation of MAP Kinase for ROS Homeostasis in Arabidopsis. Mol. Cell **41**:
826   649–660.
827   **Takahashi, F., Yoshida, R., Ichimura, K., Mizoguchi, T., Seo, S., Yonezawa, M., Maruyama,**
828   **K., Yamaguchi-Shinozaki, K., and Shinozaki, K.** (2007). The mitogen-activated protein
829   kinase cascade MKK3-MPK6 is an important part of the jasmonate signal transduction
830   pathway in Arabidopsis. Plant Cell **19**: 805–818.
831   **Torada, A., Koike, M., Ogawa, T., Takenouchi, Y., Tadamura, K., Wu, J., Matsumoto, T.,**
832   **Kawaura, K., and Ogihara, Y.** (2016). A causal gene for seed dormancy on wheat
833   chromosome 4A encodes a MAP kinase kinase. Curr. Biol. **26**: 782–787.
834   **Tuan, P.A., Kumar, R., Rehal, P.K., Toora, P.K., and Ayele, B.T.** (2018). Molecular
835   mechanisms underlying abscisic acid/gibberellin balance in the control of seed
836   dormancy and germination in cereals. Front. Plant Sci. **9**: 1–14.
837   **Yan, D. et al.** (2016). NIN-like protein 8 is a master regulator of nitrate-promoted seed
838   germination in Arabidopsis. Nat. Commun. **7**: 13179.
839   **Zhang, W. et al.** (2019). The MPK8-TCP14 pathway promotes seed germination in
840   Arabidopsis. Plant J.: 1–16.
841   **Zhou, M. et al.** (2021). Hydrogen sulfide-linked persulfidation of ABI4 controls ABA
842   responses through the transactivation of MAPKKK18 in Arabidopsis. Mol. Plant **14**: 921–
843   936.
844   **Zimmermann, P., Hirsch-Hoffmann, M., Hennig, L., and Gruissem, W.** (2004).
845   GENEVESTIGATOR. Arabidopsis Microarray Database and Analysis Toolbox. Plant
846   Physiol. **136**: 2621–2632.
847
848
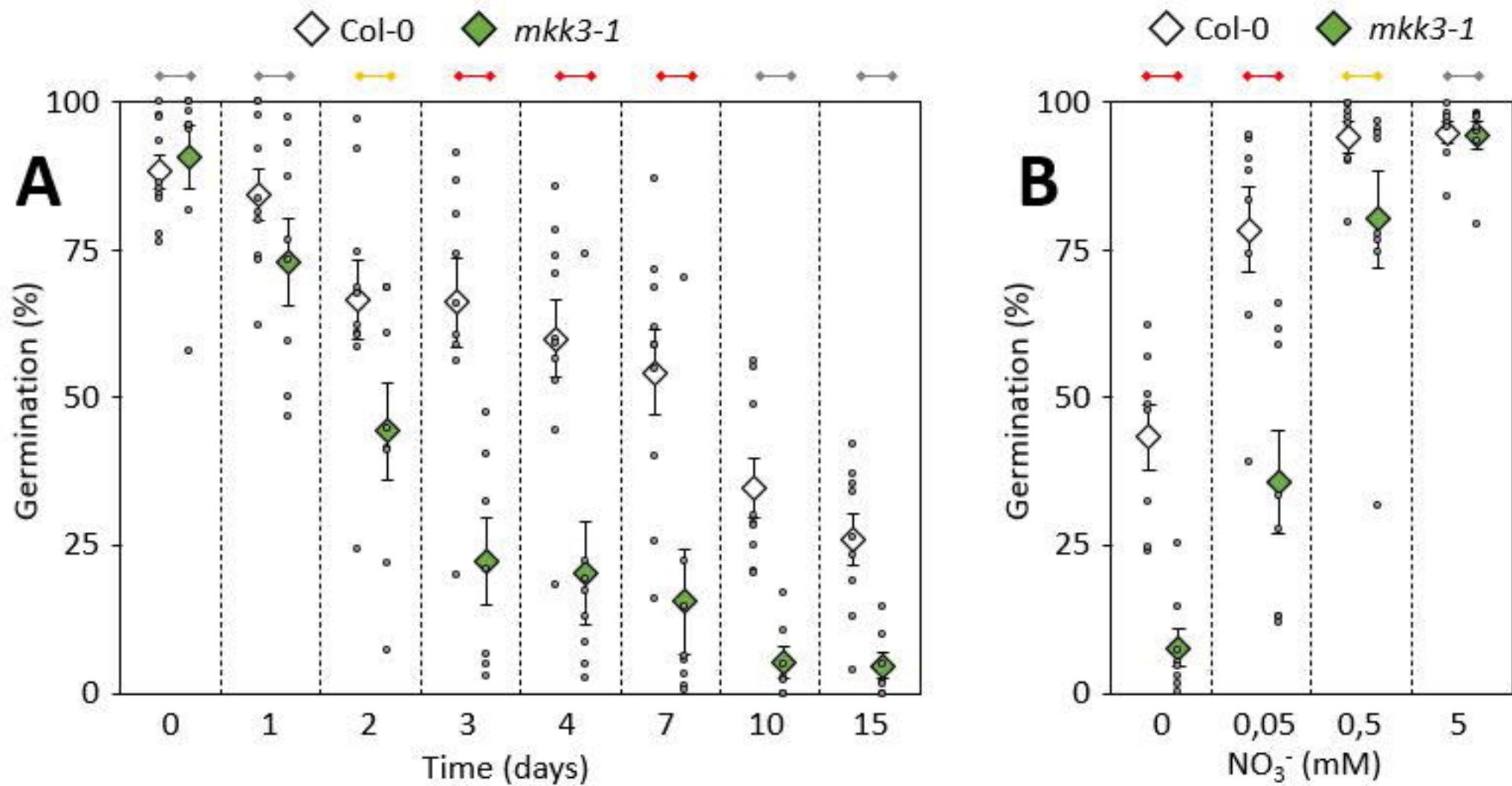
Figure 1 - *mkk3-1* seeds have a faster secondary dormancy acquisition and a reduced nitrate-triggered dormancy release

A. Col-0 and *mkk3-1* seeds were imbibed at 30°C in the dark for the indicated time to induce secondary dormancy. Germination ability was then assessed after 7 days in long day conditions. Values are mean ± SE of seven to ten biological replicates from seed batches produced independently. Values for each replicate are also shown. On the top, based on Mann-Whitney test, yellow and red sticks show differences with 1% < α < 5% and α < 1%, respectively, whereas gray sticks show no differences.

B. Col-0 and *mkk3-1* seeds were imbibed at 30°C in the dark for 10 days to induce secondary dormancy and transferred on media containing indicated NO3- concentrations. Germination ability was then assessed after 7 days in long day conditions. Values are mean ± SE of height biological replicates from seed batches largely produced independently. Values for each replicate are also shown. On the top, based on Mann-Whitney test, yellow and red sticks show differences with 1% < α < 5% and α < 1%, respectively, whereas gray sticks show no differences.
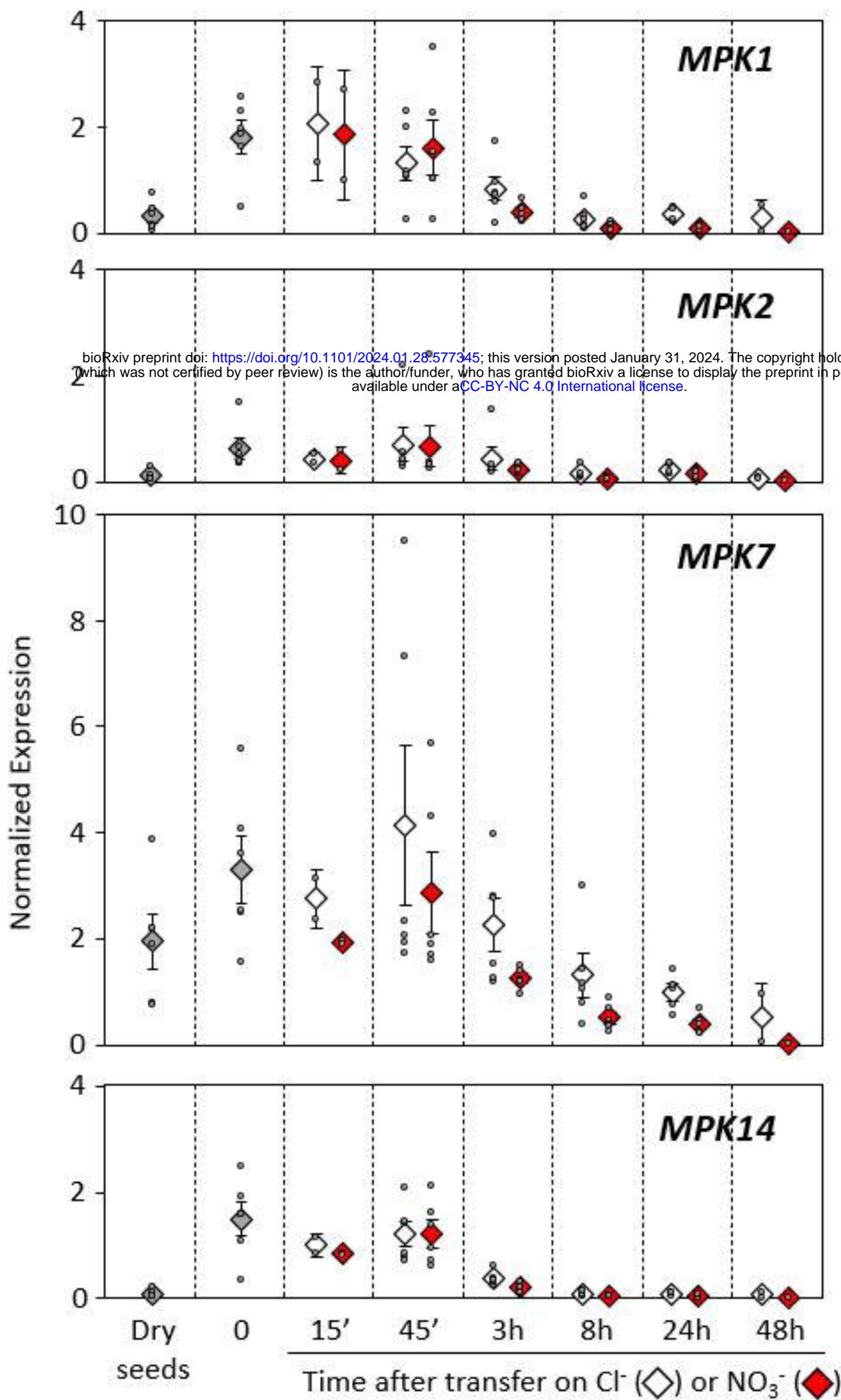
Figure 2 - Clade-C *MAPK* genes are expressed in seeds and during secondary dormancy release

RT-qPCR analysis of clade-C MAP3K genes. Transcript levels are expressed relative to ACTIN2 as reference gene. Values are mean ± SE of two to six biological replicates from seed batches produced independently. Values for each replicate are also shown.
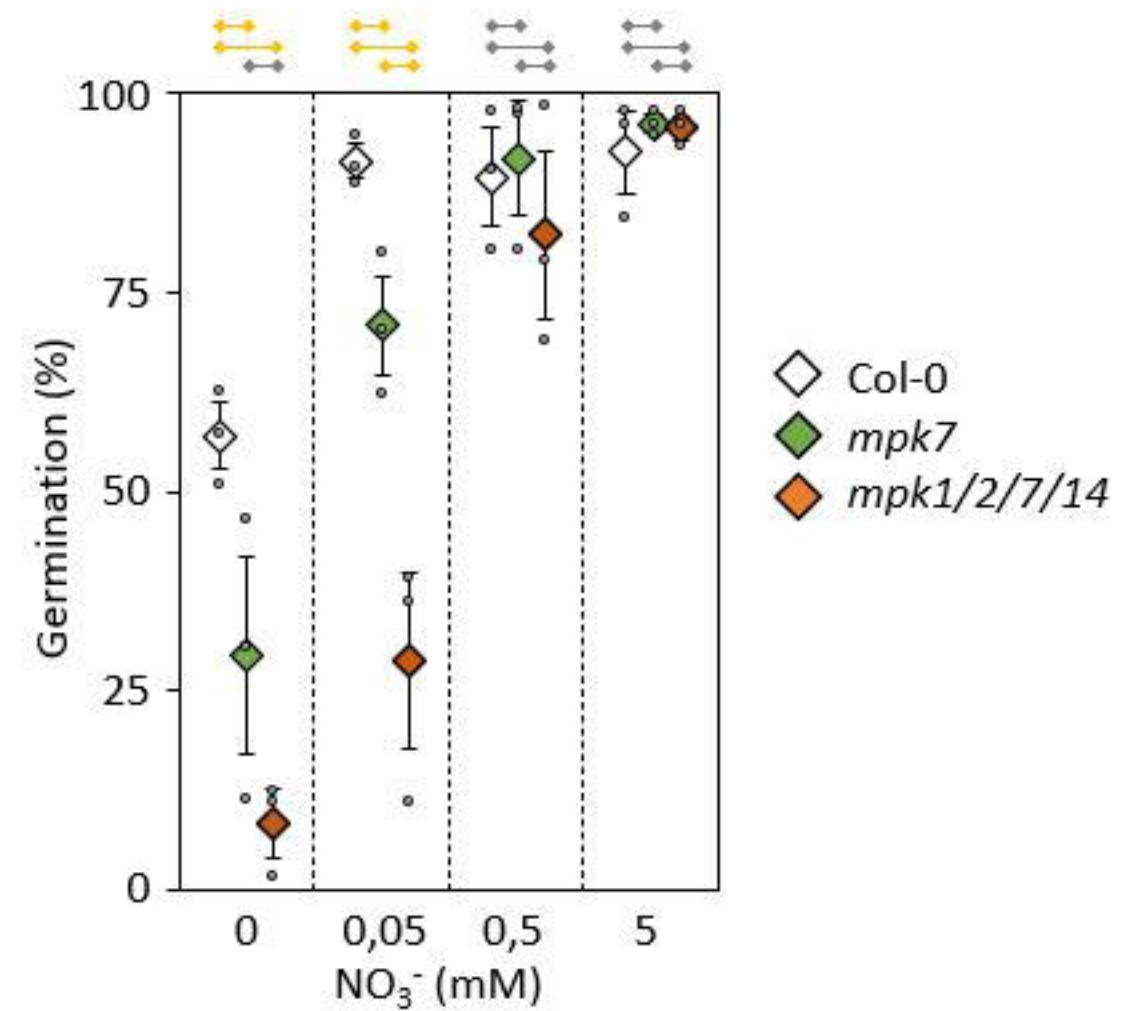
Figure 3 - Seeds impaired in C-clade MAPKs have a faster secondary dormancy acquisition and a reduced nitrate-triggered dormancy release

Col-0, *mpk7* and *mpk1/2/7/14* seeds were imbibed at 30°C in the dark for 10 days to induce secondary dormancy and transferred on media containing indicated NO3- concentrations. Germination ability was then assessed after 7 days in long day conditions. Values are mean ± SE of three biological replicates from seed batches largely produced independently. Values for each replicate are also shown. On the top, based on Mann-Whitney test, yellow sticks show differences with α < 5% whereas gray sticks show no differences.
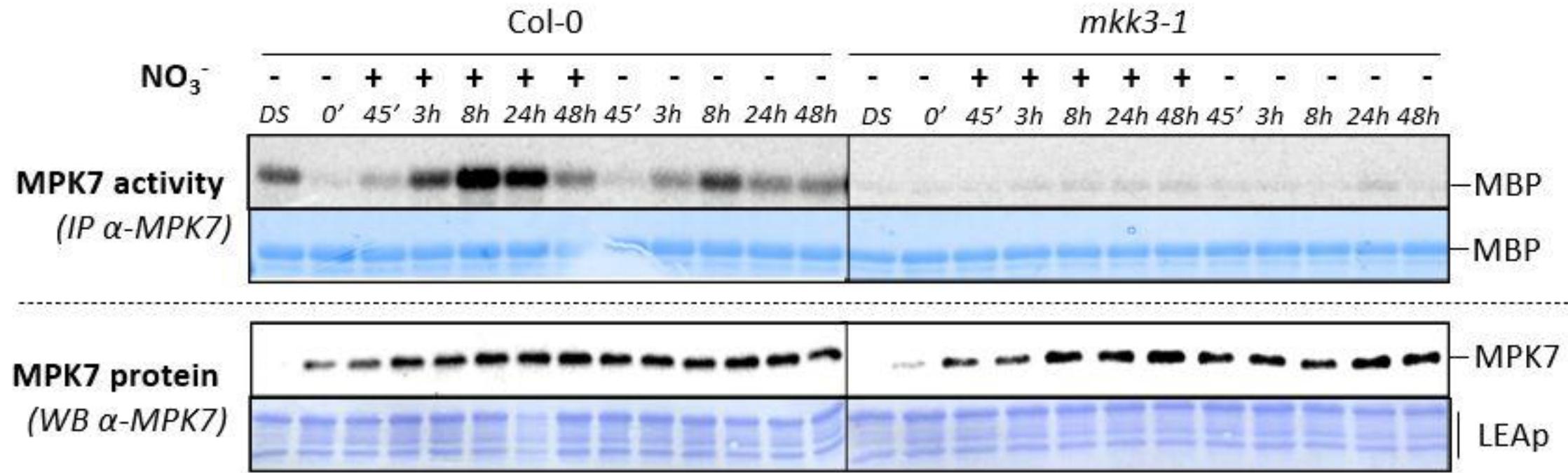
Figure 4 - MPK7 activity in dormant seeds transferred on nitrate depends on MKK3.
Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0 and mkk3-1 seeds, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl (-) or KNO3. MPK7 amount was monitored by immunoblot using an anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results were repeated two to six times depending on the time points, the quantification of these replicates being gathered in figure S3.
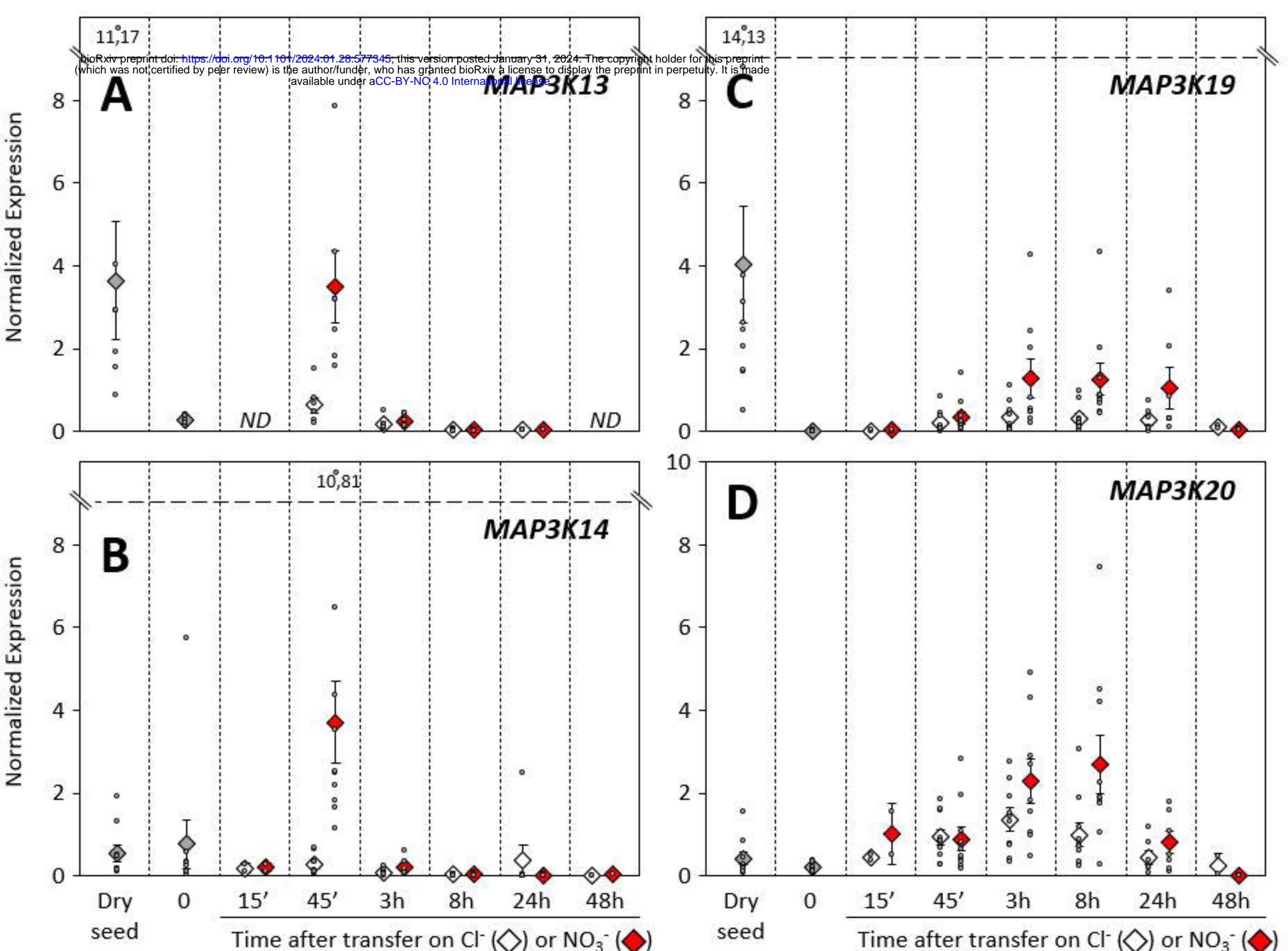
Figure 5 - *MAP3K13, MAP3K14, MAP3K19* and *MAP3K20* **are expressed in seeds and during secondary dormancy release**

RT-qPCR analysis of *MAP3K13, MAP3K14, MAP3K19* and *MAP3K20* genes expression. Transcript levels are expressed relative to ACTIN2 as reference gene. Values are mean ± SE of two to 10 biological replicates from seed batches produced independently. Values for each replicate are also shown. ND not determined.
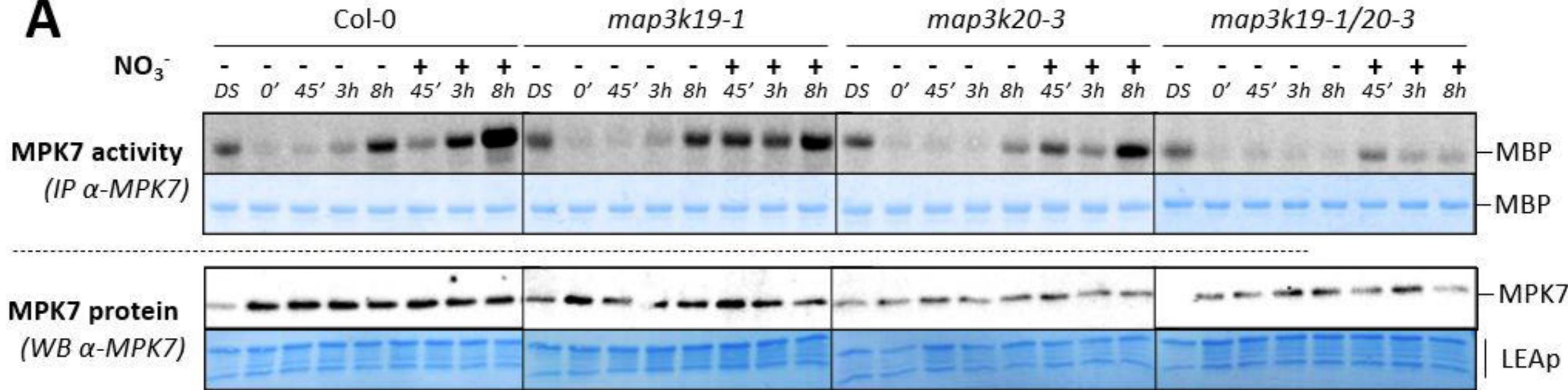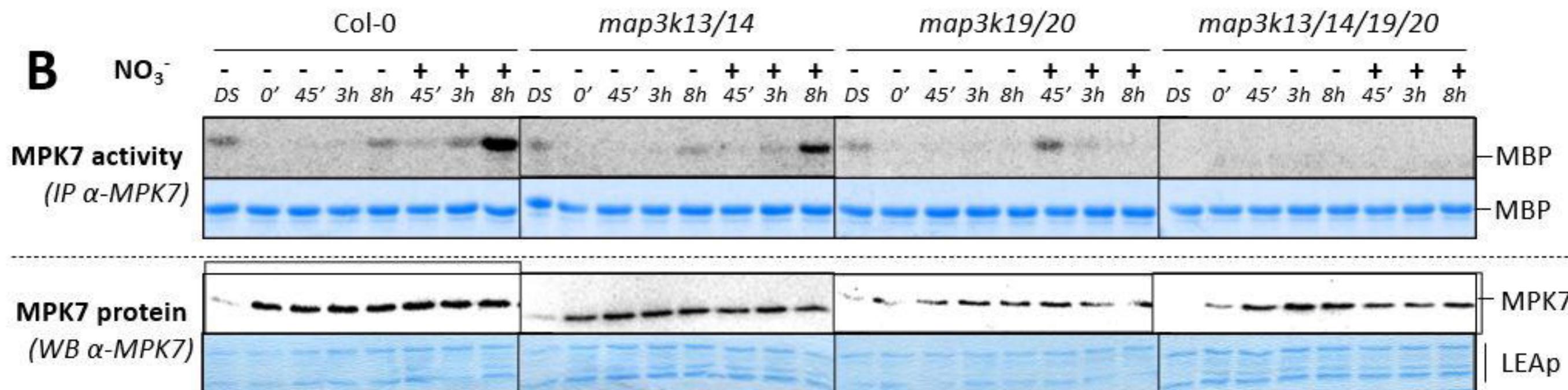
Figure 6 - MPK7 activity in dormant seeds transferred on nitrate depends on MAP3K13/14/19/20

Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from indicated background, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl or KNO3. MPK7 amount was monitored by immunoblot using an anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results were repeated 2-6 times depending of the time point an genotype for A and 3 times for B, the quantification of these replicates being gathered in figure S7A and B.

Figure 7 - *map3k13/14/19/20* seeds have a reduced nitrate-triggered dormancy release
Col-0, *map3k13CR/14CR*, *map3k19-1/20-3* and *map3k13CR/14CR/19-1/20-3* seeds were imbibed at 30°C in the dark for 10 days to induce secondary dormancy and transferred on medium containing indicated NO3 concentration. Germination ability was assessed after 7 days in long day conditions. Values are mean ± SE of three biological replicates from seed batches produced independently. Values for each replicate are also shown. On the top, based on Mann-Whitney test, yellow sticks show differences with $\alpha < 5\%$ whereas gray sticks show no differences.

Figure 8 – Constitutive expression of *MAP3K19* triggers a strong MKK3-dependent MPK7 activation and reduces the acquisition of secondary dormancy
A. Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from indicated background, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl (-) or KNO3 (+). MPK7 amount was monitored by immunoblot using an anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results were repeated two to three times depending on the time points, the quantification of these replicates being gathered in figure S8.
B. Seeds from indicated background were imbibed at 30°C in the dark for the indicated time to induce secondary dormancy. Germination ability was assessed after 7 days in long day conditions. Values are mean ± SE of 3-4 biological replicates from seed batches produced independently. Values for each replicate are also shown.
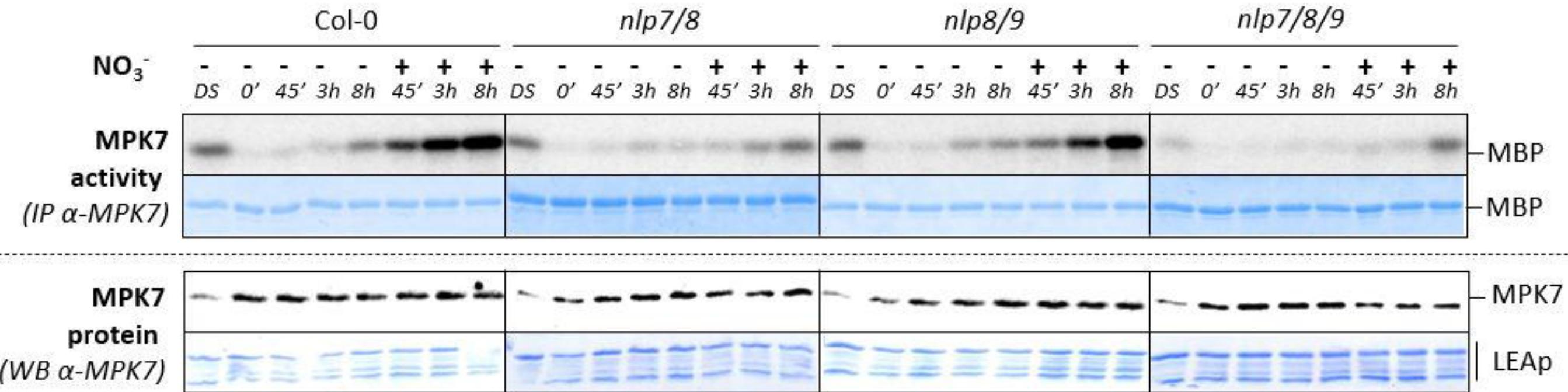
Figure 9 - MPK7 activity in dormant seeds transferred on nitrate depends on NLPs

Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from indicated background, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl or KNO3. MPK7 amount was monitored by immunoblot using an anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results were repeated 3 times, the quantification of these replicates being gathered in figure S10.
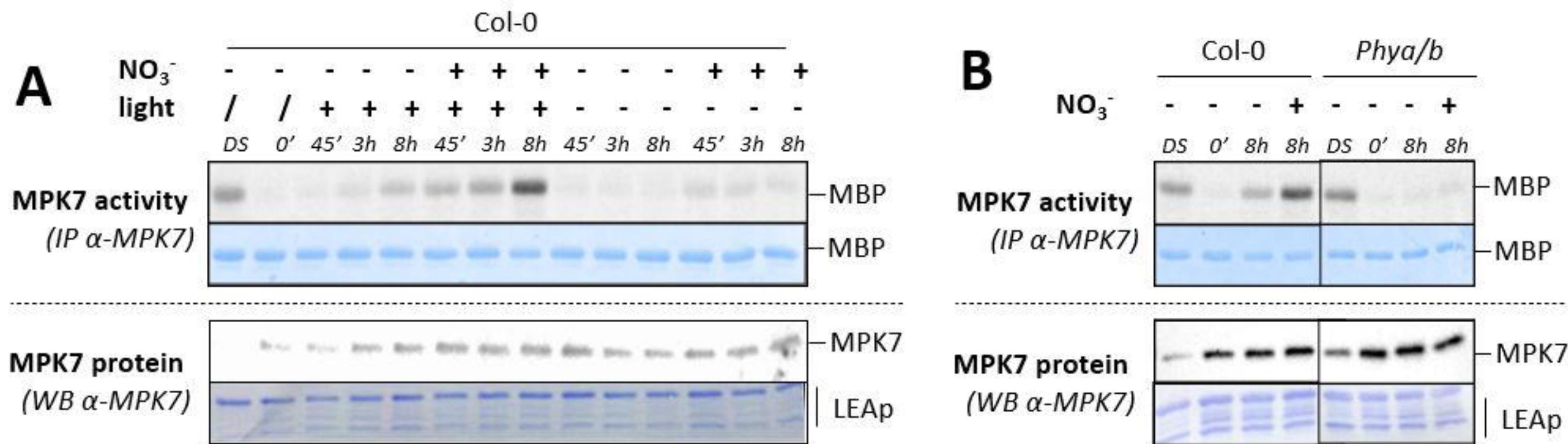
Figure 10 - MPK7 activity in dormant seeds is also triggered by light

Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0 (A and B) or *phya/b* (B) seeds, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl or KNO3 with and without whit light. MPK7 amount was monitored by immunoblot using an anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results from A were repeated two to three times depending on the time points, the quantification of these replicates being gathered in figure S7. Results from A
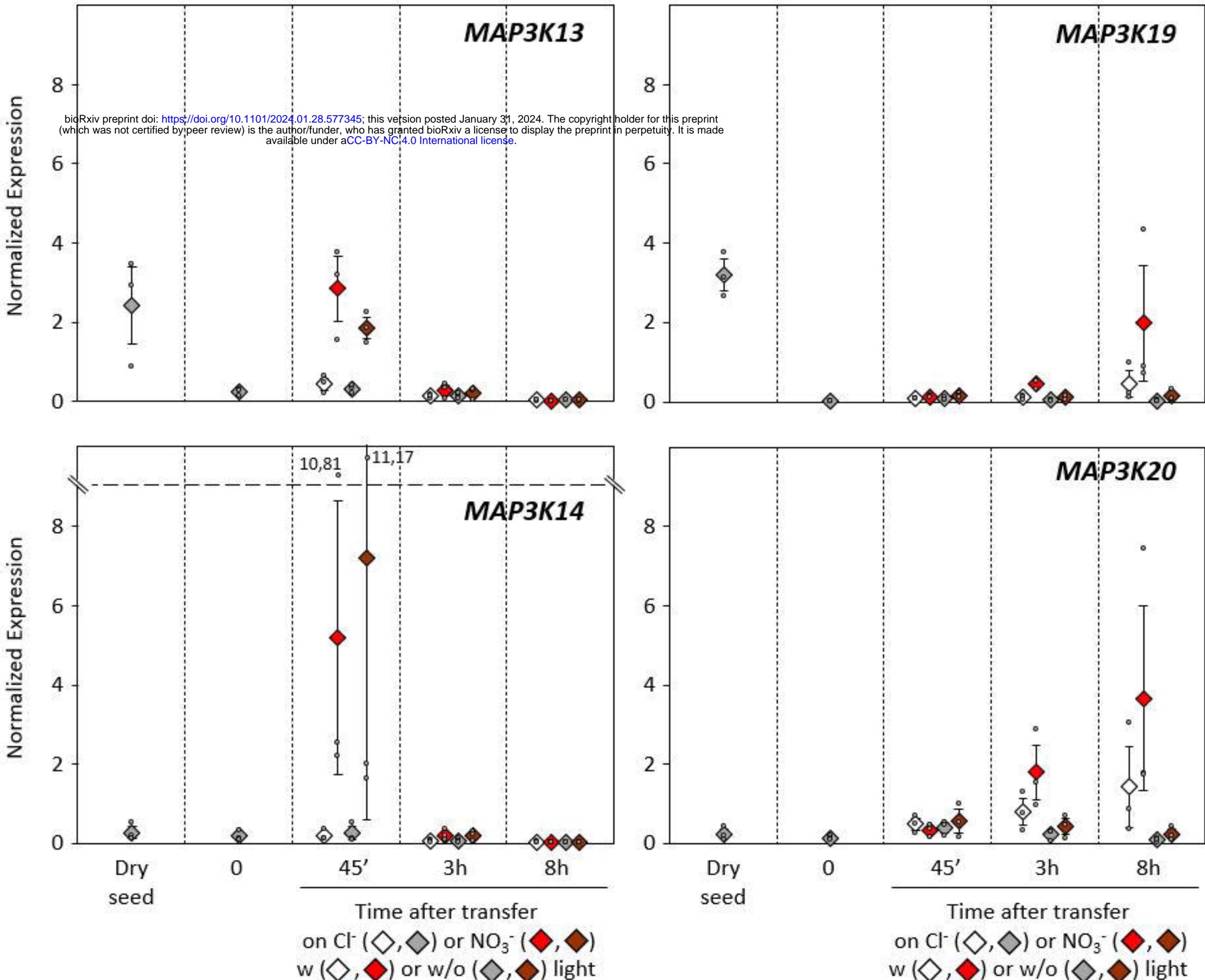
Figure 11 - *MAP3K19* **and** *MAP3K20***, but not** *MAP3K13* **and** *MAP3K14***, are differentially regulated by light**

RT-qPCR analysis of *MAP3K13*, *MAP3K14*, *MAP3K19* and *MAP3K20* genes expression. Transcript levels are expressed relative to ACTIN2 as reference gene. Values are mean ± SE of three biological replicates from seed batches produced independently. Values for each replicate are also shown. ND, not determined.
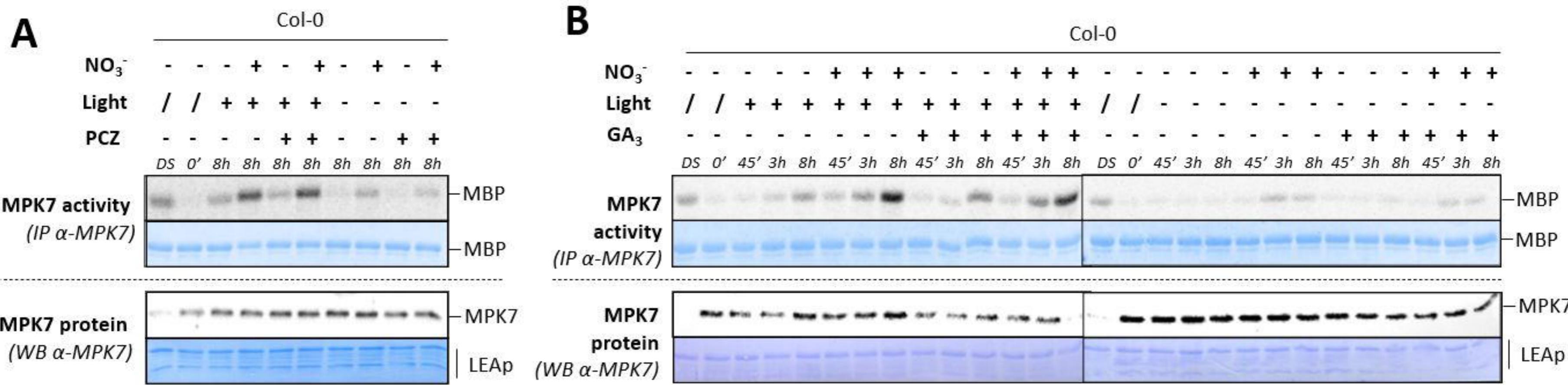
Figure 12 - MPK7 activity in dormant seeds is not modulated by GAs

A and B. Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0 seeds, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl or KNO3 with and without white light with and without Paclobutrazol 10µM (A) or with and without GA3 50µM (B). MPK7 amount was monitored by immunoblot using an anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results were repeated two to three times depending on the time points, the quantification of these replicates being gathered in figure S13.
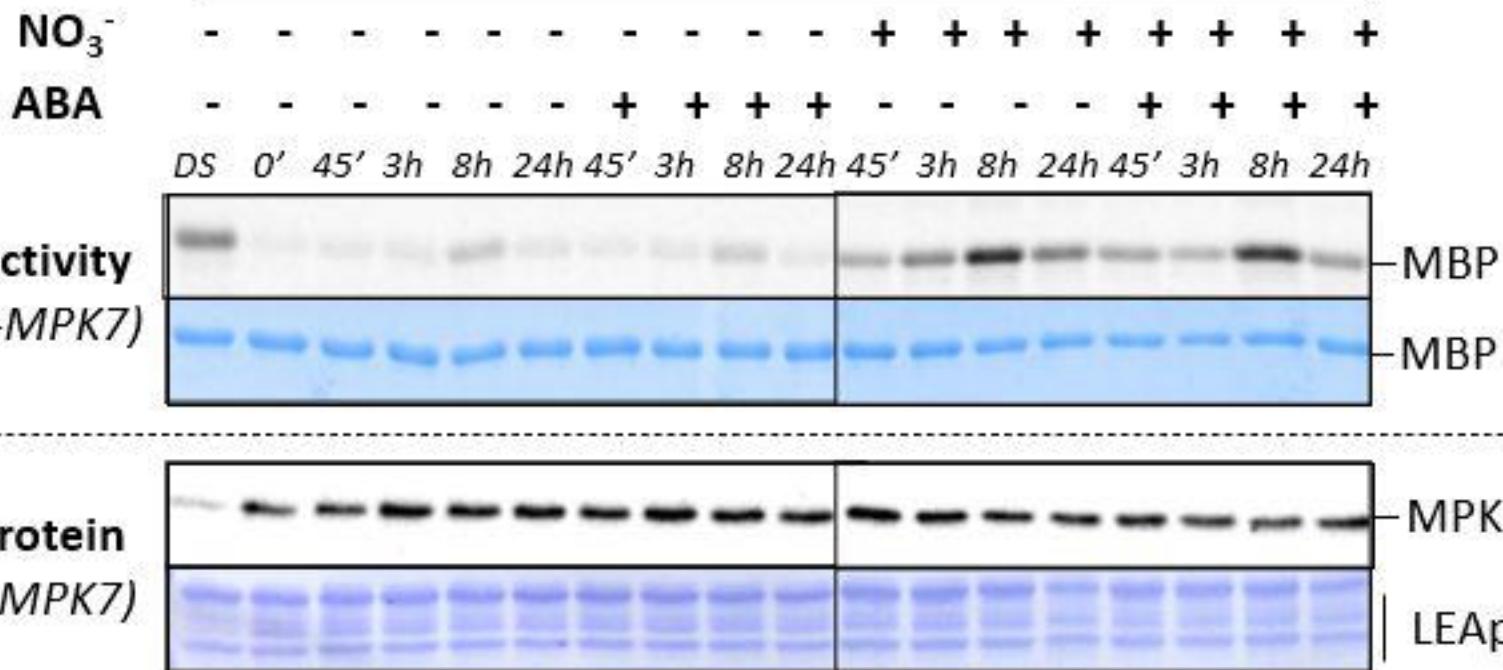
Figure 13 - MPK7 activity in dormant seeds is not modulated by ABA

Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0 seeds, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl or KNO3 with and without ABA 50µM. MPK7 amount was monitored by immunoblot using an anti-MPK7 antibody. Equal loading was controlled by Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins. Results were repeated two times depending on the time points.

Figure 13 – Working model of MKK3 module-dependent regulation of secondary seed dormancy by nitrate and light