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

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21

## 22 **Abstract**

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

39

## 40 **Introduction**

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

59 Dormancy is thought to be primarily controlled by a dynamic hormonal balance (Shu et  
60 al., 2016; Tuan et al., 2018). Abscisic acid (ABA) is a major inducer of seed dormancy,  
61 whereas gibberellins (GA) promote germination. Plants impaired in ABA/GA biogenesis or  
62 signalling pathways are affected in their ability to control dormancy and germination. After  
63 seed dispersal or harvest, the ABA catabolism in imbibed seeds contributes to the release of  
64 dormancy and allows the promotion of germination by GA. Many external cues modulate  
65 this balance. Seeds perceive and respond to changes in environmental conditions, such as  
66 temperature, light, storage periods (after-ripening), moisture content, and nitrate ( $\text{NO}_3^-$ ).  
67 External  $\text{NO}_3^-$  promotes seed germination at low concentrations in many plant species,  
68 acting as both a nutrient and signal (Alboresi et al., 2005). Furthermore, exogenous  $\text{NO}_3^-$   
69 breaks the primary dormancy efficiently and promotes the completion of seed germination  
70 by enhancing ABA catabolism and inhibiting ABA synthesis. In *Arabidopsis*, the NIN-Like

71 Protein 8 (NLP8) transcription factor is a major regulator of NO<sub>3</sub><sup>-</sup>-regulated dormancy,  
72 promoting NO<sub>3</sub><sup>-</sup>-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<sub>3</sub><sup>-</sup> (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<sub>3</sub><sup>-</sup> 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<sub>3</sub><sup>-</sup> 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<sub>3</sub><sup>-</sup> concentrations and incubated for seven days in  
114 permissive conditions (16 h; photoperiod 22 °C). NO<sub>3</sub><sup>-</sup> was provided in the form of KNO<sub>3</sub>, and  
115 KCl was added to reach a KCl + KNO<sub>3</sub> 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<sub>3</sub><sup>-</sup> concentrations (0.05 and 0.5 mM) than Col-0. 5 mM KNO<sub>3</sub> 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<sub>3</sub><sup>-</sup> although not insensitive,  
122 suggesting that an MKK3-containing MAPK module could mediate a part of the NO<sub>3</sub><sup>-</sup>  
123 signalling to promote dormancy breaking.

124 ***MPK7 is expressed in seeds and activated by NO<sub>3</sub><sup>-</sup> 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<sub>3</sub> (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  $\text{NO}_3^-$  than to  $\text{Cl}^-$ , 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  $\text{KNO}_3$  than agar supplemented  
146 with 5 mM  $\text{KCl}$  (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  $\text{NO}_3^-$  in an *MKK3*-dependent way. Other activators may be responsible  
154 for the high activity background observed when seeds were transferred on  $\text{KCl}$ .

### 155 ***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

160 of the samples. *MAP3K13* and *MAP3K14* were rapidly induced in seeds by  $\text{NO}_3^-$ , 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  $\text{KNO}_3$   
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  $\text{KNO}_3$ . 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  $\text{NO}_3^-$ -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  $\text{NO}_3^-$  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).

#### 197 ***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<sub>3</sub><sup>-</sup>-induced MPK7 activation either,  
204 suggesting that the NO<sub>3</sub><sup>-</sup> 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<sub>3</sub><sup>-</sup>-  
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<sub>3</sub><sup>-</sup> 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<sub>3</sub><sup>-</sup> 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).

#### 214 ***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<sub>3</sub><sup>-</sup>- 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 KNO<sub>3</sub>, combined or not with 10 μM  
220 paclobutrazol (PCZ), a potent blocker of GA biosynthesis. In these conditions, we did not

221 measure any variation in MPK7 activity (Figures 12A and S13A). We also confirmed that an  
222 active GA, GA<sub>3</sub>, 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 KNO<sub>3</sub>, with or without ABA, for 8 hours,  
225 and MPK7 activity was assayed. ABA did not affect the NO<sub>3</sub><sup>-</sup>-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<sub>3</sub><sup>-</sup>. 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<sub>3</sub><sup>-</sup>; 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<sub>3</sub><sup>-</sup>- nor MKK3-dependent, whereas *Ga3ox* seemed to be promoted by NO<sub>3</sub><sup>-</sup>  
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

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 (Dóczy 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 MAP3Ks. Of 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 Geninvestigator 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.

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

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 (Dóczy 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 (Bigéard 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  $\mu\text{E m}^{-2} \text{s}^{-1}$  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  $\mu\text{M KH}_2\text{PO}_4$ ; 250  $\mu\text{M MgSO}_4$ ; 750  
406  $\mu\text{M KNO}_3$ ; 125  $\mu\text{M Ca(NO}_3)_2$ ; 125  $\mu\text{M CaCl}_2$ ; 10  $\text{mG L}^{-1}$  Sequestrene138FE 100SG (Syngenta);  
407 0,4  $\mu\text{M (NH}_4)_6\text{Mo}_7\text{O}_{24}$ ; 243  $\mu\text{M H}_3\text{BO}_3$ ; 118  $\mu\text{M MnSO}_4$ ; 34,8  $\mu\text{M ZnSO}_4$ ; 10  $\mu\text{M CuSO}_4$ )  
408 containing 1  $\text{mM NO}_3^-$  (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  $\text{KNO}_3$ , KCl, 50  $\mu\text{M ABA}$  (Sigma Aldrich, ref: A1049, in ethanol), 50  $\mu\text{M GA}_3$   
421 (Sigma Aldrich, ref: G7645), or 10  $\mu\text{M Paclobutrazol}$  (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 [80–100 $\mu\text{E m}^{-2}\text{s}^{-1}$ ] 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  $\mu\text{g}$  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  $\alpha$ -MPK7 antibodies was also prepared for this study, raised against the  
448 LYYHPEAEISNA epitope. For kinase assays, immunoprecipitated kinases were resuspended in  
449 15  $\mu\text{L}$  of kinase buffer containing 0.1 mM ATP, 1  $\text{mg ml}^{-1}$  MBP, and 2  $\mu\text{Ci}$  ATP [ $\gamma$ -33P]. After 30  
450 min of reaction at room temperature, reaction was stopped with 15 $\mu\text{L}$  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:  $\alpha$ -HA (Roche

458 11867431001; 1: 10,000 dilution),  $\alpha$ -Myc (Sigma-Aldrich C3956; 1:10,000 dilution),  $\alpha$ -rat  
459 (Sigma-Aldrich A9037, 1:10,000),  $\alpha$ -rabbit (Sigma-Aldrich A6154, 1/20,000), and  $\alpha$ -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).

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481 manuscript.

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	GAGTTCGATAACCCACCG
MAP3K15	AT5G55090	F	ATCGTCGATTTGGTTGTGC
		R	CTTACCACGTGCTACCTCC
MAP3K16	AT4G26890	F	TTGAAAATCGCCGACATGGG
		R	TCACCACGAGCAACTTCTGG
MAP3K17	AT2G32510	F	TACTCGGAGAGGATCGGACG
		R	TGTTCTTCACACCTCGCTC
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	TTGTTTGCCGTAGTGAAGCT
		R	AGACTCGATATCCGCTGGTATAAAA
ABA2	AT1G52340	F	GGAGGAGCCACAGGGATAGG
		R	GCAGATCAACAATGCAGACTTTG
ABA3	AT1G16540	F	TCCTGAAGATTACAGTTGCTTATTCAC
		R	TGGGTCCACGGAAAAGTCTCT
AAO1	AT5G20960	F	GACGGGCTCGGCAACAG

		R	CATGAAAACCGCGGATACG
AAO2	AT3G43600	F	TGTCATGAAAAACGCGTACTCTCT
		R	CGCAGTGCACCGAAGCT
AAO3	AT2G27150	F	GAAGGTCTTGGAAACACGAAGAA
		R	GAAATACACATCCCTGGTGTACAAAAC
AAO4	AT1G04580	F	CAATGTTTTGGATCAGACGAGGTA
		R	CTCTATCTTTGCCAGGGTTGGTT
NCED2	AT4G18350	F	GCGGCTGAGCGTGCAATTA
		R	GGGAATAATTCCCGCAATCT
NCED3	AT3G14440	F	GCTGCGGTTTCTGGGAGAT
		R	GTCGGAGCTTTGAGAAGACGAT
NCED5	AT1G30100	F	CCTCCGTTAGTTTACCAACACT
		R	GGTGTGTCGGAGACGGAGTT
NCED6	AT3G24220	F	CGTTATTCTATGGAGCAGAATCG
		R	GGAGCGAAGTTACCTGATAATTGAA
NCED8	AT1G78390	F	GGAAAACGCCATGATCTCACA
		R	AGGATCCGCCGTTTTAGGAT
CYP707A1	AT4G19230	F	CTCACTCTTCGCCGGAAG
		R	GGAGGGAGTGGGAGTTTGGAA
CYP707A2	AT2G29090	F	CGTCTCTCACATCGAGCTCCTT
		R	GAGGGTGTTGATGGACTTTTGG
CYP707A3	AT5G45340	F	CATGCCTTTTGGTAGTGGGATTCAT
		R	CGGCCATACTGAATTCATCG
CYP707A4	AT3G19270	F	CCTGAAACCATCCGTAACATCAT
		R	TTGGCCCAAGATTGTAAGGAA
Ga20ox1	AT4G25420	F	GCCTGTAAGAAGCACGGTTTCT
		R	CTCGTGTATTCATGAGCGTCTGA
Ga20ox2	AT5G51810	F	CCCAAGGCTTTCGTTGTCAA
		R	CCGCTCTATGCAAACAGCTCT
Ga20ox3	AT5G07200	F	TCGTGGACAACAAATGGCA
		R	TGAAGGTGTCGCCTATGTTAC
Ga3ox1	AT1G15550	F	CTTGGGGTGCCTTCCAAATC
		R	AACCTTCGGACCACATTTGC
Ga3ox2	AT1G80340	F	GTTTCACCGTTATTGGCTCTCC
		R	TCACAGTATTTGAGGTGGTGGC
Ga2ox2	AT1G30040	F	CCTAAAACCTCCGCCGTTTT
		R	CCTTCATGTA CTCTCCACCGA

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486

487

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  $\pm$  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  $1\% < \alpha < 5\%$  and  $\alpha < 1\%$ ,  
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 NO<sub>3</sub>- concentrations. Germination  
499 ability was then assessed after 7 days in long day conditions. Values are mean  $\pm$  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  $1\% < \alpha < 5\%$  and  $\alpha < 1\%$ , 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  $\pm$  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 NO<sub>3</sub>- concentrations.  
515 Germination ability was then assessed after 7 days in long day conditions. Values are mean  $\pm$   
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  $\alpha < 5\%$  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 (O') and after  
523 transfer on either 5mM KCl (-) or KNO<sub>3</sub>. 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.

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  $\pm$  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 KNO<sub>3</sub>. 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 NO<sub>3</sub> concentration. Germination ability was assessed after 7  
550 days in long day conditions. Values are mean  $\pm$  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  $\alpha < 5\%$  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 KNO<sub>3</sub> (+). 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  $\pm$  SE of 3-4 biological replicates from seed batches produced  
567 independently. Values for each replicate are also shown.

568

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 KNO<sub>3</sub>. 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 KNO<sub>3</sub> 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 KNO<sub>3</sub> 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 KNO<sub>3</sub> 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

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

613

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

615 A and B. Seeds from indicated genetic background were imbibed at 30°C in the dark for 10  
616 days to induce secondary dormancy and transferred on medium containing indicated NO<sub>3</sub>  
617 concentration. Germination ability was assessed after 7 days in long day conditions. Values  
618 are mean ± SE of two biological replicates from seed batches produced independently.  
619 Values for each replicate are also shown. On the top, based on Mann-Whitney test, yellow  
620 sticks show differences with  $\alpha < 5\%$  whereas gray sticks show no differences.

621

622 **Figure S2 - MPK7 is expressed in dry seeds**

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

627

628 **Figure S3 - Quantification of MPK7 activity in dormant Col-0 and *mkk3-1* seeds transferred**  
629 **on nitrate.**

630 MPK7 activities of replicates were quantified and normalized to the one of Col-0 8h NO<sub>3</sub> (\*).  
631 Values are mean ± SE of two to six biological replicates from seed batches produced  
632 independently. Values for each replicate are also shown.

633

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

636 Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0  
637 and *mpk7-1* seeds, either dry (DS), after acquisition of secondary dormancy (O') and after  
638 transfer on either 5mM KCl or KNO<sub>3</sub>. Seeds were produced in the green house with non  
639 limiting nitrogen fertilizer, probably explaining why there is no NO<sub>3</sub> effect.

640

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

643 Kinase activity of MPK7 after immunoprecipitation with an anti-HA antibody from  
644 *MPK7locus-HA* and *mkk3-1 MPK7locus-HA* seeds, either dry (DS), after acquisition of  
645 secondary dormancy (O') and after transfer on either 5mM KCl or KNO<sub>3</sub>. MPK7-HA amount  
646 was monitored by immunoblot using an anti-HA antibody. Equal loading was controlled by  
647 Coomassie staining of the membrane. LEAp, Late Embryogenesis Abundant proteins.

648

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

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  $\pm$  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 NO<sub>3</sub> (\*).  
659 Values are mean  $\pm$  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 NO<sub>3</sub> (\*).  
665 Values are mean  $\pm$  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 NO<sub>3</sub> (\*).  
671 Values are mean  $\pm$  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 NO<sub>3</sub> (\*).  
676 Values are mean  $\pm$  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 NO<sub>3</sub> (\*).  
681 Values are mean  $\pm$  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  $\pm$  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 NO<sub>3</sub> (\*). 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.

722

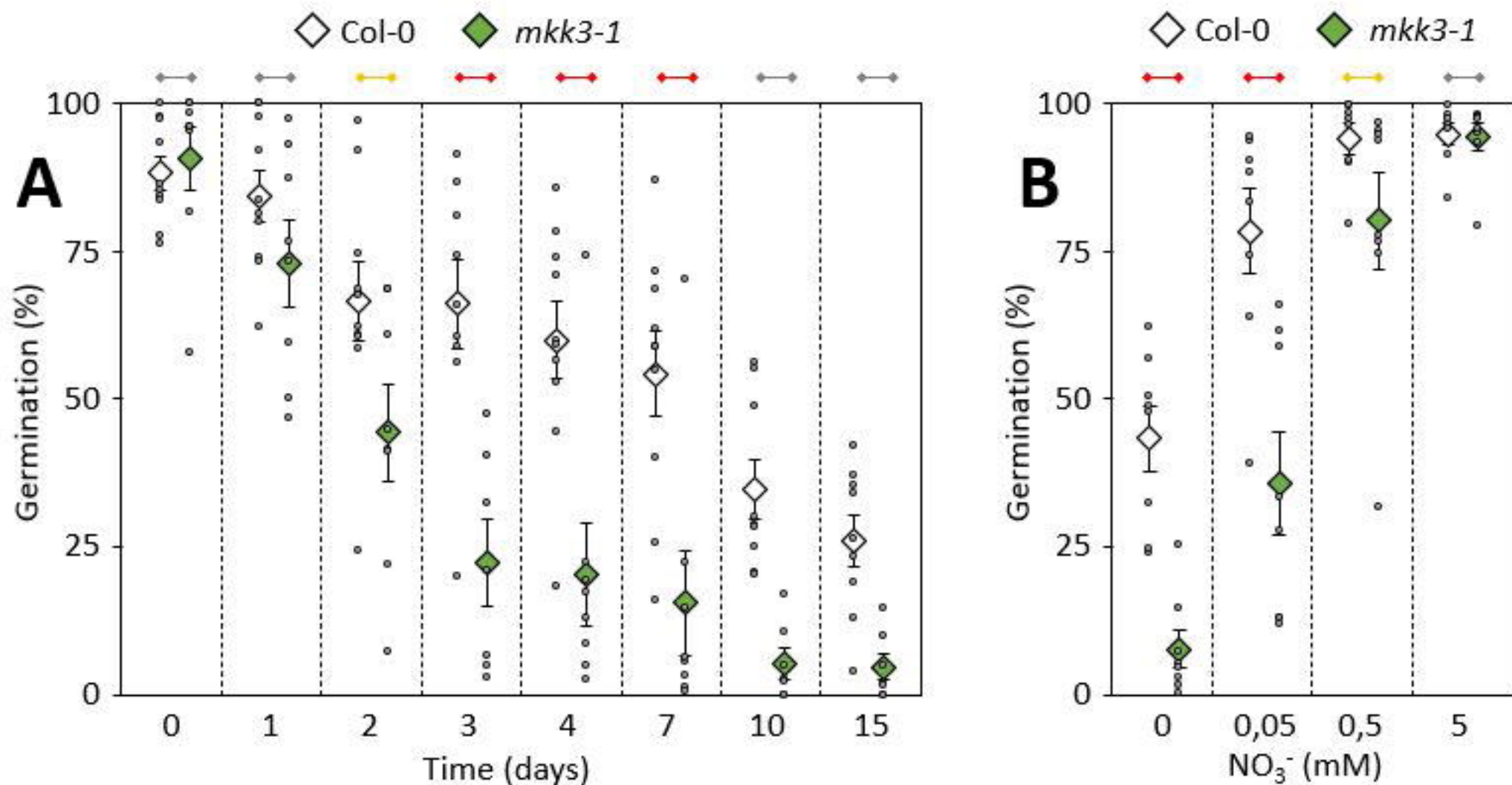
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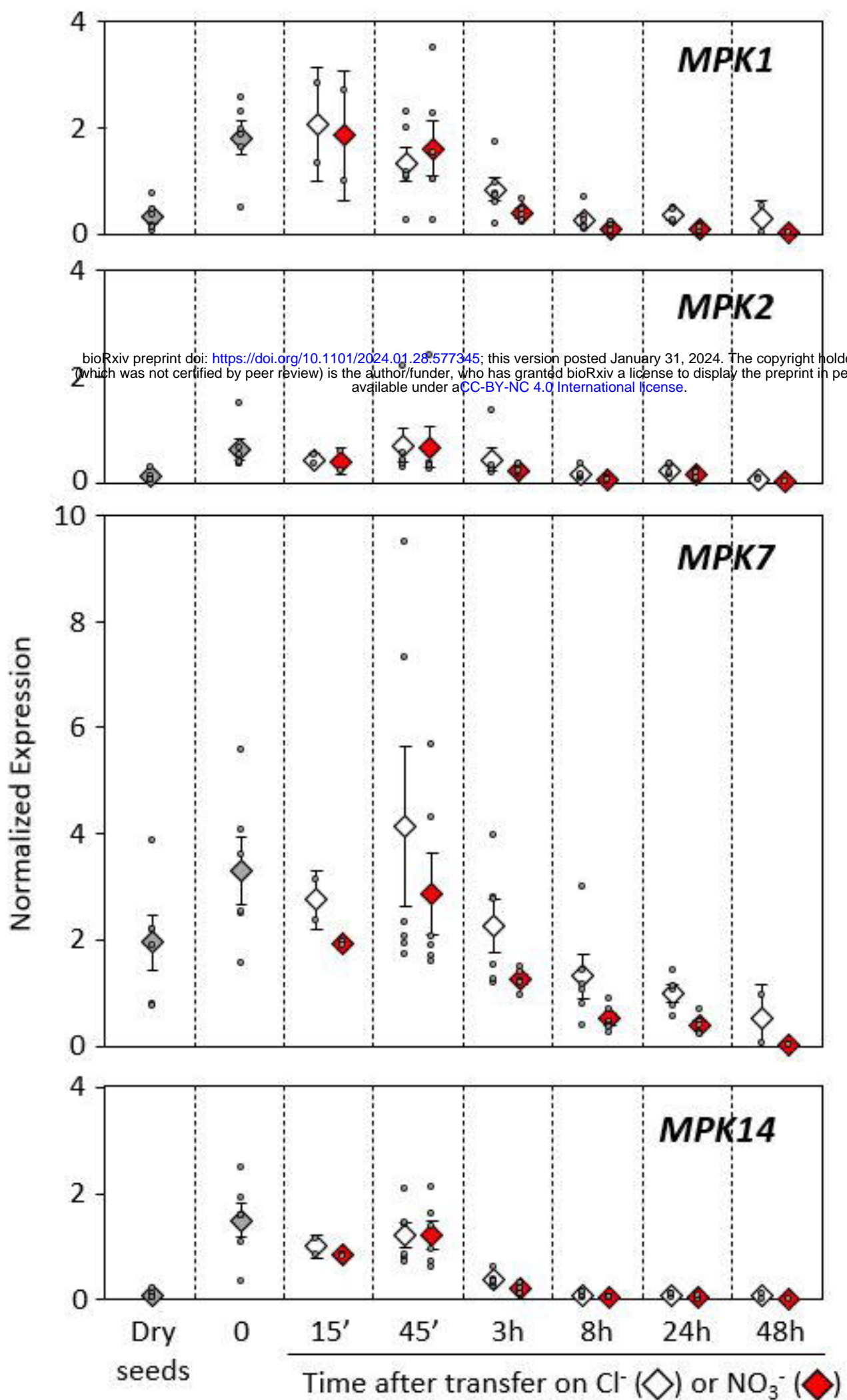
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- 847
- 848



**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  $1\% < \alpha < 5\%$  and  $\alpha < 1\%$ , respectively, whereas gray sticks show no differences.

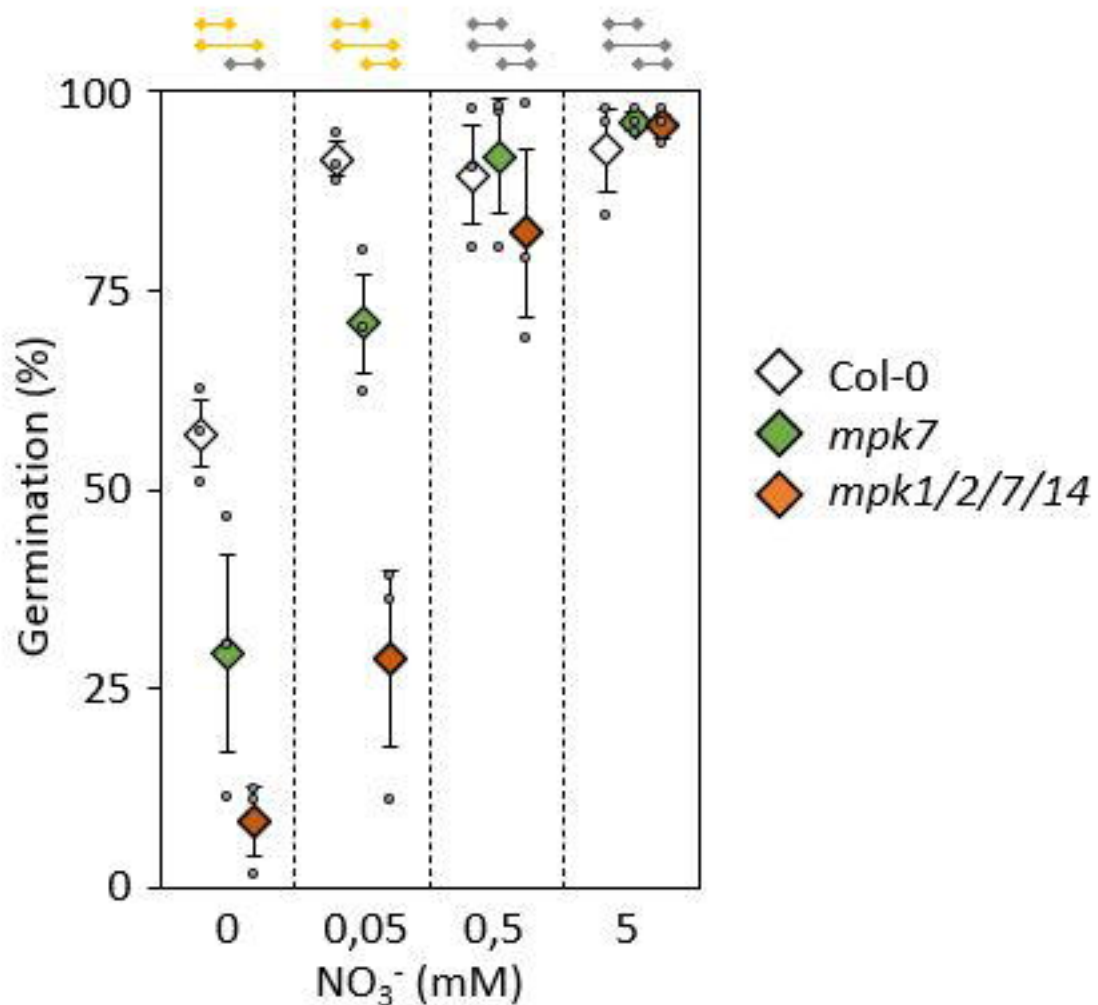
B. Col-0 and *mkk3-1* seeds were imbibed at 30°C in the dark for 10 days to induce secondary dormancy and transferred on media containing indicated NO<sub>3</sub><sup>-</sup> concentrations. Germination ability was then assessed after 7 days in long day conditions. Values are mean ± SE of height biological 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  $1\% < \alpha < 5\%$  and  $\alpha < 1\%$ , respectively, whereas gray sticks show no differences.



**Figure 2 - Clade-C MAPK genes are expressed in seeds and during secondary dormancy release**

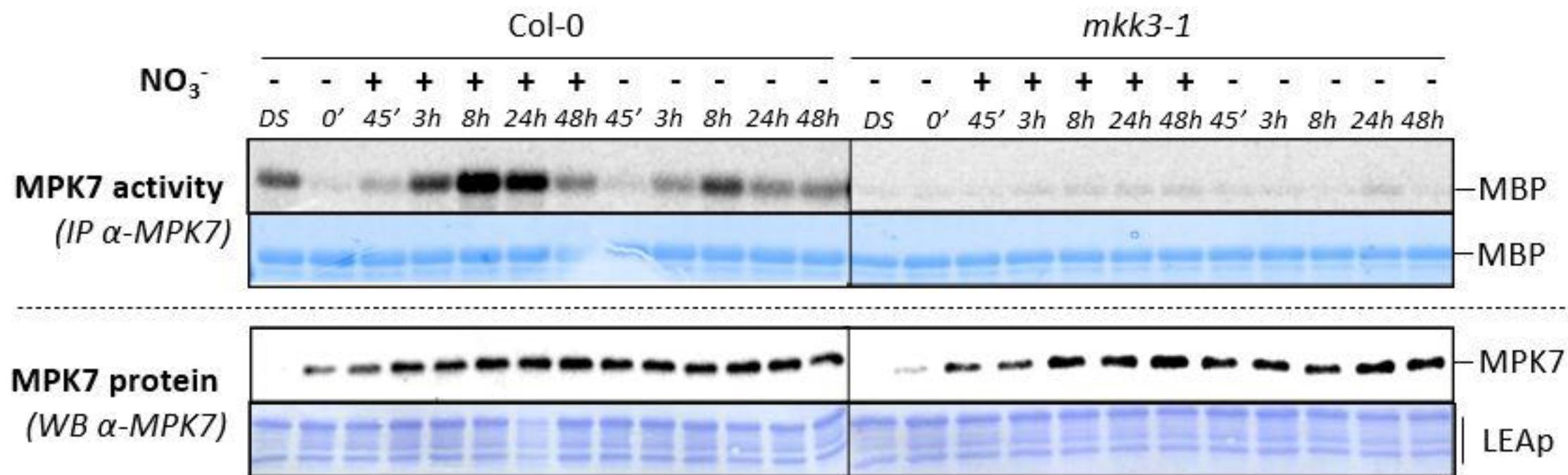
RT-qPCR analysis of clade-C MAP3K genes. Transcript levels are expressed relative to ACTIN2 as reference gene. Values are mean  $\pm$  SE of two to six biological replicates from seed batches produced independently. Values for each replicate are also shown.





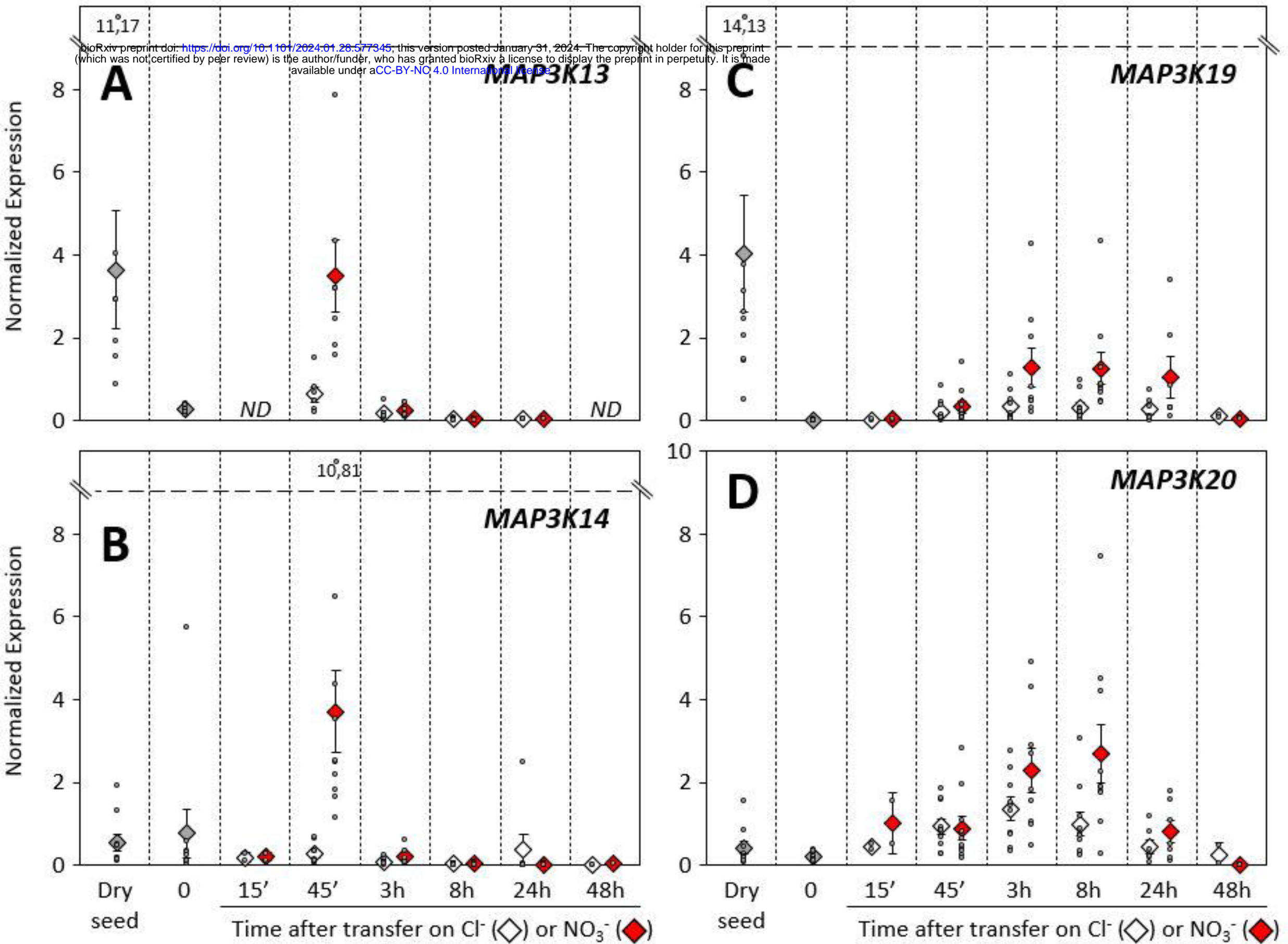
**Figure 3 - Seeds impaired in C-clade MAPKs have a faster secondary dormancy acquisition and a reduced nitrate-triggered dormancy release**

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  $\text{NO}_3^-$  concentrations. Germination ability was then assessed after 7 days in long day conditions. Values are mean  $\pm$  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  $\alpha < 5\%$  whereas gray sticks show no differences.



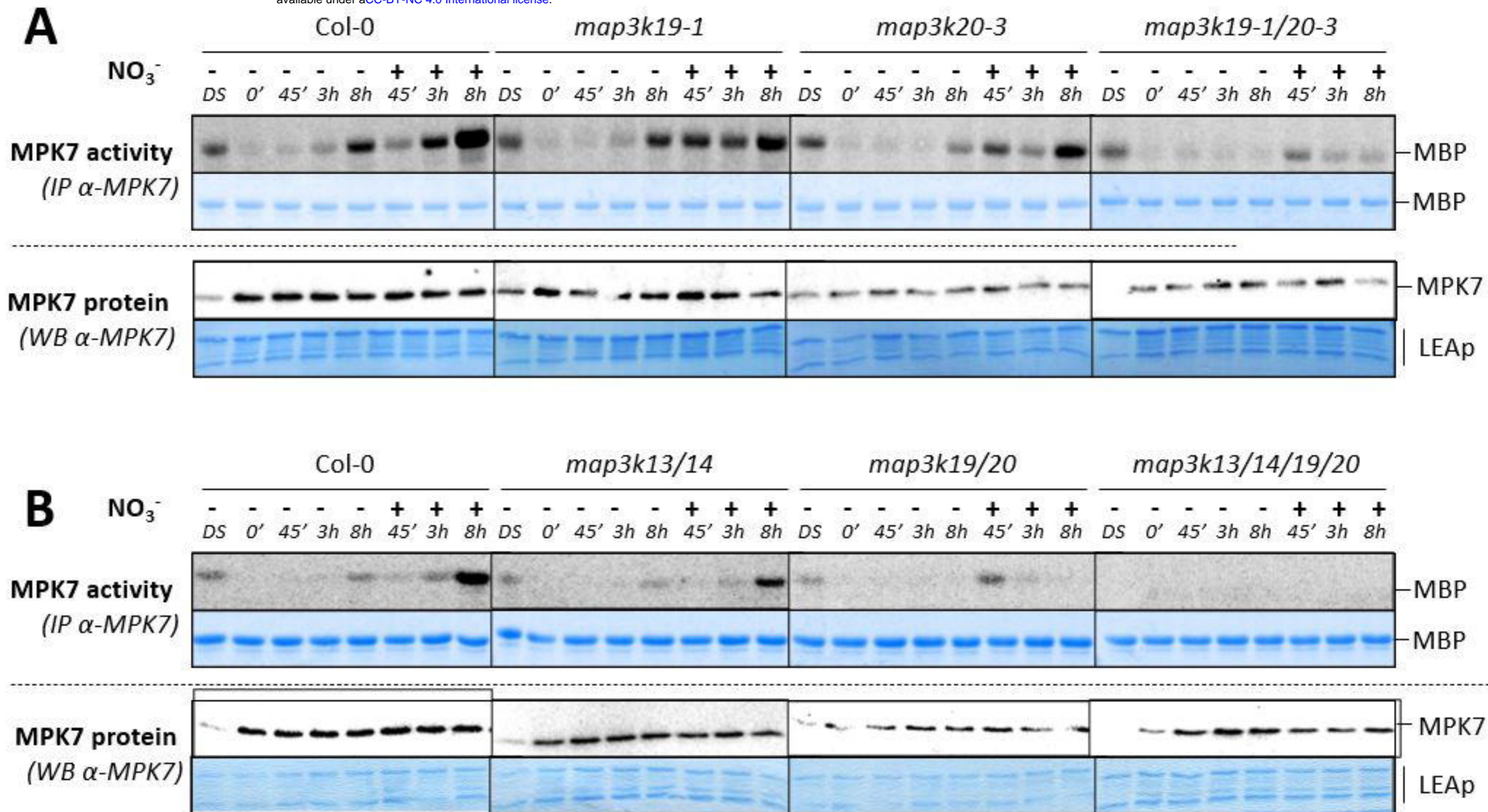
**Figure 4 - MPK7 activity in dormant seeds transferred on nitrate depends on MKK3.**

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 KNO<sub>3</sub>. 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.



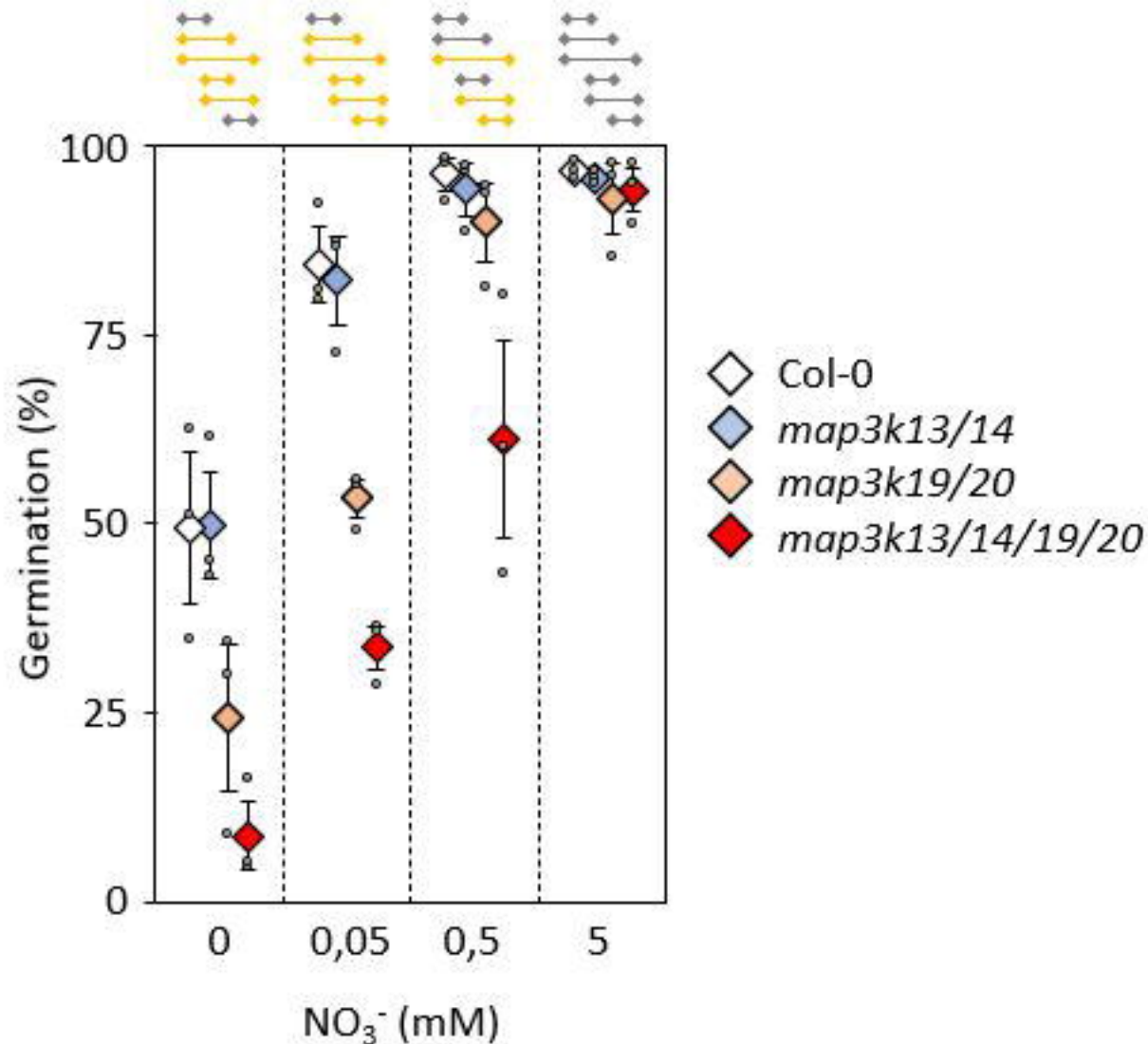
**Figure 5 - *MAP3K13*, *MAP3K14*, *MAP3K19* and *MAP3K20* are expressed in seeds and during secondary dormancy release**

RT-qPCR analysis of *MAP3K13*, *MAP3K14*, *MAP3K19* and *MAP3K20* genes expression. Transcript levels are expressed relative to ACTIN2 as reference gene. Values are mean  $\pm$  SE of two to 10 biological replicates from seed batches produced independently. Values for each replicate are also shown. ND not determined.

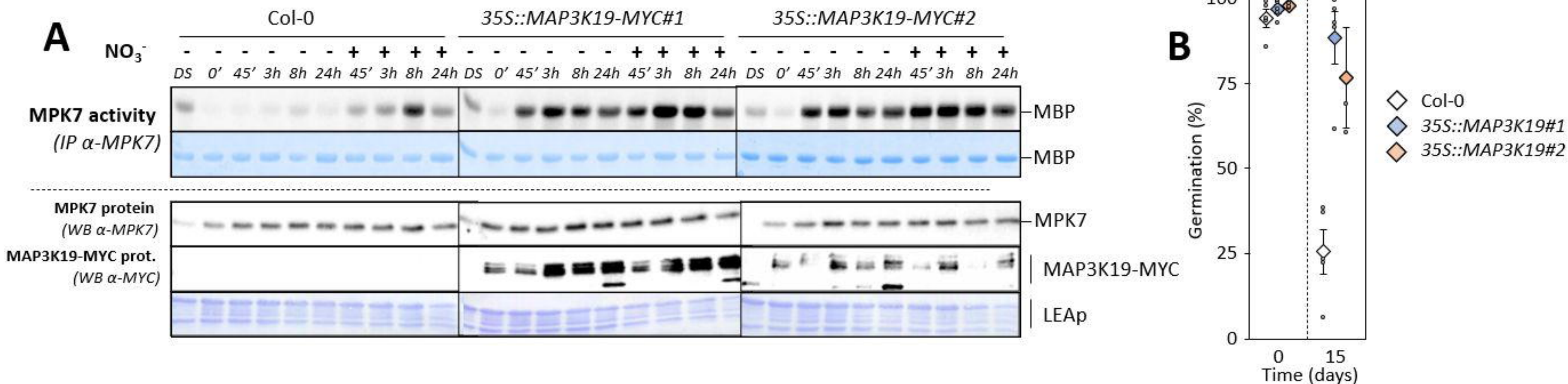


**Figure 6 - MPK7 activity in dormant seeds transferred on nitrate depends on MAP3K13/14/19/20**

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 KNO<sub>3</sub>. 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 and genotype for A and 3 times for B, the quantification of these replicates being gathered in figure S7A and B.



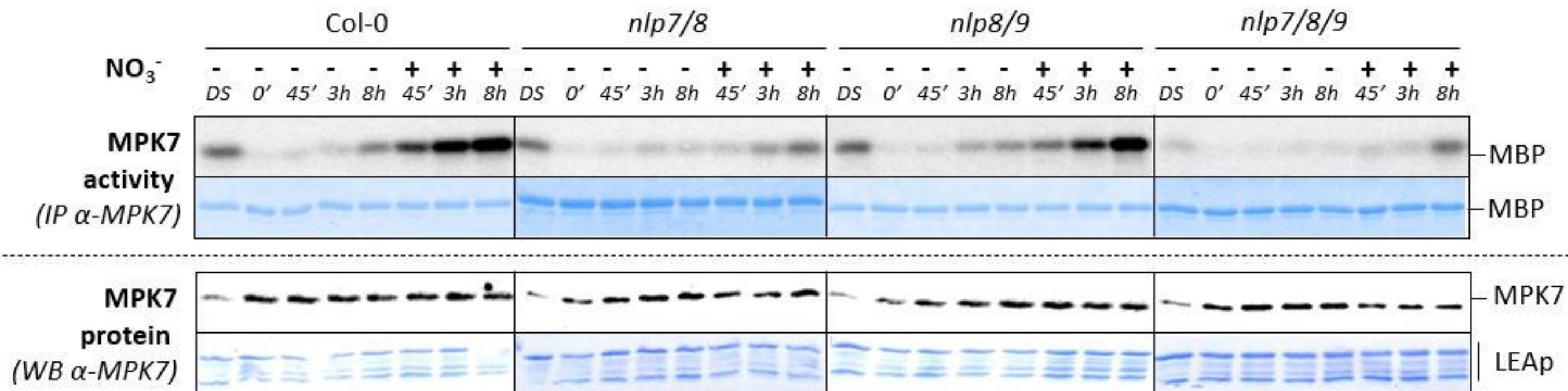
**Figure 7 - *map3k13/14/19/20* seeds have a reduced nitrate-triggered dormancy release**  
 Col-0, *map3k13CR/14CR*, *map3k19-1/20-3* and *map3k13CR/14CR/19-1/20-3* seeds were imbibed at 30°C in the dark for 10 days to induce secondary dormancy and transferred on medium containing indicated  $\text{NO}_3^-$  concentration. Germination ability was assessed after 7 days in long day conditions. Values are mean  $\pm$  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 KNO<sub>3</sub> (+). 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.

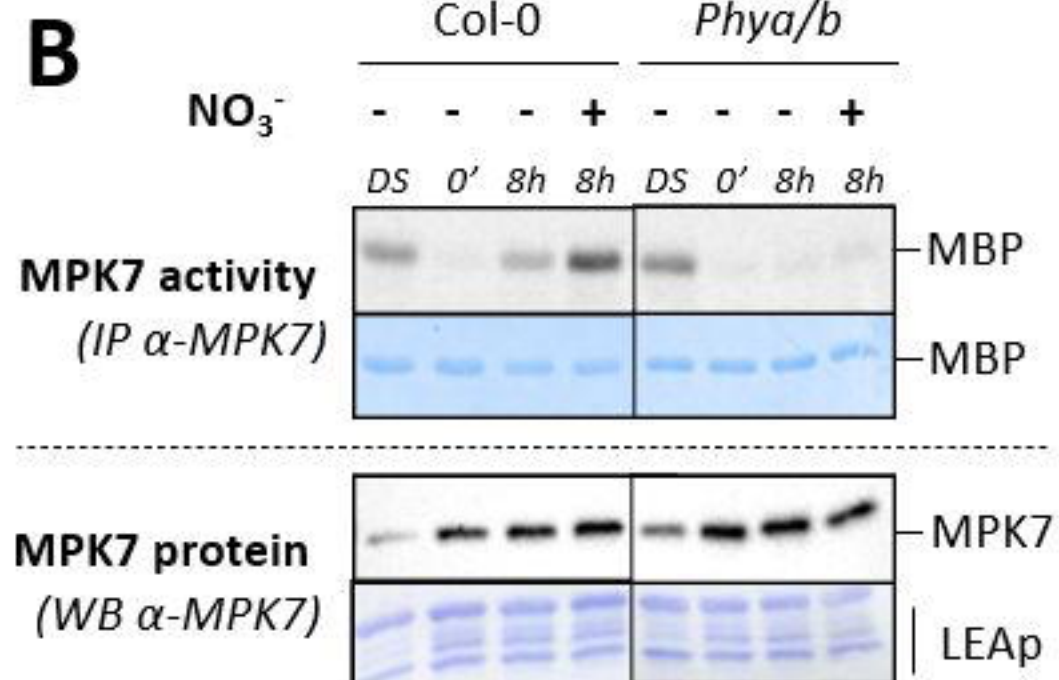
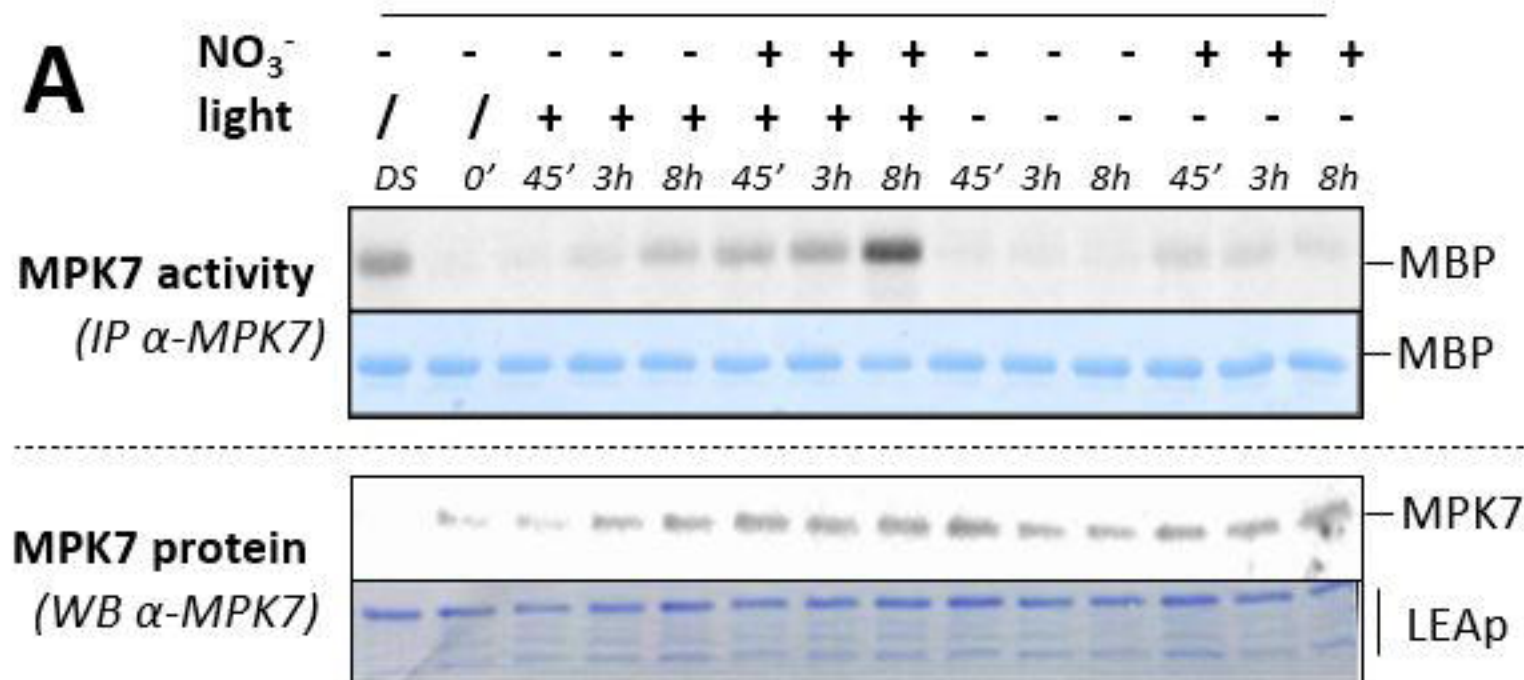
B. Seeds from indicated background were imbibed at 30°C in the dark for the indicated time to induce secondary dormancy. Germination ability was assessed after 7 days in long day conditions. Values are mean ± SE of 3-4 biological replicates from seed batches produced independently. Values for each replicate 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 KNO<sub>3</sub>. 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.

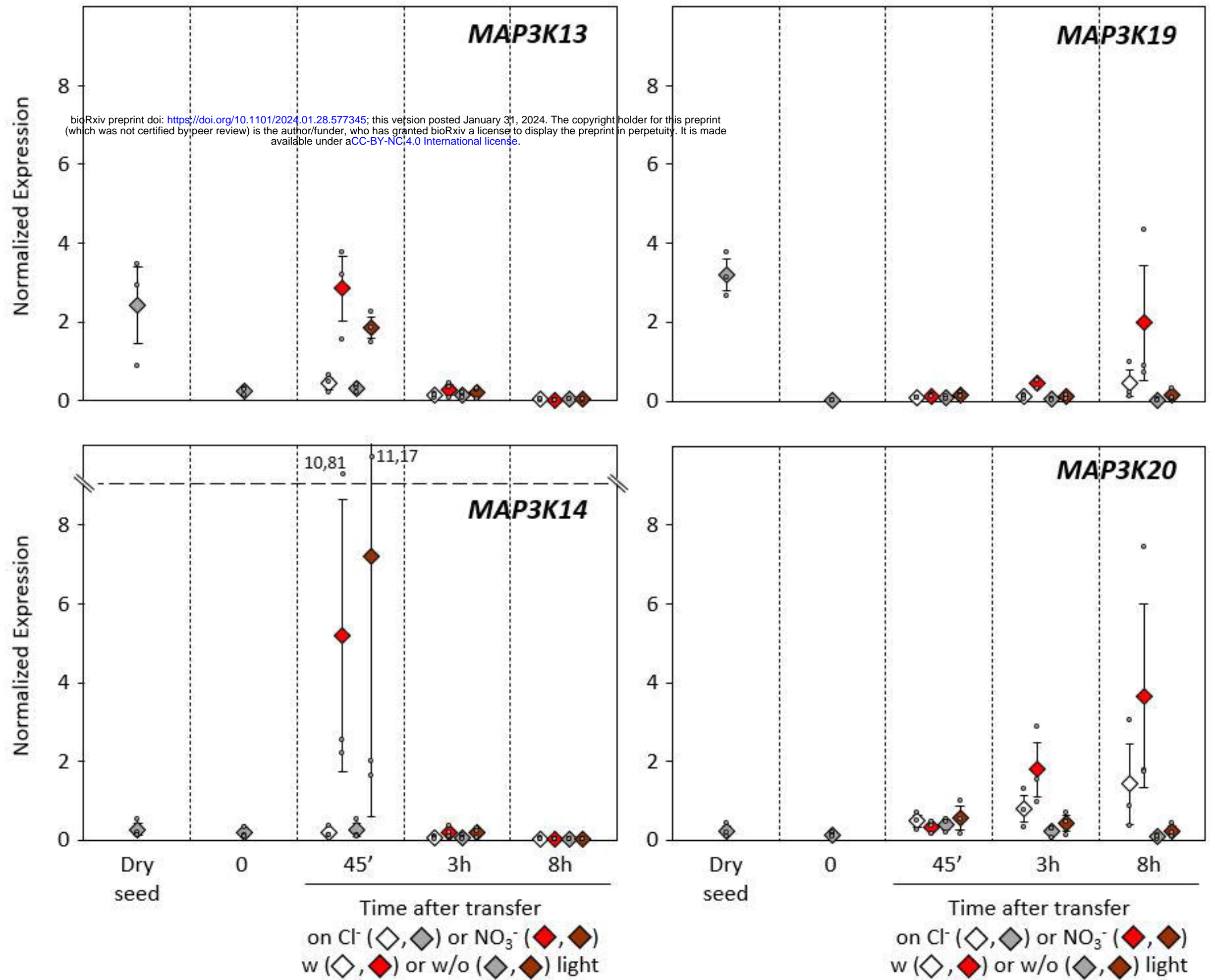
Col-0



### Figure 10 - MPK7 activity in dormant seeds is also triggered by light

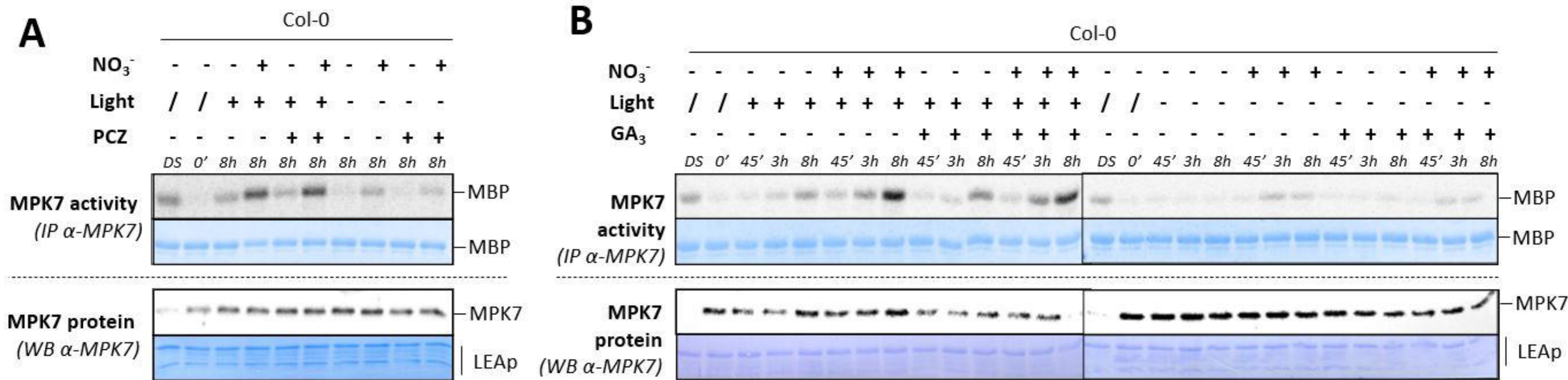
Kinase activity of MPK7 after immunoprecipitation with an anti-MPK7 antibody from Col-0 (A and B) or *phya/b* (B) seeds, either dry (DS), after acquisition of secondary dormancy (0') and after transfer on either 5mM KCl or KNO<sub>3</sub> with and without white light. MPK7 amount was monitored by immunoblot 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





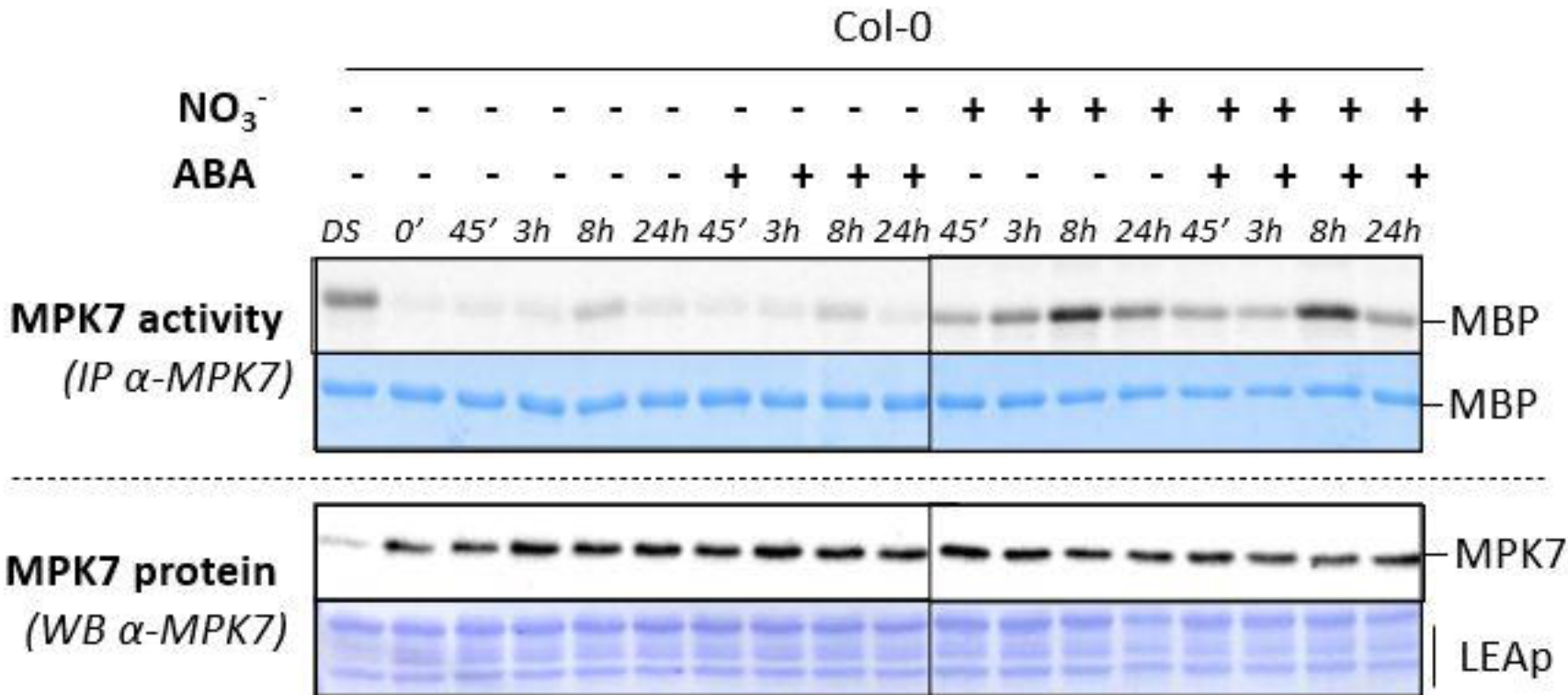
**Figure 11 - *MAP3K19* and *MAP3K20*, but not *MAP3K13* and *MAP3K14*, are differentially regulated by light**

RT-qPCR analysis of *MAP3K13*, *MAP3K14*, *MAP3K19* and *MAP3K20* genes expression. Transcript levels are expressed relative to ACTIN2 as reference gene. Values are mean  $\pm$  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 KNO<sub>3</sub> with and without white light with and without Paclobutrazol 10μM (A) or with and without GA<sub>3</sub> 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.

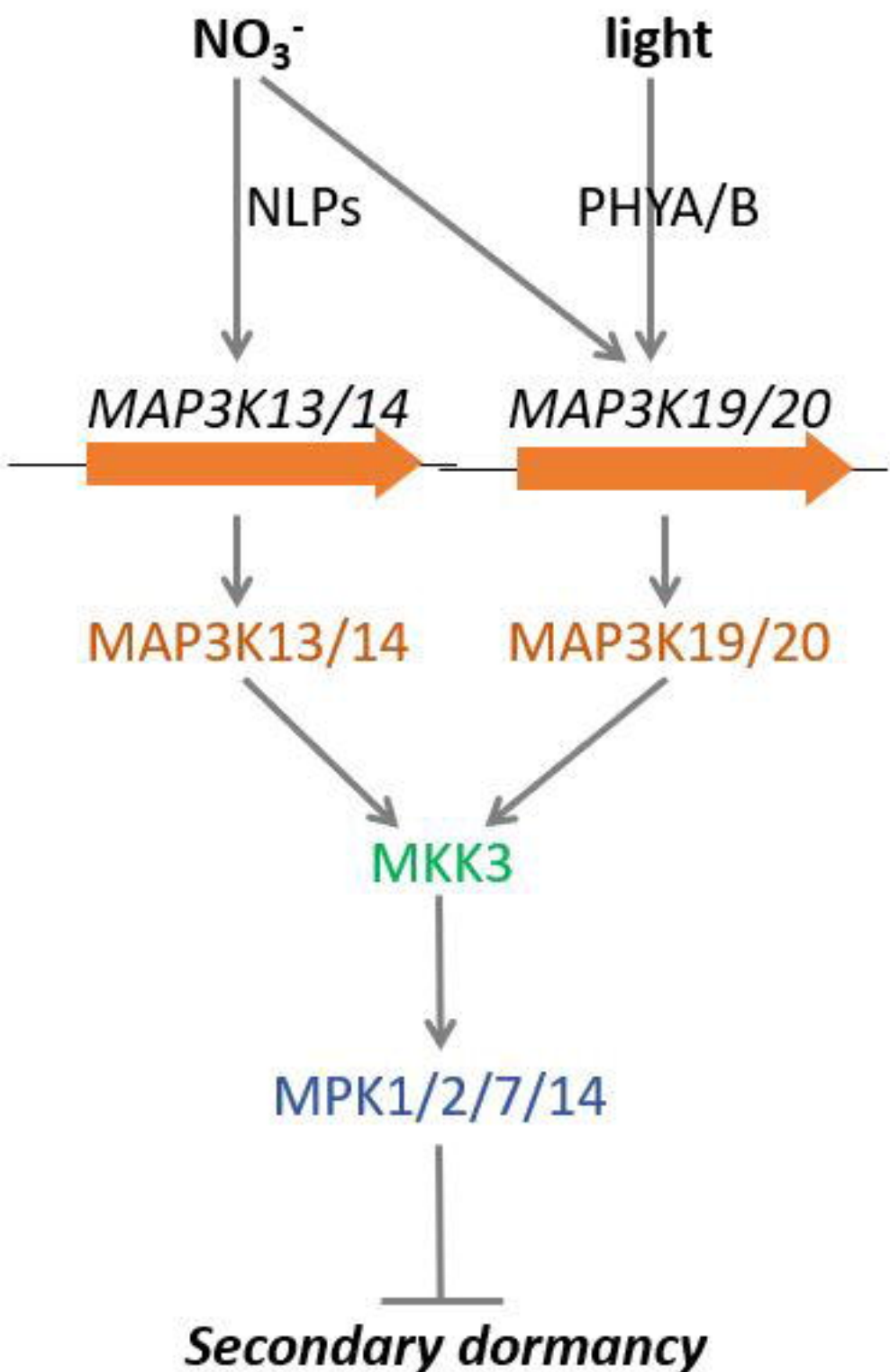


Figure 13 – Working model of MKK3 module-dependent regulation of secondary seed dormancy by nitrate and light