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The MPK8-TCP14 pathway promotes seed germination in Arabidopsis

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SUMMARY

The accurate control of dormancy release and germination is critical for successful plantlet establishment. Investigations in cereals hypothesized a crucial role for specific MAP kinase (MPK) pathways in promoting dormancy release, although the identity of the MPK involved and the downstream events remain unclear. In this work, we characterized mutants for *Arabidopsis thaliana* MAP kinase 8 (MPK8). *Mpk8* seeds presented a deeper dormancy than wild-type (WT) at harvest that was less efficiently alleviated by after-ripening and gibberellic acid treatment. We identified Teosinte Branched1/Cycloidea/Proliferating cell factor 14 (TCP14), a transcription factor regulating germination, as a partner of MPK8. *Mpk8 tcp14* double-mutant seeds presented a deeper dormancy at harvest than WT and *mpk8*, but similar to that of *tcp14* seeds. MPK8 interacted with TCP14 in the nucleus *in vivo* and phosphorylated TCP14 *in vitro*. Furthermore, MPK8 enhanced TCP14 transcriptional activity when co-expressed in tobacco leaves. Nevertheless, the stimulation of TCP14 transcriptional activity by MPK8 could occur independently of TCP14 phosphorylation. The comparison of WT, *mpk8* and *tcp14* transcriptomes evidenced that whereas no effect was observed in dry seeds, *mpk8* and *tcp14* mutants presented dramatic transcriptomic alterations after imbibition with a sustained expression of genes related to seed maturation. Moreover, both mutants exhibited repression of genes involved in cell wall remodeling and cell cycle G1/S transition. As a whole, this study unraveled a role for MPK8 in promoting seed germination, and suggested that its interaction with TCP14 was critical for regulating key processes required for germination completion.

Keywords: MAP kinase 8, seed, dormancy, germination, Teosinte Branched1/Cycloidea/Proliferating cell factor 14, *Arabidopsis thaliana*, gibberellins.

INTRODUCTION

Seed germination is a major step of plant development and life cycle. It is critical for species competition and spreading capacity in ecosystems, and eventually impacts crop growth and yield in agrosystems. To prevent inappropriate germination, seeds from temperate species are generally dormant at maturity. Dormancy is a physiological mechanism that blocks seed germination under favorable conditions, and dormancy release is therefore required for germination (Penfield, 2017). In this respect, dormancy can be considered as a major process for seed plant evolution and for plant adaptation to global change. A range of

environmental factors, for example temperature or light, and endogenous hormonal signals regulate these processes. Consequently, germination completion, i.e. the early emergence of radicle from seed envelope, can be achieved only when promoting mechanisms exceed inhibiting processes (Graeber *et al.*, 2012). In that sense, the balance between the two antagonistic hormones abscisic acid (ABA) and gibberellins (GA), which inhibit and stimulate seed germination, respectively, promotes either dormancy (high ABA and low GA contents) or germination (low ABA and high GA contents; Finkelstein *et al.*, 2008).

Protein phosphorylation is a key post-translational modification regulating diverse aspects of seed germination, including hormone responses. In particular, it is at the heart of ABA signaling (Cutler *et al.*, 2010). Several SNF1-related protein kinases subfamily 2 (SnRK2), i.e. SnRK2.2, SnRK2.3 and SnRK2.6, are activated by ABA and participate in ABA signaling in seeds (Fujii and Zhu, 2009; Nakashima *et al.*, 2009). SnRK2 activation leads to the phosphorylation of transcription factors such as ABA-INSENSITIVE 5 (ABI5) and the activation of ABA-responsive genes (Nakashima *et al.*, 2009). Conversely, ABA signaling is repressed by protein phosphatases of the PP2C family, including ABA-INSENSITIVE 1 (ABI1) and ABI2 that dephosphorylate and inhibit SnRK2s (Umezawa *et al.*, 2009). Upon ABA perception, PYR/PYL/RCAR ABA receptors bind to and inhibit PP2Cs, thereby releasing active SnRK2s (Fujii *et al.*, 2009). Although less investigated, several lines of evidence indicate that protein phosphorylation might also be involved in GA signaling in seeds. In contrast to ABA signaling, GA synthesis and signaling are inactivated in dormant seeds (Shu *et al.*, 2016). GA signaling is repressed by transcriptional repressors of the DELLA family, such as RGA-LIKE2 (RGL2; Davière and Achard, 2016). In the presence of GA, DELLA proteins get degraded, releasing DELLA-dependent repression of gene expression and allowing germination (Davière and Achard, 2016). Although the relevance of DELLA regulation by phosphorylation has not been established in seeds yet, it has been reported that RGL2 is stabilized when phosphorylated, and that the phosphorylation of the rice DELLA SLR1 negatively regulates GA signaling (Hussain *et al.*, 2005; Dai and Xue, 2010).

A body of evidence supports the involvement of mitogen-activated protein kinase (MAPK/MPK) pathways in regulating seed dormancy and dormancy release. MPK pathways are versatile signaling modules operating in all eukaryotic species (Colcombet and Hirt, 2008). The core of the module is composed of three kinases, i.e. an upstream MPK kinase (MAP3K) that phosphorylates and activates an intermediate MPK kinase (MKK) that itself regulates the downstream MPK. MPKs are implicated in plant response to abiotic and biotic environmental cues, and in the control of plant development, from cell division and differentiation to organ formation and senescence (Colcombet and Hirt, 2008; Xu and Zhang, 2015). They also play a critical role during plant reproduction before, during and after fertilization, by regulating ovule and pollen formation, pollen tube guidance, and embryo and seed development (Xu and Zhang, 2015). For instance, diverse alterations of embryo development leading to abnormal root architecture and seed size have been reported in *atmpk6* mutants (López-Bucio *et al.*, 2014). In rice, seed size is also controlled by the module OsMKKK10-OsMKK4-OsMAPK6, that includes the MPK6 ortholog OsMAPK6 (Xu *et al.*, 2018). As reported by Xing *et al.* (2009), the role of MPK6 is not restricted to seed development, but also includes the regulation of seed germination. Indeed, *mpk6* seeds exhibit low dormancy at harvest and are hyposensitive to ABA treatment. These phenotypes are also observed in *mkk1* and *mkk1mpk6* mutants, suggesting that MKK1 acts upstream of MPK6 in seeds. In addition, the different mutant seed lines presented a reduced ABA content, suggesting that the MKK1/MPK6 module promotes ABA synthesis and ABA signaling leading to dormancy (Xing *et al.*, 2009). MYB44 was proposed as a downstream effector of this module as it could be phosphorylated by MPK6 and MPK3 *in vitro* and as its phosphorylation participated in ABA-mediated inhibition of seed germination (Nguyen *et al.*, 2012). In addition, two MAP3K, Raf10 and Raf11, have been identified as positive regulators of dormancy (Lee *et al.*, 2015). As for the MKK1-MPK6 module, *raf10*, and to a lesser extent *raf11*, mutant seeds are hyposensitive to ABA. Moreover, the overexpression of Raf10 and Raf11 led to the induction of ABA- and dormancy-associated genes such as *ABI3* and *ABI5* that were repressed in *raf10raf11* double-mutant (Lee *et al.*, 2015). Whether Raf10/11 are implicated in the regulation of MKK1-MPK6 module in seeds is currently unknown. Nevertheless, MPK pathways appear important for ABA signal transduction in seeds, and participate in seed dormancy establishment and/or maintenance. In contrast, distinct MPK pathways may promote dormancy exit and germination. Indeed, natural variation of dormancy level in wheat and barley has been attributed to allelic variations in *MKK3* gene leading to impaired MPK kinase activity (Nakamura *et al.*, 2016; Torada *et al.*, 2016). Strikingly, seeds displaying *MKK3* mutations exhibit deeper dormancy, indicating that MKK3 promotes dormancy release and germination (Nakamura *et al.*, 2016; Torada *et al.*, 2016). Nevertheless, the identity of the MPKs that could be involved and the mechanisms by which MPK pathways could regulate dormancy release are currently unknown.

In the present study, we identified Arabidopsis MPK8 as a positive regulator of dormancy release. Two independent *mpk8* mutants presented a deeper seed dormancy at harvest and a delayed dormancy release during after-ripening. Interestingly, *mpk8* seeds had a wild-type (WT) sensitivity to ABA treatment but were impaired in GA response. We

investigated the link between MPK8 and TCP14, a basic-Helix-Loop-Helix (bHLH) transcription factor operating in GA response during seed germination (Tatematsu *et al.*, 2008; Resentini *et al.*, 2015). The phenotype of *mpk8 tcp14* seeds suggested that MPK8 functioned upstream of TCP14 in a common pathway. Moreover, MPK8 interacted with TCP14 *in vivo*, phosphorylated TCP14 *in vitro* and enhanced TCP14 transcriptional activity. The analysis of dry and imbibed seed transcriptomes by RNA sequencing (RNA-seq) highlighted the extensive and common upregulation of genes associated with dormancy in *tcp14* and *mpk8* imbibed seeds, conversely with a massive repression of germination-related genes. Altogether, our data suggest a role for the MPK8-TCP14 module in the regulation of dormancy-to-germination transition.

RESULTS

MPK8 is expressed in seeds and promotes dormancy release

MPK8 transcript abundance was analyzed during imbibition in freshly harvested (dormant) and after-ripened (non-dormant) seeds by quantitative reverse transcriptase-polymerase chain reaction (RT-qPCR; Figure 1). In non-dormant seeds, transcript abundance slightly increased 6 h after imbibition, and progressively decreased back to dry seed level after 24 h. Transcript abundance decreased after 6 h of imbibition in dormant seeds and progressively increased to dry seed level after 24 h. Although the profiles were slightly different in dormant and non-dormant seeds, *MPK8* transcripts were detected in dry seeds and all over the duration of imbibition.

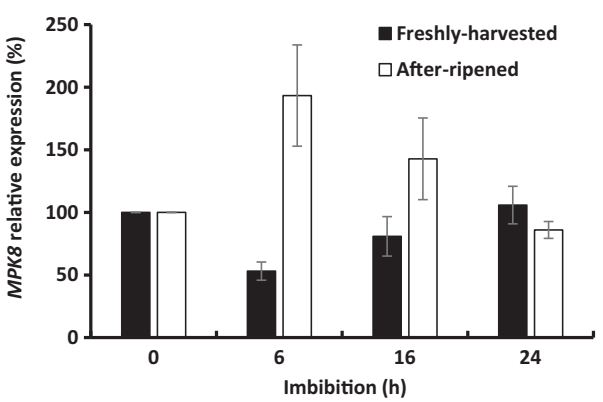

Figure 1. *MPK8* transcript levels in dry and imbibed seeds.
Transcript levels were compared by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) in freshly harvested (black bars) and 5-week after-ripened seeds (white bars) in dry state (0 h) and at different times of imbibition at 25°C in darkness. Normalization was realized with three housekeeping genes (At4g34270, At4g26410, At5g53560). Results from one biological experiment are expressed relative to transcript levels in dry seeds and are representative of three biological repeats. No significant difference was observed for the different imbibition durations compared with dry seeds.

To investigate the role of MPK8 kinase in seed germination and dormancy, the germination capacity of Arabidopsis *mpk8* mutant seeds was analyzed. We compared two independent mutant lines designated *mpk8.1* and *mpk8.2* previously described (Takahashi *et al.*, 2011). Freshly harvested mutant seeds germinated at the same rate as WT at 15°C, which alleviates dormancy, ruling out defects of seed viability (Figure S1). In contrast, when assayed at 25°C, the germination of *mpk8.1* and *mpk8.2* seeds was significantly lower than the WT all over the duration of imbibition, indicating that both mutants exhibited a higher dormancy level at harvest (Figure 2a). To alleviate dormancy, seeds were submitted to after-ripening and dormancy release was examined over the storage duration. As shown in Figure 2(b), dormancy was released faster and completed within 3 weeks in WT, whereas a proportion of *mpk8.1* and *mpk8.2* seeds remained dormant even after 4 weeks of after-ripening. Taken together, these results indicated that MPK8 was necessary for dormancy release.

MPK8 mutation affects seed sensitivity to GA but not to ABA

Abscisic acid is a major regulator of dormancy and germination. We therefore compared the sensitivity of WT and *mpk8* mutants to ABA. As shown in Figure 3, WT, *mpk8.1* and *mpk8.2* stratified seeds efficiently germinated at 25°C in the absence of treatment. ABA treatment inhibited seed germination in a similar dose-dependent manner in WT and *mpk8* mutants. MPK8 was therefore not required for the inhibition of seed germination by ABA.

In parallel, the response of WT, *mpk8.1* and *mpk8.2* seeds to GA was compared (Figure 4a). The germination of freshly harvested WT seeds was stimulated by GA, with 92% seeds germinating upon 1 mM GA treatment (Figure 4a). Although a stimulation of germination by GA was also observed for both *mpk8* mutants, it was significantly lower at any concentration tested and did not exceed 75% (Figure 4a). Conversely, *mpk8.1* and *mpk8.2* seeds showed a higher sensitivity to paclobutrazol, an inhibitor of GA synthesis (Figure 4b). As a whole, these results indicated that the two *mpk8* mutants were affected in GA response, which likely accounted for their deeper dormancy.

MPK8 interacts with and phosphorylates the bHLH transcription factor TCP14

TCP14 is a transcription factor involved in GA response during germination (Tatematsu *et al.*, 2008; Resentini *et al.*, 2015). Interestingly, the phenotype of *mpk8* seeds shared common features with that of *tcp14* mutants, including an exacerbated dormancy at harvest (Figures 2 and S2b; Tatematsu *et al.*, 2008). Moreover, TCP14 has been identified as a putative MPK8 substrate in a large-scale screen using protein arrays (Popescu *et al.*, 2009). We therefore investigated if TCP14 could constitute a target for MPK8.

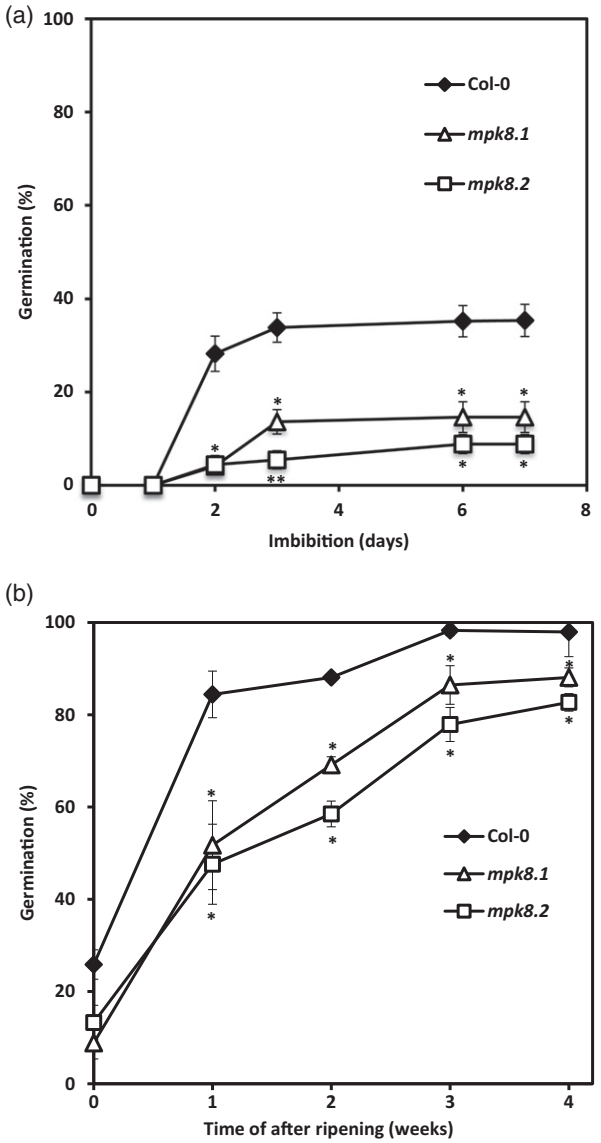

Figure 2. Germination of wild-type (WT) and *mpk8* mutant seeds.
(a) WT (Col-0; close diamonds), *mpk8.1* (open triangles) and *mpk8.2* (open squares) freshly harvested were imbibed at 25°C in darkness. Results represent germination means ± SE of six replicates.
(b) Germination rate of freshly harvested or after-ripened WT (Col-0; close diamonds), *mpk8.1* (open triangles) and *mpk8.2* (open squares) seeds. Seeds were assayed for germination at 25°C in darkness at harvest (0) and after 1–4 weeks of after-ripening treatment. For each condition, the percentage of germination is scored after 7 days of imbibition. Results represent means ± SE of three replicates. Asterisks indicate statistical differences between WT and mutant seeds at each time point, as determined by Wilcoxon–Mann–Whitney test (*$P < 0.05$; **$P < 0.01$).

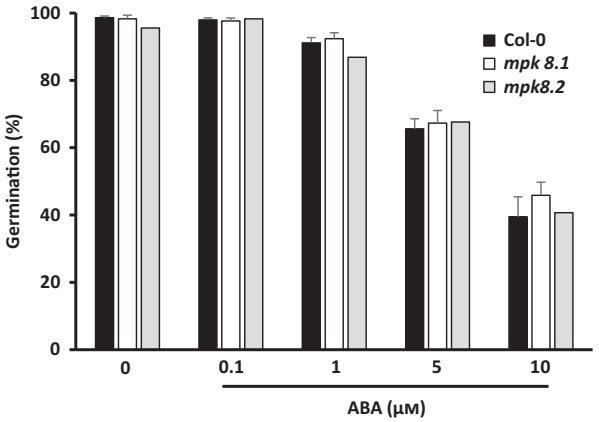

Figure 3. Abscisic acid (ABA) sensitivity of wild-type (WT) and *mpk8* mutant seeds.
Seeds were stratified 3 days at 4°C in the presence of different concentrations of ABA, and the germination rate of WT (Col-0; black bars), *mpk8.1* (white bars) and *mpk8.2* (gray bars) seeds was scored 7 days after seeds were switched to 25°C in darkness. Results represent means ± SE of six replicates. No statistical difference between WT and mutant seeds was observed using Wilcoxon–Mann–Whitney test.

We first studied if MPK8 and TCP14 could function in a common pathway. Thus, we generated homozygous *mpk8.1 tcp14.4* double-mutant seeds by crossing *mpk8.1* and *tcp14.4* single-mutants (Figure S2a). The germination capacity of freshly harvested *mpk8 tcp14* seeds was compared with that of WT and *tcp14* and *mpk8* seeds. All seed batches analyzed fully germinated at 15°C, but presented a deep dormancy when assayed at 25°C that did not bring information on the relative dormancy of WT and mutant seeds (Figure S2b). We therefore assayed germination at an intermediate temperature (Figure S2b). As shown in Figure 5, when assayed at 20°C, *mpk8 tcp14* seeds had a lower germination rate than WT seeds, indicating a deeper dormancy similar to that of *tcp14* seeds. As *tcp14* seeds were also more dormant than *mpk8* seeds, the phenotype of *mpk8 tcp14* seeds supported the hypothesis that MPK8 functioned upstream of TCP14 in a common pathway.

To determine if MPK8 shared common subcellular localizations with TCP14, GFP-tagged MPK8 was transiently expressed in tobacco leaves (Figure 6). As shown in Figure 6(a), GFP fluorescence was detected in both cytosol and nucleus, which was confirmed by the localization of Fib2-mRFP, a nucleolar marker (Figure 6b,c). MPK8 location thereby overlapped that of TCP14, which is exclusively nuclear (Kieffer *et al.*, 2011; Kim *et al.*, 2014).

The possible interaction between MPK8 and TCP14 *in planta* was investigated by bimolecular fluorescence complementation (BiFC) experiments in tobacco leaves (Figure 7). YFP fluorescence was detected in tobacco epidermal cells when MPK8-YFP^C and TCP14-YFP^N were co-expressed (Figure 7a; panel a). YFP fluorescence was specifically localized in the nucleus, the localization being confirmed by the detection of the nucleolar marker Fib2-mRFP (Figure 7a; panels b,c). Furthermore, no fluorescence was observed when MPK8-YFP^C was co-expressed with YFP^N alone or when TCP14-YFP^N was co-expressed with YFP^C alone (Figure S3). To further confirm MPK8-TCP14 interaction, co-immunoprecipitation experiments were carried out.

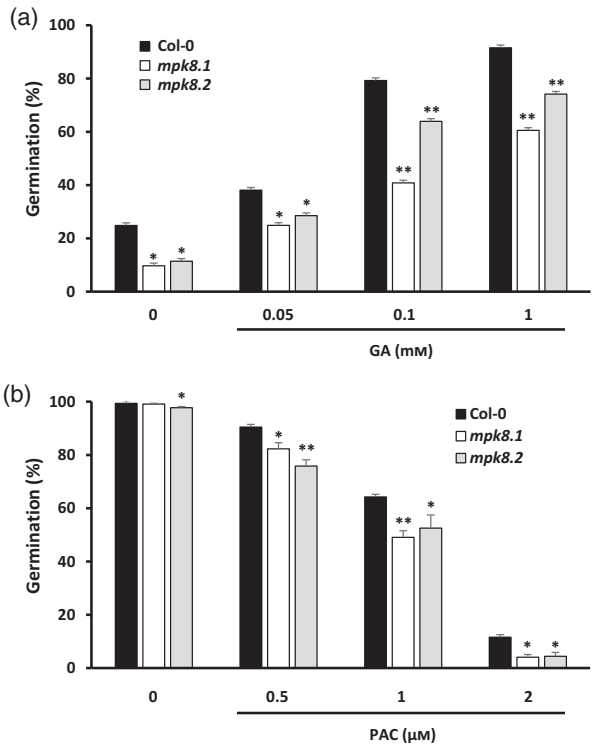

Figure 4. Gibberellin (GA) sensitivity of wild-type (WT) and *mpk8* mutant seeds.
(a) Freshly harvested WT (Col-0; black bars), *mpk8.1* (white bars) and *mpk8.2* (gray bars) seeds were germinated at 25°C in darkness in the presence of different concentrations of GA. Germination rate was scored after 3 days and results represent means ± SE of six replicates.
(b) Seeds were stratified 3 days at 4°C in the presence of different concentrations of paclobutrazol (PAC) and the germination rate of WT (Col-0; black bars), *mpk8.1* (white bars) and *mpk8.2* (gray bars) seeds was scored 7 days after seeds were transferred to 25°C in darkness. Results represent means ± SE of six replicates. Asterisks indicate statistical differences between WT and mutant seeds at each concentration, as determined by Wilcoxon–Mann–Whitney test (*$P < 0.05$; **$P < 0.01$).

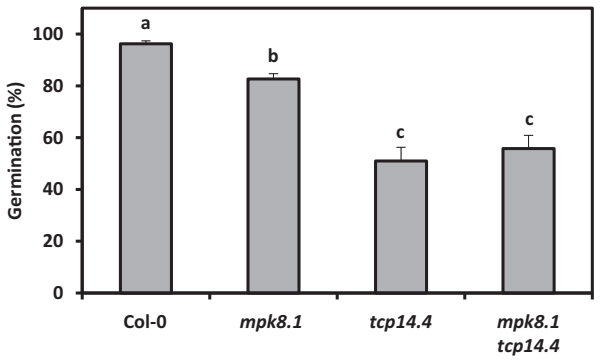

Figure 5. Germination rate of freshly harvested *mpk8 tcp14* double-mutant seeds after 7 days of imbibition at 20°C in darkness.
Results represent germination means ± SE of six replicates. Different letters indicate significantly different means (Tukey's HSD, $P < 0.05$).

HA-tagged MPK8 and Myc-tagged TCP14 were co-expressed in tobacco leaves, and TCP14-c-myc protein was immunoprecipitated with anti-Myc antibodies. As shown in Figure 7(b), MPK8-HA was subsequently detected by Western blot, indicating that MPK8 was co-immunoprecipitated with TCP14. To address the specificity of the association between TCP14 and MPK8, the interaction of MPK8 with TCP15, another TCP factor structurally related to TCP14 and regulating seed germination together with TCP14 (Resentini *et al.*, 2015), was analyzed. In these experiments, no YFP fluorescence was detected after co-expression of MPK8-YFP^C and TCP15-YFP^N in tobacco leaves (Figures 7a; panels d–f; and S3), although MPK8 and TCP15 proteins were indeed expressed in leaves (Figure S4).

The ability of MPK8 to phosphorylate TCP14 was subsequently analyzed. MPK8-GFP was immunoprecipitated from transformed tobacco leaves using anti-GFP antibody. As shown in Figure 7(c), when myelin basic protein (MBP) was used as a universal MPK substrate, MBP phosphorylation was detected with extracts from MPK8-GFP-expressing leaves, but not with untransformed leaf extracts, indicating that MPK8-GFP had a kinase activity. When MBP was replaced by recombinant GST-TCP14, a phosphorylated band corresponding to phospho-GST-TCP14 was visualized, indicating that MPK8 expressed *in planta* efficiently phosphorylated TCP14. To identify the TCP14 residues phosphorylated by MPK8, the same experiments were carried out in the presence of unlabeled ATP. Following incubation with proteins immunoprecipitated from untransformed or MPK8-GFP transformed leaves, GST-TCP14 was trypsin-digested and phosphopeptides were enriched and analyzed by nanoLC-MS/MS. Three phospho-islands were confidently detected (Table S2). Only the phosphopeptide ^{94}ELLQTQEEpSAVVAAK^{108} was systematically identified in all samples corresponding to GST-TCP14 incubation with MPK8-GFP (Figure 7d), while not detected when GST-TCP14 was incubated with extracts from untransformed leaves. The corresponding phosphosite pS^{102} is localized in the N-terminal domain of TCP14, outside of the conserved TCP domain and was not found in a [S/T-P] motif targeted by MPKs (Figure 7d).

As a whole, these data indicated that MPK8 specifically interacted with TCP14 *in vivo* and phosphorylated TCP14 *in vitro*, and that MPK8 and TCP14 operated in a common pathway.

MPK8 stimulates TCP14 transcriptional activity

We then examined the ability of MPK8 to regulate TCP14 transcriptional activity. Transcriptional activity was assayed in transiently transformed *Nicotiana benthamiana* leaves using firefly luciferase (LUC) under the control of a synthetic promoter activated by Class-I TCPs as a reporter (Resentini *et al.*, 2015) and a combination of effectors (Figure 8a). LUC activity was significantly increased (4.7-fold)

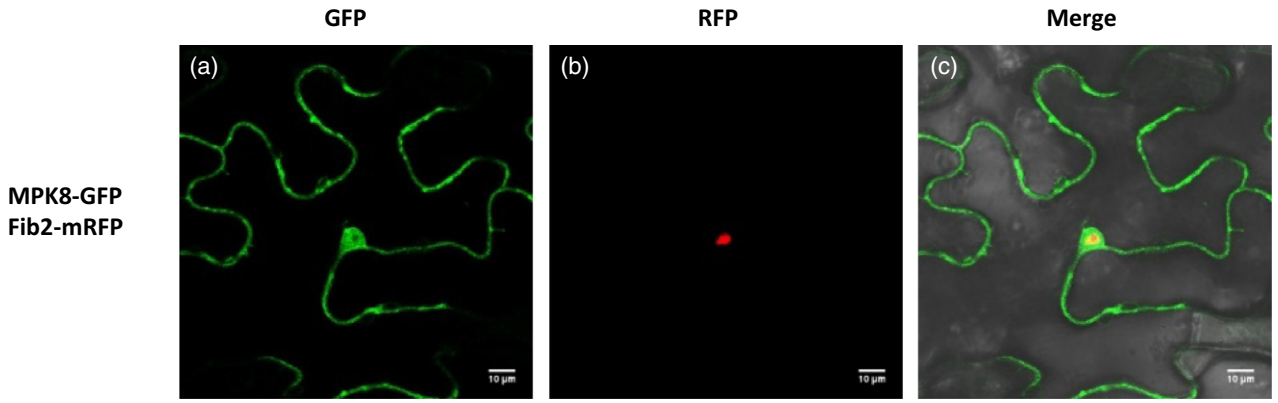

Figure 6. Subcellular localization of MPK8-GFP fusion protein. Tobacco epidermal cells were transiently transformed with MPK8-GFP and Fibrillarin-mRFP. (a) MPK8-GFP imaging; (b) Fibrillarin2-mRFP (Fib2-mRFP) imaging identifying nucleolus; (c) merged brightfield, MPK8-GFP and Fibrillarin2-mRFP imaging. Scale bar: 10 μm.

by the expression of TCP14 fused with the transcriptional activator VP16 (Figure 8b). Transcriptional activity was further stimulated (1.36-fold) when MPK8-GFP was expressed together with VP16-TCP14 (Figure 8b). To evaluate the importance of phosphorylation for the stimulation of TCP14 activity by MPK8, we mutated S^{102} residue that was systematically detected as phosphorylated (Table S2). As shown in Figure 8(b), MPK8 stimulated similarly TCP14 and $TCP14^{S102A}$ transcriptional activity (1.36- and 1.28-fold, respectively). In addition, the mutation of the other residues identified repeatedly as phosphorylated (T^5, S^6 and S^7; Table S2), alone or together with S^{102}, did not modify TCP14 stimulation by MPK8 (Figure S5), indicating that TCP14 regulation by MPK8 was achieved independently of the phosphorylation of the major sites identified by LC-MS/MS.

mpk8 and *tcp14* seeds exhibit extensive and common transcriptomic deregulation during imbibition

To investigate the importance of the interplay between MPK8 and TCP14 in seeds, we compared the transcriptomes of WT, *mpk8.1* and *tcp14.4* freshly harvested seeds by a RNA sequencing approach, in dry state and after 24 h of imbibition. As shown in Figure 9(a), the transcriptomes of the three genotypes closely associated on principal component analysis in dry seeds, due to a low number of genes differentially expressed between WT and mutants (Table S3). Indeed, only 30 genes were significantly deregulated in *mpk8* dry seeds compared with WT (Table S3). Similarly, the expression of 167 genes was significantly deregulated in *tcp14* dry seeds compared with WT (Table S3).

After 24 h of imbibition, WT seed transcriptome was deeply modified, with 4456 genes being upregulated and 3599 genes downregulated (Table S4). In this condition, a dramatic segregation between WT and both *tcp14* and *mpk8* seed transcriptomes was observed (Figure 9a). As a whole, 1978 and 1149 genes were deregulated after 24 h imbibition in *mpk8* and *tcp14* compared with WT, respectively (Table S3; Figure 9b). Moreover, *tcp14* and *mpk8* transcriptomes were closely associated (Figure 9a). Indeed, 949 genes were commonly deregulated in both mutants (Figure 9b; Table S3). Moreover, 947 of these genes were deregulated the same way in the two mutants. The majority (800) was downregulated compared with WT, and only 147 were upregulated (Figure 9b). The presence of the consensus binding motif (KHGGGVC) targeted by class-I TCPs (the structural TCP class to which TCP14 belongs) was searched in the promoter regions of the 947 deregulated genes. We thereby identified 251 motifs found in the promoter of 215 genes (Table S5), suggesting that 23% of the genes might be direct targets for TCP14. These data supported the hypothesis that MPK8 and TCP14 controlled a large set of common genes, and therefore might operate in a common signaling pathway.

To evaluate the overall impact of this modification of gene expression for seed capacity to germinate, deregulated genes were compared with SeedNet, a gene co-expression network model designed from seed germination and dormancy transcriptomic data (Bassel *et al.*, 2011). As shown in Figure 9(c), the genes upregulated in *tcp14* and *mpk8* mutants essentially gathered together in SeedNet region 1, which is associated with dormancy, whereas downregulated genes essentially gathered together in regions 2 and 3, associated with germination, indicating a close correlation between the phenotypes and the deregulation of gene expression observed in the mutants.

An overview of the putative functions of the genes commonly deregulated in both mutants has been obtained using Gene Ontology (GO) TAIR categorization (Figure 9d). Genes from down- and upregulated subgroups fell in most Cellular Functions categories. Transcript assignment was essentially in 'Metabolic Processes' and 'Cellular Processes', 'Response to Stress' and 'Abiotic or Biotic Stimulus' categories. Interestingly, only downregulated genes

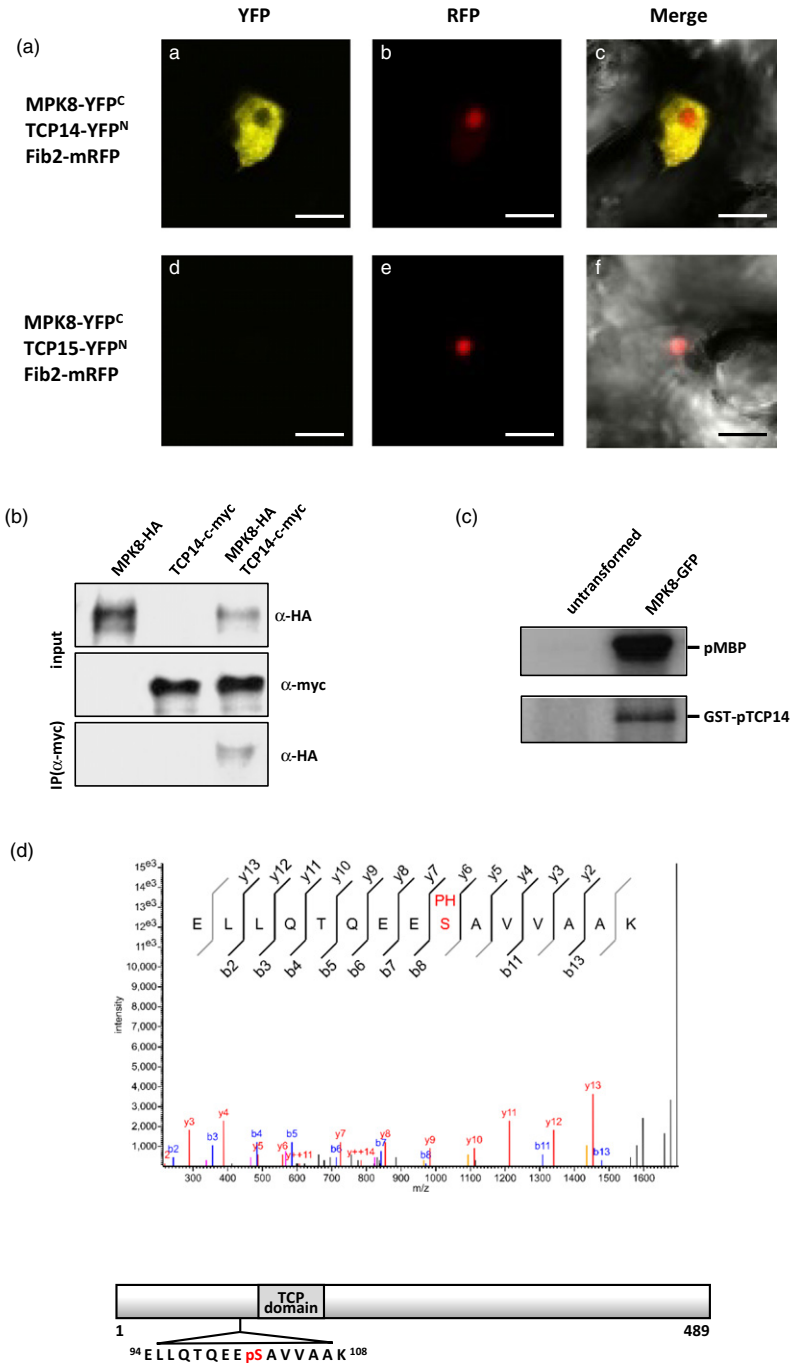

Figure 7. MPK8 interacts with TCP14 *in planta* and phosphorylates TCP14 *in vitro*.
(a) Bimolecular fluorescence complementation (BiFC) assays in transiently transformed tobacco epidermal cells. MPK8 and TCP14 or TCP15 were fused to the C- or N-terminal (YFP^C or YFP^N) fragments of YFP, and co-transfected together with mRFP-tagged Fibrillarin2. Panels (a) and (d): YFP imaging; panels (b) and (e): RFP imaging; panels (c) and (f), merged brightfield, YFP and RFP imaging. Scale bar: 10 μm.
(b) Co-IP of MPK8 and TCP14 from leaf extracts. Tobacco epidermal cells were transiently transformed with MPK8-HA or TCP14-c-myc constructs alone, or with MPK8-HA/TCP14-c-myc construct mix. Upper lane, detection of MPK8-HA in total protein extracts; middle lane, detection of TCP14-c-myc in total protein extracts; bottom lane, detection of MPK8-HA after immunoprecipitation with anti-c-myc antibodies.
(c) *In vitro* kinase assays for MPK8 activity. MPK8-GFP immunoprecipitated with anti-GFP antibodies from transiently transformed tobacco leaf extracts was assayed for kinase activity *in vitro*, with myelin-binding protein (MBP, upper lane) or recombinant GST-TCP14 (bottom lane) as substrates. Proteins immunoprecipitated from untransformed leaves were used as control. Phosphorylated MBP (pMBP) and GST-TCP14 (GST-pTCP14) were detected by autoradiography following sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE).
(d) Representative MS/MS spectrum of the phosphorylated peptides ^{94}ELLQTQEEpSAVVAAK^{108} containing the S^{102} phosphorylated residue. As indicated, S^{102} is situated in TCP14 N-terminal domain, before the conserved TCP domain.

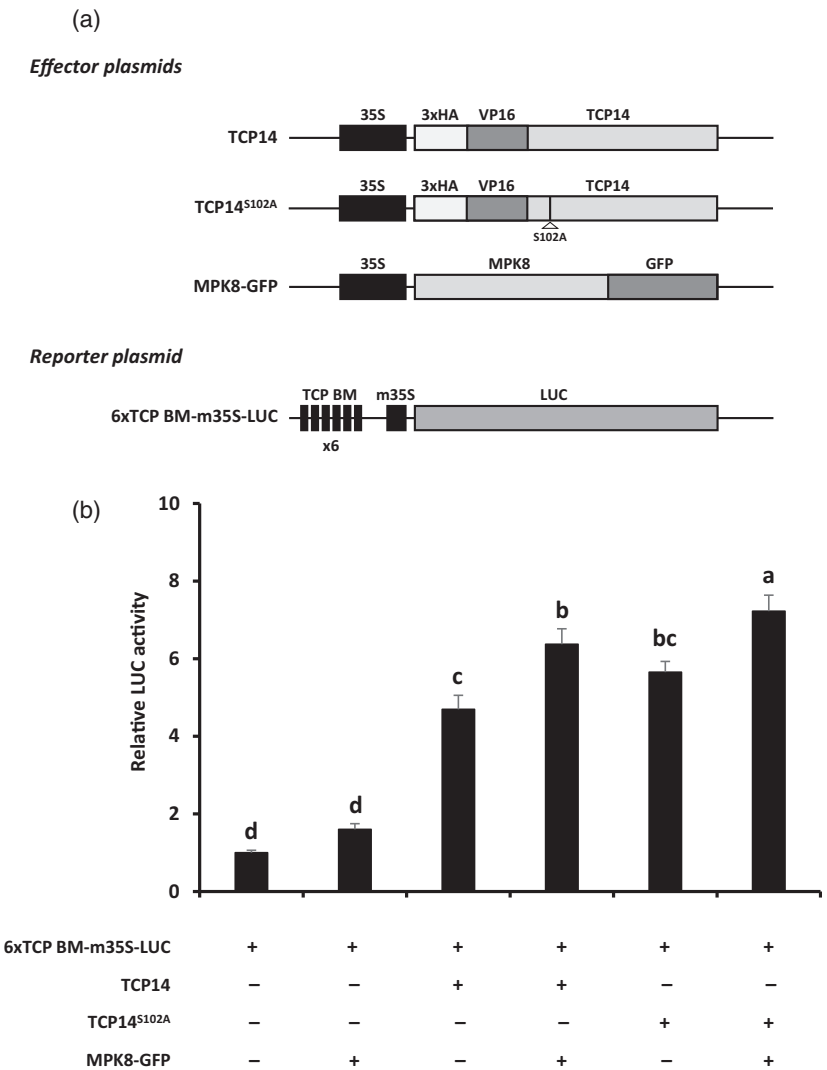

Figure 8. Modulation of the transcriptional activity of TCP14 by MPK8.
(a) Schematic diagram of the effector and reporter constructs used in co-transfection experiments. TCP BM: consensus Class I TCP-binding motif; m35S: minimal 35S promoter.
(b) *Nicotiana benthamiana* leaves were agroinfiltrated with the reporter construct alone or together with wild-type (WT) TCP14 (TCP14), mutated TCP14 ($TCP14^{S102A}$) and /or MPK8-GFP. Values represent the relative activity of firefly luciferase under the control of a synthetic promoter harboring tandem repeats of Class I TCP-binding motifs versus the control *Renilla* luciferase, and are normalized with respect to LUC relative activity in the absence of effector constructs. Means ± SE (n = 30–40 infiltrated leaves from three biological repeats) are represented, and different letters indicate significantly different means (Tukey's HSD, $P < 0.05$).

were assigned to 'DNA and RNA Metabolism'. They were also particularly abundant in the 'Cell Organization and Biogenesis' category (5.6% versus 0.7% for upregulated genes). Using REVIGO application, we further identified 29 and 79 GO terms that were significantly over-represented in up- and downregulated genes, respectively (Figure S6; Table S6). For upregulated genes, many over-represented GO terms referred to plant response to internal and environmental cues such as 'Response to Stimulus', 'Response to Stress' or 'Response to Oxidative Stress', and included genes coding for antioxidant enzymes (CAT3, MSD2) or enzymes involved in galactinol biosynthesis (GolS1, GolS2; Figures 10 and S6a). They also included a large range of heat-shock proteins and heat-shock factors, for example HSFA2, HSFA9, HSP90, HSP70 or HSP20a that were shared with 'Protein Folding' GO term. In addition to *HSP/HSF* genes specific for seed maturation (HSFA9, HSP101, HSP17), another GO term, 'Oilbody Biogenesis', that includes oleosin proteins (OLEO1, OLEO4) was over-represented. The upregulation of these gene categories that were directly associated with the maturation phase of seed development was in agreement with *mpk8* and *tcp14* exacerbated dormancy phenotypes.

Distinct over-represented GO terms were associated with commonly downregulated genes (Figure S6b; Table S6). The GO term 'Cell Wall Organization or Biogenesis' encompassed 59 genes including expansins, pectin methyl esterases or xyloglucan endotransglycosidase/hydrolase enzymes, required for cell elongation (Figure 10). Similarly, the GO terms 'Cell Cycle' and 'DNA Replication' gathered 44 genes largely involved in the completion of replication and cell cycle. In addition, a range of DNA damage repair proteins (DRT100, DRT112) and tubulins (TUA2, TUA4, TUB1, TUB5 and TUB7) involved in germination were present in either 'Response to Abiotic Stimulus' or 'Cell Cycle' GO terms (Figure 10; Table S6). These data suggested that MPK8 participated in the regulation of replication and cell cycle in seeds, as reported for TCP14. To confirm this

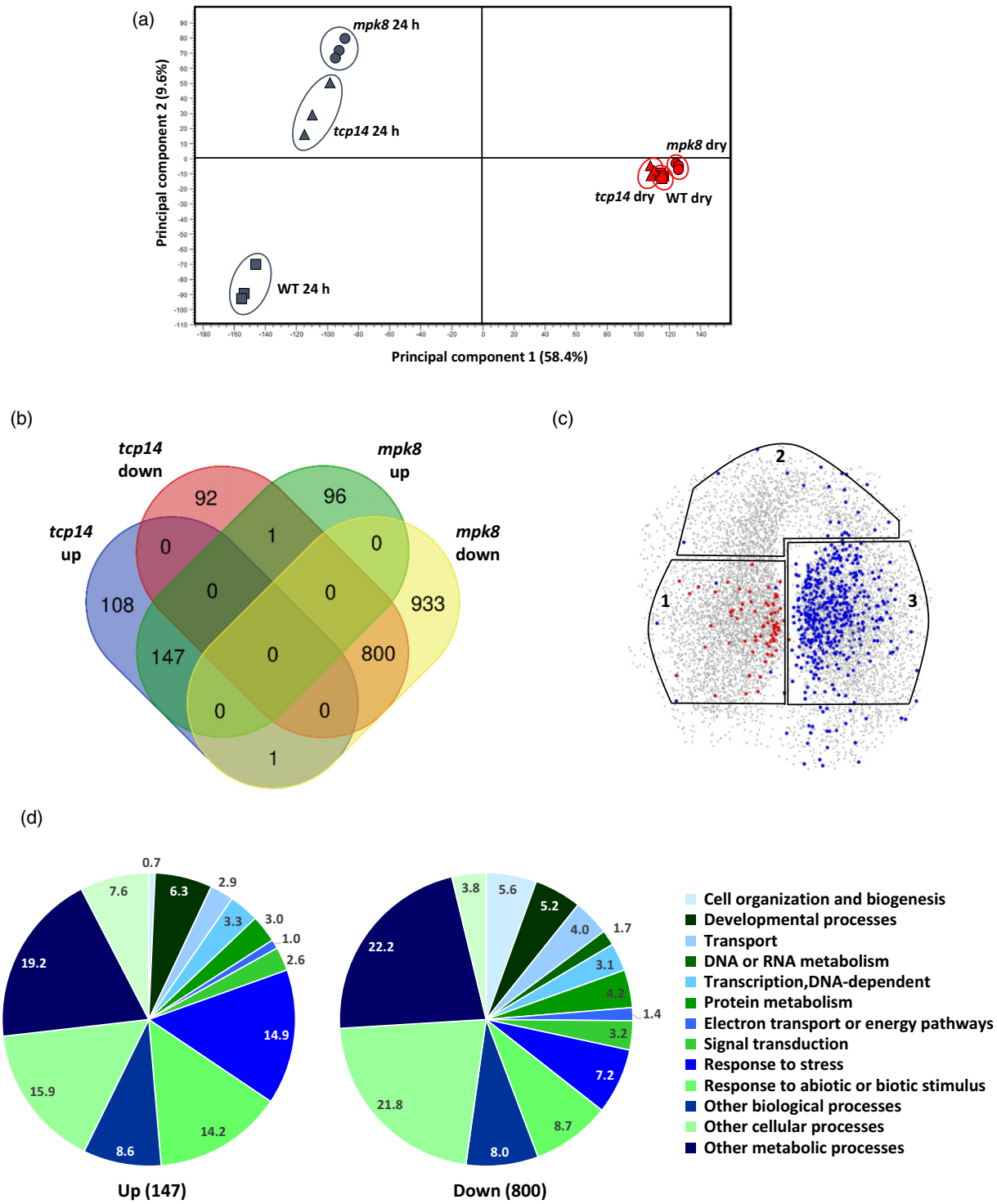

Figure 9. Changes in wild-type (WT), *mpk8.1* and *tcp14.4* seed transcriptome analyzed by RNAseq.
(a) Principal component analysis plot of transcript profiles in dry (red) and imbibed (24 h, 25°C; blue) freshly harvested *mpk8.1* (circles), *tcp14.4* (triangles) and wild-type (WT; Col-0; squares) seeds. Each point represents a biological sample.
(b) Venn diagram of the genes up- and downregulated in imbibed *mpk8.1* and *tcp14.4* compared with WT seeds. Intersections represent genes deregulated in both mutants.
(c) Localization of up- (red dots) and downregulated (blue dots) genes in the SeedNet coexpression network (www.vseed.nottingham.ac.uk). The delimitated regions correspond to clusters associated with dormancy (region 1) or germination (regions 2 and 3; Bassel *et al.*, 2011).
(d) Functional categorization (GOSlim by TAIR database) of the 947 genes similarly overexpressed (up) or repressed (down) in both mutants compared with WT seeds.

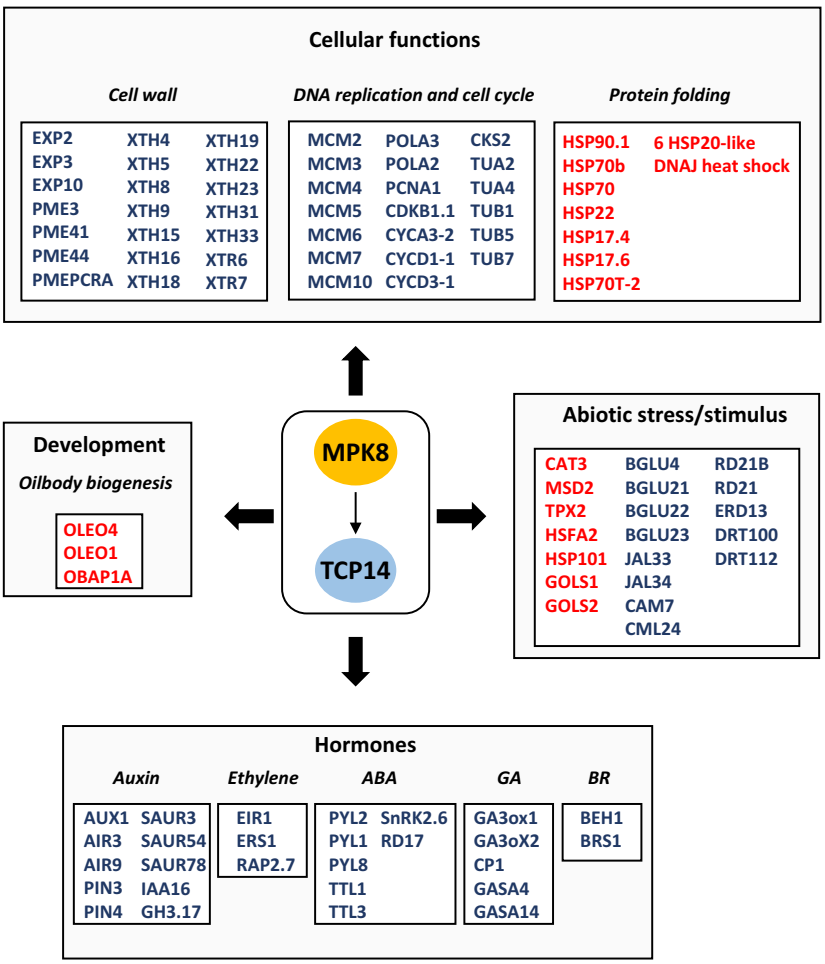

Figure 10. Overview of over-represented biological functions and relevant genes controlled by the MPK8-TCP14 pathway in imbibed seeds. The principal cellular functions targeted by the MPK8-TCP14 pathway, together with a selected list of characterized genes, are presented. Full data are compiled in Tables S3 and S6. Genes overexpressed and repressed in *mpk8.1* and *tcp14.4* seeds are indicated in red and blue, respectively.

commonality between MPK8 and TCP14 functions, the abundance of replication-associated gene transcripts was analyzed in WT, *tcp14* and *mpk8* imbibed seeds by quantitative RT-PCR (Figure 11a). Transcripts for three replicative helicase *MINI CHROMOSOME MAINTENANCE* (*MCM2*, *MCM3* and *MCM7*) genes were less abundant in *tcp14* and *mpk8* seeds. The same pattern was observed for *DNA POLYMERASE ALPHA 2* (*POLA2*), which is required for the initiation of replication, and *PROLIFERATING CELL NUCLEAR ANTIGEN1* (*PCNA1*), a key component of DNA replication machinery (Figure 11a). These data indicated that MPK8 and TCP14 played an essential and common role in cell cycle regulation during seed germination, especially at the transition between G1 and S phase.

Finally, the 'Response to Hormones' GO term gathered 81 downregulated genes involved in auxin, brassinosteroids, ethylene, ABA and GA biosynthesis or signaling (Figures 10 and S6b; Table S6). As *mpk8* and *tcp14* seeds exhibited impaired sensitivity to GA, we examined transcript abundance of two genes involved in GA synthesis and four genes involved in GA response in imbibed seeds. As shown in Figure 11(b), transcripts of *GA3ox1* and *GA3ox2*, two hydrolases required for GA synthesis, were less abundant in mutant seeds, suggesting a defect for endogenous GA synthesis. Consistently, transcript abundance for the GA-responsive genes *EXPA3*, *CP1*, *GASA4* and *GASA14* was strongly decreased in mutants. Furthermore, we established that 165 of the 947 deregulated genes identified in our study (17.4%) had been previously reported as GA-dependent in seeds (Ogawa *et al.*, 2003; Table S7), thus strengthening the link between enhanced dormancy in *mpk8* and *tcp14* mutants and GA signaling.

DISCUSSION

Seeds are dispersed in a dormant state that allows for their survival until appropriate environmental conditions are met for successful germination. The transition from dormant to non-dormant state is therefore critical for subsequent plant development. In the present study, we identified Arabidopsis MAP kinase 8 (MPK8) as a positive regulator of seed germination, and propose that this process is mediated by the transcription factor TCP14. Using two independent mutant lines, we observed that *mpk8* seeds presented a deeper dormancy level at harvest. In

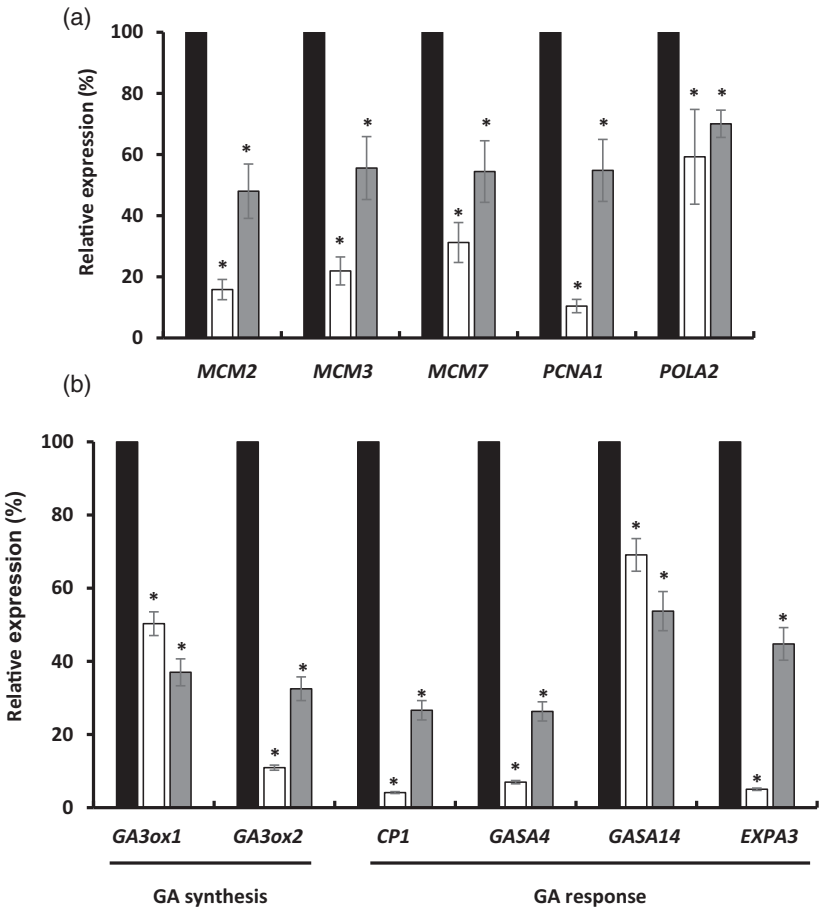

Figure 11. Transcript abundance of replication-associated (a) and gibberellin (GA) signaling (b) genes in *mpk8* and *tcp14* imbibed seeds. Transcript levels were compared by reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) in wild-type (WT; Col-0; black bars), *mpk8.1* (white bars) and *tcp14.4* (gray bars) seeds after 24 h imbibition at 25°C. Expression was normalized in relation to three housekeeping genes (At4g34270, At4g26410, At5g53560). Results from one biological experiment are expressed relative to transcript levels in WT imbibed seeds and are representative of three biological repeats. Asterisks indicate statistical differences between relative transcript abundances in WT and mutant seeds, as determined by Wilcoxon–Mann–Whitney test (*$P < 0.05$).

agreement, dormancy was less efficiently released by after-ripening treatment in *mpk8* seeds. As germination defect was only observed for freshly harvested seeds imbibed at 20–25°C, but not at lower temperature or for non-dormant seeds, MPK8 likely participated in dormancy release mechanisms. MPK8 is one of the 20 MPKs present in Arabidopsis and belongs to the poorly studied MPK subgroup D (Xu and Zhang, 2015). Subgroup D is the largest MPK subgroup with eight isoforms (MPK8, MPK9, MPK15-20) that share common structural features, including an atypical TDY motif for activation by upstream MKKs (Takahashi *et al.*, 2011). So far, MPK8 function had only been associated with plant response to wounding (Takahashi *et al.*, 2011). Our study extends MPK8 function to the regulation of seed germination. The involvement of MPK8 in dormancy release also broadens the current data available for MPK functions in seeds. Indeed, MPK6 participated to the inhibition of germination and the maintenance of dormancy triggered by ABA (Xing *et al.*, 2009). We established that *mpk8* and WT responded similarly to ABA, but that GA metabolism and response were impaired in *mpk8* mutant during imbibition. Indeed *mpk8* seed germination was less sensitive to GA and more affected by paclobutrazol than WT. In addition, imbibed *mpk8* seeds also presented decreased transcript levels of GA synthesis and response genes. As a whole, these data suggest that at least two MPK pathways coexist, with MPK6 regulating dormancy establishment/maintenance and MPK8 promoting dormancy release. The mechanism of MPK8 activation during seed imbibition is currently unknown, but it unlikely relies on changes of *MPK8* expression during imbibition as transcript levels were hardly modified. Recent genetic studies have identified TaMKK3-A and HvMKK3, the orthologs of Arabidopsis MKK3 in wheat and barley, respectively, as negative regulators of dormancy (Nakamura *et al.*, 2016; Torada *et al.*, 2016). Whether MKK3 may activate MPK8 in seeds is currently unknown. Noteworthy, when expressed in tobacco leaves, MPK8-GFP exhibited a high basal activity towards MBP, although no phosphorylation at the TDY motif was detected using LC-MS/MS. Moreover, inconsistent results have been reported concerning the ability of MKK3 to activate MPK8 *in vivo* (Takahashi *et al.*, 2011; Danquah *et al.*, 2015). Although MPK8 activation by MKK3 cannot be excluded in seeds, these observations speak for the existence of other regulatory mechanisms, for example based on calmodulins as evidenced by Takahashi *et al.* (2011) that will require further investigations.

Whereas many substrates of subgroup A and B MPKs have been identified, no target of subgroup D MPKs has been characterized so far in Arabidopsis (Dóczi and Bögre, 2018). In this study, we identified the transcription factor TCP14 as a potential downstream effector of MPK8. Firstly, *mpk8tcp14* double-mutant seeds presented a deeper dormancy at harvest, similar to that of *tcp14* seeds and deeper than that of *mpk8* seeds. As no additive effect was observed in *mpk8tcp14* double-mutant, this phenotype was consistent with the hypothesis that MPK8 and TCP14 operated in a common pathway and that MPK8 functioned upstream of TCP14. Secondly, MPK8 and TCP14 specifically interacted in the nucleus in BiFC experiments and could be co-immunoprecipitated when co-expressed in tobacco leaves. These results are consistent with the detection of MPK8-GFP in both cytosol and the nucleus, and with the specific localization of TCP14 in the nucleus previously reported (Kieffer *et al.*, 2011). Thirdly, we evidenced that MPK8 phosphorylated TCP14. GFP-tagged MPK8 expressed *in planta* was an active kinase and exhibited activity towards MBP *in vitro*. More importantly, it also efficiently phosphorylated recombinant TCP14 in the same assays. Based on protein microarray analysis, TCP14 had been proposed as a specific substrate for recombinant MPK8, and was not phosphorylated by the other MPKs tested (MPK1-7, 10, 16; Popescu *et al.*, 2009). Our data further establish that TCP14 is a *bona fide* substrate for MPK8. Out of the three phospho-islands identified, only S^{102} residue was systematically detected as phosphorylated. This phosphosite is located outside the conserved TCP domain, in the N-terminal domain that is highly variable among class-I TCPs. Noteworthy, S^{102} was followed by an alanine residue instead of a proline. Similarly, MPK9, another D-type MPK, was able to autophosphorylate on S^{464} that is also followed by an alanine (Nagy *et al.*, 2015). As the other TCP14 phosphoresidues detected were followed by various amino acids but proline, the sequences targeted by MPK8 and other D-type MPKs might significantly diverge from the canonical [S/T-P] motif, and the identification of additional substrates of these MPKs would help unravel their motif specificity. Finally, MPK8 significantly enhanced TCP14 transcriptional activity in transactivation assays, which established a direct functional link between MPK8 and TCP14. Nevertheless, the mutation of S^{102} and/or T^5/S^6/S^7, the only TCP14 phosphoresidues repeatedly identified using LC-MS/MS, did not alter TCP14 stimulation by MPK8. Although the involvement of other phosphoresidues only marginally or even not detected by LC-MS/MS analyses cannot be ruled out, these data suggest that MPK8 might regulate TCP14 via phosphorylation-independent mechanisms. Compelling evidence has highlighted the non-catalytic regulatory functions of protein kinases that allosterically modify target conformation or function as scaffold to recruit additional regulators (Kung and Jura, 2016). For instance, although GSK3β efficiently phosphorylates E2F1 transcription factor, the regulation of E2F1 transcriptional activity by GSK3β is independent of phosphorylation and only requires direct interaction between the two partners (García-Alvarez *et al.*, 2007). Similar mechanisms may underlie the regulation of TCP14 transcriptional activity by MPK8, and future investigations using a catalytically deficient version of MPK8 should help decipher the requirement of TCP14 phosphorylation for this process *in vivo*. On the other hand, TCP14 phosphorylation by MPK8 may regulate mechanisms that could not be revealed by the experimental approach used, for example TCP14 stability or interaction with additional regulatory partners. As TCP14 function is regulated via its interaction with different repressors of seed germination (Rueda-Romero *et al.*, 2012; Resentini *et al.*, 2015), it will be interesting to evaluate if TCP14 phosphorylation may affect these associations.

TCP14 is a transcription factor of the Teosinte Branched1/Cycloidea/Proliferating cell factor family that gathers 24 members in Arabidopsis (Martín-Trillo and Cubas, 2010; Nicolas and Cubas, 2016). Together with TCP15, TCP14 is an important regulator of seed germination (Tatematsu *et al.*, 2008; Resentini *et al.*, 2015). As for *mpk8*, *tcp14* mutant seeds presented exacerbated dormancy at harvest and paclobutrazol hypersensitivity (Tatematsu *et al.*, 2008). Our study further evidenced an extensive and common deregulation of GA pathway in *mpk8* and *tcp14* that likely accounted for the exacerbated dormancy observed. Qualitatively, important GA synthesis (*GA3ox1*, *GA3ox2*) and response (e.g. *GASA4*, *GASA14*) genes were dramatically downregulated in *mpk8* and *tcp14* imbibed seeds. Quantitatively, RNA-seq analyses revealed that about 45% of the genes reported as GA-responsive in seeds (Ogawa *et al.*, 2003) were deregulated in both mutants. These analyses also evidenced that the two mutants presented a dramatic overlap of transcriptome deregulation far beyond the GA pathway alone. Indeed, more than 900 genes were similarly deregulated in both mutants. Strikingly, 82% of the genes deregulated in *tcp14* were also affected in *mpk8*, supporting the hypothesis that the regulation of TCP14 by MPK8 participated in gene expression in imbibed seeds. In contrast, MPK8 did not interact *in vivo* with TCP15, the other class-I TCP regulating seed germination (Resentini *et al.*, 2015), suggesting that TCP14 was specifically targeted by MPK8. Nevertheless, the large proportion of genes uniquely deregulated in *mpk8* seeds suggested that additional transcription factors currently unidentified were also regulated by MPK8.

The MPK8-TCP14 pathway appeared particularly crucial during dormancy-to-germination transition. Indeed, whereas the mutations of *MPK8* and *TCP14* deeply modified the transcriptome of imbibed seeds, they hardly affected that of dry seeds. Similarly, only few genes were

deregulated in *tcp14* plantlets (Yang *et al.*, 2017). The distribution of the genes deregulated in *mpk8* and *tcp14* in SeedNet network, together with their biological functions, provided clues on the processes dependent on the MPK8-TCP14 pathway in imbibed seeds. On the one hand, it would participate in the acquisition of germination capacity via the repression of seed maturation and desiccation programs activated during seed development. In this view, the expression of genes participating in the acquisition of desiccation tolerance, for example *HsfA9*, *HSPs*, *GolS1-GolS2* (Leprince *et al.*, 2017), was maintained in *mpk8* and *tcp14* seeds. These data confirmed and extended the regulation of *HSP* gene expression by TCP14 in seeds (Rueda-Romero *et al.*, 2012). A similar dysregulation was detected for oleosins involved in oilbody biogenesis during late embryonic development (Siloto *et al.*, 2006). Although ABA plays a major role in controlling seed maturation, its relation with the MPK8-TCP14 pathway is not established. Indeed, although several genes related to ABA were deregulated in mutant seeds (Table S6), they did not include key regulators of ABA response such as ABI3 or ABI5. Furthermore, in contrast to *tcp14* (Tatematsu *et al.*, 2008), *mpk8* seeds displayed a WT ABA sensitivity. Further investigations are therefore required to establish if and how ABA signaling is interconnected with the MPK8-TCP14 pathway. On the other hand, major processes associated with germination were dependent on the functionality of the MPK8-TCP14 pathway. In this view, cell expansion is the critical mechanism responsible for radicle growth and protrusion. Strikingly, genes involved in cell wall organization and biogenesis constituted the largest functional class repressed in *mpk8* and *tcp14* mutants. Cell wall remodeling enzymes such as XHTs, EXPs or PMEs participate in both cell expansion in the embryo and cell wall loosening in the endosperm (Müller *et al.*, 2013; Sechet *et al.*, 2016). Through their regulation, the MPK8-TCP14 pathway may therefore participate in a coordinated remodeling of cell wall properties in endosperm and embryo. Beside cell expansion, cell proliferation is likely dependent on the functionality of the TCP14-MPK8 pathway. Among the deregulated genes identified, more than 40 were associated with DNA replication and cell cycle. Although cell division is not required for germination, a switch from G1 to S phase progressively occurs during imbibition, correlated with the expression of DNA replication markers (Barrôco *et al.*, 2005; Velappan *et al.*, 2017). Several studies have evidenced the role of TCP14 in regulating cell proliferation and cyclin gene expression (Tatematsu *et al.*, 2008; Kieffer *et al.*, 2011; Rueda-Romero *et al.*, 2012; Davière *et al.*, 2014; Resentini *et al.*, 2015). Our data highlighted that this regulation relied, at least in part, on the MPK8-TCP14 pathway in seeds. As several tubulin genes were also deregulated in *mpk8* and *tcp14* mutants, the MPK8-TCP14 pathway might participate in diverse aspects of cell cycle regulation, including DNA replication during G1/S transition, microtubule organization and cyclin expression, before and during radicle protrusion.

In conclusion, MPK8 appears as a regulator of seed germination through its functional interplay with TCP14. Future work will aim at unravelling the molecular mechanisms underlying the regulation of TCP14 by MPK8.

EXPERIMENTAL PROCEDURES

Plant material

Arabidopsis thaliana ecotype Col-0 was used as WT, together with previously described mutant lines, in the same genetic background: *mpk8.1* and *mpk8.2* (SALK_129553 and SALK_037501, respectively (Takahashi *et al.*, 2011) and *tcp14.4* (Kieffer *et al.*, 2011). The double-mutant *mpk8.1 tcp14.4* was generated by crossing *mpk8.1* and *tcp14.4* and genotyping their F2 progeny. All primers used for genotyping are listed in Table S1. WT and mutant seeds were produced and harvested simultaneously, and stored as described (Leymarie *et al.*, 2012).

Germination assays

Germination was assayed in darkness, as described (Basbouss-Serhal *et al.*, 2015). Germination was scored daily, according to radicle emergence through testa. For ABA and paclobutrazol treatments, seeds were stratified for 3 days at 4°C before incubation at 25°C.

For dormancy release by after-ripening, freshly harvested seeds were stored in darkness at 20°C and 56% humidity. Seed samples were collected each week and assayed for germination at 25°C. Fully non-dormant seed batches were typically obtained after 4 weeks of treatment and subsequently stored at −20°C.

RNA extraction and RT-qPCR

RNAs were extracted from 50 mg of dry or imbibed seeds according to Chang *et al.* (1993) and treated with Turbo DNA-free DNase (ThermoFisher, Waltham, MA, USA). RNAs were subsequently purified on Nucleospin XS columns (Macherey-Nagel, Hoerdt, France) according to manufacturer's instructions. Total RNA (2 μg) was reversely transcribed with RevertAid reverse transcriptase (ThermoFisher). Real-time PCR amplification was then performed in Mastercycler epgradients Realplex^2 (Eppendorf, Hambourg, Germany), using maxima SYBR Green/quantitative PCR (qPCR) Master Mix (ThermoFisher) as described by the manufacturer. The qPCR program was as follow: 95°C/10 min followed by 30 cycles (95°C/30 sec; 56°C/30 sec; 72°C/30 sec). Primer pair efficiencies and critical thresholds were calculated using Realplex^2 software (Eppendorf). The results of three biological replicates were normalized using three genes (At4g26410, At4g34270, At5g53560) exhibiting a steady-state level of transcription. An arbitrary value of 100% was assigned to the WT seeds (dry seed or 24 h imbibition) for the normalization of the relative transcript abundance. Primers used are listed in Table S1.

Localization and BiFC experiments

All clones used have been obtained as described in Data S1. Agroinfection of 3-week-old *N. benthamiana* was performed as described (Valsecchi *et al.*, 2013). Fluorescent signals were detected by confocal microscopy using a Leica TCS-SP5 inversed microscope. For localization, GFP fluorescence (λ_{exc} = 488 nm; λ_{em} = 511 nm) was detected after 36 h. For BiFC assays, YFP

(λ_{exc} = 515 nm; λ_{em} = 525 nm) and mRFP (λ_{exc} = 560 nm; λ_{em} = 575 nm) fluorescence was detected after 48 h. Images were subsequently processed with Fiji software.

Co-immunoprecipitation assays

Nicotiana benthamiana leaves transfected for BiFC experiments were collected 36 h after infiltration and ground in liquid nitrogen. Proteins were extracted in IP buffer (50 mM Tris-HCl buffer, pH 7.4 containing 2 mM DTT, 5 mM EDTA, 5 mM EGTA, 50 mM β-glycerophosphate, 10 mM NaF, 1 mM orthovanadate, 150 mM NaCl, 1% Triton, 1 × Protease Inhibitor Cocktail [Sigma-Aldrich, Saint-Louis, MO, USA]). Extracts were centrifuged (13 000 ***g***, 4°C, 15 min) and supernatant protein content was determined using Bradford protein assay. Five-hundred micrograms of proteins (540 μl final) were incubated on a wheel with 40 μl of 50% protein A-Sepharose slurry (Sigma-Aldrich) for 1 h at 4°C. Following centrifugation (13 000 ***g***, 4°C, 1 min), 5 μg anti-c-myc antibody (mouse monoclonal antibody, clone 9E10, Sigma-Aldrich) was added to the supernatant and the mixture was incubated at 4°C for 2 h. After addition of 40 μl of 50% protein A-Sepharose slurry, proteins were incubated for 1 h at 4°C. Immunoprecipitated proteins were washed three times with 500 μl IP buffer and twice with 500 μl washing buffer (20 mM Tris-HCl pH 7.4, 1 mM DTT, 5 mM EGTA, 15 mM $MgCl_2$). Immunoprecipitated proteins were solubilized in 40 μl of Laemmli loading buffer, separated by 7.5% sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred onto nitrocellulose membrane. HA-tagged proteins were detected by Western blot with mouse anti-HA antibody (monoclonal 12CA5, Sigma-Aldrich, 1:1000) and revealed by ECL.

Production of GST-TCP14 recombinant protein

TCP14 full-length cDNA was cloned into pGEX-2T (GE Healthcare, Chicago, IL, USA) for a GST fusion at the N-terminus. GST-TCP14 protein production and purification were performed as described (Boudsocq *et al.*, 2012).

Kinase assay

MPK8-GFP protein was immunoprecipitated from 500 μg soluble proteins extracted from *N. benthamiana* infiltrated leaves as described for co-immunoprecipitation experiments, using 5 μg of anti-GFP antibody (mouse monoclonal antibody, clones 7.1 and 13.1, Sigma-Aldrich). An additional wash of beads with 500 μl KAB buffer (50 mM Tris-HCl pH 7.5, 1 mM DTT, 10 mM $MgCl_2$) was performed. Kinase reaction was performed at 30°C in 20 μl of KAB buffer supplemented with 0.1 mM ATP, 185 kBq [γ-^{32}P] ATP and 1 μg of myelin-binding protein (MBP) or recombinant GST-TCP14. After 30 min, reactions were stopped by adding 5 μl of 5 × SDS–PAGE loading buffer, heated at 95°C for 5 min and separated on 8 or 15% SDS–PAGE, for GST-TCP14 and MBP-containing reactions, respectively. Radiolabeled MBP and GST-TCP14 were detected by autoradiography on dried gels.

Mass spectrometry phosphorylation site identification

TCP14-GST was phosphorylated as described above, using non-radioactive ATP. Proteins were submitted to cysteine alkylation in 50 mM ammonium bicarbonate and 4 M urea. After dilution by 4, digestion was carried out with 0.3 mg of trypsin (Promega, gold, Madison, WI, USA) at 37°C overnight. Samples were desalted and dried out.

Digested proteins were enriched for phosphopeptides with TiO_2 in 80% acetonitrile, 6% trifluoroacetic acid, as described (Matheron *et al.*, 2014).

Digests were analyzed with a timsTOF Pro mass spectrometer (Bruker, Billerica, MA, USA) coupled to a nanoElute UHPLC (Bruker), equipped with a C18 PepMap100 pre-column (5 mm, 300 μm i.d, 100 Å, 5 μm, Thermo) and an analytical column RP-C18 Odyssey (25 cm, 75 μm i.d., 120 Å, 1,6 μm IonOpticks). Separation was performed at a flow rate of 400 nl min^{-1}, at 50°C. Elution gradient was run from 2 to 15% B in 18 min, 15 to 25% B in 9 min, and 25 to 37% B in 3 min.

Mass spectrometry acquisition was run in DDA mode with PASEF. A collision energy stepping was applied during each time MS/MS separation event. Dynamic exclusion (0.4 min) was activated. Low-abundance precursors were selected several times for PASEF-MS/MS until the target value. Parent ion selection was achieved with a two-dimensional m/z and 1/k0 selection area filter allowing exclusion of singly charged ions. Total cycle time was 1.15 sec with 10 PASEF cycles.

Mgf files were generated using Data Analysis 5.1 (Bruker) and processed with X!Tandem pipeline 3.4.3 (Langella *et al.*, 2017). Search parameters included: mass tolerance of 50 ppm; Ser, Thr and Tyr phosphorylation as variable modifications. Searches (normal and decoy) were performed against a concatenated Uniprot database of *A. thaliana*, *N. tabacum* and contaminants. The phosphopeptide mode was used. *P*-values of peptides and proteins were adjusted for FDR < 1%, with ≥2 peptides per protein. Detailed procedures are presented in Data S2.

LUC activity assay

Transient transcriptional activity assays were performed as described (Resentini *et al.*, 2015). All constructs used have been obtained as described in Data S1. Leaves of 4-week-old *N. benthamiana* were infiltrated with a 1:4 ratio of cells with reporter and effector constructs. Firefly and the control Renilla LUC activities were assayed on leaf extracts 72 h after infiltration with the Dual-Glo Luciferase Assay System (Promega) according to manufacturer's instructions and quantified with TriStar^2 S LB 942 Multimode Microplate Reader (Berthold, Thoiry, France).

Transcriptome studies

Total RNA was extracted from 50 mg of seeds from three independent cultures using RNEAsy kit (Qiagen, Hilden, Germany) according to the supplier's instructions. RNA-seq libraries were generated following the TruSeq Stranded protocol (Illumina®, California, USA) with a sizing of 260 bp. RNA-seq libraries were then sequenced in paired-end (PE) and a read length of 75 bases on an Illumina NexSeq500 (IPS2 POPS platform). Eighteen samples per lane of NextSeq500 were pooled using individual bar-coded adapters, which generated approximately 20 million of PE reads per sample. All steps of the experiment, from growth conditions to bioinformatics analyses, were saved in CATdb database (Gagnot *et al.*, 2008; http://tools.ips2.u-psud.fr/CATdb/; projectID: NGS2016_10_Dormance) according to the international MINSEQE standard 'minimum information about a high-throughput sequencing experiment'. RNA-seq bioinformatic treatment and analysis are described in Data S3.

Data deposition

RNA-seq project is submitted into the international repository GEO (Gene Expression Omnibus; http://www.ncbi.nlm.nih.gov/geo (Edgar *et al.*, 2002). ProjectID: GSE119344.

The mass spectrometry proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository (Vizcaíno *et al.*, 2013) with the dataset identifier PXD013544.

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AUTHOR CONTRIBUTIONS

JP and EB designed the research; WZ, CB, JP and EB wrote the manuscript; WZ, FC, SL, CP, LM, SH, MB, JP and EB performed experiments; WZ, MP, CP, LM, FR, MAB, JP and EB analyzed data. JP and EB contributed equally to this work.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. Germination of freshly harvested WT and *mpk8* seeds at 15°C.

Figure S2. Germination of freshly harvested WT, *mpk8*, *tcp14* and *mpk8 tcp14* double-mutant seeds at different temperatures.

Figure S3. BIFC assay controls for Figure (a).

Figure S4. Immunodetection of MPK8-HA, TCP14-c-myc, TCP15-c-myc in transiently transformed tobacco epidermal cell extracts.

Figure S5. Modulation of the transcriptional activity of $TCP14^{S102A}$, $TCP14^{T5A/S6A/S7A}$ and $TCP14^{T5A/S6A/S7A/S102A}$ by MPK8.

Figure S6. GO terms significantly over-represented in genes similarly overexpressed and repressed in *mpk8.1* and *tcp14.4* imbibed seeds compared with WT.

Table S1. List of primers used in the study.

Table S2. TCP14 phospho-islands identified by LC-MS/MS.

Table S3. Differentially expressed genes in dry and imbibed seeds in *mpk8* and *tcp14* mutant compared with WT.

Table S4. Differentially expressed genes in WT seeds after 24 h imbibition.

Table S5. Identified TCP motifs in the promoter of differentially expressed genes.

Table S6. Over-represented GO terms in genes deregulated commonly in *mpk8* and *tcp14* seeds after 24 h imbibition.

Table S7. GA-dependent genes deregulated commonly in *mpk8* and *tcp14* seeds after 24 h imbibition.

Data S1. Cloning procedures.

Data S2. Detailed procedure for mass spectrometry phosphorylation site identification.

Data S3. RNA-seq bioinformatic treatment and analysis.

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