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.
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 SnRKs (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 (M KK) 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 atm mpk6 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 hypomensitive 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 hypomensitive 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 vitro, 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.

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.

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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<sup>C</sup> and TCP14-YFP<sup>N</sup> 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<sup>C</sup> was co-expressed with YFP<sup>N</sup> alone or when TCP14-YFP<sup>N</sup> was co-expressed with YFP<sup>C</sup> alone (Figure S3). To further confirm MPK8-TCP14 interaction, co-immunoprecipitation experiments were carried out.

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).
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-YFPc and TCP15-YFPN 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 94ELLQTQEEpSAVVAAK108 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 phospho-site 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)
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 S102 residue that was systematically detected as phosphorylated (Table S2). As shown in Figure 8(b), MPK8 stimulated similarly TCP14 and TCP14S102A transcriptional activity (1.36- and 1.28-fold, respectively). In addition, the mutation of the other residues identified repeatedly as phosphorylated (T5, S6 and S7; Table S2), alone or together with S102, did not modify TCP14 stimulation by MPK8, 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 ELLOQTEEpsAVVAAK containing the S102 phosphorylated residue. As indicated, S102 is situated in TCP14 N-terminal domain, before the conserved TCP domain.
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 (HSFA8, 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 delimited 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.
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 EXP3, CP1, GAS4 and GAS14 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
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.

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 (\({* P < 0.05}\)).
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 (Döczi 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 upstream of TCP14. Noteworthy, 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 (Martin-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 HsfA8, 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 (Barróco 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 (Basbous-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 epgradient (Eppendorf, Hamburg, 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 Realplex² 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 imbibed) for the normalization of the relative transcript abundance.

**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 inverted microscope. For localization, GFP fluorescence (λ_{exc} = 488 nm; λ_{em} = 511 nm) was detected after 36 h. For BiFC assays, YFP fluorescence (λ_{exc} = 494 nm; λ_{em} = 520 nm) was detected after 48 h.

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respectively. Radiolabeled MBP and GST-TCP14 were detected by Western blot with mouse anti-HA antibody (clone 9E10, Sigma-Aldrich) was added to the washing buffer (20 mM Tris-HCl pH 7.4, 1 mM DTT, 5 mM EGTA, 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 MgCl2). 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 MgCl2) was performed. Kinase reaction was performed at 30°C in 20 µl of KAB buffer supplemented with 0.1 mM ATP, 185 kBq [32P]-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 mM urea. After dilution by 4, digestion was carried out with 0.3 µg of trypsin (Promega, gold, Madison, WI, USA) at 37°C overnight. Samples were desalted and dried out.

Digested proteins were enriched for phosphopeptides with TiO₂ 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 A, 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/đó 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 TriStar² S LB 942 Multi-mode Microplate Reader (Berthold, Thoiry, France).

Transcriptome studies

Total RNA was extracted from 50 mg of seeds from three independent cultures using RNEAasy 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 280 bp. RNA-seq libraries were then sequenced in paired-end (PE) and a read length of 75 bases on an Illumina NexSeq500 (IP52 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_Dorman) 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 TCP14S102A, TCP14S102A, TCP14S66/60/7A and TCP14S66/60/7A/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.

REFERENCES


