Plant Physiology and Biochemistry 135 (2019) 242–252

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

Research article

Differential regulation of the durum wheat MAPK phosphatase 1 by calmodulin, bivalent cations and possibly mitogen activated protein kinase 3

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ARTICLE INFO

Keywords:
Calmodulin
Cations
Durum wheat
Mitogen-activated protein kinase (MPK)
Mitogen-activated protein kinase phosphatase (MKP)
Phosphorylation

ABSTRACT

MAPK phosphatases (MKPs) are relevant negative regulators of MAPKs in eukaryotes as they mediate the feedback control of MAPK cascades in multiple cellular processes. Despite their relevance, our knowledge on the role of cereal MKPs in stress tolerance is scarce and TMKP1 remains today the only studied MKP in wheat. TMKP1 was previously reported to be involved in plant salt stress tolerance. Moreover, TMKP1 was shown to interact with calmodulin (CaM), 14-3-3 and TMPK3/TMPK6 proteins, which regulate its catalytic activity. To further understand the functional properties of TMKP1, we investigate here the contribution of its phosphorylation status, and of TMPK3 together with CaM and bivalent cations on the catalytic activity. In-gel kinase assays revealed that TMKP1 can be phosphorylated by similar wheat and *Arabidopsis* MAPKs, including most likely MPK3 and MPK6. In addition, we provide evidence for the capacity of wheat TMPK3 to bind to TMKP1 via a conserved Kinase Interacting Domain (KID) located on its C-terminal part. This interaction leads to a stimulation of TMKP1 activity in the presence of Mn^{2+} or Mg^{2+} ions, but to its inhibition in the presence of Ca^{2+} ions. However, the phosphorylation status of TMKP1 seems to be dispensable for TMKP1 activation by TMPK3. Remarkably, in assays combining TMPK3 with CaM/Ca^{2+} complex, we registered rather an inhibition of TMKP1 activity which however can be suppressed by Mn^{2+} cations. Our data are in favor of complex differential regulation of TMKP1 by its MPK substrates, metallic cations that might help in fine-tuning the plant cellular responses to various stresses.

1. Introduction

Mitogen-activated protein kinase (MAPK) cascades are highly conserved signaling modules in eucaryotes, and play key role in the control of diverse aspects of growth, development, cytokinesis, senescence, programmed cell death, and stress signaling (Ellis, 2012; Rasmussen et al., 2012). The MAPK cascade consists of three sequentially activated protein kinases, i.e., MAP3K (MAPKKK), MAP2K (MAPKK), and MAPK (MAPK group, 2002). Once activated, a MAP3K in turn phosphorylates its downstream MAP2K which phosphorylates and activates its downstream MAPK (Rodriguez et al., 2010). MAPKs are activated by dual phosphorylation of Thr and Tyr residues within their T-X-Y consensus sequence by the dual-specificity MAPKKs. The magnitude and the duration of MAPK activation play a decisive role in determining the signaling specificity and both must be tightly regulated. This depends on protein phosphatases such as the MAPK phosphatases (MKPs) MKPs are dual-specificity phosphatases (DSP) dephosphorylating both Tyr and Thr residues, and exhibit high specificity for MAPKs (Bartels et al., 2010).

It was previously reported that MKPs undergo also phosphorylation which then may influence their functional properties. The phosphorylation of the yeast Msg5 MAPK phosphatase by its substrate Slt2 MAPK affects the interaction between the two proteins (Sohaskey and Ferrell, 2002). Also, mammalian MKP-1 is phosphorylated by ERK proteins and this phosphorylation stabilizes MKP-1 protein by reducing its proteolytic degradation (Brondello et al., 1999). Besides, MKP-1 phosphorylation does not modify its intrinsic ability to dephosphorylate ERK (Brondello et al., 1999) but depends upon the nuclear accumulation of active ERK (Reffas and Schlegel, 2000). A similar scenario was observed on *Arabidopsis* AtMKP1 which can be phosphorylated in vitro and in

Abbreviations: DSP, dual specificity phosphatase; MAPK (MPK), Mitogen-activated protein kinase; MKP, MAP kinase phosphatase; OMFP, 3-O-methylfluorescein phosphate; CaM, Calmodulin; CaMBD, Calmodulin Binding Domains; MAPKs, Mitogen Activated Protein Kinase; NLS, Nuclear localization sequence; TMKP1, Triticum MAPK phosphatases 1

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https://doi.org/10.1016/j.plaphy.2018.12.016
Received 8 November 2018; Received in revised form 16 December 2018; Accepted 17 December 2018
Available online 18 December 2018
0981-9428/

vivo by endogenous MPK6. In fact, using TiO2 chromatography and mass spectrometric analysis, Park et al. (2011) found that four Ser/Thr residues in AtMKP1, each followed by a proline residue (Thr64, Thr109, Ser558, and Ser572) were potentially phosphorylated by AtMPK6. This phosphorylation stimulates the phosphatase activity of AtMKP1 in vitro (Park et al., 2011) and was reported to be required for its function in response to UV-B stress as it promotes its stability under UV-B treatment (Gonzalez-Beistero and Ulm, 2013). The phosphorylation of AtMKP1 was also found to be required for its stabilization and accumulation during PAMP (Pathogen-Associated Molecular Patterns) responses and bacterial resistance (Gonzalez-Beistero and Ulm, 2013; Jiang et al., 2017). Although AtMKP1 remains until now the only plant MKP known to be regulated by phosphorylation, it was demonstrated that the catalytic activity of other plant orthologs such as NtMKP1 of tobacco, are activated by their own MAPK partners (Katou et al., 2007).

TMKP1 isolated from a Tunisian *Triticum durum* variety Om Rabia3 is the only studied wheat MKP so far (Zaidi et al., 2010; Ghorbel et al., 2015, 2017) and was reported to act as a positive regulator of salt stress tolerance in *Arabidopsis* (Zaidi et al., 2016). TMKP1 exhibits a phosphatase activity in vitro (Zaidi et al., 2010, 2012) that is specifically enhanced by Mn^{2+} and Mg^{2+}, but without any synergistic effect between the two bivalent cations (Ghorbel et al., 2015). Moreover, TMKP1 interacts with CaM in calcium dependant manner through at least one putative CaMBD located at the C-terminal part of TMKP1 (Ghorbel et al., 2015) as reported for its homologues AtMKP1, NtMKP1 and OsMKP1 (Yamakawa et al., 2004; Katou et al., 2005; Lee et al., 2008). TMKP1 is the only DSP to be inhibited by the CaM/Ca^{2+} complex in vitro. However, in the presence of Mn^{2+}, the phosphatase activity of TMKP1 is enhanced by CaM/Ca^{2+} complex, outlining the relevance of this bivalent cation in the activation of TMKP1. 14-3-3 proteins are also able to bind to TMKP1 in phospho-dependent manner and stimulate its catalytic activity (Ghorbel et al., 2017). On another hand, a phosphatase treatment decreased about 45% the catalytic activity of TMKP1 demonstrating that its phosphorylation status is important for an optimal phosphatase activity (Ghorbel et al., 2017).

In this study, we provide experimental evidence for TMKP1 phosphorylation by plant MPKs including most likely MPK3 and MPK6. Moreover we identified on the C-terminal part of TMKP1 a conserved kinase binding motif (566–575 a.a) through which TMPK3 interacts with the wheat MKP and stimulates its activity in vitro. In the presence of a mixture of TMPK3 with CaM/Ca^{2+} complex, the TMKP1 activity was rather inhibited but this inhibition was suppressed by the addition by Mn^{2+}. All these data outline not only the role of MPK substrates and bivalent cations in the control of plant MKP activity but also the complexity of the mechanism regulating this class of phosphatases that may offer plants the capacity to adjust their response to rapidly changing environments.

2. Materials and methods

2.1. Plant material and stress treatments

Seeds of durum wheat (*Triticum turgidum* L. subsp. durum) cultivar Om Rabiaa3 were supplied by INRAT, Laboratoire de PhysiologieVégétale (Tunis, Tunisia). The seeds were sterilized in 0.5% NaClO solution for 15 min, then washed three times with sterile water and germinated on wet Whatman paper in Petri dishes. Seedlings were grown in a greenhouse at 25 ± 2 °C, under photosynthetically active radiation of 280 μmol $m^{-2}.s^{-1}$, 16 h photoperiod and 60 ± 10% relative humidity. Salt stress (200 mM NaCl) treatments were applied on 10 day-old seedlings for 24 h.

Seeds of *Arabidopsis thaliana* (Col-0 Accession) were sterilized in 20% NaClO; 0.05% Tween; 80% ethanol solution for 10 min, washed three times with ethanol (96%) and three times with sterile water. Seeds were germinated on Murashig and Skoog (MS) medium in Petri dishes after 2 days of vernalization at 4 °C. Seedlings were grown in a greenhouse at 22 ± 2 °C, under photosynthetically active radiation of 280 μmol $m^{-2}.s^{-1}$, 16 h photoperiod and 60 ± 10% relative humidity during ten days. Salt stress (200 mM NaCl) treatments were applied on 10 day-old seedlings for 3 h.

2.2. Plant protein extraction

Leaves from 10-days old wheat or *Arabidopsis* seedlings were ground in liquid nitrogen and proteins were extracted in a buffer containing 10 mM $MgCl_2$, 1 mM DTT, 100 μM PMSF, 2 mM leupeptin, 50 μM MG132 and 1% (v/v) protease inhibitor cocktail for plant extracts (Sigma) and phosphatase inhibitors (50 mM HEPES-NaOH pH 7.5; 50 mM $K_4P_2O_7$; 10 mM Na_3VO_4; 5 mM NaF). Insoluble material was removed by centrifugation at 3000*g* for 30 min at 4 °C and protein concentration was determined using the Bradford assay (Interchim).

2.3. In gel kinase assays

lmmunocomplex kinase assays using total protein extracts from wheat (20 μg) or *Arabidopsis* (40 μg) leaves and a recombinant His-TMKP1 as a substrate were performed as formerly described (Li et al., 2017). Briefly, plant protein samples were run on 10% SDS-PAGE that was previously polymerized with 2 mg of purified His-TMKP1 and then subjected to a kinase assay in 15 μl reaction buffers (20 mM HEPES, 1 mM Na_2-VO_4, 0.5 mM PMSF, 2 mM EDTA, 2 mM DTT, 200 nM ATP, 1 μCi [γ^{32}]-P-ATP). After an incubation at 30 °C for 30 min, the reaction was stopped by adding 3 μl of sodium dodecyl sulfate (SDS) sample buffer (250 mM Tris–HCl pH 6.8, 10% SDS, 30% glycerol, 0.02% bromophenol blue), immediately followed by boiling at 95 °C for 5 min. The incorporation of [γ^{32}]-P was visualized by autoradiography and quantified with a phosphorimager and Image Quant software (Molecular Dynamics). Band intensities in western blots were quantified when necessary by ImageJ software (https://imagej.nih.gov/ij/).

2.4. Protein expression and purification

The recombinant proteins GST-TMPK3 (accession no. AAC28850) GST-TMKP1 and GST-ΔNTMKP1 were produced in the Rosetta *E. coli* strain (DE3) (Novagen) after cloning of the corresponding ORF in-frame with a GST tag into the pGEX-2TK expression vector (GE Healthcare; Zaidi et al., 2010). Similarly, His-TMKP1, His-TMKP1^{C214G} (dead phosphatase mutant form), His-ΔCTMKP1 (isoform harboring the first 387 aa), His-KIDTMKP1 (harboring the first 616 aa), and His- No-KIDTMKP1 (containing the first 566 aa without KID domain) were produced as recombinant proteins using pET28a expression vector (Novagen, Madison; Zaidi et al., 2012; Ghorbel et al., 2017). TdCaM1.1 (Genbank KJ5000051.1) was also cloned in frame to poly-His tags in pET28a expression vector (Novagen, Madison). Recombinant proteins were purified by affinity chromatography on either Glutathione Sepharose 4B beads (Amersham Biosciences) or nickel columns (GE Healthcare) according to described procedures (Ghorbel et al., 2015). Protein quantification was performed using the Bradford method (Bradford, 1976) and the correct size of recombinant proteins was checked by SDS-PAGE.

2.5. In vitro phosphatase assays

The phosphatase assays were performed as previously described (Ghorbel et al., 2015, 2017) using 1 μg of purified recombinant forms of His-TMKP1, His-ΔCTMKP1, GST-TMKP1, GST-ΔNTMKP1, His-KIDTMKP1, His-NoKIDTMKP1 or His-TMKP1^{C214G}, in presence of different amounts of GST-TMPK3, and 3-O-methylfluorescein phosphate (OMFP, Sigma) as a substrate. Phosphatase activity was carried out in the presence or absence of various concentrations of bivalent cations (Mn^{2+}, Mg^{2+} and Ca^{2+}) in 0.8 ml of reaction buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 500 μM OMFP) at 30 °C for

various time periods (0, 10, 20 and 30 min). All reactions were assayed at saturating substrate quantities (500 μM) as previously described (Ghorbel et al., 2015) and at which the initial velocity (V0) becomes constant. The reaction was quenched by addition of 200 μl of NaCl 5M and the amount of 3-O-methylfluorescein (OMF) released was measured by absorbance at 477 nm.

2.6. Phosphatase treatments

To generate a dephosphorylated His-TMKP1 (dpHis-TMKP1) protein, 10 μg of the purified protein were treated with 400 Units of λ−phosphatase (New England Biolabs) at 30 °C for 30 min in 50 mM HEPES, 10 mM NaCl, 2 mM DTT, and 1 mM Mn^{2+}. The reaction was stopped by heating at 65 °C for 15 min in the presence of 3 mM Na_2EDTA.

2.7. GST pull down assays

Prior to binding, Gluthatione Sepharose 4B beads were washed with the appropriate buffer (Tris-HCl 20 mM; pH 7.4, EDTA 1 mM, DTT 0.5 mM, NaCl 150 mM, 0.5% Triton, PMSF 1 mM) and equilibrated with the same buffer. Then, the beads were incubated with 15 μg of GST-TMPK3 or GST for 3 h at 4 °C, and washed three times to discard the unfixed proteins. Twenty micrograms of the different recombinant forms of TMKP1 proteins were then incubated with the immobilized proteins overnight at 4 °C. After extensive washes, proteins were dissociated from the beads by boiling in Tris-HCl 50 mM, pH 6.8, DTT 1 mM, SDS 2%, glycerol 10%, bromophenol blue 0.1% and separated by SDS-PAGE (10%). The His-TMKP1 and the other deleted forms were finally detected by western blot using the anti-TMKP1 antibody as previously described (Ghorbel et al., 2015).

2.8. Antibodies

Commercial antibodies recognizing *Arabidopsis* AtMPK3 (Sigma product number M8318) and AtMPK6 (Sigma product number A7104) were used. Rabbit polyclonal antibodies were raised against TMKP1 (Zaidi et al., 2010).

2.9. Statistical analysis

The results were compared statistically by using a Student's *t*-test and differences were considered significant at $p < 0.01$.

3. Results

3.1. TMKP1 is phosphorylated by similar wheat and Arabidopsis protein kinases

The binding of TMKP1 to MPKs may result not only in their dual dephosphorylation at Thr and Tyr residues but also in a post-translational modification of TMKP1 by phosphorylation as it was reported for the *Arabidopsis* counterpart AtMKP1 (Park et al., 2011). To check this hypothesis, we performed in gel kinase assays using a recombinant His-TMKP1 as a substrate. For this purpose, 20 μg of proteins extracted from leaves of the Tunisian wheat Om Rabiaa3 variety were loaded on SDS-PAGE. Protein extracts from wheat grown under standard or salt stress conditions (200 mM NaCl during 24 h) were used to see whether the stress treatment can influence the phosphorylation status of TMKP1. Similar assays were performed on 40 μg of *Arabidopsis* seedlings protein extracts submitted or not to salt stress. Using wheat protein extracts, two bands of 43 and 48 kDa were detected (Fig. 1a) which may correspond to TMPK3 and TMPK6 proteins respectively, according to their calculated sizes (42.57 kDa for TMPK3 and 47.6 kDa for TMPK6). Although weak, the signals detected within wheat proteins extracted from non stressed or salt treated samples were not significantly different. Similarly, using *Arabidopsis* total protein extracts, two bands of 43 and 48 kDa were clearly detected and they should also correspond, according to their size, to AtMPK3 and AtMPK6 respectively. The intensity of the 48 kDa band is weaker compared to the 43 kDa band, which seems to be even more intense after salt stress treatment (with ≈20% increase in band intensity compared to non stressed conditions, as measured with ImageJ software; Fig. 1b). To further confirm the contribution of AtMPK3 and AtMPK6 into TMKP1 phosphorylation in *Arabidopsis*, we immunoprecipitated total protein extracts using anti-AtMPK3 or anti-AtMPK6 antibodies and we used those immunoprecipitates to perform a new in gel kinase assays on His-TMKP1. Our results show that with the first antibody, a single band of 43 kDa corresponding to the size of AtMPK3 protein is detected (Fig. 1c). Whereas with the second antibody, a single band of 48 kDa that may correspond to AtMPK6 protein is detected (Fig. 1c). Therefore, these in gel kinase assays indicate that TMKP1 can be phosphorylated by similar wheat and *Arabidopsis* MPKs that may include MPK3/6 and this phosphorylation seems to be only moderately influenced by salt stress treatments.

3.2. Activation of TMKP1 by TMPK3 occurs independently of its phosphorylation status but is promoted by Mn^{2+} and Mg^{2+}

The in vitro phosphatase activity of TMKP1 was reported to decrease about 45% after a treatment with λ-phosphatase (Ghorbel et al., 2017), indicating that the phosphorylation status is essential for optimal TMKP1 activity. This activity was also stimulated in the presence of GST-TMPK3 (Zaidi et al., 2010). To investigate whether the stimulatory effects of TMPK3 on TMKP1 activity is dependent on TMKP1 phosphorylation status, we performed OMFP-based phosphatase assays on either phosphorylated (His-TMKP1) or dephosphorylated (dpHis-TMKP1) recombinant forms mixed with a purified GST-TMPK3. In the absence of GST-TMPK3, the phosphatase activity increased with time (Fig. 2a); and after 30 min it was two-fold higher for His-TMKP1 compared to dpHis-TMKP1 as previously shown (Ghorbel et al., 2017, Fig. 2a). When mixed with GST-TMPK3 (using a TMKP1/TMPK3 molar ratio of 1:4), not only His-TMKP1 but also dpHis-TMKP1 activity was increased. Interestingly, the fold induction caused by GST-TMPK3 is similar in both cases indicating that TMKP1 activation by this wheat MPK seems to occur independently of its initial TMKP1 phosphorylation status.

Moreover, some divalent cations like Mg^{2+} and especially Mn^{2+} were reported to stimulate the phosphatase activity of TMKP1 in vitro, whereas others such as Fe^{2+}; Zn^{2+}; Cu^{2+} and Ca^{2+} have no effect (Ghorbel et al., 2015). Therefore, we investigated here the effect of TMPK3 on TMKP1 activity in the absence or in presence of different metallic cations. Zn^{2+}, Fe^{2+} and Cu^{2+} did not modify TMKP1 activity in presence of TMPK3 but surprisingly the addition of Ca^{2+} resulted in a significant decrease of TMKP1 activity (Fig. 2b). The inhibitory effect of Ca^{2+} cations started with Ca^{2+} concentrations as low as 0.5 mM and inhibition reached its maximum level using 2 mM Ca^{2+} for which a decrease of ~80% of the initial rate (V0) was observed (Fig. 2b). This inhibition seemed to be specific to Ca^{2+} since the addition of EGTA a well known Ca^{2+} chelator, restored the activity of TMKP1 in presence of TMPK3 (Fig. 2b).

In contrast, phosphatase assays performed in a buffer containing 2 mM of Mn^{2+}, revealed that the addition of TMPK3 increased TMKP1 activity by about 3-fold comparatively to that measured in the presence of Mn^{2+} alone (Fig. 3a). The Mn^{2+} stimulatory effect can be detected also with concentrations of Mn^{2+} as low as 1 mM. In the presence of TMPK3 and Mg^{2+}, the V0 of TMKP1 activity also increased in cation-dose-dependent manner, but less efficiently than with Mn^{2+} (with a maximum of 2 fold increase using 3 mM Mg^{2+} compared to the basal activity (Fig. 3b)). Therefore, Mn^{2+} appeared to trigger more TMKP1 phosphatase activity than Mg^{2+} when used either alone (as shown previously Ghorbel et al., 2015) or in combination with GST-TMPK3

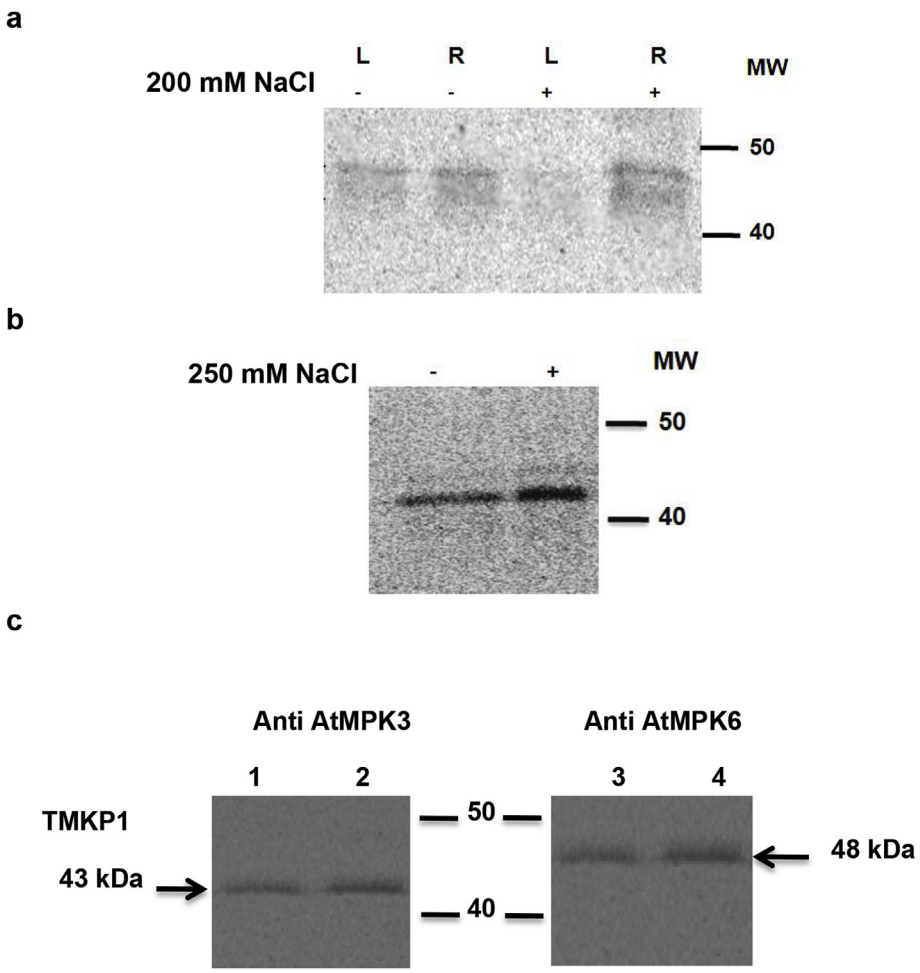

Fig. 1. TMKP1 is phosphorylated in vitro by plant protein kinases. In gel kinase assays of TMKP1 were performed on total protein extracts from wheat and *Arabidopsis*. Two mg of His-TMKP1 were polymerized with 10% SDS-PAGE on which durum wheat **(a)** and *Arabidopsis* **(b)** total protein extracts were loaded. For wheat, 20 µg of proteins extracted from leaves (L) and roots (R) of seedlings treated (+) or not (−) with 200 mM NaCl for 24 h were loaded. Fourty µg of total protein extracts of *Arabidopsis* seedlings submitted (+) or not (−) to 250 mM NaCl for 3 h were used. Involvement of AtMPK3 and AtMPK6 in the phosphorylation of TMKP1 (1c). AtMPK3 and AtMPK6 proteins were first immunoprecipitated using specific commercial antibodies from *Arabidopsis* total protein extracts. The immunoprecipates were then used in-gel-kinase assays as indicated in 1a.

(Fig. 3).

3.3. TMPK3 activates the catalytic activity of TMKP1 via its binding to a conserved domain in the C-terminal region

Structural, biochemical and genetic data have shown that docking motifs from interacting proteins are necessary for MAPKs to bind to and phosphorylate their targets. MAPK docking sites are ubiquitous and have been demonstrated for yeast and mammals MAPK (Kusari et al., 2004). The best-characterized MAPK-docking motifs are the so-called Kinase Interacting Domain (KID) or D-motifs. The consensus D-motif $[(R/K)_{(1-2)}\text{-}(X)_{(2-6)}\text{-}\Phi\text{-}x\text{-}\Phi$; where Φ denotes a hydrophobic residue] is found in activators (MAPKKs), negative regulators (phosphatases), scaffolding proteins and various substrates (Remenyi et al., 2006; Gaestel, 2008). Sequence alignment analyses show that, similarly to other plant MKPs, TMKP1 contains at its C-terminal part (566–575 a.a) a conserved sequence (RRGGFSCLKL) that can serve as a potential KID or D motif (Suppl. Fig. 1).

In order, to assess the role of this motif in TMPK3 binding and its subsequent putative regulatory role on TMKP1 activity, we have generated recombinant C-terminal-truncated versions with deletions at the C-terminal region including (His- NoKIDTMKP1) or not (His-KIDTMKP1) the D-motif. These truncated forms together with full-length (His-TMKP1) and dead phosphatase mutant His-TMKP1^{C214G} (Zaidi et al., 2012) were produced, purified and used in GST pull down assays using GST-TMPK3. Subsequently, the pulled down proteins were analyzed by western blotting using anti-TMKP1 antibody (Zaidi et al., 2010). As shown in Fig. 4b, His-TMKP1 is able to interact with GST-TMPK3 (lane 4) but not with GST alone (lane 1) nor with beads (lane 2) indicating that TMKP1-TMPK3 interaction is specific. Moreover, the His-TMKP1^{C214G} dead phosphatase form is able to interact with TMPK3 (lane 5) suggesting that the catalytic cystein is not required for this interaction. TMPK3 binds also to His-KIDTMKP1 (lane 6) but not to His-NoKIDTMKP1 truncated forms (lane 7). These results indicate that the D-motif might be required for the TMKP1-TMPK3 interaction.

Next, as TMPK3 was shown to enhance TMKP1 catalytic activity, we naturally investigated the impact of D-motif removal on this stimulatory effect. Therefore new series of phosphatase assays on either His-KIDTMKP1 or His-NoKIDTMKP1 incubated together with GST-TMPK3 and using OMFP as a substrate were performed. Without TMPK3 in the medium, the phosphatase activities of His-NoKIDTMKP1 and His-KIDTMKP1 had 4-fold higher levels than the full-length protein in presence or in absence of divalent cations Mn^{2+} and Mg^{2+} (Fig. 5a) as previously shown for another truncated form His-ΔCTMKP1 (Ghorbel et al., 2015) which harbors only the first 380 amino acids. This finding suggests once more that TMKP1 C-terminal part exerts a constitutive and still unknown inhibitory effect on its enzymatic activity. When mixed with TMPK3, the catalytic activity of His-KIDTMKP1 increased more than 3 times while those of His-ΔCTMKP1 or His-NoKIDTMKP1 remained unchanged (Fig. 5b), providing further evidence that the stimulatory effect of TMPK3 on TMKP1 activity might be exerted through its binding to the conserved D-motif.

3.4. TMKP1 harbors at its N-terminal region a conserved ion binding motifs required for its activations by Mn^{2+} and Mg^{2+}

The observed stimulatory effects of Mn^{2+} and Mg^{2+} on TMKP1 activity suggest that wheat phosphatase may harbor cation binding

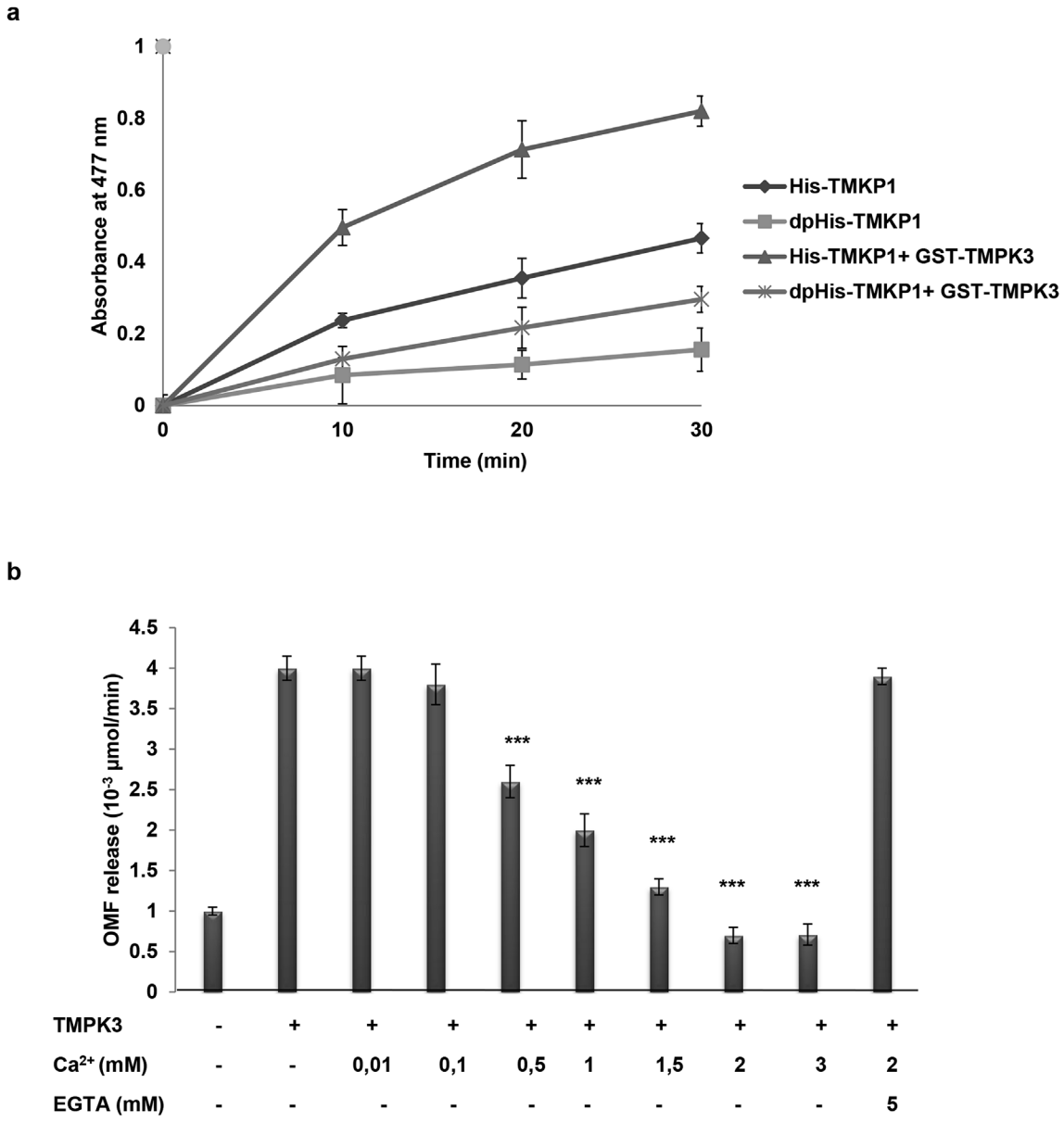

Fig. 2. The phosphatase activity of TMKP1 is stimulated by TMPK3. (a) The hydrolysis of the OMFP was assayed for 30min in the presence of 1 µg of recombinant His-TMKP1, or dpHis-TMKP1 (diamonds and closed squares as indicated), used alone or mixed with GST-TMPK3 (triangles and stars as indicated), TMKP1 and TMPK3 were mixed using a molar ratio of 1:4. All data are mean values ± S.E of absorbance (λ = 477 nm) from four independent assays. (b) Effect of TMPK3/Ca^{2+} on His-TMKP1 phosphatase activity. TMKP1 activity was measured using a TMKP1/TMPK3 molar ratio of 1:4 either without or with increasing concentrations of Ca^{2+} (0–3 mM) and EGTA (5 mM) as indicated. All data are mean values ± S.E of initial rate V0 (10^{-3} µmol of OMF/min) from three independent assays. (***) indicates values significantly different from the control. (basal phosphatase activity of TMKP1). Statistical significance was assessed using the student t-test at $p < 0.01$.

motifs. Alignment with well known Mn^{2+} or Mg^{2+} binding proteins (http://www.uniprot.org) revealed the presence of putative Mg^{2+} and Mn^{2+} binding sites on the N-terminal region of TMKP1 sequence (position 71–79 for Mg^{2+} and 132–141 for Mn^{2+}, Suppl. Fig. 2). The same motifs were also found within the N-terminal regions of other plant MKPs including *Hordeum vulgare* (HvMKP1), *Zea mays* (ZmMKP1), *Oryza sativa* (OsMKP1), *Brachypodium distachyon* (BdMKP1) *Arabidopsis thaliana* (AtMKP1), *Nicotiana tabacum* (NtMKP1), *Glycine max* (GmMKP1) and *Gossypium arboretum* (GaMKP1) (Suppl. Fig. 2b and c). The presence of these well conserved motifs suggests that the activity of TMKP1 and other plant MKPs could be modulated by common regulatory mechanisms involving divalent cations. To reinforce our hypothesis, we carried out additional phosphatase assays on a N-terminal truncated form of TMKP1 lacking the first 135 amino acids (GST-ΔNTMKP1) that was previously characterized (Zaidi et al., 2010). This deletion leads to the removal of the putative Mg^{2+} and Mn^{2+} binding motifs. Our results showed that in the absence of any cation, GST-ΔNTMKP1 exhibited a weaker activity (~15% decrease) compared to the full length protein, as previously reported (Zaidi et al., 2010). However, the stimulatory effects of Mg^{2+} or Mn^{2+} on the truncated form were either abolished or significantly attenuated respectively, demonstrating the importance of these cation-binding sites at the N-terminal region of TMKP1 (Fig. 6).

The addition of TMPK3 resulted in higher phosphatase activities of GST-ΔNTMKP1 but the fold increase is lower compared to that observed on GST-TMKP1 (1.75 vs 3.6 fold at maximum). Moreover, the induction of GST-ΔNTMKP1 activity by TMPK3 remained almost identical in presence or absence of Mn^{2+} or Mg^{2+} confirming that Mn^{2+} and Mg^{2+}

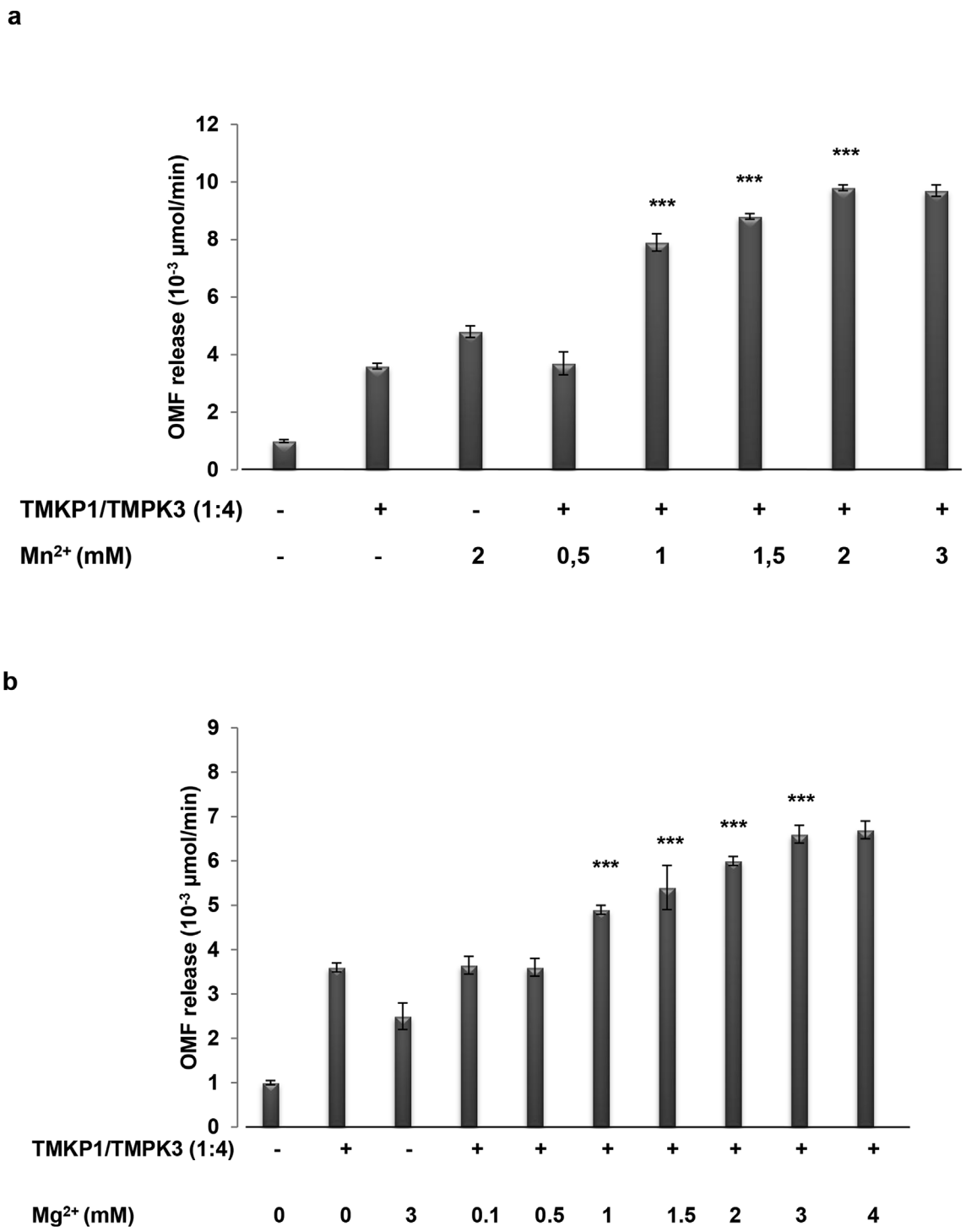

Fig. 3. Stimulatory effects of Mn^{2+} and Mg^{2+} on TMKP1 activity. In vitro phosphatase activity was assayed on 1 µg of His-TMKP1 using 500 µM OMFP as substrate. Assays were performed in the presence of TMPK3 (with TMKP1/TMPK3 molar ratio of 1:4) and increasing concentrations of Mn^{2+} (a), or Mg^{2+}(b). Values are means of initial rates V0 (10^{-3} µmol of OMF/min) ± S.E from three independent experiments. (***) indicates statistical significance compared to the control, as assessed by the student t-test at $p < 0.01$.

binding sites were abolished in this truncated form. Altogether, these data raise again the relevance of divalent cations Mg^{2+} and especially Mn^{2+} in stimulating TMKP1 activity and indicate that the optimal activation of TMKP1 by TMPK3 requires the binding of these metallic cations to conserved motifs at its N-terminal end.

3.5. Phosphatase activity of TMKP1 is differentially regulated by TMPK3 and TdCaM1.1/Ca^{2+} complex

We have previously demonstrated that TMKP1 phosphatase activity is inhibited by the *Arabidopsis* calmodulin (AtCaM1) in a calcium-dependent manner (Ghorbel et al., 2015). This inhibitory effect was also observed here with a durum wheat TdCaM1.1 (GenBank: KJ500005.1) that shares 98% of identity with AtCaM1. Like *Arabidopsis* ortholog, a

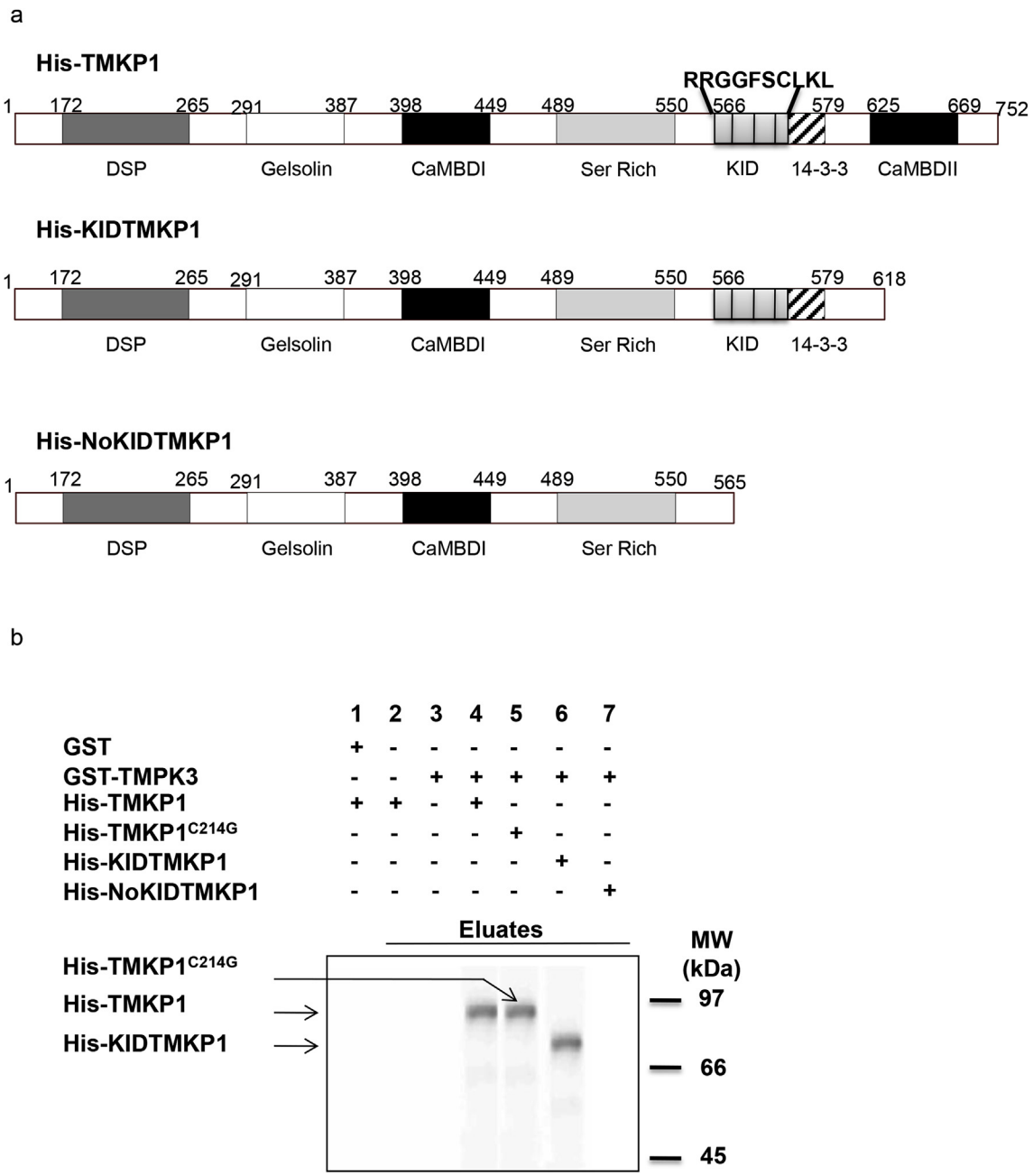

Fig. 4. TMKP1 interacts with TMPK3 via its C terminal part. (a) Schematic presentation of the different TMKP1 isoforms used in binding assays, full length TMKP1 (1–752; up) KIDTMKP1 (1-618aa; middle) and NoKIDTMKP1 (1-565aa; below). The conserved domains of TMKP1 including the catalytic dual specificity phosphatase (DSP), gelsolin, calmodulin-binding domains I and II (CaMBDI, CaMBDII), the serine-rich region, the Kinase interacting domain (KID) 14-3-3-binding motif are presented by boxes with distinct patterns. (b) GST pull-down assays for the TMKP1/TMPK3 interaction. Pulled down proteins were loaded on 10% SDS-PAGE, and analyzed by immunoblotting with anti-TMKP1 antibody. Eluates from GST grafted beads incubated with purified His-TMKP1 (lane 1); from ungrafted beads incubated with purified His-TMKP1 (lane 2); GST-TMPK3-bound sepharose beads alone (lane 3); GST-TMPK3 bound sepharose beads incubated with His-TMKP1 (lane 4), His-TMKP1^{C214G} (lane 5), His-KIDTMKP1 (lane 6), or His-NoKIDTMKP1 (lane 7). Positions of molecular weight markers are indicated on the right side of the panel. Experiments were repeated three times with identical results.

recombinant form (His-TdCaM1.1) alone had no effect but mixed with Ca^{2+}, it decreased the phosphatase activity of TMKP1. This inhibition was also shown to be TdCaM1.1 dose-dependent. Indeed by increasing the TMKP1/TdCaM1.1 M ratios from 1:2 to 1:6 the phosphatase activity decreased from 1 to ~5 times (Suppl. Fig. 3a). Moreover, the addition of EGTA, restored the activity of TMKP1 in presence of TdCaM1.1/Ca^{2+} whereas EGTA alone did not alter TMKP1 activity (Suppl. Fig. 3a), indicating that Ca^{2+} is necessary for the calmodulin inhibition of TMKP1 as previously shown for AtCaM1 (Ghorbel et al., 2015). Moreover, the inhibition of TMKP1 activity in presence of TdCaM1.1/Ca^{2+} complex was suppressed as with AtCaM1, when Mn^{2+} was added to the reaction mixture (Suppl. Fig. 3b). Together these results outline that TMKP1 is under the control of a conserved CaM1 mediated mechanism.

Finally, we investigated the effect of combining CaM/Ca^{2+} complexes and TMPK3 on the phosphatase activities of TMKP1. Our results showed that TMKP1 catalytic activity in the presence of TMKP1/TMPK3 molar ratio of 1:4, and increasing amounts of CaM/Ca^{2+} complex (with molar TMKP1/TdCaM1.1 ratios ranging from 1:1 to 1:8), was inhibited (Fig. 7a) and this inhibition was more pronounced with increasing Ca^{2+} concentrations in the reaction buffer (Fig. 7b).

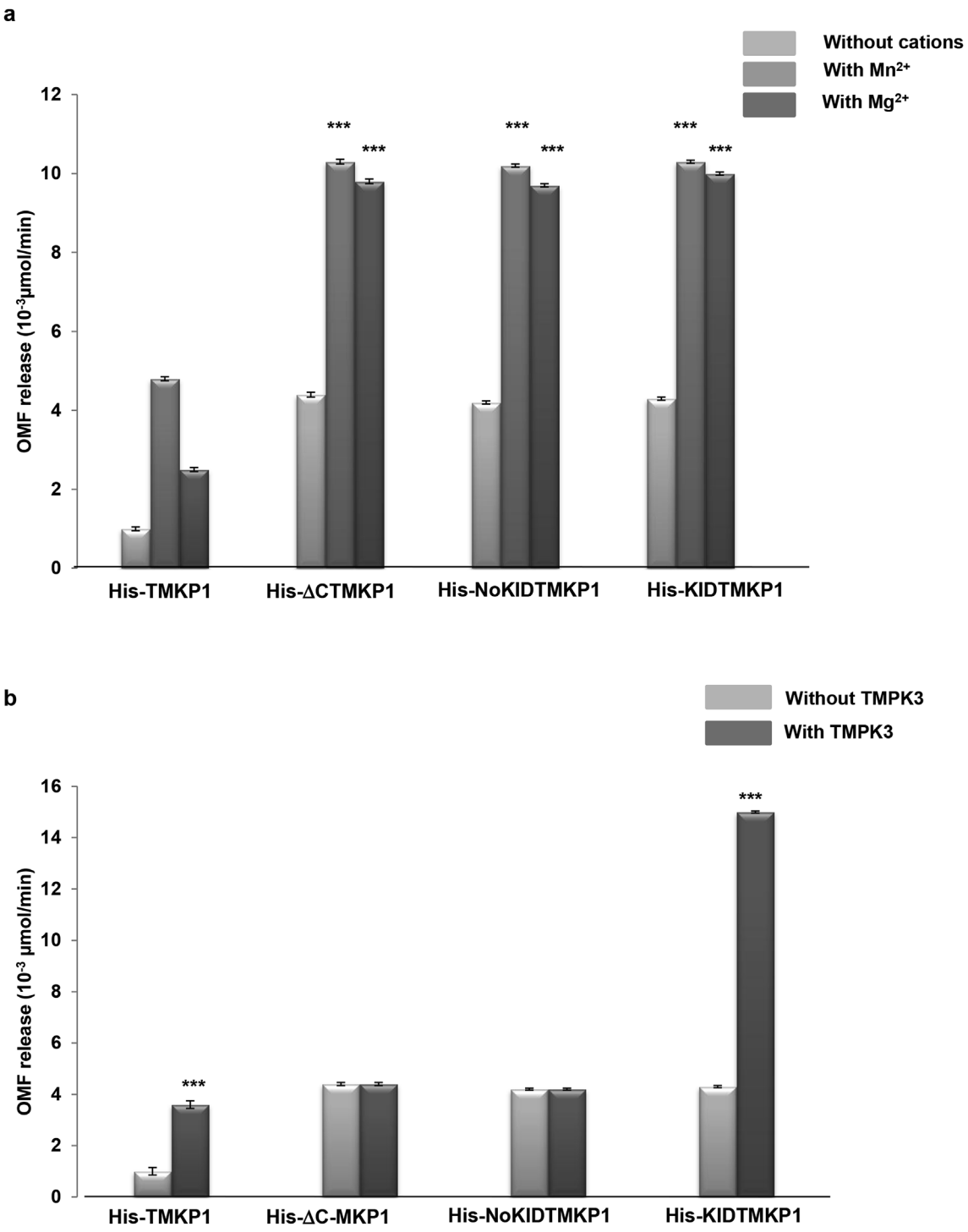

Fig. 5. Comparative analysis of the phosphatase activities of His-TMKP1 and its deletion derivatives. (a) Phosphatase assays were performed as indicated in Fig. 3, on 1 µg of His-TMKP1, His-ΔCTMKP1, His-NoKIDTMKP1 or His-KIDTMKP1, in the absence or the presence of 2 mM Mn^{2+} or Mg^{2+}. (b) Effect of TMPK3 on the phosphatase activity of TMKP1 or its deletion derivatives with molar ratios of 1:4. Values are means of initial rates (10^{-3} µmol of OMF/min) ± S.E from four independent experiments. (***) indicates statistical significance compared to the control, as assessed by the student t-test at $p < 0.01$.

However, the addition of Mn^{2+} to the mixture including TMKP3/TdCaM1.1/Ca^{2+}, resulted in the stimulation of TMKP1 phosphatase activity. In fact, the V0 of the reaction raised from 9.7 to 13.8 µmol/min in presence of Mn^{2+} (Fig. 7 c) reinforcing the relevance of Mn^{2+} ions in TMKP1 activity stimulation.

4. Discussion

Several studies describing how the binding of plant MKPs to various factors (Calmodulin, MAPKs, 14-3-3 proteins) resulted in a pronounced modulation of their activities, were reported (Katou et al., 2005; Yoo et al., 2004; Zaidi et al., 2010; Park et al., 2011; Ghorbel et al., 2015, 2017). In particular, the role of MPK substrates in increasing the MKP catalytic activities was documented in plants (Katou et al., 2005; Park

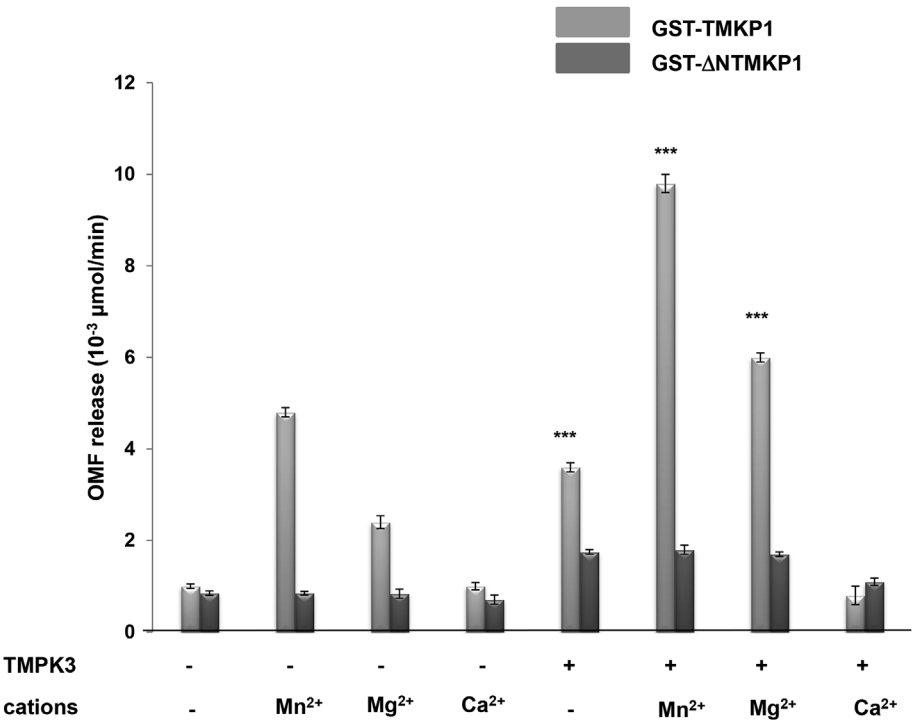

Fig. 6. Deletion of putative Mg^{2+} and Mn^{2+} binding sites suppressed the positive effect of the cations on TMKP1 phosphatase activity. The effects of addition of 2 mM Ca^{2+}, Mg^{2+} or Mn^{2+} divalent cations on phosphatase activity of 1 µg of recombinant GST-TMKP1 or GST-ΔNTMKP1. Values are means of initial rates (10^{-3} µmol of OMF/min) ± S.E from three independent experiments. Statistical (***) indicates values significantly different from the control. Statistical significance was assessed using the student t-test at $p < 0.01$.

et al., 2011). Recently, a question regarding phosphorylation of MKPs by their MPK substrates and how this post-translational modification can contribute to MAPK signaling specificity and physiological responses starts to emerge. In *Arabidopsis*, AtMKP1 was shown to be phosphorylated by its substrate, AtMPK6, in vitro and in planta (Park et al., 2011; Gonzelez-Beistero and Ulm, 2013), which resulted in enhanced phosphatase activity of AtMKP1 (Park et al., 2011). This phosphorylation occurs at four sites (Thr64, Thr109, Ser558, and Ser572) that are also conserved in several MKPs proteins in plants (Gonzalez-Beistero and Ulm, 2013). Most interestingly, the phosphorylation of AtMKP1 was shown to be required for its stabilization and accumulation under either UV stress or during PAMP (Pathogen-Associated Molecular Patterns) responses and bacterial resistance (Anderson et al., 2014; Gonzalez-Beistero and Ulm, 2013; Jiang et al., 2017).

Here, we provide through in gel kinase assays, evidence for a possible phosphorylation of TMKP1 under standard and salt stress conditions by similar wheat and *Arabidopsis* MAPKs including most likely MPK3 and MPK6 (Fig. 1). Bio-informatic analysis revealed the presence of 9 putative phosphorylation sites in TMKP1 sequence (T43; T84; S274; S288; S481; S486; S508; S536; S556) that have a proline amino acid at position −1; those amino acids are able to be phosphorylated by MPK3 as revealed by scansite3 server (http://scansite3.mit.edu). We showed here that TMPK3 stimulates the phosphatase activity of TMKP1 (Fig. 2a). Similarly, AtMKP2 was shown to be catalytically activated by AtMPK3 and AtMPK6 (Lee and Ellis, 2007). However, this MPK-mediated activation of plant MKPs cannot be considered as a general rule since in tobacco the binding of SIPK, homolog of AtMPK3, has no effect on NtMKP1 phosphatase activity (Katou et al., 2005). In our assays, we did not have an experimental evidence for TMKP1 activation by TMPK3 via its phoshorylation. Nevertheless, we have noticed that this activation occurs independently on its phosphorylation status (Fig. 2a). The stimulatory effect of TMPK3 observed on TMKP1 could be due simply to its binding which may result in a conformational change conferring hence higher catalytic activity. To test our hypothesis, we checked whether the binding of TMPK3 to TMKP1 is a prerequisite to its activation. The conserved D-motif at the C-terminal part of TMKP1 motif (566–575; Suppl. Fig.1a) is the most likely binding site for TMPK3. Indeed, we demonstrated by GST pull down assays performed on different truncated forms of TMKP1 that the D-motif might be required for TMKP1/TMPK3 interaction as was previously reported for AtMKP1 (Gonzalez-Beistero and Ulm, 2013, Fig. 4). Consequently, the deletion of the D-motif results in the abolition of the stimulatory effect of TMPK3 on TMKP1 activity. Therefore, it seems that TMPK3 activates TMKP1 perhaps through its direct binding to the D-motif at the C-terminal region.

The effect of TMPK3 was also investigated in the presence of divalent cations and/or CaM/Ca^{2+} that were previously shown to regulate the phosphatase activity of TMKP1 (Ghorbel et al., 2015). We showed here for the first time that the phosphatase activity of a plant MKP could be modulated by MAPKs and cations (Fig. 2 b, c; Fig. 3). Remarkably, our assays revealed that MKP1 can be inhibited by TMPK3 in presence of Ca^{2+} but stimulated by Mn^{2+} and with a lower extend Mg^{2+} cations (Fig. 3). Moreover, the positive effects of Mn^{2+} and Mg^{2+} cations were suppressed on a truncated form of TMKP1 where the first 135 aa were deleted (Fig. 6). This finding fits with the bio-informatic analyses that show the presence of Mg^{2+} (71–79) and Mn^{2+} (132–141) binding motifs in the N-terminal part of TMKP1 (Suppl. Fig. 2). Mn^{2+} and Mg^{2+} binding sites have almost the same sequence composition by sharing the same predominant motif of secondary structure. Moreover, both metal cations are frequently bound to Asp and Glu residues in “beta strand – random coil – beta strand” and “beta strand – random coil – alpha helix” structural motifs (Khrustalev et al., 2016). In association with Asp and Glu residues, His residues are considered as major binder of Mn^{2+} cations (Khrustalev et al., 2016).

On additional phosphatase assays, we found that the concomitant presence of TMPK3 and CaM/Ca^{2+} resulted in an inhibition of TMKP1 activity. Most interestingly, this inhibitory effect was suppressed by adding the Mn^{2+}cation to this mixture, indicating again the relevance of this cation in stimulating TMKP1 activity. From our data, it can be concluded that TMPK3 and CaM/Ca^{2+} complex exert differential regulatory effects on TMKP1 activity, depending on presence or absence of Mn^{2+} cations. How can Mn^{2+} mediate the activation of the TMKP1 activity in the presence of CaM/Ca^{2+} and/or TMPK3 remains an open question. It is plausible that Mn^{2+}cations might modify the TMKP1 conformation to promote or facilitate CaM and/or MPKs binding in a way that leads ultimately to the activation of the phosphatase activity.

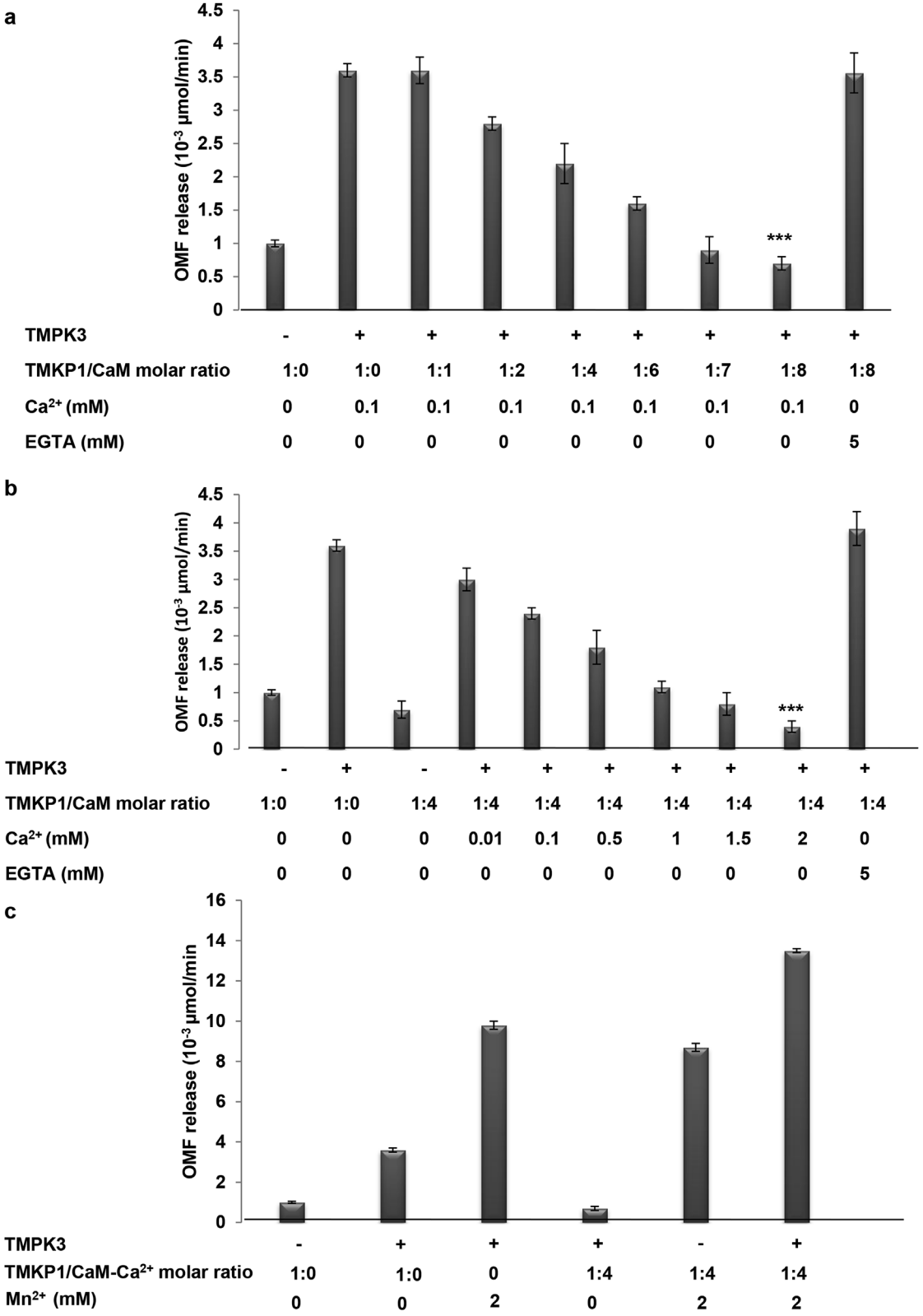

Fig. 7. Dual effects of the TdCaM1.1/Ca^{2+} complex on TMKP1 activity in presence of recombinant TMPK3. (a) Inhibitory effect of the TdCaM1.1/Ca^{2+} complex on His-TMKP1 activity. TMKP1 activity was measured using a fixed TMKP1/TMPK3 molar ratio of 1:4 and an increasing TMKP1/TdCaM1.1 M ratios ranging from 1:1 to 1:8, in the presence of 2 mM Ca^{2+} and EGTA (5 mM) as indicated. (b) Inhibitory effect of TdCaM1.1/Ca^{2+} complex on His-TMKP1 activity in presence of a fixed amount of GST-TMPK3 and increasing concentrations of Ca^{2+} (0–2 mM) (c) Effect of TdCaM1.1/Ca^{2+} complex on His-TMKP1 activity in presence of a fixed amount GST-TMPK3 in presence of 2 mM Mn^{2+}. Assays were carried out as indicated in Fig. 3. All data are mean values ± S.E of initial rate (10^{-3} µmol of OMF/min) from at least three independent assays. (***) indicate values significantly different from the control. Statistical significance was assessed by applying the student t-test at $p < 0.01$.

5. Conclusion

In conclusion, the data obtained in this study regarding TMKP1 provide evidence for complex regulatory mechanisms exerted on TMKP1 where TMPK3 and CaM/Ca^{2+} act in a differential manner depending on the presence of specific cations. Most importantly, our results strongly suggest that Mn^{2+} acts as a key cofactor in these regulatory mechanisms. Future work is needed to i) check whether a Mn^{2+}-mediated regulatory mechanism (involving also other partners such as MPK and CaM/Ca^{2+}) exists for other plant MKPs and ii) investigate its physiological significance especially during plant stress responses.

Conflicts of interest

The authors declare that they have no conflict of interest.

CRediT authorship contribution statement

Mouna Ghorbel: Investigation, Writing – original draft, Conceptualization, Writing – review & editing. **Ikram Zaidi:** Investigation, Methodology. **Chantal Ebel:** Conceptualization, Writing – review & editing, Supervision. **Moez Hanin:** Conceptualization, Writing – review & editing, Supervision.

Acknowledgments

We thank Elena Najar-Duran and Agnese Rabissi, (CRAG) for technical help in performing In gel Kinase assays. This work was supported by the core funding provided by the Ministry of Higher Education and Scientific Research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2018.12.016.

References

Anderson, Jeffrey C., Wan, Y., Kim, Y.-M., Pasa-Tolic, L., Metz, T.O., Peck, S.C., 2014. Decreased abundance of type III secretion system inducing signals in *Arabidopsis* mkp1 enhances resistance against Pseudomonas syringae. Proc. Natl. Acad. Sci. Unit. States Am. 111, 6846–6851.

Bartels, S., González Besteiro, M.A., Lang, D., Ulm, R., 2010. Emerging functions for plant MAP kinase phosphatases. Trends Plant Sci. 15, 322–329.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Ann. Biochem. 72, 248–254.

Brondello, J.M., Pouyssegur, J., McKenzie, F.R., 1999. Reduced MAP kinase phosphatase-1 degradation after p42/p44MAPK-dependent phosphorylation. Science 286, 2514–2517.

Ellis, E.B., 2012. Postal code for a plant MAPK. Biochem. J. 446, 5–7.

Gaestel, M., 2008. Specificity of signaling from MAPKs to MAPKAPKs: kinases' tango nuevo. Front. Biosci. 13, 6050–6059.

Ghorbel, M., Zaidi, I., Robe, E., Ranty, B., Mazars, C., Galaud, J.P., Hanin, M., 2015. The activity of the wheat MAP kinase phosphatase 1 is regulated by manganese and by calmodulin. Biochimie 108, 13–19.

Ghorbel, M., Cotelle, V., Ebel, C., Zaidi, I., Ormancey, M., Galaud, J.P., Hanin, M., 2017. Regulation of the wheat MAP kinase phosphatase 1 by 14-3-3 proteins. Plant Sci. 257, 37–47.

González Besteiro, M.A., Ulm, R., 2013. Phosphorylation and stabilization of *Arabidopsis* MAP kinase phosphatase 1 in response to UV-B stress. J. Biol. Chem. 288, 480–486.

Jiang, L., Anderson, J.C., González Besteiro, M.A., Peck, S.C., 2017. Phosphorylation of *Arabidopsis* MAP kinase phosphatase 1 (MKP1) is required for PAMP responses and resistance against bacteria. Plant Physiol. 175, 1839–1852.

Katou, S., Karita, E., Yamakawa, H., Seo, S., Mitsuhara, I., Kuchitsu, K., Ohashi, Y., 2005. Catalytic activation of the plant MAPK phosphatase NtMKP1 by its physiological substrate salicylic acid-induced protein kinase but not by calmodulins. J. Biol. Chem. 280, 39569–39581.

Katou, S., Kuroda, K., Seo, S., Yanagawa, Y., Tsuge, T., Yamazaki, M., Miyao, A., Hirochika, H., Ohashi, Y., 2007. A calmodulin-binding mitogen-activated protein kinase phosphatase is induced by wounding and regulates the activities of stress-related mitogen-activated protein kinases in rice. Plant Cell Physiol. 48, 332–344.

Khrustalev, V.V., Barkovsky, E.V., Khrustaleva, T.A., 2016. Magnesium and manganese binding sites on proteins have the same predominant motif of secondary structure. J. Theor. Biol. 395, 174–185.

Kusari, A.B., Molina, D.M., Sabbagh Jr., W., Lau, C.S., Bardwell, L., 2004. A conserved protein interaction network involving the yeast MAP kinases Fus3 and Kss1. J. Cell Biol. 164, 267–277.

Lee, J.S., Ellis, E.B., 2007. Arabidopsis MAPK phosphatase 2 (MKP2) positively regulates oxidative stress tolerance and inactivates the MPK3 and MPK6 MAPKs. J. Biol. Chem. 282, 25020–25029.

Lee, K., Song, E.H., Kim, H.S., Yoo, J.H., Han, H.J., Jung, M.S., Lee, S.M., Kim, K.E., Kim, M.C., Cho, M.J., Chung, W.S., 2008. Regulation of MAPK phosphatase 1 (AtMKP1) by calmodulin in *Arabidopsis*. J. Biol. Chem. 283, 23581–23588.

Li, H., Ding, Y., Shi, Y., Zhang, X., Zhang, S., Gong, Z., Yang, S., 2017. MPK3- and MPK6-mediated ICE1 phosphorylation negatively regulates ICE1 stability and freezing tolerance in Arabidopsis. Dev. Cell 43, 1–13.

MAPK group, 2002. Mitogen-activated protein kinase cascades in plants: a new nomenclature. Trends Plant Sci. 7, 1360–1385.

Park, H.C., Song, E.H., Nguyen, X.C., Lee, K., Kim, K.E., Kim, H.S., Lee, S.M., Kim, S.H., Bae, D.W., Yun, D.J., Chung, W.S., 2011. *Arabidopsis* MAP kinase phosphatase 1 is phosphorylated and activated by its substrate AtMPK6. Plant Cell Rep. 30, 1523–1531.

Rasmussen, M.W., Roux, M., Petersen, M., Mundy, J., 2012. MAP kinase cascades in *Arabidopsis* innate immunity. Front. Plant Sci. 3, 169.

Reffas, S., Schlegel, W., 2000. Compartment-specific regulation of extracellular signal-regulated kinase (ERK) and c-Jun N-terminal kinase (JNK) mitogen-activated protein kinases (MAPKs) by ERK-dependent and non-ERK-dependent inductions of MAPK phosphatase (MKP)-3 and MKP-1 in differentiating P19 cells. Biochem. J. 352, 701–708.

Remenyi, A., Good, M.C., Lim, W.A., 2006. Docking interactions in protein kinase and phosphatase networks. Curr.Op.in Struc. Biol. 16, 676–685.

Rodriguez, M.C., Petersen, M., Mundy, J., 2010. Mitogen-activated protein kinase signaling in plants. Annu. Rev. Plant Biol. 61, 621–649.

Sohaskey, M.L., Ferrell Jr., J.E., 2002. Activation of p42 mitogen-activated protein kinase (MAPK), but not c-Jun NH2-terminal kinase, induces phosphorylation and stabilization of MAPK phosphatase XCL100 in Xenopus oocytes. Mol. Biol. Cell 13, 454–468.

Yamakawa, H., Mitsuhara, I., Ito, N., Seo, S., Kamada, H., Ohashi, Y., 2004. Transcriptionally and post-transcriptionally regulated response of 13 calmodulin genes to tobacco mosaic virus-induced cell death and wounding in tobacco plant. Eur. J. Biochem. 168, 3916–3929.

Yoo, J.H., Cheong, M.S., Park, C.Y., Moon, B.C., Kim, M.C., Kang, Y.H., Park, H.C., Choi, M.S., Lee, J.H., Jung, W.Y., Yoon, H.W., Chung, W.S., Lim, C.O., Lee, S.Y., Cho, M.J., 2004. Regulation of the dual specificity protein phosphatase, DsPTP1, through interactions with calmodulin. J. Biol. Chem. 279, 848–858.

Zaidi, I., Ebel, C., Touzri, M., Herzog, E., Evrard, J.L., Schmit, A.C., Masmoudi, K., Hanin, M., 2010. TMKP1 is a novel wheat stress responsive MAP kinase phosphatase localized in the nucleus. Plant Mol. Biol. 73, 325–338.

Zaidi, I., González, A., Touzri, M., Alvarez, M.C., Ramos, J., Masmoudi, K., Ariño, J., Hanin, M., 2012. The wheat MAP kinase phosphatase 1 confers higher lithium tolerance in yeast. FEMS Yeast Res. 12, 774–784.

Zaidi, I., Ebel, C., Belgaroui, N., Ghorbel, M., Amara, I., Hanin, M., 2016. The wheat MAP kinase phosphatase 1 alleviates salt stress andincreases antioxidant activities in *Arabidopsis*. J. Plant Physiol. 193, 12–21.