

# TMKP1 is a novel wheat stress responsive MAP kinase phosphatase localized in the nucleus

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**Abstract** The regulation of plant signalling responses by Mitogen-Activated Protein Kinases (MAPKs)-mediated protein phosphorylation is well recognized. MAP kinase phosphatases (MKPs) are negative regulators of MAPKs in eukaryotes. We report here the identification and the characterization of TMKP1, the first wheat MKP (*Triticum turgidum* L. *subsp.* *Durum*). Expression profile analyses performed in two durum wheat cultivars showing a marked difference in salt and drought stress tolerance, revealed a differential regulation of *TMKP1*. Under salt and osmotic stress, *TMKP1* is induced in the sensitive wheat variety and repressed in the tolerant one. A recombinant TMKP1 was shown to be an active phosphatase and capable to interact specifically with two wheat MAPKs (TMPK3 and TMPK6). In BY2 tobacco cells transiently expressing GFP::TMKP1, the fusion protein was localized into the nucleus. Interestingly, the deletion of the N-terminal non catalytic domain results in a strong accumulation of the truncated fusion protein in the cytoplasm. In addition, when expressed in BY2 cells, TMPK3 and TMPK6 fused to red fluorescent protein (RFP) were shown to be present predominantly in the nucleus. Surprisingly, when co-expressed with the N-terminal truncated TMKP1 fusion

protein; both kinases are excluded from the nuclear compartment and accumulate in the cytoplasm. This strongly suggests that TMKP1 interacts *in vivo* with TMPK3 and TMPK6 and controls their subcellular localization. Taken together, our results show that the newly isolated wheat MKP might play an active role in modulating the plant cell responses to salt and osmotic stress responses.

**Keywords** Abiotic stress · Durum wheat · MAP kinase · MAP kinase phosphatase · GFP · RFP · Nuclear localization

## Introduction

To cope with highly variable environmental stresses, plants have to set a series of adaptation mechanisms ranging from cellular metabolism to physiological and developmental responses. It is well established that signal transduction based on protein phosphorylation plays a pivotal role in response to extracellular stimuli. The mitogen activated protein kinases (MAPK) cascade is perhaps the most important of these routes. The MAPK-based signal transduction pathways orchestrate a variety of cellular processes such as cell growth and death, differentiation, and stress responses (Mishra et al. 2006; Jonak et al. 2002; Qi and Elion 2005; Morris 2001). Each MAPK pathway is composed of three functionally linked kinase modules, MAPKKK, MAPKK and MAPK. MAPKKKs are serine/threonine kinases that activate MAPKKs through the phosphorylation of two Serine/threonine residues in a conserved S/T-X<sub>3-5</sub>-S/T motif. MAPKK are dual specificity kinases that phosphorylate MAPKs on threonine and tyrosine residues in the T-X-Y motif. MAPKs are serine/threonine kinases that activate by phosphorylation various

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substrates including other protein kinases, transcription factors and cytoskeletal proteins. Although MAPK signalling cascades exist in all eukaryotes, the composition and function of specific components may differ significantly (Morris 2001). In yeast and animals, the MAPKs comprise three major groups (or subfamilies), the extracellular signal-regulated kinases (ERK), the c-Jun amino (NH<sub>2</sub>)-terminal kinases or stress-activated protein kinases (JNK/SAPK) and high osmolarity glycerol response or p38 kinases (Hog/p38) (Qi and Elion 2005). In contrast, the majority of plant MAP kinases described belong to the ERK group, whereas only few belong to the Hog/p38 and none so far to the JNK subfamilies (Morris 2001; Nakagami et al. 2005).

Plant MAPK-encoding genes were first reported in pea (Stafstrom et al. 1993), alfalfa (Duerr et al. 1993; Jonak et al. 1993), tobacco (Wilson et al. 1993), and *Arabidopsis* (Mizoguchi et al. 1993). Since then, many MAPKs were described in rice, petunia and others (Nakagami et al. 2005; Jonak et al. 2002). The involvement of MAPKs in abiotic and biotic stress responses was extensively studied (Nakagami et al. 2005; Mishra et al. 2006). Previous studies have shown that two tobacco MAPKs WIPK (wound-induced protein kinase) and SIPK, (salicylic acid-induced protein kinase), as well as their corresponding MAPKs homologs in other dicotyledonous species including *Arabidopsis*, alfalfa, tomato and parsley, are activated by a wide range of biotic and abiotic stresses such as wounding, osmotic shock, high salinity, drought, UV irradiation, ozone exposure, extreme temperature, oxidative stress and pathogen infection (Seo et al. 1999; Ichimura et al. 2000; Zhang and Klessig 2001; Tena et al. 2001; Asai et al. 2002; Jonak et al. 2002; MAPK group 2002; Holley et al. 2003; Kroj et al. 2003; Del Pozo et al. 2004). In addition, the rice OsMPK5a and OsMPK6 are also activated by a variety of stresses such as wounding, drought, high salinity, cold and pathogen infection (Agrawal et al. 2003; Xiong and Yang 2003; Kurusu et al. 2005; Lieberherr et al. 2005). Combined to transient expression assays, yeast two-hybrid analyses of MAPK cascades in *Arabidopsis*, tobacco and *Medicago* showed that MAPK pathway components seem to function in different combinations and might have distinct functions in different biological contexts. In *Arabidopsis*, MEKK1 (MAPKKK) interact with MPKK4 and MPKK5 in pathogen defence (Asai et al. 2002), but can also activate MKK2 during abiotic stress (Teige et al. 2004).

Accumulating evidence has demonstrated that differences in the duration and the magnitude of MAPK activities regulate signalling specificity (Ebisuya et al. 2005; Pouyssegur and Lenormand 2003). In other words, fine tuning the MAPK activity is essential in determining the signalling outcome. Thus, inactivation of MAPK plays a

crucial role in various physiological processes. Active MAPKs are inactivated through dephosphorylation of threonine and/or tyrosine residues within the activation loop. The dephosphorylation could be achieved by serine/threonine phosphatases (including PP2A (Kristensson and Andersson 2005) and PP2C), tyrosine phosphatases and dual-specificity MAPK phosphatases (MKPs) which are highly specific to MAPK (for review see Schweighofer and Meskiene 2008). The MKPs act as negative regulators of MAPK and dephosphorylate both serine/threonine and tyrosine residues (Keyse 2000; Theodosiou and Ashworth 2002).

In contrast to many members of the MAPK family, plant MKPs perhaps form a small gene family. For 20 putative MAPKs, only five MKPs are predicted in the *Arabidopsis* genome (Ulm et al. 2002). While MAPKs were significantly studied in plants, only few reports dealing with the relevance of MKPs activity/function/role were published. In *Arabidopsis*, mutations of the *AtMKP1* cause sensitivity to genotoxic stress but increased tolerance to salt stress (Ulm et al. 2001; Ulm et al. 2002). *AtMKP1* interacts with MPK6 and to a lesser extent with MPK3 and MPK4. These results suggest that MKP1 regulates genotoxic and salt stress response pathways, with MPK6 as a common component for both routes. The ability of MKP1 to dephosphorylate MPK6 remains, however, to be proven. Silencing of *AtMKP2*, another *Arabidopsis* MKP gene, results in ozone sensitivity indicating its role in oxidative stress response. In addition, it has been shown that overexpression of *NtMKP1*, a putative tobacco ortholog of *AtMKP1*, compromised wound-induced activation of SIPK and WIPK (Yamakawa et al. 2004). On the other hand, the rice homolog of *AtMKP1*, *OsMKP1* is induced by wounding and seems to be involved in the negative regulation of wound response in rice (Katou et al. 2007). Despite the relevance of plant MKPs in the regulation of stress response, *OsMKP1* remains up to now, the unique example described in economically relevant monocotyledonous crop.

In this study we report the identification and the characterization of the first wheat MKP (TMKP1), the ortholog of *AtMKP1* and *OsMKP1*. We investigated gene expression of *TMKP1* in response to salt and osmotic stress, in two *durum* wheat varieties showing marked difference in their salt and drought stress tolerance. In vitro phosphatase activity of TMKP1 as well as its interaction with two wheat MAPKs, TMPK3 and TMPK6 are reported. We further show that TMKP1 has a nuclear localization and contributes into the control of the sub-cellular localization of the two interacting wheat MAPK. All the data presented in this report raise the importance of TMKP1 as a potential regulator of stress signalling pathways in wheat.

## Materials and methods

### Plant materials and stress treatments

Two cultivars of *durum* wheat (*Triticum turgidum* L. subsp. *durum*) Mahmoudi and Om Rabia3 were supplied by INRAT, Laboratoire de Physiologie Végétale (Tunis, Tunisia). Seeds of each line were sterilized in 0.5% NaOCl solution supplemented with 0.5 mg/ml prodazim, for 15 min, then washed 3 times with sterile water and placed on Petri dishes with wet Wattman 3MM filter paper for germination. Ten-days old seedlings were treated, when indicated, with NaCl (200 mM) or 15% polyethylene glycol 8000 (PEG 8000) for 6 and 24 h. Naproxen was added during salt stress at final concentration of 1 mM.

### Isolation and cloning of the full-length TMKP1 cDNA

Total RNA was extracted using the RNeasy Plant mini Kit (Qiagen) according to the manufacturer's instructions. Approximately, 100 mg of frozen leaves of 10 days-old seedlings of *durum* wheat Mahmoudi variety treated with 200 mM NaCl for 24 h, was used for RNA extraction.

The primers TMKP1-MF2 (5'-GTAGCTGGAGTTTAC TAAGG-3') and TMKP1-MR33 (5'GGACGGAGAGCG TATTGATCCCA-3') based on a previously published EST (Accession no. AJ606016) allowed the amplification of a ~350 bp cDNA fragment corresponding to TMKP1 sequence. The full length cDNA of *TMKP1* was generated using the Gene Racer Kit (Invitrogen) based on RNA ligase-mediated (RLM-RACE) and oligo-capping rapid amplification of cDNA ends (RACE) methods. This technique allows the production of 5' and 3' ends of cDNA. The 5'RACE was achieved with the primers Gene Racer 5'-Nested (5'-GGACACTGACATG-GACTGAAGGAGT A-3') and TMKP1-MR40 (5'-GGGAGCTAGTTGAAG GTGA-3') giving rise to 1.7 kb fragment then cloned in pCR4Blunt-TOPO vector (Invitrogen) to create the plasmid p5'TM. For the 3'RACE, the two primers Gene Racer 3'-Nested (5'-CGCTACGTAACGGCATGACAGTG-3') and TMKP1-MF2 were used. The resulting PCR fragment of 1.5 kb was then cloned in pCR4Blunt-TOPO vector giving rise to the construct p3'TM. The inserts in the two constructs have an overlapping sequence within the ORF of *TMKP1* including a single *Bam*HI site. In order to assemble the whole *TMKP1* cDNA, p5'TM was digested by *Bam*HI and a fragment of 1.5 kb was inserted in the same site in p3'TM resulting in a construct pCTM containing the full length cDNA. Finally the *TMKP1* ORF was amplified from the pCTM plasmid with the primers OTM-N (5'-GCGGCCCATGGCCAACCCCG-3') and TRV1 (5'-GCTCTAGATTAACGAGCA-TCCAGCACCTC-3') harbouring *Nco*I and *Xba*I sites, respectively. The PCR

amplification yielded a ≈2.4 kb fragment which was cloned in pCR4Blunt-TOPO vector giving rise to the plasmid pZNX-B harbouring the ORF of *TMKP1*. All indicated constructs were sequenced in both orientations using the dye terminator cycle sequencing method (Applied Biosystems). The sequence data of *TMKP1* has been deposited in GenBank database (Accession #EU502843). Sequence homologies against databases were performed using BLAST programs (Altschul et al. 1997).

### Reverse transcription-PCR analyses

For the semi-quantitative assay of transcript levels in the two *durum* wheat varieties Mahmoudi and Om Rabia3, the following protocol was used. Total RNA was extracted from roots of both varieties using Trizol reagent (Invitrogen) according to the manufacturer's protocol. The RNA was treated with DNaseI (Promega) at 37°C for 15 min. DNase-treated RNA samples (2 µg) were reverse-transcribed using M-MLV reverse transcriptase (Invitrogen). The reverse transcription (RT) reactions were performed at 37°C for 1 h using 2 µM oligo-dT<sub>18</sub>. Diluted aliquots (1:20) of the resulting RT-reaction product were then employed as template for PCR amplification generating a *TMKP1* specific fragment of 550 bp using the following primers: TMKP1-MF4: 5'-CACCTGTTAATTCAGCAGCA-3' and TMKP1-MR33. A wheat *Actin* gene fragment used as an internal control was amplified with the primers: ActF: 5'-GGC GATGAAGCTCAATCCAAACG-3' and ActR: 5'-GGTCACGACCAGCAAGAT CAAGACG-3'. For PCR reactions, samples were denatured for 5 min at 94°C and then run for 30 cycles of 30 s at 94°C, 30 s at 56°C, and 30S at 72°C, with a final extension of 5 min at 72°C. The PCR products were then separated by electrophoresis in 1% (w/v) agarose gel. For each stress treatment, at least three independent experiments have been performed.

### Southern blot analysis

Genomic DNA (15 µg) extracted from wheat leaves using Nucleon Phytopure DNA extraction and purification kit (Amersham) was digested separately with *Eco*RI, *Xba*I, *Bgl*II and *Sal*I restriction enzymes. The digested fragments were fractionated by electrophoresis in 0.8% (w/v) agarose gel and transferred to a Hybond N+ nylon membrane (Amersham). The full length *TMKP1* ORF labelled with α<sup>32</sup>P-dCTP was used as a probe. The blotted membrane was prehybridized for 4 h and then hybridized with the labelled probe overnight at 42°C in a buffer containing 4× SSC, 0.1% (w/v) SDS, 50% formamide, 10% (w/v) dextrane sulphate and 200 µg/ml salmon sperm DNA. Following hybridization, the membrane was washed twice

with 2× SSC, 0.1% SDS at 42°C for 30 min and twice with 0.2× SSC, 0.1% SDS at 42°C for 30 min and exposed to X-ray film (Kodak).

#### Expression and purification of the recombinant TMKP1 and TMPK3 proteins in *Escherichia coli*

The full length, truncated ORFs of TMKP1 and the full length ORF of TMPK3 (accession no. AAC28850, Takezawa 1999) were amplified by PCR, digested by the appropriate restriction enzyme, and cloned in-frame with GST into the pGEX-2TK expression vector (Amersham). The resulting constructs pGST-TMKP1, pGST-TMKP1ΔN<sub>1–135</sub>, pGST-TMKP1-N<sub>1–135</sub> and pGST-TMPK3 were introduced into the Rosetta *E. coli* strain (DE3) (Novagen, Madison, WI, USA). The production of the different recombinant proteins (GST::TMKP1, GST::TMKP1ΔN<sub>1–135</sub>, GST::TMKP1-N<sub>1–135</sub> and GST::TMPK3) was induced by 100 μM isopropyl β-D-thiogalactopyranoside (IPTG) for 3 h at 37°C. Protein extraction and purification of recombinant proteins on Glutathione Sepharose 4B beads (Amersham Biosciences) were performed as described previously (Janski et al. 2008). Protein quantification was performed using the Bradford method (1976).

#### Antibody production and immunoblot analyses

A polyclonal antiserum was raised in rabbits using 2 mg of purified GST::TMKP1 as an antigen. The first and second immunization injections were performed with complete Freund adjuvant (CFA) and incomplete Freund adjuvant (IFA), respectively. An initial blood collection is performed on a rabbit before immunization to be used as a pre-immune serum control.

For immunoblot analyses, proteins were extracted from 0.3 g wheat leaf tissues in 2 ml of a buffer containing HEPES–NaOH (pH 7.5), 50 mM, 5 mM MgCl<sub>2</sub>, 1 mM DTT, 0.1% CHAPS, 5% glycerol and protease inhibitors cocktail (Complete EDTA-free, Roche Diagnostics). The homogenate was placed on ice for 2 min and centrifuged at 14,000 g for 10 min at 4°C. The resulting supernatant was sampled for proteins which were quantified according to the Bradford method (Bradford 1976). Proteins samples were then electrophoretically separated on 10% polyacrylamide gel and transferred to polyvinylidene difluoride membrane (Millipore Corp). Following blocking with milk, membranes were probed with different dilutions of the rabbit anti-TMKP1 (1/5,000, 1/10,000 and 1/20,000) serum individually using the MiniSlot™ system (Immunetics®, Boston, Massachusetts, USA.). After extensive washing in PBS × 1, 0.01% Tween 20, the horseradish peroxidase—conjugated goat anti-rabbit IgG (Molecular Probes, 1/10,000 dilution) was added as a secondary antibody. Signal detection was

performed as described in the ECL western detection kit (Amersham Biosciences). For stress treatments, immunoblot analyses using 1/5,000 dilution of the anti-TMKP1 serum were performed on total proteins (10 μg) extracted from roots of MH and OR varieties as described above.

#### GST pull down assay

TMKP1 and its deletion derivatives (GST::TMKP1, GST::TMKP1ΔN<sub>1–135</sub> and GST::TMKP1-N<sub>1–135</sub>) were used for in vitro interaction with two wheat MAPKs (TMPK3 and TMPK6) by a GST pull down assay. *NcoI* and *EcoRI* sites were introduced by PCR into the TMPK3-ORF using K<sub>3</sub>AD<sub>1</sub> (5'-TTACCATGGAGATGGACGGCGCTCCGG-3') and K<sub>3</sub>AD<sub>2</sub> (5'-TTAGAATTCCT-AGTATCGGAAGTTGGG-3') primers, and into TMPK6 ORF (accession no. AY173962) using K<sub>6</sub>AD<sub>1</sub> (5'-TTACCATGGAGATGGACGCCGGCGGGG-3') and K<sub>6</sub>AD<sub>2</sub> (5'-TTA GAATTCCTACTGGTAATCAGGGTTG-3') primers. Both PCR fragments were then digested by *NcoI* and *EcoRI* and inserted into the same sites on the pGADT7 expression vector (Clontech). The resulting recombinant plasmids were employed to produce in vitro [<sup>35</sup>S]-methionine-labelled TMPK3 and TMPK6 using the TNT coupled Reticulocyte Lysate System (Promega), in the presence of T7 RNA polymerase. Prior binding, GST::TMKP1, GST::TMKP1ΔN<sub>1–135</sub> and GST::TMKP1-N<sub>1–135</sub> proteins were immobilized on Glutathione Sepharose 4B beads. The radiolabelled TMPK3 or TMPK6 proteins were then incubated with the immobilized proteins for 1 h 30 min at 4°C. After five washes with the STE buffer (Tris–HCl 10 mM; pH 8, EDTA 1 mM, NaCl 150 mM), proteins were dissociated from the beads by incubation (5 min at 100°C) in Tris–HCl 50 mM, pH 6.8, DTT 100 mM, SDS 2%, glycerol 10%, bromophenol blue 0.1% and separated by SDS–PAGE. The labelled TMPK3 and TMPK6 were detected by autoradiography.

#### Phosphatase activity

The phosphatase assay was performed on purified GST::TMKP1, GST::TMKP1ΔN<sub>1–135</sub> using the 3-*O*-methylfluorescein phosphate (OMFP, Sigma) as substrate. Phosphatase activity was measured at 30°C during 1 h in 0.8 ml of reaction buffer (50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 1 mM EDTA, 500 μM OMFP). The amount of 3-*O*-methylfluorescein product was measured by absorbance at 477 nm.

#### Subcellular localization of TMKP1

The *TMKP1* ORF was amplified from pZNX-B by the primers Ph1Mlu-5' (5'TTAACGCGTAATGGCCAACC



CCGACG-3') and Ph1Mlu-3' (5'TTAACGCG-TATTAAC GAGCATCCAGCA-3'). For the amplification of the 5'-deleted *TMKP1* ORF, the Ph1Mlu-5' was replaced with Ph1Mlu- $\Delta$ 5' primer (5'-TTAACGCGTATACCTTGGCGG CGACG-3'). The N terminal region corresponding to the first 135 a.a of *TMKP1*, was also amplified using Ph1Mlu-5' and NPh1Mlu3' (5'TTAACGCGTAGACGTGCTCG GCCACCTT3') primers. All primers harbour extension with a *MluI* restriction site to facilitate the cloning of the PCR products in-frame with GFP into pCKeGFP derived expression vector (Heim et al. 1995) digested with the same enzyme. *TMPK3* and *TMPK6* ORFs were cloned in pNER- $\times$ 1 vector (Jean-Luc Evrard unpublished) in frame with a red monomeric fluorescent protein (RFP). To this end, *TMPK3* and *TMPK6* ORFs were first amplified from pGADT7 based constructs by FMPK3 (5'CATGCCATGG ACGGCGCTCCGGT-GGCCGAG3') with RMPK3 (5'CC GCTCGAGCGTATCGGAAGTTGGGGTTCA-3') primers and by FMPK6 (5'CATGCCATGGACGCCGCGGGG CGCAGCC-G3') with RMPK6 (5'GGAA-GATCTCTGG TAATCAGGGTTGAACG3') primers, respectively. Both PCR products were then digested with *NcoI/XhoI* (for *TMPK3* ORF) or with *NcoI/BglIII* (for *TMPK6* ORF) before their ligation into pNER- $\times$ 1 vector, digested with appropriate enzymes. The resulting constructs GFP::TMKP1, GFP-TMKP1 $\Delta$ N<sub>1-135</sub>, GFP-TMKP1-N<sub>1-135</sub>, *TMPK3*-RFP and *TMPK6*-RFP were introduced into BY2 tobacco cells (Nagata et al. 1992) by the particle delivery method using a Bio-Rad Biolistic PDS 1000/He system according to the instruction manual. The bombarded cells were incubated on Murashige and Skoog (MS) media containing plates for 6 h at 25°C. Visualization was performed by Zeiss LSM510 confocal laser scanning microscope (CLSM) equipped with argon and helium/neon lasers, standard filters, and a 63 $\times$ , 1.2 numerical aperture (NA) C-Apochromat water-immersion lens. Images were processed with LSM510 version 2.8 (Zeiss).

## Results

### Isolation of *TMKP1*, a *durum* wheat MAP kinase phosphatase

Interested in decoding stress signal transduction via MAP Kinase phosphatases in wheat, we used specific primers designed according to a previously published EST of 397 bp (Accession no. AJ606016) from common wheat (*Triticum aestivum*) spikelets, and showing high homology with other plant MKPs (Ciaffi et al. 2005). By performing RT-PCR on total RNA from *durum wheat* variety Mahmoudi, we amplified a cDNA fragment showing sequence identity to the published EST. After 5' and 3'

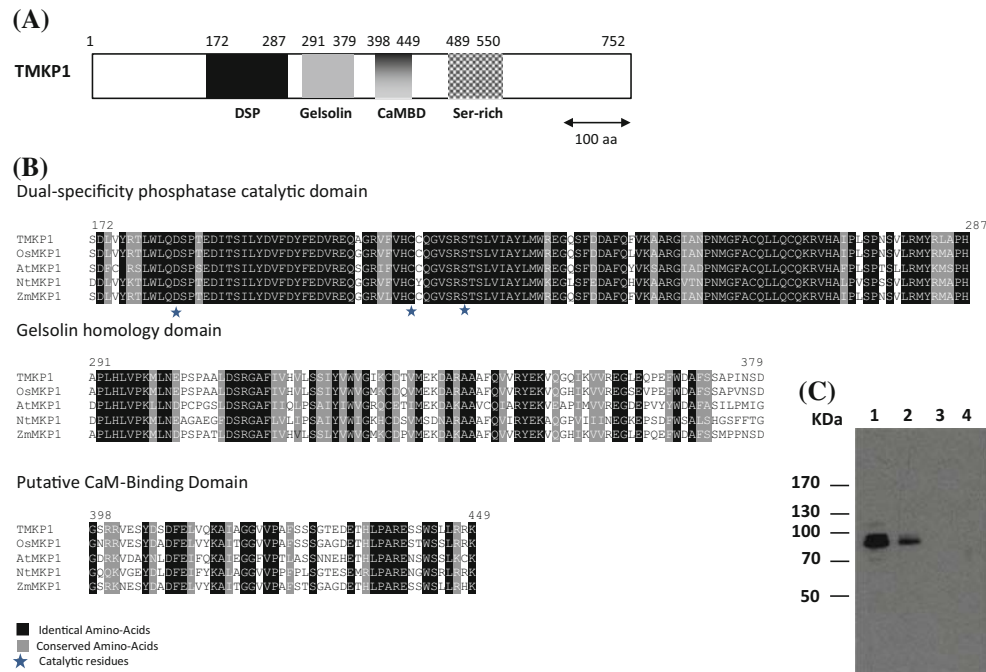
RACE (see materials and methods) we reconstituted a full length cDNA named *TMKP1* (Accession no. EU502843) of 2,866 bp. Sequence analyses revealed an ORF of 2,259 bp with a 5' UTR of 52 bp and a 3'UTR of 555 bp. The deduced protein sequence has a size of 752 amino acids, with a predicted molecular mass of 81,8 kDa.

The amino acid sequence of *TMKP1* shows high homology with known plant MKPs including those of *Z. mays* (*ZmMKP*), *O. sativa* (*OsmMKP1*), *A. thaliana* (*AtMKP1*) and *N. tabacum* (*NtMKP1*). The highest homology was found with the rice monocotyledonous counterpart (79% identity and 84% homology).

The isolated *TMKP1* shares all common features of other plant MKPs (Fig. 1A, B). The N-terminal region displays the catalytic domain with the PTP-DSP overlapping motifs (172–287 a.a). Particularly, the DSPs signature motif (VHCcQGsRSTSLVIA YLM; amino acids 212–230) with the conserved cysteine (Cys<sup>214</sup>) was identified in this domain. Indeed, this well conserved cysteine is essential for the catalytic activity of all known DSPs since it is required for the nucleophilic attack of substrates' phosphorus and the formation of thiol-phosphate intermediate. Two additional residues (Asp<sup>183</sup> and Ser<sup>221</sup>) were also found within the catalytic domain of *TMKP1*. The Aspartate enhances catalysis by protonating the leaving oxygen group and the serine is necessary for the hydrolysis of the thiol-phosphate intermediate (Theodosiou and Ashworth 2002). The catalytic part is followed by a domain similar to the actin-binding protein gelsolin (291–379 a.a). In addition, a region enriched in basic and hydrophobic residues (398–449 a.a) shows high homology with the previously characterized calmodulin-binding domains of *NtMKP1* and *OsmMKP1* (Yamakawa et al. 2004; Katou et al. 2005). The two amino acids that were shown to be critical for CaM binding are also conserved (Trp<sup>442</sup> and Leu<sup>445</sup>). Finally, a serine tract region (especially between residues 489–550) is also present at the C-terminal part of *TMKP1*.

The gene product of *TMKP1* was analyzed by western blot performed on total protein extracts prepared from wheat (*T. durum Desf.*) leaves and using a polyclonal antibody raised against a recombinant *TMKP1* protein (see below). Our result showed a specific band slightly higher than 80 kDa and close to the predicted molecular weight of *TMKP1* (Fig. 1C).

Genomic organization of *TMKP1* locus was accessed by Southern blot hybridizations of DNA isolated from wheat (*T. durum Desf.*) leaves and digested by *BglIII*, *EcoRI*, *Sall* and *XbaI* endonucleases. Southern blot analysis revealed two bands with *Sall*, *BglIII*, and *EcoRI* but not with *XbaI* digested DNA, when probed with the full length *TMKP1* ORF, suggesting that the wheat genome might have 1 or 2 copies of the *TMKP1* gene (Fig. 2).



**Fig. 1** Schematic presentation and western blot analysis of TMKP1 of *Triticum durum*. **A** Structure of TMKP1 protein showing the positions of the conserved domains including the catalytic dual specificity (DSP) domain, gelsolin, the CaMBD, and the serin rich region. **B** alignment of the conserved domains shared by plant MKPs. Identical, conserved and catalytic amino acids are indicated with *black boxes*, *grey boxes*, and *stars*, respectively. The DDBJ/GenBank/EMBL accession numbers of the *durum* wheat TMKP1, tobacco NtMKP1, *Arabidopsis* AtMKP1, maize ZmMKP1, and *rice* OsMKP1,

are EU502843, AB117525, AF312745, AF312746, and BAF46959, respectively. **C** Accumulation of TMKP1 protein by western blot analysis. Ten micrograms of total proteins extracted from leaves of the wheat variety Mahmoudi were loaded and the protein gel blot was probed with an anti-serum against GST::TMKP1 (see “Materials and methods”). Lane 1, 2, and 3: immunoblots using 1/5,000, 1/10,000 and 1/20,000 dilutions of anti-TMKP1 serum, respectively, Lane 4: immunoblot using a pre-immune serum as negative control

### Expression pattern of TMKP1

We were interested in comparing the expression profile of *TMKP1* in two *durum* wheat varieties exhibiting a marked difference in salt and drought stress tolerance (Brini et al. 2008): a sensitive variety called Mahmoudi (MH) and a tolerant variety called Om Rabia3 (OR).

RT-PCR experiments were performed on root tissues of both varieties submitted to salt (NaCl) and osmotic stress treatments (PEG) (Fig. 3). Under standard growth conditions, *TMKP1* transcript seems to accumulate at distinct levels in OR and MH. Considering the sensitive MH variety and following salt treatment, *TMKP1* transcripts accumulation started after 6 h reaching higher levels after 24 h of treatment. Comparatively, in the tolerant variety OR, the *TMKP1* expression profile is seemingly the opposite. Indeed, in roots, *TMKP1* seems to be repressed rather than induced after salt and osmotic stress.

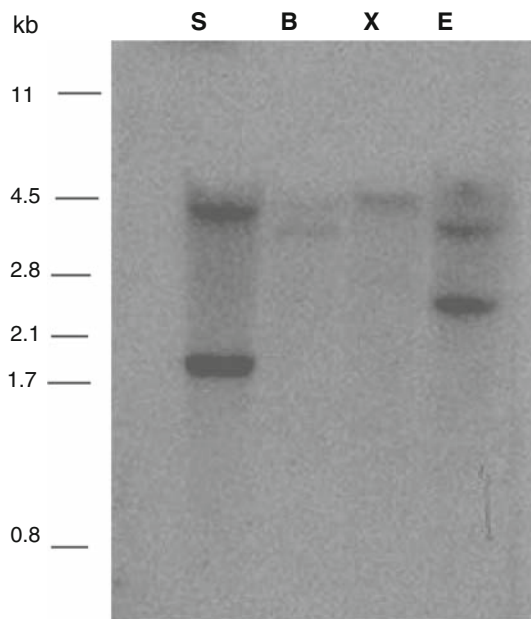
Knowing that abscisic acid (ABA) is a key phytohormone regulating abiotic stress response including salinity (Hirayama and Shinozaki 2007), we were interested in investigating whether salt induction observed in sensitive MH variety roots depends on ABA. Therefore, we performed

a salt stress assay on MH in the presence of naproxen, an inhibitor of the dioxygenase responsible for the generation of xanthoxal, one of the last steps in the ABA biosynthesis pathway (Lee and Milborrow 1997; Hansen and Grossmann 2000). Our results showed that naproxen suppresses the salt induced expression of *TMKP1* in roots (Fig. 3). These data suggest that the salt induction of *TMKP1* seems to be ABA-dependent in the roots of MH variety.

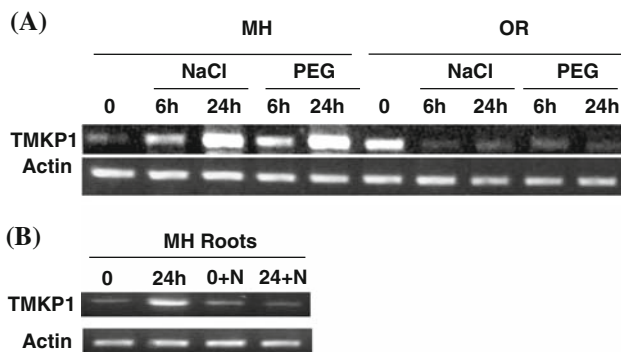
Furthermore, in order to monitor the accumulation of *TMKP1* protein following stress exposure, immunoblots were carried out on protein extracts of roots of the two wheat varieties. Higher amounts of *TMKP1* accumulate in MH variety after 24 h of salt and PEG stress treatment. Inversely, these treatments result in a strong reduction of *TMKP1* proteins in the OR variety (Fig. 4). The immunoblots are in agreement with RT-PCR data, showing that upon stress, *TMKP1* is indeed differentially regulated in the two wheat varieties, i.e. induced in MH and repressed in OR.

### TMKP-1 interacts with TMPK 3 and TMPK 6

*Arabidopsis* MPK3 and MPK6 are known to be involved in diverse abiotic and biotic stress responses and to be targets



**Fig. 2** Southern blot analysis of the genomic organisation of *TMKP1* gene. Genomic DNA extracted from the leaves of *Triticum durum* Desf. (Variety Mahmoudi) was cleaved with *EcoRI*, *XbaI*, *BglII* and *SalI* and the membrane was probed with the full length *TMKP1* ORF. DNA fragments of lambda DNA digested with *PstI* were used as molecular markers (indicated on the left)



**Fig. 3** RT-PCR analyses of *TMKP1* expression. **A** The effects of salt stress (200 mM NaCl) and osmotic stress PEG (15%). RT-PCR products were generated from RNA samples extracted from treated roots of two wheat varieties Oum rabiia (*OR*) and Mahmoudi (*MH*). Times of treatments (0, 6, and 24 h) are indicated. **B** The effect of naproxen on salt (24 h treatment) regulated expression of *TMKP1* in roots of *MH* variety. All data presented are representative of at least three independent experiments

of AtMKP1 (Nakagami et al. 2005; Ulm et al. 2001). Looking for potential wheat MAPKs (TMPK) interacting with TMKP1, we naturally chose TMPK3 (known as WCK-1, accession no. AAC28850) and TMPK6 (accession no. AY173962), highly homologous to AtMKP3 and AtMKP6, respectively.

To investigate the specificity of interaction of TMKP1 with TMPK3 and TMPK6, full length (GST::TMKP1) and

truncated forms of TMKP1 were produced in *E. coli* and employed in a GST pull down assay (Fig. 4). We used two truncated forms: the N-terminal deleted version (GST::TMKP1 $\Delta$ N<sub>1–135</sub>) and a large C-terminal deleted version where only the 135 first amino acids were maintained (GST::TMKP1-N<sub>1–135</sub>).

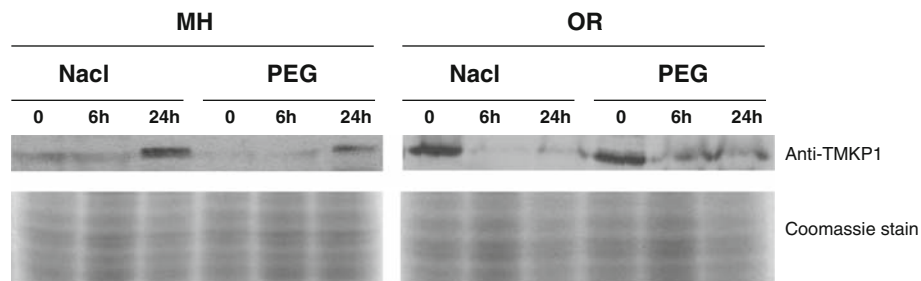
In parallel, both TMPK3 and TMPK6 were translated in vitro using a Reticulocyte Lysate based system in the presence of [<sup>35</sup>S] methionine (Fig. 5). The radiolabelled TMPK3 or TMPK6 were incubated with either full length or truncated forms of TMKP1 immobilized on the glutathion Sepharose beads. After SDS-PAGE and autoradiography, the radiolabelled TMPK3 or TMPK6 were detected in the fractions containing GST::TMKP1 and GST::TMKP1 $\Delta$ N<sub>1–135</sub>, but not in those containing GST::TMKP1-N<sub>1–135</sub> or GST alone (used as a negative control) (Fig. 5). These results show that TMKP1 interacts in vitro with TMPK3 and TMPK6 and the N-terminal domain (at least the first 135 a.a) is not required for these interactions.

#### TMKP1 has a phosphatase activity

To determine whether TMKP1 is an active phosphatase, the recombinant forms of this protein (GST::TMKP1, GST::TMKP1 $\Delta$ N<sub>1–135</sub>) produced in *E. coli*, were purified on glutathion Sepharose beads (Fig. 6A) and used for a phosphatase assay using OMFP as a substrate. The phosphatase activity is reflected by the hydrolysis of the OMFP releasing the 3-*O*-methylfluorescein adducts measurable by its absorbance at 477 nm. As shown in Fig. 6B, we observed a time dependent increase of the reaction product, indicative of a constant phosphatase activity of TMKP1. The N-terminal deleted form (GST::TMKP1 $\Delta$ N<sub>1–135</sub>) has ~20% less phosphatase activity compared to the full length protein. This result shows that indeed the wheat MKP1 has a phosphatase activity but the N-terminal domain is not essential for that activity in vitro. Moreover, the addition of a recombinant TMPK3 (GST::TMPK3) results in a twofold increase of the phosphatase activity of TMKP1. Similar fold increase in the activity of the N-terminal truncated form of TMKP1 was allocated to GST::TMPK3 (Fig. 6C).

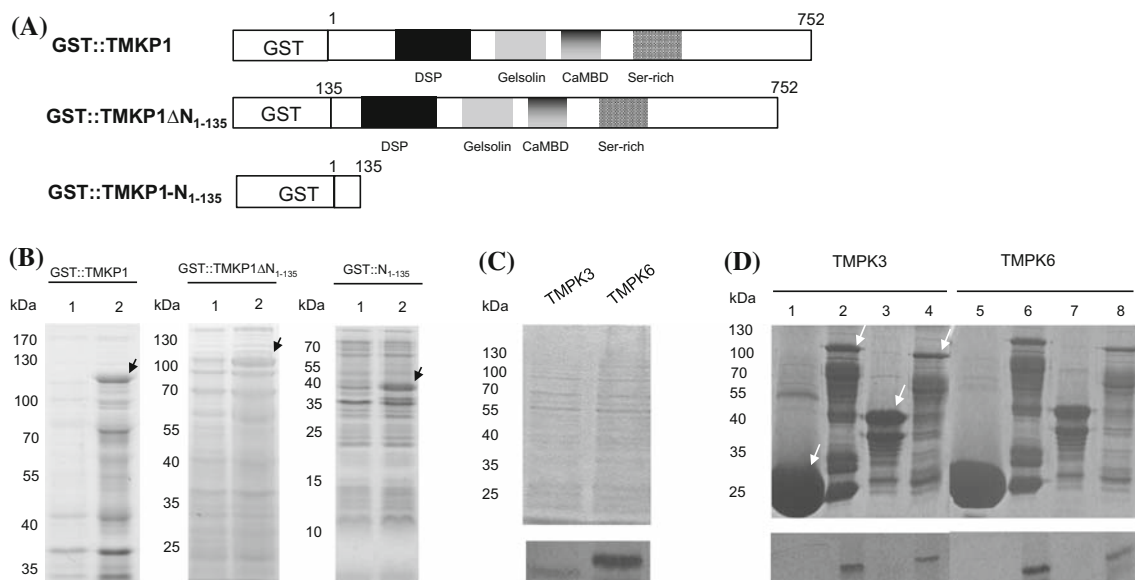
#### TMKP1 protein is localised in the nucleus

As mentioned earlier, plant MKPs are not well characterized and especially no data on subcellular localization of plant MKPs have been published so far. To better understand the biological function of TMKP1, we were interested to know where TMKP1 exerts its function. We therefore performed transient expression assays by particle bombardment of BY2 tobacco cultured cells with



**Fig. 4** Immunoblot analysis of TMKP1 protein levels in roots of MH and OR wheat varieties, with anti-TMKP1 serum. Both varieties were exposed to 200 mM NaCl or 15% PEG for indicated times (0, 6, and

24 h). On lower panels, protein loading controls are shown on 10% SDS-PAGE stained with Coomassie blue



**Fig. 5** TMKP1 interacts in vitro with TMPK3 and TMPK6. **A** Schematic representation of TMKP1 fragments fused in frame to GST. **B** SDS-PAGE analysis using Coomassie Brilliant Blue R 250 of protein extracts from bacterial cultures producing GST::TMKP1, GST::TMKP1 $\Delta$ N<sub>1-135</sub> and GST::TMKP1-N<sub>1-135</sub> proteins. Total proteins from non induced (lane 1) and IPTG-induced cells (lane 2) are presented. Arrows indicate the position of the band corresponding to the expected recombinant protein. **C** Production of [<sup>35</sup>S]-methionine radiolabelled TMPK3 and TMPK6 proteins in vitro. The upper panel is a Coomassie blue-stained SDS-PAGE gel and the lower panel is the autoradiogram where the bands of both proteins detected.

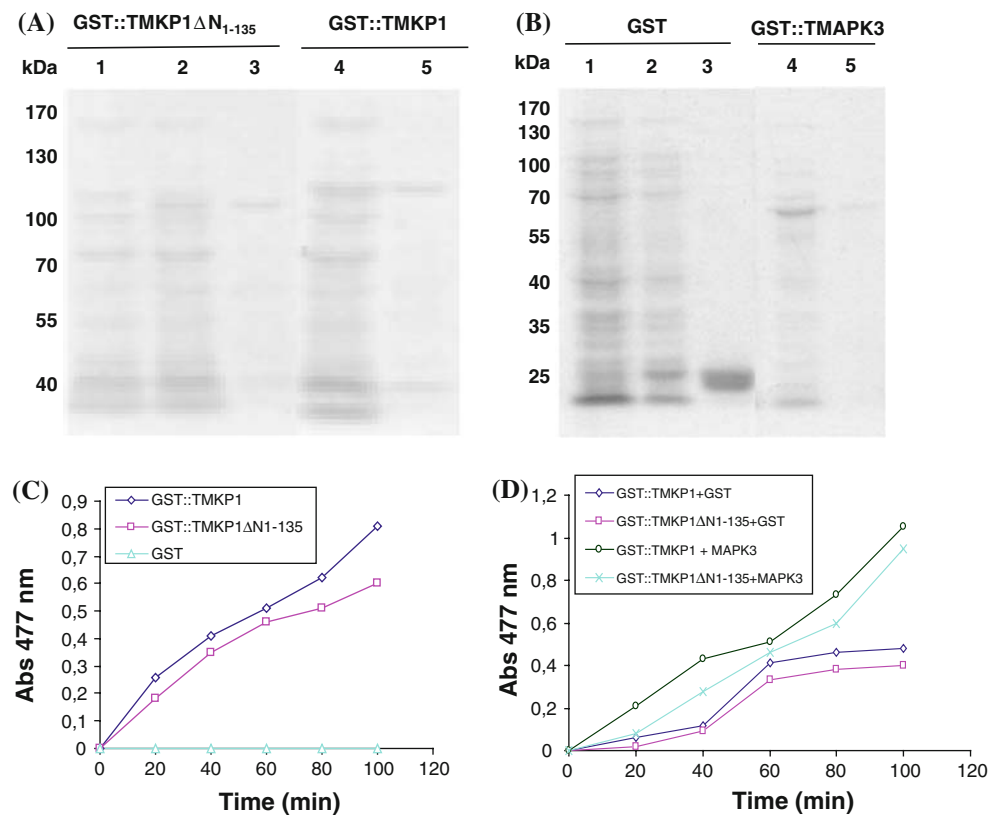
**D** GST pull down analysis of full-length and truncated forms of TMKP1 with TMPK3, and TMPK6, respectively. GST (lanes 1, 5), GST::TMKP1 (lanes 2, 6), GST::TMKP1-N<sub>1-135</sub> (lanes 3, 7) and GST::TMKP1 $\Delta$ N<sub>1-135</sub> (lanes 4, 8) are indicated by white arrows. Proteins were immobilized on Glutathione-Sepharose 4B beads to which [<sup>35</sup>S]-methionine radiolabelled TMPK3 and TMPK6 were added. Beads-bound proteins were analyzed on Coomassie Blue-stained SDS/PAGE gel (upper panel). The pulled-down TMPK3 and TMPK 6 are detected by autoradiography (lower panel). The size of protein standards is given in kDa on the left of each SDS-PAGE gel

constructs expressing N-terminal TMKP1 fusions to eGFP. Confocal microscopy analyses of the transformed cells showed that, unlike eGFP distributed evenly in the nucleus and the cytoplasm, GFP::TMKP1 accumulates in the nucleus (Fig. 7). The size of GFP::TMKP1 (109 kDa), above the exclusion limit of the nuclear pore (about 60 kDa, Mattaj and Englmeier 1998), indicates an active nuclear import. TMKP1 sequence analysis did not reveal any typical nuclear localization signal. To determine the sequence requirement for the nuclear localization, we transformed BY2 cells with GFP::TMKP1 $\Delta$ N<sub>1-135</sub>. The confocal microscopy observations revealed a strong

accumulation of the truncated-TMKP1 in the cytoplasm indicating that the N-terminal part is required for the nuclear localization of TMKP1. This finding was further confirmed when the N-terminal domain was fused to eGFP (GFP::TMKP1-N<sub>1-135</sub>). In this case, the fluorescence regains the nuclear compartment of BY-2 cells with even a stronger intensity in the nucleolus.

Since TMKP1 interacts in vitro with TMPK6 and TMPK3, we looked for TMKP1/TMPK3 and TMKP1/TMPK6 co-localizations in plant cell; we performed a novel series of transient expression assays on BY-2 cells including translational fusions of TMPK3 and TMPK6 with red





**Fig. 6** Phosphatase activity of the recombinant TMKP1 proteins. **A** SDS–PAGE analyses of total proteins isolated from non induced (lane 1), IPTG induced (lanes 2, 4) bacterial cells and after partial purification of GST::TMKP1 $\Delta$ N<sub>1-135</sub> (lane 3) and GST-TMKP1 (lane 5) proteins. **B** SDS–PAGE analyses of total proteins isolated from non induced (lane 1), IPTG induced (lanes 2, 4) bacterial cells and after partial purification of GST (lane 3) and GST::TMKP3 (lane

5) proteins. Positions of molecular weight markers are indicated on the left; **C**, **D** time courses of TMKP1 phosphatase activity using OMFP as a substrate. Activities registered on GST-TMKP1, GST::TMKP1 $\Delta$ N<sub>1-135</sub> and GST proteins in the absence (**C**) or in the presence of GST::TMKP3 (**D**) are presented. All experiments were repeated three times, and the data from one representative assay are shown here

fluorescent protein (RFP) (TMPK3::RFP, 66 kDa and TMPK6::RFP, 70 kDa). Bombardment of BY-2 cells with TMPK3::RFP or TMPK6::RFP constructs results in a significant (intense) signal in the nucleus (Fig. 8). When expressed together with GFP::TMKP1, TMPK3::RFP and TMPK6::RFP seem to accumulate also intensively in the nucleus. Unexpectedly, after the co-transformation of BY-2 cells with either TMPK3::RFP or TMPK6::RFP and GFP::TMKP1 $\Delta$ N<sub>1-135</sub>, both wheat kinases are excluded from the nucleus and accumulate mainly, like the truncated TMKP1, in the cytoplasm (Fig. 9). These results suggest that the interaction of TMKP1 with TMPK3 or TMPK6, detected *in vitro* (see above), also occurs *in vivo*. Moreover, and interestingly, they also suggest that the cellular distribution of both TMPKs is under the control of TMKP1.

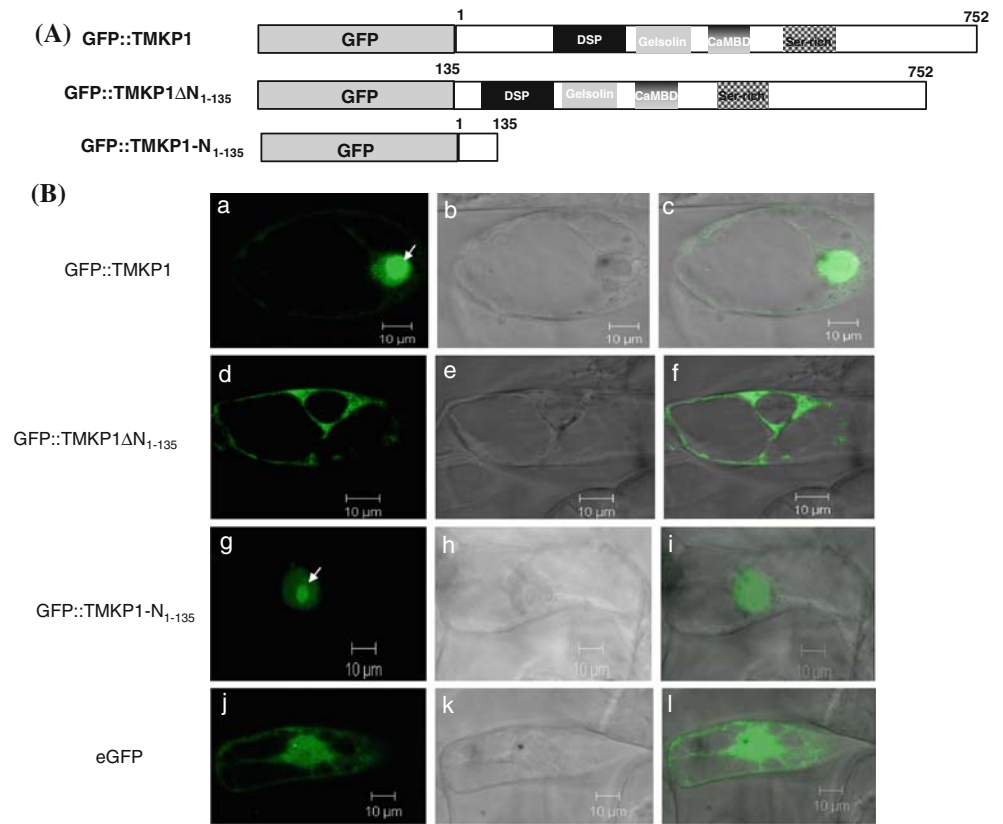
## Discussion

An important determinant of the final biological response in a cell is the magnitude and duration of the activation of a

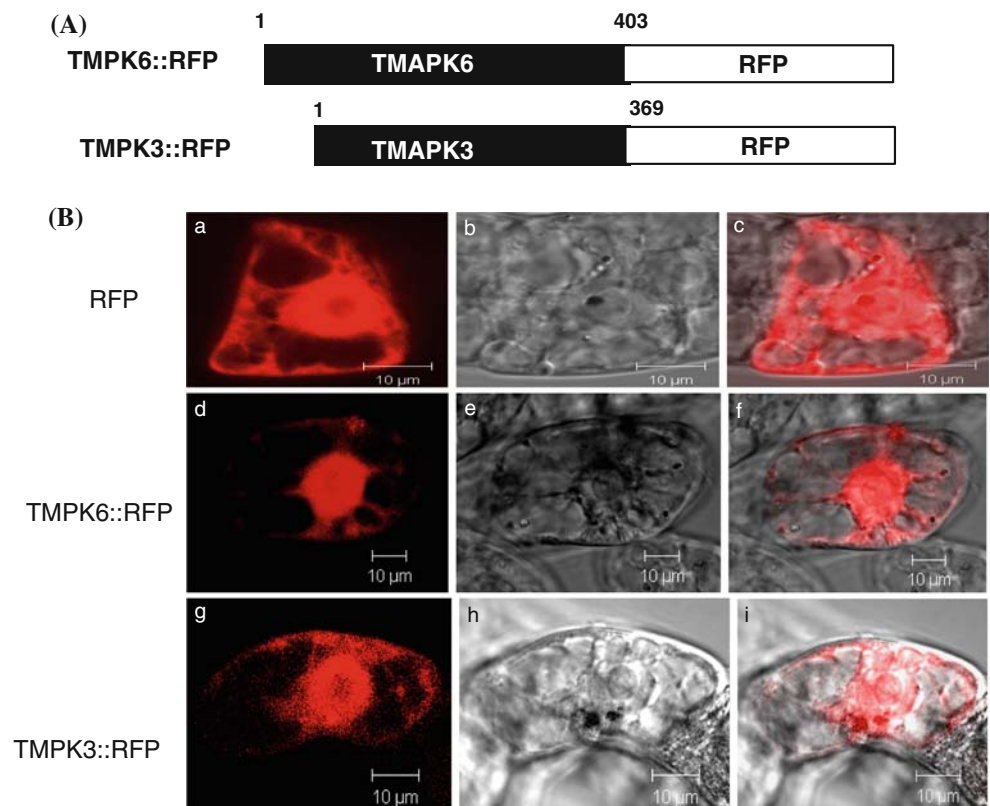
set of MAPKs in a given stress situation, which is governed by the upstream activating MAP kinases and the deactivating phosphatases. This concept is supported by a vast amount of biochemical and genetic data from mammals on the importance of MKPs in setting appropriate stress responses (Camps et al. 2000). In plants, our understanding about the biological roles of MKPs in controlling MAPK signalling pathways is still in its infancy.

In the present study, we report the identification and the molecular characterization of the first MKP in wheat (TMKP1). The amino acid sequence analysis of TMKP1, shows that it shares all common features with other plant MKPs. The N-terminal region displays the catalytic domain (172–287 a.a) including the signature motif for DSPs (VHC<sub>c</sub>qGvsRSTSLVIAAYLM) with the conserved cysteine (Cys<sup>214</sup>). The catalytic domain of TMKP1 is followed by a gelsolin homology domain (291–374 a.a) suggesting a potential role in cytoskeleton regulation. To our knowledge, this domain was not described in any MKP other than those from plants. As gelsolin is involved in actin remodeling when activated by calcium (Kwiatkowski

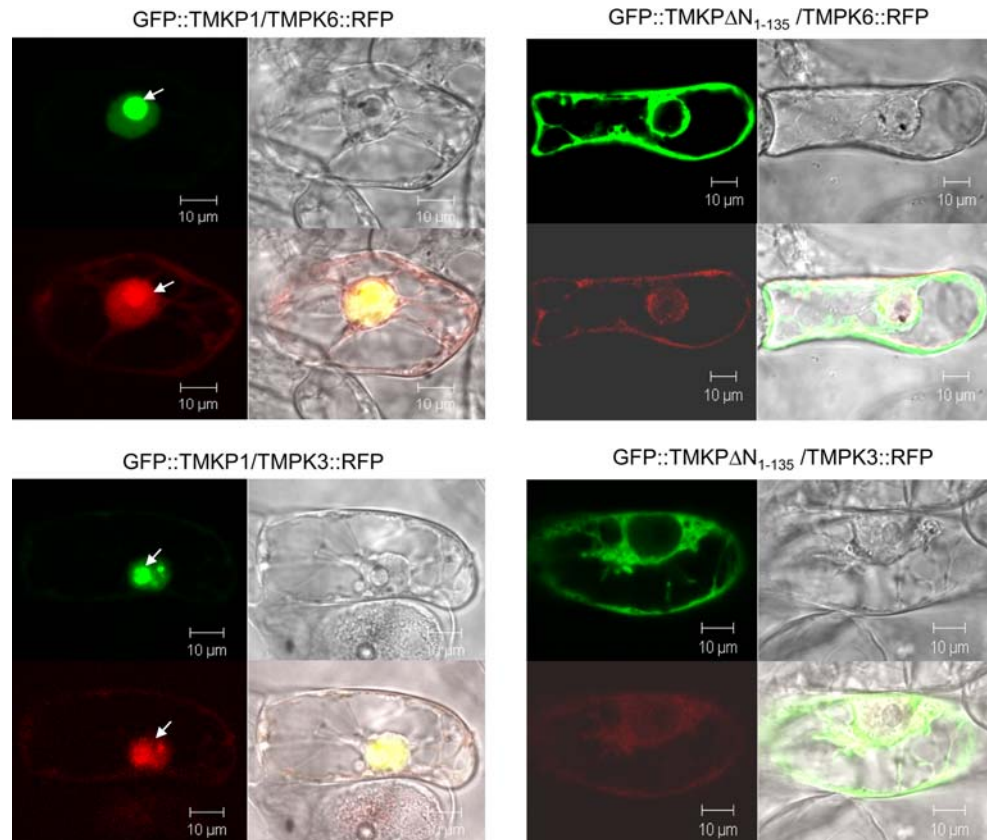
**Fig. 7** Subcellular localization of the TMKP1 protein. **A** Schematic representation of TMKP1 fragments fused in frame with GFP. N-terminal and C-terminal amino acid positions and functional domains are indicated. **B** Subcellular localizations were investigated in BY2 tobacco cells by confocal microscopy after particle bombardment with pCKeGFP empty vector (used as a control), or constructs containing the various GFP fusions (GFP::TMKP1, GFP::TMKP1 $\Delta$ N<sub>1–135</sub>, and GFP::TMKP1-N<sub>1–135</sub>). Confocal GFP images (*a, d, g, j*) as well as DIC (*b, e, h, k*) and composite images of GFP and DIC (*c, f, i, l*) are shown. Nucleoli are indicated by white arrows. Scale bar, 10  $\mu$ m



**Fig. 8** Subcellular localization of TMPK3 and TMPK6. **A** Schematic representation of TMPK3 and TMPK6 full length ORFs fused to RFP (TMPK3::RFP and TMPK6::RFP) N-terminal and C-terminal amino acid positions are indicated. **B** Both fusions as well as RFP alone used as a control, were transiently expressed into BY-2 tobacco cells and observed under confocal microscope 6 h post-bombardment. Confocal RFP images (*a, d, g*) as well as DIC (*b, e, h*) and composite images of RFP and DIC (*c, f, i*) are shown. Scale bar, 10  $\mu$ m



**Fig. 9** Cellular co-localization of TMKP1 with TMPK3 and TMPK6. BY-2 cells co-expressing various combinations of GFP::TMKP1 or GFP::TMKP1 $\Delta$ N<sub>1-135</sub> and TMPK3::RFP or TMPK6::RFP as indicated, were observed under confocal microscope. Images on the *left* show GFP or RFP detection and those on the *right* are composite images of GFP or RFP and DIC. Nucleoli are indicated by *white arrows*. Scale bar, 10  $\mu$ m



1999), speculating that plant MKPs might be required for both stress response and cytoskeleton dynamics exploring both phosphorylation cascades and calcium fluxes, is an attractive model. Furthermore, a putative calmodulin (CaM) binding domain is identified (398–449 a.a). CaM, a small acidic protein, plays a central role in transducing  $\text{Ca}^{2+}$  signals by modulating activities of numerous target proteins (Kao et al. 2000). In animal cells,  $\text{Ca}^{2+}$ /CaM-dependent protein phosphorylation and dephosphorylation are implicated in the regulation of a number of cellular processes. However, little is known on the functions of  $\text{Ca}^{2+}$ /CaM-dependent protein kinases and phosphatases in  $\text{Ca}^{2+}$  signalling in plants. Yet, the CaM binding domain seems to be another unique feature for plant MKPs and phosphatases. Specific binding of CaM to recombinant NtMKP1, AtMKP1 and OsMKP1 proteins was demonstrated (Katou et al. 2005, 2007; Lee et al. 2008). However, this binding is not essential for the inactivation of SIPK by NtMKP1 *in vivo* and *in vitro* (Katou et al. 2005). In contrast to NtMKP1, the phosphatase activity of *Arabidopsis* DsPTP1 depending on the substrate, can be stimulated or inhibited by CaM (Yoo et al. 2004). The phosphatase activity of AtMKP1 was increased by CaM in a  $\text{Ca}^{2+}$ -dependent manner (Lee et al. 2008). Therefore, a role of CaM binding in modulating the function of plant MKPs and other phosphatases cannot be ruled out.

We investigated in this report the expression profile of *TMKP1* in two *durum* wheat varieties with marked differences in drought and salinity stress tolerance. Our data showed that *TMKP1* expression is not constitutive but regulated by salt and osmotic stress. At both transcript and protein levels, increasing and decreasing amounts of *TMKP1* were observed in MH and OR varieties, respectively. This indicates that it may be involved in the regulation of the response to these stresses in wheat. This finding contrasts what was previously reported in *Arabidopsis* where the expression of *AtMKP1* is not significantly affected by salinity, or osmotic stress (Ulm et al. 2001; <https://3.met.genevestigator.com>). The homologues of *TMKP1* in rice (*OsMKP1*) and in tobacco (*NtMKP1*) were induced so far by wounding only (Katou et al. 2007; Yamakawa et al. 2004). In mammals, several *MKP* genes playing critical roles in stress responses, were induced by various stress treatments; e.g. the mouse *MKP1* was transcriptionally induced by UV-C, heat shock and  $\text{H}_2\text{O}_2$  (Li et al. 2001; Kondoh and Nishida 2007). It is therefore not excluded that *TMKP1* can have distinct roles in different environmental stress-responses including salt and osmotic stress. Interestingly, a differential expression pattern of *TMKP1* was observed between the sensitive (MH) and the tolerant (OR) wheat varieties. In fact, *TMKP1* is induced by salt and osmotic stresses in roots of MH,

whereas it is repressed by both treatments in OR roots. It is possible that TMKP1 can act as a negative regulator of salt and osmotic stress responses in MH variety where its induction could contribute partially to its relative stress sensitivity. In respect to this hypothesis, the inhibition of *TMKP1* expression in the tolerant variety might then result in an improved stress tolerance. It might be of interest to see if other wheat genotypes of various geographical origins with specific tolerance towards salt stress show similar inhibition of *TMKP1*. If a correlation between stress tolerance and repression of *TMKP1* exists, *TMKP1* could be a good biological indicator for screening of tolerant/sensitive wheat varieties.

A comprehensive approach to decipher the role of *TMKP1* in wheat exposed to environmental constraints requires the investigation of its cellular partners notably the MAPKs. In this context, our in vitro protein interaction-assay showed that *TMKP1* specifically interacts with two stress responsive wheat MAPKs, *TMPK3* and *TMPK6* which are highly homologous to the *Arabidopsis* counterparts *MPK3* and *MPK6*, respectively. These two MAPKs are among the most important MAPKs involved in diverse stress signalling pathways in *Arabidopsis* (Nakagami et al. 2005). It has been previously shown that the bread wheat (*Triticum aestivum*) *TMPK3* is induced by fungal elicitors (Takezawa 1999) and that *TMPK3* and *TMPK6* are differentially regulated during the interaction with the fungal pathogen *Mycosphaerella graminicola* (Rudd et al. 2008). Our GST-pull down assays revealed that *TMKP1* specifically interacts with either *TMPK3* or *TMPK6*. These interactions do not require the N-terminal non catalytic domain of *TMKP1*. In contrast, in the tobacco counterpart *NtMKP1*, this domain was shown to be required for binding to and inactivation of its *SIPK* target in vitro and in vivo (Katou et al. 2005). Although the N-terminal non catalytic domains are highly conserved between *NtMKP1* and *TMKP1*, binding data are seemingly contradictory. However, observations cannot be directly compared considering the different experimental setups. The binding assay with *NtMKP1* is based on activated (phosphorylated) *SIPK* whereas in this current work in vitro translated and non phosphorylated *MPK3* or *6* were used. Nevertheless, it is premature to rule out a possible role of the non catalytic N-terminal domain of *TMKP1* in the interaction with other unknown *TMPKs*.

The phosphatase activity of *TMKP1* was assessed through an in vitro assay performed on the recombinant protein (GST::*TMKP1*) and using OMFP as a substrate. The truncated form of *TMKP1* (GST::*TMKP1* $\Delta$ <sub>N<sub>1–135</sub></sub>) was also active but show slightly lower phosphatase activity. This result indicates that the N-terminal non catalytic region is not essential for the phosphatase activity in vitro but this does not exclude the contribution of this

domain in dephosphorylating its MAPK targets. In addition, the phosphatase activity of *TMKP1* was found to be markedly increased by its co-incubation with *TMPK3*. Recently, the phosphatase activity of *NtMKP1* was also shown to be stimulated by co-incubation with *SIPK* (a tobacco MAPK orthologue of *Arabidopsis* *MPK6*). Similarly, the phosphatase activity of *Arabidopsis* *AtMKP2* (specific for oxidative stress response) was also found to be enhanced by co-incubation with *MPK3* or *MPK6*, but neither with *MPK4* nor *MPK12* (Lee and Ellis 2007). In mammals, the binding of two different mammalian MKPs to their substrate MAPKs results in significantly increase of their in vitro phosphatase activity (Kyriakis and Avruch 2001; Camps et al. 1998). Therefore, the association of MKPs with their MAPK substrate(s) may generally enhance the catalytic activity of MKPs in both animals and plants.

While dispensable for the phosphatase activity and for the in vitro interactions with *TMPK3* and *TMPK6*, the N-terminal domain of *TMKP1* seems to play an important role in its subcellular localization. Once expressed in BY2 tobacco cells, the fusion GFP::*TMKP1* protein was shown to accumulate in the nucleus, whereas the truncated form GFP::*TMKP1* $\Delta$ <sub>N<sub>1–135</sub></sub> is predominately cytoplasmic. No *bona fide* NLS sequence was identified in this region and in the remaining amino acid sequence of *TMKP1*. Knowing the molecular size of GFP::*TMKP1* (109 kDa), its travelling into the nucleus by a free diffusion through the nuclear pores, as small molecules can do (less than 50 kDa), is rather excluded.

It is still possible that *TMKP1* may travel from the cytoplasm to the nucleus by associating with NLS-containing proteins as was previously proposed for MAPKs that do not have any conserved NLS sequence (Cyert 2001).

It has been recently shown that *AtMKP1* is localized in the cytoplasm (Bartels et al. 2009). This difference with our localization data suggests that *TMKP1* may have a regulatory function different from that of *AtMKP1*, perhaps by interacting with specific nuclear components.

We have also investigated the subcellular localization of *TMPK3* and *TMPK6* and as potential in vivo substrates for *TMKP1*, we addressed the question whether *TMPK3*/*TMKP1* and *TMPK6*/*TMKP1* co-localize in the plant cell. In the absence of *TMKP1*, *TMPK3*::RFP and *TMPK6*::RFP were shown to accumulate predominately into the nucleus. The nuclear localization of these two wheat MAPKs was not reported before. We observed that together with GFP::*TMKP1*, *TMPK3*::RFP or *TMPK6*::RFP were shown also to accumulate into the nucleus. Interestingly, in the presence of GFP::*TMKP1* $\Delta$ <sub>N<sub>1–135</sub></sub>, the pattern of localization of both kinases changes and follows the truncated form of *TMKP1* in the cytoplasm. This finding not only



reinforces the hypothesis that TMKP1 binds in the plant cell to TMPK3 and/or TMPK6 but also suggest that it may affect their shuttling between the nucleus and the cytoplasm. It is well established in yeast and mammalian cells that MAPKs shuttle between the cytoplasm and the nucleus and that their cytoplasmic return tightly correlates with their dephosphorylation (Cyert 2001). In our case, the phosphatase activity of TMKP1 on TMPK3 and TMPK6 has not been investigated. Regardless its dephosphorylating action on TMPK3 or TMPK6, it is also possible for TMKP1 and TMKP1 $\Delta$ N<sub>1–135</sub> to have an impact on their subcellular localization by simply acting, respectively, as a nuclear and cytoplasmic tether for these MAPKs. Similar scenario was already described for several MAPKs such as Hog1 (MAPK specific for the high osmolarity growth pathway) of *S. cerevisiae*, which shows a nuclear and cytoplasmic localization depending on binding to phosphatases PTP2 and PTP3, respectively.

In conclusion, we describe here the molecular characterization of the first MKP identified in wheat as an active phosphatase able to interact with two TMPKs. Future work will be directed to enhance our understanding of the dynamics of interactions between TMKP1 and TMPKs and to elucidate their role in regulating the physiological outcome of MAPK signalling in stress response in plants.

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