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ZmMPK6, a novel maize MAP kinase that interacts with 14-3-3 proteins

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Abstract

Although an increasing body of evidence indicates that plant MAP kinases are involved in a number of cellular processes, such as cell cycle regulation and cellular response to abiotic stresses, hormones and pathogen attack, very little is known about their biochemical properties and regulation mechanism. In this paper we report on the identification and characterization of a novel member of the MAP kinase family from maize, ZmMPK6. The amino acid sequence reveals a high degree of identity with group D plant MAP kinases. Recombinant ZmMPK6, expressed in *Escherichia coli*, is an active enzyme able to autophosphorylate. Remarkably, ZmMPK6 interacts *in vitro* with GF14-6, a maize 14-3-3 protein and the interaction is dependent on autophosphorylation. The interacting domain of ZmMPK6 is on the C-terminus and is comprised between amino acid 337 and amino acid 467. Our results represent the first evidence of an interaction between a plant MAP kinase and a 14-3-3 protein. Possible functional roles of this association *in vivo* are discussed.

Abbreviations: ERK, Extracellular-signal Regulated Kinase; GST, glutathione *S*-transferase; MAPK, mitogen-activated protein kinase; MAPKKK, mitogen-activated protein kinase kinase kinase; MEK, *M*itogen-activated protein kinase/*E*xtracellular signal-regulated kinase *K*inase); MHA2, *M*aize H^+-*A*TPase isoform *2*; PAGE, polyacrylamide gel electrophoresis; PVDF, polyvinylidene fluoride; RT-PCR, reverse transcriptase polimerase chain reaction

Introduction

Mitogen-activated protein kinases (MAPKs) are proline-directed serine/threonine kinases that phosphorylate substrates containing the consensus motif ΨX[S/T]P, where Ψ represents a proline or an aliphatic amino acid (Clark-Lewis *et al.*, 1991).

In eukaryotes the MAPK cascades mediate the transmission and amplification of extracellular stimuli induced by hormones, growth factors, cytokines and environmental stresses. A typical MAPK three-step cascade consists of a MAPK kinase kinase that phosphorylates on serine or threonine residues a MAPK kinase, which, in turn,

phosphorylates a MAPK, on threonine and tyrosine residues, in the invariant sequence TXY (Cobb and Goldsmith, 1995).

MAPKs can be subdivided in three groups: Extracellular-signal Regulated Kinase (ERKs), characterized by the phosphorylation motif TEY, are activated by mitogenic signals and are involved in differentiation and cell cycle regulation (Robinson and Cobb, 1997); JNKs (cJun N-terminal Kinase) and p38s/Hog1, that collectively represents the Stress Activated Protein Kinase (SAPKs), containing the TPY and TGY phosphorylation motif respectively, are activated by many cellular stresses and apoptotic signals (Weston and Davis, 2002) the protozoal MAPK3s, containing the canonical dual phosphorylation motifs (TEY or TDY) or the single phosphorylation motifs THE or SEG (Kultz, 1998) whose functions are still poorly understood.

In plants, a number of MAPKs has been identified and characterized from different species. Completion of Arabidopsis genome sequence has revealed the existence of 23 MAPK genes (Jonak *et al*., 2002). Recently, a division of plant MAPKs in 4 subgroups (A–D), according to amino acid sequence homology and phosphorylation motif (TEY and TDY) has been proposed (Ichimura *et al*., 2002).

Emerging evidence suggests that plant MAPKs are involved in the cell cycle regulation (Calderini *et al*., 1998). They, in turn, respond to a wide range of stimuli (Tena *et al*., 2001) such as abiotic stresses (Jonak *et al*., 1996; Ichimura *et al*., 2000), hormones (Kieber *et al*., 1993; Mockaitis and Howell, 2000; Ouaked *et al*., 2003) and pathogen attack (Zhang and Klessig, 2001; Song and Goodman, 2002). However very little is known about the molecular mechanism of MAPK regulation in plants. It has been demonstrated that they can be activated by plant Mitogen-activated protein kinase/Extracellular signal-regulated kinase Kinase (MEK) homologs and de-activated by tyr-phosphatase (Huang *et al*., 2000) or dual specificity phosphatase (Gupta *et al*., 1998), similarly to animal MAPKs.

In animals binding of 14-3-3 proteins to some elements of the MAP kinase cascade has been reported (Fu *et al*., 2000*;* van Hemert *et al*., 2001), even though the functional significance of this binding is not fully understood.

14-3-3 proteins are a family of highly conserved eukaryotic dimeric proteins. They play a central role in the regulation of cell cycle, differentiation, apoptosis, and the coordination of multiple signal transduction pathways in animals (Fu *et al*., 2000*;* van Hemert *et al*., 2001; Tzivion and Avruch, 2002) as well as in plants (Finnie *et al*., 1999; Aducci *et al*., 2002). 14-3-3s bind their targets in a phosphorylation-dependent manner (Muslin *et al*., 1996; Yaffe *et al*., 1997) even though proteins interacting in a phosphorylation-independent manner have also been identified (Fu *et al*., 2000).

Besides the well-studied interaction between 14-3-3s and the MAPKKK Raf-1, in animals association of 14-3-3 ε and ζ isoforms with MEKK1, MEKK2 and MEKK3 has been reported (Fanger *et al*., 1998). In *Saccharomyces cerevisiae* 14-3-3s have been found to bind the MAPKKK Ste20, and 14-3-3 suppression abolishes MAPK signalling during pseudohyphal development (Roberts *et al*., 1997). In cultured fibroblasts, inactivation of 14-3-3s leads to apoptosis induced via JNK and p38 MAPKs (Xing *et al*., 2000).

In this paper, we identified and characterized a novel MAP kinase from maize, ZmMPK6, which is able to interact with 14-3-3 proteins. To our knowledge, these data represent the first evidence of a possible involvement of 14-3-3 proteins in the regulation of MAP cascade in plants.

Materials and methods

Chemicals

[γ-^{32}P]ATP (specific activity 110 TBq/mmol), thrombin and Pre-scission protease were from Amersham Biosciences (Upssala, Sweden). Protein kinase A, catalytic subunit, myelin basic protein (MBP) and histone were from Sigma (St. Louis, Missouri, USA). L15Vp peptide LKGLDIDTIQQNYTpV (Tp, phosphothreonine) and L15V peptide LKGLDIDTIQQNYTV were synthesized by Neosystem (Strasbourg, France). Chemicals for gel electrophoresis were from Bio-Rad (Hercules, California, USA). All other reagents were of analytical grade.

Cloning of cDNA

The cDNA of ZmMPK6 was PCR amplified from clone 104 with primers MPK6forw (5′-**GGATC-**

CATGCAGCACGACCAGAAGAAG-3′), corresponding to the sequence 1–21, spanning the ATG codon (underlined), and MPK6rev (5′-**GGATCC**CTACTACCAGTGACCACCACGAC T-3′), corresponding to the sequence 1653–1671 and containing the original and an adjunctive stop codon (underlined). In bold, *Bam*HI restriction site is shown. The amplified cDNA was cloned into a pGEM-T vector according to the manufacturer's instruction (Promega). After sequencing, the *Bam*HI fragment was recovered and subcloned into *Bam*HI-cut pGEX-6P1 (Amersham Biosciences) or pET28a$^+$ (Novagen) vectors, using 'DNA ligation system' kit (Amersham Biosciences) according to manufacturer's protocol. Products of ligase reactions were used to transform *Escherichia coli* DH5α competent cells. pGEX-6P1 and pET28a$^+$ vectors, carrying the ZmMPK6 cDNA, were referred as pMPK6-X and pMPK6-H, respectively. For protein expression pMPK6-X and pMPK6-H vectors were transferred in *E. coli* BL21(DE3) strain.

ZmMPK6 mutant construction

Site directed mutagenesis of Ser-411 of ZmMPK6 was performed by using the "QuickChange site-directed mutagenesis methods" (Stratagene) and pMPK6-X as the template. The mutagenic primers were (mutations generated are underlined): S411Aforw: 5′-CCTGAGAGGCAACATAATGC ATTGCCGCGGCC-3′ and S411Arev 5′-CCGCG GCAATGCATTATGTTGCCTCTCAGGTG-3′.

After 18 cycles of PCR (30 s at 95 °C, 1 min at 53 °C, 8 min at 68 °C) 10 units of *Dpn*I were added to the mixture to digest the DNA template and reaction was carried out at 37 °C for 2 h. 20 μl of reaction mixture were used to transform *E. coli* DH5α competent cells. Incorporation of mutation was controlled by DNA sequencing.

In order to obtained the deletion mutant Δ220, lacking of the last 220 residues, we took advantage of the presence of a *Eco*RI site at the 1009 position of ZmMPK6 cDNA and a *Eco*RI site in the multicloning site of the pGEX-6P1 vector. The *Eco*RI-*Eco*RI cassette was removed from pMPK6-X and after re-circularization, plasmid was used to transform *E. coli* DH5α competent cells.

Deletion mutants lacking of the last 10, 50 and 90 residues were obtained by PCR using a 5′oligonucleotide annealing at 1009–1035 of ZmMPK6 cDNA, containing *Eco*RI site (underlined) 5′-GAATTCGAGAGGCGGAGAGTTA-CAAAG-3′ and the following 3′ oligonucleotides

Δ10: 5′-CTCGAG**CTA**AGGTAGTTTGTTC GCTGC-3′ annealing at 1608–1622.

Δ50: 5′-CTCGAG**CTA**AGGGATATTGTTT GGGG-3′ annealing at 1485–1501.

Δ90: 5′-CTCGAG**CTA**TGCAACATCACCTT GAGG-3′ annealing at 1364–1381.

all containg a *Xho*I site (underlined) and a stop codon (in bold)

Fragments amplified were cloned into pGEM-T vector according to the manufacturer's instruction (Promega). After sequencing, the *Eco*RI-*Xho*I cassettes were recovered and cloned into *Eco*RI-*Xho*I-cut pMPK6-X using 'DNA ligation system' kit (Amersham Biosciences) according to manufacturer's protocol. The ligation products were used to transform *E. coli* DH5α competent cells.

For mutated protein expression recombinant vectors were transferred in *E. coli* BL21(DE3) strain.

Expression of recombinant protein in E. coli

ZmMPK6 was expressed as GST and 6 His-tag fusion protein using pGEX-6P1 and in pET28a$^+$ vectors, respectively. ZmMPK6 mutants were expressed as GST fusion proteins, using pGEX-6P1.

BL21(DE3) transformants were grown at 37 °C to $OD_{600} = 0.6$ and then induced with 1 mM isopropyl thio-β-D-galactoside (IPTG) for 3,5 h at 30 °C. GST-fusion protein was purified on glutathione-sepharose 4B (Amersham Biosciences) according to manufacturer's instructions. When indicated, the purified fusion protein was digested with 2 units of Pre-scission protease (Amersham Biosciences) in the cleavage buffer (50 mM Tris-HCl, pH 7.5, 15 mM NaCl, 1 mM DTT, 1 mM EDTA) for 16 h at 4 °C. 6His-tag fusion protein was purified with 'His-Bind Purification kit' (Novagen) according to manufacturer's instructions.

GF14-6 wild type and the K56E mutant were expressed as GST-fusion protein as described (Visconti *et al.*, 2003).

Proteins concentration was measured by the method of Bradford (Bradford, 1976) with BSA as a standard.

Immunoblotting

For immunoblotting analysis, proteins were separated by SDS-PAGE (Laemmli, 1970) using a mini-gel apparatus (Bio-Rad), then electroblotted by semidry apparatus onto PVDF membrane with 39 mM glycine, 48 mM Tris, 0.1% SDS, 10% methanol. After blocking for 1 h in TTBS (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 0.05% Tween 20) with 5% no-fat dried milk at room temperature, the membranes were incubated with the HRP-conjugated anti-GST antibody (1:2000; Sigma-Aldrich) or the anti-polyHis antibody (1:3000; Sigma-Aldrich). Following three washes with TTBS, the membranes pre-incubated with anti-polyHis were incubate with HRP-conjugated anti-mouse secondary antibody (1:2000; Sigma-Aldrich).

Overlay assay

The overlay assays were carried out according to Fullone *et al.* (1998), with minor modifications. The GST-fused 14-3-3s were labelled with [γ^{32}P]-ATP (110 TBq/mmol, Amersham Biosciences) on cAMP-dependent protein kinase phosphorylation site present at junction between GST and cloned proteins, as reported (Fullone *et al.*, 1998). ^{32}P-labelled GF14-6 wild type and K56E mutant were used as probes in the overlay experiments.

Two micrograms of wild type or mutated ZmMPK6 expressed as GST-fusion proteins were separated on SDS-PAGE and blotted on nitrocellulose membrane by semidry electroblotting. The membranes were blocked in buffer HT (25 mM Hepes-OH, 75 mM KCl, 5 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.05% Tween 20, pH 7.5) containing 5% no-fat dried milk and then incubated overnight at 4 °C in buffer HT with 2% no-fat dried milk, 3 μg of ^{32}P-labelled GF14-6 wild type or K56E mutant (9 kBq/ml). Then the membranes were extensively washed with buffer HT and radioactivity detected with autoradiography at −80 °C. For phosphorylation and dephosphorylation experiments, before subjecting to the SDS-PAGE, 2 μg of Pre-scission digested ZmMPK6 were incubated with 30 μM ATP for 1 h at 30 °C or with 2 units of calf intestinal alkaline phosphatase (AP) for 45 min at 37 °C, respectively.

Pull-down assay

Fifteen micrograms of glutathione-sepharose immobilised GST or GST-GF14-6 were incubated with 0.5 mg of His-ZmMPK6-expressing bacterial lysate or with a control lysate, in buffer HT for 1 h a 4 °C. After extensive washes with buffer HT, beads bound proteins were recovered by means of two incubation with 50 μl each of 10 mM glutathione. The two elutions were pulled and an aliquot was run in SDS-PAGE and subjected to immunoblotting with anti-GST or anti-polyHis antibody, as previously described.

Kinase assay

Kinase assays were performed in a reaction mixture containing 25 mM Tris-MES pH 7.5, 12 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT, 25 μM ATP and 2.5 μCi [γ^{32}P]-ATP and 1.5 μg of MBP; approximately 1 μg of recombinant protein was used for each reaction in a final volume of 15 μl. After incubation for 30 min at 30 °C, reaction was stopped by addition of 5 mM EDTA. Samples were run in SDS-PAGE and phosphorylated MBP was detected by autoradiography of dried gels.

Results

Identification of a new maize MAPK from a EST clone

A DNA fragment of approximately 2500 bp from an EST library of maize (clone 104, from the collection of maize silk ESTs of the Max Planck Institute in Koeln; *http://www.mpiz-koeln.mpg.de/~riehl/ArrayDB/MzArrayDB.htm*; Bhat *et al.*, 2003) showing sequence identity with plant MAPK was selected and re-sequenced. It contains a complete ORF of 1671 bp. The ORF codes for a polypeptide of 557 amino acids, with a calculated molecular mass of 63.5 kDa and p*I* of 8.7. Amino acid sequence comparison with FASTA program (Pearson, 1990) revealed a high degree of identity with group D plant MAPKs (ranging 60–70%) (Ichimura *et al.*, 2002). In contrast, alignment with two maize MAPK, ZmMPK4 and ZmMPK5, belonging the group A of plant MAPKs, revealed a similarity of only 40%, limited to N-terminus

containing the kinase domain (Berberich *et al.*, 1999). We have named this new MAPK ZmMPK6 (GenBank accession number AY425817). Amino acid sequence alignment of ZmMPK6 with other plant MAPKs, obtained with the CLUSTAL W program (Thompson *et al.*, 1994), is shown in Figure 1. In accordance with other members of the group D of plant MAPK, ZmMPK6 contains, in addition to the 11 subdomains conserved in ser/thr protein kinases, an extended C-terminus of approximately 220 residues and it is characterized by the presence of an aspartic acid residue in the dual phosphorylation motif, TDY.

RT-PCR analysis demonstrates that ZmMPK6 is expressed in roots and leaves of maize seedlings (data not shown).

ZmMPK6 cDNA was expressed in *E. coli* as GST or 6-His tag fusion protein. Recombinant ZmMPK6 is catalitically active, being able to autophosphorylate and to phosphorylate Myelin basic protein (MBP) and histone The calculated specific activity towards MBP is 2 nmol min^{-1} mg^{-1} (data not shown).

ZmMPK6 interacts with 14-3-3 proteins in vitro

Analysis of the ZmMPK6 primary structure revealed the presence of the sequence RQHNS^{411}LP, related to the 14-3-3 mode-2 binding motif RX_{1-3}(S/T)XP (Yaffe *et al.*, 1997). This finding prompted us to investigate whether ZmMPK6 could interact with 14-3-3 proteins.

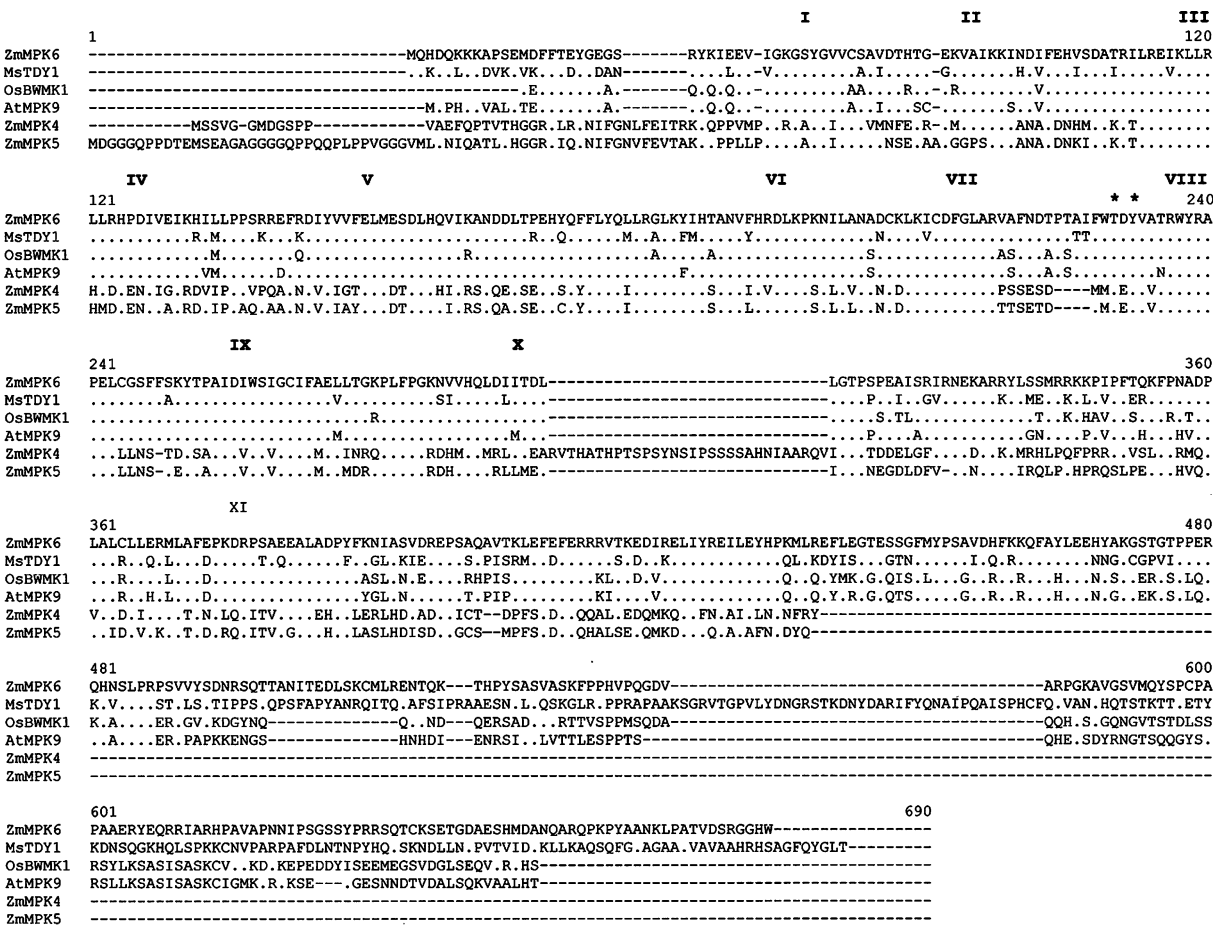

Figure 1. ZmMPK6 sequence and comparison. Alignment of the deduced ZmMPK6 protein sequence (GenBank AY425817) with other members of group D MAPK from *Medicago sativa* (MsTDY1, AAD28617), *Oryza sativa* (OsBWMK1, AF177392), *Arabidopsis thaliana* (AtMPK9, AB038694) and with ZmMPK4 (AB016801) and ZmMPK5 (AB016802) from *Zea mays*. Dot represents amino acid residues that match ZmMPK6. Gaps were introduced to maximize alignment. The 11 MAP kinase subdomains are labelled by roman numerals. The TDY phosphorylation motif is indicated by * *.

To this purpose an *in vitro* overlay assay, using a radiolabelled maize GF14-6 14-3-3 isoform as a probe (Fullone *et al.*, 1998), was performed. The autoradiography (Figure 2A, right panel) shows that ^{32}P-labelled GF14-6 bound both to the GST-ZmMPK6 (lane 1) and to the Pre-scission cleaved ZmMPK6 (lane 2), whereas it did not interact with the GST alone (lane 3). These results were confirmed by pull-down experiments. The GST-GF14-6, or the GST as a control, were immobilized onto glutathione sepharose beads and incubated with a bacterial lysate containing or not the His-ZmMPK6. Proteins bound to the beads were eluted with glutathione and subjected to SDS-PAGE and immunoblotting with antipoly-His antibodies. Results demonstrate that the His-ZmMPK6 specifically bound to the immobilized GST-GF14-6 (Figure 2B, right panel, lane 1). In order to check that the same amount of GF14-6 or GST was used, PVDF membrane was Coomassie stained (left panel) or immunodecorated with anti-GST antibodies (central panel).

Specificity of binding was demonstrated by a competition experiment. The addition of the

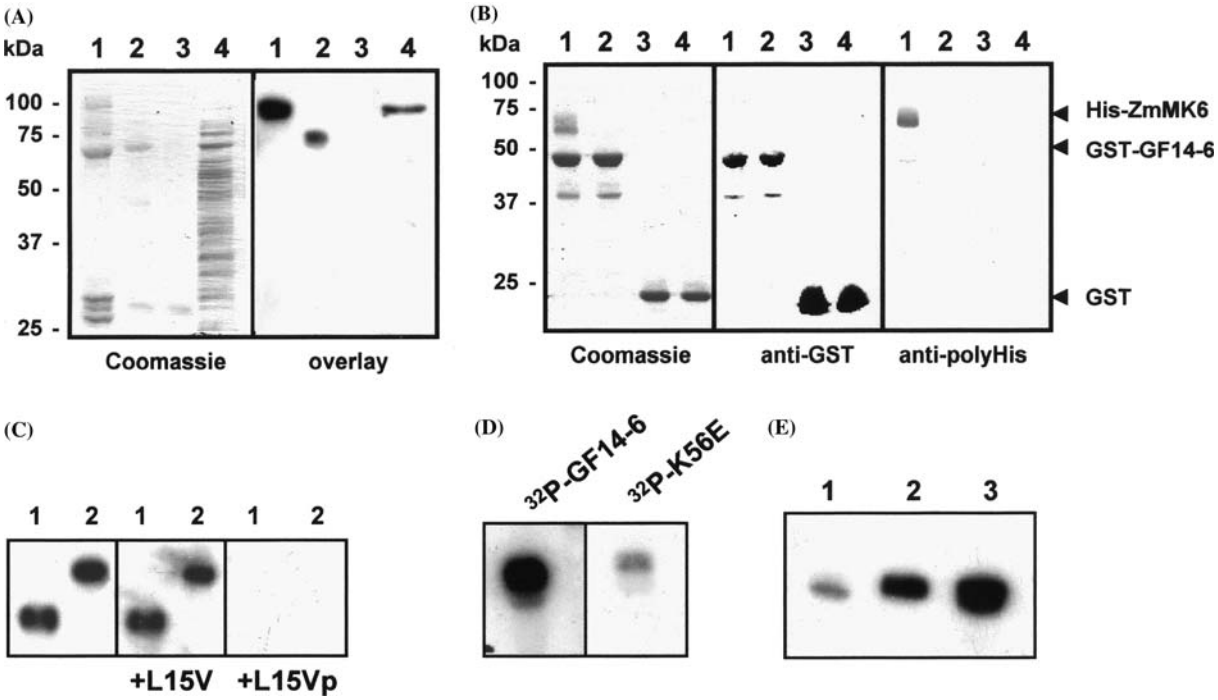

Figure 2. In vitro interaction of ZmMPK6 with GF14-6. (A) Overlay assay. 1 μg of GST-ZmMPK6 (lane 1), pre-scission cleaved ZmMPK6 (lane 2) or GST alone (lane 3) were subjected to SDS-PAGE, transferred onto nitrocellulose membrane and incubated with ^{32}P-labelled GF14–6. 20 μg plasma membranes from maize roots, containing the 14-3-3 target H^+-ATPase, were loaded as a positive control (lane 4). (B) Pull-down assay. 0.5 mg of protein lysate from induced His-ZmMPK6 expressing bacteria (lane 1 and 3) or not induced (lane 2 and 4) were incubated with GST-GF14-6 (lane 1 and 2) or GST (lane 3 and 4) immobilized on glutathione-sepharose beads. Beads bound proteins were recovered, separated on 12% SDS-PAGE, transfered on PVDF membrane and subjected to Coomassie staining (left panel) or immunoblotting with anti-GST (central panel) and anti-polyHis antibody (right panel). (C) Competition assay with a peptide reproducing a 14-3-3 binding site. 1 μg of GST-ZmMPK6 (lanes 1), pre-scission cleaved ZmMPK6 (lanes 2) were subjected to SDS-PAGE, transferred onto nitrocellulose membrane and incubated with ^{32}P-labelled GF14-6. Where indicated, 1 μM of L15V and L15Vp, synthetic peptides reproducing the unphosphorylated and the phosphorylated binding motif of the H^+-ATPase, respectively, were added. Radioactivity was detected by autoradiography. (D) Overlay assay with GF14-6 mutant K56E. One microgram of Pre-scission cleaved ZmMPK6 was subjected to SDS-PAGE, transferred onto nitrocellulose membrane and incubated with ^{32}P-labelled GF14-6 or ^{32}P-labelled K56E mutant. Radioactivity was detected by autoradiography. (E) Role of phosphorylation of ZmMPK6 on the interaction with GF14-6. One microgram of Pre-scission cleaved ZmMPK6 was incubated with alkaline phosphatase (lane 1), in the absence (lane 2) or in the presence of ATP (lane 3), then subjected to SDS-PAGE, transferred onto nitrocellulose membrane and incubated with ^{32}P-labelled GF14-6. Radioactivity was detected by autoradiography.

L15Vp peptide, reproducing the 14-3-3 binding site in the MHA2 H^+-ATPase, as a competing substrate in overlay assay, completely abolished interaction of ^{32}P-labelled GF14-6 with both Pre-scission cleaved ZmMPK6 (Figure 2C, lane 1) and GST-ZmMPK6 (Figure 2C, lane 2). No effect was observed using the corresponding unphosphorylated peptide L15V. These results suggest that binding of 14-3-3 protein to ZmMPK6 involves the same residues of 14-3-3 proteins as those involved in binding other target proteins. A confirmation to that was obtained by using the K56E mutant of GF14-6, which is unable to interact with the H^+-ATPase (Visconti *et al.*, 2003), as a probe in the overlay experiment. Autoradiography, reported in Figure 2D, shows a very reduced interaction of ^{32}P-labelled K56E with the Pre-scission cleaved ZmMPK6 compared to the wild type ^{32}P-labelled GF14-6.

Since it is known that 14-3-3 binding generally requires serine/threonine phosphorylation of targets, we investigated whether also the interaction of GF14-6 with ZmMPK6 was phosphorylation dependent. To this purpose, the overlay assay was performed after subjecting Pre-scission cleaved ZmMPK6 to dephosphorylation with alkaline phosphatase or phosphorylation in the presence of ATP. Results, reported in Figure 2E, show that alkaline phosphatase treatment strongly reduced 14-3-3 binding (lane 1) whereas the pre-incubation with ATP significantly increased the ability of ZmMPK6 to interact with ^{32}P-labelled GF14-6 (lane 3).

In order to test whether ZmMPK6 activity was affected by 14-3-3 binding, a kinase assay using MBP and [γ-^{32}P]ATP as substrates was carried out after pre-incubating the enzyme with GF14-6. The autoradiography shows that MBP was phosphorylated at the same extent both in the absence (Figure 3, lane 1) and in the presence (lane 2) of GF14-6, thus indicating that 14-3-3 binding did not affect the catalytic activity of ZmMPK6.

To verify whether the putative 14-3-3 binding site RQHNS^{411}LP could account for the association of ZmMPK6 with 14-3-3 protein, site-directed mutagenesis of the serine 411 was performed. The S411A mutant was expressed in *E. coli* as GST-fusion protein and its catalytic activity tested. S411A mutant is also catalytically active and retains the autophosphorylating ability (data not shown). S411A ZmMPK6 was used in overlay assay as a bait in order to test its ability to bind 14-3-3 protein. S411A mutation did not hamper the interaction of ZmMPK6 with the 14-3-3 protein (Figure 4A). This negative result prompted us to undertake a more systematic approach. Different deletions of the C-terminal region, which is an unique feature of D-group MAPKs, such as ZmMPK6, were produced and tested for the ability to interact with the 14-3-3 probe. ZmMPK6(1-547), ZmMPK6(1-507), ZmMPK6(1-467) and ZmMPK6(1-337) mutants, lacking the last 10, 50, 90 and 220 amino acids, respectively, were expressed at levels comparable to wild type protein and hence tested in the overlay assay. As shown in Figure 4B, 14-3-3 binding is maintained in the Δ10, Δ50 and Δ90 mutants, whereas it is completely abolished in the Δ220 mutant, lacking the entire C-terminal domain. These results demonstrate that the 14-3-3 binding site is located in the region 337–467. However, attempts to identify the binding motif by other mutants were unsuccessful, due to poor expression levels of deletion mutants comprised between residues 337 and 467 (data not shown).

The reported results suggest that 14-3-3 binding involves a non-canonical 14-3-3 binding motif,

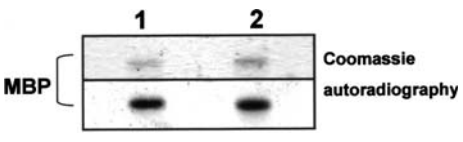

Figure 3. Effect of GF14-6 binding on the ZmMPK6 activity. One microgram of Pre-scission cleaved ZmMPK6 was incubated with [γ^{32}P]ATP and 1.5 μg of MBP as the substrate in the absence (lane 1) or in the presence (lane 2) of 2 μg of GF14-6. After 12% SDS-PAGE proteins were subjected to autoradiography for 1 h at room temperature.

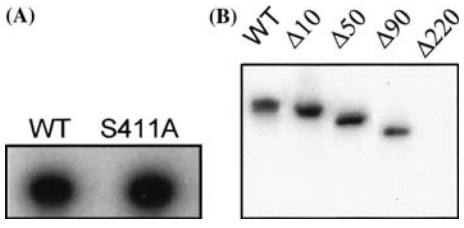

Figure 4. Interaction of GF14-6 with ZmMPK6 mutants. One microgram of Pre-scission cleaved wild type or mutated ZmMPK6 was subjected to SDS-PAGE, transferred onto nitrocellulose membrane and incubated with ^{32}P-labelled GF14-6. Radioactivity was detected by autoradiography. (A) Overlay assay of S411A mutant. (B) Overlay assay of ZmMPK6 deletion mutants.

since 14-3-3 consensus sequences are not occurring in the identified region.

Discussion

MAP kinases are the terminal components of a three-step cascade utilized by eukaryotic cells to transduce a wide array of extracellular signals. Regulation of these enzyme activities is well characterized in animals and yeast, whereas less is known in plants.

In this paper we report on a novel MAPK from *Zea mays*, termed ZmMPK6, which remarkably interacts *in vitro* with 14-3-3 proteins. The kinase has been identified from a clone of a maize EST library.

The amino acid sequence comparison reveals that ZmMPK6 belongs to the group D of plant MAPK, characterized by the presence of the TDY activation motif in their T-loop and by an extended C-terminal region compared to the other plant MAPK groups.

Recombinant ZmMPK6 displays a specific activity of 2 nmol min^{-1} mg^{-1}, in accordance with values reported for MAPK expressed in *E. coli* (Huang *et al*., 2000).

In animals the association of 14-3-3s with MAPK and MAPKK kinases is well documented and recently also the interaction of 14-3-3s with the Big Mitogen-activated protein kinase 1 (BMK1) has been reported (Zheng *et al*., 2004). These pieces of evidence prompted us to test whether the novel kinase ZmMPK6 could interact with plant 14-3-3 proteins. Results demonstrate that ZmMPK6 is able to bind 14-3-3 proteins in overlay and pull-down experiments. Interestingly, both ZmMPK6 and BMK1 contain an extended C terminus. This common feature might underlie a peculiar regulatory mechanism involving 14-3-3 proteins.

In accordance with the general mechanism underlying 14-3-3 binding to target proteins, we found that the interaction is dependent upon ZmMPK6 phosphorylation.

Two different 14-3-3 binding motifs, R[S/Ar][+/Ar]Sp[L/E/A/M]P and RX[S/Ar][+]Sp[L/E/A/M]P (where Ar indicates an aromatic residue and + a basic residue), defined as mode-1 and mode-2 respectively, have so far been identified (Yaffe *et al*., 1997). However these motifs do not account for all 14-3-3 interactions, as 14-3-3 binding to target proteins containing phosphorylated sequences different from mode-1 and mode-2 have also been reported (Liu *et al*., 1997; Ku *et al*., 1998). Sequence analysis had revealed the presence of a putative 14-3-3 mode-2 binding motif in ZmMPK6. Thus the putative phosphorylation site at serine 411 within this motif was mutated to test its actual involvement in 14-3-3 binding. The S411A mutant, however, retains the ability to bind 14-3-3s *in vitro*. Results from the deletion mutants allow to identify a binding domain falling in the region 337–467. It is possible to infere that a non-canonical phosphorylation-dependent motif within the ZmMPK6 sequence is involved in 14-3-3 binding, as no canonical sequence is present in the identified domain. Such alternative motifs have already been found in other cases, e.g. for the plasma membrane H^+-ATPase in plants (Fuglsang *et al*., 1999).

Binding of 14-3-3 proteins to ZmMPK6 does not alter the catalytic activity of the enzyme. However, the low activity of recombinant ZmMPK6 could potentially mask a regulatory effect of 14-3-3 binding. Unfortunately, attempts to activate ZmMPK6 with commercially available MEKs were uneffective (data not shown). Alternatively, 14-3-3 association might modulate the intracellular localization of ZmMPK6. In this respect, it is worth noting that 14-3-3 proteins regulate the nuclear shuttling of the RSG (Repression of Shoot Growth), a bZIP transcriptional factor involved in shoot growth by controlling transcription of genes for gibberellin biosynthesis (Igarashi *et al*., 2001). Intriguingly, MAP kinases can migrate into the nucleus where they act as transcription factor regulators; this has been documented with animal and yeast cells (Davis, 2000) and recently for BWMK1, a MAPK from rice. This kinase is found in the nucleus and mediates pathogenesis-related gene expression by activating the OsEREBP1 transcription factor (Cheong *et al*., 2003).

In conclusion, evidence reported in the present study clearly demonstrates that plant MAP kinase, ZmMPK6, is a novel target for 14-3-3 proteins. As for the regulatory role of this interaction, further work will be necessary in order to ascertain whether it involves modulation of the kinase activity or alternative mechanisms.

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