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ORIGINAL ARTICLE

Identity of an ABA-activated 46 kDa mitogen-activated protein kinase from *Zea mays* leaves: partial purification, identification and characterization

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Abstract Mitogen-activated protein kinase (MAPK) cascades have been shown to be important components in abscisic acid (ABA) signal transduction pathway. In this study, a 46 kDa MAPK (p46MAPK) induced by ABA was partially purified from maize (*Zea mays*) by Q-Sepharose FF, Phenyl-Sepharose FF, Resource Q, Mono QTM 5/50 GL, poly-L-lysine-agarose, and Superdex 75 prep-grade columns, and was identified as ZmMAPK5 (gil4239889) by the matrix-assisted laser desorption/ionization time-of-flight/time-of-flight (MALDI-TOF/TOF) mass spectrometry. Furthermore, the kinase showed optimal activity at pH 8.0, 30°C, and 10 mM $MgCl_2$; the K_m for myelin basic protein (MBP) substrate and ATP were 0.13 μg μl^{-1} and 62 μM, respectively. MBP was the preferred substrate, of which the threonine residue was phosphorylated. Finally, the kinase was found to respond to diverse extracelluar stimuli. These results enable us to further reveal the function of the ZmMAPK5 in ABA signaling.

Keywords Abscisic acid · Mass spectrometry · Mitogen-activated protein kinase · Protein purification · *Zea*

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Abbreviations

ABA	Abscisic acid
MALDI-TOF/TOF	Matrix-assisted laser desorption/ionization time-of-flight/time-of-flight
MAPK	Mitogen-activated protein kinase
MAPKK	Mitogen-activated protein kinase kinase
MAPKKK	Mitogen-activated protein kinase kinase kinase
MBP	Myelin basic protein
MS	Mass spectrum
SA	Salicylic acid

Introduction

The mitogen-activated protein kinases (MAPKs), a family of serine/threonine protein kinases, are one of the major and evolutionally conserved signaling pathways by which extracellular stimuli are transduced into intracellular responses in eukaryote cells (Tena et al. 2001; Zhang and Klessig 2001; Jonak et al. 2002; Nakagami et al. 2005). The basic assembly of an MAPK cascade is a three interlinked protein kinase module (MAPKKK–MAPKK–MAPK). MAPK, the last kinase in the cascade, is activated by dual phosphorylation of the threonine and tyrosine residues in a tripeptide motif (T–X–T) located between subdomains VII and VIII of the kinase catalytic domain, where X could be Glu, Gly, Pro or Asp. This phosphorylation is mediated by upstream activators, MAPK kinase (MAPKK or MEK) and MAPKK kinase (MAPKKK or MEKK). Activated MAPK can phosphorylate a variety of substrates including transcription factors, other protein kinases and cytoskeleton-associated proteins (Nakagami et al. 2005). An increasing body of evidence indicate that MAPKs are

involved in plant signal transduction in response to pathogens, drought, salinity, cold, wounding, O_3, ROS, heavy metal and hormone stimuli (Tena et al. 2001; Zhang and Klessig 2001; Jonak et al. 2002; Lu et al. 2002; Mittler 2002; Moon et al. 2003; Xiong and Yang 2003; Nakagami et al. 2005; Yeh et al. 2007; Zhang et al. 2006, 2007; Xing et al. 2008; Zong et al. 2009).

The phytohormone, abscisic acid (ABA), plays critical roles in plant response to environmental stress. It induces a myriad of cellular responses in plants through complex signal transduction cascades, leading to tolerance towards these stress conditions (Finkelstein et al. 2002; Zhu 2002). Many studies have shown that MAPK cascades are involved in ABA signaling. ABA treatment can activate several MAPK isoforms between 40 and 43 kDa in barley (*Hordeum vulgare*) aleurone protoplasts (Knetsch et al. 1996), p45MAPK in pea (*Pisum sativum*, Burnett et al. 2000), p38MAPK in moss (*Funaria hygrometrica*, D'Souza and Johri 2002), AtMPK3, p46MAPK (Lu et al. 2002), AtMPK6 and AtMPK4 (Xing et al. 2008) in *Arabidopsis* (*Arabidopsis thaliana*), OsMAPK5 in rice (*Oryza sativa*, Xiong and Yang 2003), and p46MAPK in maize (*Zea mays*, Zhang et al. 2006, 2007). Apart from the MAPKs in *Arabidopsis* and rice, however, the MAPKs induced by ABA in other plants are not been well known. As the MAPK cascade is suggested to be the converging point of multiple signals, identification and characterization of the MAPKs become essential to dissect the plant self defense mechanisms.

In recent studies, using pharmacological and biochemical approaches, our results showed that ABA activated a 46 kDa MAPK (p46MAPK), which in turn induced the expression and activities of antioxidant enzymes in maize (Zhang et al. 2006, 2007). H_2O_2 and NO are involved in the p46MAPK signaling pathway. The activation of MAPK also enhances the production of H_2O_2, forming a positive feedback loop. We do not know, however, the identity of the p46MAPK induced by ABA in maize leaves. In this study, the p46MAPK induced by ABA in maize leaves was purified, identified and characterized. Our results show that the ABA-activated p46MAPK is ZmMAPK5, which enables us to further reveal the function of the ZmMAPK5 in ABA signaling.

Materials and methods

Plant material and treatments

Seeds of maize (*Zea mays* L. cv Nongda 108; from Nanjing Agricultural University, China) were sown in trays of sand in a light chamber at a temperature of 22–28°C, with photosynthetic active radiation of 200 μmol m^{-2} s^{-1} and a photoperiod of 14/10 h (day/night), and watered daily. When the second leaves were fully expanded, they were collected and used for all investigations.

For drought, salt and heavy metal treatments, the cut ends of the stems, respectively, were placed in the different beakers wrapped with aluminum foil containing 10% PEG 6000 solution, 250 mM NaCl and 10 mM $CdCl_2$ for 4 h at 22°C with a continuous light intensity of 100 μmol m^{-2} s^{-1}. For plant hormone treatments, the concentrations of the three different plant hormones, ABA, ethephon (ETH) and salicylic acid (SA), were 100 μM, 1 mM and 100 μM, respectively. For H_2O_2 treatment, the solution concentration was 10 mM. Ultraviolet light (UV-C, 254 nm) was irradiated from a distance of 15 cm using a germicidal lamp. The detached plants were placed in water under 4°C for the cold stress treatment, or the plant leaf cut with sterile scissors for wounding treatment. Leaf segments were sampled at the times indicated and immediately frozen under liquid N_2.

Partial purification of p46MAPK

Leaves treated with 100 μM ABA for 2 h were harvested, frozen and stored at −80°C. Frozen maize leaves (1,000 g) were ground to a fine powder in the presence of liquid N_2 and mixed with 1.5 volume extraction buffer (100 mM Hepes, pH 7.5, 5 mM EDTA, 5 mM EGTA, 10 mM dithiothreitol (DTT), 10 mM Na_3VO_4, 10 mM NaF, 50 mM *β*-glycerophosphate, 1 mM PMSF, 5 μg ml^{-1} leupeptin, 5 μg ml^{-1} aprotinin, 5% glycerol). The crude homogenate was filtered through four layers of Miracloth (Calbiochem), centrifuged at 23,000*g* for 1 h, and the resulting supernatant fraction was brought to 30% $(NH_4)_2SO_4$ saturation. After stirring slowly for 30 min, the precipitant was collected by centrifugation at 23,000*g* for 10 min. The pellets were then dissolved in a total of 80 ml of buffer A (25 mM Tris, pH 7.5, 1 mM EGTA, 10 mM *β*-glycerophosphate, 0.1 mM Na_3VO_4, 1 mM DTT, 5% glycerol) plus 1 mM PMSF, 5 μg ml^{-1} leupeptin and 5 μg ml^{-1} aprotinin. After ultracentrifugation (Beckman Optimak L-80XP) at 100,000*g* for 1 h, the supernatant was loaded onto an 80 ml Sephadex G 25 M column for desalting and buffer exchange with buffer A plus 50 mM NaCl. All chromatographic runs were carried out on the AKTA Purifier 100 system and the AKTA Prime System (GE-Healthcare).

Q-Sepharose fast flow

The fractions were loaded onto a 40 mL Q-Sepharose fast flow anion exchange column equilibrated with buffer A plus 50 mM NaCl (Fig. 2a). After washing with 80 ml of buffer A containing 50 mM NaCl, the column was eluted with 80 ml of buffer A containing 192.5 mM NaCl, and

then eluted with a 300 ml linear gradient of 192.5 to 440 mM NaCl in buffer A. The kinase activity eluted at ~310 mM (conductivity: 22.8 mS/cm).

Phenyl-Sepharose fast flow

The fractions containing the highest kinase activity were pooled, adjusted to a final concentration of 300 mM NaCl, and loaded onto a 20 ml Phenyl-Sepharose fast flow (HS) hydrophobic interaction column equilibrated with buffer A plus 300 mM NaCl (Fig. 2b). After washing with 40 ml of buffer A containing 300 mM NaCI, the column was then eluted with 40 ml each of buffer A plus 180, 60 and 0 mM NaCl and step eluted with 40 ml each of buffer A plus 24, 48 and 60% ethylene glycol in buffer A. The active fractions (eluting at 48% ethylene glycol) were pooled and loaded onto an 80 ml Sephadex G 25 M column for buffer exchange with buffer A plus 50 mM NaCl.

Resource Q

The fractions were then loaded onto a 6 ml Resource Q anion exchange column equilibrated with buffer A plus 50 mM NaCl, After washing with 18 ml of buffer A containing 50 mM NaCl, the column was eluted with a 60-ml linear gradient of 50–430 mM NaCl in buffer A (Fig. 2c). The kinase activity eluted at ~288 mM (conductivity: 20 mS/cm).

Mono Q™ 5/50 GL

The active fractions were pooled and loaded onto an 50 ml Sephadex G 25 M column for buffer exchange with buffer A plus 145 mM NaCl, and then loaded onto a 1 ml Mono Q™ 5/50 GL anion exchange column equilibrated with buffer A plus 145 mM NaCl (Fig. 2d). After washing with 5 ml of buffer A plus 145 mM NaCl, the column was then eluted with a 20 ml linear gradient of 145–335 mM NaCl in buffer A. The kinase activity eluted at ~263 mM (conductivity: 23.9 mS/cm).

Poly-L-lysine-agarose column

The peak fractions containing the p46MAPK were pooled, adjusted to a final concentration of 10 mM $MgCl_2$, and exchanged with buffer B (25 mM Tris, pH 7.5, 10 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 10 mM *p*-glycerophosphate, 0.1 mM Na_3VO_4, 0.02% Triton X-100) plus 145 mM NaCl. The above-mentioned sample was then loaded onto a 3.5 ml poly-L-lysine-agarose column (Sigma). After washing with buffer B plus 145 mM NaCl, the column was eluted with a 40-ml gradient of 145–810 mM NaCl in buffer B (Fig. 2e). The kinase activity eluted at ~760 mM (conductivity: 43 mS/cm).

Superdex 75 prep-grade column

The active fractions were pooled and concentrated with a concentrator (10,000 molecular weight cut-off, Vivaspin 2, Vivascience AG). To further purify the p46MAPK, the above-mentioned concentrated sample was loaded onto a Superdex 75 prep-grade column (120) equilibrated with buffer B plus 250 mM NaCl, and the column was eluted with the same buffer at 0.5 ml/min (Fig. 2f).

Protein determination

Protein content was determined according to the method of Bradford (1976) with bovine serum albumin as standard.

In-solution kinase activity assay

Protein kinase activity was measured by the incorporation of radioactive phosphate from ATP into MBP. Unless specifically indicated, assays were performed at room temperature for 30 min in a final volume of 40 µl containing 0.5 mg ml^{-1} MBP, 50 µM [γ-^{32}P] ATP, 25 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, and enzyme. At the end of the incubation period, 20 µl reaction mixture was spotted on 1 cm × 1 cm Whatman P81 phosphocellulose paper pieces. These were then washed three times with 75 mM H_3PO_4 (for 5 min each), rinsed for 5 min in ethanol, air dried, and placed in vials with scintillation liquid, and levels of radioactivity were determined.

In-gel kinase activity assay

In-gel kinase activity assays were performed using the method as described by Zhang and Klessig (1997) with slight modifications. Extracts or fractions were resolved in 12% SDS-polyacrylamide gels embedded with 0.5 mg ml^{-1} MBP in the separating gel as a substrate. After electrophoresis, SDS was removed by washing the gel with a washing buffer (25 mM Tris, pH 7.5, 0.5 mM DTT, 0.1 mM Na_3VO_4, 5 mM NaF, 0.5 mg ml^{-1} bovine serum albumin, and 0.1% Triton X-100) three times for 30 min each at room temperature. The proteins were then denatured for 1 h at room temperature in 6 M guanidine–HC1, 25 mM Tris–HCl (pH 7.0), and 5 mM 2-mercaptoethanol. The kinases were allowed to renature in 25 mM Tris, pH 7.5, 1 mM DTT, 0.1 mM Na_3VO_4, and 5 mM NaF at 4°C overnight with six changes of buffer. The gel was then incubated at room temperature in 20 ml of reaction buffer (25 mM Tris, pH 7.5, 2 mM EGTA, 12 mM $MgCl_2$, 1 mM DTT, and 0.1 mM Na_3VO_4) with 200 nM ATP plus 1.85 × 10^6 Bq

[γ-^{32}P] ATP (1.11×10^{14} Bq mM^{-1}) for 60 min. The reaction was stopped by transferring the gel into 5% trichloroacetic acid (w/v)/1% sodium pyrophosphate (w/v). The unincorporated [γ-^{32}P] ATP was removed by washing with the same solution for at least 6 h with five changes. The gel was dried onto Whatman 3 MM paper and exposed to Kodak XAR-5 film.

Analysis of proteins by mass spectrometry

Proteins to be analyzed by mass spectrometry (MS) were separated by 12% SDS-PAGE as described. Gels were stained with Coomassie Brilliant Blue R250, and the 46 kDa band was excised and sent to the National Center of Biomedical Analysis, Academy of Military Medical Sciences (Beijing, China) for mass spectrometry analyses. Briefly, Protein in gel fragment was digested with trypsin and the tryptic digest was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Ultraflex, Brucker Daltonics, Bremen, Germany). Proteins were identified using MS/MS ion search of Mascot search engine (http://www.matrixscience.com, Matrix Science, London, England) and Viridiplantae (Green Plants) protein database (NCBI, 20071116). The Mascot search parameters were as follows: type of search, MS/MS ion search; enzyme, trypsin; variable modifications, carbamidomethyl (C), oxidation (M); mass values, monoisotopic; protein mass, unrestricted; peptide mass tolerance, ±0.3 Da; fragment mass tolerance, ±0.9 Da; max missed cleavages, 2; instrument type, MALDI-TOF-TOF.

Effect of temperature, pH and metal ions concentration on purified p46MAPK activity

The kinase activity was assayed in in-solution, as described above. When one reaction condition was changed, the other condition was invariable. The optimum temperature was determined by incubation at 0, 10, 20, 30, 40, 50 and 70°C. The effect of pH adjusted to 3, 4, 5, 6, 7, 8, 9 and 10 was tested. The activities at 0, 1, 2, 5, 10, 15 and 20 mM $MgCl_2$ were evaluated. The activities at 0, 0.25, 0.5, 1, 2 and 4 mM $MnCl_2$ were also evaluated.

Identification of phosphorylated amino acids in the substrates

Protein substrates were labeled by phosphorylation in the presence of [γ-^{32}P] ATP and precipitated by using 10% (w/v) TCA. After washing with 10% (w/v) TCA and acetone, the pellets were hydrolyzed in 6 M HCl for 2 h at 110°C, dried in an evaporator, and then dissolved in 10 µl of a phosphoamino acids standard (1 mg ml^{-1} each of L-phosphoserine, L-phosphothreonine, and L-phosphotyrosine). To separate the phosphoamino acids, the thin-layer chromatography was performed on cellulose plates according to a described procedure (Duclos et al. 1991). Solvent A: 5 volume isobutyric acid, 3 volume 0.5 M NH_4OH; Solvent B: 7 volume 2-propanol, 1.5 volume HCl, 1.5 volume H_2O. The position of the standards was visualized by ninhydrin (0.2% [w/v] in acetone), and the labeled amino acids were detected by autoradiography.

Semiquantitative RT-PCR expression analysis

Total RNA was isolated from leaves and subjected to RT-PCR amplification with 25 cycles; actin gene was used as the control to show the normalization of the amount of templates in PCR reactions. The two primers for ZmMAPK5 are as follows: forward GCCGCAGCAGCCACTGCC and reverse TGAATGCAGCCCTCTGCGC.

Antibody production and immunoprecipitation kinase activity assay

The peptides for ZmMAPK5-C (EEQMKDLIYQEALA FNPDYQ) corresponding to the carboxy terminus of ZmMAPK5 were synthesized as described in Berberich et al. (1999) and conjugated to the keyhole limpet hemacyanin (KLH) carrier by Jinsite Corporation. The ZmMAPK5 polyclonal antibody was raised in rabbits and purified by affinity chromatography. The specificity of the antibody for ZmMAPK5 was proven earlier by Berberich et al. (1999).

Protein extract (100 µg) was incubated with anti-ZmMAPK5 polyclonal antibody (diluted 1:10,000, v/v) in immunoprecipitation buffer (20 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 2 mM EGTA, 1 mM Na_3VO_4, 1 mM NaF, 10 mM β-glycerophosphate, 5 µg ml^{-1} leupeptin, 5 µg ml^{-1} aprotinin, 0.5% [v/v] Triton X-100, and 0.5% [v/v] NP-40) overnight in a rocker at 4°C. About 20 µl packed volume of protein A agarose was added, and the incubation was continued for 2 h. Agarose bead–protein complexes were pelleted by brief centrifugation. After washing with immunoprecipitation buffer three times, 1× SDS sample buffer was added and boiled for 3 min. After centrifugation, the supernatant fraction was electrophoresed on 12% SDS-polyacrylamide gels, and the in-gel kinase assay was performed.

Immunoblots

The protein extracts (20 µg) were separated by 12% SDS-PAGE. After electrophoresis, the gel was transferred to a polyvinylidene difluoride (PVDF) membrane at 100 V for 70 min at 4°C in a Mini Trans-Blot Electrophoretic Transfer Cell (Bio-Rad, Hercules, CA, USA). The membrane was incubated in blocking solution containing TBST

(20 mM Tris-base, 137 mM NaCl, 0.1% Tween-20, pH 7.6) supplemented with 5% (w/v) non-fat dry milk for 1 h at room temperature. Then it was washed three times for 10 min with TBST buffer. The blots were probed with anti-ZmMAPK5 polyclonal antibodies. The immune complexes were detected by a horseradish peroxidase (HRP)-conjugated secondary antibody (Pierce, Appleton, WI, USA). Antibodies were used at 1:2,000, and secondary antibodies were used at 1:4,000.

Results

Purification of the p46MAPK

In previous studies, we reported that a 46 kDa mitogen-activated protein kinase (p46MAPK) activated by ABA is involved in ABA-induced antioxidant defense in the leaves of maize (Zhang et al. 2006, 2007). To identify the nature of the p46MAPK, the protein was purified from leaves of maize seedlings (1,000 g). Samples were prepared and subjected to $(NH_4)_2SO_4$ fractionation as described in "Materials and methods", followed by ultracentrifugation and chromatography on Q-Sepharose FF, Phenyl-Sepharose FF, Resource Q, Mono Q^{TM} 5/50 GL, poly-L-lysine-agarose, and Superdex 75 prep-grade columns. Elution from $(NH_4)_2SO_4$ fraction was analyzed with in-solution kinase assay, using myelin basic protein (MBP) as a substrate. Proteins precipitated by 0–30% $(NH_4)_2SO_4$ fraction contained almost all of the MBP kinase activity compared with the proteins precipitated by 40–80% (Fig. 1a). The same elution was also analyzed with in-gel kinase assay, using MBP as a substrate. Only one significant band of kinase activity was detected in gel and the molecular mass was about 45.4 kDa (Fig. 1b), correlated with the 46 kDa MAPK induced by ABA. Typical elution profiles of the subsequent six chromatography steps are shown in Fig. 2a–f, and only a single major kinase activity peak was detected by the in-solution assay. The purified enzyme preparation had a specific acticity of 20,420 pmol min^{-1} mg^{-1} and the yield was 1.1% (summarized in Table 1).

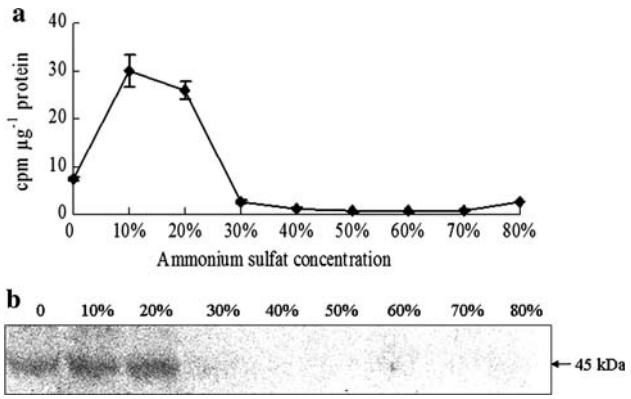

Fig. 1 The p46MAPK activity of $(NH_4)_2SO_4$ fraction. **a** In-solution kinase activity of each precipitant fraction. **b** In-gel kinase activity of each precipitant fraction. The protein extract of maize leaves was fractionated by $(NH_4)_2SO_4$ added sequentially to 10, 20, 30, 40, 50, 60, 70 and 80% saturation. The precipitant was dissolved, centrifuged and then desalted with a Sephadex G 25 M column. MAPK activity was analyzed by in-solution kinase assay (**a**) or in-gel kinase assay (**b**) with MBP as the substrate. MBP phosphorylation was visualized by autoradiography

After SDS-PAGE analysis and Coomassie Brilliant Blue R-250 staining, the gel showed the protein profile at typical stages of purification, but the aimed protein was not seen clearly (Fig. 3a). The sliver staining was used to stain the gel; the enzymes from Superdex 75 prep-grade column showed three bands in the 45–66.2 kDa range (Fig. 3b). Only a 45.4-kDa protein band was close to the molecular weight of p46MAPK. To ensure that the solution contained the kinase of interest, we performed in-gel kinase assays with MBP as a substrate (Fig. 3c). Only one band of kinase activity was detected, and its molecular mass was about 45.4 kDa. However, there was little enzyme activity in band 4. The reason for this might be that the active fractions from phenyl-sepharose fast flow contained 48% ethylene glycol, which affects the concentration of fractions. Thus, the concentration of the aimed protein might be key to the kinase activity. These results suggest that this polypeptide was the 46 kDa ABA-activated MAPK.

Separation of p46MAPK on the Mono QTM 5/50 GL and Superdex 75 pg column

To further determine whether the purified kinase was associated with the kinase activated by ABA, we purified the kinase from the ABA-treated and water-treated maize leaves at the same time. After the ultracentrifugation step and desalting with Sephadex G 25 M column, protein samples prepared from either 100 μM ABA-treated or water-treated maize leaves were loaded onto a Mono Q^{TM} 5/50 GL column connected to an AKTA Purifier 10 system (Fig. 4a). The kinase activity was determined by the in-solution kinase assay, using MBP as the substrate. The peak of kinase activity of treatment was higher than that of the control. Its identity was confirmed by the in-gel kinase activity assay, which revealed a kinase of 45.4 kDa in the Mono Q^{TM} 5/50 GL fractions with high kinase activity (Fig. 4b).

The Superdex 75 prep-grade gel filtration chromatography was also employed to estimate the molecular mass, because it provided sufficient separation of proteins from 3.0 to 70.0 kDa. The p46MAPK enriched by chromatography migrated with an apparent molecular mass of approximately 45.7 kDa, indicating that ABA-induced p46MAPK is a monomeric 46 kDa enzyme (data not shown).

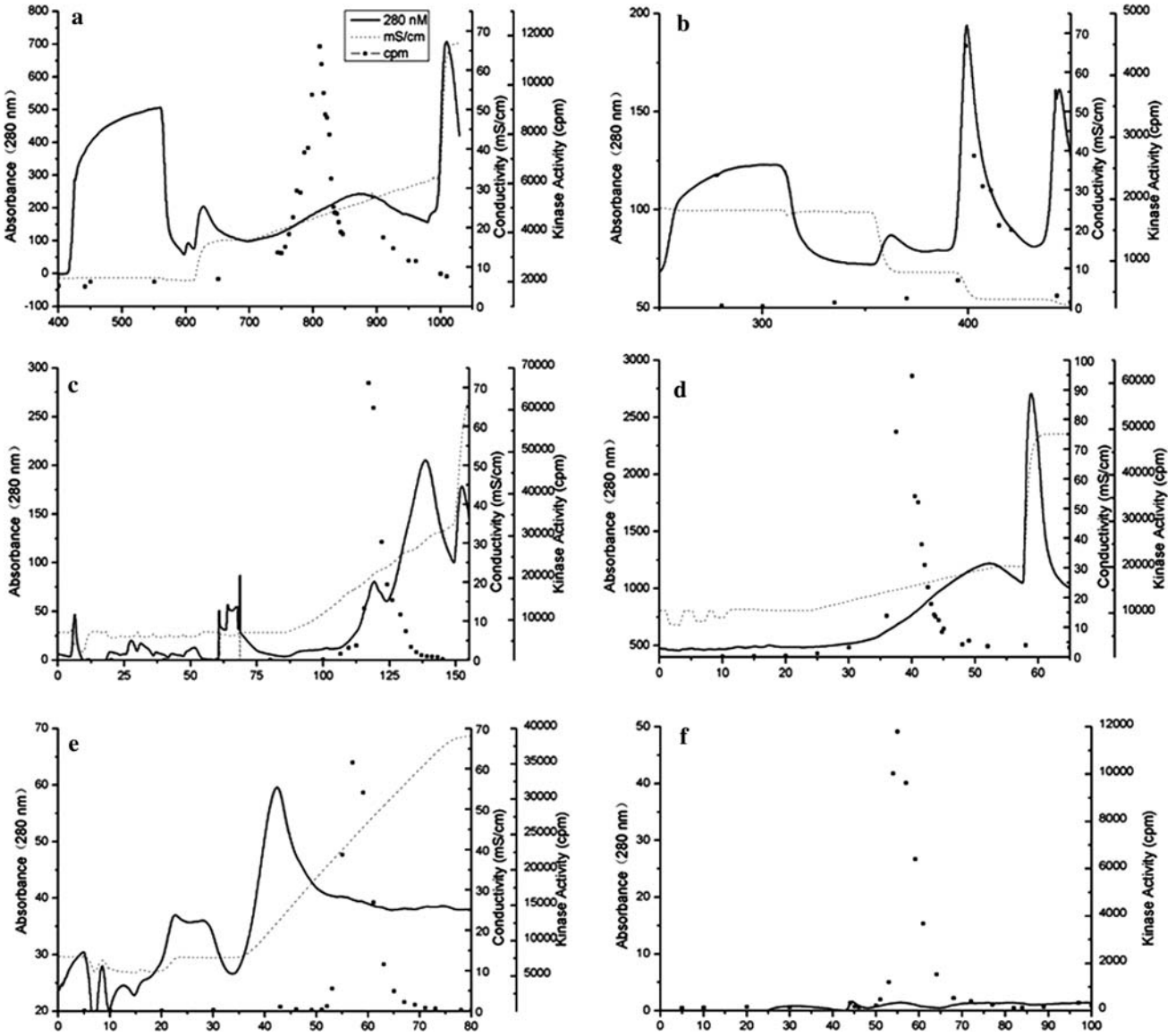

Fig. 2 Elution profiles of protein concentration and kinase activity from each chromatography step. **a** Chromatography on a Q-Sepharose FF column. **b** Chromatography on a Phenyl-Sepharose FF (HS) column. **c** Chromatography on a Resource Q column. **d** Chromatography on a Mono Q™ 5/50 GL column. **e** Chromatography on a poly-L-lysine-agarose column. **f** Chromatography on a Hiload 16/60 Superdex 75 pg column. The kinase activity (*filled circles*) was determined by in-solution kinase assay with MBP as the substrate. The MBP phosphorylation was calculated by scintillation counter. The *dashed lines* indicate the NaCl gradient profiles (as indicated by conductivity, mS/cm). The *black lines* indicate the absorbance at 280 nm

Identification of proteins by mass spectrometry

In order to identify the p46MAPK, we undertook the sequencing by tandem mass spectrometry, a well-documented technique for protein sequencing analysis. The purified protein was sliver stained on an SDS-PAGE gel showing a band corresponding to 46 kDa (Fig. 3b), and was in-gel digested by trypsin, followed by MALDI-TOF/TOF-MS/MS analyses. The resulting spectrum was used to search for matching proteins in the NCBI database, using the Mascot search program. The search yielded a top score of 207 for gi|4239889, MAP kinase 5 [*Zea mays*], (protein scores greater than 69 are significant; $P < 0.05$). Figure 5a showed the sequences of peptides obtained by MS/MS. Sequences of 17 peptides were successfully determined by MS/MS, which could be assigned to ZmMAPK5. The sequence coverage of the peptides shown in Fig. 5a against the whole sequences of ZmMAPK5 was 37% (data not shown). The nominal mass of the kinase was found to be 44.9 kDa, which is similar to the physicochemical properties found in the purified MAPK (45.4 and 45.7 kDa). The difference between the

Table 1 Purification protocol of p46MAPK from *Zea mays*

Sample	Protein (mg)	Total activity (pmol min^{-1})	Specific activity (pmol min^{-1} mg^{-1})	Purification fold	Yield %
Homogenate	3,740	7,106	2	1	100.0
$(NH_4)_2SO_4$ 30%	258	4,489	17	9	63.2
100, 000*g*	171	3,454	20	10	48.6
Q-Sepharose FF	11.56	983	85	43	13.8
Phenyl-Sepharose FF	1.73	415	240	120	5.8
Resource Q	0.368	307	834	417	4.3
Mono Q™ 5/50 GL	0.138	249	1,804	902	3.5
Poly-L-lysine-agarose	0.021	189	9,005	4,502	2.7
Hiload 16/60Superdex 75 pg	0.004	82	20,420	10,210	1.1

The starting homogenate was prepared from 1,000 g of maize leaves treated with 100 μM ABA for 2 h. Proteins from different stages of purification were analyzed for MAPK activity by in-solution kinase assay with MBP as the substrate

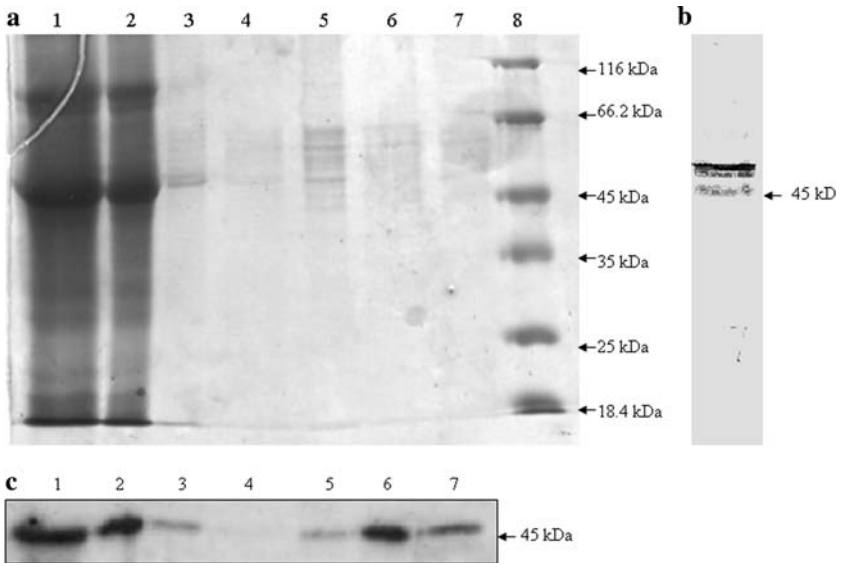

Fig. 3 Analysis of purification pools by gel electrophoresis and in-gel kinase assay. **a** Proteins from different stages of purification were resolved in a 12% polyacrylamide gel containing SDS and visualized by Coomassie blue. *Lane 1* crude extract; *lane 2* supernatant after 130,000*g* ultracentrifugation; *lane 3* pooled fractions from Q-Sepharose FF column; *lane 4* pooled fractions from Phenyl-Sepharose FF column; *lane 5* pooled fractions from Mono Q™ 5/50 GL column; *lane 6* pooled fraction from poly-L-lysine-agarose column; *lane 7* pooled fractions from Hiload 16/60 Superdex 75 pg column; *lane 8* molecular mass marker standards. The position of molecular mass markers in kilodaltons is indicated on the *right*. **b** Proteins from Hiload 16/60 Superdex 75 pg column were resolved in a 12% SDS-polyacrylamide gel and stained with sliver. **c** In-gel kinases assay of purified protein kinase from different stages of purification. The fractions were loaded onto a 12% SDS-polyacrylamide gel embedded with MBP. MBP phosphorylation was visualized by autoradiography

putative molecular weight and the one found by the SDS-PAGE and the gel filtration might be related to the post-translational modifications. Mascot search of the sequences obtained by MS/MS analysis against NCBI database resulted in the identification of other plant MAPKs such as *Triticum aestivum* (gil27542952), *Zea mays* (gil39599010), *Cocos nucifera* (gil29603441), *Nicotiana attenuate* (gil134254740), *Nicotiana tabacum* (gil78096654) and *Cocos nucifera* (gil29603439). Furthermore, the selected tryptic peptide (*m*/*z* 1779.841) sequenced by MS/MS revealed an amino acid sequence of TTSETDFMTEYVVTR, corresponding to residues 218–232 of ZmMAPK5. These results clearly indicate that the ABA-activated p46MAPK is ZmMAPK5.

Biochemical characterization of the ZmMAPK5

Because of the easy loss of kinase activity during the purification step, the partially purified ZmMAPK5 from the poly-L-lysine-agarose column was used for characterization. Although the partially purified ZmMAPK5 preparation contained other proteins, it did not contain other contaminant

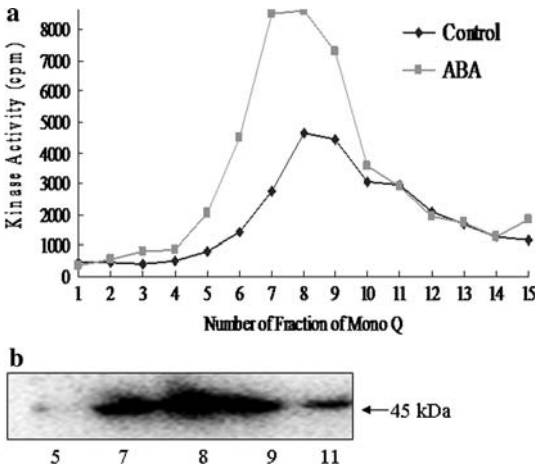

Fig. 4 Elution profile of protein kinase from the Mono QTM 5/50 GL column corresponds to the p46 MAPK. **a** After the ultracentrifugation step and desalting with Sephadex G 25 M column, protein samples prepared from either ABA-treated (ABA) or water-treated maize (control) were loaded onto a Mono QTM 5/50 GL column connected to an AKTA Purifier 10 system. The kinase activity was determined by the in-solution kinase assay, with MBP as a substrate. **b** Selected fractions from the Mono QTM 5/50 GL column chromatography of the protein sample from ABA-treated maize were assayed by the in-gel kinase method, with MBP as substrate. The mass of the kinase in kilodaltons is indicated on the *left*

kinases and therefore allowed the partial characterization of the p46MAPK.

The partially purified ZmMAPK5 showed complete dependence on Mg^{2+} for its activity. The kinase was active at Mg^{2+} from 1 to 20 mM. The optimum kinase activity was obtained at 15 mM Mg^{2+} (Fig. 6a), which was higher than that of the tobacco SIP kinase (Zhang and Klessig 1997). The SIP kinase activity was strongly stimulated by Mg^{2+} up to a total concentration of 2 mM and then decreased slightly as the concentration of Mg^{2+} increased. The Mn^{2+} had no effect on the kinase activity. The pH activity profile of the purified kinase is shown in Fig. 6a. The purified enzyme showed activity in a very broad pH range (5.0–10.0). Even at a pH of 5.0 or 10, it remained with high activity, which was consistent with a previous study (Zhang and Klessig 1997). The effect of temperature on the kinase activity was determined by assaying enzyme activity at different temperatures (Fig. 6a). The kinase from maize was active at temperatures from 0 to 70°C, with an optimum around 35°C. Using different substrates (MBP, histone III-S and casein), our results showed that only MBP was a good phosphate acceptor, histone was weakly phosphorylated, and phosphorylation of casein was not be detected using in-solution kinase assay (Fig. 6b). Three substrates were also analyzed by in-gel kinase assay, and only MBP phosphorylated band could be detected (data not shown). These results indicated that ZmMAPK5 is a substrate-specific kinase. The K_m for MBP was determined by varying its concentration between 0.05 and 1.0 μg μl^{-1}, whereas the K_m for ATP ranged between 10 and 100 μM. The apparent K_m values of purified kinase for MBP and ATP were 0.13 μg μl^{-1} and 62 μM, respectively. Michaelis–Menton and Lineweever–Burk plots for MBP and ATP are shown in Fig. 6b. Phosphoamino acid analysis using thin-layer chromatography demonstrated that only threonine residue was phosphorylated on MBP by ZmMAPK5 (Fig. 6c), showing that it belongs to the serine/threonine family of protein kinase.

The ZmMAPK5 responses to diverse extracelluar stimuli

Berberich et al. (1999) reported that ABA did not induce the expression of ZmMAPK5 and the activity of ZmMAPK5. However, recent reports have shown that ABA and H_2O_2 can activate p46MAPK (Zhang et al. 2006, 2007), which has been identified as ZmMAPK5 in this study. In order to further confirm whether the ZmMAPK5 is regulated by ABA and H_2O_2, the polyclonal antibody that recognizes the C-terminal region of ZmMAPK5 was raised in rabbits, and the immunoprecipitation kinase assay was used. Both 100 μM ABA and 10 mM H_2O_2 induced the expression of the ZmMAPK5 gene and activation of ZmMAPK5 (Fig. 7). To determine whether ZmMAPK5 is activated in response to other signals, the leaves were treated with salicylic acid (SA), ethephon (ETH), PEG, NaCl, $CdCl_2$, cold, wounding and UV, and the expression and the activity of ZmMAPK5 were analyzed. All these treatments induced the expression and the activity of ZmMAPK5, although the kinetics of the responses was different (Fig. 7). To measure the levels of the ZmMAPK5 proteins during activation, immunoblot analyses with the ZmMAPK5 antibody were performed. The amounts of proteins remained constant under the stress conditions (Fig. 7c).

Discussion

During the last decade, the role of the plant hormone ABA in the induction of antioxidant defense and the mechanism of ABA signal transduction in this process have been the subject of extensive research (Jiang and Zhang 2002a, b, 2003, 2004; Hung and Kao 2004; Park et al. 2004; Hu et al. 2005, 2007; Zhang et al. 2006, 2007). However, many of the components in the ABA signal transduction pathway remain to be elucidated. Our previous studies showed that a 46-kDa MBP kinase activated by ABA was involved in ABA-induced antioxidant defense. The MBP kinase activation was associated with Tyr phosphorylation, and inhibited by pretreatments with the specific MAPKK inhibitors PD98059 and U0126. The size of the kinase was in the

a

gi|4239889 Mass: 44863 Score: 207 Expect: 8.4e-16 Queries matched: 17

MAP kinase 5 [Zea mays]

Observed	Mr(expt)	Mr(calc)	Delta	Start	End	Miss	Ions	Peptide
814.4766	813.4693	813.4861	-0.0168	266	272	0	---	R.KPLFPGR.D
879.4366	878.4293	878.4320	-0.0027	333	339	0	---	K.MLTFDPR.Q
894.4728	893.4655	893.4429	0.0226	210	217	0	---	K.ICDFGLAR.T
904.4685	903.4613	903.4675	-0.0062	273	279	0	---	R.DHVHQLR.L
909.5182	908.5109	908.5080	0.0029	132	139	0	---	R.DIIPPAQR.A
951.4674	950.4601	950.4644	-0.0043	210	217	0	---	K.ICDFGLAR.T + Carbamidomethyl (C)
1179.6260	1178.6187	1178.5866	0.0321	333	341	1	---	K.MLTFDPRQR.I + Oxidation (M)
1334.6904	1333.6831	1333.6561	0.0270	121	131	0	---	R.HMDHENIVAIR.D
1350.6815	1349.6742	1349.6510	0.0232	121	131	0	---	R.HMDHENIVAIR.D + Oxidation (M)
1475.7788	1474.7715	1474.7892	-0.0177	99	111	2	---	K.IANAFDNKIDAKR.T
1507.6792	1506.6719	1506.8419	-0.1700	181	193	1	---	R.GLKYIHSANVLHR.D
1585.7097	1584.7024	1584.8848	-0.1824	306	318	2	---	R.QLPRHPRQSLPEK.F
1699.8606	1698.8533	1698.9430	-0.0897	266	279	1	---	R.KPLFPGRDHVHQLR.L
1716.8734	1715.8661	1715.9253	-0.0592	118	131	1	---	K.LLRHMDHENIVAIR.D
1779.8340	1778.8267	1778.8033	0.0234	218	232	0	126	R.TTSETDFMTEYVVTR.W
1795.8299	1794.8226	1794.7982	0.0244	218	232	0	---	R.TTSETDFMTEYVVTR.W + Oxidation (M)
2460.2305	2459.2232	2459.2002	0.0230	280	301	0	---	R.LLMELIGTPNEGDLDFVNENAR.R

b

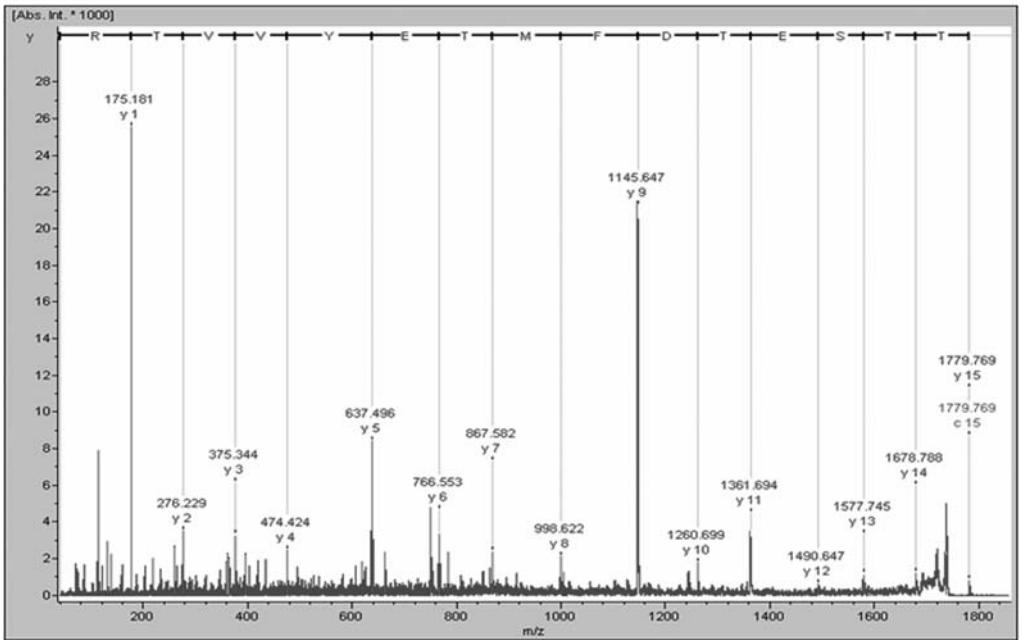

Fig. 5 Results of MALDI-TOF/TOF-MS for the p46MAPK. **a** Observed and expected monoisotopic $[M + H]^+$ masses of ions from the tryptic digest of the p46 MAPK. *Numbers* in *superscript* indicate overlapping peptide sequences. **b** Results of MS/MS of the selected peptide (*m/z* 1779.841). The amino acid sequence is TTSETDFMTEYVVTR. Protein in-gel fragment was digested with trypsin and the tryptic digest was analyzed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (Ultraflex, Brucker Daltonics, Bremen, Germany) using BioTools software, version 3.0 to search the NCBI database using the online program Mascot. The Mascot search parameters were as follows. Type of search: MS/MS ion search; enzyme: trypsin; Variable modifications: carbamidomethyl (C), oxidation (M); mass values: monoisotopic; protein mass: unrestricted; peptide mass tolerance: ±0.3 Da; fragment mass tolerance: ±0.9 Da; max missed cleavages: 2; instrument type: MALDI-TOF-TOF

range of known members of the MAPK family (38–55 kDa). All these evidence demonstrated that the 46-kDa MBP kinase belongs to the MAPK family (Zhang et al. 2006, 2007). However, the identity of the ABA-activated p46MAPK in maize leaves is not clear. One of the first rigorous demonstrations that a particular MAPK gene encodes the activated enzyme relied on purification of the activated enzyme and the cloning of corresponding gene based on the partial amino acid sequences. Zhang and Klessig (1997) for the first time purified an SA-induced protein kinase (SIPK) from tobacco suspension cells, and further cloned and characterized the SIPK. Katou et al. (2005) purified a 51-kDa MAPK, which was activated in potato (*Solanum tuberosum*) tubers treated with hyphal wall elicitor, identified

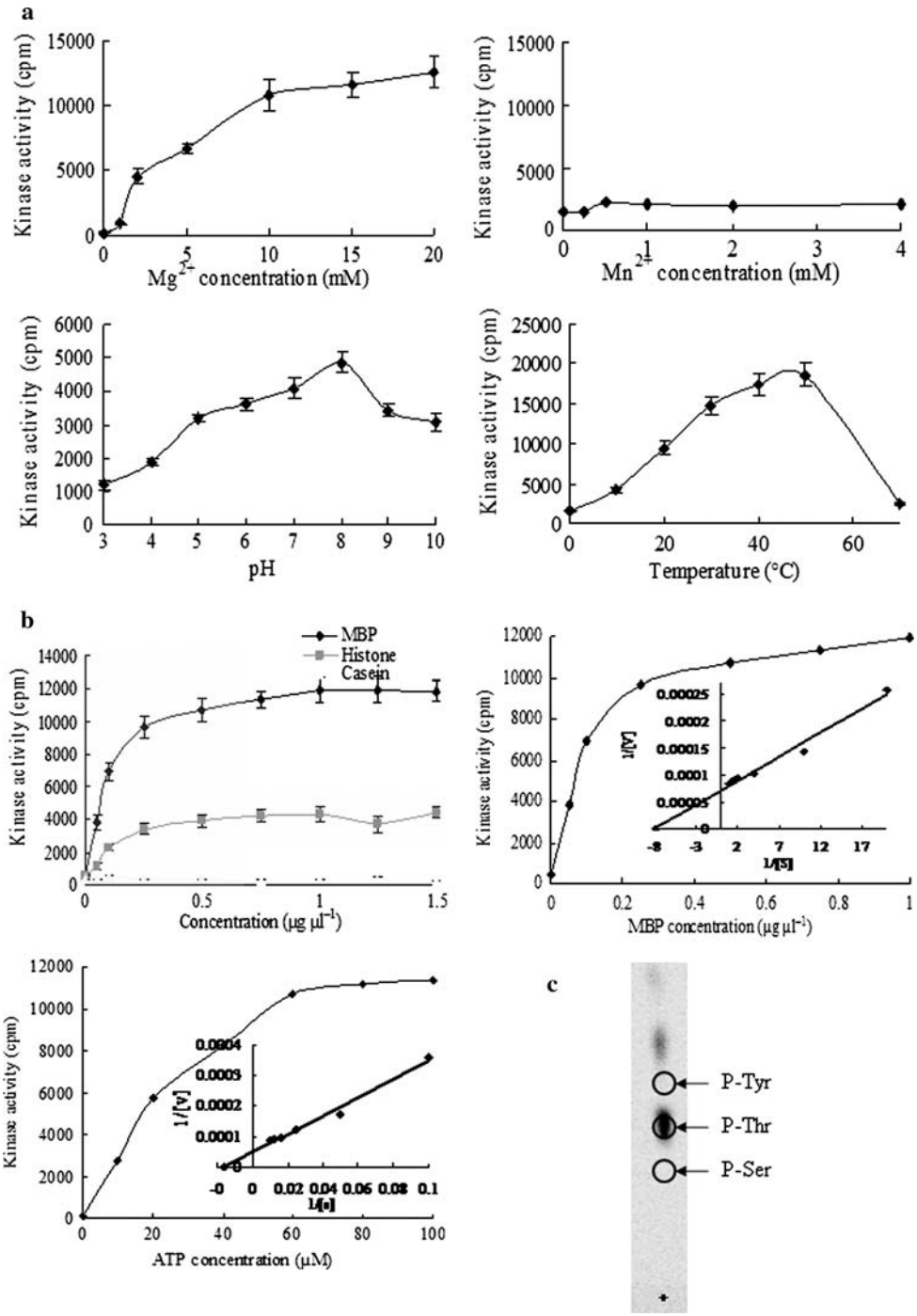

Fig. 6 Biochemical characterization of ZmMAPK5. **a** Effects of various Mg^{2+} concentrations, Mn^{2+} concentrations, reaction pH and reaction temperatures on the activities of ZmMAPK5. **b** Kinetic analysis with ZmMAPK5 using different substrates. The assay conditions were as described in "Materials and methods", except that various concentrations of MBP, histone III-S, and casein were used. Michaelis–Menten parameters were determined using different concentrations of MBP and ATP. The *inset* shows a Lineweaver–Burk plot for the same values. **c** Thin-layer chromatography of the amino acid phosphorylation in MBP by the ZmMAPK5. The phosphoamino acids were separated by thin-layer chromatography. The *circles* indicate the positions of markers of phosphotyrosine, phosphoserine and phosphothreonine as visualized by ninhydrin staining. The labeled amino acids were detected by autoradiography that matched phosphothreonine visualized by ninhydrin staining

partial amino acid sequence and isolated the cDNA designated *StMPK1*. To reveal the identity and function of the p46MAPK, in this study we purified, identified and characterized the enzyme.

The complete purification scheme presented for p46MAPK from maize is the first report for this enzyme from natural leaf tissues. The maize p46MAPK was purified to near-homogeneity by $(NH_4)_2SO_4$ fractionation, ultracentrifugation and six-column chromatography steps (see "Matrials and methods" for details). Elution profiles of the six chromatography steps are shown in Fig. 2a–f. The partially purified p46MAPK preparation had a specific activity of 20,420 pmol min^{-1} mg^{-1}, using MBP as a substrate. This is 17-fold lower than the specific activity of SIPK purified from tobacco suspension culture cells (Zhang and Klessig 1997). The yield of purification was 1.1%, which was also lower than that of SIPK, but was near that of StMPK1 (Katou et al. 2005). The low level of purification is due to the fact that the enzyme activity is lost in the procedure of purification. The SIPK was purified from

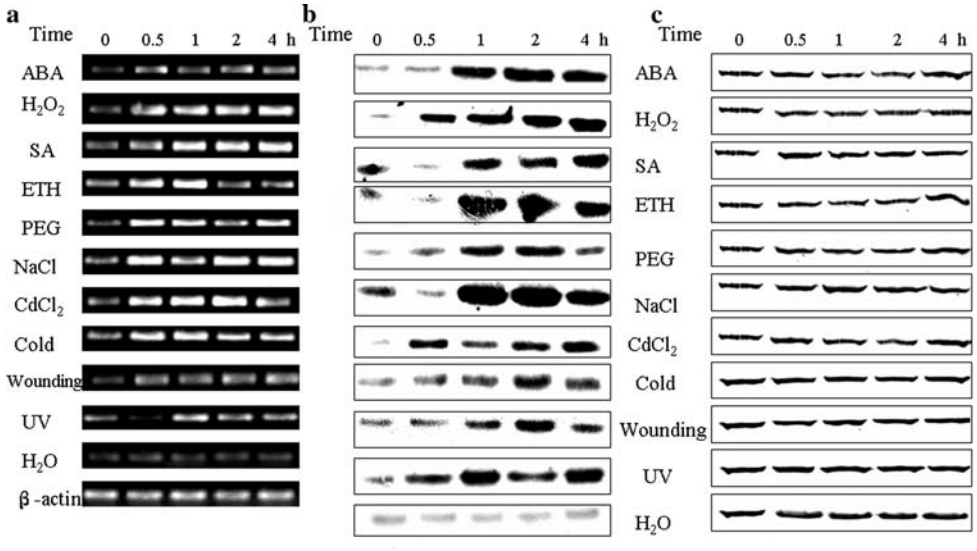

Fig. 7 Activation of ZmMAPK5 by various abiotic stimuli. **a** Effects of abiotic stimuli on the expression of the ZmMAPK5 gene analyzed by semi-quantitative RT-PCR. **b** Effects of abiotic stimuli on the activity of the ZmMAPK5. The ZmMAPK5 was immunoprecipitated from leaves after treatment. The ZmMAPK5 activity was measured in immunoprecipitation kinase assay using myelin basic protein (MBP) as a substrate. **c** Immunoblot analysis with anti-ZmMAPK5 antibody. Protein extracts were separated by 12% SDS-PAGE and detected by immunoblot analysis using anti-ZmMAPK5 antibody. The experiments were repeated at least five times (**a**) or three times (**b**, **c**) with similar results. The detached plants were treated with 100 μM ABA, 10 mM H_2O_2, 100 μM SA, 100 μM ethephon, 10% PEG, 250 mM NaCl, 500 μM $CdCl_2$, 4°C, wounding and UV for various times up to 4 h at 25°C with a continuous light intensity of 100 μmol m^{-2} s^{-1}. Plants treated with distilled water under the same conditions during the whole period served as controls

tobacco suspension culture cells, while p46MAPK was from green leaves. The purification of the SIPK was carried out in 3 days, but 5–7 days were needed for the purification of the p46MAPK. Moreover, it was difficult to eliminate the pigment in elution, which especially affected the purification efficiency. Rapid purification is critical because the phosphatase inhibitors included in the buffer were unable to completely inhibit all of the phosphatases that can inactivate the MAPK (Zhang and Klessig 1997).

Development in mass spectrometry (MS) technology has dramatically accelerated the application of proteomics in recent years. The recent MALDI-TOF/TOF mass spectrometry technology allows for rapid acquisition of MS/MS data with high sensitivity (Reyzer and Caprioli 2005; Hortin 2006). The partially purified protein was sliver stained on an SDS-PAGE gel showing a band corresponding to 46 kDa (Fig. 3) and was in-gel digested by trypsin, followed by MALDI-TOF/TOF-MS/MS analyses. The resulting spectrum was used to search for matching proteins in the Viridiplantae (green plants) protein database (NCBI, 20071116), using MS/MS ion search of Mascot search engine (http://www.matrixscience.com, Matrix Science, London, England). The search yielded a top score of 207 for ZmMAPK5 (protein scores greater than 69 are significant; $P < 0.05$; Fig. 5). Furthermore, the selected peptide (m/z 1779.841) sequenced by MS/MS revealed an amino acid sequence of TTSETDFMTEYVVTR, corresponding to residues 218–232 of ZmMAPK5. Therefore, it was confirmed that the ABA-activated p46MAPK was ZmMAPK5, which was found involved in the process of senescence and in recovery from low-temperature stress in maize plants (Berberich et al. 1999).

Though Berberich et al. (1999) found this kinase early and cloned the gene, there were no further studies on ZmMAPK5. Therefore, we investigated the partial physicochemical properties and the responses to diverse extracellular stimuli. In its active form and in the absence of other contaminating kinase activities, the partially purified ZmMAPK5 allowed the primary characterization. The molecular mass of the ZmMAPK5 was found to be 45.4, 45.74 and 44.9 kDa by SDS-PAGE, gel-filtration and MS, respectively, suggesting that the activation of ZmMAPK5 may be a post-translation modification. The kinase showed activity in the range of 20–50°C in temperature, 2.5–15 mM $MgCl_2$, and a broad pH of 5.0–9.0. The K_m for MBP substrate and ATP were 0.13 μg μl^{-1} and 62 μM, respectively. The threonine residue of MBP was phosphorylated by ZmMAPK5. The purified ZmMAPK5 showed an absolute requirement for Mg^{2+} and could only utilize Mg^{2+}-ATP as a phosphate donor, which was in consistent with the SIPK (Zhang and Klessig 1997). The substrate specificity of ZmMAPK5 was similar to that of the SIPK. They strongly phosphorylated MBP, weakly phosphorylated histone and did not phosphorylate casein.

The characterizations of ZmMAPK5 obtained in this study are partially consistent with those of tobacco SIPK. These may be explained by analysis of the phylogenetic tree, which indicates that ZmMAPK5 is most homologous to AtMPK6 and NtSIPK (Berberich et al. 1999; Zong et al. 2009). However, it was reported that ABA did not induce the expression and activity of ZmMAPK5 in maize leaves (Berberich et al.1999). It was also shown that the activities of SIPK and AtMPK6 were not affected by ABA treatment (Hoyos and Zhang 2000; Ichimura et al. 2000). By contrast, a recent study showed that AtMPK6 in *Arabidopsis* leaves can be activated by ABA treatment (Xing et al. 2008). In this study, to confirm whether the ZmMAPK5 is regulated by ABA, the polyclonal antibody that recognizes the C-terminal 20 amino acids of ZmMAPK5 was raised in rabbits, and the immunoprecipitation kinase and immunoblot analyses were used. The specificity of the antibody was proven previously (Berberich et al.1999). Our data show that both the gene expression and the activity of ZmMAPK5 are induced by ABA in maize leaves, although the levels of the proteins remained constant (Fig. 7). It is possible that different plant tissues or cells may have different response to ABA stimulus. However, our data from this study and previous studies (Zhang et al. 2006, 2007) provide unequivocal evidence for the involvement of ZmMAPK5 in ABA signaling in maize leaves.

Though AtMPK6 was implicated in responses to various abiotic stimuli, little or no stress-induced gene expression of ATMPK6 was observed in any of the treatments (Ichimura et al. 2000; Ahlfors et al. 2004). However, OsSIPK, homologous to AtMPK6, can be activated by plant hormones and various stresses at transcriptional levels (Lee et al. 2008). In the present study, our results showed that the amount of proteins remained constant under stress conditions. Both transcriptional and post-translational levels of ZmMAPK5 can be regulated by H_2O_2, SA, ethephon (ETH), PEG, NaCl, $CdCl_2$, cold, wounding and UV, and only the kinetics of their responses differed (Fig. 7). In a previous study, our results suggest that the activation of p46MAPK (ZmMAPK5) is dependent on endogenous ABA in maize leaves (Zhang et al. 2007). Xing et al. (2008) also demonstrated that AtMKK1-AtMPK6 as a key module is involved in ABA signaling causing stress responses. Though ABA plays critical roles in the plant's responses to environmental stresses, we do not know whether MAPK cascades responses to these stresses are ABA-dependent or not.

In summary, we have described the isolation and purification of a 46-kDa MAPK activated by ABA in maize leaves, which was identified as the ZmMAPK5 by mass spectrometry. The partial physicochemical properties of ZmMAPK5 and the ability to respond to diverse extracellular stimuli were studied. These results can help us to further reveal the function of the ZmMAPK5 in ABA signaling pathways.

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