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RESEARCH PAPER

ZmCPK11 is involved in abscisic acid-induced antioxidant defence and functions upstream of ZmMPK5 in abscisic acid signalling in maize

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Abstract

Calcium-dependent protein kinases (CDPKs) have been shown to be involved in abscisic acid (ABA)-mediated physiological processes, including seed germination, post-germination growth, stomatal movement, and plant stress tolerance. However, it is not clear whether CDPKs are involved in ABA-induced antioxidant defence. In the present study, the role of the maize CDPK ZmCPK11 in ABA-induced antioxidant defence and the relationship between ZmCPK11 and ZmMPK5, a maize ABA-activated mitogen-activated protein kinase (MAPK), in ABA signalling were investigated. Treatments with ABA and H_2O_2 induced the expression of *ZmCPK11* and increased the activity of ZmCPK11, while H_2O_2 was required for the ABA-induced increases in the expression and the activity of ZmCPK11. The transient gene expression analysis and the transient RNA interference (RNAi) test in protoplasts showed that ZmCPK11 is involved in ABA-induced up-regulation of the expression and the activities of superoxide dismutase (SOD) and ascorbate peroxidase (APX), and in the production of H_2O_2. Further, ZmCPK11 was shown to be required for the up-regulation of the expression and the activity of ZmMPK5 in ABA signalling, but ZmMPK5 had very little effect on the ABA-induced up-regulation of the expression and the activity of ZmCPK11. Moreover, the transient gene expression analysis in combination with the transient RNAi test in protoplasts showed that ZmCPK11 acts upstream of ZmMPK5 to regulate the activities of antioxidant enzymes. These results indicate that ZmCPK11 is involved in ABA-induced antioxidant defence and functions upstream of ZmMPK5 in ABA signalling in maize.

Key words: Abscisic acid, antioxidant defence, calcium-dependent protein kinase, maize, mitogen-activated protein kinase, signal transduction.

Introduction

Abscisic acid (ABA) is a plant hormone that plays critical roles in adaptive responses to environmental stresses such as drought and salt stress. ABA accumulates in plant cells under water stress, stimulates stomatal closure, and regulates the expression of many genes, thus increasing the plant's capacity to cope with stress conditions (Cutler *et al.*, 2010; Hubbard *et al.*, 2010; Umezawa *et al.*, 2010; Joshi-Saha *et al.*, 2011). Accumulating evidence indicates that ABA-enhanced water stress tolerance is associated with the induction of antioxidant defence systems, including reactive oxygen species (ROS)-scavenging enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX), and glutathione reductase (GR), and non-enzymatic antioxidants such as ascorbic acid, glutathione, α-tocopherol, and carotenoids (Jiang and Zhang, 2002*a*, *b*; Hu *et al.*, 2005; Miao *et al.*, 2006; Zhang *et al.*, 2006,

2007; Neill *et al*., 2008; Xing *et al*., 2008; Miller *et al*., 2010; Ye *et al*., 2011; Ozfidan *et al*., 2012). Ca^{2+}, calmodulin (CaM), NADPH oxidase, H_2O_2, nitric oxide (NO), mitogen-activated protein kinase (MAPK), and Ca^{2+}/CaM-dependent protein kinase (CCaMK) are important intermediate components in ABA-induced antioxidant defence (Jiang and Zhang, 2002*a*, *b*, 2003; Hu *et al*., 2005, 2007; Zhang *et al*., 2006, 2007; Neill *et al*., 2008; Xing *et al*., 2008; Ye *et al*., 2011; Ma *et al*., 2012; Shi *et al*., 2012).

Calcium-dependent protein kinases (CDPKs) are serine/threonine protein kinases that include a Ca^{2+}-binding CaM-like domain and are one of the best characterized Ca^{2+} sensors in plants. CDPKs constitute a large multigene family consisting of 34 genes in *Arabidopsis* (Cheng *et al*., 2002; Hrabak *et al*., 2003) and 29 genes in rice (Asano *et al*., 2005). A number of studies have shown that CDPKs are involved in the responses of plants to various abiotic stresses, including drought, salt, hormonal stimuli, and oxidative stress (Saijo *et al*., 2000; Xing *et al*., 2001; Choi *et al*., 2005; Ludwig *et al*., 2005; Mori *et al*., 2006; Kobayashi *et al*., 2007, 2012; Ma and Wu, 2007; Zhu *et al*., 2007; Boudsocq *et al*., 2010; Mehlmer *et al*., 2010; Xu *et al*., 2010; Zou *et al*., 2010; Asano *et al*., 2011, 2012; Franz *et al*., 2011; Zhao *et al*., 2011; Boudsocq and Sheen, 2012). In *Arabidopsis*, AtCPK3 and AtCPK6 (Mori *et al*., 2006), AtCPK4 and AtCPK11 (Zhu *et al*., 2007), AtCPK10 (Zou *et al*., 2010), and AtCPK32 (Choi *et al*., 2005) have been shown to be positive regulators of ABA-mediated physiological processes, including seed germination, post-germination growth, stomatal movement, and plant stress tolerance. In rice, OsCPK12 (Asano *et al*., 2012) and OsCPK21 (Asano *et al*., 2011) are involved in positive regulation of the ABA signalling pathway. However, a recent study showed that AtCPK12 is a negative ABA signalling regulator in seed germination and post-germination growth (Zhao *et al*., 2011). CDPKs have also been shown to be associated with the production of ROS. Ectopic expression of *AtCPK1* increases NADPH oxidase activity and ROS production in tomato protoplasts (Xing *et al*., 2001). AtCPK4, AtCPK5, AtCPK6, and AtCPK11 redundantly regulate the bacterial elicitor flg22-induced ROS burst and pathogen defence (Boudsocq *et al*., 2010). When potato plants are attacked by pathogens, StCDPK4 and StCDPK5 regulate ROS production by phosphorylating NADPH oxidase (StRbohB) and inducing the oxidative burst (Kobayashi *et al*., 2007). A further study demonstrated that StCDPK5 can activate StRbohA–D, which mediate the StCDPK5-induced ROS burst (Kobayashi *et al*., 2012). In rice, a recent study showed that OsCPK12 can induce the expression of the antioxidant genes *OsAPX2* and *OsAPX8* under salt stress, and reduce the salt-induced accumulation of H_2O_2 (Asano *et al*., 2012). These results suggest that OsCPK12 positively regulates ROS detoxification by controlling the expression of antioxidant genes. However, whether CDPKs are involved in ABA-induced antioxidant defence remains to be determined.

The MAPK cascade has been shown to be another major signal transduction pathway that is widely used to adapt cellular metabolism to a changing environment (Colcombet and Hirt, 2008; Pitzschke *et al*., 2009; Wurzinger *et al*., 2011). In *Arabidopsis*, it has been shown that *AtMPK3*, *AtMPK6*, *AtMPK9*, and *AtMPK12* are involved in ABA signalling (Colcombet and Hirt, 2008; Xing *et al*., 2008; Jammes *et al*., 2009; Liu, 2012). AtMPK6 in *Arabidopsis* (Xing *et al*., 2008), ZmMPK5 and ZmMPK3 in maize (Lin *et al*., 2009; Wang *et al*., 2010), and OsMPK1 and OsMPK5 in rice (Zhang *et al*., 2012) are required for ABA-induced antioxidant defence. Although both CDPKs and MAPKs have been shown to be involved in ABA signalling, it is not clear whether there exists a link between the CDPK pathway and the MAPK pathway in ABA signalling.

In this study, the role of the maize CDPK ZmCPK11, which belongs to group I of the CDPK family and is closely related to AtCPK4 and AtCPK11 (Boudsocq and Sheen, 2012), in ABA-induced antioxidant defence was investigated. Previous studies have shown that ZmCPK11 is a component of touch- and wound-induced pathway(s), participating in early stages of local and systemic responses (Szczegielniak *et al*., 2005, 2012). Moreover, the relationship between ZmCPK11 and ZmMPK5, which is required for the ABA-induced antioxidant defence and for the positive feedback regulation of NADPH oxidase activity (Zhang *et al*., 2006; Ding *et al*., 2009; Lin *et al*., 2009), in ABA signalling was also examined. Here, evidence is provided to show that ZmCPK11 is involved in ABA-induced antioxidant defence and acts upstream of ZmMPK5 in ABA signalling in maize.

Materials and methods

Plant materials 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 °C (night) to 28 °C (day), photosynthetic active radiation of 200 μmol m^{-2} s^{-1}, and a photoperiod of 14/10 h (day/night), and watered daily. For protoplast isolation, maize plants were grown at 26 °C under dark conditions. When the second leaves were fully expanded, they were collected and used for investigations.

The plants were excised at the base of the stem and placed in distilled water for 4 h to eliminate wound stress. After treatment, the cut ends of the stems were placed in beakers wrapped with aluminium foil containing 100 μM ABA, 10% (w/v) polyethylene glycol (PEG 6000), 10 mM H_2O_2, or 10 mM $CaCl_2$ for the indicated time, with a continuous light intensity of 200 μmol m^{-2} s^{-1}. To study the effects of inhibitors, the detached plants were pre-treated with 100 μM diphenyleneiodonium chloride (DPI), 10 mM dimethylthiourea (DMTU), 200 U of CAT, 100 μM trifluoperazine (TFP), 10 mM ethylene glycol-bis(2-aminoethyl ether)-*N*,*N*,*N*′,*N*′- tetraacetic acid (EGTA), 100 μM 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), and 10 μM 1,4-diamino-2,3-dicyano-1,4-bis(*o*-aminophenyl-mercapto) butadiene (U0126) for 4 h, and then subjected to 100 μM ABA treatment. For fluridone treatment, maize seeds were soaked in 100 μM fluridone for 16 h, then germinated and grown under the same conditions as described above. Detached plants were treated with distilled water under the same conditions for the whole period and served as controls for the above. After treatments of detached maize plants, the second leaves were sampled and immediately frozen under liquid N_2 for further analysis.

Protein extraction and immunocomplex kinase activity assay

Protein was extracted from leaves or protoplasts with an extraction buffer as described previously (Zhang *et al*., 2006), but without

5 mM EGTA in the case of ZmCPK11 assay. After centrifugation at 12 000 *g* for 30 min at 4 °C, the supernatants were transferred into new tubes, immediately frozen with liquid N_2, and stored at –80 °C. Protein content was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

For immunocomplex kinase assay, protein extract (100 µg) was incubated with anti-ZmCPK11 antibody (2 µg) or anti-ZmMPK5 antibody (2 µg) in an immunoprecipitation buffer as described previously (Zhang *et al*., 2006), but without 2 mM EGTA in the case of ZmCPK11 assay, at 4 °C for 12 h on a rocker. An ~25 µl volume of protein G–garose was added, and the incubation was continued for another 3 h. Agarose bead–protein complexes were pelleted by brief centrifugation. After washing with immunoprecipitation buffer three times, reaction buffer {25 mM TRIS, pH 7.5, 100 µM Na_3VO_4, 1 mM dithiothreitol (DTT), 12 mM $MgCl_2$, 1 mM $CaCl_2$ (not in the ZmMPK5 assay), 200 nM ATP plus 50 µCi of [γ-^{32}P]ATP (3000 Ci mM^{-1}), 0.25 µg µl^{-1} histone S-III for ZmCPK11 or 0.25 mg ml^{-1} MBP for ZmMPK5} was added and reacted for 30 min at room temperature. Ten loading samples were then added and boiled for 5 min. After centrifugation, the supernatant fraction was electrophoresed on SDS–polyacrylamide gels. The gel was dried onto Whatman 3 MM paper and exposed to Kodak XAR-5 film. Relative activation levels of ZmCPK11 and ZmMPK5 proteins, detected by immunocomplex kinase activity assay and quantificated by Quantity One software (Bio-Rad Laboratories Inc., USA), are presented as values relative to those of the corresponding controls.

RNA preparation and cDNA synthesis

Total RNA was isolated from leaves or protoplasts using RNAiso Plus (TaKaRa Bio Inc., China) according to the manufacturer's instructions. DNase treatment was included in the isolation step using RNase-free DNase (TaKaRa Bio Inc.). Approximately 2 µg of total RNA was reverse transcribed using oligo $d(T)_{16}$ primer and M-MLV reverse transcriptase (TaKaRa Bio Inc.) at 42 °C for 75 min.

Real-time quantitative RT–PCR expression analysis

Real-time quantitative reverse transcription–PCRs (RT–PCRs) were performed in a DNA Engine Opticon 2 real-time PCR detection system (Bio-Rad Laboratories Inc., USA) using SYBR *Premix Ex Taq*™ (TaKaRa Bio Inc.) according to the manufacturer's instructions. cDNA was amplified by PCR using the following primers: *ZmCPK11* (GenBank accession no. AAP57564.2), forward CCTCCACGACCCCGACAATG and reverse ACCTCTCCGAG CACCCCAAC; *ZmMPK5* (GenBank accession no. BAA74734.1), forward ACTGATGGACCGCAAACC and reverse GGGTGACG AGGAAGTTGG; *SOD4* (GenBank accession no. X17565), forward TGGAGCACCAGAAGATGA and reverse CTCGTGTCC ACCCTTTCC; *cAPX* (GenBank accession no. EU969033), forward TGAGCGACCAGGACATTG and reverse GAGGGCTTTGTCA CTTGGT; and *β-actin* (GenBank accession no. J01238), forward GTTTCCTGGGATTGCCGAT and reverse TCTGCTGCTGA AAAGTGCTGAG.

Each PCR (20 µl) contained 10 µl of 2× Real-time PCR Mix (containing SYBR Green), 0.2 µM of each primer, and appropriately diluted cDNA. The thermal cycling conditions were 94 °C for 30 s followed by 40 cycles of 94 °C for 10 s, 60 °C for 20 s, and 72 °C for 20 s. To standardize the data, the ratio of the absolute transcript level of the target genes to the absolute transcript level of *β-actin* was calculated for each sample. The relative expression levels of the target genes were calculated as γ-fold changes relative to the appropriate control experiment for the different chemical treatments.

Plasmids

The full-length cDNA fragment was amplified with the *Kpn*I sites and then cloned between the *Cauliflower mosaic virus* (CaMV) 35S promoter and yellow fluorescent protein (YFP) of the pXZP008 vector. The primer pairs were: forward *GGTA CC*CGGGTTTGCTGGGATTCAAGAGTTCGCCG and reverse *GGTACC*GAATGCAGCCGGACCCGAGCGGGAA.

In vitro *synthesis of dsRNA*

DNA templates were produced by PCR using primers containing the T7 promoter sequence (5'-TTAATACGACTCACTATAGGG AGG-3') on both the 5' and 3' ends. The primers used to amplify DNA of *ZmCPK11* were: forward TAATACGACTCACTATA GGGAGACCACTGACTTTGGGCTTTCC and reverse TAATA CGACTCACTATA GG GAGACAGCTTCTGGACCATAGCAT. The PCR conditions were as follows: denaturing step at 94 °C for 5 min, followed by 34 cycles of 94 °C for 30 s, 58 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. The primers used to amplify DNA of *ZmMPK5* were: forward TAATACGACTCACTATAGGGAGAACCTGGTGGAAAAGA TGCT and reverse TAATACGACTCACTATAGGGAGACATG CTGCTCGAAGTCAAA. The PCR conditions were as follows: denaturing step at 94 °C for 5 min, followed by 34 cycles of 94 °C for 30 s, 53 °C for 30 s, 72 °C for 30 s, and a final extension at 72 °C for 10 min. After PCR product clean up, the DNA templates were used for *in vitro* synthesis of double-stranded RNAs (dsRNAs) using the Ribomax Express kit (Promega). The dsRNAs were purified by phenol–chloroform–isopropanol extraction, dissolved in RNase-free water, and quantified by UV spectrophotometry.

Protoplast isolation

Maize plants were grown in the dark at 26 °C for ~7 d. When the second leaves were fully expanded, the protoplasts from the second leaf were isolated according to the method described by Ma *et al*. (2012).

Transfection of protoplasts with plasmid DNA or dsRNAs

The CaMV35S-ZmCPK11-YFP plasmid or dsRNAs were delivered into protoplasts using a PEG–calcium-mediated method described previously (Yoo *et al*., 2007; Zhai *et al*., 2009). About 10 µg of plasmid DNA or dsRNAs per 100 µl ×10^5 protoplasts were used for transient expression analysis. The DNA or dsRNA and protoplast mixtures were added to 40% PEG solution (40% PEG 4000, 0.4 mM mannitol, and 100 mM $CaCl_2$, adjusted to pH 7.0 with 1 M KCl), mixed gently, and incubated for 15 min at room temperature in the dark. Protoplasts were washed by 440 µl of W5 solution, and incubated in W5 medium containing 0.1% (w/v) glucose in the dark overnight.

Subcellular localization of ZmCPK11

The protoplasts expressing the ZmCDPK11–YFP fusion protein after 16 h incubation were observed using a laser confocal microscope (TCS-SP2, Leica, Bensheim, Germany), with excitation at 530 nm and emission at 525 nm. For the nuclear staining, 4',6-diamino-2-phenylindole (DAPI; 1 µg µl^{-1}) was added to the culture medium and incubated for 1 h. For the plasma membrane staining, *N*-[3-triethyl-ammoniumpropyl]-4-[*p*-diethylaminophenylhexatrienyl] pyridinium dibromide (FM4-64, 5 µg µl^{-1}) was added to the culture medium and incubated for 30 min. FM4-64 fluorescence was observed under a microscope using an RFP filter, with excitation at 543 nm and emission at 580 nm.

H_2O_2 detection by confocal laser scanning microscopy

H_2O_2 production in protoplasts was monitored using the H_2O_2-sensitive fluorescent probe 2′,7′-dichlorofluorescein diacetate (H_2DCF-DA; Molecular Probes, Leiden, The Netherlands) using the method described by Bright *et al*. (2006). Images acquired were analysed using Leica IMAGE software. Data are presented as mean pixel intensities. A total of 120 protoplasts are observed per treatment for three independent replicates.

Enzyme assays

Protoplasts were homogenized in a solution of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone. The homogenate was centrifuged at 12 000 *g* for 30 min at 4 °C and the supernatant was immediately used for the antioxidant enzyme assays. The total activities of SOD and APX were determined as described previously (Jiang and Zhang, 2001). Protein content was determined according to the method of Bradford (1976) with BSA as standard.

Results

ABA and H_2O_2 induce the expression of ZmCPK11 *and increase the activity of ZmCPK11 in maize leaves*

To investigate the effects of ABA and H_2O_2 on the induction of the expression of *ZmCPK11* and the activity of ZmCPK11 in leaves of maize plants, relative quantitative real-time PCR analysis and immunocomplex kinase activity assay were used. Treatments with ABA (100 µM) and H_2O_2 (10 mM) induced a rapid increase in the expression of *ZmCPK11* (Fig. 1A). A biphasic response in the expression of *ZmCPK11* in maize leaves exposed to ABA and H_2O_2 treatments was observed, in which the first peak occurred after 20 min of treatment, and the second peak appeared after 90 min of H_2O_2 treatment or 120 min of ABA treatment (Fig. 1A). Treatments with ABA and H_2O_2 also caused a rapid increase in the activity of ZmCPK11 in maize leaves (Fig. 1B). Time-course analysis showed that ABA treatment led to a significant increase in the activity of ZmCPK11 within 30 min, maximized at 60 min, remained high for 90 min after ABA treatment, and then decreased to the control level after 120 min of ABA treatment. Compared with the ABA treatment, H_2O_2 treatment also caused a similar change in the activity of ZmCPK11, but the activity of ZmCPK11 returned to the control level after 90 min of H_2O_2 treatment. As a positive control, $CaCl_2$ treatment also induced the increases in the expression of *ZmCPK11* (Fig. 1A) and the activity of ZmCPK11 (Fig. 1B).

To investigate whether the expression of *ZmCPK11* can be regulated by endogenous ABA, maize seeds were pretreated with an inhibitor of ABA biosynthesis, fluridone,

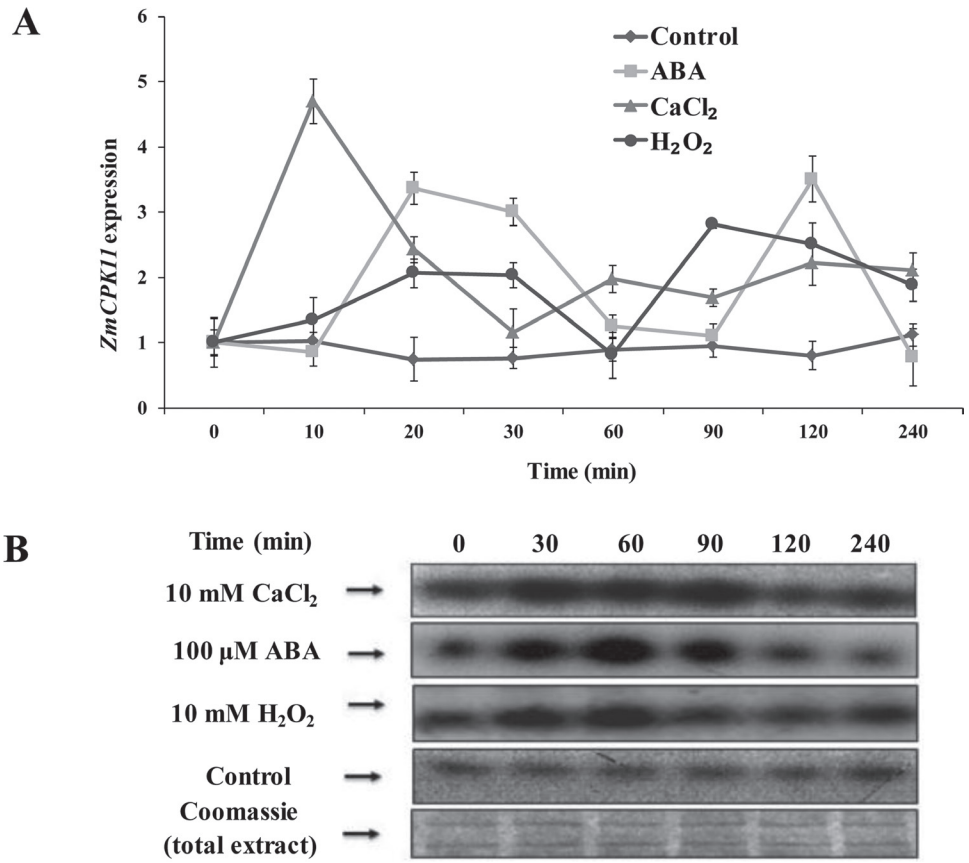

Fig. 1. ABA, H_2O_2, and $CaCl_2$ induce the expression of *ZmCPK11* and the activity of ZmCPK11 in maize leaves. (A) Expression analysis of *ZmCPK11* in leaves of maize plants exposed to ABA, H_2O_2, and $CaCl_2$ treatments. The detached maize plants were treated with ABA (100 µM), H_2O_2 (10 mM), and $CaCl_2$ (10 mM) for various times as indicated. The relative expression levels of the *ZmCPK11* gene were analysed by real-time quantitative PCR. Values are means ±SE of three independent experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test. (B) Induction of the activity of ZmCPK11 by ABA, H_2O_2, and $CaCl_2$. The detached plants were treated as described in (A). ZmCPK11 was immunoprecipitated from leaves after treatments, and the activity of ZmCPK11 was measured by immunoprecipitation kinase assay using histone S-III as a substrate. Corresponding Coomassie staining was also shown as indicated. Experiments were repeated at least three times with similar results.

and then the pre-treated plants were exposed to PEG treatment. PEG treatment induced an increase in the expression of *ZmCPK11*, but the increase was inhibited by the pre-treatment with fluridone (Fig. 2A). The effect of fluridone on the expression of *ZmCPK11* was overcome by the application of 100 μM ABA. Pre-treatment with fluridone alone had very little effect on the expression of *ZmCPK11* in leaves of maize plants (Fig. 2A). These results suggest that ABA is involved in the up-regulation of *ZmCPK11* expression in the leaves of maize plants exposed to water stress.

To determine whether ABA-induced increases in the expression of *ZmCPK11* and the activity of ZmCPK11 are related to the action of endogenous H_2O_2, several ROS manipulators, such as DPI, an inhibitor of NADPH oxidase, DMTU, a trap for H_2O_2, and CAT, the enzyme eliminating H_2O_2, were applied. Pre-treatments with DPI, DMTU, and CAT substantially suppressed the ABA-induced increases in the expression of *ZmCPK11* (Fig. 2B) and the activity of ZmCPK11 (Fig. 2C), suggesting that H_2O_2 is required for the ABA-induced up-regulation of the expression and the activity of ZmCPK11 in maize leaves.

Subcellular localization of ZmCPK11 *in maize protoplasts*

Previous studies showed that different CDPKs have different subcellular localizations, including the plasma membrane, endoplasmic reticulum, actin cytoskeletal system, mitochondria, peroxisomes, cytosol, nucleus, and oil bodies (Zou *et al.*, 2010; Wurzinger *et al.*, 2011; Boudsocq and Sheen, 2012; Kobayashi *et al.*, 2012). To investigate the intracellular localization of ZmCPK11, a reporter gene encoding YFP was fused to *ZmCPK11* (*ZmCPK11-YFP*), which is driven by the *35S:ZmCPK11-YFP* promoter, and then transformed into maize protoplasts by PEG–calcium-mediated transformation (Yoo *et al.*, 2007). The nucleus was stained by DAPI, and the plasma membrane was marked by FM4-64. The results showed that ZmCPK11–YFP was localized in the nucleus and the cytoplasm (Fig. 3).

ZmCPK11 is involved in ABA-induced up-regulation of the expression and the activities of antioxidant enzymes and the production of H_2O_2

To investigate whether ZmCPK11 is involved in the ABA-induced antioxidant defence response, a transient gene expression analysis (Yoo *et al.*, 2007) and a transient RNA interference (RNAi) test in protoplasts (Zhai *et al.*, 2009), which have been proven to be suitable for functional analysis of plant genes (An *et al.*, 2005; Bart *et al.*, 2006; Chen *et al.*, 2006; Yoo *et al.*, 2007; Zhai *et al.*, 2009; Ma *et al.*, 2012; Shi *et al.*, 2012; Zhang *et al.*, 2012), were used for the functional analysis of *ZmCPK11* in ABA signalling. Protoplast transfection with *35S:ZmCPK11-YFP* plasmid caused a significant increase in the expression of *ZmCPK11* (Fig. 4C), but transfection in protoplasts with an *in vitro*-synthesized dsRNA against *ZmCPK11* (RNAi) resulted in a substantial suppression of the expression of *ZmCPK11* (Fig. 5C). Transient expression of *ZmCPK11* in protoplasts resulted in significant increases in the expression of the antioxidant genes *SOD4*, encoding a cytosolic isoform of SOD, and *cAPX*, encoding a cytosolic isoform of APX, and the activities

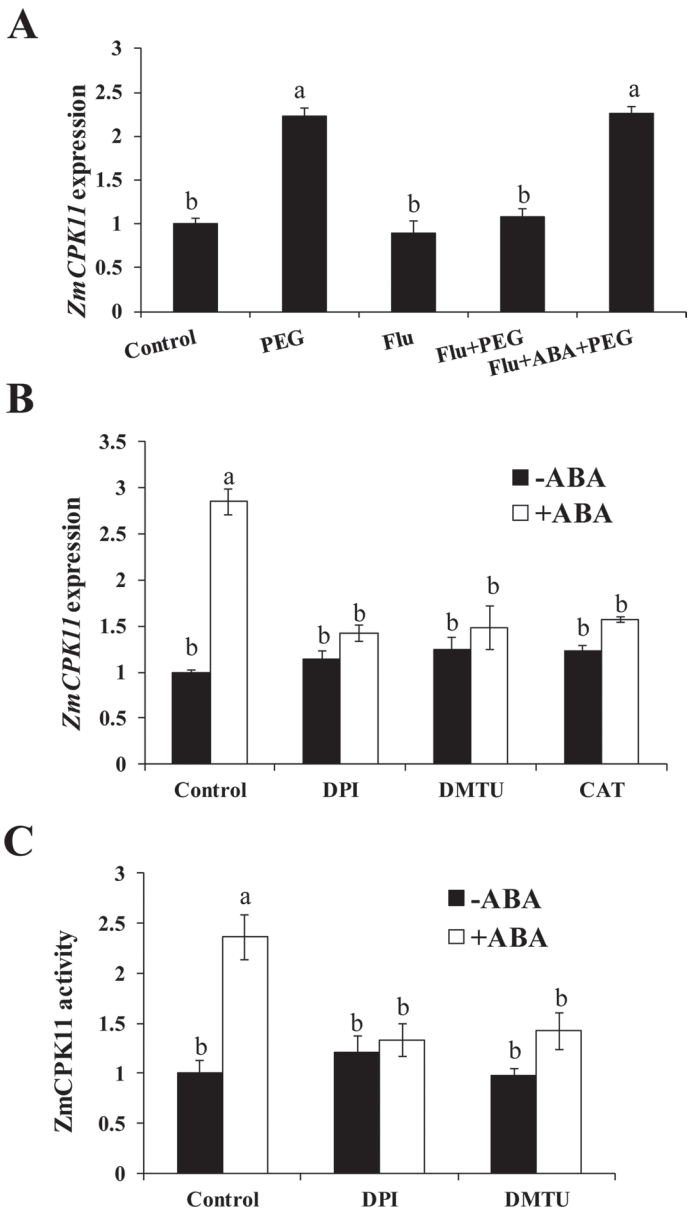

Fig. 2. H_2O_2 is required for ABA-induced activation of ZmCPK11 in maize leaves. (A) Effect of pre-treatment with the ABA biosynthetic inhibitor fluridone (Flu) on the expression of *ZmCPK11* in maize leaves exposed to PEG treatment. The fluridone-treated and -untreated seedlings were exposed to 10% PEG treatment for 1 h. ABA (100 μM) was added to overcome the effects of fluridone. (B, C) Effects of pre-treatments with the ROS manipulators DMTU, DPI, and CAT on the expression of *ZmCPK11* (B) and the activity of ZmCPK11 (C) in maize leaves exposed to ABA treatment. The detached maize plants were pre-treated with 10 mM DMTU, 100 μM DPI, and 200 U of CAT for 4 h, and then exposed to 100 μM ABA for 30 min (B) or 60 min (C). Values are means ±SE of three independent experiments. Means denoted by the same letter did not significantly differ at $P<0.05$ according to Duncan's multiple range test.

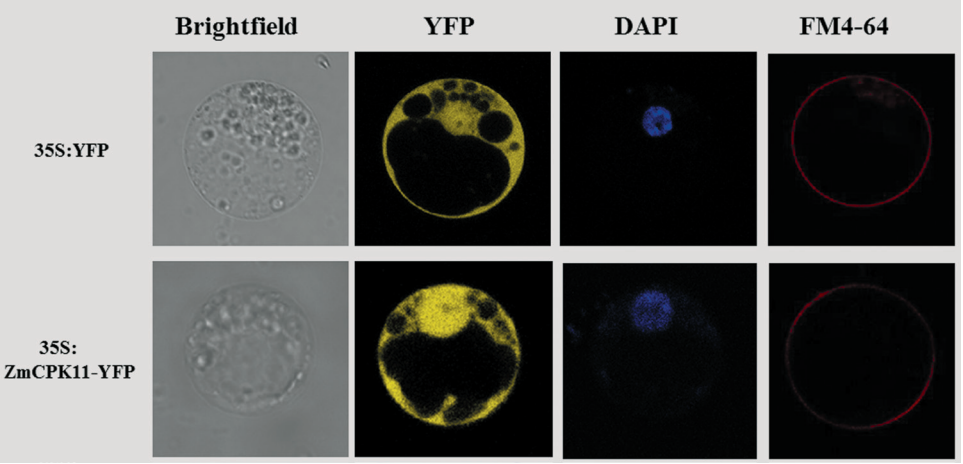

Fig. 3. Subcellular localization of ZmCPK11 in maize protoplasts. Constructs carrying *35S:ZmCPK11-YFP* or *35S:YFP* were introduced into protoplasts prepared from the leaves of maize by PEG–calcium-mediated transformation. Transfected protoplasts were observed after 16 h incubation by a laser confocal microscope. Nuclei are shown with DAPI staining (blue). The plasma membrane was labelled with FM4-64 (red). Experiments were repeated at least five times with similar results.

of the antioxidant enzymes SOD and APX, when compared with those in protoplasts transfected with the empty vector (Fig. 4A, B), but RNAi-mediated silencing of *ZmCPK11* decreased the expression of *SOD4* and *cAPX* and the activities of SOD and APX (Fig. 5A, B). Further, treatment with 10 μM ABA induced significant increases in the expression of *ZmCPK11* (Fig. 5C), *SOD4*, and *cAPX* (Fig. 5A) and the activities of SOD and APX (Fig. 5B) in the control protoplasts, and the increases were blocked by the RNAi silencing of *ZmCPK11*. These results indicate that ZmCPK11 is required for ABA-induced increases in the expression of *SOD4* and *cAPX* and the activities of SOD and APX.

To investigate whether ABA-activated ZmCPK11 also affects ABA-induced H_2O_2 production, protoplasts transfected with dsRNA against *ZmCPK11* were used, and H_2O_2 production in the protoplasts was monitored using the fluorescent probe H_2DCF-DA. The RNAi silencing of *ZmCPK11* in the protoplasts not only decreased the H_2O_2-mediated fluorescence under the control condition, but also blocked the ABA-induced increase in the fluorescence (Fig. 6). The specificity of the H_2O_2-mediated fluorescence was proven by the application of CAT. These results indicate that ZmCPK11 is involved in ABA-induced H_2O_2 production.

ZmCPK11 *regulates the expression of* ZmMPK5 *and the activity of ZmMPK5 in ABA signalling*

Previous studies showed that ABA and H_2O_2 induced the expression of *ZmMPK5* and the activity of ZmMPK5 in leaves of maize plants, and ZmMPK5 is required for ABA-induced antioxidant defence (Zhang *et al*., 2006; Ding *et al*., 2009; Lin *et al*., 2009). To establish a possible link between ZmCPK11 and ZmMPK5 in ABA signalling, the detached maize plants were pre-treated with the Ca^{2+} chelator EGTA and the CDPK inhibitor TFP, and the MAPK kinase (MAPKK) inhibitors PD98059 and U0126, respectively, and then exposed to ABA treatment. Experimental results showed that pre-treatments with EGTA and TFP substantially suppressed the ABA-induced increase in the activity of ZmCPK11 in maize leaves (Fig. 7B, right), and also blocked the ABA-induced increases in the expression of *ZmMPK5* (Fig. 7A, left) and the activity of ZmMPK5 (Fig. 7B, left). However, pre-treatments with PD98059 and U0126 almost completely blocked the ABA-induced increase in the activity of ZmMPK5 (Fig. 7C, left), but had very little effect on the ABA-induced up-regulation of the expression of *ZmCPK11* (Fig. 7A, right) and the activity of ZmCPK11 (Fig. 7C, right).

To determine the relationship between ZmCPK11 and ZmMPK5 in ABA signalling, transient RNAi analysis in maize protoplasts was used. RNAi-mediated silencing of *ZmCPK11* in protoplasts decreased the expression of *ZmCPK11* (Fig. 8B) and the activity of ZmCPK11 (Fig. 8C) under control conditions, and also decreased the expression of *ZmMPK5* (Fig. 8A) and the activity of ZmMPK5 (Fig. 8C). In contrast, RNAi silencing of *ZmMPK5* inhibited the expression of *ZmMPK5* (Fig. 8A) and the activity of ZmMPK5 (Fig. 8D), but had very little effect on the expression of *ZmCPK11* (Fig. 8B) and the activity of ZmCPK11 (Fig. 8D). Further, ABA-induced increases in the expression of *ZmMPK5* (Fig. 8A) and the activity of ZmMPK5 (Fig. 8C) were substantially suppressed by RNAi silencing of *ZmCPK11*, but RNAi silencing of *ZmMPK5* had very little effect on the ABA-induced increases in the expression of *ZmCPK11* (Fig. 8B) and the activity of ZmCPK11 (Fig. 8D). These results suggest that ZmCPK11 regulates the expression of *ZmMPK5* and the activity of ZmMPK5 in ABA signalling, and ZmMPK5 does not mediate the ABA-induced up-regulation of the expression and activity of ZmCPK11.

ZmCPK11 functions upstream of ZmMPK5 to regulate the activities of antioxidant enzymes

To determine the relationship between ZmCPK11 and ZmMPK5 in the regulation of antioxidant enzyme activity,

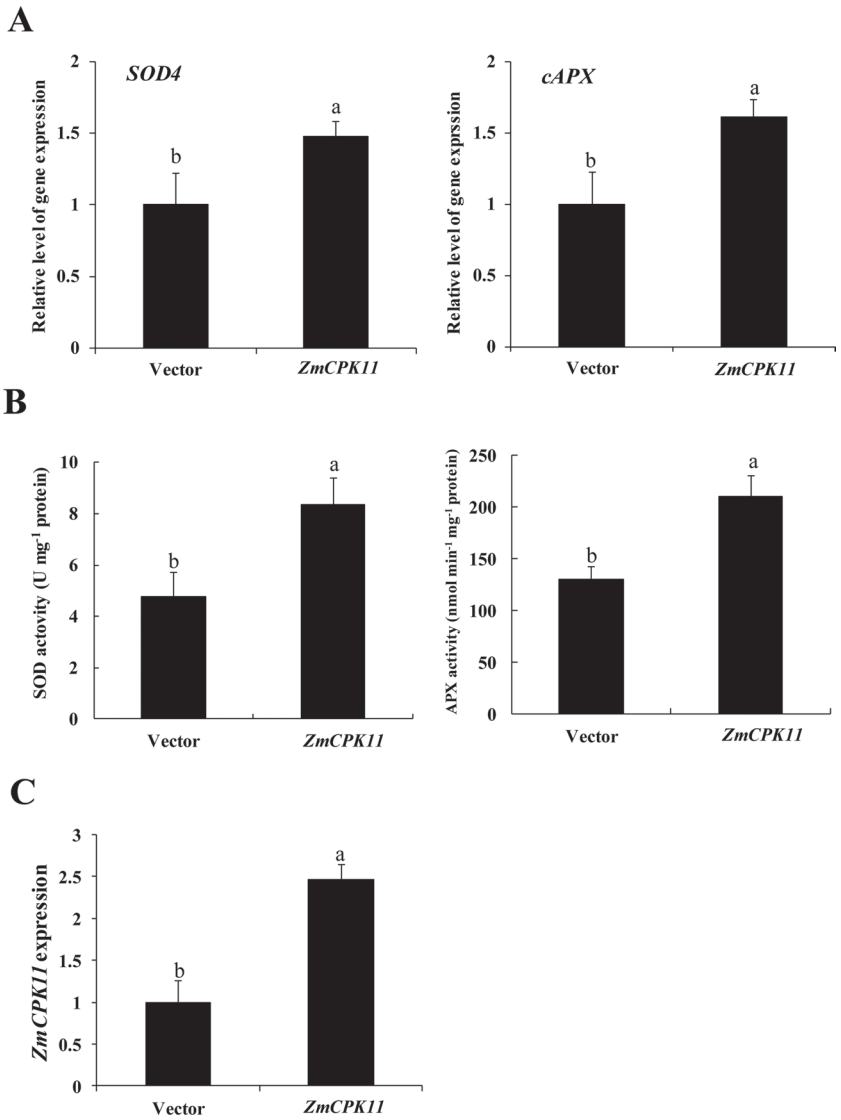

Fig. 4. Transient expression of *ZmCPK11* up-regulates the expression and the activities of SOD and APX in maize protoplasts. (A) The expression of *SOD4* and *cAPX* in protoplasts with transiently expressed *ZmCPK11*. (B) The activities of SOD and APX in protoplasts with transiently expressed *ZmCPK11*. (C) The expression of *ZmCPK11* in protoplasts with transiently expressed *ZmCPK11*. Protoplasts were transfected with *ZmCPK11* or empty vector (control) and incubated for 16 h. Values are means ±SE of three independent experiments. Means denoted by the same letter did not differ significantly at $P<0.05$ according to Duncan's multiple range test.

transient RNAi analysis in combination with the transient expression test in maize protoplasts was conducted. Transient expression of *ZmCPK11* or *ZmMPK5* in protoplasts induced the increases in the activities of SOD and APX, but RNAi-mediated silencing of *ZmCPK11* or *ZmMPK5* decreased the activities of SOD and APX (Fig. 9A, B). However, in the protoplasts with transiently silenced *ZmMPK5*, the transient expression of *ZmCPK11* could hardly induce the increases in the activities of SOD and APX, but in the protoplasts with transiently silenced *ZmCPK11*, the transient expression of *ZmMPK5* induced a similar increase in the activities of SOD and APX, compared with those in the protoplasts with transiently expressed *ZmMPK5* alone (Fig. 9A, B). These results suggest that ZmCPK11 acts upstream of ZmMPK5 to regulate the activities of antioxidant enzymes.

Discussion

With 34 members in *Arabidopsis* (Cheng *et al.*, 2002; Hrabak *et al.*, 2003) and 29 members in rice (Asano *et al.*, 2005), CDPKs constitute a large multigene family. In *Arabidopsis* and rice, several CDPKs, such as AtCPK3 and AtCPK6 (Mori *et al.*, 2006), AtCPK4 and AtCPK11 (Zhu *et al.*, 2007), AtCPK10 (Zou *et al.*, 2010), AtCPK32 (Choi *et al.*, 2005), and OsCPK12 (Asano *et al.*, 2012) and OsCPK21 (Asano *et al.*, 2011) have been reported to be positive regulators of ABA-mediated physiological processes, including seed germination, post-germination growth, stomatal movement, and plant stress tolerance. Under conditions of high salinity, the accumulation of H_2O_2 in *OsCPK12*-overexpressing plants was less than that in wild-type plants, whereas the accumulation was more in *oscpk12* mutant and *OsCPK12* RNAi plants

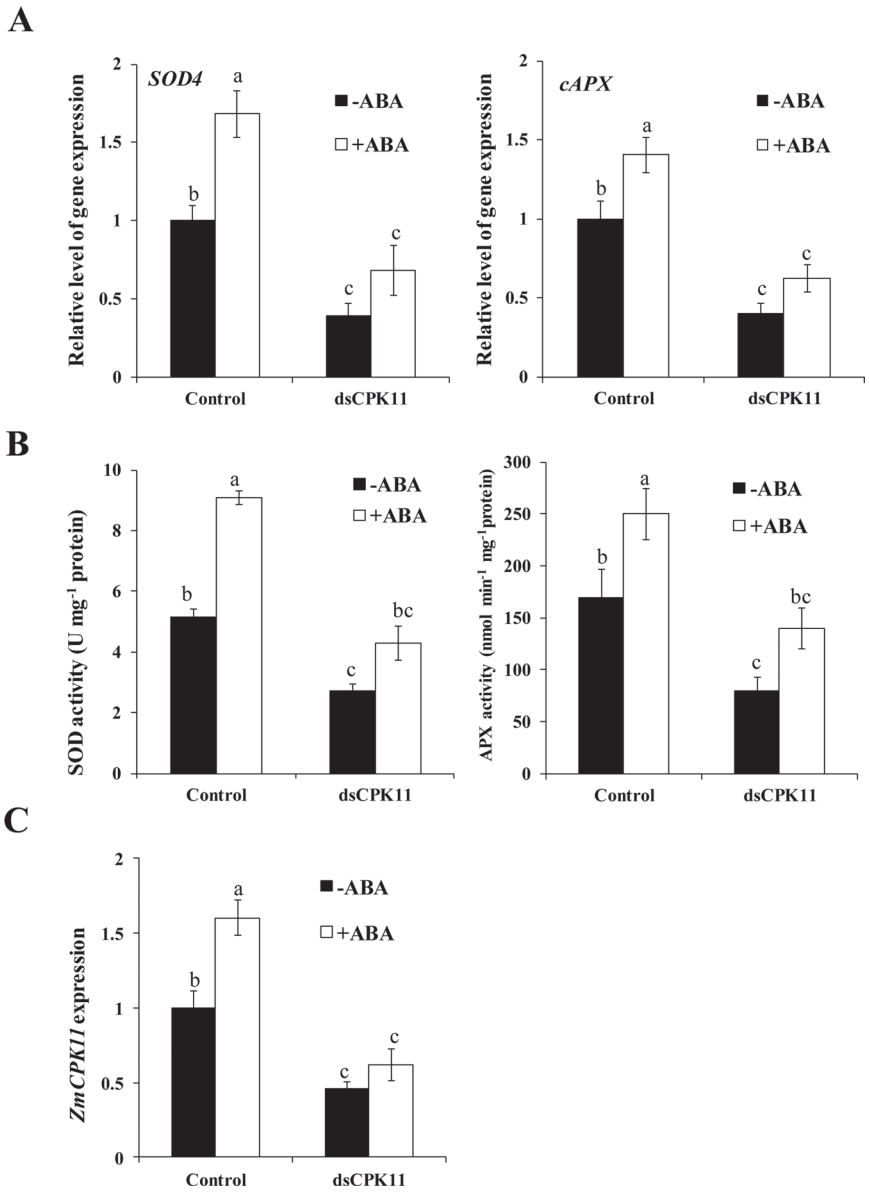

Fig. 5. RNAi-mediated silencing of *ZmCPK11* inhibits the ABA-induced increases in the expression and activities of SOD and APX in maize protoplasts. (A) The expression of *SOD4* and *cAPX* in protoplasts with transiently silenced *ZmCPK11.* Protoplasts were treated with 10 μM ABA for 5 min, and the relative expression levels of *SOD4* and *cAPX* were analysed by real-time quantitative PCR. (B) The activities of SOD and APX in protoplasts with transiently silenced *ZmCPK11*. The protoplasts were treated with 10 μM ABA for 5 min, and the activities of SOD and APX were measured as described in the Materials and methods. (C) The expression of *ZmCPK11* in protoplasts with transiently silenced *ZmCPK11*. Protoplasts were transfected with dsRNA against *ZmCPK11* (dsCPK11) or with water (control) and incubated for 24 h. Values are means ±SE of three independent experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

(Asano *et al*., 2012). The levels of ROS accumulation were correlated with altered expression levels of the antioxidant genes *OsAPX2* and *OsAPX8* in the *OsCPK12*-overexpressing and loss-of-function plants under the conditions of high salinity. These results suggest that OsCPK12 positively regulates ROS detoxification by controlling the expression of *OsAPX2* and *OsAPX8*. However, the detoxification of ROS regulated by OsCPK12 under salt stress seems to be ABA independent (Asano *et al*., 2012). Therefore, whether CDPKs are involved in ABA-induced antioxidant defence is not yet clear. In this study, a functional analysis was performed of ZmCPK11, which belongs to group I of the CDPK family and is closely related to AtCPK4 and AtCPK11 (Boudsocq and Sheen, 2012), in ABA-induced antioxidant defence in maize. The results showed that ABA treatment induced increases in the expression of *ZmCPK11* and the activity of ZmCPK11 in leaves of maize (Fig. 1), and ABA was required for the PEG-induced increase in the expression of *ZmCPK11*

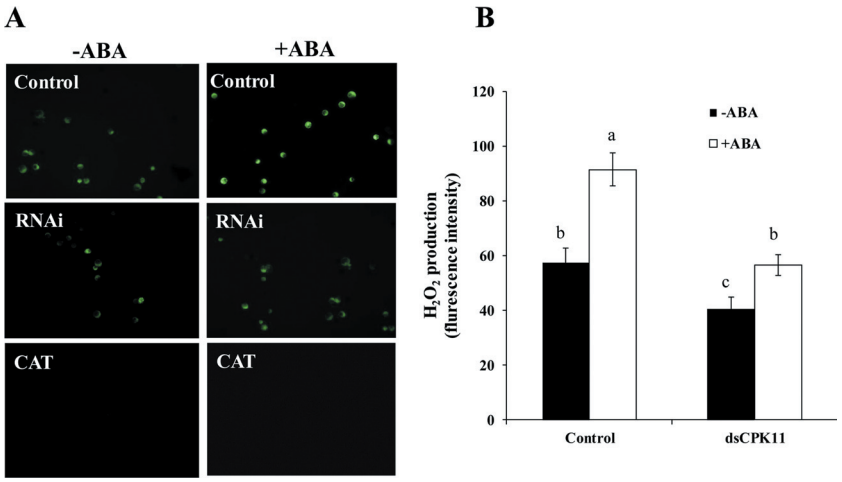

Fig. 6. ZmCPK11 mediates ABA-induced production of H_2O_2 in maize protoplasts. (A) H_2O_2 fluorescence in protoplasts with transiently silenced *ZmCPK11*. The protoplasts were treated with 10 μM ABA (+ABA) or the incubation medium (–ABA) for 5 min, and then loaded with H_2DCF-DA for 10 min. CAT (20 U) was also added to the control protoplasts in the presence or absence of ABA. The protoplasts transfected with water were used as controls. H_2O_2 was visualized by confocal microscopy. Experiments were repeated at least three times with similar results. (B) Changes in the fluorescence intensity in (A). Values are means ±SE of three independent experiments. Means denoted by the same letter did not significantly differ at $P<0.05$ according to Duncan's multiple range test.

(Fig. 2A). The transient expression of *ZmCPK11* in maize protoplasts enhanced the expression of the antioxidant genes *SOD4* and *cAPX* and the activities of SOD and APX (Fig. 4). In contrast, the RNAi silencing of *ZmCPK11* in protoplasts decreased the expression and the activities of SOD and APX (Fig. 5). Further, ABA treatment induced increases in the expression and activities of these antioxidant enzymes in control protoplasts, and the increases were inhibited in protoplasts transfected with dsRNA against *ZmCPK11* (Fig. 5). These results clearly indicate that ZmCPK11 is involved in ABA-induced up-regulation of the expression and activities of antioxidant enzymes in maize.

The MAPK cascade has been demonstrated to play a crucial role in plant responses to environmental stresses (Colcombet and Hirt, 2008; Pitzschke *et al*., 2009). AtMPK6 in *Arabidopsis* and its homologues in maize and rice, ZmMPK5 and OsMPK1, have been shown to be involved in ABA-induced antioxidant defence (Xing *et al*., 2008; Lin *et al*., 2009; Zhang *et al*., 2012). However, it is not clear whether there exists a cross-talk between the CCaMK pathway and the MAPK pathway in ABA signalling. Previous studies showed that the application of the CDPK inhibitor *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide hydrochloride (W7) inhibited the activation of MAPKs induced by cold and heat (Sangwan *et al*., 2002) or by heavy metals (Yeh *et al*., 2007), suggesting that the stress-induced activation of MAPKs may occur through the action of CDPKs. However, W7 is also a well-known CaM antagonist. This implies that the inhibition of the activities of MAPKs by W7 under environmental stresses is not certain from the action of CDPKs. Genetic evidence shows that there exists a complex relationship between CDPKs and MAPKs in plant responses to environmental stresses. In an early study addressing cross-talk between CDPK and MAPK signalling in response to biotic stress, it was found that elevated CDPK signalling inhibited stress-induced MAPK activation (Ludwig *et al*., 2005). However, several recent studies demonstrated that, in response to pathogen- or microbe-associated molecular patterns (Boudsocq *et al*., 2010; Kobayashi *et al*., 2012) and salt stress (Mehlmer *et al*., 2010), CDPKs and MAPKs act in parallel and no direct cross-talk exists between them. Therefore, to elucidate the relationship between CDPKs and MAPKs in ABA signalling appears to be particularly interesting. In the present study, three lines of evidence indicate that there exists a link between ZmCPK11 and ZmMPK5 in ABA signalling in maize. First, pre-treatments with EGTA and TFP suppressed the ABA-induced increase in the activity of ZmCPK11 in maize leaves, and also inhibited the ABA-induced increases in the expression of *ZmMPK5* and the activity of ZmMPK5 (Fig. 7). Secondly, RNAi-mediated silencing of *ZmCPK11* in maize protoplasts not only decreased the expression of *ZmMPK5* and the activity of ZmMPK5 under control conditions, but also blocked the ABA-induced increases in the expression and the activity of ZmMPK5 (Fig. 8). In contrast, RNAi-mediated silencing of *ZmMPK5* in protoplasts affected neither the expression and activity of ZmCPK11 under control conditions, nor the ABA-induced increases in the expression and activity of ZmCPK11. Finally, in the protoplasts with transiently silenced *ZmMPK5*, the transient expression of *ZmCPK11* could hardly induce the increases in the activities of SOD and APX, but the RNAi silencing of *ZmCPK11* had very little effect on the ZmCPK5-induced increases in the activities of these antioxidant enzymes (Fig. 9). Taken together, these results clearly indicate that ZmCPK11 functions upstream of ZmMPK5 in ABA-induced antioxidant defence in maize. Moreover, recent studies showed that treatments with ABA, H_2O_2, and PEG induced the expression of the rice CCaMK gene *OsDMI3* and the maize CCaMK gene *ZmCCaMK* in the leaves of these

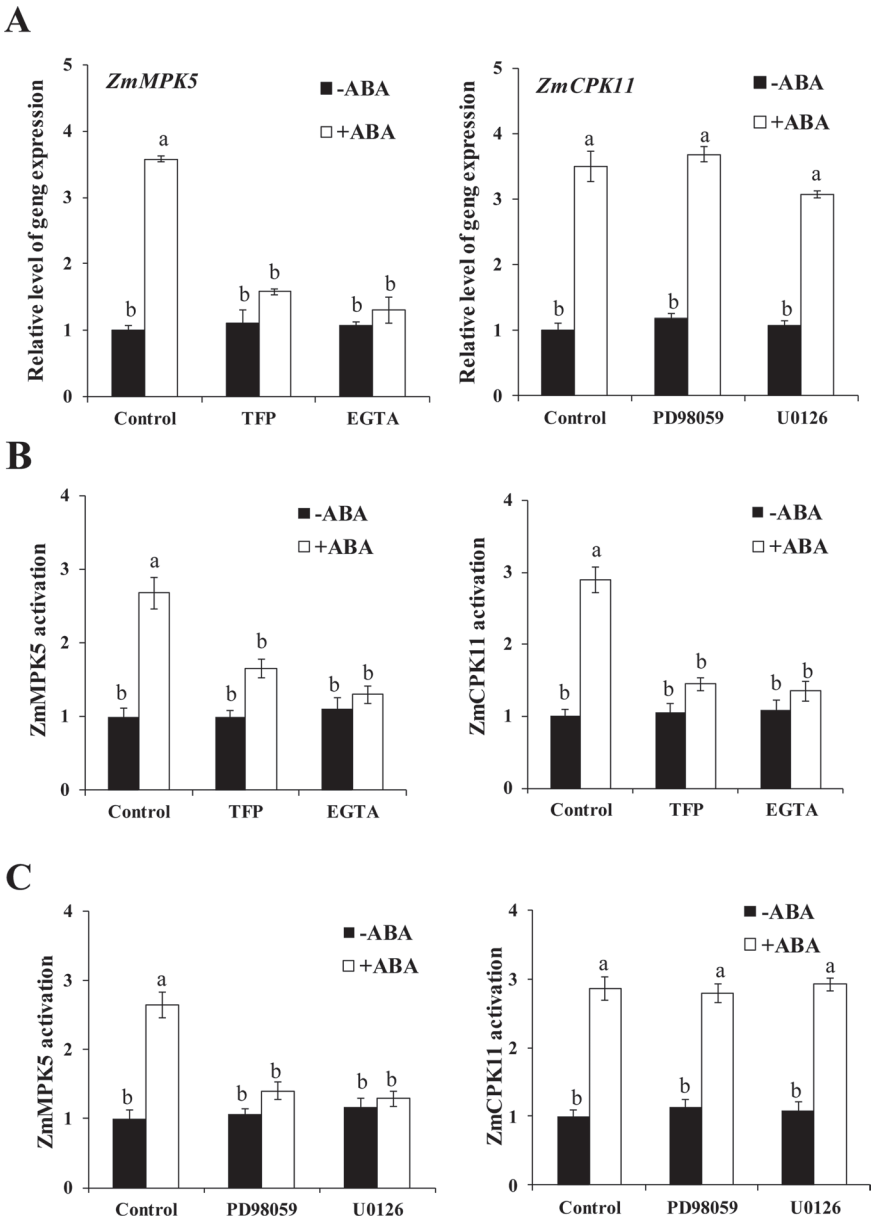

Fig. 7. Effects of pre-treatments with EGTA and TFP on the ABA-induced activation of ZmMPK5 and the effects of pre-treatments with PD98059 and U0126 on the ABA-induced activation of ZmCPK11 in maize leaves. The detached maize plants were pre-treated with 10 mM EGTA, 100 μM TFP, 100 μM PD98059, and 10 μM U0126 for 4 h, and then exposed to 100 μM ABA treatment. Values are means ±SE of three independent experiments. Means denoted by the same letter did not differ significantly at $P<0.05$ according to Duncan's multiple range test.

plants, and these CCaMKs were required for ABA-induced antioxidant defence and oxidative stress tolerance under water stress (Ma *et al*., 2012; Shi *et al*., 2012). A further study revealed that in rice leaves, OsDMI3 is required for the up-regulation of the expression and activity of the MAPK OsMPK1 in ABA signalling (unpublished data), indicating that there also exists a cross-talk between the CCaMK pathway and the MAPK pathway in ABA signalling. The present results suggest that MAPK is a convergence point of the CDPK pathway and the CCaMK pathway in ABA signalling.

In ABA signalling, ROS are important signal molecules (Neill *et al*., 2008; Wang and Song, 2008; Mittler *et al*., 2011) and NADPH oxidase is a major source of ROS (Kwak *et al*., 2003). In this study, H_2O_2 treatment induced the expression of *ZmCPK11* and the activity of ZmCPK11 in maize leaves (Fig. 1), and H_2O_2 is required for ABA-induced increases in the expression and the activity of ZmCPK11 (Fig. 2), suggesting that H_2O_2 might function upstream of ZmCPK11 in ABA signalling. In addition, another important signal molecule, NO, has also been shown to be involved in the regulation of CDPK and CCaMK. The NO donor sodium nitroprusside (SNP) induced the activation of a 50 kDa CDPK in cucumber (Lanteri *et al*., 2006) and ZmCCaMK in maize (Ma *et al*., 2012). H_2O_2-dependent NO production plays an

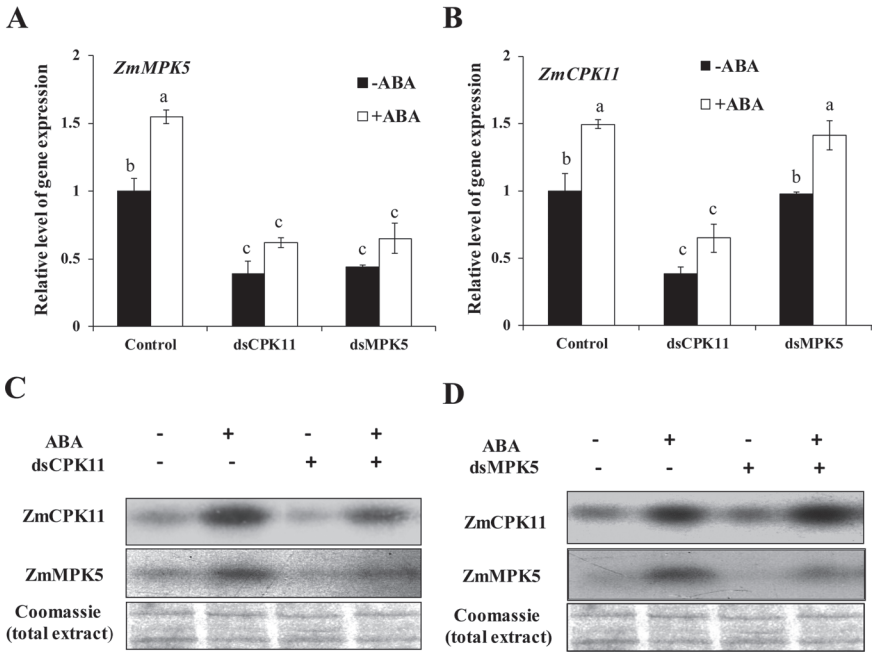

Fig. 8. ZmCPK11 regulates the expression of *ZmMPK5* and the activity of ZmMPK5 in ABA signalling. (A) The expression of *ZmMPK5* in protoplasts with transiently silenced *ZmCPK11* (dsCPK11) and *ZmMPK5* (dsMPK5). The protoplasts were treated with 10 μM ABA for 5 min, and the relative expression level of *ZmMPK5* was analysed by real-time quantitative PCR. (B) The expression of *ZmCPK11* in protoplasts with transiently silenced *ZmCPK11* (dsCPK11) and *ZmMPK5* (dsMPK5). The protoplasts were treated with 10 μM ABA for 5 min, and the relative expression level of *ZmCPK11* was analysed by real-time quantitative PCR. (C) The activity of ZmCPK11 and ZmMPK5 in maize protoplasts with transiently silenced *ZmCPK11* (dsCPK11). (D) The activity of ZmCPK11 and ZmMPK5 in maize protoplasts with transiently silenced *ZmMPK5* (dsMPK5). In A and B, values are means ±SE of three independent experiments. Means denoted by the same letter did not significantly differ at $P<0.05$ according to Duncan's multiple range test. In C and D, experiments were repeated at least three times with similar results.

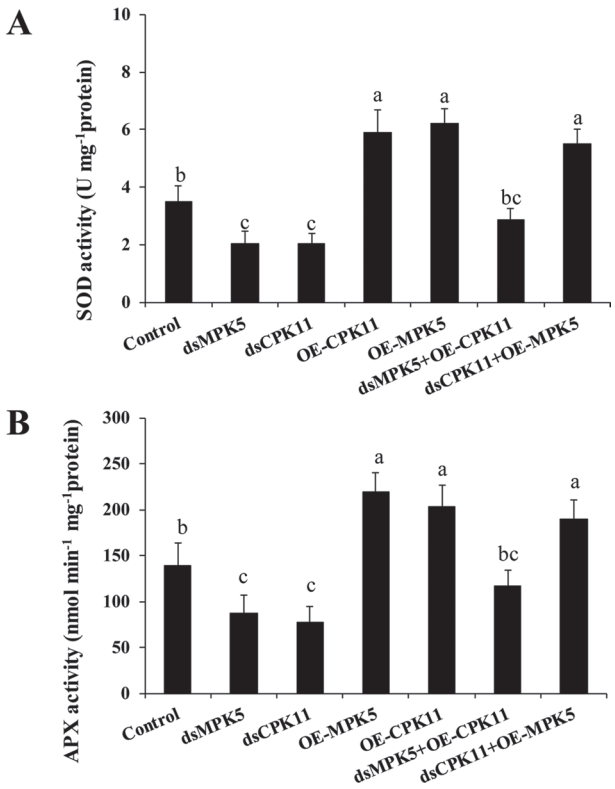

Fig. 9. ZmCPK11 functions upstream of ZmMPK5 to regulate the activities of antioxidant enzymes. (A) The activity of SOD in protoplasts with transiently expressed *ZmCPK11* (OE-CPK11) and *ZmMPK5* (OE-MPK5), transiently silenced *ZmCPK11* (dsCPK11) and *ZmMPK5* (dsMPK5), transiently expressed *ZmCPK11* in combination with transiently silenced *ZmMPK5* (dsMPK5+OE-CPK11), or transiently expressed *ZmMPK5* in combination with transiently silenced *ZmCPK11* (dsCPK11+OE-MPK5). (B) The activity of APX in protoplasts with transiently expressed *ZmCPK11* (OE-CPK11) and *ZmMPK5* (OE-MPK5), transiently silenced *ZmCPK11* (dsCPK11) and *ZmMPK5* (dsMPK5), transiently expressed *ZmCPK11* in combination with transiently silenced *ZmMPK5* (dsMPK5+ OE-CPK11), or transiently expressed *ZmMPK5* in combination with transiently silenced *ZmCPK11* (dsCPK11+OE-MPK5). Values are means ±SE of three independent experiments. Means denoted by the same letter did not differ significantly at $P<0.05$ according to Duncan's multiple range test.

important role in ABA-induced activation of ZmCCaMK (Ma *et al.*, 2012). These results suggest that NO might also be involved in the regulation of ZmCPK11 in ABA signalling. On the other hand, CDPKs have also been shown to be associated with the production of ROS. In *Arabidopsis*, several CDPKs such as AtCPK4, AtCPK5, AtCPK6, and AtCPK11 from group I have been demonstrated to play a key role in defence-induced ROS production (Boudsocq *et al.*, 2010). StCDPK4 and StCDPK5, close homologues of

AtCPK5/AtCPK6, can directly phosphorylate the NADPH oxidases StRbohB and StRbohC to induce ROS production (Kobayashi *et al*., 2007, 2012). Interestingly, ROS and NO production induced by ABA was not impaired in the *cpk6* guard cells of *Arabidopsis* (Munemasa *et al*., 2011). In this study, RNAi-mediated silencing of *ZmCPK11* in maize protoplasts blocked the ABA-induced increase in the production of H_2O_2 (Fig. 6), suggesting that ZmCPK11 mediates the ABA-induced up-regulation of the production of H_2O_2. Previous studies showed that H_2O_2 is required for the activation of ZmMPK5 in maize leaves (Ding *et al*., 2009; Lin *et al*., 2009). This might be a reason why ZmCPK11 induced the activation of ZmMPK5 in ABA signalling. However, in this study, the expression of *ZmMPK5* was also up-regulated by ZmCPK11 in ABA signalling, suggesting that the CDPK can activate the transcription of the MAPK. The mechanism whereby ZmCPK11 regulates the expression of *ZmMPK5* and the activity of ZmMPK5 remains to be elucidated.

In conclusion, the present data indicate that ZmCPK11 is required for ABA-induced antioxidant defence in maize leaves. ABA-induced H_2O_2 production activates ZmCPK11, which induces the activation of ZmMPK5, thus resulting in the up-regulation of the expression and the activities of antioxidant enzymes in ABA signalling.

Acknowledgements

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