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 H2O2 induced the expression of ZmCPK11 and increased the activity of ZmCPK11, while H2O2 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 H2O2. 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, 2002a, b; Hu et al., 2005; Miao et al., 2006; Zhang et al., 2006,
Calcium-dependent protein kinases (CDPKs) are serine/threonine protein kinases that include a Ca\textsuperscript{2+}-binding CaM-like domain and are one of the best characterized Ca\textsuperscript{2+} sensors in plants. CDPKs constitute a large multigene family consisting of local and systemic responses (Szczegielniak et al., 2005, 2012). Moreover, the relationship between ZmCPK11 and ZmMPK5, which is required for 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\textsuperscript{-2} s\textsuperscript{-1}. To study the effects of inhibitors, the detached plants were pre-treated with 100 µM diphenyleleniodonium chloride (DPI), 10 mM dimethylthiourea (DMTU), 200 µM of CAT, 100 µM triluoperoxizine (TFP), 10 mM ethylene glycol-bis(2-aminoethyl ether)-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(e-aminoethyl-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\textsubscript{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-agarose 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$_3$VO$_4$, 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-32P]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 immunoblotting, were quantified and expressed relative to those of the corresponding controls.

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., 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.

In vitro synthesis of dsRNA
DNA templates were produced by PCR using primers containing the T7 promoter sequence (5’-TTATACGACTCACTATAGG AG-3’) on both the 5’ and 3’ ends. The primers used to amplify DNA of ZmCPK11 were: forward TAATACGACTCACTATA TGGAGACCGACCTTGGGCTTTCC and reverse TAATA CGACTCATATA GGAGAGCCTCTCGAGGAGGAGA. The PCR conditions were as follows: denaturing 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 TAATACGACTCATATA TGGAGACCGACCTTGGGCTTTCC and reverse TAATACGACTCATATA GGAGAGCCTCTCGAGGAGGAGA. The PCR conditions were as follows: denaturing 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 of protoplasts were used for transient expression analysis. The DNA or dsRNA and protoplast mixtures were incubated for 40% PEG solution (40% PEG 4000, 0.4 M 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-diamidino-2-phenylindole (DAPI; 1 μg ml$^{-1}$) was added to the culture medium and incubated for 1 h. For the plasma membrane staining, N-[3-triethyl-ammoniumpropyl]-4-[p-diethylaminophenylhexatrieny] pyridinium dibromide (FM4-64, 5 μg ml$^{-1}$) 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$_2$O$_2$ detection by confocal laser scanning microscopy
H$_2$O$_2$ production in protoplasts was monitored using the H$_2$O$_2$-sensitive fluorescent probe 2′,7′-dichlorofluorescin diacetate (H$_2$DCF-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 H₂O₂ induce the expression of ZmCPK11 and increase the activity of ZmCPK11 in maize leaves
To investigate the effects of ABA and H₂O₂ 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 H₂O₂ (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 H₂O₂ treatments was observed, in which the first peak occurred after 20 min of treatment, and the second peak appeared after 90 min of H₂O₂ treatment or 120 min of ABA treatment (Fig. 1A). Treatments with ABA and H₂O₂ 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, H₂O₂ 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 H₂O₂ treatment. As a positive control, CaCl₂ 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,
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 H2O2, several ROS manipulators, such as DPI, an inhibitor of NADPH oxidase, DMTU, a trap for H2O2, and CAT, the enzyme eliminating H2O2, 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 H2O2 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 H2O2

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 of the corresponding enzymes.

Fig. 2. H2O2 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.
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 H2O2 production, protoplasts transfected with dsRNA against ZmCPK11 were used, and H2O2 production in the protoplasts was monitored using the fluorescent probe H2DCF-DA. The RNAi silencing of ZmCPK11 in the protoplasts not only decreased the H2O2-mediated fluorescence under the control condition, but also blocked the ABA-induced increase in the fluorescence (Fig. 6). The specificity of the H2O2-mediated fluorescence was proven by the application of CAT. These results indicate that ZmCPK11 is involved in ABA-induced H2O2 production.

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

Previous studies showed that ABA and H2O2 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 Ca2+ 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 ZmCPK11 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,
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$_2$O$_2$ in OsCPK12-overexpressing plants was less than that in wild-type plants, whereas the accumulation was more in oscpk12 mutant and OsCPK12 RNAi plants.
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. 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.
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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 CCAK 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 (Ye 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, H2O2, and PEG induced the expression of the rice CCAK gene OsDM13 and the maize CCAK gene ZmCCaMK in the leaves of these

Fig. 6. ZmCPK11 mediates ABA-induced production of H2O2 in maize protoplasts. (A) H2O2 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 H2DCF-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. H2O2 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.
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$_2$O$_2$ treatment induced the expression of ZmCPK11 and the activity of ZmCPK11 in maize leaves (Fig. 1), and H$_2$O$_2$ is required for ABA-induced increases in the expression and the activity of ZmCPK11 (Fig. 2), suggesting that H$_2$O$_2$ 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$_2$O$_2$-dependent NO production plays an

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**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.
ZmCPK11 functions in ABA signalling

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

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.

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 5min, 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 5min, 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
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$_2$O$_2$ (Fig. 6), suggesting that ZmCPK11 mediates the ABA-induced up-regulation of the production of H$_2$O$_2$. Previous studies showed that H$_2$O$_2$ 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$_2$O$_2$ 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.

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