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RESEARCH PAPER

Nitric oxide-activated calcium/calmodulin-dependent protein kinase regulates the abscisic acid-induced antioxidant defence in maize

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Abstract

Nitric oxide (NO), hydrogen peroxide (H_2O_2), and calcium (Ca^{2+})/calmodulin (CaM) are all required for abscisic acid (ABA)-induced antioxidant defence. Ca^{2+}/CaM-dependent protein kinase (CCaMK) is a strong candidate for the decoder of Ca^{2+} signals. However, whether CCaMK is involved in ABA-induced antioxidant defence is unknown. The results of the present study show that exogenous and endogenous ABA induced increases in the activity of ZmCCaMK and the expression of *ZmCCaMK* in leaves of maize. Subcellular localization analysis showed that ZmCCaMK is located in the nucleus, the cytoplasm, and the plasma membrane. The transient expression of *ZmCCaMK* and the RNA interference (RNAi) silencing of *ZmCCaMK* analysis in maize protoplasts revealed that ZmCCaMK is required for ABA-induced antioxidant defence. Moreover, treatment with the NO donor sodium nitroprusside (SNP) induced the activation of ZmCCaMK and the expression of *ZmCCaMK*. Pre-treatments with an NO scavenger and inhibitor blocked the ABA-induced increases in the activity and the transcript level of ZmCCaMK. Conversely, RNAi silencing of *ZmCCaMK* in maize protoplasts did not affect the ABA-induced NO production, which was further confirmed using a mutant of *OsCCaMK*, the homologous gene of *ZmCCaMK* in rice. Moreover, H_2O_2 was also required for the ABA activation of ZmCCaMK, and pre-treatments with an NO scavenger and inhibitor inhibited the H_2O_2-induced increase in the activity of ZmCCaMK. Taken together, the data clearly suggest that ZmCCaMK is required for ABA-induced antioxidant defence, and H_2O_2-dependent NO production plays an important role in the ABA-induced activation of ZmCCaMK.

Key words: ABA, antioxidant defence, H_2O_2, NO, ZmCCaMK.

Introduction

Plants are subjected to various biotic and abiotic stresses during their growth and development and have evolved various mechanisms to adapt to these stresses. The phytohormone abscisic acid (ABA) represents a key signal to regulate plant responses to biotic and abiotic stresses (Zhu, 2002; Cutler *et al*., 2010). ABA can enhance the antioxidant defence system to protect cells and subcellular systems from the damage caused by excess reactive oxygen species (ROS) (Jiang and Zhang, 2001; Park *et al*., 2004; Neill *et al*., 2008). Previous studies have shown that hydrogen peroxide (H_2O_2), nitric oxide (NO), calcium (Ca^{2+})/calmodulin (CaM), and mitogen-activated protein kinase (MAPK) are required for ABA-induced up-regulation of the expression and

the activities of antioxidant enzymes such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), and glutathione reductase (GR) in plants (Jiang and Zhang, 2002; Zhang *et al*., 2006, 2007; Sang *et al*., 2008; Zhang *et al*., 2009). However, the detailed mechanisms of ABA-induced antioxidant defence remain unclear.

Ca^{2+} is a ubiquitous and pivotal second messenger in the signal transduction networks (Pei *et al*., 2000; Dodd *et al*., 2010; Kim *et al*., 2010; Kudla *et al*., 2010). Various stimuli, such as salinity, drought, cold, heat shock, mechanical disturbances, ABA, H_2O_2, and pathogen elicitors, trigger changes in the cytosolic Ca^{2+} concentration, and the transient Ca^{2+} elevations are recognized by several Ca^{2+} sensors such as CaM and CaM-like protein (CML), calcium-dependent protein kinase (CDPK), calcineurin B-like protein (CBL), and Ca^{2+}/CaM-dependent protein kinase (CCaMK) (Harmon *et al*., 2000; Luan *et al*., 2002; Yang and Poovaiah, 2003; Zhang and Lu, 2003; Harper *et al*., 2004; Bouché *et al*., 2005; Harper and Harmon, 2005; Yano *et al*., 2008; DeFalco *et al*., 2010). These Ca^{2+} sensors convert the Ca^{2+} signals into various physiological responses.

CCaMK is a strong candidate for the decoder of Ca^{2+} spiking. A CCaMK structure includes a serine/threonine kinase domain, a CaM-binding domain, and three EF-hand motifs, similar to the visinin-like domain (Patil *et al*., 1995; Takezawa *et al*., 1996). Many studies have shown that CCaMK is a common symbiosis signalling pathway component and regulates both arbuscular mycorrhiza (AM) and rhizobial symbioses in legumes and non-legumes (Lévy *et al*., 2004; Godfroy *et al*., 2006; Tirichine *et al*., 2006; Chen *et al*., 2007, 2008; Capoen *et al*., 2009; Hayashi *et al*., 2010; Kang *et al*., 2011). CCaMK was also suggested to play roles in meiosis and mitosis (Patil *et al*., 1995; Poovaiah *et al*., 1999; Yang and Poovaiah, 2003). In addition to its development-related roles, CCaMK may also be involved in abiotic stress responses. The wheat CCaMK gene *TaCCaMK* was down-regulated by ABA, as well as NaCl and polyethylene glycol (PEG) treatments in wheat seedling roots (Yang *et al*., 2011). Overexpressing *TaCCaMK* in *Arabidopsis* reduced their sensitivity to ABA treatment during seed germination and enhanced the seed germination rate under high-salt conditions. These results suggest that *TaCCaMK* is a negative regulator of ABA signalling involved in abiotic stress responses in wheat.

Previous studies have shown that Ca^{2+}/CaM is required for ABA-induced antioxidant defence, and the cross-talk between Ca^{2+}/CaM and H_2O_2 and NO plays a pivotal role in the ABA signalling in leaves of maize seedlings (Jiang and Zhang, 2003; Sang *et al*., 2008). However, it is unknown whether Ca^{2+}/CaM-mediated up-regulation in the antioxidant defence is through the action of CCaMK activated by Ca^{2+}/CaM in ABA signalling and, if so, what the relationship between ZmCCaMK and H_2O_2 and NO in ABA signalling is.

In this study, the role of CCaMK in ABA-induced up-regulation in the expression of antioxidant genes such as *SOD4*, encoding a cytosolic isoform of SOD, *cAPX*, encoding a cytosolic isoform of APX, the total activities of antioxidant enzymes SOD and APX in leaves of maize plants, and the relationship between CCaMK and H_2O_2 and NO were investigated. By combining pharmacological and biochemical analysis with a genetic approach, evidence is provided to show that ZmCCaMK is required for ABA-induced antioxidant defence, and H_2O_2-induced NO production plays an important role in the activation of ZmCCaMK in ABA signalling.

Materials and methods

Plant materials and treatments

Maize (*Zea mays* L. cv. Nongda 108; from Nanjing Agricultural University, China), rice (*Oryza sativa*) cultivar Nipponbare, and the rice mutant line NF8513 were used in this study. In the mutant experiments, Nipponbare was used as the wild-type control. Seeds of maize were sown in trays of sand, and rice plants were grown hydroponically with a nutrient solution in a light chamber at a temperature of 22–28 °C, with a photosynthetically active radiation of 200 μmol m^{-2} s^{-1} and a photoperiod of 14/10 h (light/dark), and watered daily. For protoplast isolation, maize plants were grown at 25 °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 1 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% PEG, 100 μM sodium nitroprusside (SNP), or 10 mM H_2O_2 solution for various times at 25 °C, with a continuous light intensity of 200 μmol m^{-2} s^{-1}. In order to study the effects of various inhibitors or scavengers, the detached plants were pre-treated with 200 μM *N*-(6-aminohexyl)-5-chloro-1-naphthalene sulphonamide hydrochloride (W7), 100 μM trifluoperazine (TFP), 5 mM EGTA, 5 mM $LaCl_3$, 10 mM dimethylthiourea (DMTU), 200 U of catalase (CAT), 100 μM diphenyleneiodonium (DPI), 200 μM 2-4-carboxyphenyl-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (cPTIO), and 200 μM N^G-nitro-L-Arg methyl ester (L-NAME) for 4 h, then exposed to 100 μM ABA or 10 mM H_2O_2 treatment for various times 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. For fluridone treatment, the seeds soaking in 80 μM fluridone for 14 h were sown in trays of sand. After treatments of detached maize plants, the second leaves were sampled and immediately frozen under liquid N_2 for further analysis.

Antibody production and immunoprecipitation kinase activity assay

The peptides for ZmCCaMK-C (GDITEPGKLDEVFD) corresponding to the C-terminus of ZmCCaMK were synthesized and conjugated to the keyhole limpet haemocyanin carrier. The ZmCCaMK polyclonal antibody was raised in rabbits and purified by affinity chromatography. Protein was extracted from maize leaves or protoplasts as described previously (Zhang *et al*., 2006). Protein content was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as standard. For immunocomplex kinase assay, protein extract (200 μg) was incubated with anti-ZmCCaMK antibody (7.5 μg) in an immunoprecipitation buffer as described previously (Zhang *et al*., 2006). Kinase activity in the immunocomplex was determined by an in-gel kinase assay using histone S-III as the substrate (Takezawa *et al*., 1996; Zhang *et al*., 2002). The immunocomplex was incubated in reaction buffer [25 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 2.5 mM $CaCl_2$, 2 μM CaM, 1 mg ml^{-1} histone S-III] with 200 nM ATP plus 1 μCi of [γ-^{32}P]ATP (3000 Ci mM^{-1}) for 30 min. An equal volume of SDS sample buffer was added to stop the reaction. The reaction mix was boiled at 100 °C for 5 min and resolved by SDS–PAGE. The unincorporated [γ-^{32}P]ATP was removed by washing with 5% trichloroacetic acid (w/v)/1% sodium pyrophosphate (w/v) at least three times. The gel was dried onto Whatman 3 MM paper and exposed to Kodak XAR-5 film. Pre-stained size markers (Bio-Rad) were used to calculate the size of the kinases. Relative activation levels of ZmCCaMK protein were quantitated by Quantity One software (Bio-Rad Laboratories Inc., USA).

Isolation of total RNA and RT-PCR analysis

Total RNA was isolated from leaves or protoplasts using RNAiso Plus (TaKaRa Bio Inc., China) according to the instructions supplied by the manufacturer. Approximately 2 μg of total RNA were reverse transcribed using oligo$(dT)_{18}$ primer and M-MLV reverse transcriptase (TaKaRa Bio Inc., China). Transcript levels of several genes were measured by RT-PCR using the following primers: *ZmCCaMK* (GenBank accession no. DQ403196; size of product, 457 bp), forward CGCCGTTCCATGCACCA and reverse AGCCTCATCGCCCTCAGCAC; *SOD4* (GenBank accession no.. X17565; size of product, 404 bp), forward GGGCACAATCTTCTTCACC and reverse GTCCGATGATCCCACAAG; *cAPX* (GenBank accession no. EU969033; size of product, 450 bp), forward TCACACCCTGGGAAGATG and reverse GCTTCATATCAAACCTTCTCC; *GAPDH* (glyceraldehyde phosphate dehydrogenase; GenBank accession no. X07156; size of product, 264 bp), forward CAACGACCCCTTCATCACC and reverse ACCTTCTTGGCACCACCCT. To standardize the results, the relative abundance of *GAPDH* was determined and used as the internal standard. The cycle number of the PCRs was adjusted for each gene to obtain barely visible bands in agarose gels. Aliquots of the PCRs were loaded on agarose gels and stained with ethidium bromide.

Real-time quantitative RT-PCR expression analysis

The expression of *ZmCCaMK* was also measured by qRT-PCR using the DNA Engine Opticon 2 real-time PCR detection system (Bio-Rad Laboratories Inc., USA) with the SYBR® Premix Ex Taq™ (TaKaRa Bio Inc., China) according to the manufacturer's instructions. The cDNA was amplified by PCR using the following primers: *ZmCCaMK* (size of product, 172 bp), forward CTCAAGCCCGAGAACTGCC and reverse TGGCAGCCGAGACATCC; *β-actin* (GenBank accession no. J01238; size of product, 152 bp), forward GTTTCCTGGGATTGCCGAT and reverse TCTGCTGCTGAAAAGTGCTGAG. To standardize the results, the amplification of *β-actin* was determined and used as the internal standard. The data were normalized to the amplification of a maize *β-actin* gene. For each sample, the mean value from three qRT-PCRs was adapted to calculate the expression abundance, and the mean values were then plotted with their SE.

Vector construction and in vitro *transcription of the* ZmCCaMK *gene double-stranded RNA*

The full-length cDNA fragment was amplified with the addition of a *Bsr*GI site and then inserted in-frame with yellow fluorescent protein (YFP) into the pXZP008 vector driven by the *Cauliflower mosaic virus* (CaMV) 35S promoter. The primers used for the PCR amplification were: forward 5′-TGTACAAGATTCCTCGCACAAACCTGCCACA-3′, and reverse 5′-TGTACAGGTGGGAATGAAGTTGAACGAGTTGGAAT-3′ (underlining indicates the *Bsr*GI site).

A template partial-length DNA fragment was amplified by PCR using primers flanked by a T7 promoter, forward (5′-TAATACGACTCACTATAGGGCAAGCCCGAGAACTGCC-3′) and reverse (5′-TAATACGACTCACTATAGGGTGGCAGCCGAGACATCC-3′) (the T7 promoter site is underlined). The PCR amplification consisted of initial denaturation at 94 °C for 3 min, then 35 cycles of 94 °C for 20 s, 60 °C for 15 s, and 72 °C for 15 s, and a final extension at 72 °C for 2 min. Double-stranded RNA (dsRNA) of *ZmCCaMK* was synthesized *in vitro* using the RiboMAX™ Large Scale RNA Production System-T7 (Promega, Madison, WI, USA) according to the manufacturer's instructions. The purity and concentration of synthesized dsRNA were checked by 2% agarose gel electrophoresis and spectrophotometry.

Protoplast preparation and transfection with constructs or dsRNAs

Protoplast isolation and transfection with constructs or dsRNAs were based on the protocol for maize mesophyll protoplasts provided online by J. Sheen's laboratory http://genetics.mgh.harvard.edu/sheenweb with minor modifications. For transfection, 1 ml of maize protoplasts (usually 5×10^5 cells ml^{-1}) were transfected with 150 μg of YFP–ZmCCaMK fusion constructs (pXZP008 vector as control) or dsRNAs (H_2O as control) using a PEG–calcium-mediated method. Then the transfected protoplasts were incubated in incubation solution overnight in the dark at 25 °C. After that, protoplasts were collected and used for further analysis.

Localization

Expression of YFP–ZmCCaMK fusion constructs and YFP was monitored using a confocal microscope (TCS-SP2, Leica, Bensheim, Germany). The nucleus was stained with the 4′,6-diamidino-2-phenylindole (DAPI) dye, and the plasma membrane was visualized by staining with *N*-(3-triethylammoniumpropyl]-4-[*p*-diethyl- aminophenylhexatrienyl) pyridinium dibromide (FM4-64).

Western blot assay

Proteins were extracted from protoplasts tansfected with *YFP* or *YFP-ZmCCaMK*, and 20 μg of total protein was subjected to SDS–PAGE. Western blot analysis was performed as described by Sambrook and Russell (2001). Anti-YFP antibody was used to detect the YFP protein or YFP–ZmCCaMK protein.

Antioxidant enzyme assays

Protoplasts were homogenized in 0.5 ml of 50 mM potassium phosphate buffer (pH 7.0) containing 1 mM EDTA and 1% polyvinylpyrrolidone, with the addition of 1 mM ascorbate in the case of APX assay. The homogenate was centrifuged at 12 000 *g* for 15 min at 4 °C and the supernatant was immediately used for the susequent antioxidant enzyme assays. The total activities of antioxidant enzymes were determined as previously described (Jiang and Zhang, 2001). Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitro blue tetrazolium. One unit of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of nitro blue tetrazolium, as monitored at 560 nm. Total APX activity was measured by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized.

Nitric oxide detection by confocal laser scanning microscopy (CLSM)

Measurement of NO was performed with the specific NO dye 4,5-diaminofluorescein diacetate (DAF-2DA), using the method as described by Corpas *et al*. (2004) with slight modifications. Leaf segments of ~0.5 cm^2 or protoplasts were incubated in loading buffer [0.1 mM $CaCl_2$, 10 mM KCl, 10 mM 2-(*N*-morpholino)ethanesulphonic acid (MES)-TRIS, pH 5.6] and DAF-2DA at a final concentration of 10 μM for 1 h in the dark at 25 °C, followed by washing with loading buffer for 1 h. All images were visualized using confocal microscopy (excitation 495 nm, emission 515 nm). To enable the comparison of changes in signal intensity, confocal images were taken under identical exposure conditions for all the samples. The green fluorescence intensity of images acquired was quantified using Leica IMAGE software to integrate the intensity over all pixels within the boundary of each image. The value of each image was normalized to a reference image of the basal state. Data are presented as average fluorescence intensity.

Results

ABA induces increases in the activity and the expression of ZmCCaMK in leaves of maize

In order to investigate the effect of ABA on the activation of ZmCCaMK in leaves of maize plants, an antibody against the C-terminus of ZmCCaMK was raised and an

immunoprecipitation kinase assay was performed on protein extracts from the leaves of maize plants treated or not with ABA, using histone S-III as a substrate (Takezawa *et al.*, 1996; Zhang *et al.*, 2002). Under control conditions, a low background level of ZmCCaMK activity was observed (Fig. 1A). However, when 100 μM ABA was applied, a significant increase in the activation of ZmCCaMK was detected after 20 min treatment, was maximizal at 30 min, and then decreased (Fig. 1A). Moreover, the ABA-induced activation of ZmCCaMK occurred in a dose-dependent manner in the concentration range of 10–100 μM ABA (Fig. 1D).

To investigate whether the activation of ZmCCaMK can be induced by endogenous ABA, maize seeds were pre-treated by fluridone, an inhibitor of carotenoid biosynthesis, and hence of ABA biosynthesis (Nagamune *et al.*, 2008), and then the pre-treated plants were exposed to PEG treatment. PEG-induced activation of ZmCCaMK was significantly inhibited in 80 μM fluridone-pre-treated plants, but this effect of fluridone was

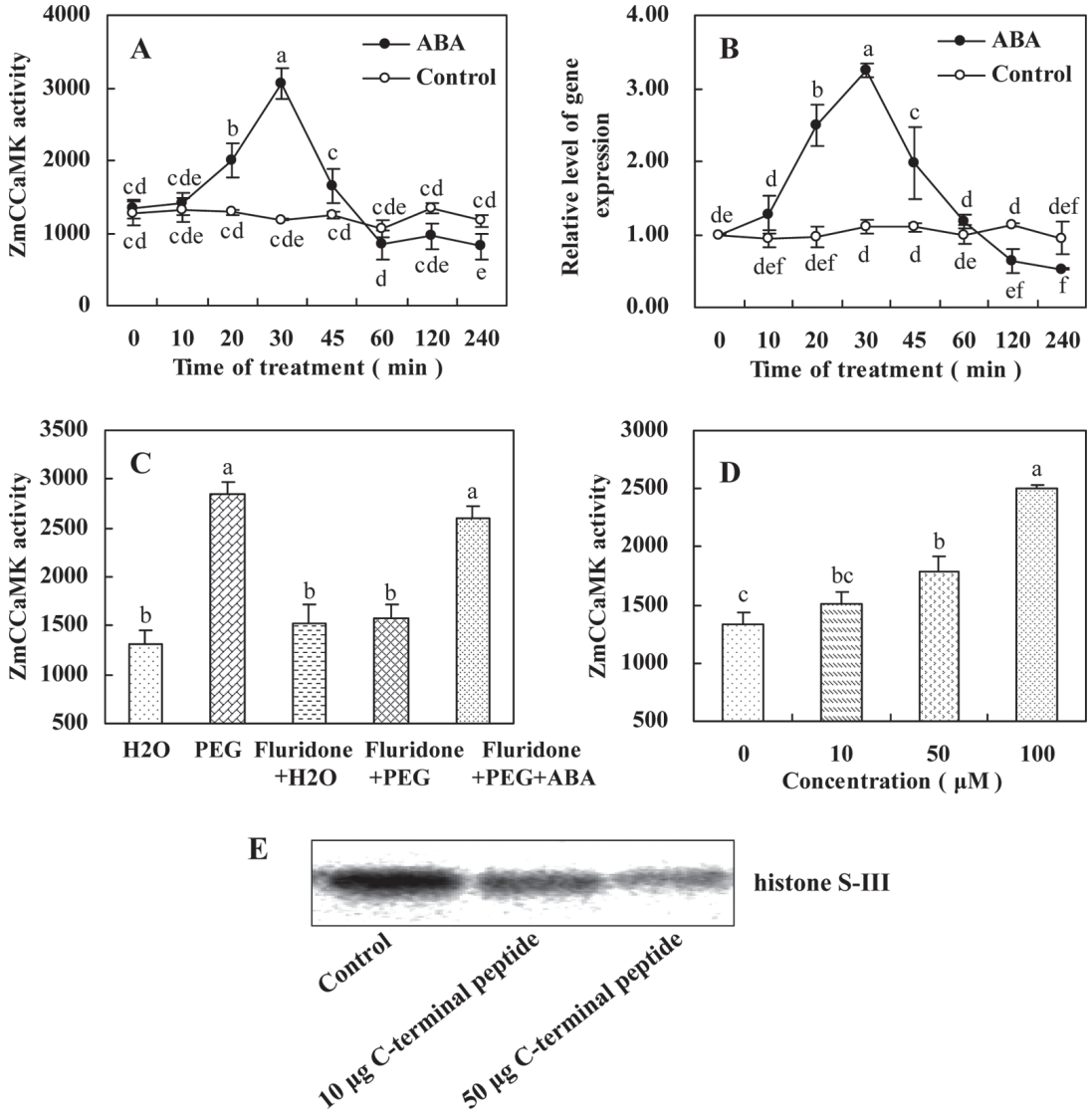

Fig. 1. ABA induces the up-regulation in the activity of ZmCCaMK and the expression of *ZmCCaMK* in leaves of maize plants. (A) Time course of ABA-induced ZmCCaMK activation. The detached plants were treated with 100 μM ABA for various times as indicated. Protein extracts from control or ABA-treated leaves were immunoprecipitated with ZmCCaMK antibody and then subjected to an in-gel kinase assay. (B) Time course of ABA-induced gene expression of *ZmCCaMK*. The detached plants were treated as described in A. Relative expression levels of the *ZmCCaMK* gene, analysed by qRT-PCR, are normalized to *β-actin* transcript levels. (C) PEG-induced ABA activates ZmCCaMK. The detached plants were treated as follows: distilled water (control); 10% PEG; 80 μM fluridone+H_2O; 80 μM fluridone+10% PEG; 80 μM fluridone+10% PEG+100 μM ABA. Protein extracts were subjected to immunoprecipitation kinase assay. (D) Dose dependence for ABA-induced ZmCCaMK activation. The detached plants were treated with 0, 10, 50, and 100 μM ABA for 30 min. Protein extracts were subjected to immunoprecipitation kinase assay. (E) Kinase activity assay of immune complexes. Immunoprecipitation was performed in the absence or presence of 10 μg or 50 μg of competitor peptides corresponding to the C-terminal peptide of ZmCCaMK. Immunocomplex kinase activity was measured using an in-gel kinase assay. Experiments were repeated at least three times with similar results. Values are means ±SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

overcome by the application of 100 μM ABA (Fig. 1C), indicating that water stress-induced endogenous ABA accumulation can activate ZmCCaMK.

The effects of ABA on the induction of *ZmCCaMK* gene expression in leaves of maize plants were further examined, and relative quantitative real-time PCR analysis was performed using total RNA extracted from maize plants treated or not with 100 μM ABA. The experimental results showed that the *ZmCCaMK* gene was up-regulated by the treatment with 100 μM ABA (Fig. 1B), and the changed pattern of *ZmCCaMK* gene expression was similar to that of ZmCCaMK activation in leaves of maize exposed to ABA treatment (Fig. 1A, B).

To prove the specificity of the antibody, immunoprecipitations with or without peptide competitors were carried out and immune complexes were assayed for kinase activity. Proteins that could phosphorylate histone S-III were precipitated from extracts of leaves of maize plants. The immune complexes were competed out by the peptide used to raise the antibody against the C-terminal region of ZmCCaMK (Fig. 1E). Further, pre-treatments with the Ca^{2+} chelator EGTA, the Ca^{2+} channel blocker $LaCl_3$, and the CaM antagonists TFP and W7 significantly reduced the ABA-induced activation of ZmCCaMK in leaves of maize plants treated with ABA (data not shown).

Subcellular localization of ZmCCaMK in maize mesophyll protoplasts

To gain evidence indicative of function, the subcellular localization of ZmCCaMK was investigated in transfected maize protoplasts by using confocal microscopy. YFP fusion constructs for ZmCCaMK were generated under the control of the CaMV 35S promoter. The results showed that YFP–ZmCCaMK was localized in the nucleus, the cytoplasm, and the plasma membrane in maize mesophyll protoplasts (Fig. 2A, B). Nuclear location

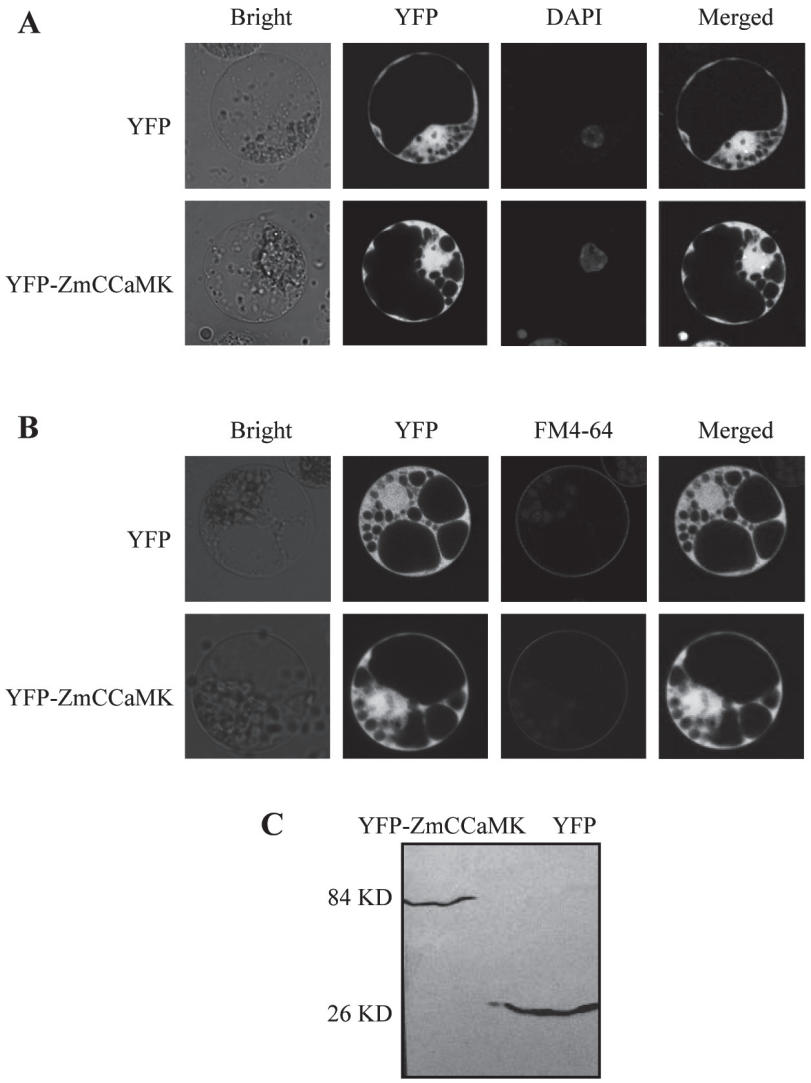

Fig. 2. Subcellular localization of ZmCCaMK in maize protoplasts. (A, B) Protoplasts were isolated from the leaves of maize, and then were transfected with constructs carrying *35S:YFP-ZmCCaMK* or *35S:YFP* by PEG–calcium-mediated transformation. Fluorescence micrographs were taken after 16 h of incubation by a laser confocal microscope. The nucleus was stained with DAPI dye (blue, A). The plasma membrane was labelled with FM4-64 steryl dye (red, B). Experiments were repeated at least five times with similar results. (C) Western blot analysis for YFP–ZmCCaMK fusion proteins with an anti-YFP antibody. Experiments were repeated at least five times with similar results. (This figure is available in colour at *JXB* online.)

was confirmed by means of DAPI staining for DNA (Sakamoto *et al*., 2004), and the plasma membrane location was confirmed by co-localization with the plasma membrane marker, FM4-64 (Levy *et al*., 2007; Zelazny *et al*., 2007). In addition, the localization of YFP–ZmCCaMK was not regulated by ABA treatment in maize mesophyll protoplasts (data not shown). Furthermore, the expression of the effector proteins was confirmed by western blot analysis using an anti-YFP antibody in maize mesophyll protoplasts transfected with constructs (Fig. 2C).

ZmCCaMK modulates the ABA-induced antioxidant defence

To investigate whether ZmCCaMK mediates the ABA-induced antioxidant defence in maize, a transient gene expression analysis and a transient RNA interference (RNAi) analysis in maize mesophyll protoplasts, which have been proven to be efficient for functional analysis of plant genes (Sheen, 2001; Chen *et al*., 2006; Zhai *et al*., 2009; Kim and Somers, 2010; Gao *et al*., 2011), were used for the functional analysis of *ZmCCaMK* in ABA-induced antioxidant defence.

As anticipated, transient expression of *ZmCCaMK* in protoplasts resulted in a significant enhancement in the transcript levels of *ZmCCaMK*, and the antioxidant genes, *SOD4* and *cAPX*, when compared with those in the protoplasts transfected with the empty vector (Fig. 3A). Similar to the expression of antioxidant genes, the total activities of the antioxidant enzymes SOD and APX were also obviously increased in protoplasts showing transient expression of *ZmCCaMK* (Fig. 3B, C). On the other hand, RNAi-mediated silencing of *ZmCCaMK*, which resulted in an obvious reduction in the expression of *ZmCCaMK* (Fig. 4A) and the activity of ZmCCaMK (Fig. 4B), significantly decreased the activities of SOD and APX compared with control (Fig. 4D, E). Further, treatment with 10 μM ABA only slightly increased the expression of *ZmCCaMK* and the activities of ZmCCaMK, SOD, and APX in protoplasts subjected to RNAi silencing of *ZmCCaMK* (Fig. 4B–E). However, treatment with 10 μM ABA induced significant increases in the expression of *ZmCCaMK* and the activities of ZmCCaMK, SOD, and APX in the control protoplasts (Fig. 4B–E). Taken together, these data demonstrate unequivocally that ZmCCaMK is required for ABA-induced antioxidant defence in maize protoplasts.

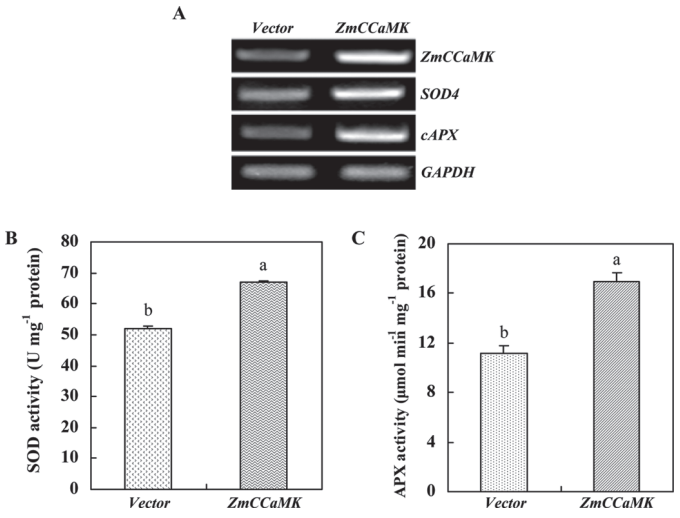

Fig. 3. The gene expression and the total activities of antioxidant enzymes in maize mesophyll protoplasts transiently expressing *ZmCCaMK*. (A) The expression of *ZmCCaMK*, *SOD4*, and *cAPX*. Protoplasts isolated from the leaves of maize were transfected with constructs carrying *YFP-ZmCCaMK* (*ZmCCaMK*), and control protoplasts were transfected with empty vector (Vector). Relative expression of antioxidant enzymes was analysed by RT-PCR, and was normalized to *GAPDH* transcript levels. Experiments were repeated at least three times with similar results. (B and C) The activities of SOD and APX. Protoplasts were isolated as described in A, and the activities of SOD and APX were measured as described in the Materials and methods. Values are means ±SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

NO induces the activation and gene expression of ZmCCaMK in leaves of maize plants

It has been reported that NO is involved in the ABA-induced antioxidant defence system and NO functions both upstream and downstream of Ca^{2+}/CaM in plants (Zhang *et al*., 2007; Sang *et al*., 2008; Aboul-Soud *et al*., 2009). To establish a link between NO and ZmCCaMK in ABA signalling, the NO donor SNP was used. Treatment with SNP led to a rapid activation of ZmCCaMK (Fig. 5A). A rapid increase in the activity of ZmCCaMK was detected within 10 min and maximized at 45 min after SNP treatment which has been shown to result in NO effects itself by using sodium ferricyanide as control (Zhang *et al.*, 2007). The activity of ZmCCaMK decreased after 60 min of SNP treatment. SNP treatment also induced a significant increase in the expression of *ZmCCaMK* in a similar manner as the activity of ZmCCaMK in leaves of maize plants (Fig. 5B). In addition, the activation of ZmCCaMK occurred at SNP concentrations as low as 10 μM and the activity of ZmCCaMK appeared to reach a maximum at 100 μM SNP (Fig. 5C).

In order to determine that the induction of ZmCCaMK by ABA is related to ABA-induced NO generation, the effects of the NO scavenger cPTIO and the NOS inhibitor L-NAME on ABA-induced activation of ZmCCaMK were assessed. The detached plants were pre-treated with cPTIO and L-NAME, and then exposed to ABA treatment. ABA-induced increases in the activity of ZmCCaMK and the gene expression of *ZmCCaMK* were greatly inhibited in the presence of cPTIO and L-NAME, as shown in Fig. 5D and E. Alone, cPTIO and L-NAME has little effect on the activation of ZmCCaMK and the gene expression of *ZmCCaMK*. Together these data suggest that ABA-induced NO production is required for ABA-induced activation of ZmCCaMK.

ABA-activated ZmCCaMK does not mediate the ABA-induced NO production

The role of ZmCCaMK in ABA-induced NO production was also examined by monitoring NO synthesis in response to ABA

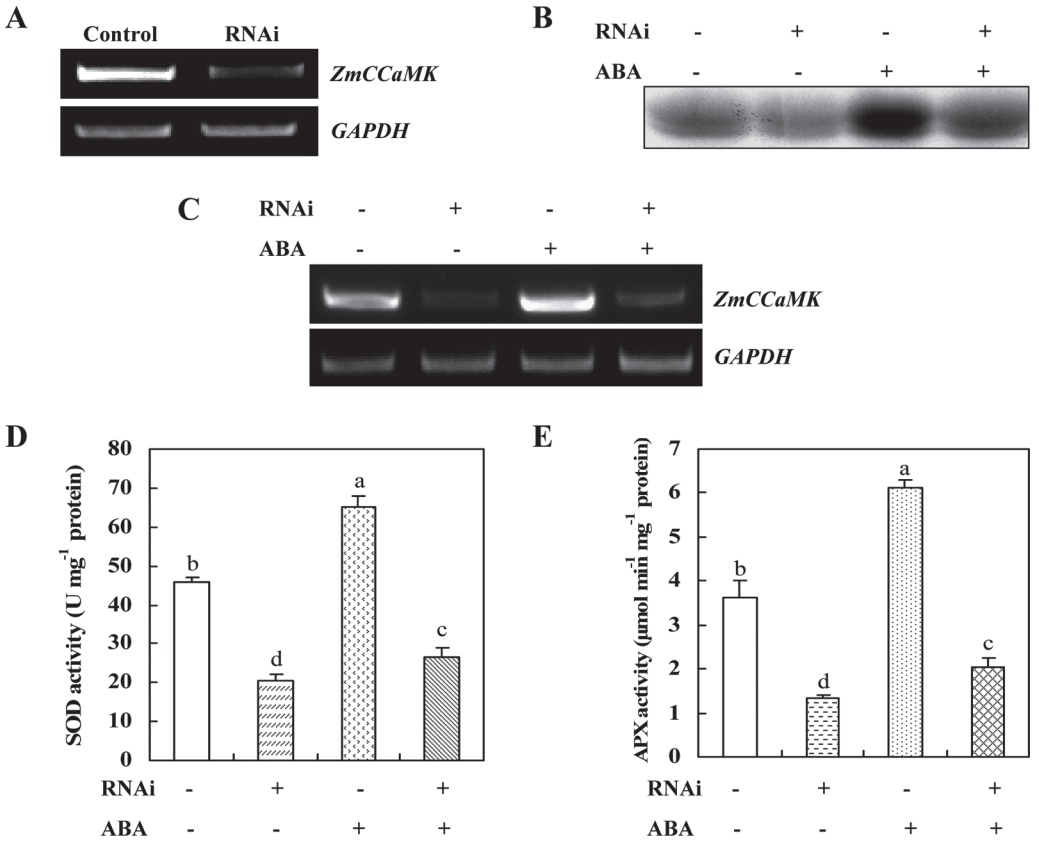

Fig. 4. The expression of *ZmCCaMK* and the activities of ZmCCaMK and antioxidant enzymes in the protoplasts with transiently silenced *ZmCCaMK*. (A) The expression of *ZmCCaMK*. Protoplasts were transfected with dsRNA against *ZmCCaMK* (RNAi) or with water (Control) and incubated for 24 h. Silencing of *ZmCCaMK* was analysed by RT-PCR, and was normalized to *GAPDH* transcript levels. Experiments were repeated at least three times with similar results. (B) ABA-induced activation of ZmCCaMK. The protoplasts were treated with 10 μM ABA for 5 min. Protein extracts from ABA-treated or untreated protoplasts were immunoprecipitated with ZmCCaMK antibody and then subjected to an in-gel kinase assay. Experiments were repeated at least three times with similar results. (C) ABA-induced gene expression of *ZmCCaMK*. Protoplasts were treated with 10 μM ABA for 15 min, and the expression of *ZmCCaMK* was analysed by RT-PCR. Experiments were repeated three times with similar results. (D, E) ABA increased the activities of SOD (D) and APX (E). Protoplasts were treated with 10 μM ABA for 15 min, and the activities of SOD and APX were measured as described in the Materials and methods. Values are means ±SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

treatment, using protoplasts transfected with dsRNA against *ZmCCaMK*. Protoplasts were loaded with the NO-specific fluorescent dye DAF-2DA, and CLSM was used to monitor changes in NO-induced fluorescence in mesophyll protoplasts in maize leaves. Treatment with 10 μM ABA led to a rapid increase in NO-induced fluorescence in mesophyll protoplasts compared with control, and a similar induction was also observed in protoplasts transfected with dsRNA against *ZmCCaMK* (Fig. 6), suggesting that ABA-induced ZmCCaMK activation is not required for ABA-induced NO production.

To gain further evidence that ZmCCaMK is not essential for ABA-induced NO production, the rice mutant line NF8513 ('Nipponbare') containing the *Tos17* insertion in *OsDMI3*, a homologous gene of *ZmCCaMK* in rice, was screened and the homozygous mutant in NF8513 was isolated. As shown in Fig. 7, a significant increase in NO-induced fluorescence was observed in ABA-treated leaves of the wild type compared with the control leaves. NO production was observed as early as 1 h after the addition of 100 μM ABA, was maximizal after 2 h of ABA treatment, and then decreased after 4 h treatment. Importantly, leaves of the rice mutant line NF8513 also exhibited a significant increase in the level of NO in response to exogenous ABA in the same manner as did those of the wild type, and ABA-induced NO production in the wild type and mutant was not significantly different (Fig. 7). These data further confirm that ZmCCaMK is not involved in ABA-induced NO accumulation in ABA signalling.

H_2O_2 is required for ABA-induced activation of ZmCCaMK and the activation is regulated by NO

Previous work showed that ABA-induced NO generation was mediated by H_2O_2 in ABA signalling in leaves of maize (Zhang *et al*., 2007). To investigate whether H_2O_2 also plays a role in NO-induced activation of ZmCCaMK in ABA signalling, the activation and gene expression of ZmCCaMK induced by H_2O_2

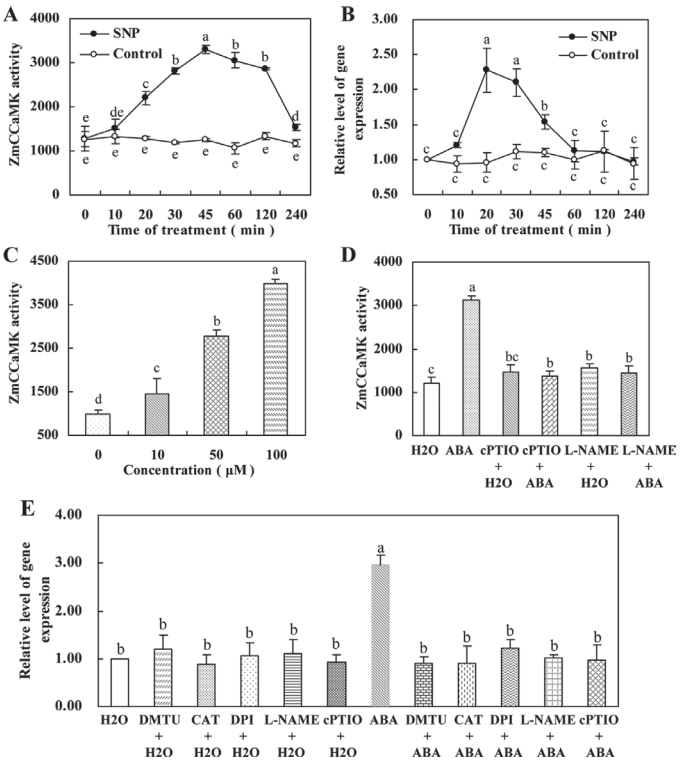

Fig. 5. NO induces the activation of ZmCCaMK and the expression of *ZmCCaMK* in leaves of maize plants. (A) Time course of SNP-induced ZmCCaMK activation. The detached plants were treated with 100 μM SNP for various times as indicated. Protein extracts from control or SNP-treated leaves were immunoprecipitated with ZmCCaMK antibody and then subjected to an in-gel kinase assay. (B) Time course of SNP-induced gene expression of *ZmCCaMK*. The detached plants were treated as described in A. Relative expression levels of the *ZmCCaMK* gene, analysed by qRT-PCR, are normalized to *β-actin* transcript levels. (C) Dose dependence for SNP-induced ZmCCaMK activation. The detached plants were treated with 0, 10, 50, and 100 μM SNP for 45 min and then subjected to immunoprecipitation kinase assay. (D) Effects of pre-treatments with cPTIO and L-NAME on ABA-induced ZmCCaMK activation. The detached plants were treated as follows: distilled water (control); 100 μM ABA; 200 μM cPTIO+H_2O; 200 μM cPTIO+100 μM ABA; 200 μM L-NAME+H_2O; 200 μM L-NAME+100 μM ABA. The detached plants were pre-treated with the scavenger or inhibitor for 4 h then exposed to 100 μM ABA treatment for 30 min. (E) Effects of pre-treatments with the H_2O_2 scavengers or inhibitor, DMTU, CAT, and DPI, and the NO scavenger or inhibitor, cPTIO and L-NAME, on the expression of *ZmCCaMK* in leaves of maize plants exposed to ABA treatment. The detached plants were treated as follows: distilled water (control); 10 mM DMTU+H_2O; 200 U of CAT+H_2O; 100 μM DPI+H_2O; 200 μM L-NAME+H_2O; 200 μM cPTIO+H_2O; 100 μM ABA; 10 mM DMTU+100 μM ABA; 200 U of CAT+100 μM ABA; 100 μM DPI+100 μM ABA; 200 μM L-NAME+100 μM ABA; 200 μM cPTIO+100 μM ABA. The detached plants were pre-treated with scavengers or inhibitors for 4 h then exposed to 100 μM ABA treatment for 30 min. Values are means ±SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

were examined in leaves of maize plants. Exogenous 10 mM H_2O_2 treatment rapidly induced an increase in the activity of ZmCCaMK in leaves of maize plants (Fig. 8A). Several ROS manipulators, such as DPI, an inhibitor of NADPH oxidase, and DMTU and CAT, H_2O_2 scavengers, were used to assess the effect of endogenous H_2O_2 on the induction of ZmCCaMK. Pre-treatments with DPI, DMTU, and CAT clearly blocked the ABA-induced activation of ZmCCaMK in maize plants (Fig. 8C), suggesting that H_2O_2 is required for the activation of ZmCCaMK in ABA signalling. Similarly, the gene expression of *ZmCCaMK* was also significantly up-regulated by both exogenous and endogenous H_2O_2 (Figs 5E, 8B). Furthermore, pre-treatment with cPTIO and L-NAME significantly blocked the H_2O_2-induced activation of ZmCCaMK in leaves of maize (Fig. 8D). Moreover, a previous study showed that exogenous H_2O_2 and ABA-induced H_2O_2 could induce the production of NO in leaves maize (Zhang *et al.*, 2007). Together with these data, the present results suggest that NO is required for H_2O_2-induced activation of ZmCCaMK in ABA signalling in leaves of maize plants.

Discussion

In animal cells, CaMKs have been shown to be involved in H_2O_2 signal transduction that results in the regulation of various cellular processes (Nguyen *et al*., 2004; Bouallegue *et al*., 2009; Palomeque *et al*., 2009). In plants, CCaMKs have high homology to mammalian CaMKs in both the kinase and CaM-binding domains (Yang *et al*., 2007) and are thought to function in a manner analogous to CaMKII (Mitra *et al*., 2004; Yang *et al*., 2007). It has been well documented that CCaMKs play important roles in mediating symbiotic relationships with bacteria and fungi (Lévy *et al*., 2004; Godfroy *et al*., 2006; Tirichine *et al*., 2006; Chen *et al*., 2007, 2008; Capoen *et al*., 2009; Hayashi *et al*., 2010; Kang *et al*., 2011). CCaMKs were also suggested to play roles in meiosis and mitosis (Yang and Poovaiah, 2003) and in abiotic stress responses (Yang *et al*., 2011). CCaMKs were obviously regulated at the transcriptional level by ABA, NaCl, or PEG treatment (Yang *et al*., 2011). However, it is not clear whether CCaMKs are involved in oxidative stress responses in plants as CaMKs are in animals. In the present study, the results showed that ABA treatment induced the expression of *ZmCCaMK* and the activity of ZmCCaMK in leaves of maize plants (Fig. 1A, B, D) and in maize mesophyll protoplasts (Fig. 4B, C). Water stress-induced endogenous ABA also increased the activity of ZmCCaMK (Fig. 1C). These results suggest that ZmCCaMK is very likely to participate in ABA signalling as reported by Yang *et al.* (2011). To investigate further the involvement of ZmCCaMK in ABA signalling, the role of ZmCCaMK in ABA-induced antioxidant defence was investigated by means of transient overexpression or transient silencing of *ZmCCaMK* in maize mesophyll protoplasts. Transiently expressing *ZmCCaMK* in maize mesophyll protoplasts significantly enhanced the activities of SOD and APX (Fig. 3). In contrast, the activities of SOD and APX were reduced in the protoplasts transfected with dsRNA against *ZmCCaMK* (Fig. 4). More importantly, ABA treatment failed to induce an increase in the activities of SOD and APX

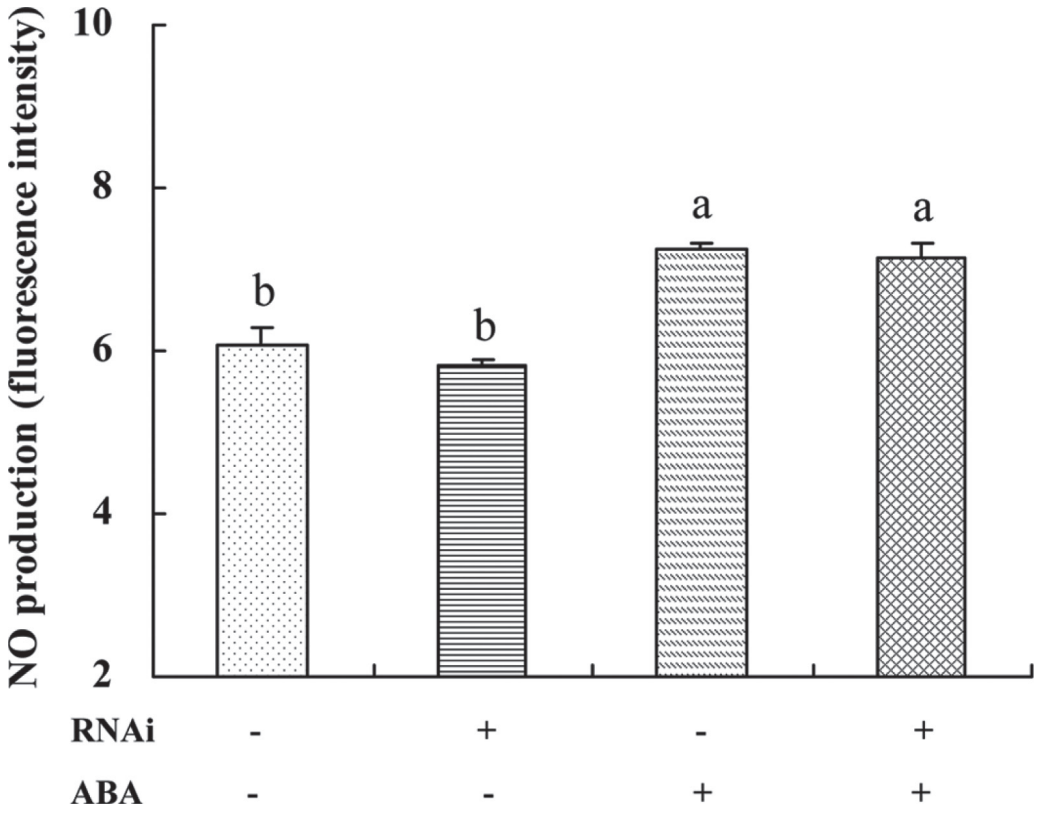

Fig. 6. NO production in transiently silenced *ZmCCaMK* protoplasts. The protoplasts were treated with 10 μM ABA for 30 min, and then loaded with 4,5-diaminofluorescein diacetate (DAF-2DA) and detected by confocal laser scanning microscopy. Values are the mean ±SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

in the protoplasts transfected with dsRNA against *ZmCCaMK*, although ABA treatment significantly increased the activities of SOD and APX in the control protoplasts (Fig. 4). These results provide conclusive evidence that ZmCCaMK is required for the enhancement of ABA-induced antioxidant defence. Furthermore, in a rice mutant of OsCCaMK (*OsDMI3*), a gene homologous to *ZmCCaMK* in rice, ABA treatment was also not able to induce the increases in the activities of these antioxidant enzymes in the leaves of rice (unpublished data). These data clearly indicate that ZmCCaMK and its orthologue in rice play an important role in the ABA-induced antioxidant defence.

However, a recent study showed that in wheat, the expression of *TaCCaMK*, which is closely related to maize *ZmCCaMK* and rice *OsCCaMK*, was down-regulated by ABA, as well as NaCl and PEG treatments in wheat seedlings roots (Yang *et al*., 2011). Overexpression of *TaCCaMK* in *Arabidopsis* reduced ABA sensitivity in seed germination and enhanced the seed germination rate under high-salt conditions. These results suggest that *TaCCaMK* is, as a negative regulator for ABA signalling, involved in abiotic stress responses in wheat. The conclusion seems to be in contrast to the conclusion of this study. There exist several explanations for the discrepancy. In the study by Yang *et al*. (2011), the expression of *TaCCaMK* in wheat roots exposed to ABA, NaCl, and PEG treatments was analysed over a 3 h period, and a transient change in the expression of *TaCCaMK* at times >3 h was not analysed. In the present study, ABA treatment induced a significant increase in the expression of *ZmCCaMK* within 20 min (Fig. 1B). The expression of *ZmCCaMK* reached the maximum after 30 min of ABA treatment, and then decreased to the control level within 60 min of ABA treatment. After 2 h of ABA treatment, the expression of *ZmCCaMK* induced by ABA was significantly lower than that in the control. Moreover, CCaMK does not exist in the *Arabidopsis* genome (Harper *et al*., 2004; DeFalco *et al.*, 2010). Overexpression of *TaCCaMK* in *Arabidopsis* could interfere with the function of Ca^{2+} sensors in *Arabidopsis*. The phenotypes of transgenic plants that reduced ABA sensitivity in seed germination and enhanced the seed germination rate under high-salt conditions might be not from the direct role of TaCCaMK. Another possibility is that the difference between the study by Yang *et al*. (2011) and the present study may be related to the different physiological process. It is also possible that different plant species or organs have different responses to ABA.

In *Medicago trunculata*, CCaMK (DMI3) was localized in the nucleus in epidermal root cells and root hairy cells (Kaló *et al*., 2005; Smit *et al*., 2005). However, in *Triticum aestivum*, TaCCaMK with a 3′ end green fluorescent protein (GFP) fusion has been shown to be located both on the plasma membrane and in the nucleus (Yang *et al*., 2011). In this study, transient

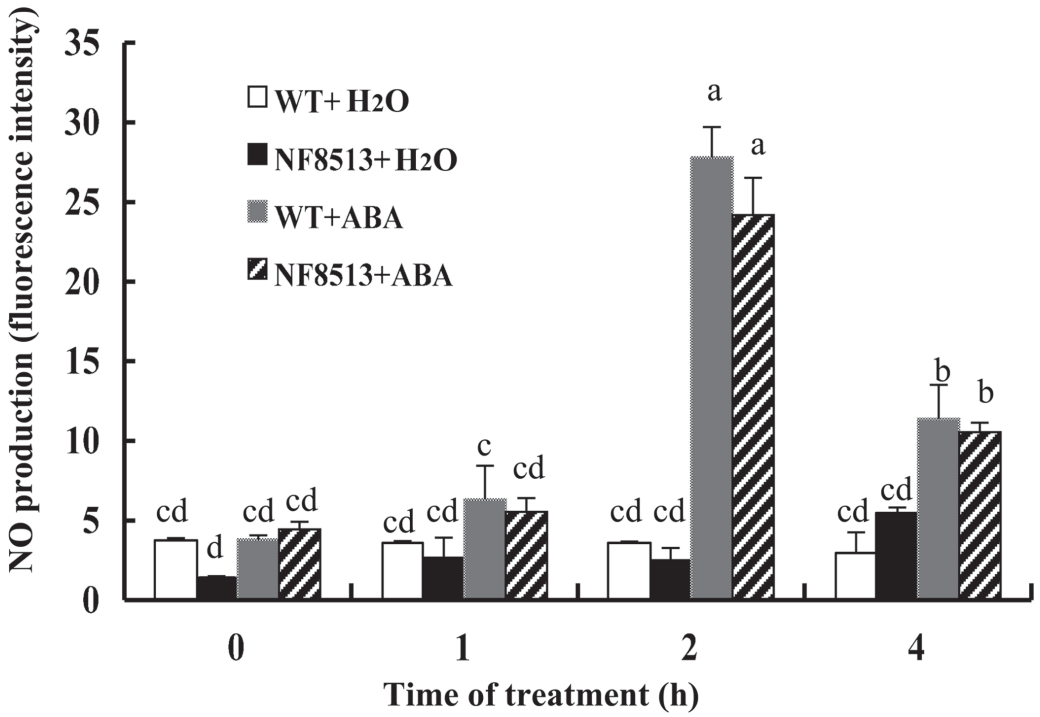

Fig. 7. Time course analysis of NO production in mesophyll cells of the rice mutant of *OsDMI3* and the wild type exposed to ABA treatment. The detached plants were treated with 100 μM ABA or distilled water for various times as indicated, and then the leaf segments were loaded with 4,5-diaminofluorescein diacetate (DAF-2DA) and detected by confocal laser scanning microscopy. Values are means ±SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

expression of *YFP-ZmCCaMK* revealed that the fluorescence of this construct was detected in the nucleus, the cytoplasm, and the plasma membrane in maize protoplasts (Fig. 2). The fluorescence of OsDMI3–YFP was also detected in the nucleus, the cytoplasm, and the plasma membrane in rice protoplasts that were transformed with an *OsDMI3-YFP* fusion construct under the control of the 35S promoter or the *OsDMI3* native promoter (unpublished data). These results suggest that ZmCCaMK is located not only in the nucleus, but also in the cytosol and the plasma membrane. Different subcellular localizations of CCaMKs may be related to their distinct functions in plants.

Previous studies showed that ABA-induced NO production up-regulated the expression and the activities of antioxidant enzymes in ABA signalling, and there exists a cross-talk mechanism between Ca^{2+}/CaM and NO in ABA-induced antioxidant defence in maize leaves (Jiang and Zhang, 2003; Zhang *et al*., 2007; Sang *et al*., 2008). To investigate further the mechanisms of ZmCCaMK in ABA-induced antioxidant defence, the relationship between NO and ZmCCaMK was studied. The results showed that treatment with the NO donor SNP induced an increase in the activity of ZmCCaMK and the expression of *ZmCCaMK* in maize leaves (Fig. 5A–C), and pre-treatment with an NO scavenger or inhibitor substantially reduced the ABA-induced increases in the activity and the transcription level of ZmCCaMK (Fig. 5D, E). These results suggest that NO is involved in the activation of ZmCCaMK in ABA signalling. Conversely, the expression of *ZmCCaMK* was down-regulated through RNAi to investigate the effects of ZmCCaMK on the production of NO. The results showed that the RNAi silencing of *ZmCCaMK* in maize protoplasts did not block the ABA-induced increase in the production of NO (Fig. 6). Similarly, the mutant of *OsDMI3*, a homologous gene of *ZmCCaMK* in rice, also did not affect the ABA-induced increase in the production of NO within 4 h of ABA treatment (Fig. 7). These results provide conclusive evidence that NO is required for the ABA-induced up-regulation in the expression and the activity of ZmCCaMK, and ZmCCaMK does not mediate the ABA-induced NO production in maize leaves.

ROS have also been demonstrated to be important signal transduction molecules (Miller *et al*., 2008, 2010; Mittler *et al*., 2011). In ABA signalling, ROS play an important role in the regulation of stomatal closure, stress survival, and growth processes (Neill *et al*., 2008; Mittler *et al*., 2011). It has been well established that H_2O_2 induces NO synthesis and accumulation in ABA signalling, and the ABA–H_2O_2–NO cascade is involved in ABA-induced stomatal closure (Bright *et al*., 2006; Neill *et al*., 2008) and antioxidant defence (Zhang *et al*., 2007). However, it is not clear whether the ABA–H_2O_2–NO cascade is involved in the ABA-induced activation of ZmCCaMK in ABA signalling. In this study, H_2O_2 treatment obviously induced increases in the activity of ZmCCaMK and gene expression of *ZmCCaMK* (Fig. 8A, B). Pre-treatments with H_2O_2 scavengers or inhibitor significantly blocked the ABA-induced activation of ZmCCaMK and the gene expression of *ZmCCaMK* (Figs 5E, 8C). These results suggest that ABA-induced H_2O_2 production is required for the ABA-induced activation of ZmCCaMK. A previous study

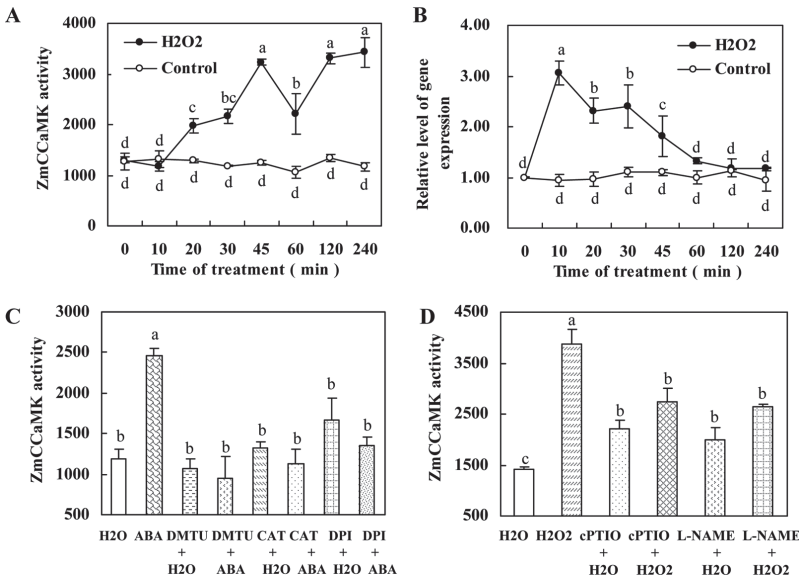

Fig. 8. H_2O_2 is involved in the ABA-induced activation of ZmCCaMK and the activation is mediated by NO. (A) Time course of H_2O_2-induced ZmCCaMK activation. The detached plants were treated with 10 mM H_2O_2 for various times as indicated. Protein extracts from control or H_2O_2-treated leaves were immunoprecipitated with ZmCCaMK antibody and then subjected to an in-gel kinase assay. (B) Time course of H_2O_2-induced gene expression of *ZmCCaMK*. The detached plants were treated as described in A. Relative expression levels of the *ZmCCaMK* gene, analysed by qRT-PCR, are normalized to *β-actin* transcript levels. (C) Effects of H_2O_2 scavengers or an inhibitor on ABA-activated ZmCCaMK. The detached plants were treated as follows: distilled water (control); 100 μM ABA; 10 mM DMTU+H_2O; 10 mM DMTU+100 μM ABA; 200 U of CAT+H_2O; 200 U of CAT+100 μM ABA; 100 μM DPI+H_2O; 100 μM DPI+100 μM ABA. The detached plants were pre-treated with scavengers or inhibitor for 4 h then exposed to 100 μM ABA treatment for 30 min. (D) Effects of an NO scavenger or inhibitor on H_2O_2-activated ZmCCaMK. The detached plants were treated as follows: distilled water (control); 10 mM H_2O_2; 200 μM cPTIO+H_2O; 200 μM cPTIO+10 mM H_2O_2; 200 μM L-NAME+H_2O; 200 μM L-NAME+10 mM H_2O_2. The detached plants were pre-treated with scavengers or inhibitors for 4 h then exposed to 10 mM H_2O_2 treatment for 45 min. Values are means ±SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

showed that exogenous H_2O_2 and ABA-induced H_2O_2 could increase the accumulation of NO in leaves of maize plants (Zhang *et al*., 2007). Furthermore, in the present study, pre-treatments with an NO scavenger and inhibitor inhibited the H_2O_2-induced increase in the activity of ZmCCaMK (Fig. 8D). All these data suggest that the H_2O_2-NO pathway is involved in ABA-induced activation of ZmCCaMK.

In conclusion, the present data indicate that ZmCCaMK is required for ABA-induced antioxidant defence in maize leaves. ABA-induced NO production mediated by H_2O_2 activates ZmCCaMK, thus resulting in the up-regulation of the expression and the activities of antioxidant enzymes in ABA signalling.

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