RESEARCH PAPER

ZmMPK5 is required for the NADPH oxidase-mediated self-propagation of apoplastic $H_2O_2$ in brassinosteroid-induced antioxidant defence in leaves of maize

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Abstract

Brassinosteroids (BRs) have been shown to induce hydrogen peroxide ($H_2O_2$) accumulation, and BR-induced $H_2O_2$ up-regulates antioxidant defence systems in plants. However, the mechanisms by which BR-induced $H_2O_2$ regulates antioxidant defence systems in plants remain to be determined. In the present study, the role of ZmMPK5, a mitogen-activated protein kinase, in BR-induced antioxidant defence and the relationship between the activation of ZmMPK5 and $H_2O_2$ production in BR signalling were investigated in leaves of maize ($Zea mays$) plants. BR treatment activated ZmMPK5, induced apoplastic and chloroplastic $H_2O_2$ accumulation, and enhanced the total activities of antioxidant enzymes. Such enhancements were blocked by pre-treatment with mitogen-activated protein kinase kinase (MAPKK) inhibitors and $H_2O_2$ inhibitors or scavengers. Pre-treatment with MAPKK inhibitors substantially arrested the BR-induced apoplastic $H_2O_2$ production after 6 h of BR treatment, but did not affect the levels of apoplastic $H_2O_2$ within 1 h of BR treatment. BR-induced gene expression of NADPH oxidase was also blocked by pre-treatment with MAPKK inhibitors and an apoplastic $H_2O_2$ inhibitor or scavenger after 120 min of BR treatment, but was not affected within 30 min of BR treatment. These results suggest that the BR-induced initial apoplastic $H_2O_2$ production activates ZmMPK5, which is involved in self-propagation of apoplastic $H_2O_2$ via regulation of NADPH oxidase gene expression in BR-induced antioxidant defence systems.

Key words: Antioxidant defence system, brassinosteroid, hydrogen peroxide, MAPK cascade, NADPH oxidase, maize ($Zea mays$).

Introduction

Brassinosteroids (BRs) are a group of naturally occurring steroidal plant hormones that regulate plant growth and development (Li et al., 1996; Li and Chory, 1999; Li et al., 2009; Ren et al., 2009; Tanaka et al., 2009). They are also shown to ameliorate various biotic and abiotic stress effects (Mazorra et al., 2002; Ozdemir et al., 2004; Bajguz and Hayat, 2009; Liu et al., 2009; Xia et al., 2009). Although much effort have been made to recommend this phytohormone as a plant growth regulator for widespread utilization in agricultural production, the mechanisms by which BR influences plant growth and development, and stress tolerance are still poorly understood.

Plant responses to various types of stresses are associated with generation of reactive oxygen species (ROS) (Mittler, 2002; Apel and Hirt, 2004; Breusegem et al., 2008). There are many potential sources of ROS in plant cells, including chloroplasts, mitochondria, peroxisomes, plasma membrane NADPH oxidases, cell wall peroxidases, apoplastic oxalate
oxidases, and amine oxidases (Mittler, 2002; Neill et al., 2002; Foyer and Noctor, 2003; Apel and Hirt, 2004; Bartoli et al., 2004; Hu et al., 2006). Genetic evidence shows that ROS generated by NADPH oxidase play important roles in plant defence response, abiotic stress, and hormonal response (Torres et al., 2002; Yoshioka et al., 2003; Torres and Dangl, 2005; Kwak et al., 2006; Torres, 2010). The activity of NADPH oxidase can be regulated by Ca$^{2+}$, calcium-dependent protein kinase (CDPK), and Rac GTPase (Sagi and Fluhr, 2006; Kobayashi et al., 2007; Wong et al., 2007; Ogasawara et al., 2008). In plants, a biphasic ROS accumulation response to pathogens (Torres et al., 2006; Yamamizo et al., 2007) and abscisic acid (ABA) (Razem and Hill, 2007) has been reported. NADPH oxidase is involved in the H$_2$O$_2$ bursts (Yoshioka et al., 2001; Yamamizo et al., 2007; Lin et al., 2009). Although it has been shown that BR causes the generation of ROS resulting from enhanced NADPH oxidase activity in cucumber (Xia et al., 2009), the mechanisms by which BR-induced ROS production up-regulates antioxidant defence have yet to be determined.

The mitogen-activated protein kinase (MAPK) cascade is one of the major pathways by which extracellular stimuli are transduced into intracellular responses in all eukaryotic cells (Tena et al., 2001; Nakagami et al., 2005; Pitzschke and Hirt, 2006). MAPK and immediate upstream activators, MAPK kinase (MAPKK) and MAPKK kinase (MAPKKK), constitute a functionally interlinked MAPK
activated MAPK can phosphorylate a variety of substrates including transcription factors, other protein kinases, and cytoskeleton-associated proteins (Nakagami et al., 2005; Pitzschke and Hirt, 2006). It has been shown that MAPKs are involved in plant signal transduction in response to various stimuli (Tena et al., 2001; Mittler, 2002; Mittler et al., 2004; Nakagami et al., 2005; Pitzschke and Hirt, 2006; Zhang et al., 2007). Recent studies showed that BR could transiently activate MAPK within 15–30 min in rice (Sharma et al., 2001), and induce MAPK1 and MAPK3 gene expression in cucumber (Xia et al., 2009), suggesting that MAPK may be involved in BR signalling. A previous study showed that a 46 kDa MAPK is involved in ABA-induced antioxidant defence and acts downstream of ROS production (Zhang et al., 2006), and the ABA-activated 46 kDa MAPK has been identified to be maize ZmMPK5 (Ding et al., 2009). However, it is not clear whether ZmMPK5 is involved in the BR-induced antioxidant defence and, if so, what the relationship is between ZmMPK5 activation and H₂O₂ production in BR signalling.

In this study, the role of ZmMPK5 in BR-induced antioxidant defence and the relationship between BR, ZmMPK5, and H₂O₂ production in BR signalling were investigated. ZmMPK5 is generally activated by BR treatment. Several MAPKK inhibitors and ROS manipulators were used to assess the possible links between ZmMPK5 activation, H₂O₂ production, and the antioxidant defence system in BR signalling. It was found that the BR-induced initial apoplastic H₂O₂ production activates ZmMPK5, which is involved in the self-propagation of apoplastic H₂O₂ via the regulation of NADPH oxidase gene expression in the BR-enhanced antioxidant defence systems.

### Materials and methods

#### Plant material and treatments

Seeds of maize (Zea mays L. cv Nongda 108; from Nanjing Agricultural University, China) were sown in trays of sand in a light chamber at a temperature of 22 °C (night) and 28 °C (day), photosynthetic active radiation (PAR) of 200 μmol m⁻² s⁻¹, and a photoperiod of 14 h light/10 h dark, and watered daily. When the second leaves were fully expanded, they were collected and used for all 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 in aluminium foil containing 10 nM BR solution for 4, 8, or 24 h at 25 °C, with a continuous light intensity of 200 μmol m⁻² s⁻¹. To study the effects of various inhibitors or scavengers, the detached plants were pre-treated with 100 μM 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), 10 μM 1,4-diamino-2,3-dicyano-1,4-bis (α-aminophenylmercapt) butadiene (U0126), 100 μM diphenylene iodonium (DPI), 200 U of catalase (CAT), 1 mM 3-(3,4-dichlorophenyl)-1,1-dimethyleurea (DCMU), or distilled water for 4 h, and then exposed to 10 nM BR treatment for 1 h. The plants treated with distilled water under the same conditions during the whole period served as controls. Experiments were repeated at least five times with similar results.

#### Protein extraction and in-gel kinase activity assay

Protein was extracted from leaves with an extraction buffer (100 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), pH 7.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 5 mM ethylene glycol-bis(β-aminoethyl ether)-N,N',N',N''-tetraacetic acid (EGTA), 10 mM diethiothreitol (DTT), 10 mM Na₃VO₄, 10 mM NaF, 1 mM phenylmethylsulphonyl fluoride (PMSF), 5 μg ml⁻¹ leupeptin, 5 μg ml⁻¹ aprotonin, 50 mM β-glycerophosphate) using the method of Zhang and Klessig (1997) with minor modifications. After centrifugation at 15 000 g for 30 min at 4 °C, the supernatants were transferred into clean tubes and immediately frozen with liquid N₂, and stored at −80 °C. Protein content was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as standard.

In-gel kinase activity assays were performed using the method described by Zhang and Klessig (1997). Extracts containing 20 μg of protein were electrophoresed on 10% SDS–polyacrylamide gels embedded with 0.25 mg ml⁻¹ of myelin basic protein (MBP) in the separating gel as a kinase substrate. After electrophoresis, SDS gels were removed by washing the gel with washing buffer (25 mM TRIS, pH 7.5, 0.5 mM DTT, 0.1 mM Na₃VO₄, 5 mM NaF, 0.5 mg ml⁻¹ BSA, and 0.1% Triton X-100) three times for 30 min each at room temperature. The kinases were allowed to renature in 25 mM TRIS, pH 7.5, 1 mM DTT, 0.1 mM Na₃VO₄, and 5 mM NaF at 4 °C overnight with three changes of buffer. The gel was then incubated at room temperature in 30 ml of reaction buffer (25 mM TRIS, pH 7.5, 2 mM EGTA, 12 mM MgCl₂, 1 mM DTT, and 0.1 mM Na₃VO₄) with 200 nM ATP plus 50 μCi of [γ-³²P]ATP (3000 Ci mm⁻¹) for 60 min. The reaction was stopped by transferring the gel into 5% (w/v) trichloroacetic acid (TCA) and 0.1 M sodium pyrophosphate. The unincorporated [γ-³²P]ATP was removed by washing with the same solution for at least 6 h with five changes. The gel was dried and exposed to Kodak XAR-5 film (Rochester, NY, USA). Pre-stained size markers (Bio-Rad, Hercules, CA, USA) were used to calculate the size of the kinases.

#### Antibody production and immunoprecipitation kinase activity assay

The peptides for ZmMPK5-C (EEQMKDLIYYEALAFNPDYQ) corresponding to the C-terminus of ZmMPK5 were synthesized as
described in Berberich et al. (1999) and conjugated to the keyhole limpet haemocyanin carrier. The ZmMPK5 polyclonal antibody was raised in rabbits and purified by affinity chromatography. The specificity of the antibody for ZmMPK5 was proven earlier by Berberich et al. (1999). Protein extract (100 µg) was incubated with anti-ZmMPK5 antibody (diluted 1:10 000, v/v) in an immunoprecipitation buffer as described previously (Zhang et al., 2006). Kinase activity in the immunocomplex was determined by an in-gel kinase assay as described above.

Isolation of total RNA and qRT-PCR analysis

Total RNA was isolated from leaves by using an RNaseasy Plant mini kit (Qiagen, Valencia, CA, USA) according to the instructions supplied by the manufacturer. A 2 µg aliquot of RNA was reverse transcribed to cDNA with a SuperScriptIII RTS First-Strand cDNA Synthesis Kit (Invitrogen, Carlsbad, CA, USA). Transcript levels of each gene were measured by qRT-PCR using an iCycler (Bio-Rad, Hercules, CA, USA) with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA). To standardize the results, the amplification of Zmactin was also determined and used as the internal standard. The data were normalized to the amplification of a maize actin gene. For each sample, the mean value from three qRT-PCRs was adapted to calculate the transcript abundance, and the mean values were then plotted with their SE. The primers that were used in qRT-PCR were: ZmMPK5 (GenBank accession no. AB016802) (forward TCTGCTCGGCGGTCAACT; reverse AAGGCGTTGGC-GATCTTCTT); ZmrbohA (GenBank accession no. DQ855284) (forward CACACGTGACCTGCGACTTC; reverse CCCCAAGGTGGCCATGA); ZmrbohB (GenBank accession no. EU807966) (forward GGCCAGTACTTCGGAGACA; reverse ATTACACCTGTGACCTTCCA); ZmrbohC (GenBank accession no. DQ897930) (forward TTCTCTTGCTGTATGCCCG; reverse CTTCGATATTCGGCAGCCA); ZmrbohD (GenBank accession no. EF364442) (forward CCGTGCTGCAGACGCCTT; reverse GCCGATCCTGGCATGCCTGAAA); and Zmactin (GenBank accession no. EU952376) (forward GTCGACATCTGATTGAGTTG).

Antioxidant enzyme assays

Frozen leaf segments (0.5 g) were homogenized in 10 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

Fig. 4. Cytochemical localization of brassinosteroid (BR)-induced hydrogen peroxide (H₂O₂) accumulation in mesophyll cells of maize leaves with CeCl₃ staining and transmission electron microscopy. (A) H₂O₂ accumulation in apoplasts. (B) H₂O₂ accumulation in chloroplasts. (C) H₂O₂ accumulation in mitochondria and peroxisomes. The detached plants were treated with 10 nM BR for various times as indicated. All experiments were repeated at least three times with similar results. Arrows indicate CeCl₃ precipitates. C, chloroplast; CW, cell wall; M, mitochondrion; N, nucleus; P, peroxisome; V, vacuole; IS, intercellular space. Bar = 1 µm.
the case of ascorbate peroxidase (APX) assay. The homogenate was centrifuged at 15 000 \( \times g \) for 20 min at 4°C and the supernatant was immediately used for the following antioxidant enzyme assays. The total activities of antioxidant enzymes were determined as described previously (Jiang and Zhang, 2001). Total superoxide dismutase (SOD) (EC 1.15.1.1) activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. One unit of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of nitroblue tetrazolium as monitored at 560 nm. Total CAT (EC 1.11.1.6) activity was assayed by measuring the rate of decomposition of \( \text{H}_2\text{O}_2 \) at 240 nm. Total APX (EC 1.11.1.11) activity was measured by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized. Total glutathione reductase (GR) (EC 1.6.4.2) activity was measured by following the change in \( A_{340} \) as oxidized glutathione-dependent oxidation of NADPH.

**Histochemical detection of \( \text{H}_2\text{O}_2 \)**

\( \text{H}_2\text{O}_2 \) was visually detected in the leaves of plants by using 3,3-diaminobenzidine (DAB) as substrate (Fryer et al., 2002). Briefly, plants were excised at the base of stems with a razor blade and supplied through the cut stems with a 1 mg ml\(^{-1}\) solution of DAB, pH 3.8, for 8 h under light at 25°C, and then exposed to various treatments. After these treatments, the second leaves were decolorized by immersion of leaves in boiling ethanol (96%) for 10 min. This treatment decolorized the leaves except for the deep brown polymerization product produced by the reaction of DAB with \( \text{H}_2\text{O}_2 \). After cooling, the leaves were extracted at room temperature with fresh ethanol, and photographed.

**Cytochemical detection of \( \text{H}_2\text{O}_2 \)**

\( \text{H}_2\text{O}_2 \) was visualized at the subcellular level using CeCl\(_3\) for localization (Bestwick et al., 1997). Electron-dense CeCl\(_3\) deposits
are formed in the presence of H2O2 and are visible by transmission electron microscopy. Tissue pieces (1–2 mm2) were excised from the treated and untreated leaves and incubated in freshly prepared 5 mM CeCl3 in 50 mM 3-(N-morpholino) propanesulphonic acid (MOPS) at pH 7.2 for 1 h. The leaf sections were then fixed in 1.25% (v/v) glutaraldehyde and 1.25% (v/v) paraformaldehyde in 50 mM sodium cacodylate buffer, pH 7.2, for 1 h. After fixation, tissues were washed twice for 10 min in the same buffer and post-fixed for 45 min in 1% (v/v) osmium tetroxide, and then dehydrated in a graded ethanol series (30–100%; v/v) and embedded in Eponaraldite (Agar Aids, Bishop’s Storford, UK). After 12 h in pure resin, followed by a change to fresh resin for 4 h, the samples were polymerized at 60°C for 48 h. Blocks were sectioned (70–90 nm) on a Reichert-Ultracut E microtome (Reichert-Jung, Leica, Bensheim, Germany) and mounted on uncoated copper grids (300 mesh). Sections were examined using a transmission electron microscope at an accelerating voltage of 75 kV.

**Results**

**ZmMPK5 is involved in BR-induced antioxidant defence systems**

To determine whether MAPK is involved in BR signalling, protein extracts from BR-treated and untreated leaves were prepared and subjected to in-gel kinase assay, using MBP as a substrate. A rapid activation of a 46 kDa MBP kinase was detected in samples treated with BR (Fig. 1A). The MBP kinase activity rapidly reached its highest level at 15 min and was maintained at a high level for 30 min, and then decreased after 1 h of BR treatment. A previous study showed that the ABA-activated a 46 kDa MBP kinase is ZmMPK5 in leaves of maize plants (Ding et al., 2009). To investigate whether the 46 kDa MBP kinase activated by BR is ZmMPK5, an antibody against specific peptides (EEQMKDLIYQEALAFNPYDQ) corresponding to the C-terminus of ZmMPK5 was raised and an immunoprecipitation kinase assay was carried out. As shown in Fig. 1B, the immunoprecipitated ZmMPK5 activity was correlated with the MBP kinase activity in the protein extracts, suggesting that the 46 kDa MBP kinase induced by BR is ZmMPK5. However, the expression analysis of ZmMPK5, using qRT-PCR, showed that treatment with BR only slightly induced the expression of ZmMPK5 (Fig. 1C).

To determine whether the activation of ZmMPK5 contributes to BR-induced antioxidant defence systems, the effects of PD98059 and U0126, two widely used specific inhibitors of MAPK (Favata et al., 1998; Romeis et al., 1999; Lu et al., 2002; Samuel and Ellis, 2002), on the activities of BR-induced antioxidant enzymes, such as APX, SOD, GR, and CAT, were examined. The results showed...
that pre-treatment with PD98059 and U0126 significantly blocked the increases in the total activities of APX, SOD, GR, and CAT induced by BR treatment in leaves of maize plants (Fig. 2B), and almost completely inhibited the activation of ZmMPK5 induced by BR (Fig. 2A). These results suggest that BR-activated ZmMPK5 is involved in BR-induced antioxidant defence in leaves of maize plants.

**BR-induced H2O2 accumulation in the apoplast and chloroplast up-regulates the antioxidant defence systems**

To investigate possible roles of H2O2 in BR-induced antioxidant defence, the accumulation of H2O2 in situ in leaves of maize plants exposed to BR was determined. The reaction of DAB with H2O2, producing a brown polymerization product in the presence of peroxidases (Fryer et al. 2002), was studied. This histochemical method has been widely used for the detection of H2O2 generated in plant tissues in response to biotic and abiotic stresses (Thordal-Christensen et al. 1997; Orozco-Cárdenas and Ryan 1999; Orozco-Cárdenas et al. 2001; Fryer et al. 2002; Pellinen et al. 2002; Zhang et al. 2006). The results showed that the DAB staining substantially increased in BR-treated maize leaves (Fig. 3). Further, the subcellular localization of H2O2 accumulation in leaves of maize plants exposed to BR treatment was also investigated, using a cytochemical technique with CeCl3, which reacts with H2O2 to produce electron-dense deposits of cerium perhydroxides (Bestwick et al. 1997). In BR-treated leaves of maize plants, CeCl3 deposits, indicative of the accumulation of H2O2, were observed predominantly in apoplasts and chloroplasts in the mesophyll cells (Fig. 4A, B). In apoplasts, 10 nM BR treatment rapidly induced H2O2 accumulation within 0.5 h of BR treatment, and kept increasing up to 6 h of BR treatment in leaves of maize plants (Fig. 4A). Similarly, in chloroplasts, H2O2 accumulation was visible as early as within 0.5 h BR treatment but was maximal at 1 h of BR treatment (Fig. 4B). No H2O2 accumulation was observed in mitochondria and peroxisomes in the BR-treated leaves of maize plants (Fig. 4C).

To determine the roles of apoplastic and chloroplastic H2O2 in BR-induced antioxidant defence systems, several apoplastic and chloroplastic H2O2 manipulators, such as DPI, an inhibitor of plasma membrane NADPH oxidase (Jiang and Zhang 2002, 2003), CAT, which cannot cross the plasma membrane, and DCMU, which has been shown to inhibit chloroplastic H2O2 production (Gao et al. 2008), were used. Pre-treatment with DPI, CAT, and DCMU significantly blocked the increase in the total activities of several antioxidant defence enzymes induced by BR treatment (data not shown), suggesting that the BR-induced apoplastic and chloroplastic H2O2 accumulation up-regulates the BR-induced antioxidant defence systems.
Apoplastic but not chloroplastic \( \text{H}_2\text{O}_2 \) accumulation is required for the activation of ZmMPK5, and apoplastic \( \text{H}_2\text{O}_2 \) accumulation is not related to chloroplastic \( \text{H}_2\text{O}_2 \) accumulation

A previous study showed that \( \text{H}_2\text{O}_2 \) can activate ZmMPK5 in leaves of maize plants (Lin et al., 2009). To investigate the effects of BR-induced \( \text{H}_2\text{O}_2 \) accumulation on the BR-induced activation of ZmMPK5 in leaves of maize plants, the detached plants were pre-treated with several ROS manipulators, such as DPI, CAT, and DCMU, and then exposed to BR treatment. The immunoprecipitation kinase assay showed that pre-treatment with DPI and CAT almost completely blocked the BR-induced ZmMPK5 activation, but pre-treatment with DCMU had very little effect on the ZmMPK5 activation induced by BR treatment in leaves of maize plants (Fig. 5A). These results suggest that it is apoplastic \( \text{H}_2\text{O}_2 \) but not chloroplastic \( \text{H}_2\text{O}_2 \) that induces the BR-induced activation of ZmMPK5 in BR-induced antioxidant defence.

In order to determine whether the apoplastic \( \text{H}_2\text{O}_2 \) accumulation is related to the chloroplastic \( \text{H}_2\text{O}_2 \) accumulation induced by BR treatment in leaves of maize plants, the detached plants were pre-treated with the apoplastic or chloroplastic \( \text{H}_2\text{O}_2 \) manipulators, and then exposed to BR treatment. Pre-treatment with DPI and CAT had little effect on the chloroplastic \( \text{H}_2\text{O}_2 \) accumulation in leaves of maize plants exposed to BR treatment (Fig. 5B). Similarly, pre-treatment with DCMU also had little effect on the BR-induced apoplastic \( \text{H}_2\text{O}_2 \) accumulation (Fig. 5C), although pre-treatment with DCMU almost completely blocked the chloroplastic \( \text{H}_2\text{O}_2 \) accumulation induced by BR in leaves of maize plants (Fig. 3). These results suggest that the apoplastic \( \text{H}_2\text{O}_2 \) accumulation is not related to the chloroplastic \( \text{H}_2\text{O}_2 \) accumulation induced by BR treatment in leaves of maize plants.

ZmMPK5 is required for NADPH oxidase-mediated enhancement of apoplastic \( \text{H}_2\text{O}_2 \)

To investigate whether BR-induced apoplastic and chloroplastic \( \text{H}_2\text{O}_2 \) accumulation is regulated by ZmMPK5, the effects of pre-treatment with the MAPKK inhibitors on the BR-induced \( \text{H}_2\text{O}_2 \) accumulation were examined. Pre-treatment with PD98059 and U0126 abolished the majority of apoplastic \( \text{H}_2\text{O}_2 \) accumulation, detectable with CeCl\(_3\) staining at 6 h after BR treatment (Fig. 6), but had little effect on the chloroplastic \( \text{H}_2\text{O}_2 \) accumulation during the whole duration of BR treatment (Fig. 7). These results suggest that the activation of ZmMPK5 mediates the BR-induced apoplastic \( \text{H}_2\text{O}_2 \) accumulation.

To investigate further the effects of the BR-activated ZmMPK5 on the BR-induced apoplastic \( \text{H}_2\text{O}_2 \) accumulation, the kinetics of inhibition by the MAPKK inhibitors in the BR-induced apoplastic \( \text{H}_2\text{O}_2 \) accumulation were examined. Pre-treatment with PD98059 and U0126 had very little effect on the BR-induced apoplastic \( \text{H}_2\text{O}_2 \) accumulation at 10 min, 30 min, and 1 h of BR treatment, but substantially blocked the apoplastic \( \text{H}_2\text{O}_2 \) accumulation at 6 h of BR treatment (Fig. 6). These results suggest that the BR-induced initial apoplastic \( \text{H}_2\text{O}_2 \) burst is independent of the BR-induced activation of ZmMPK5, but the BR-induced enhancement of apoplastic \( \text{H}_2\text{O}_2 \) requires the BR-induced ZmMPK5 activation in leaves of maize plants.

A previous study has suggested that NADPH oxidase might be an important source of apoplastic \( \text{H}_2\text{O}_2 \) accumulation in BR signalling (Xia et al., 2009). To elucidate further the relationship between the activation of ZmMPK5 and the accumulation of apoplastic \( \text{H}_2\text{O}_2 \) in BR signalling, NADPH oxidase gene expression in BR-treated leaves of maize plants was examined. BR treatment significantly elevated the expression of ZmrbohA–ZmrbohD in leaves of maize plants. The expression of ZmrbohA–ZmrbohD increased after 15 min of BR treatment, and peaked after 120 min or 240 min of BR treatment, then declined (Fig. 8). Pre-treatment with DPI and CAT dramatically abolished the expression of ZmrbohA–ZmrbohD at 120 min of BR treatment, but had little effect at 30 min of BR treatment in leaves of maize plants (Fig. 9A). These results suggest that BR-induced apoplastic \( \text{H}_2\text{O}_2 \) accumulation can induce NADPH oxidase gene expression to produce more \( \text{H}_2\text{O}_2 \) in BR signalling. Furthermore, pre-treatment with the
Fig. 9. Effects of pre-treatment with the apoplastic hydrogen peroxide (H₂O₂) inhibitor or scavenger and the mitogen-activated protein kinase kinase (MAPKK) inhibitors on the expression of ZmrbohA–ZmrbohD in leaves of maize plants exposed to brassinosteroid (BR) treatment. (A) Effects of pre-treatment with the apoplastic H₂O₂ inhibitor or scavenger. The detached plants were pre-treated with 100 nM diphenylene iodonium (DPI), 200 U of catalase (CAT), or distilled water for 4 h, and then exposed to 10 nM BR treatment for 30 min and 120 min, respectively. (B) Effects of pre-treatment with MAPKK inhibitors. The detached plants were pre-treated with 100 nM 2-(2-amino-3-methoxyphenyl)-4H-1-benzopyran-4-one (PD98059), 10 μM 1,4-diamino-2,3-dicyano-1,4-bis (o-aminophenylmercapto) butadiene (U0126), or distilled water for 4 h, and then exposed to 10 nM BR for 30 min and 120 min, respectively. The plants
MAPKK inhibitors showed similar effects to the H₂O₂ inhibitors on NADPH oxidase gene expression. Pre-treatment with PD98059 and U0126 did not affect the increase in the expression of Zmrboha-ZmrbohD at 30 min, but significantly blocked the increase in the expression of Zmrboha-ZmrbohD at 120 min of BR treatment (Fig. 9B), suggesting that BR-induced activation of ZmMPK5 up-regulates the gene expression of NADPH oxidase, which results in the accumulation of apoplastic H₂O₂ induced by BR in leaves of maize plants.

Discussion

It has been well documented that BR can induce antioxidant defence systems to enhance stress tolerance (Mazorra et al., 2002; Ozdemir et al., 2004; Bajiguz and Hayat, 2009; Liu et al., 2009). However, the mechanisms by which BR induces antioxidant defence have yet to be determined. It has been shown that MAPKs are involved in plant signal transduction in response to various stimuli (Tena et al., 2001; Mittler, 2002; Mittler et al., 2004; Nakagami et al., 2005; Pitzschke and Hirt, 2006). BR could transiently activate MAPK within 15–30 min of treatment in rice (Sharma et al., 2001) and induce MAPK7 and MAPK3 gene expression in cucumber (Xia et al., 2009). However, it is not clear whether a MAPK pathway is involved in BR-enhanced antioxidant defence in plants. In this study, the results showed that BR induced activation of a 46 kDa MBP kinase in leaves of maize plants (Fig. 1A) and the immunoprecipitation kinase assay suggested that the 46 kDa MBP kinase is ZmMPK5 (Fig. 1B). The inhibition of BR-induced ZmMPK5 substantially blocked the increases in the total activities of the antioxidant enzymes induced by BR treatment (Fig. 2A, B) in leaves of maize plants. These results clearly suggest that BR-activated ZmMPK5 is involved in the BR-induced antioxidant defence systems.

H₂O₂ is considered as a central signalling molecule in plant responses to biotic and abiotic stresses (Foyer et al., 1997; Neill et al., 2002). Increased production of H₂O₂ induced the expression of antioxidant genes, and enhanced the capacity of antioxidant defence systems in plants (Jiang and Zhang, 2001). In the present study, the results showed that BR induced an increase in H₂O₂ production in leaves of maize plants (Fig. 3) and the BR-induced H₂O₂ accumulation up-regulated the BR-induced antioxidant defence systems (data not shown). These data support the findings that BR can cause the increased generation of ROS and enhance the capacity of antioxidant defence systems in plants (Xia et al., 2009). However, Xia et al. (2009) reported that H₂O₂ only accumulated on the cell walls of mesophyll cells but not in chloroplasts in cucumber exposed to BR treatment; in contrast, the present results showed that BR could induce H₂O₂ accumulation not only in apoplasts but also in chloroplasts in leaves of maize plants (Fig. 4A–C). The difference in the subcellular localization of H₂O₂ accumulation induced by BR between the study by Xia et al. (2009) and the present study may be related to the sampling time of BR treatment. The absence of H₂O₂ accumulation in chloroplasts at 6 h of BR treatment in cucumber could be due to the capacity of these organelles to remove ROS produced using their efficient antioxidant systems, as has been reported earlier (Orozco-Cárdenas et al., 2001; Romero-Puertas et al., 2004). In comparison with chloroplasts, the apoplast has relatively little antioxidant defence and, hence, H₂O₂ accumulates when H₂O₂ synthesis is increased (Neill et al., 2002, Pastori and Foyer, 2002). This might be the reason why only the apoplastic H₂O₂ accumulated with BR treatment in the study by Xia et al. (2009). It is also possible that different plant species may have different responses to BR.

For years H₂O₂ has been considered to be a mobile signal molecule (Mittler, 2002; Neill et al., 2002; Pastori and Foyer, 2002) and to play profound roles in mediating rapid, long-distance, cell–cell propagating signals in plants (Miller et al., 2009). Previous studies showed that H₂O₂ generated in chloroplasts, mitochondria, and peroxisomes may move into the cytosol (Neill et al., 2002, Shigeoka et al., 2002). However, a recent study showed that H₂O₂ generated in the apoplast could not diffuse freely into the cytosol and other subcellular compartments within the tested time frame (Hu et al., 2006). It is not clear whether the accumulation of apoplastic H₂O₂ induced by BR can move into chloroplasts. In the present study, pre-treatment with DPI and CAT, which almost completely blocked the apoplastic H₂O₂ accumulation, did not affect the chloroplastic H₂O₂ accumulation, and pre-treatment with DCMU, which almost completely blocked the chloroplastic H₂O₂ accumulation, also did not affect the apoplastic H₂O₂ accumulation induced by BR in mesophyll cells of maize leaves (Figs. 3, 5B, C). These results suggest that the apoplastic and the chloroplastic H₂O₂ accumulation induced by BR are not related to each other and may be regulated by different signalling pathways in leaves of maize plants.

In a recent study, it has been shown that the apoplastic H₂O₂ induced by BR is involved in the BR-induced increase in the expression of MAPK1 in cucumber (Xia et al., 2009). However, these authors did not investigate whether MAPK activation also affects the H₂O₂ accumulation. In this study, the results showed that pre-treatment with the apoplastic H₂O₂ inhibitor or scavenger, DPI or CAT, which almost completely inhibited the BR-induced apoplastic H₂O₂ accumulation (Fig. 5B), significantly blocked treated with distilled water under the same conditions during the whole period served as controls. Relative expression levels of Zmrboha–ZmrbohD genes, analysed by qRT-PCR, are normalized to Zmactin transcript levels. Values are means ± SE of three different experiments. Means denoted by the same letter did not significantly differ at P <0.05 according to Duncan’s multiple range test.
the BR-induced ZmMPK5 activation (Fig. 5A). However, pre-treatment with DCMU, which decreased the BR-induced chloroplastic H$_2$O$_2$ accumulation to the base level (Fig. 5C), had little effect on BR-induced ZmMPK5 activation (Fig. 5A). A previous study showed that exogenous H$_2$O$_2$ induced ZmMPK5 activation in leaves of maize (Lin et al., 2009). Moreover, pre-treatment with two specific MAPKK inhibitors significantly blocked the exogenous H$_2$O$_2$-induced antioxidant defence in leaves of maize plants (Zhang et al., 2006). Taken together, the data suggest that BR-induced apoplastic H$_2$O$_2$ accumulation activates ZmMPK5, which in turn leads to the up-regulation of antioxidant defence systems in leaves of maize plants. On the other hand, the results also showed that pre-treatment with the specific MAPKK inhibitors PD98059 and U0126 abolished the majority of BR-induced apoplastic H$_2$O$_2$ accumulation after 6 h of BR treatment (Fig. 6), suggesting that the activation of ZmMPK5 is also involved in the BR-induced apoplastic H$_2$O$_2$ accumulation in leaves of maize plants. However, pre-treatment with PD98059 and U0126 did not affect the BR-induced apoplastic H$_2$O$_2$ accumulation within 1 h of BR treatment (Fig. 6). These results suggest that the initial apoplastic H$_2$O$_2$ accumulation does not require ZmMPK5 activation and the ZmMPK5-dependent increase in BR-induced H$_2$O$_2$ accumulation could be an amplification loop in BR signalling. Pre-treatment with PD98059 and U0126, during the 6 h treatment with BR, did not affect the chloroplastic H$_2$O$_2$ accumulation (Fig. 7), suggesting that the chloroplastic H$_2$O$_2$ accumulation induced by BR does not require the activation of ZmMPK5.

The possible existence of positive amplification loops in ROS signalling has recently been reported in plants in response to elicitor (Yoshioka et al., 2003) and oxidative stress (Rizhsky et al., 2004). In this study, four lines of evidence indicate that ZmMPK5 is required for NADPH oxidase-mediated self-propagation of apoplastic H$_2$O$_2$ in BR signalling. First, BR treatment significantly induced the expression of the NADPH oxidase gene (Fig. 8) and the inhibition of NADPH oxidase activity by pre-treatment with DPI, an inhibitor of NADPH oxidase, led to a significant decrease in the BR-induced apoplastic H$_2$O$_2$ accumulation (Fig. 5B), suggesting that BR-induced apoplastic H$_2$O$_2$ accumulation is caused by increased NADPH oxidase activity. Secondly, BR-induced endogenous apoplastic H$_2$O$_2$ accumulation (Fig. 9A) and exogenous H$_2$O$_2$ up-regulated the later stage of NADPH oxidase gene expression (Lin et al., 2009), suggesting that BR-induced H$_2$O$_2$ accumulation up-regulates NADPH oxidase gene expression, which further enhances the apoplastic H$_2$O$_2$ accumulation, forming a self-propagation loop of apoplastic H$_2$O$_2$ in BR signalling. Thirdly, the BR-induced apoplastic H$_2$O$_2$ accumulation is involved in the BR-induced ZmMPK5 activation (Fig. 5A), and the BR-induced ZmMPK5 activation is also required for the BR-induced apoplastic H$_2$O$_2$ accumulation only after 6 h but not within 1 h of BR treatment (Fig. 6), suggesting that the initial apoplastic H$_2$O$_2$ accumulation activates ZmMPK5, which enhances the apoplastic H$_2$O$_2$ accumulation, forming an amplification loop in BR signalling. Fourthly, the inhibition of BR-induced ZmMPK5 activation almost completely blocked the later but not the initial stage of BR-induced increase in NADPH oxidase gene expression (Fig. 9B). Taken together, the data strongly suggest that ZmMPK5 activated by BR-induced apoplastic H$_2$O$_2$ accumulation induces NADPH oxidase gene expression, which in turn enhances H$_2$O$_2$ accumulation in BR signalling in leaves of maize plants.

In conclusion, the results clearly suggest that ZmMPK5 is required for NADPH oxidase-mediated self-propagation of apoplastic H$_2$O$_2$ in BR-induced antioxidant defence systems in leaves of maize plants. Following perception of the BR signal, apoplastic H$_2$O$_2$ produced by NADPH oxidase may activate ZmMPK5, which in turn enhances apoplastic H$_2$O$_2$ accumulation via the induction of NADPH oxidase gene expression, forming a positive amplification loop. BR-induced H$_2$O$_2$ accumulation up-regulates the activities of antioxidant defence enzymes, which conversely controls ROS levels, resulting in the suppression of ROS. Further studies are needed to provide genetic evidence for the involvement of ZmMPK5 in BR-induced apoplastic H$_2$O$_2$ self-propagation and to elucidate how chloroplastic H$_2$O$_2$ up-regulates antioxidant defence systems in BR signalling.

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