MAP65-1a positively regulates H$_2$O$_2$ amplification and enhances brassinosteroid-induced antioxidant defence in maize

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Abstract

Brassinosteroid (BR)-induced antioxidant defence has been shown to enhance stress tolerance. In this study, the role of the maize 65 kDa microtubule-associated protein (MAP65), ZmMAP65-1a, in BR-induced antioxidant defence was investigated. Treatment with BR increased the expression of ZmMAP65-1a in maize (Zea mays) leaves and mesophyll protoplasts. Transient expression and RNA interference silencing of ZmMAP65-1a in mesophyll protoplasts further revealed that ZmMAP65-1a is required for the BR-induced increase in expression and activity of superoxide dismutase (SOD) and ascorbate peroxidase (APX). Both exogenous and BR-induced endogenous H$_2$O$_2$ increased the expression of ZmMAP65-1a. Conversely, transient expression of ZmMAP65-1a in maize mesophyll protoplasts enhanced BR-induced H$_2$O$_2$ accumulation, while transient silencing of ZmMAP65-1a blocked the BR-induced expression of NADPH oxidase genes and inhibited BR-induced H$_2$O$_2$ accumulation. Inhibiting the activity and gene expression of ZmMPK5 significantly prevented the BR-induced expression of ZmMAP65-1a. Likewise, transient expression of ZmMPK5 enhanced BR-induced activities of the antioxidant defence enzymes SOD and APX in a ZmMAP65-1a-dependent manner. ZmMPK5 directly interacted with ZmMAP65-1a in vitro. These results suggest that BR-induced antioxidant defence in maize operates through the interaction of ZmMPK5 with ZmMAP65-1a. Furthermore, ZmMAP65-1a functions in H$_2$O$_2$ self-propagation via regulation of the expression of NADPH oxidase genes in BR signalling.

Key words: Antioxidant defence, brassinosteroid, H$_2$O$_2$, NADPH oxidase, ZmMAP65-1a, ZmMPK5.

Introduction

Brassinosteroids (BRs) are a class of steroid hormones controlling various growth and developmental processes in plants, including cell division and expansion, photomorphogenesis, xylem differentiation, floral development, and seed germination (Clouse and Sasse, 1998; Bishop and Koncz, 2002; Bajguz, 2007; Choudhary et al., 2012). In addition, BRs have
also been demonstrated to regulate biotic and abiotic stress responses in plants (Kagale et al., 2007; Divi and Krishna, 2009; Xia et al., 2009; Zhang et al., 2011; Wang, 2012). Several studies have shown that exogenously applied BR enhances the tolerance to oxidative, Cu and Cr, and cold stress, and is accompanied by the accumulation of H$_2$O$_2$ and the enhancement of antioxidants enzymes, such as superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX) and glutathione reductase (Xia et al., 2009; Choudhary et al., 2010, 2011, 2012; Zhang et al., 2010; Cui et al., 2011), which scavenge excessive reactive oxygen species (ROS) in distinct organs (Foyer and Noctor, 2005; Tan et al., 2011). However, it remains largely unknown how BRs induce ROS production and upregulate antioxidant defence.

The plant cytoskeleton can be readily remodelled in response to a variety of intracellular and external stimuli. As an important component of the cytoskeleton, microtubules (MTs) have been well documented to be essential for intra- and extracellular signalling, and the regulation of the dynamic instability of MTs plays a critical role in MT function, such as in the plant’s ability to withstand salt and osmotic stress (Mathur and Chua, 2000; Nogales, 2000; Lü et al., 2007; Sedbrook and Kaloriti, 2008; Wang et al., 2011a). MT dynamic instability is precisely regulated by microtubule-associated proteins (MAPs) (Desai and Mitchell, 1997).

MAP65 is one of the most abundant plant MAPs (Jiang and Sonobe, 1993). The first members of the MAP65 family of proteins were isolated from tobacco BY2 cells as a group of 60–65 kDa proteins that co-purified with MTs (Jiang and Sonobe, 1993). Subsequently, MAP65 proteins were identified respectively in other plants. Arabidopsis has nine MAP65 proteins with predicted molecular masses between 54 and 80 kDa (Hussey et al., 2002). The rice genome encodes 11 members of the MAP65 family (Guo et al., 2009). These proteins have evolved to take on distinct tasks required for multifaceted cellular activities. MAP65 has been shown to be responsible for the bundling of cortical MTs during secondary cell-wall formation in xylogenesis and during the expansion of primary cell walls (Mao et al., 2006). In addition, many studies have shown that MAP65 proteins not only play critical roles for cell division and elongation, root growth, and leaf senescence (Keech et al., 2010; Lucas et al., 2011; Soares et al., 2011; Dhouokushe et al., 2012), but are also required for stabilizing MTs during low temperature and NaCl stress (Smertenko et al., 2004; Mao et al., 2005). In addition, MAP65 is essential for giant-cell development during root knot nematode infection (Caillaud et al., 2008). These findings show that MAP65 proteins are required both for developmental processes and for responses to biotic and abiotic stress. However, little is known about how MAP65 proteins function in response to stresses.

Recent studies have reported that cytoskeleton (i.e. actin filament) reconfiguration is sufficient to activate BR signalling (Lanza et al., 2012), BR treatment induced H$_2$O$_2$ production and enhanced the activities of antioxidant enzymes (Zhang et al., 2010), and disturbance of ROS homeostasis resulted in MT and atypical tubulin formation and aggregation of MAP65 (Livanos et al., 2012). These observations suggest that there might be a link between MAP65 and BR signalling. Here, this hypothesis was tested and our experimental results showed that maize MAP65, ZmMAP65-1a, interacts with ZmMPK5 and is required for BR-induced antioxidant defence.

### 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 growth chamber at a temperature of 22–28 °C, photosynthetic active radiation of 200 µmol m$^{-2}$ s$^{-1}$, and a photoperiod of 14/10h (day/night), and were watered daily. 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 aluminum foil containing 10 nM BR or 10 mM H$_2$O$_2$ 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 100 µM 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), 5 mM dimethylthiourea (DMTU), 100 µM diphenyle iodonium (DPI) or 200 U CAT for 4 h prior to treatment with 10 nM BR 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 treatment of the detached plants, the second leaves were sampled and immediately frozen in liquid N$_2$.

#### Isolation of total RNA and real-time quantitative reverse transcriptase-PCR (qRT-PCR) expression analysis

Total RNA was isolated from leaves or protoplasts using a RNAiso Plus kit (TaKaRa, Dalian, China) according to the instructions supplied by the manufacturer. DNase treatment was included in the isolation step using the RNase-free DNase (TaKaRa). Approximately 2 µg of total RNA was reverse transcribed using an oligo(dT)$_{18}$ primer and Moloney murine leukemia virus reverse transcriptase (TaKaRa). Transcript levels of several genes were measured by qRT-PCR using a DNA Engine Opticon 2 real-time PCR detection system (Bio-Rad, USA) with SYBR® Premix Ex Taq™ (TaKaRa) according to the manufacturer’s instructions. The cDNA was amplified by PCR using the following primers: ZmSOD4 (GenBank accession no. NM_001112234), forward 5′-TGGAGACACCAGAAAGATGA-3′ and reverse 5′-CCTCGTGTCCTACCCCTTCC-3′; ZmMAP65-1a (EU972930), forward 5′-TGAGAGCGACCAGGACATTG-3′ and reverse 5′-GAGGCTTTGTCACCTGT-3′; ZmMAP65-1b (EU972934), forward 5′-AAGAGGAGTTTACC-3′ and reverse 5′-TTCTCTTGCCTGTATGCCGC-3′; ZmMPK5 (AB016802), forward 5′-TCTGCTCGGCGGTCAACT-3′ and reverse 5′-AAGCCGTGCGGCCTTCTCTTT-3′; ZmRbohA (DQ855284), forward 5′-CACACGTGACCTGCGACTTC-3′ and reverse 5′-CCCAAGGGTGCCCATGA-3′; ZmRbohB (EU807966), forward 5′-GGCCAGATTTCCGTAAGACAC-3′ and reverse 5′-ATTACACCAGTGATGCTTCTCC-3′; ZmRbohC (DQ897930), forward 5′-TTCGCTGCGGACTGCC-3′ and reverse 5′-CCCAGGATGGGGATCA-3′; ZmRbohD (EF364442), forward 5′-CCGCGGTCAAGACGCTTCT-3′ and reverse 5′-CCTGATCCCGTATCCCTGAAA-3′; ZmACTIN (EU952376), forward 5′-GCCATCATGATCGTGATG-3′ and reverse 5′-GTCGACACTTCTAGTGGAGTTG-3′. To standardize the results, amplification of ZmACTIN was determined and used as the internal standard. The data were normalized to amplification of the internal standard. 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 standard error (SE).
Vector construction and in vitro transcription of ZmMAP65-1a dsRNA
The full-length cDNA fragment was amplified with the addition of a *Kpn*I site and then inserted in frame with yellow fluorescent protein (YFP) into the pXZP008 vector driven by the cauliflower mosaic virus 35S promoter. The primers used for the PCR amplification were: 5′-GGTACCAGATGCCTAGCTCATATATGGC-3′ and 5′-GGTACCCTGTGTTGTGCCTGAAACCGGATC-3′.

DNA templates were produced by PCR using primers containing the T7 promoter sequence (5′-TATAACGACTCACTATAGGC-3′) on both 5′ and 3′ ends. The primers used to amplify DNA of ZmMAP65-1a were: 5′-TATAACGACTCACTATAGGCCTCACAATACGACTCACTATAGGGC-3′ and 5′-TATAACGACTCACTATAGGCCTCTTGTCCTTGTATTGTCCCT-3′. The PCR conditions were as follows: denaturing step at 94 °C for 5 min, followed by 35 cycles of 94 °C for 15 s, 67 °C for 15 s, and 72 °C for 15 s, with a final extension at 72 °C for 10 min. After PCR product clean-up, the DNA templates were used for *in vitro* synthesis of dsRNA using a Ribomax Express kit (Promega, USA). The dsRNA was purified by phenol/chloroform/isopropanol extraction, dissolved in RNase-free water, and quantified by UV spectrophotometry.

Protoplast preparation and transfection with DNA constructs or dsRNAs
Maize plants were grown at 25 °C under dark conditions. When the second leaves were fully expanded, protoplasts were isolated from leaves used for transfection with DNA constructs or dsRNAs 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 × 10⁵ cells mL⁻¹) were transfected with 100 μg of 35S-ZmMAP65-1a–YFP fusion construct (using the pXZP008 vector as a control), 35S-ZmMAP65-1a–mCherry (using the pXZP008 vector as a control), or 150 μg of dsRNAs (using H₂O as a control) using a polyethylene glycol/calcium-mediated method. The transfected protoplasts were incubated in incubation solution overnight in the dark at 25 °C, and the protoplasts were then collected and used for further analysis.

Antioxidant enzyme assays
Protoplasts were homogenized in 0.7 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 the APX assay. The homogenate was centrifuged at 15 000 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 previously described (Zhang et al., 2006). For transfection, 1 mL of maize protoplasts (usually 5 × 10⁵ cells mL⁻¹) were transfected with 100 μg of 35S-ZmMAP65-1a–YFP fusion construct (using the pXZP008 vector as a control), 35S-ZmMAP65-1a–mCherry (using the pXZP008 vector as a control), or 150 μg of dsRNAs (using H₂O as a control) using a polyethylene glycol/calcium-mediated method. The transfected protoplasts were incubated in incubation solution overnight in the dark at 25 °C, and the protoplasts were then collected and used for further analysis.

*H₂O₂ detection by confocal laser-scanning microscopy*
H₂O₂ production in protoplasts was monitored using the H₂O₂-sensitive fluorescent probe H2DCF-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 fluorescence intensity.

Expression and purification of recombinant ZmMAP65-1a
Full-length ZmMAP65-1a was cloned into *EcoRI*/XhoI-digested pGEX4T-1 vector to generate a glutathione S-transferase (GST)–ZmMAP65-1a construct. Fusion proteins were expressed in *Escherichia coli* strain BL21(DE3) according to the manufacturer’s instructions. Protein expression was induced with isopropyl β-D-1-thiogalactopyranoside for 4 h in Luria–Bertani liquid medium. The bacteria were collected at 5000 g for 15 min, resuspended in PBS (pH 8.0), sonicated, and centrifuged at 12 000 g for 10 min. The resulting supernatant was used for protein purification with GST-affinity agarose (Genscript, Nanjing, China) according to the manufacturer’s instructions. Purified proteins were used for immunoblotting and an immunoprecipitation kinase activity assay.

Immunoblotting
Purified proteins were subjected to SDS-PAGE. Immunoblotting was performed as described by Ma et al. (2012). Anti-GST antibody (Abmart, Shanghai, China) was used to detect the GST–ZmMAP65-1a protein.

Antibody production and immunoprecipitation kinase activity assay
The peptide ZmMPK5-C (EEQLKDIYQEAFLNPDYQ) corresponding to the C terminus of ZmMPK5 was synthesized as described by Berberich et al. (1999) and conjugated to keyhole limpet haemocyanin. ZmMPK5 polyclonal antibody was raised in rabbits and purified by affinity chromatography.

Protein was extracted from maize leaves as described previously (Zhang et al., 2006). Protein content was determined according to the method of Bradford (1976) with BSA as a standard. For the immunoprecipitation kinase assay, protein extract (200 μg) was incubated with anti-ZmMPK5 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 GST–ZmMAP65-1a fusion protein as the substrate. The immunocomplex and GST–ZmMAP65-1a were incubated in reaction buffer (25 mM Tris/HCl, pH 7.5, 5 mM MgCl₂, 1 mM DTT, 1 mM EGTA) with 200 nM ATP and 1 μCi of [γ-³²P]ATP (3000 Ci mmol⁻¹) for 30 min. An equal volume of SDS sample buffer was added to stop the reaction. The reaction mix was boiled for 5 min and resolved by SDS-PAGE. Unincorporated [γ-³²P]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 3MM paper and exposed to Kodak XAR-5 film. Pre-stained size markers (Bio-Rad) were used to calculate the apparent molecular mass.

Binuclear fluorescence complementation (BiFC) analysis of the interaction between ZmMPK5 and ZmMAP65-1a
Onion epidermal cells were co-transfected with the expression vectors YFP³–ZmMPK5 and YFP–ZmMAP65-1a by DNA particle bombardment according to the manufacturer’s instructions (Biologic PDS-1000/He Particle Delivery System; Bio-Rad). Co-expression of YFP³ and YFP³, YFP³–ZmMPK5 and YFP³–ZmMAP65-1a, and YFP³ and YFP³–ZmMAP65-1a in onion epidermal cells were used as negative controls. YFP fluorescence was detected after 12–16 h of transfection.

Results
BR upregulates the expression of ZmMAP65-1a
To investigate whether ZmMAP65-1a participates in BR signalling, total RNA was isolated from maize leaves or protoplasts treated with 10 nM BR, and the expression of ZmMAP65-1a was analysed by real-time qRT-PCR analysis. As shown in Fig. 1A, 10 nM BR treatment induced a rapid
increase in the expression of ZmMAP65-1a in maize leaves. The expression of ZmMPP65-1a was upregulated after 20 min, peaked after 30 min, and then decreased after 45 min of BR treatment in the leaves of maize plants (Fig. 1A). In protoplasts, the expression of ZmMAP65-1a was even more rapidly upregulated by the treatment of 10 nM BR (Fig. 1B).

ZmMAP65-1a is required for BR-induced antioxidant defence

Previous studies have showed that BR can induce antioxidant defence to enhance stress tolerance (Xia et al., 2009; Zhang et al., 2010) and that MAP65 plays a role in the regulation of MTs against stresses (Livanos et al., 2012; Zhang et al., 2012). Therefore, we wanted to investigate whether ZmMAP65-1a is involved in BR-induced antioxidant defence. To elucidate the relationship between ZmMAP65-1a and antioxidant defence, we used transient gene expression and transient RNA interference (RNAi) in maize mesophyll protoplasts. This approach has been proven to be efficient for functional analysis of plant genes (Sheen, 2001; Zhai et al., 2009; Kim and Somers, 2010).

The results showed that transient expression of ZmMAP65-1a in mesophyll protoplasts caused significant increases in the expression of ZmMAP65-1a and the antioxidant genes SOD4 and APX2 (Fig. 1C) when compared with that in protoplasts transfected with empty vector. Transient silencing of ZmMAP65-1a resulted in a marked reduction in the expression of ZmMAP65-1a and substantially decreased the gene expression of SOD4 and APX2 compared with the control (Fig. 1D). These results suggested that ZmMAP65-1a can induce the expression of antioxidant genes.

To investigate further the role of ZmMAP65-1a in BR-induced antioxidant defence, the activities of SOD and APX were determined. In agreement with the effects on gene expression, transient expression of ZmMAP65-1a in

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**Fig. 1.** BR induces the expression of ZmMAP65-1a, and ZmMAP65-1a is required for the expression of SOD4 and APX2 in maize. (A, B) Expression analysis of ZmMAP65-1a in maize leaves (A) or mesophyll protoplasts (B) exposed to BR treatment. The maize seedlings or protoplasts were treated with 10 nM BR for various times as indicated. Seedlings treated with distilled water and protoplasts treated with culture medium under the same conditions served as controls. Relative expression level of ZmMAP65-1a was analysed by real-time qRT-PCR. (C) Expression analysis of ZmMAP65-1a, SOD4 and APX2 in protoplasts transiently expressing ZmMAP65-1a. Protoplasts isolated from maize leaves were transfected with constructs carrying 35S–ZmMAP65-1a–YFP (ZmMAP65-1a). Protoplasts were transfected with empty vector as a control. The relative expression levels of ZmMAP65-1a, SOD4 and APX2 were analysed by real-time qRT-PCR. (D) Expression analysis of ZmMAP65-1a, SOD4 and APX2 in protoplasts transiently silencing ZmMAP65-1a. Protoplasts were transfected with dsRNA against ZmMAP65-1a (RNAi) or distilled water as a control. The relative expression levels of ZmMAP65-1a, SOD4 and APX2 were analysed by real-time qRT-PCR. 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.
protoplasts also caused significant increases in the total activities of SOD and APX (Fig. 2C), and transient silencing of ZmMAP65-1a resulted in significant decreases in the activities of SOD and APX compared with the control (Fig. 3B). Furthermore, treatment with 10nM BR significantly induced the expression of ZmMAP65-1a (Figs 2B and 3A) and the activities of SOD and APX in control protoplasts (Figs 2A, C and 3B), which were further promoted in protoplasts transiently expressing ZmMAP65-1a (Fig. 2B, C). However, in protoplasts transiently silencing ZmMAP65-1a, BR treatment was no longer able to induce SOD and APX (Fig. 3). Taken together, these data demonstrated unequivocally that ZmMAP65-1a is required for BR-induced antioxidant defence in maize mesophyll protoplasts.

Altered H$_2$O$_2$ levels affect the BR-induced expression of ZmMAP65-1a

As described above, BR induced the expression of ZmMAP65-1a. In addition, BR also induced H$_2$O$_2$...
production, which subsequently enhanced antioxidant defence (Zhang et al., 2010). In order to reveal the relationship between ZmMAP65-1a and H₂O₂, the effect of treatment with 10mM H₂O₂ on the transcript level of ZmMAP65-1a in maize leaves. Treatment with 10mM H₂O₂ induced a biphasic response, in which the first peak occurred after 20 min of treatment, and the second peak appeared within 60 min of treatment, in the expression of ZmMAP65-1a. Moreover, H₂O₂ treatment also rapidly induced the expression of ZmMAP65-1a in maize mesophyll protoplasts (Fig. 4B).

In order to study the possible role of endogenous H₂O₂ induced by BR in the expression of ZmMAP65-1a, H₂O₂ scavengers, such as DMTU and CAT, were used. Furthermore, as NADPH oxidase is a key generator of H₂O₂ in plant cells (Xia et al. 2009), we also used DPI, an inhibitor of NADPH oxidase. Pre-treatments with DMTU, DPI, and CAT substantially reduced the BR-induced increase in the expression of ZmMAP65-1a in leaves, whereas the pre-treatments had little effect on the expression of ZmMAP65-1a in the absence of BR treatment (Fig. 4C).

These data suggested that BR-induced H₂O₂ production is required for the BR-induced expression of ZmMAP65-1a in maize leaves.

ZmMAP65-1a affects BR-induced H₂O₂ production

Crosstalk between H₂O₂ and other components, such as mitogen-activated protein kinase (MAPK), nitric oxide (NO) and calcium (Ca²⁺), has been demonstrated to operate in abscisic acid- or BR-induced antioxidant defence (Zhang et al., 2006, 2010; Sang et al., 2008). Therefore, the effect of BR-induced ZmMAP65-1a on BR-induced H₂O₂ production was investigated by determining the level of H₂O₂ production in response to BR in protoplasts transiently expressing or silencing ZmMAP65-1a. The results showed that transient expression of ZmMAP65-1a substantially increased H₂O₂ accumulation (Supplementary Fig. S1 at JXB online), while transient silencing of ZmMAP65-1a significantly reduced H₂O₂ accumulation (Fig. 5). BR treatment could further enhance the H₂O₂ level only in protoplasts transiently expressing ZmMAP65-1a (Supplementary Fig. S1) but not in protoplasts where ZmMAP65-1a was silenced (Fig. 5). These results suggested that ZmMAP65-1a is also required for BR-induced H₂O₂ production and that there is a crosstalk between H₂O₂ and ZmMAP65-1a in BR signalling.

ZmMAP65-1a affects the expression of NADPH oxidase genes

NADPH oxidase is an important source of apoplastic H₂O₂ accumulation (Xia et al., 2009) and mediates H₂O₂...
self-propagation in BR signalling (Zhang et al., 2010). To elucidate further the role of ZmMAP65-1a in the regulation of H$_2$O$_2$ accumulation in BR signalling, the expression of NADPH oxidase genes (rboh) was analysed. BR treatment induced significant increases in the expression of ZmrbohA, ZmrbohB, ZmrbohC, and ZmrbohD in protoplasts (Supplementary Fig. S2 at JXB online). RNAi silencing of ZmMAP65-1a in protoplasts reduced the expression of ZmrbohA, ZmrbohC, and ZmrbohD, and it could no longer be upregulated by BR treatment. In contrast, the BR-induced expression of ZmrbohB was only slightly decreased by ZmMAP65-1a silencing (Fig. 6A). These data suggested that ZmMAP65-1a is involved in the regulation of the gene expression of NADPH oxidase in BR signalling. However, transient expression of ZmMAP65-1a in protoplasts had little if any effect on the expression of ZmrbohA–D in either BR-treated or untreated protoplasts (Fig. 6B).

ZmMPK5 is also required for the regulation of expression of NADPH oxidase gene in BR signalling in leaves of maize (Zhang et al., 2010). To determine whether there is a link between ZmMAP65-1a and ZmMPK5, the effect of ZmMPK5 on the expression of ZmMAP65-1a in BR signalling was tested. First, two inhibitors of MAPK kinase (MAPKK), PD98059 and U0126, which almost completely inhibit the activation of ZmMPK5 in response to BR (Zhang et al., 2010), were used. As shown in Fig. 7A, pre-treatment with PD98059 and U0126 strongly inhibited the BR-induced expression of ZmMAP65-1a in leaves but had no significant effect on the expression of ZmMAP65-1a in the absence of BR treatment. Next, the BR response was studied in protoplasts where ZmMPK5 was transiently silenced. Our results showed that BR treatment no longer caused increased...

Fig. 4. H$_2$O$_2$ is required for the BR-induced expression of ZmMAP65-1a in maize. (A, B) Expression analysis of ZmMAP65-1a in maize leaves (A) or mesophyll protoplasts (B) exposed to H$_2$O$_2$ treatment. The seedlings or protoplasts were treated with 10 mM H$_2$O$_2$ (A) or 1 mM H$_2$O$_2$ (B) for various times as indicated. Seedlings treated with distilled water and protoplasts treated with culture medium, under the same conditions during the whole period served as controls. The relative expression level of the ZmMAP65-1a gene was analysed by real-time qRT-PCR. (C) Effects of pre-treatments with ROS manipulators DMTU, DPI, and CAT on the expression of ZmMAP65-1a in response to BR treatment. The detached plants were pre-treated with 5 mM DMTU, 100 μM DPI, or 200 U CAT for 4 h, and then exposed to 10 nM BR treatment for 0.5 h. Plants treated with distilled water under the same conditions served as a control. After treatment, the relative expression level of ZmMAP65-1a gene was analysed by real-time qRT-PCR. 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 ZmMAP65-1a in protoplasts where ZmMPK5 was transiently silenced (Fig. 7B). These results suggested that BR-induced ZmMPK5 activation regulates ZmMAP65-1a at the transcriptional level.

To investigate further the link between ZmMAP65-1a and ZmMPK5 in BR-induced antioxidant defence, both ZmMPK5 and dsRNA of ZmMAP65-1a were transfected into mesophyll protoplasts. The results showed that transient expression of ZmMPK5 alone significantly increased the activities of the antioxidant defence enzymes SOD and APX in control mesophyll protoplasts, which were further enhanced by BR treatment. However, the activities of SOD and APX were only partly upregulated in mesophyll protoplasts transfected with both ZmMPK5 and dsRNA of ZmMAP65-1a (Fig. 8). These results suggested that the interaction between ZmMAP65-1a and ZmMPK5 functions in BR-induced antioxidant defence.

MAPks have been shown to phosphorylate MAPs and the phosphorylated MAPs participated in many cell processes (Komis et al., 2011). To study whether there is a direct interaction between ZmMAP65-1a and ZmMPK5, the in vivo interaction between ZmMAP65-1a and ZmMPK5 was analysed by BiFC. In this system, YFP is split into N-terminal (YFPN) and C-terminal (YFPC) halves, and fluorescence is observed when two proteins fused to each YFP half interact with each other. Our experimental results showed that strong YFP fluorescence could be observed when YFPN–ZmMPK5 and YFPC–ZmMAP65-1a
were co-expressed in onion epidermal cells (Fig. 9D). In contrast, no YFP signal was observed when no-fusion YFP<sup>C</sup> and no-fusion YFP<sup>N</sup>, YFP<sup>N</sup>-ZmMPK5 and no-fusion YFP<sup>C</sup>, and no-fusion YFP<sup>N</sup> and YFP<sup>C</sup>-ZmMAP65-1a, as the controls, were co-transformed. These results are consistent with an interaction between ZmMAP65-1a and ZmMPK5 in vivo.

Fig. 6. ZmMAP65-1a affects the expression of NADPH oxidase genes in protoplasts. (A) Expression analysis of ZmrbohA–D in protoplasts transiently silencing ZmMAP65-1a. Protoplasts were transfected with dsRNA against ZmMAP65-1a (RNAi) or with distilled water as a control. Protoplasts were treated with 10nM BR for 10 min, and the relative expression levels of ZmrbohA–D were analysed by real-time qRT-PCR. (B) Expression analysis of ZmrbohA–D in protoplasts transiently expressing ZmMAP65-1a. Protoplasts were transfected with constructs carrying 35S–ZmMAP65-1a–YFP (ZmMAP65-1a), and control protoplasts were transfected with empty vector. Protoplasts were treated with 10nM BR for 10 min and the relative expression levels of ZmrbohA–D were analysed by real-time qRT-PCR. 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.
To investigate whether ZmMPK5 can phosphorylate ZmMAP65-1a in vitro, immunocomplex kinase assays were performed using recombinant GST–ZmMAP65-1a as substrate. The GST–ZmMAP65-1a fusion protein with an apparent molecular mass of ~90 kDa was expressed in E. coli and affinity purified (Fig. 9A). The same band was also detected with an anti-GST antibody (Fig. 9B). An antibody was raised against a peptide sequence in the C terminus of ZmMPK5 and used for the in vitro immunocomplex kinase assays. As shown in Fig. 9C, a strong phosphorylation band was detected, suggesting that ZmMPK5 directly phosphorylates ZmMAP65-1a in maize.

Discussion

MT organization and dynamics play a vital role in enhancing plant tolerance to abiotic stresses, such as drought, salt, and low temperature (Wang et al., 2011). MAP65 is one of the most abundant plant MT-associated proteins, and tightly regulates MT organization (Amos and Schlieper, 2005). In the presence of MAP65-1, microtubule bundles are more resistant to cold treatment in Arabidopsis (Mao et al., 2005). A recent study also revealed an important role of MAP65-1 in salt stress tolerance. Knockout of MAP65-1 results in microtubule depolymerization under salt stress and overexpression of MAP65-1 increases salt tolerance in Arabidopsis cells (Zhang et al., 2012). These results indicate an essential role of MAP65 in regulating MT organization in plant tolerance to abiotic stress.

Komorisono et al. (2005) reported a dwarf rice mutant with altered MT organization and upregulated gibberellin biosynthesis, suggesting a link between MTs and gibberellin signalling. More recent studies showed that the BR-induced antioxidant defence system enhanced plant tolerance to abiotic stress (Xia et al., 2009; Zhang et al., 2010). However, there is no report about a connection between MTs and BR signalling so far. Here, we discovered a novel function of MAP65 in BR-enhanced antioxidant defence. Exogenously applied BR upregulated the expression of ZmMAP65-1a in leaves (Fig. 1A) and mesophyll protoplasts of maize (Fig. 1B), suggesting that ZmMAP65-1a is very likely to participate in BR signalling. Furthermore, transient expression of ZmMAP65-1a in protoplasts significantly increased expression of the major antioxidant genes SOD4 and APX2 and the activities of the corresponding enzymes, which were further enhanced by BR treatment (Figs 1C and 2C). Conversely, compared with the control, RNAi silencing of ZmMAP65-1a in mesophyll protoplasts substantially decreased the expression of SOD4 and APX2 and the activities of SOD and APX, which could no longer be induced by BR treatment (Figs 1D and 3). These results indicate the crucial importance of ZmMAP65-1a in BR-induced antioxidant defence in leaves of maize plants.

H2O2 accumulation induced by various stimuli can induce antioxidant defence to scavenge abundant H2O2, protecting plants from damage (Miller et al., 2010). Here, we described the complex relationship between ZmMAP65-1a and H2O2 in BR signalling. Exogenous H2O2 treatment induced the expression of ZmMAP65-1a in both leaves and protoplasts (Fig. 4A, B). Scavenging or inhibiting the endogenous H2O2 level produced by BR inhibited the BR-induced increase in ZmMAP65-1a expression in maize leaves (Fig. 4C). These results suggest that H2O2 is required for the expression of ZmMAP65-1a in BR signalling. Previous work found that H2O2 treatment resulted in MT depolymerization in human
Recently, Livanos et al. (2012) demonstrated that ROS signalling pathways are implicated in MT organization in plant cells, and they found that plant MTs are sensitive to both ROS overproduction and low ROS levels. Disturbance of ROS homeostasis induced atypical tubulin formation, which was a more stable structure than MTs, while MAP65-1, acting as a ‘tubulin-associated protein’, may underlie the bundling and/or the assembly of the atypical tubulin polymers. Taking these results together, it is possible that BR-produced H$_2$O$_2$ induces ZmMAP65-1a expression, subsequently regulating MT reorganization, leading to enhanced disturbance of ROS homeostasis tolerance.

Our previous studies showed that there is crosstalk between H$_2$O$_2$ and NO, Ca$^{2+}$/calmodulin and MAPK (Sang et al., 2008; Zhang et al., 2010). In the present study, crosstalk was detected between H$_2$O$_2$ and ZmMAP65-1a in BR signalling. BR-induced H$_2$O$_2$ accumulation also was regulated by transient expression and transient silencing of ZmMAP65-1a in maize mesophyll protoplasts (Fig. 5 and Supplementary Fig. S1). Thus, H$_2$O$_2$ appears to operate both upstream and downstream of ZmMAP65-1a, most likely indicating a positive feedback by ZmMAP65-1a on H$_2$O$_2$ production. A suggested model for the interaction between components described in this report is shown in Fig. 10.

NADPH oxidase is a main source of BR-induced apoplastic H$_2$O$_2$ accumulation (Zhang et al., 2010). In the present study, BR treatment rapidly induced the expression of four NADPH oxidase genes in protoplasts (Supplementary Fig. S2). Transient RNAi silencing of ZmMAP65-1a in the protoplasts arrested the BR-induced upregulation in the expression of NADPH oxidase genes (Fig. 6A). Considering the crosstalk between BR-induced H$_2$O$_2$ accumulation and BR-induced ZmMAP65-1a expression described above, we propose that ZmMAP65-1a induced by BR-produced H$_2$O$_2$ increases NADPH oxidase gene expression, which in turn enhances H$_2$O$_2$ accumulation, forming a H$_2$O$_2$ self-propagation loop, in BR signalling in leaves of maize plants.
Surprisingly, transient expression of \textit{ZmMAP65-1a} did not enhance the expression of the NADPH oxidase genes, except for a slight increase for \textit{ZmrbohC} (Fig. 6B). One possible explanation for this disparity is that BR treatment induces not only the expression of the \textit{ZmMAP65-1a} gene but also modification of the \textit{ZmMAP65-1a} protein, and modification of \textit{ZmMAP65-1a} is essential for the regulation of NADPH oxidase gene expression in BR signalling. A similar pattern of post-translational regulation has been reported for the \textit{Arabidopsis} and tobacco homologues \textit{AtMAP65-1}, \textit{AtMAP65-3}, and \textit{NtMAP65-1a} (Smertenko \textit{et al.}, 2004; Sasabe \textit{et al.}, 2006; Caillaud \textit{et al.}, 2008). Besides that, in addition to NADPH oxidases, cell-wall peroxidase and polyamine oxidase are also sources of apoplastic H$_2$O$_2$ production (Mittler, 2002). Moreover, the chloroplast is another location of BR-induced H$_2$O$_2$ production (Zhang \textit{et al.}, 2002).
BR-induced \( \text{H}_2\text{O}_2 \) production from these different sources is not synchronous with that from NADPH oxidase, as described by Lin et al. (2009). Therefore, transient expression of ZmMAP65-1a may induce \( \text{H}_2\text{O}_2 \) production from other sources and thereby cause the upregulation of antioxidant defence enzymes in BR signalling.

Recent studies demonstrated a link between BR and MAPK. Both BR signalling and MAPK regulated the transcription factor SPEECHLESS in stomatal development in Arabidopsis (Gudesblat et al., 2012). AtMKK4 and AtMKK5 acted downstream of BR signalling as targets of the BIN2 kinase (Khan et al., 2013). BR regulated stomatal development by activating the MAPK cascade (Kim et al., 2012). Inhibiting the expression (Nie et al., 2013) and activity (Zhang et al., 2010) of MAPK reduced \( \text{H}_2\text{O}_2 \) accumulation and the activities of antioxidant defence enzymes in BR signalling. Moreover, a recent study revealed that MAPK is involved in MT rearrangements, probably via MAP65-1 (Beck et al., 2011). Here, we connected the MAPK, MAP65, and BR signalling. Our experimental results showed that inhibiting the activity and expression of ZmMPK5 by pre-treatments with inhibitors or RNAi silencing blocked the BR-induced expression of ZmMAP65-1a (Fig. 7). Furthermore, transient expression of ZmMPK5 could increase the activities of SOD and APX, but this effect was partly blocked by transient silencing of ZmMAP65-1a (Fig. 8). These data indicate that ZmMAP65-1a and ZmMPK5 need to interact to assert their role in BR-induced antioxidant defence. Obviously, the interaction between ZmMAP65-1a and ZmMPK5 might modulate other process as well.

Previous studies have shown that MAPKs phosphorylate a variety of substrates including transcription factors, other protein kinases, and cytoskeleton-associated proteins in response to various stimuli (Nakagami et al., 2005; Pitzschke and Hirt, 2006). MAP65 phosphorylation by MAPK was found to affect its MT bundling activity during cytokinesis and interphase (Sasabe and Machida, 2006, 2012). An MAPK mutant showed prominent cytokinetic defects (Beck et al., 2011), suggesting an important role of MAPK phosphorylation in cytokinesis. Some studies have shown that MAP65-1 from Arabidopsis is phosphorylated by MPK4 and MPK6 (Smertenko et al., 2006; Beck et al., 2010), while MAP65-2 and MAP65-3 are phosphorylated by the heterologous protein in tobacco, NRK1 (Komis et al., 2011). However, there is no evidence as to whether MAP65 is also phosphorylated by MAPK in plant response to BR or stress. The present study not only revealed the in vivo protein interaction between ZmMPK5 and ZmMAP65-1a but also provided evidence of phosphorylation of ZmMAP65-1a by ZmMPK5 (Fig. 9). The phosphorylation of MAP65 by MAPK renders it incapable of MT bundling and enhances destabilization and turnover of MTs at the phragmoplast equator in mitosis (Sasabe et al., 2006; Smertenko et al., 2006). During salt stress, both depolymerization and reorganization of MTs are believed to play a vital role in the stress response (Wang et al., 2011b). Under oxidative stress, ROS overproduction induces disruption of MTs and the formation of atypical tubulin polymers, which is a common adaptation and protection against stress (Livanos et al., 2012). Thus, these studies and our results suggest that BR induces \( \text{H}_2\text{O}_2 \) accumulation, which then results in depolymerization of MTs and atypical tubulin polymer formation, which is promoted by ZmMPK5 phosphorylation of ZmMAP65-1a (Fig. 10). Besides phosphorylation of MAP65-1, Zhang et al. (2012) found that phosphatidic acid binds to MAP65-1, increasing its activity in enhancing MT polymerization and bundling, thereby enhancing salt stress tolerance. Therefore, there should be multiple ways to regulate MAP65 activity in modulating the depolymerization and reorganization of MTs during stresses.

In summary, our experimental results indicate that ZmMAP65-1a is required for BR-induced antioxidant defence. In this process, BR-induced expression of ZmMAP65-1a is mediated by BR-induced \( \text{H}_2\text{O}_2 \) production. Conversely, the increase in the expression of ZmMAP65-1a amplifies \( \text{H}_2\text{O}_2 \) production via induction of NADPH oxidase genes, forming a positive \( \text{H}_2\text{O}_2 \) amplification loop. ZmMPK5 regulates ZmMAP65-1a gene expression and phosphorylation in BR signalling. Our results clearly suggest that ZmMAP65-1a is an important component in BR-induced antioxidant defence in maize. Whether there are other kinases or signal transduction pathways that regulate MAP65 should be addressed in future studies.

Supplementary data

Supplementary data are available at JXB online

Fig. S1. Transient expression of ZmMAP65-1a enhances BR-induced \( \text{H}_2\text{O}_2 \) production.

Fig. S2. Time course of changes in the expression of NADPH oxidase genes in response to BR treatment.

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quantitation of microgram quantities of protein utilizing the principle of Bradford MM.

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