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MAP65-1a positively regulates H_2O_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_2O_2 increased the expression of *ZmMAP65-1a*. Conversely, transient expression of *ZmMAP65-1a* in maize mesophyll protoplasts enhanced BR-induced H_2O_2 accumulation, while transient silencing of *ZmMAP65-1a* blocked the BR-induced expression of NADPH oxidase genes and inhibited BR-induced H_2O_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 vivo* and phosphorylated 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_2O_2 self-propagation via regulation of the expression of NADPH oxidase genes in BR signalling.

Key words: Antioxidant defence, brassinosteroid, H_2O_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_2O_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 organelles (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 Mitchison, 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; Dhonukshe *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_2O_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 14/10 h (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_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 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 diphenylene 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 an 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)$_{16}$ 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′-TGGAGC ACCAGAAGATGA-3′ and reverse 5′-CTCGTGTCCAC CCTTTCC-3′; *ZmAPX2* (EU969033), forward 5′-TGAGCGACC AGGACATTG-3′ and reverse 5′-GAGGGCTTTGTCACTT GGT-3′; *ZmMAP65-1a* (EU972149), forward 5′-AAGAGGA AAGTTGACC-3′ and reverse 5′-TGCTTGATTGTCCCTGT-3′; *ZmMPK5* (AB016802), forward 5′-TCTGCTCGGCGGTCAACT-3′ and reverse 5′-AAGGCGTTGGCGATCTTCTT-3′; *ZmrbohA* (DQ855284), forward 5′-CACACGTGACCTGCGACTTC-3′ and reverse 5′-CCCCAAGGTGGCCATGA-3′; *ZmrbohB* (EU807966), forward 5′-GGCCAGTACTTCGGTGAAACA-3′ and reverse 5′-ATTACACCAGTGATGCCTTCCA-3′; *ZmrbohC* (DQ897930), forward 5′-TTCTCTTGCCTGTATGCCGC-3′ and reverse 5′-CTTTCGTATTCCGCAGCCA-3′; *ZmrbohD* (EF364442), forward 5′-CCGGCTGCAGACGTTCTT-3′ and reverse 5′-CCTGATCCGTGATCTTCGAAA-3′; *ZmACTIN* (EU952376), forward 5′-GCCATCCATGATCGGTATGG-3′ and reverse 5′-GTCGCACTTCATGATGGAGTTG-3′. To standardize the results, amplification of *ZmACTIN* was determined and used as the internal standard. The data were normalized to amplification of the maize *ZmACTIN* 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 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′-GGTACCGATGGCCGGTGACATTACATGCGG-3′ and 5′-GGTACCCGTGGTGTGCTCGGAACCGGATC-3′.

DNA templates were produced by PCR using primers containing the T7 promoter sequence (5′-TAATACGACTCACTATAGGC-3′) on both 5′ and 3′ ends. The primers used to amplify DNA of *ZmMAP65-1a* were: 5′-TAATACGACTCACTATAGGCGCGTCT CAAACGGCACT-3′ and 5′-TAATACGACTCACTATAGGC TGTCTTTCTTGCTATCCTTCC-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 5×10^5 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 H$_2$O 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 000g 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). 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.

H$_2$O$_2$ detection by confocal laser-scanning microscopy

H$_2$O$_2$ production in protoplasts was monitored using the H$_2$O$_2$-sensitive fluorescent probe 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 *Eco*RI/*Xho*I-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 5000g for 15 min, resuspended in PBS (pH 8.0), sonicated, and centrifuged at 12 000g 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 (EEQMKDLIYQEALAFNPDYQ) 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 MgCl$_2$, 1 mM DTT, 1 mM EGTA) with 200 nM ATP and 1 µCi of [γ-^{32}P]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-^{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 3MM paper and exposed to Kodak XAR-5 film. Pre-stained size markers (Bio-Rad) were used to calculate the apparent molecular mass.

Bimolecular fluorescence complementation (BiFC) analysis of the interaction between ZmMPK5 and ZmMAP65-1a

Onion epidermal cells were co-transfected with the expression vectors YFP^N–*ZmMPK5* and YFP^C–*ZmMAP65-1a* by DNA particle bombardment according to the manufacturer's instructions (Biolistic PDS-1000/He Particle Delivery System; Bio-Rad). Co-expression of YFP^N and YFP^C, YFP^N–*ZmMPK5* and YFP^C, and YFP^N and YFP^C–*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
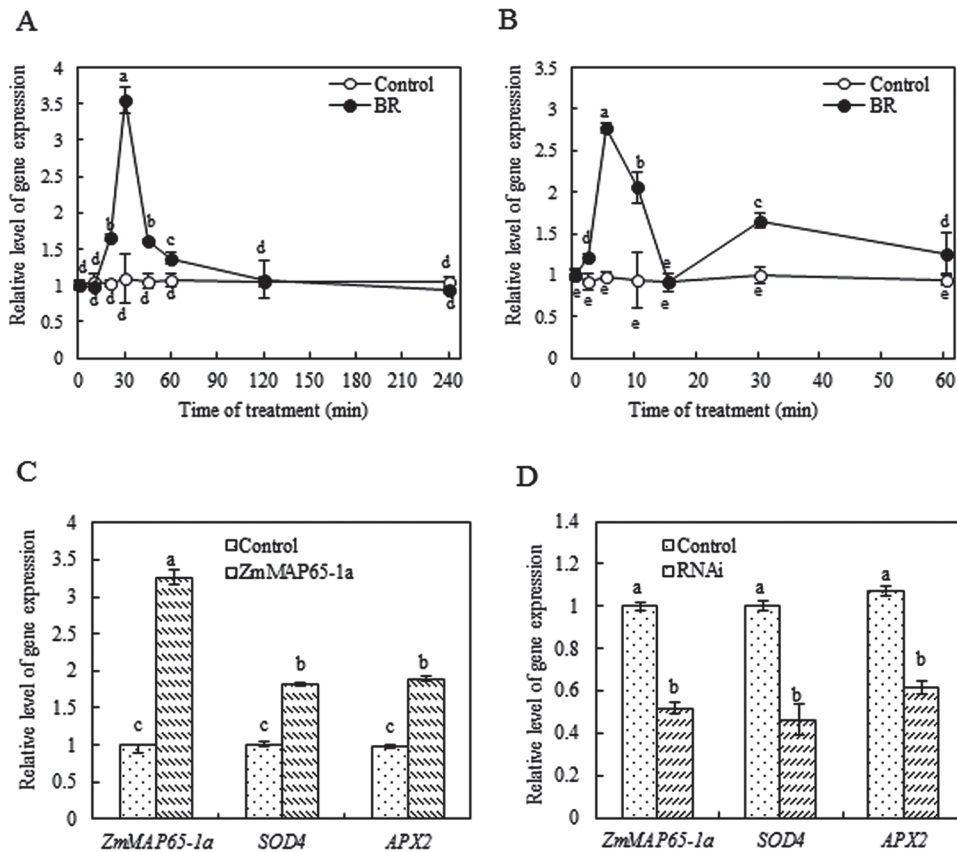

Fig. 1. BR induces the expression of *ZmMAP65-1a*, and ZmMAP65-1a is required for the expression of *SOD4* and *APX2* in maize. (A, B) Expression analysis of *ZmMAP65-1a* in maize leaves (A) or mesophyll protoplasts (B) exposed to BR treatment. The maize seedlings or protoplasts were treated with 10 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 10 nM 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$_2$
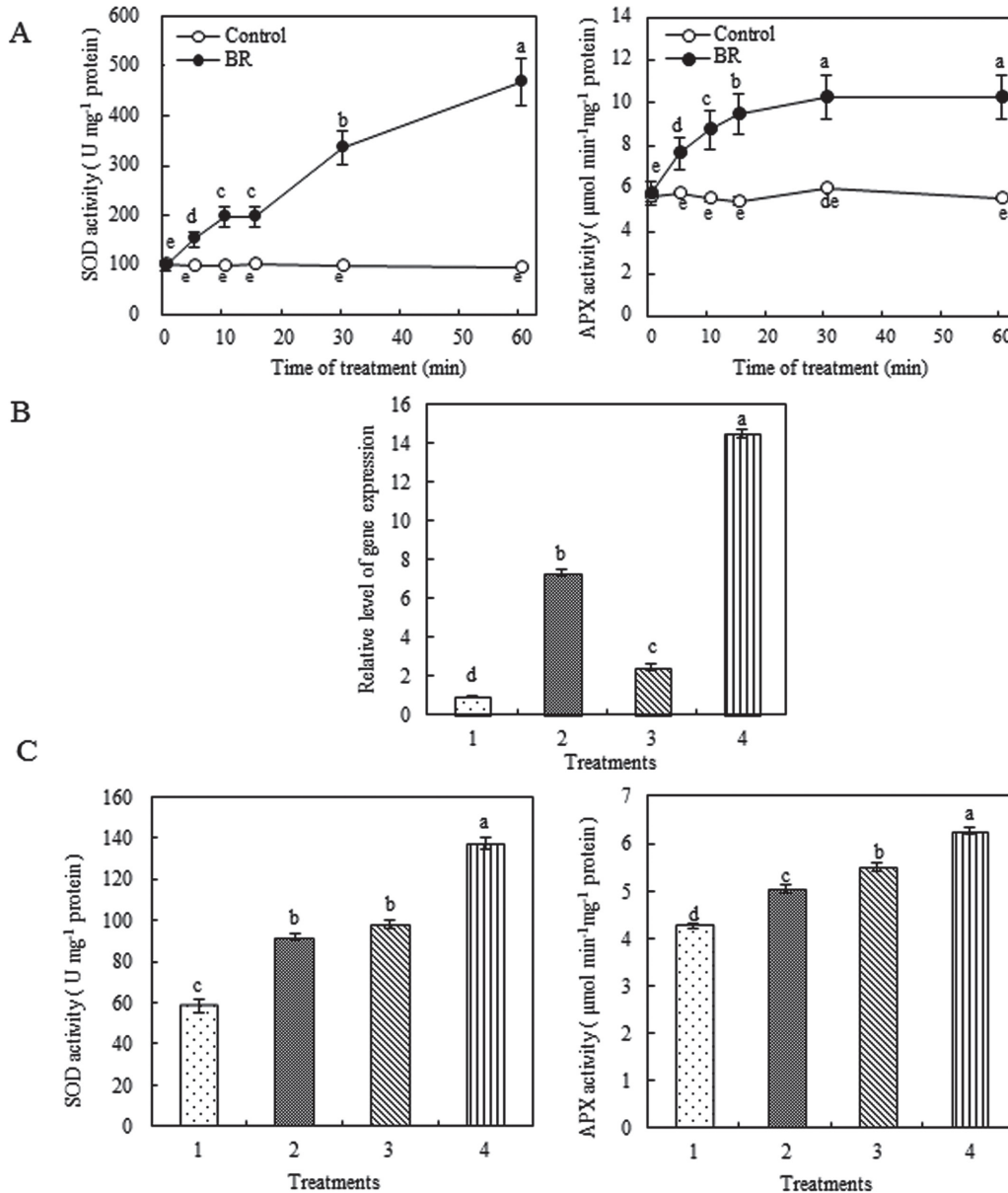

Fig. 2. Transient expression of *ZmMAP65-1a* upregulates the activities of SOD and APX in protoplasts. (A) Time course of changes in the activities of the antioxidant enzymes SOD and APX in the protoplasts of maize leaves. Protoplasts were treated with 10 nM BR for various times as indicated. Protoplasts treated with culture medium under the same conditions served as controls. (B) Expression analysis of *ZmMAP65-1a* in protoplasts. The protoplasts were treated as follows: 1, empty vector; 2, 35S–*ZmMAP65-1a*–YFP; 3, empty vector+BR; 4, 35S–*ZmMAP65-1a*–YFP+BR. The protoplasts were treated with 10 nM BR for 10 min, and the relative expression level of *ZmMAP65-1a* was analysed by real-time qRT-PCR. (C) Activities of SOD and APX in protoplasts transiently expressing *ZmMAP65-1a*. The protoplasts were treated as described in (B), and the activities of SOD and APX were measured. 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.
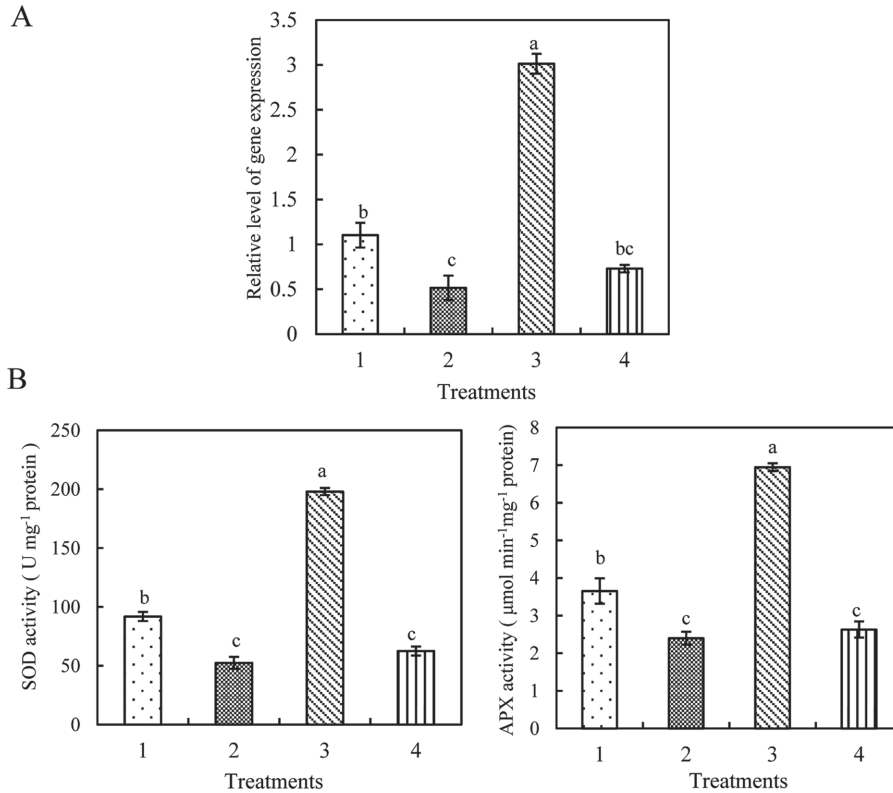
Fig. 3. Transient silencing of *ZmMAP65-1a* downregulates the activities of SOD and APX in protoplasts. (A) Expression analysis of *ZmMAP65-1a* in protoplasts. The protoplasts were treated as follows: 1, distilled water; 2, RNAi; 3, distilled water+BR; 4, RNAi+BR. The protoplasts were treated with 10 nM BR for 10 min, and the relative expression level of *ZmMAP65-1a* was analysed by real-time qRT-PCR. (B) Activities of SOD and APX in protoplasts transiently silencing *ZmMAP65-1a*. The protoplasts were treated as described in (A), and the activities of SOD and APX were measured. 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.

production, which subsequently enhanced antioxidant defence (Zhang *et al.*, 2010). In order to reveal the relationship between ZmMAP65-1a and H_2O_2, the effect of treatment with 10 mM H_2O_2 on the transcript level of *ZmMAP65-1a* was investigated. As shown in Fig. 4A, H_2O_2 treatment induced a significant increase in the expression of *ZmMAP65-1a* in maize leaves. Treatment with 10 mM H_2O_2 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, H_2O_2 treatment also rapidly induced the expression of *ZmMAP65-1a* in maize mesophyll protoplasts (Fig. 4B).

In order to study the possible role of endogenous H_2O_2 induced by BR in the expression of *ZmMAP65-1a*, H_2O_2 scavengers, such as DMTU and CAT, were used. Furthermore, as NADPH oxidase is a key generator of H_2O_2 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 H_2O_2 production is required for the BR-induced expression of *ZmMAP65-1a* in maize leaves.

ZmMAP65-1a affects BR-induced H_2O_2 production

Crosstalk between H_2O_2 and other components, such as mitogen-activated protein kinase (MAPK), nitric oxide (NO) and calcium (Ca^{2+}), 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 H_2O_2 production was investigated by determining the level of H_2O_2 production in response to BR in protoplasts transiently expressing or silencing *ZmMAP65-1a*. The results showed that transient expression of *ZmMAP65-1a* substantially increased H_2O_2 accumulation (Supplementary Fig. S1 at *JXB* online), while transient silencing of *ZmMAP65-1a* significantly reduced H_2O_2 accumulation (Fig. 5). BR treatment could further enhance the H_2O_2 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 H_2O_2 production and that there is a crosstalk between H_2O_2 and ZmMAP65-1a in BR signalling.

ZmMAP65-1a affects the expression of NADPH oxidase genes

NADPH oxidase is an important source of apoplastic H_2O_2 accumulation (Xia *et al.*, 2009) and mediates H_2O_2
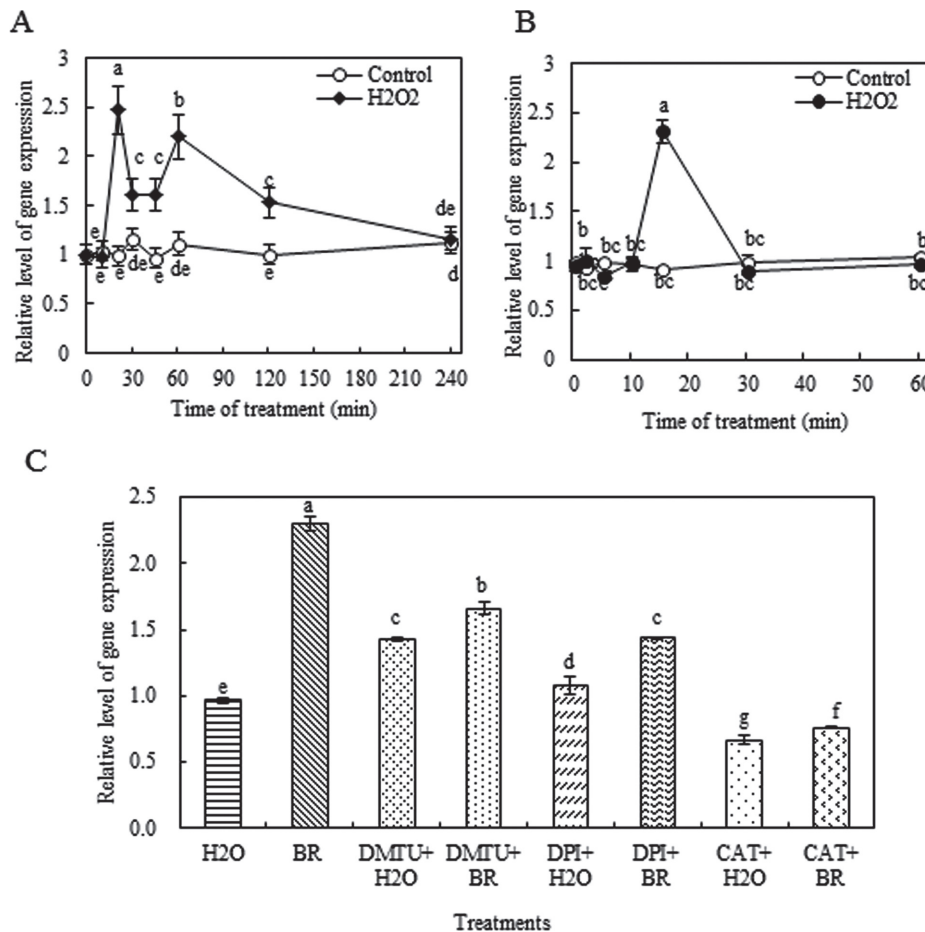
A

B

C

Fig. 4. H_2O_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_2O_2 treatment. The seedlings or protoplasts were treated with 10 mM H_2O_2 (A) or 1 mM H_2O_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 the *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.

self-propagation in BR signalling (Zhang *et al.*, 2010). To elucidate further the role of ZmMAP65-1a in the regulation of H_2O_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 interacting with ZmMAP65-1a *promotes BR-induced antioxidant defence*

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
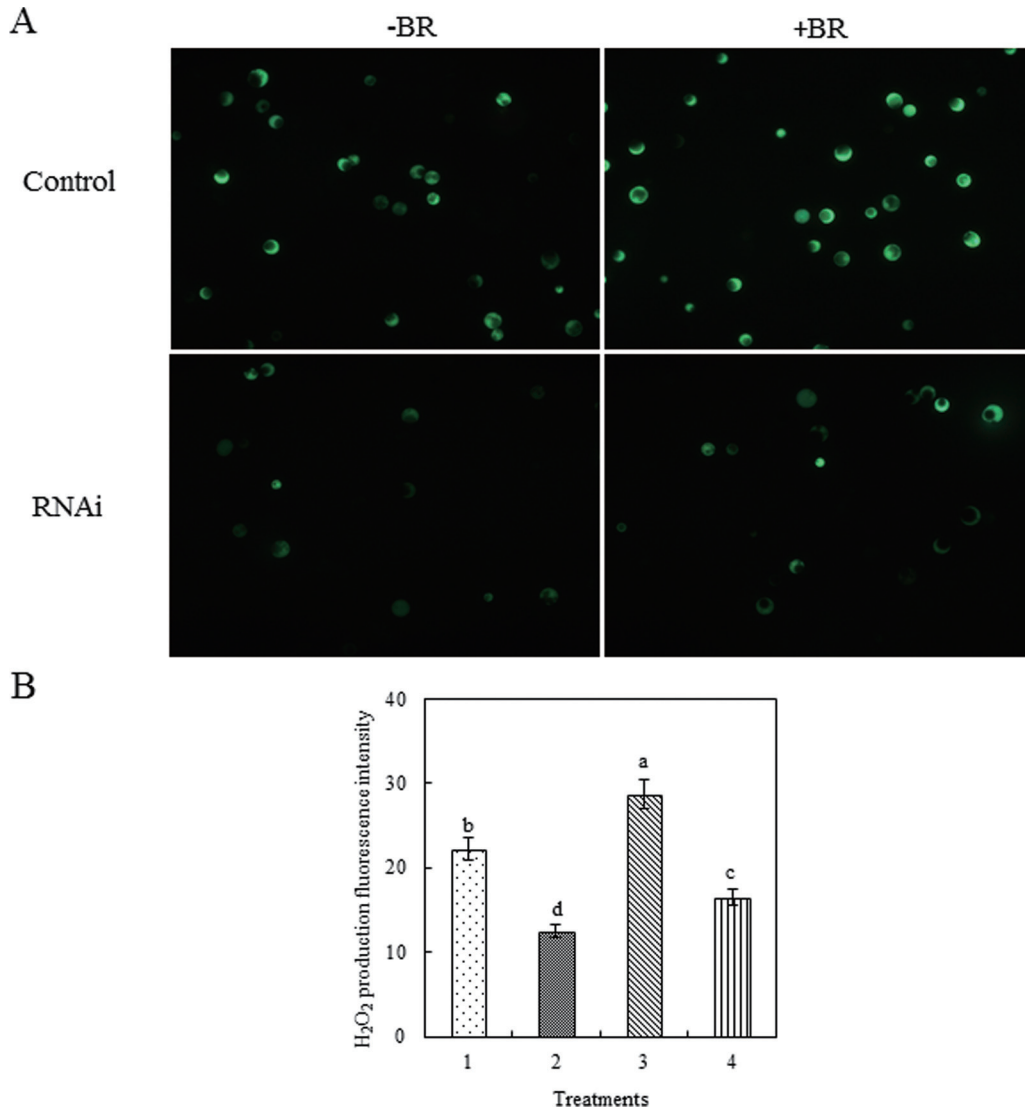
A

B

Fig. 5. Transient silencing of *ZmMAP65-1a* reduces BR-induced H_2O_2 production. (A) H_2O_2 fluorescence in protoplasts transiently silencing *ZmMAP65-1a*. Protoplasts transfected with dsRNA against *ZmMAP65-1a* (RNAi) or distilled water as a control were treated with 10 nM BR (+BR) or incubation medium (–BR) for 10 min and then loaded with H2DCF-DA for 10 min. H_2O_2 was visualized by confocal microscopy. Experiments were repeated at least three times with similar results. (B) Quantitation of the fluorescence intensity in (A). The protoplasts were treated as follows: 1, distilled water; 2, RNAi; 3, distilled water+BR; 4, RNAi+BR. 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.

ZmMAP65-1a interacts directly with ZmMPK5

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 (YFP^N) and C-terminal (YFP^C) 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 YFP^N–*ZmMPK5* and YFP^C–*ZmMAP65-1a*
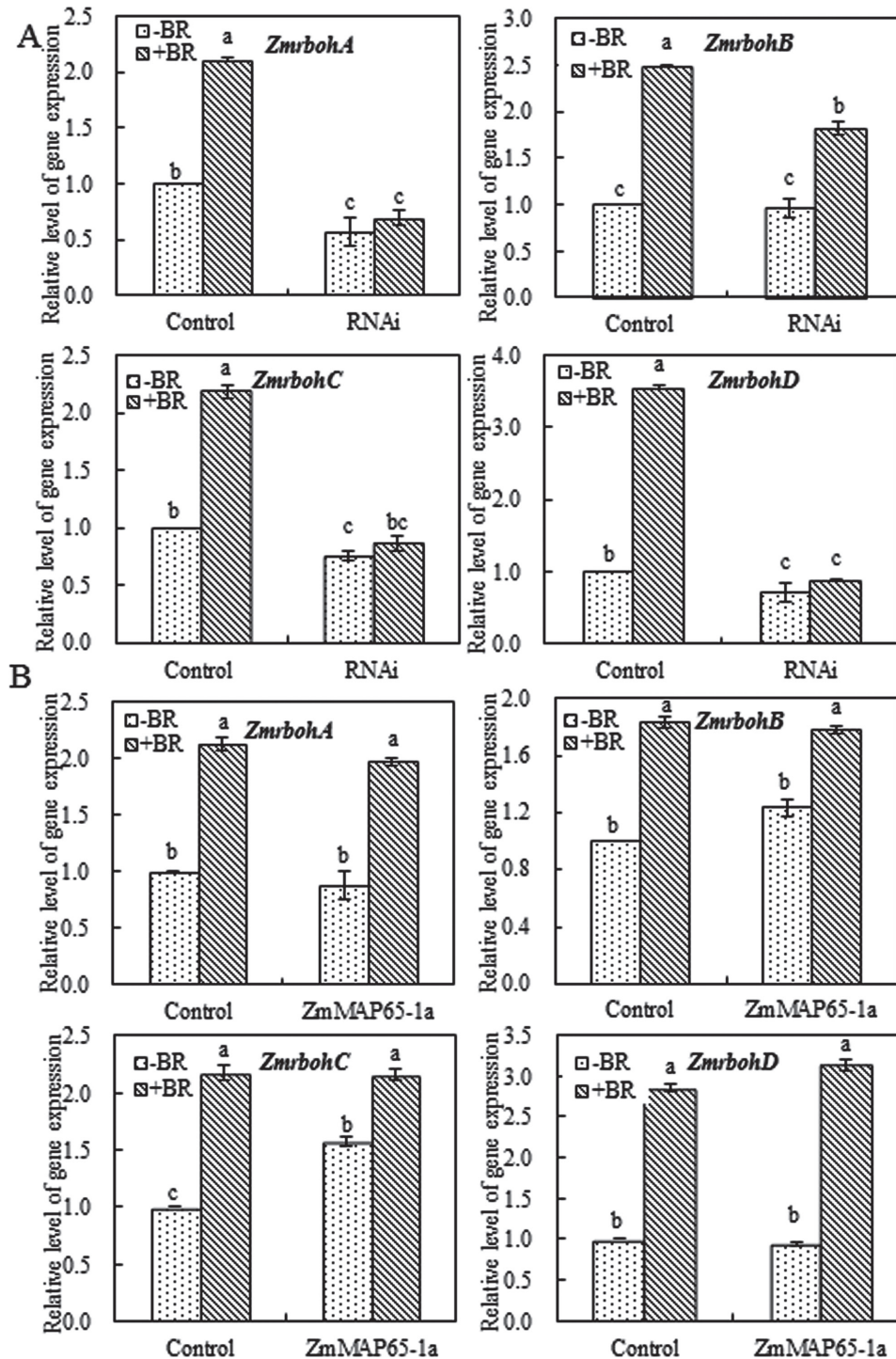
Fig. 6. ZmMAP65-1a affects the expression of NADPH oxidase genes in protoplasts. (A) Expression analysis of *ZmrbohA–D* in protoplasts transiently silencing *ZmMAP65-1a*. Protoplasts were transfected with dsRNA against *ZmMAP65-1a* (RNAi) or with distilled water as a control. Protoplasts were treated with 10 nM 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 10 nM 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.

were co-expressed in onion epidermal cells (Fig. 9D). In contrast, no YFP signal was observed when no-fusion YFP^C and no-fusion YFP^N, YFP^N–*ZmMPK5* and no-fusion YFP^C, and

no-fusion YFP^N and YFP^C-*ZmMAP65-1a*, as the controls, were co-transformed. These results are consistent with an interaction between ZmMAP65-1a and ZmMPK5 *in vivo*.
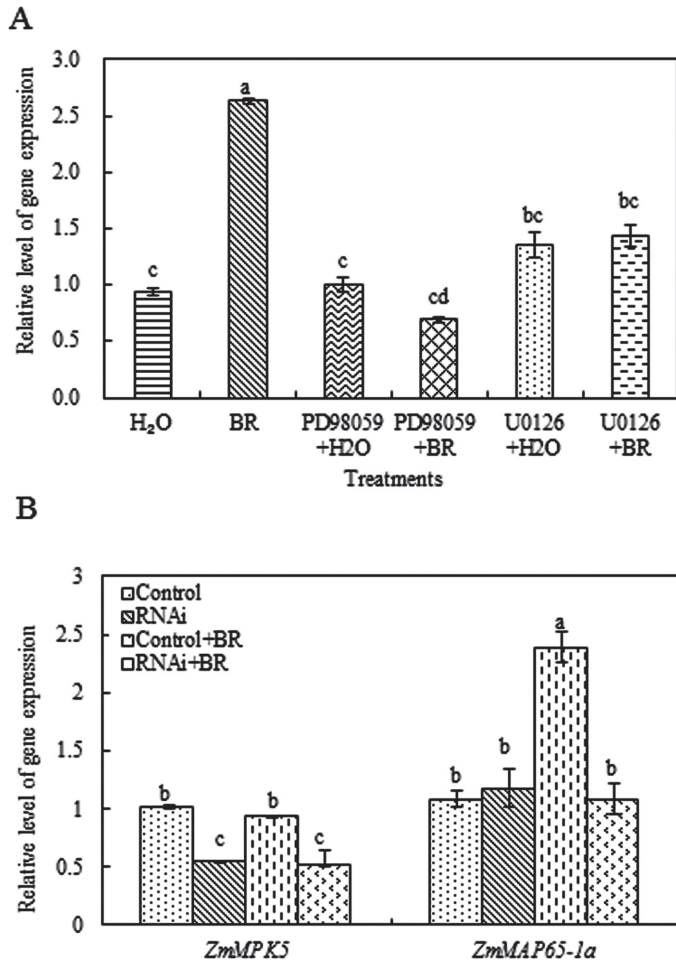
Fig. 7. ZmMPK5 regulates the expression of *ZmMAP65-1a*. (A) Effects of pre-treatment with the MAPKK inhibitors PD98059 and U0126 on the expression of *ZmMAP65-1a* in leaves of maize plants exposed to BR treatment. The detached plants were pre-treated with distilled water, 10 μM U0126 or 100 μM PD98059 for 4 h, and then exposed to treatment with 10 nM BR or distilled water for 30 min. The relative expression level of *ZmMAP65-1a* was analysed by real-time qRT-PCR. (B) Expression analysis of *ZmMAP65-1a* in protoplasts transiently silencing *ZmMPK5*. Protoplasts were transfected with dsRNA against *ZmMPK5* (RNAi) or distilled water as a control. Protoplasts were treated with 10 nM BR for 10 min, and the relative expression levels of *ZmMPK5 and ZmMAP65-1a* 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*a*). 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* increase 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.

H_2O_2 accumulation induced by various stimuli can induce antioxidant defence to scavenge abundant H_2O_2, protecting plants from damage (Miller *et al.*, 2010). Here, we described the complex relationship between ZmMAP65-1a and H_2O_2 in BR signalling. Exogenous H_2O_2 treatment induced the expression of *ZmMAP65-1a* in both leaves and protoplasts (Fig. 4A, B). Scavenging or inhibiting the endogenous H_2O_2 level produced by BR inhibited the BR-induced increase in *ZmMAP65-1a* expression in maize leaves (Fig. 4C). These results suggest that H_2O_2 is required for the expression of *ZmMAP65-1a* in BR signalling. Previous work found that H_2O_2 treatment resulted in MT depolymerization in human
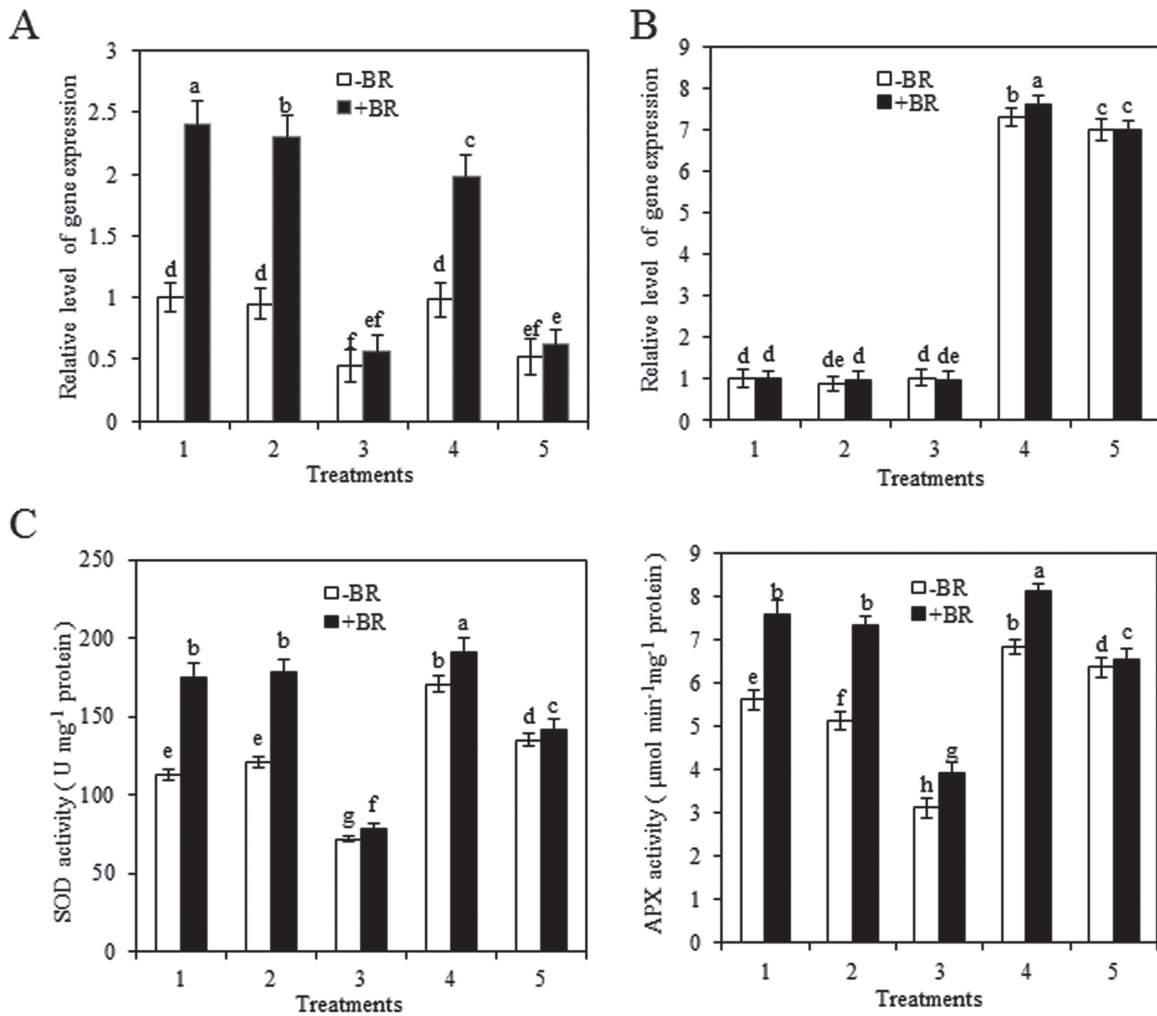
Fig. 8. The activities of SOD and APX in protoplasts transfected with 35S–*ZmMPK5*–YFP and dsRNA of *ZmMAP65-1a*. (A, B) The expression levels of *ZmMAP65-1a* (A) and *ZmMAPK5* (B) in protoplasts. Protoplasts were treated as follows: 1, distilled water; 2, empty vector; 3, dsRNA of *ZmMAP65-1a*; 4, 35S–*ZmMAPK5*–YFP; 5, dsRNA of *ZmMAP65-1a* and 35S–*ZmMAPK5*–YFP. (C) The activities of the antioxidant enzymes SOD and APX in protoplasts treated as described in (A) and (B). 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.

cells (Sponne *et al.*, 2003; Lee *et al.*, 2005). 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_2O_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_2O_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_2O_2 and ZmMAP65-1a in BR signalling. BR-induced H_2O_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_2O_2 appears to operate both upstream and downstream of ZmMAP65-1a, most likely indicating a positive feedback by ZmMAP65-1a on H_2O_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_2O_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_2O_2 accumulation and BR-induced *ZmMAPK65-1a* expression described above, we propose that ZmMAP65-1a induced by BR-produced H_2O_2 increases NADPH oxidase gene expression, which in turn enhances H_2O_2 accumulation, forming a H_2O_2 self-propagation loop, in BR signalling in leaves of maize plants.
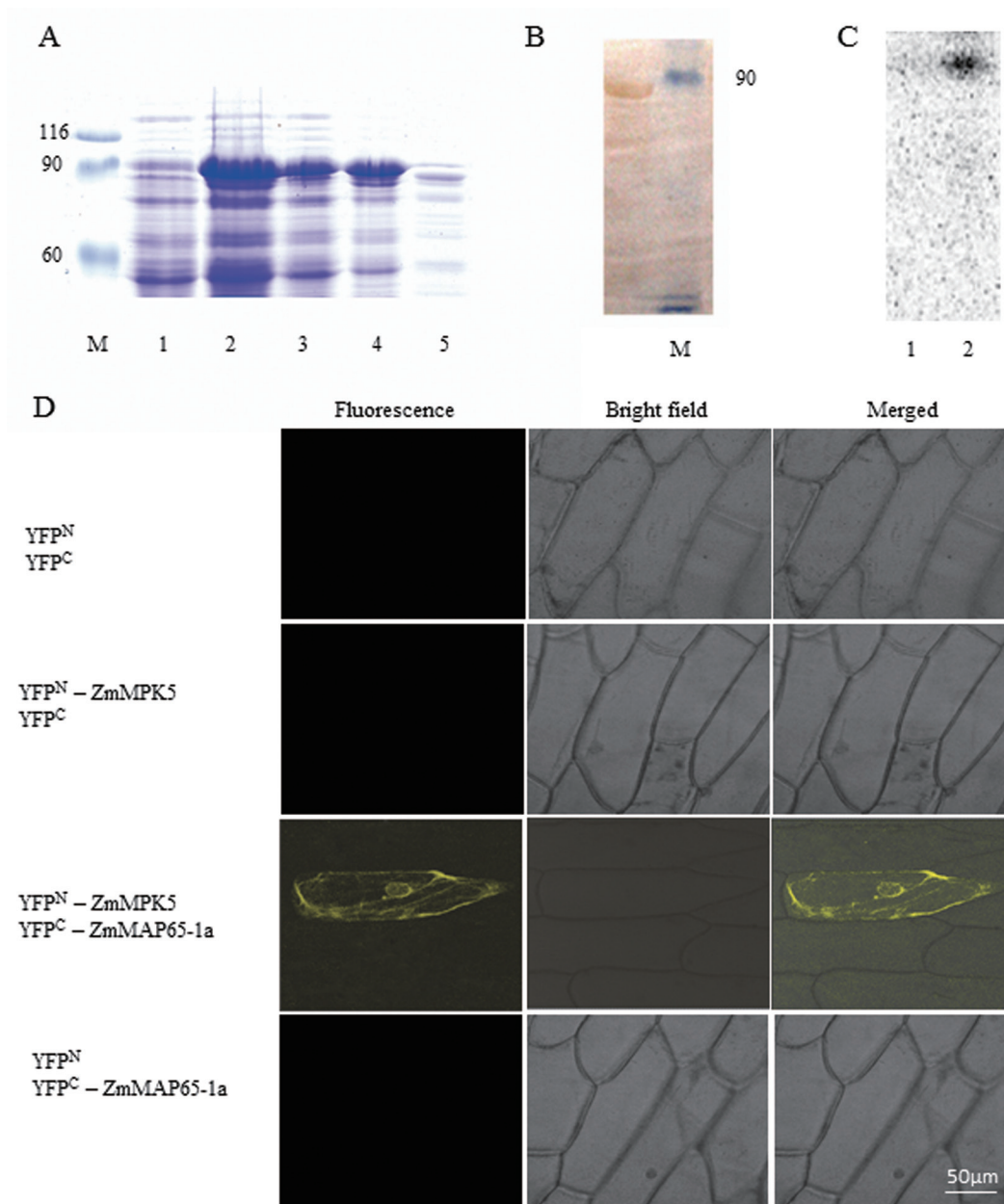
Fig. 9. ZmMPK5 interacts with ZmMAP65-1a. (A) Purification of GST-tagged ZmMAP65-1a. The fusion protein appeared as a major band of 90 kDa by SDS–PAGE. M: Marker; 1, total extract from uninduced bacterial cells; 2, total extract from transformed bacterial cells after IPTG induction; 3, pellet from induced bacterial cells; 4, supernatant from induced bacterial cells; 5, Q-Sepharose elution of fusion protein. (B) Protein immunoblots probed with anti-GST antibody. *Lane 1*, GST–ZmMAP65-1a; *Lane 2*, Marker. (C) Phosphorylation of ZmMAP65-1a by ZmMPK5 *in vitro*. Protein extract from BR-treated leaves were immunoprecipitated with ZmMPK5 antibody. Protein prepared from uninduced (*Lane 1*) or IPTG induced (*Lane 2*) cultures was used as substrate and subjected to an in-gel kinase assay. (D) BiFC detection of ZmMAP65-1a and ZmMPK5 interaction in onion epidermal cells. Experiments were repeated at least three times with similar results.

Surprisingly, transient expression of *ZmMAP65-1a* did not enhance the expression of the NADPH oxidase genes, except for a slight increase for *ZmrbohC* (Fig. 6B). One possible explanation for this disparity is that BR treatment induces not only the expression of the *ZmMAP65-1a* gene but also modification of the ZmMAP65-1a protein, and modification of 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 *Arabidopsis* and tobacco homologues AtMAP65-1, AtMAP65-3, and NtMAP65-1a (Smertenko *et al.*, 2004; Sasabe *et al.*, 2006; Caillaud *et al.*, 2008). Besides that, in addition to NADPH oxidases, cell-wall peroxidase and poly-amine oxidase are also sources of apoplastic H_2O_2 production (Mittler, 2002). Moreover, the chloroplast is another location of BR-induced H_2O_2 production (Zhang *et al.*,
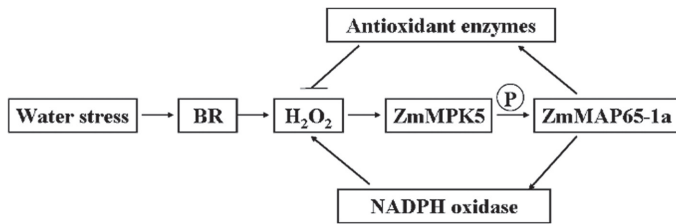
Fig. 10. Conceptual model of the role of ZmMAP65-1a in BR induce antioxidant defence. BR induces H_2O_2 production, the production of H_2O_2 increases ZmMAP65-1a accumulation, and ZmMPK5 phosphorylates ZmMAP65-1a, thus resulting in the upregulation of antioxidant defence enzymes. ZmMAP65-1a also enhances H_2O_2 production by NADPH oxidase, forming a positive amplification loop. The upregulation in the activities of antioxidant enzymes enhances the ability of cells to scavenge excess H_2O_2 under stress.

2010). BR-induced H_2O_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 H_2O_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 H_2O_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 showed 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 MT 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 H_2O_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 regulation 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 H_2O_2 production. Conversely, the increase in the expression of *ZmMAP65-1a* amplifies H_2O_2 production via induction of NADPH oxidase genes, forming a positive H_2O_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 H_2O_2 production.

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

Acknowledgments

References

Amos LA, Schlieper D. 2005. Microtubules and maps. *Advances in Protein Chemistry* **71,** 257–298.

Bajguz A. 2007. Metabolism of brassinosteroids in plants. *Plant Physiology and Biochemistry* **45,** 95–107.

Beck M, Komis G, Müller J, Menzel D, Šamaj J. 2010. *Arabidopsis* homologs of nucleus- and phragmoplast-localized kinase 2 and 3 and mitogen-activated protein kinase 4 are essential for microtubule organization. *Plant Cell* **22,** 755–771.

Beck M, Komis G, Ziemann A, Menzel D, Šamaj J. 2011. Mitogen-activated protein kinase 4 is involved in the regulation of mitotic and cytokinetic microtubule transitions in *Arabidopsis thaliana*. *New Phytologist* **189,** 1069–1083.

Berberich T, Sano H, Kusano T. 1999. Involvement of a MAP kinase, ZmMPK5, in senescence and recovery from low-temperature stress in maize. *Molecular and General Genetics* **262,** 534–542.

Bishop GJ, Koncz C. 2002. Brassinosteroids and plant steroid hormone signaling. *Plant Cell* **14,** S97–S110.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding. *Analytical Biochemistry* **72,** 248–254.

Bright J, Desikan R, Hancock JT, Weir IS, Neill SJ. 2006. ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. *The Plant Journal* **45,** 113–122.

Caillaud MC, Lecomte P, Jammes F, et al. 2008. MAP65-3 microtubule-associated protein is essential for nematode-induced giant cell ontogenesis in *Arabidopsis*. *Plant Cell* **20,** 423–437.

Choudhary SP, Bhardwaj R, Gupta BD, Dutt P, Gupta RK, Biondi S, Kanwar M. 2010. Epibrassinolide induces changes in indole-3-acetic acid, abscisic acid and polyamine concentrations and enhances antioxidant potential of radish seedlings under copper stress. *Physiologia Plantarum* **140,** 280–296.

Choudhary SP, Kanwar M, Bhardwaj R, Gupta BD, Gupta RK. 2011. Epibrassinolide ameliorates Cr (VI) stress via influencing the levels of indole-3-acetic acid, abscisic acid, polyamines and antioxidant system of radish seedlings. *Chemosphere* **84,** 592–600.

Choudhary SP, Yu JQ, Yamaguchi-Shinozaki K, Shinozaki K, Tran Lam-Son P. 2012. Benefits of brassinosteroid crosstalk. *Trends in Plant Science* **10,** 594–605.

Clouse SD, Sasse JM. 1998. Brassinosteroids: essential regulators of plant growth and development. *Annual Review of Plant Physiology and Plant Molecular Biology* **49,** 427–451.

Cui JX, Zhou YH, Ding JG, Xia XJ, Shi K, Chen SC, Asami T, Chen Z, Yu JQ. 2011. Role of nitric oxide in hydrogen peroxide-dependent induction of abiotic stress tolerance by brassinosteroids in cucumber. *Plant, Cell & Environment* **34,** 347–358.

Desai A, Mitchison TJ. 1997. Microtubule polymerization dynamics. *Annual Review of Cell and Developmental Biology* **13,** 83–117.

Dhonukshe P, Weits DA, Cruz-Ramirez A, et al. 2012. A PLETHORA-auxin transcription module controls cell division plane rotation through MAP65 and CLASP. *Cell* **149,** 383–396.

Divi UK, Krishna P. 2009. Brassinosteroid: a biotechnological target for enhancing crop yield and stress tolerance. *New Biotechnology* **26,** 131–136.

Foyer CH, Noctor G. 2005. Redox homeostasis and antioxidant signaling: a metabolic interface between stress perception and physiological responses. *Plant Cell* **17,** 1866–1875.

Gudesblat G, Schneider-Pizon J, Betti C, et al. 2012. SPEECHLESS integrates brassinosteroid and stomata signaling pathways. *Nature Cell Biology* **14,** 548–554.

Guo L, Ho CMK, Kong Z, Lee YRJ, Qian Q, Liu B. 2009. Evaluating the microtubule cytoskeleton and its interacting proteins in monocots by mining the rice genome. *Annals of Botany* **103,** 387–402.

Hussey PJ, Hawkins TJ, Igarashi H, Kaloriti D, Smertenko A. 2002. The plant cytoskeleton: recent advances in the study of the plant microtubule-associated proteins MAP-65, MAP-190 and the *Xenopus* MAP215-like protein, MOR1. *Plant Molecular Biology* **50,** 915–924.

Jiang CJ, Sonobe S. 1993. Identification and preliminary characterization of a 65 kDa higher-plant microtubule-associated protein. *Journal of Cell Science* **105,** 891–901.

Kagale S, Divi UK, Krochko JE, Keller WA, Krishna P. 2007. Brassinosteroid confers tolerance in *Arabidopsis thaliana* and *Brassica napus* to a range of abiotic stresses. *Planta* **225,** 353–364.

Keech O, Pesquet E, Gutierrez L, Ahad A, Bellini C, Smith SM, Gardeström P. 2010. Leaf senescence is accompanied by an early disruption of the microtubule network in *Arabidopsis*. *Plant Physiology* **154,** 1710–1720.

Khan M, Rozhon W, Biqeard J et al. 2013. Brassinosteroid-regulated GSK3/Shaggy-like kinases phosphorylate mitogen-activated protein (MAP) kinase kinases, which control stomata development in *Arabidopsis thaliana*. *Journal of Biological Chemistry* **288,** 7519–7527.

Kim J, Somers DE. 2010. Rapid assessment of gene function in the circadian clock using artificial microRNA in *Arabidopsis thaliana* mesophyll protoplasts. *Plant Physiology* **154,** 611–621.

Kim TW, Michniewicz M, Bergmann DC, Wang ZY. 2012. Brassinosteroid regulates stomatal development by GSKS-mediated inhibition of a MAPK pathway. *Nature* **482,** 419–422.

Komis G, Illés P, Beck M, Šamaj J. 2011. Microtubules and mitogen-activated protein kinase signalling. *Current Opinion in Plant Biology* **14,** 650–657.

Komorisono M, Ueguchi-Tanaka M, Aichi I, Hasegawa Y, Ashikari M, Kitano H, Matsuoka M, Sazuka T. 2005. Analysis of the rice mutant *dwalf and gladius leaf 1*. Aberrant katanin-mediated microtubule organization cause up-regulation of gibberellin biosynthetic genes independently of gibberellin signaling. *Plant Physiology* **138,** 1982–1993.

Lanza M, Garcia-Ponce B, Castrillo G, et al. 2012. Role of actin cytoskeleton in brassinosteroid signaling and in its integration with the auxin response in plants. *Developmental Cell* **22,** 1275–1285.

Lee CF, Liu CY, Hsieh RH, Wei YH. 2005. Oxidative stress-induced depolymerization of microtubules and alteration of mitochondrial mass in human cells. *Annals of the New York Academy of Sciences* **1042,** 246–254.

Lin F, Ding H, Wang J, Zhang H, Zhang A, Zhang Y, Tan M, Dong W, Jiang M. 2009. Positive feedback regulation of maize NADPH oxidase by mitogen-activated protein kinase cascade in abscisic acid signalling. *Journal of Experimental Botany* **60,** 3221–3238.

Livanos P, Galatis B, Quader H, Apostolakos P. 2012. Disturbance of reactive oxygen species homeostasis induces atypical tubulin polymer formation and affects mitosis in root-tip cells of *Triticum turgidum* and *Arabidopsis thaliana*. *Cytoskeleton* **69,** 1–21.

Lucas JR, Courtney S, Hassfurder M, Dhingra S, Bryant A, Shaw SL. 2011. Microtubule-associated proteins MAP65-1 and MAP65-2 positively regulate axial cell growth in etiolated *Arabidopsis* hypocotyls. *Plant Cell* **23,** 1889–1903.

Lü B, Gong Z, Wang J, Zhang J, Liang J. 2007. Microtubule dynamics in relation to osmotic stress-induced ABA accumulation in *Zea mays* roots. *Journal of Experimental Botany* **58,** 2565–2572.

Ma F, Lu R, Liu H, Shi B, Zhang J, Tan M, Zhang A, Jiang, M. 2012. Nitric oxide-activated calcium/calmodulin-dependent protein kinase regulates the abscisic acid-induced antioxidant defence in maize. *Journal of Experimental Botany* **63,** 4835–4847.

Mao T, Jin L, Li H, Liu B, Yuan M. 2005. Two microtubule-associated proteins of the *Arabidopsis* MAP65 family function differently on microtubules. *Plant Physiology* **138,** 654–662.

Mao G, Buschmann H, Doonan JH, Lloyd CW. 2006. The role of MAP65-1 in microtubule bundling during *Zinnia* tracheary element formation. *Journal of Cell Science* **119,** 753–758.

Mathur J, Chua NH. 2000. Microtubule stabilization leads to growth reorientation in *Arabidopsis* trichomes. *Plant Cell* **12,** 465–477.

Miller G, Suzuki N, Ciftci-Yilmaz S, Mittler R. 2010. Reactive oxygen species homeostasis and signalling during drought and salinity stresses. *Plant, Cell & Environment* **33,** 453–467.

Mittler R. 2002. Oxidative stress, antioxidants and stress tolerance. *Trends in Plant Science* **7,** 405–410.

Nakagami H, Pitzschke A, Hirt H. 2005. Emerging MAP kinase pathways in plant stress signaling. *Trends in Plant Science* **10,** 339–346.

Nie WF, Wang MM, Xia XJ, Zhou YH, Shi K, Chen ZX, Yu JQ. 2013. Silencing of tomato RBOH1 and MPK2 abolishes brassinosteroid-induced H_2O_2 generation and stress tolerance. *Plant, Cell & Environment* **36,** 789–803.

Nogales E. 2000. Structural insight into microtubule function. *Annual Review of Biochemisty* **69,** 277–302.

Pitzschke A, Hirt H. 2006. Mitogen-activated protein kinase and reactive oxygen species signaling in plants. *Plant Physiology* **141,** 351–356.

Sang J, Zhang A, Lin F, Tan M, Jiang, M. 2008. Cross-talk between calcium-calmodulin and nitric oxide in abscisic acid signaling in leaves of maize plants. *Cell Research* **18,** 577–588.

Sasabe M, Machida Y. 2006. MAP65: a bridge linking a MAP kinase to microtubule turnover. *Current Opinion in Plant Biology* **9,** 563–570.

Sasabe M, Machida Y. 2012. Regulation of organization and function of microtubules by the mitogen-activated protein kinase cascade during plant cytokinesis. *Cytoskeleton* **69,** 913–918.

Sasabe M, Soyano T, Takahashi Y, Sonobe S, Igarashi H, Itoh TJ, Hidaka M, Machida Y. 2006. Phosphorylation of NtMAP65-1 by a MAP kinase downregulates its activity of microtubule bundling and stimulates progression of cytokinesis of tobacco cells. *Genes and Development* **20,** 1004–1014.

Sedbrook JC, Kaloriti D. 2008. Microtubules, MAPs and plant directional cell expansion. *Trends in Plant Science* **13,** 303–310.

Sheen J. 2001. Signal transduction in maize and *Arabidopsis* mesophyll protoplasts. *Plant Physiology* **127,** 1466–1475.

Smertenko AP, Chang HY, Wagner V, Kaloriti D, Fenyk S, Sonobe S, Lloyd C, Hauser MT, Hussey PJ. 2004. The *Arabidopsis* microtubule-associated protein AtMAP65-1: molecular analysis of its microtubule bundling activity. *Plant Cell* **16,** 2035–2047.

Smertenko AP, Chang HY, Sonobe S, Fenyk SI, Weingartner M, Bögre L, Hussey PJ. 2006. Control of the AtMAP65-1 interaction with microtubules through the cell cycle. *Journal of Cell Science* **119,** 3227–3237.

Soares VLF, Rodrigues SM, de Oliveira TM, *et al.* 2011. Unraveling new genes associated with seed development and metabolism in *Bixa orellana* L. by expressed sequence tag (EST) analysis. *Molecular Biology Reports* **38,** 1329–1340.

Sponne I, Fifre A, Drouet B, Klein C, Koziel V, Pincon-Raymond M, Olivier JL, Chambaz J, Pillot T. 2003. Apoptotic neuronal cell death induced by the non-fibrillar amyloid-β peptide proceeds through an early reactive oxygen species-dependent cytoskeleton perturbation. *Journal of Biological Chemisty* **278,** 3437–3445.

Tan M, Lu J, Zhang A, Hu B, Zhu X, Li W. 2011. The distribution and cooperation of antioxidant (iso)enzymes and antioxidants in different subcellular compartments in maize leaves during water stress. *Journal of Plant Growth Regulation* **30,** 255–271.

Wang C, Zhang LJ, Chen WF. 2011*a*. Plant cortical microtubules are putative sensors under abiotic stresses. *Biochemistry* **76,** 320–326

Wang S, Kurepa J, Hashimoto T, Smalle JA. 2011*b*. Salt stress-induced disassembly of *Arabidopsis* cortical microtubule arrays involves 26S proteasome-dependent degradation of SPIRAL1. *Plant Cell* **23,** 3412–3427.

Wang ZY. 2012. Brassinosteroids modulate plant immunity at multiple levels. *Proceedings of the National Academy of the Sciences, USA* **109,** 7–8.

Xia XJ, Wang YJ, Zhou YH, Tao Y, Mao WH, Shi K, Asami T, Chen Z, Yu, JQ. 2009. Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. *Plant Physiology* **150,** 801–814.

Zhai Z, Sooksa-nguan T, Vatamaniuk OK. 2009. Establishing RNA interference as a reverse-genetic approach for functional analysis in protoplasts. *Plant Physiology* **149,** 642–652.

Zhang A, Jiang M, Zhang J, Tan M, Hu X. 2006. Mitogen-activated protein kinase is involved in abscisic acid-induced antioxidant defense and acts downstream of reactive oxygen species production in leaves of maize plants. *Plant Physiology* **141,** 475–487.

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Zhang A, Zhang J, Ye N, Cao J, Tan M, Zhang J, Jiang M. 2010. ZmMPK5 is required for the NADPH oxidase-mediated self-propagation of apoplastic H$_2$O$_2$ in brassinosteroid-induced antioxidant defence in leaves of maize. *Journal of Experimental Botany* **61,** 4399–4411.

Zhang A, **Zhang J**, **Zhang J**, **Ye N**, **Zhang H**, **Tan M**, **Jiang M**. 2011. Nitric oxide mediates brassinosteroid-induced ABA biosynthesis involved in oxidative stress tolerance in maize leaves. *Plant and Cell Physiology* **52,** 181–192.

Zhang Q, Lin F, Mao T, Nie J, Yan M, Yuan M, Zhang W. 2012. Phosphatidic acid regulates microtubule organization by interacting with MAP65-1 in response to salt stress in *Arabidopsis*. *Plant Cell* **24,** 4555–4576.