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Calcium and ZmCCaMK are involved in brassinosteroid-induced antioxidant defense in maize leaves

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Brassinosteroids (BRs) have been shown to enhance stress tolerance by inducing antioxidant defense systems. However, the mechanisms of BR-induced antioxidant defense in plants remain to be determined. In this study, the role of calcium (Ca^{2+}) and maize calcium/calmodulin-dependent protein kinase (CCaMK), ZmCCaMK, in BR-induced antioxidant defense, and the relationship between ZmCCaMK and Ca^{2+} in BR signaling were investigated. BR treatment led to a significant increase in cytosolic Ca^{2+} concentration in protoplasts from maize mesophyll, and Ca^{2+} was shown to be required for BR-induced antioxidant defense. Treatment with BR induced increases in gene expression and enzyme activity of ZmCCaMK in maize leaves. Transient overexpression and silencing of *ZmCCaMK* in maize protoplasts demonstrated that ZmCCaMK was required for BR-induced antioxidant defense. The requirement for CCaMK was further investigated using a loss-of-function mutant of *OsCCaMK*, the orthologous gene of *ZmCCaMK* in rice. Consistent with the findings in maize, BR treatment could not induce antioxidant defense in the rice *OsCCAMK* mutant. Furthermore, Ca^{2+} was required for BR-induced gene expression and activation of ZmCCaMK, while ZmCCaMK was shown to enhance the BR-induced increase in cytosolic Ca^{2+} concentration. Moreover, our results also showed that ZmCCaMK and H_2O_2 influenced each other. These results indicate that Ca^{2+} works together with ZmCCaMK in BR-induced antioxidant defense, and there are two positive feedback loops between Ca^{2+} or H_2O_2 and ZmCCaMK in BR signaling in maize.

Keywords: Antioxidant defense • Brassinosteroid • Calcium • Calcium/calmodulin-dependent protein kinase • H_2O_2 • Maize.

Abbreviations: APX, ascorbate peroxidase; BR, brassinosteroid; BRz, brassinazole; CaM, calmodulin; CAT, catalase; CCaMK, calcium/calmodulin-dependent protein kinase; DAB, 3,3′-diaminobenzidine; DMTU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DPI, diphenyleneiodonium; dsRNA, double-stranded RNA; Fluo-3/AM, Fluo-3 acetoxymethyl ester; GR, glutathione reductase; H_2DCF-DA, 2,7-dichlorofluorescein diacetate; LSCM, laser scanning confocal microscopy; MAPK, mitogen-activated protein kinase; PEG, polyethlene glycol; qRT–PCR, real-time quantitative reverse transcription–PCR; RNAi, RNA interference; ROS, reactive oxygen species; SOD, superoxide dismutase; YFP, yellow fluorescent protein.

Introduction

Brassinosteroids (BRs) are a family of growth-promoting steroidal plant hormones that are found at low levels in pollen, seeds and young vegetative tissues throughout the plant kingdom. Genetic studies in Arabidopsis have illustrated the essential roles of BRs in a wide range of developmental processes and helped to identify many genes involved in BR biosynthesis and signal transduction (Li and Chory 1999, Bajguz 2007, Kim and Wang 2010, Choudhary et al. 2012, Fàbregas et al. 2013). In addition, BRs have been demonstrated to alleviate various biotic and abiotic stress effects (Kagale et al. 2007, Wang 2012). BR enhanced the tolerance to oxidative, cadmium, salinity and copper stresses, which was accompanied by the accumulation of hydrogen peroxide (H_2O_2) and the enhancement of antioxidant enzymes, including superoxide dismutase (SOD), catalase (CAT), ascorbate peroxidase (APX), glutathione peroxidase (GPX) and glutathione reductase (GR) (Xia et al. 2009, Choudhary et al. 2010, Zhang et al. 2010, Zhang et al. 2011, Ahammed et al. 2013, Hayat et al. 2014). H_2O_2, nitric oxide (NO), NADPH oxidase and mitogen-activated protein kinase (MAPK) are required for BR-induced antioxidant defense in plants (Xia et al. 2009, Zhang et al. 2010, Zhang et al. 2011, Zhu et al. 2013). However, the detailed mechanisms of BR-induced antioxidant defense remain unclear.

Calcium (Ca^{2+}) is a universal second messenger, and the concentration of cytosolic Ca^{2+} represent a central hub where multiple signal transduction pathways intersect (McAinsh et al. 2009, Dodd et al. 2010, Kudla et al. 2010, Monshausen 2012). In plants, various stimuli, such as salinity, drought, cold, heat shock, ABA, H_2O_2 and pathogen elicitors, trigger changes in the concentration of cytosolic Ca^{2+}, and the transient Ca^{2+} elevations are recognized by several Ca^{2+} sensors such as calmodulin (CaM), calcium-dependent protein kinase

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(CDPK) and calcineurin B-like protein (CBL) (Luan et al. 2002, Yang and Poovaiah 2003, Harper and Harmon 2005, DeFalco et al. 2010, Hashimoto and Kudla 2011, Batistič and Kudla 2012, Schulz et al. 2013). In spite of extensive studies on Ca^{2+} signaling, little is currently known about the involvement of Ca^{2+} in BR signaling. A recent study showed that exogenously applied BR caused an elevation in the concentration of cytosolic Ca^{2+} in Arabidopsis (Zhao et al. 2013), suggesting a new function for Ca^{2+} in BR signaling. However, it is not clear how Ca^{2+} is translated into specific responses in BR signaling.

Calcium/calmodulin-dependent protein kinase (CCaMK) is a strong decoder of Ca^{2+} spiking. This protein is characterized by a serine/threonine kinase domain, a CaM-binding domain and three EF-hand motifs that potentially bind Ca^{2+}, and its activity is subject to dual regulation by Ca^{2+} and CaM (Gleason et al. 2006, Tirichine et al. 2006, Yang et al. 2007, Hayashi et al. 2010). CCaMKs have been isolated from lily, tobacco, maize, rice, wheat, *Lotus japonicus*, *Medicago truncatula* and *Sesbania rostrata* (Harper et al. 2004, Levy et al. 2004, Mitra et al. 2004, Chen et al. 2007, Capoen et al. 2009, Hayashi et al. 2010, Yang et al. 2011), and a growing amount of evidence reveals their functions in plants. For example, CCaMK has been demonstrated to be a key regulator of root nodule and arbuscular mycorrhizal symbioses (Levy et al. 2004, Mitra et al. 2004, Gleason et al. 2006, Chen et al. 2007, Hayashi et al. 2010, Shimoda et al. 2012), and has also been shown to be involved in responses to abiotic stress (Yang et al. 2011, Ma et al. 2012, Shi et al. 2012, Shi et al. 2014). The wheat CCaMK gene *TaCCaMK* was regulated by ABA, NaCl and polyethylene glycol (PEG) treatments in wheat seedling roots. More recent studies also showed that ABA and PEG could induce the expression and activity of CCaMK, and CCaMK is required for ABA-induced antioxidant defense in the leaves of rice and maize (Ma et al. 2012, Shi et al. 2012, Shi et al. 2014). BRs and ABA can co-regulate the expression of hundreds of genes (Nemhauser et al. 2006), and they interact physiologically in controlling many developmental processes (Steber and McCourt 2001, Chen et al. 2004, Finkelstein et al. 2008, Gao et al. 2008) and stress responses (Zhang et al. 2011). Therefore, it is reasonable to hypothesize that CCaMK may be involved in BR signaling. In the present study, this hypothesis was tested and the results showed that ZmCCaMK is involved in BR signaling and is required for BR-induced antioxidant defense. Ca^{2+} plays an important role in gene expression and activation of ZmCCaMK, and ZmCCaMK also in turn influences the cytosolic Ca^{2+} concentration in BR signaling.

Results

Ca^{2+} is required for BR-induced antioxidant defense

Ca^{2+} is a universal second messenger that acts as a mediator of stimulus–response coupling in eukaryotes (Oh et al. 2012). Here, the effect of BR on the concentration of cytosolic Ca^{2+} was investigated. To visualize cytosolic Ca^{2+}, protoplasts were prepared from mesophyll from leaves, loaded with Fluo-3 acetoxymethyl ester (Fluo-3/AM), a Ca^{2+}-sensitive fluorescent probe (Zhang et al. 1998), and subsequently treated with 10 nM BR, and observed by laser scanning confocal microscopy (LSCM). As shown in **Fig. 1**, the concentration of cytosolic Ca^{2+} increased rapidly and reached a maximum after 6 min of treatment, which was nearly 2-fold higher than untreated protoplasts.

To determine further whether BR-induced Ca^{2+} is involved in BR-induced antioxidant defense, the Ca^{2+} chelator EGTA and Ca^{2+} channel blocker $LaCl_3$ were used. BR treatment induced significant increases in the activities of APX and SOD, which were almost completely blocked by pre-treatment with EGTA or $LaCl_3$, and these pre-treatments alone did not affect the activities of APX and SOD (**Fig. 2**). These results suggest that Ca^{2+} is required for BR-induced antioxidant defense in maize leaves.

BR induces increases in gene expression and activity of ZmCCaMK in maize leaves

CCaMK is an important decoder of Ca^{2+}. In order to determine the possible involvement of ZmCCaMK in BR signaling, the effect of BR on the induction of ZmCCaMK was investigated. The expression of *ZmCCaMK* and the activity of ZmCCaMK in maize leaves were measured using quantitative real-time PCR analysis and immunocomplex kinase activity assay, respectively. Treatments with BR (10 nM) induced rapid increases in gene expression and the activity of ZmCCaMK (**Fig. 3A–C**). A biphasic response in the expression of *ZmCCaMK* was observed, in which the first peak of *ZmCCaMK* expression occurred after 30 min of BR treatment, then decreased, and the second peak of *ZmCCaMK* expression occurred after 120 min of BR treatment (**Fig. 3A**). Similar biphasic responses were observed in the expression of *ZmCCaMK* in maize mesophyll protoplasts (**Fig. 3B**) and in the activity of ZmCCaMK in maize leaves (**Fig. 3C**). The BR-induced activation of ZmCCaMK occurred in a dose-dependent manner in the concentration range of 5–20 nM BR (**Fig. 3D**).

To investigate whether the expression of *ZmCCaMK* and the activity of ZmCCaMK can be induced by endogenous BR, the specific BR biosynthesis inhibitor brassinazole (Brz) (Asami et al. 2001) was used. PEG treatment induced increases in expression of *ZmCCaMK* and activity of ZmCCaMK in maize leaves (**Fig. 3E**). Pre-treatment with Brz significantly inhibited the PEG-induced expression of *ZmCCaMK* and the activity of ZmCCaMK, which were restored by the application of exogenous BR (10 nM), while Brz pre-treatment alone had no effect on the expression of *ZmCCaMK* or the activity of ZmCCaMK (**Fig. 3F**). These results indicate that endogenous BR up-regulates the expression of *ZmCCaMK* and the activity of ZmCCaMK in leaves of maize plants in response to PEG.

ZmCCaMK is involved in BR-induced antioxidant defense

To investigate further whether ZmCCaMK mediates the BR-induced antioxidant defense in maize, we used a transient gene expression and a transient gene silencing analysis in

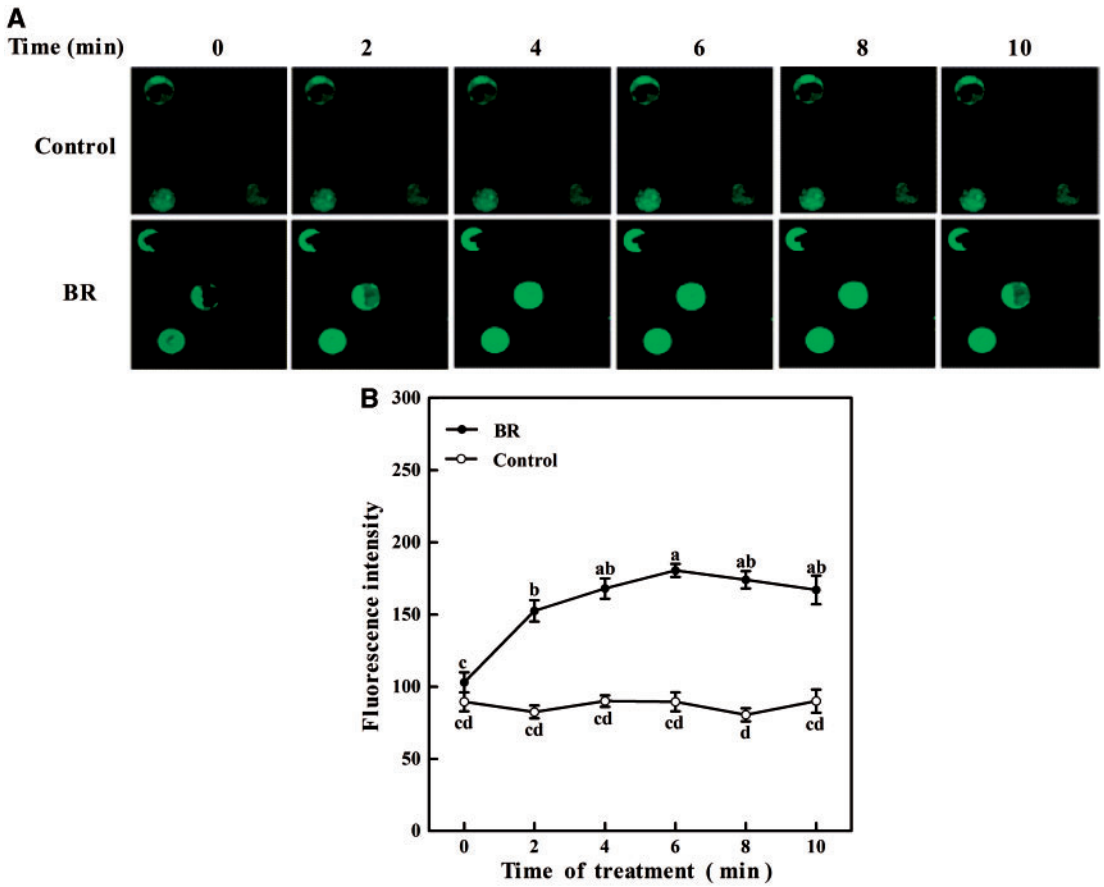

Fig. 1 BR-induced changes in cytosolic calcium concentration in protoplasts of maize (*Zea mays*) mesophyll cells. (A) Confocal images of maize mesophyll protoplasts loaded with Fluo-3/AM. The protoplasts were treated with culture medium (Control) or 10 nM BR (BR), and were observed by laser scanning confocal microscopy (LSCM). Experiments were repeated at least three times with similar results. (B) Changes in fluorescence intensity. The fluorescence intensity was measured by fluorescence microscopy and calculated as the average value of those obtained by scanning >50 protoplasts from 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 (Yoo et al. 2007, Zhai et al. 2009). This method has been shown to be suitable for functional analysis of plant genes (An et al. 2005, Ma et al. 2012, Shi et al. 2012, Ding et al. 2013). As anticipated, transient expression or silencing of *ZmCCaMK* in protoplasts resulted in a significant increase or decrease in the protein levels of ZmCCaMK (**Fig. 4A**). Transient expression of *ZmCCaMK* in protoplasts resulted in significant increases in expression of the antioxidant genes *APX2* and *SOD4* and corresponding activities of APX and SOD, when compared with control (**Fig. 4B**). Conversely, RNA interference (RNAi)-mediated silencing of *ZmCCaMK* decreased the expression of *APX2* and *SOD4* and the activities of APX and SOD (**Fig. 4C**). BR treatment could induce significant increases in expression of *APX2* and *SOD4* and activities of APX and SOD in control protoplasts (Control), but the response to BR treatment was strongly attenuated in *ZmCCaMK* transiently silenced protoplasts (RNAi) (**Fig. 4C**). These results indicate that ZmCCaMK is involved in BR-induced antioxidant defense in maize plants.

To confirm further that CCaMK functions in BR-induced antioxidant defense, the rice mutant line NF8513 ('Nipponbare') containing the *Tos17* insertion in *OsDMI3* (Os05g41090), the closest rice homolog of *ZmCCaMK*, was used. As is the case for *ZmCCaMK*, *OsDMI3* is a single-copy gene. BR treatment led to significant increases in activities of APX and SOD in leaves of wild-type rice, but could not up-regulate activities of APX and SOD in leaves of the NF8513 plants homozygous for the mutation (**Fig. 5**). These data further confirm that CCaMK is involved in BR-induced antioxidant defense.

Ca^{2+} is essential for BR-induced gene expression and activity of ZmCCaMK

As described above, both Ca^{2+} and ZmCCaMK are involved in BR-induced antioxidant defense in maize leaves. To determine the relationship between Ca^{2+} and ZmCCaMK in BR signaling, the effect of $CaCl_2$ on ZmCCaMK was determined first. Treatment with 20 mM $CaCl_2$ led to a significant increase in activity of ZmCCaMK (**Fig. 6A**). The activity of ZmCCaMK was up-regulated after 10 min, peaked at 30 min and then decreased after 45 min of $CaCl_2$ treatment.

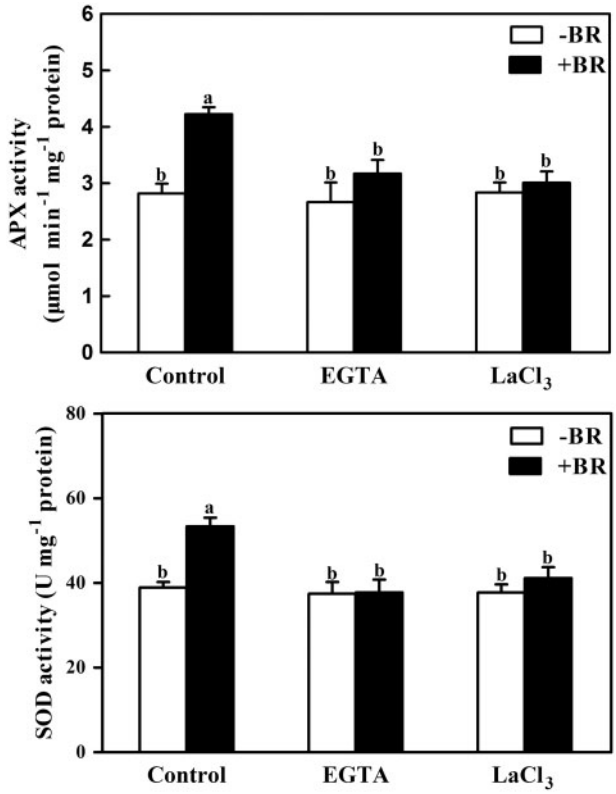

Fig. 2 Effects of pre-treatments with a Ca^{2+} chelator and a Ca^{2+} channel blocker on the activities of antioxidant enzymes APX and SOD in leaves of maize exposed to BR treatment. The detached plants were treated with 5 mM EGTA or 5 mM $LaCl_3$ for 4 h, then exposed to 10 nM BR for 12 h. Plants treated with distilled water under the same conditions served as controls. 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.

In order to investigate further whether BR-activated ZmCCaMK is related to BR-induced Ca^{2+}, the Ca^{2+} chelator EGTA and the Ca^{2+} channel blocker $LaCl_3$ were used. Pre-treatments with EGTA and $LaCl_3$ essentially eliminated BR-induced increases in gene expression and activity of ZmCCaMK, while these pre-treatments alone had no effect on gene expression and activity of ZmCCaMK (**Fig. 6B**, **C**). Together these results suggest that BR-induced Ca^{2+} is required for BR-up-regulated gene expression and activity of ZmCCaMK in maize leaves.

ZmCCaMK affects BR-induced cytosolic Ca^{2+} concentration elevation

Next, we wanted to explore if there is also a feedback regulation of ZmCCaMK on cytosolic Ca^{2+} concentration. To test this, the protoplasts transfected with ubi-*ZmCCaMK*-mCherry and double-stranded RNA (dsRNA) against *ZmCCaMK* were used. Transient expression of *ZmCCaMK* in protoplasts resulted in a significant increase in the concentration of cytosolic Ca^{2+}, which was further enhanced by BR treatment (**Fig. 7A**, **B**). Conversely, RNAi-mediated silencing of *ZmCCaMK* caused a significant decrease in the concentration of cytosolic Ca^{2+}, and BR treatment failed to induce the levels of cytosolic Ca^{2+} concentration observed in control protoplasts (**Fig. 7C**, **D**). These results indicate that ZmCCaMK mediates BR-induced elevation of cytosolic Ca^{2+} concentration.

The relationship between H_2O_2 and ZmCCaMK in BR signaling

As described above, ZmCCaMK is involved in BR-induced antioxidant defense. Our previous study showed that BR induced the production of H_2O_2, which subsequently enhanced antioxidant defense (Zhang et al. 2010). Therefore, we wanted to determine whether BR-induced increases in gene expression and activity of ZmCCaMK are related to BR-induced endogenous H_2O_2. To test this, 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DMTU) and CAT, H_2O_2 scavengers, and diphenylene iodoniun (DPI), an inhibitor of NADPH oxidase were used. Pre-treatments with DMTU, CAT and DPI substantially reduced the BR-induced increases in gene expression and activity of ZmCCaMK, while these pre-treatments alone had no effect (**Fig. 8A**), suggesting that H_2O_2 is required for the BR-induced up-regulation in gene expression and activity of ZmCCaMK.

To investigate whether BR-activated ZmCCaMK also affects BR-induced H_2O_2 production, the protoplasts transfected with ubi-*ZmCCaMK*-mCherry and dsRNA against *ZmCCaMK* were used. The results showed that transient expression of *ZmCCaMK* significantly increased H_2O_2 accumulation (**Fig. 8B**), while transient silencing of *ZmCCaMK* significantly reduced H_2O_2 accumulation (**Fig. 8C**). BR treatment could further enhance H_2O_2 production in protoplasts transiently expressing *ZmCCaMK* (**Fig. 8B**) but only to a small degree in protoplasts where *ZmCCaMK* was transiently silenced (**Fig. 8C**). These results suggest that ZmCCaMK is also required for BR-induced H_2O_2 production.

To obtain further evidence that CCaMK is required for BR-induced H_2O_2 production, the mutant of *OsDMI3* was used and H_2O_2 production was detected using 3,3′-diaminobenzidine (DAB) staining in rice leaves. As shown in **Fig. 8D**, BR treatment led to a substantial increase in the production of H_2O_2 in wild-type leaves, but could only induce a small increase in H_2O_2 in leaves of mutant line NF8513.

To determine further the mechanism of ZmCCaMK action in the regulation of H_2O_2 production in BR signaling, we analyzed the gene expression of NADPH oxidase, which is an important source of apoplastic H_2O_2 accumulation (Xia et al. 2009). As shown in **Fig. 9A**, the expression of *ZmrbohB*, *ZmrbohC* and *ZmrbohD* was substantially increased in protoplasts with transient expression of *ZmCCaMK*, and BR treatment could further enhance the expression of *ZmrbohB* and *ZmrbohC*. In contrast, RNAi-mediated silencing of *ZmCCaMK* decreased the expression of *ZmrbohB*, *ZmrbohC* and *ZmrbohD*, and the effect of BR treatment on expression was highly attenuated (**Fig. 9B**). These data indicate that not only does H_2O_2 affect ZmCCaMK, but ZmCCaMK also regulates H_2O_2 production via NADPH oxidase in BR signaling. Hence, as for Ca^{2+}, there is also a feedback loop between H_2O_2 and ZmCCaMK in BR signaling.

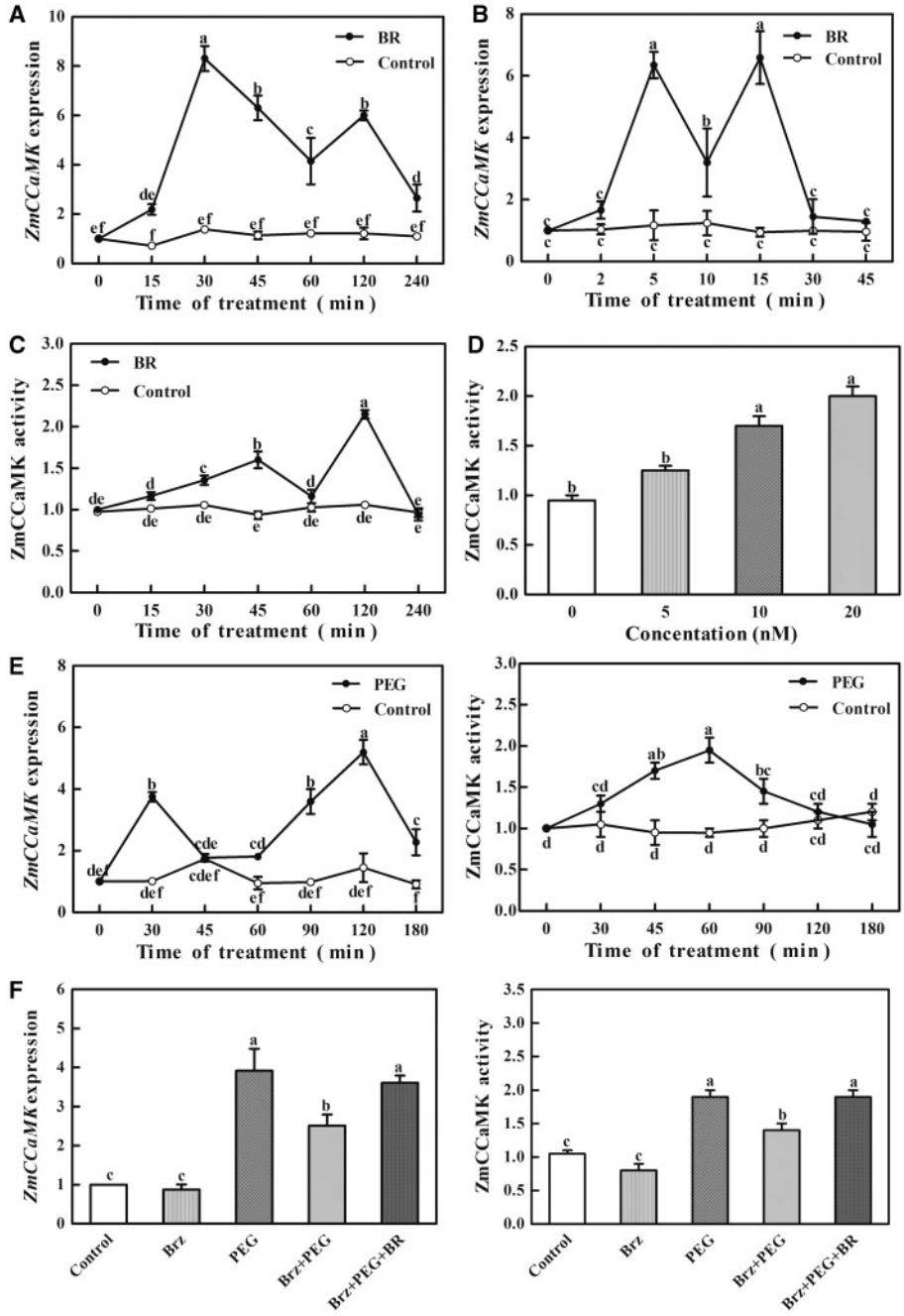

Fig. 3 BR induces expression of *ZmCCaMK* and activity of ZmCCaMK in maize. (A, B) Expression of *ZmCCaMK* in maize leaves (A) or mesophyll protoplasts (B) exposed to BR treatment. The maize seedlings or protoplasts were treated with 10 nM BR for various times as indicated. Seedlings treated with distilled water and protoplasts treated with culture medium under the same conditions served as controls. The relative expression level of *ZmCCaMK* was analyzed by qRT–PCR. (C) Induction of activity of ZmCCaMK by BR. The detached plants were treated as described in (A). Protein extracted from control or BR-treated leaves was immunoprecipitated with ZmCCaMK antibody and then subjected to an in-gel kinase assay. (D) Dose dependence for BR-induced ZmCCaMK activation. The detached plants were treated with 0, 5, 10 or 20 nM BR for 45 min. Protein extracts were subjected to immunoprecipitation kinase assay. (E) Time course of PEG-induced gene expression of *ZmCCaMK* (left) and ZmCCaMK activity (right). The maize seedlings were treated with 10% PEG for various times as indicated. Seedlings treated with distilled water under the same conditions served as controls. (F) Effect of pre-treatment with the BR biosynthesis inhibitor brassinazole (Brz) on expression of *ZmCCaMK* (left) and activity of ZmCCaMK (right) in maize leaves exposed to PEG treatment. The detached plants were pre-treated with distilled water or 5 μM Brz for 4 h, and then exposed to 10% PEG for 30 min (left) or 60 min (right). BR (10 nM) was added to overcome the effects of Brz. 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.

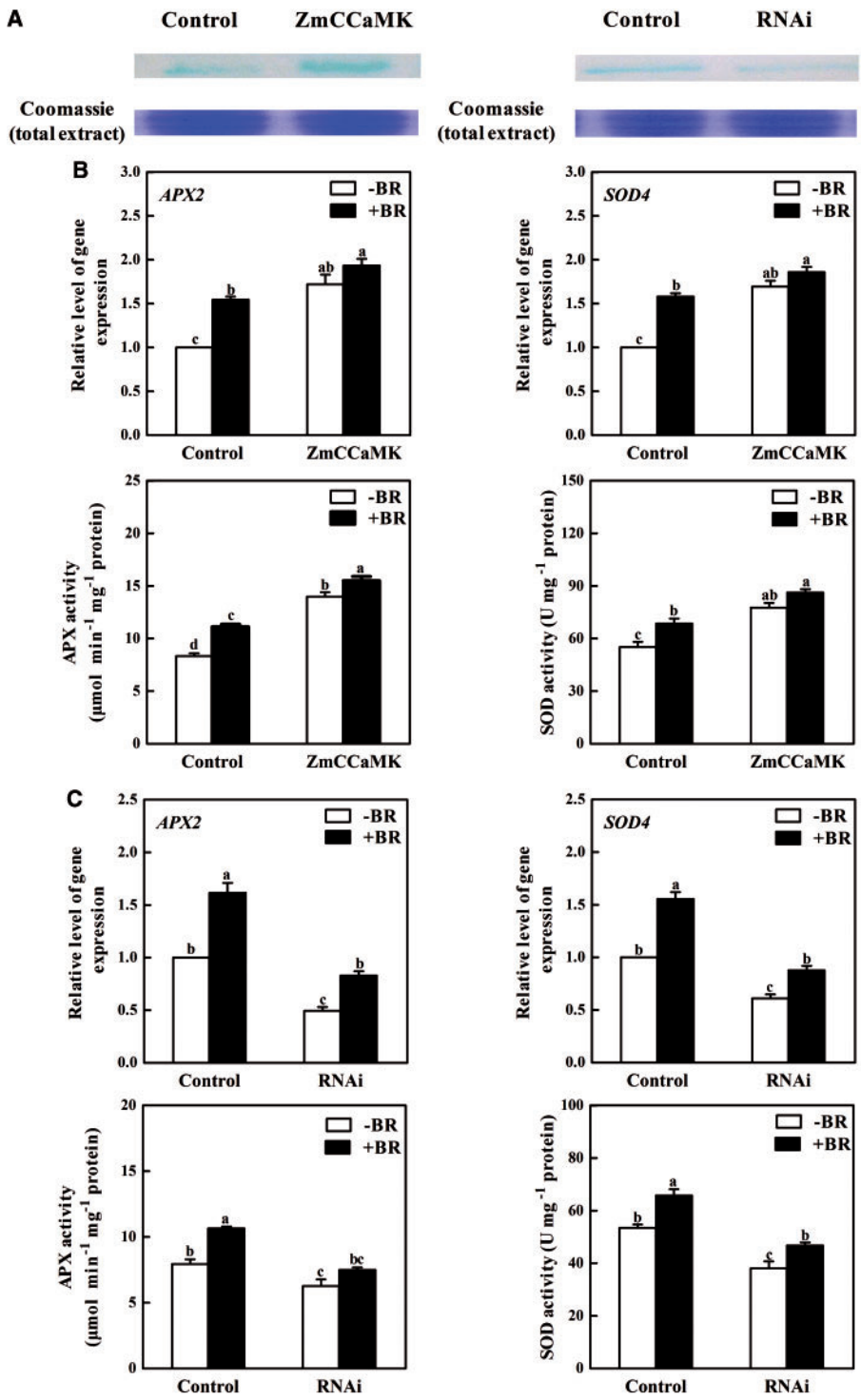

Fig. 4 ZmCCaMK is involved in the BR-induced up-regulation in gene expression and activities of antioxidant enzymes APX and SOD in maize protoplasts. (A) The protein levels of ZmCCaMK in the protoplasts transiently expressing or silencing *ZmCCaMK*. (B) The expression of *APX2* and *SOD4* and activities of APX and SOD in the protoplasts transiently expressing *ZmCCaMK*. The protoplasts were transfected with constructs carrying ubi-*ZmCCaMK*-YFP (ZmCCaMK) or empty vector (Control) and incubated for 16 h. (C) The expression of *APX2* and *SOD4* and activities of APX and SOD in the protoplasts transiently silencing *ZmCCaMK*. The protoplasts were transfected with dsRNA against *ZmCCaMK* (RNAi) or distilled water (Control) and incubated for 24 h. The protoplasts (B, C) were treated with culture medium (−BR) or 10 nM BR (+BR) for 5 min (expression of *APX2* and *SOD4*) or 10 min (activities of APX and SOD). The protein levels of ZmCCaMK and the relative expression levels of *APX2* and *SOD4* and the activities of APX and SOD were measured as described in the Materials and Methods. In (A), experiments were repeated at least three times with similar results. In (B) and (C), 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.

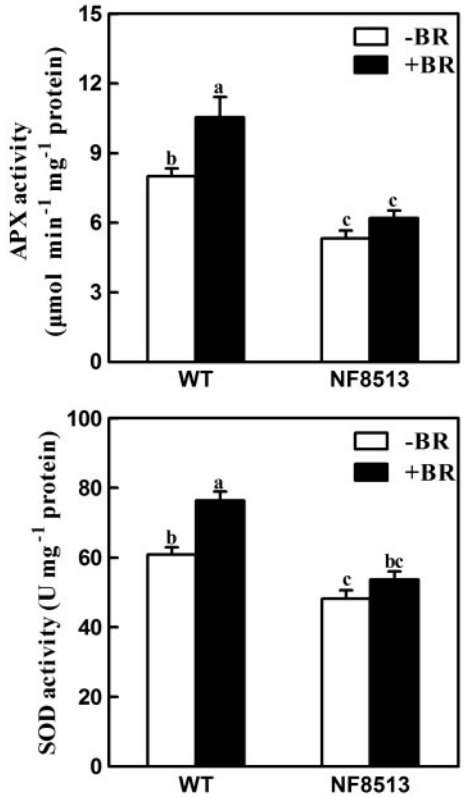

Fig. 5 Total activities of APX and SOD in leaves of the rice mutant of *OsDMI3* and the wild type exposed to BR treatment. The plants were treated with 10 nM BR or distilled water for 12 h. Values are means ± SE of three different experiments. Means denoted by the same letter did not differ significantly at $P < 0.05$ according to Duncan's multiple range test.

Discussion

Cytosolic Ca^{2+} functions as a ubiquitous second messenger in plants, and elevations in the concentration of cytosolic Ca^{2+} represent a central hub where multiple signal transduction pathways intersect (Monshausen 2012). There are only a few studies relating Ca^{2+} to BR signaling, e.g. it has been reported that Ca^{2+}-mediated signaling has a critical role in BR biosynthesis by controlling the function of DWF1 (Du and Poovaiah 2005) and both Ca^{2+} and BR increased the transcript level of *TaIAA1* (Singla et al. 2006). Recently, Oh et al. (2012) found that CaM bound to the recombinant cytoplasmic domain of BRI1 in a Ca^{2+}-dependent manner. Further studies revealed that BR directly caused an elevation in cytosolic Ca^{2+} in Arabidopsis, indicating a role for Ca^{2+} in BR signaling (Zhao et al. 2013). Through analysis of the Ca^{2+} concentration in maize protoplasts exposed to BR treatment (**Fig. 1**), we found that BR induces elevation of the cytosolic Ca^{2+} concentration in an important crop plant. Our results further suggest that Ca^{2+} functions in the BR-induced antioxidant defense (**Fig. 2**). BR signal transduction is one of the best studied signaling pathways in plants (Jaillais et al. 2011). BRI1, a leucine-rich repeat receptor kinase, perceives BR at the cell surface, and then acts through autophosphorylation and transphosphorylation to transduce the BR response. However, some recent studies point to a signaling cascade distinct from the phosphorylation/dephosphorylation cascade (Witthoft and Harter 2011, Harter et al. 2012, Zhao et al. 2013). Zhao et al. (2013) showed that BR induced Ca^{2+} and affected the expression of several genes independently of BR phosphorelay signaling downstream from the BRI1 receptor. They proposed that Ca^{2+} shuts down the BRI1-dependent phosphorelay cascade by regulating CaM or the cyclic nucleotide-gated channel. Apparently, BR-dependent Ca^{2+} signaling and the well-characterized phosphorylation/dephosphorylation system act independently to activate different BR-responsive genes. Therefore, the role of Ca^{2+} in BR-induced antioxidant defense might be independent of the phosphorelay cascade.

The key question in the field of Ca^{2+} signaling is by what means this simple ion can regulate such a wide spectrum of cellular processes. A cell must activate a unique combination of Ca^{2+} sensors to respond appropriately to a specific perturbation in cytosolic Ca^{2+} concentration induced by various stimuli. In animals, CaMKII, which has high homology to plant CCaMK (Yang et al. 2007), is an important decoder of Ca^{2+} signal (Smedlerand Uhlén 2014). CaMKII and PP1 function together as a simple molecular device that specifically translates strong Ca^{2+} signals into all-or-none potentiation of individual hippocampal synapses (Bradshaw et al. 2003). In plants, CCaMK has been demonstrated to be involved in the root nodule and arbuscular mycorrhizal symbioses, meiosis and mitosis (Yang and Poovaiah 2003, Levy et al. 2004, Mitra et al. 2004, Gleason et al. 2006, Chen et al. 2007, Hayashi et al. 2010, Shimoda et al. 2012, Takeda et al. 2012). During the last few years, significant advances in determination of the role of the CCaMK in abiotic stress tolerance have also been reported. CCaMK was significantly influenced at the transcriptional and post-translational level by ABA, PEG, NaCl, H_2O_2 and NO in maize, rice and wheat (Yang et al. 2011, Ma et al. 2012, Shi et al. 2012, Shi et al. 2014). Here we identified a Ca^{2+} decoder, ZmCCaMK, which responds to BR treatment at the transcriptional and post-translational levels in leaves of maize (**Fig. 3**). Moreover, our results also showed that ZmCCaMK functions in BR-induced antioxidant defense and requires BR-induced Ca^{2+} for the signal transduction (**Fig. 4–6**). The structure of CCaMK includes three EF-hand motifs that potentially bind Ca^{2+} ions, and it is therefore likely that Ca^{2+} binds directly to ZmCCaMK to regulate the activity of ZmCCaMK in BR signaling. However, BR-induced Ca^{2+} also affected the expression of *ZmCCaMK* (**Fig. 6**), so there is another pathway linking Ca^{2+} and the de novo synthesis of ZmCCaMK.

Our results further revealed that ZmCCaMK regulates the cytosolic Ca^{2+} concentration, forming a feedback loop in BR signaling (**Fig. 7**). The cytosolic concentration of Ca^{2+} is influenced by many factors. Ca^{2+} enters plant cells through Ca^{2+}-permeable ion channels in the plasma membrane (White 2000). Furthermore, Ca^{2+} released from intracellular compartments, such as the central vacuole and endoplasmic reticulum, also contributes to control cytosolic Ca^{2+} concentration in a variety of physiological responses (Berridge 2002, Pottosin and Schönknecht 2007). A recent study showed that BRI1

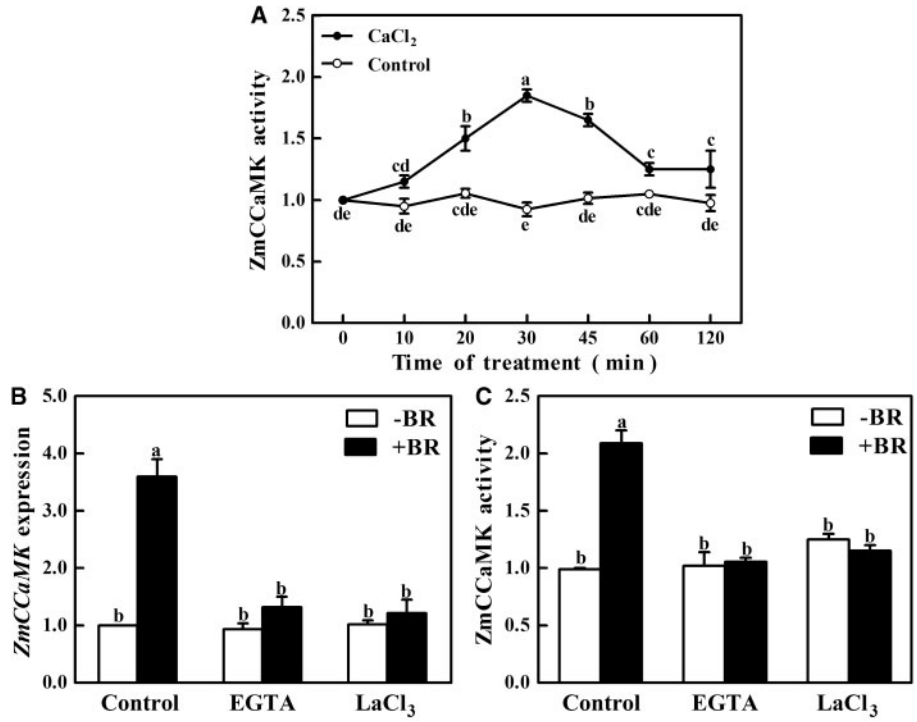

Fig. 6 Ca^{2+} is required for BR-induced gene expression and activation of ZmCCaMK in maize leaves. (A) $CaCl_2$-induced changes in activity of ZmCCaMK in maize leaves. The detached plants were treated with 20 mM $CaCl_2$ for various times as indicated, and the activity of ZmCCaMK was analyzed by immunoprecipitation kinase assay. (B) Effects of pre-treatment with a Ca^{2+} chelator and a Ca^{2+} channel blocker on expression of *ZmCCaMK* in maize leaves exposed to BR treatment. The detached plants were pre-treated with 5 mM EGTA and 5 mM $LaCl_3$ for 4 h, and then exposed to 10 nM BR for 30 min. (C) Effects of pre-treatment with a Ca^{2+} chelator and a Ca^{2+} channel blocker on the activity of ZmCCaMK in maize leaves exposed to BR treatment. The detached plants were pre-treated with 5 mM EGTA and 5 mM $LaCl_3$ for 4 h, and then exposed to 10 nM BR for 45 min. Plants treated with distilled water under the same conditions served as controls. 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.

contributes to the BR-induced Ca^{2+} signal (Zhao et al. 2013). However, there is no direct evidence to link CCaMK to regulation of the cytosolic Ca^{2+} concentration in BR signaling so far, and the mechanism by which ZmCCaMK affects the cytosolic Ca^{2+} concentration remains to be elucidated. Taken together, our results indicate that BR-induced cytosolic Ca^{2+} concentration elevation activated ZmCCaMK, which then promoted further elevation in cytosolic Ca^{2+} concentration to amplify BR signaling.

Although high concentrations of ROS are cytotoxic, lower concentrations of ROS have been considered as an important cellular signal (Miller et al. 2010, Mittler et al. 2011). Our recent study showed that H_2O_2 treatment increased gene expression and activity of ZmCCaMK in maize leaves (Ma et al. 2012), and we show here that H_2O_2 is required for the BR-induced up-regulation in gene expression and activity of ZmCCaMK (**Fig. 8A**), suggesting that H_2O_2 regulates ZmCCaMK in BR signaling. However, previous studies also showed that *OsDMI3*, the rice ortholog of *ZmCCaMK*, is involved in the regulation of H_2O_2 synthesis in ABA signaling (Shi et al. 2012). Here, our results clearly indicate that ZmCCaMK also affects H_2O_2 accumulation in BR signaling (**Fig. 8B–D**). BR-induced apoplastic H_2O_2 originated mainly from NADPH oxidase, and H_2O_2 can amplify itself by regulating NADPH oxidase (Zhang et al. 2010). In the present study, ZmCCaMK affected the expression of BR-induced NADPH oxidase genes (**Fig. 9**). These data suggest that H_2O_2 induced by BR activated ZmCCaMK, and then ZmCCaMK further stimulated the production of H_2O_2 by up-regulating NADPH oxidase gene expression, forming a positive amplification loop.

In conclusion, the central tenet of the work presented is the novel finding that Ca^{2+} works together with ZmCCaMK in BR-induced antioxidant defense. There is a close link between Ca^{2+} and ZmCCaMK and a feedback loop between ZmCCaMK and H_2O_2 in BR signaling in plants. BR induces H_2O_2 production and elevated Ca^{2+}, which up-regulate gene expression and activity of ZmCCaMK that in turn results in further Ca^{2+} accumulation and enhances the H_2O_2 production via NADPH oxidase. Ultimately the signal transduction induces antioxidant defense systems to scavenge excess H_2O_2 (**Fig. 10**).

Materials and Methods

Plant materials and treatments

Maize (*Zea mays* L. cv. Nongda 108; from Nanjing Agricultural University, China), rice (*Oryza sativa*) cultivar Nipponbare and the rice mutant line NF8513 were used in this study. In the mutant experiments, Nipponbare was used as the wild-type control. Seeds of maize were sown in trays of sand, and rice plants were grown hydroponically with a nutrient solution in a growth

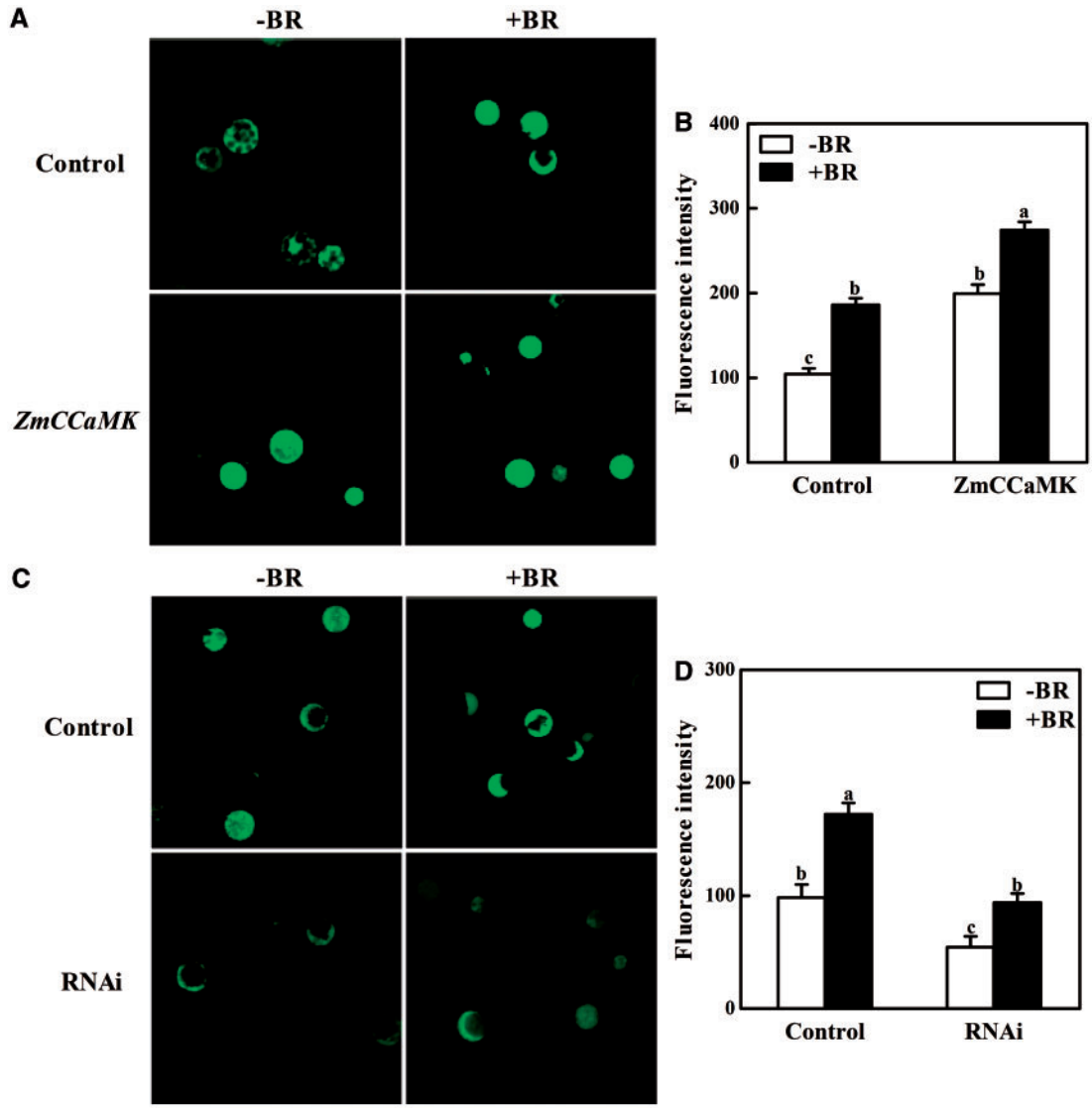

Fig. 7 ZmCCaMK mediates the BR-induced cytosolic Ca^{2+} concentration in maize protoplasts. (A) Ca^{2+} fluorescence in protoplasts transiently expressing *ZmCCaMK*. The protoplasts were transfected with constructs carrying ubi-*ZmCCaMK*-mCherry (ZmCCaMK) or empty vector (Control), then loaded with Fluo-3/AM, treated with culture medium (−BR) or 10 nM BR (+BR), and observed by laser scanning confocal microscopy (LSCM). (B) Quantitation of the fluorescence intensity in (A). (C) Ca^{2+} fluorescence in the protoplasts transiently silencing *ZmCCaMK*. The protoplasts were transfected with dsRNA against *ZmCCaMK* (RNAi) or distilled water (Control), then loaded with Fluo-3/AM, and treated with culture medium (−BR) or 10 nM BR (+BR), and observed by LSCM. (D) Quantitation of the fluorescence intensity in (C). The fluorescence intensity (B, D) was measured by fluorescence microscopy and calculated as the average value of those obtained by scanning > 50 protoplasts from three different experiments. In (A) and (C), experiments were repeated at least three times with similar results. In (B) and (D), 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.

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. For protoplast isolation, maize plants were grown at 26°C under dark conditions. When the second leaves were fully expanded, they were collected and used for investigations.

The plants were excised at the base of the stems and placed in distilled water for 2 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, 20 mM $CaCl_2$ or 10% (w/v) PEG6000 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 5 mM EGTA, 5 mM $LaCl_3$, 100 μM DPI, 200 U of CAT, 10 mM DMTU or 5 μM Brz for 4 h, and then subjected to 10 nM BR or 10% PEG treatment for various times under the same conditions as described above. Detached plants were treated with distilled water under the same conditions for the whole period to serve as controls for the above. After treatments of detached plants, the second leaves were sampled and immediately frozen under liquid N_2 for further analysis.

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

Total RNA was isolated from leaves or protoplasts using an RNAiso Plus kit (TAKARA) according to the instructions supplied by the manufacturer. DNase treatment was included in isolation step using RNase-free DNase (TAKARA).

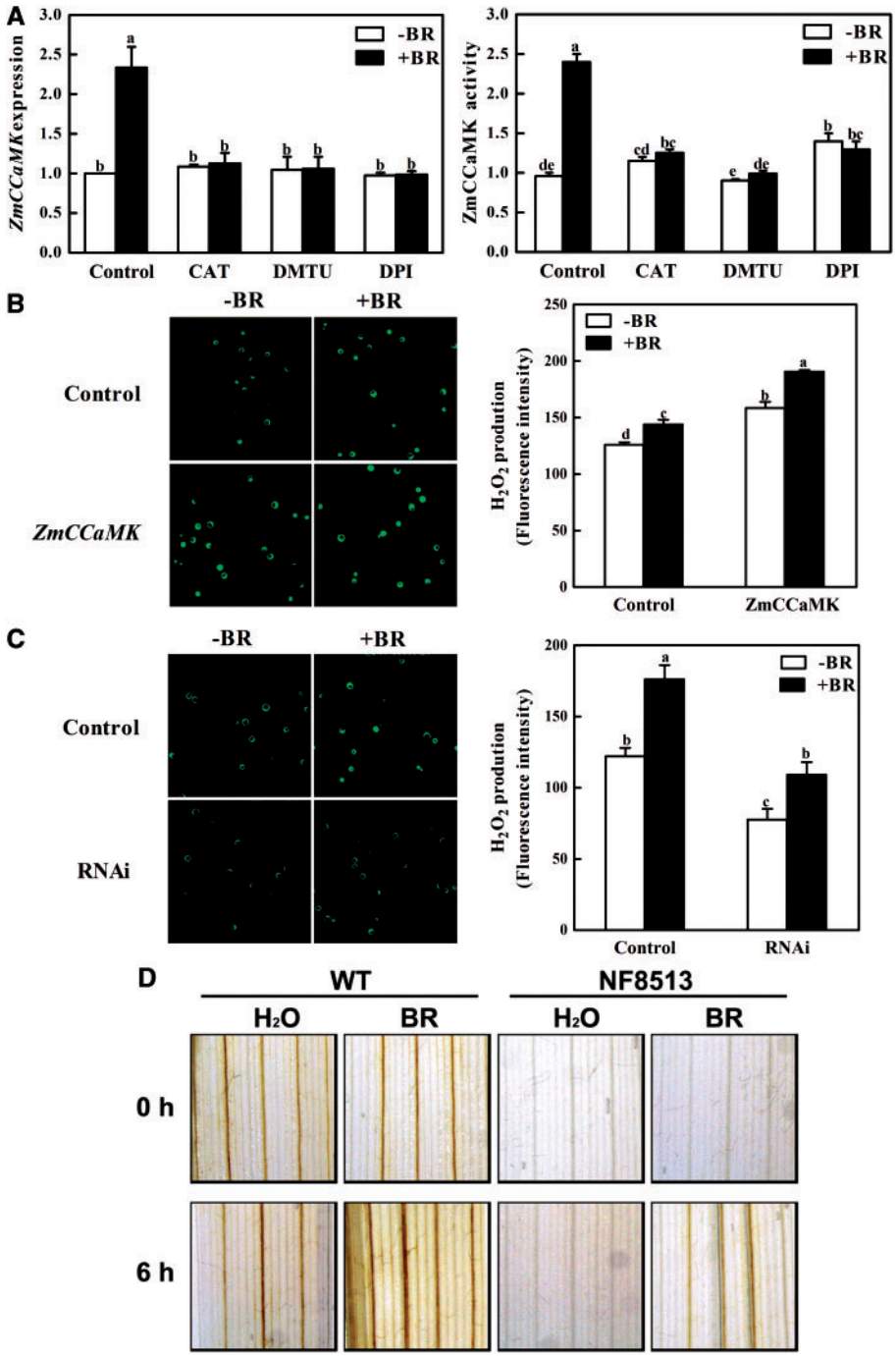

Fig. 8 Relationship between ZmCCaMK and H_2O_2 in BR signaling. (A) Effects of pre-treatment with the H_2O_2 scavenger or inhibitor CAT, DMTU and DPI on expression of *ZmCCaMK* (left) and activity of ZmCCaMK (right) in maize leaves exposed to BR treatment. The detached plants were pre-treated with 200 U of CAT, 10 mM DMTU or 100 μM DPI for 4 h, and then exposed to 10 nM BR for 30 min (left) or 45 min (right). Plants treated with distilled water under the same conditions served as controls. (B) H_2O_2 fluorescence (left) and quantitation of the fluorescence intensity (right) in the protoplasts transiently expressing *ZmCCaMK*. The protoplasts transfected with constructs carrying ubi-*ZmCCaMK*-mCherry (ZmCCaMK) or empty vector (Control) were treated with 10 nM BR (+BR) or incubation medium (−BR) for 10 min and then loaded with H_2DCF-DA for 10 min. H_2O_2 was visualized by confocal microscopy. (C) H_2O_2 fluorescence (left) and quantitation of the fluorescence intensity (right) in the protoplasts transiently silencing *ZmCCaMK*. The protoplasts transfected with dsRNA against *ZmCCaMK* (RNAi) or distilled water (Control) were treated with 10 nM BR (+BR) or incubation medium (−BR) for 10 min and then loaded with H_2DCF-DA for 10 min. H_2O_2 was visualized by confocal microscopy. (D) Histochemical detection of leaf H_2O_2 production in the rice mutant of *OsDMI3* and the wild type exposed to BR treatment. The plants were excised at the base of the stem and the detached plants were treated with 10 nM BR or distilled water for 6 h; H_2O_2 production in leaves was detected by DAB staining. In (B–D), experiments were repeated at least three times with similar results. In (A), 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.

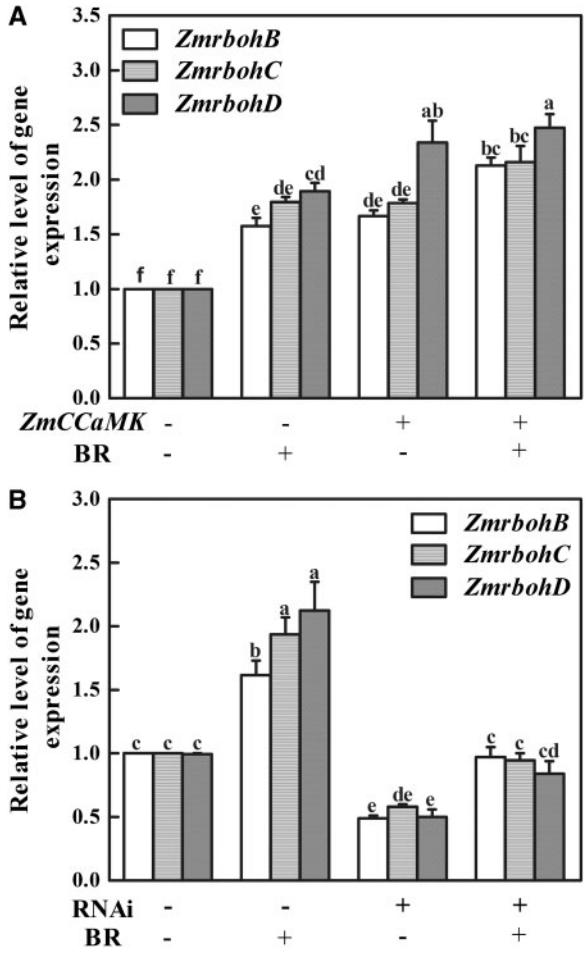

Fig. 9 ZmCCaMK affects the expression of NADPH oxidase genes in maize protoplasts. (A) Expression analysis of *ZmrbohB*–*ZmrbohD* in the protoplasts transiently expressing *ZmCCaMK*. The protoplasts were transfected with constructs carrying ubi-*ZmCCaMK*-YFP (ZmCCaMK+) or empty vector (ZmCCaMK−). (B) Expression analysis of *ZmrbohB*–*ZmrbohD* in the protoplasts transiently silencing *ZmCCaMK*. The protoplasts were transfected with dsRNA against *ZmCCaMK* (RNAi+) or distilled water (RNAi−). The protoplasts (A, B) were treated with culture medium (BR−) or 10 nM BR (BR+) for 10 min, and the relative expression level of *ZmrbohB-D* was analyzed by 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.

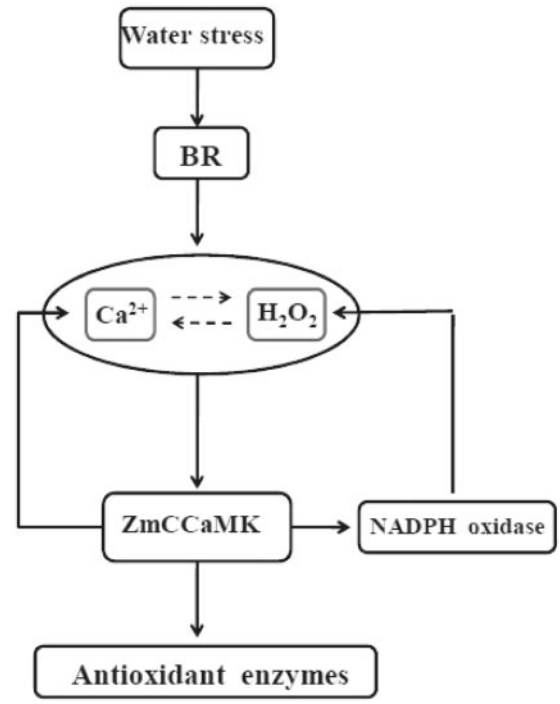

Fig. 10 Model of the relationship of H_2O_2, Ca^{2+} and ZmCCaMK in BR-induced antioxidant defense. BR induces H_2O_2 production and Ca^{2+} accumulation, which cause an increase in both transcription and activity of ZmCCaMK, leading to up-regulation of antioxidant defense enzymes. Meanwhile, ZmCCaMK affects Ca^{2+} accumulation and also enhances the H_2O_2 via NADPH oxidase, forming two positive feedback loops.

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) with SYBR® Premix Ex Taq™ (TAKARA) according to the manufacturer's instructions. The cDNA was amplified by PCR using the following primers: *ZmCCaMK* (GenBank accession No. DQ403196), forward CTCAAGCCCGAGAACTGCC and reverse T GGCAGCCGAGACATCC; *SOD4* (GenBank accession No. X17565), forward TG GAGCACCAGAAGATGA and reverse CTCGTGTCCACCCTTTCC; *APX2* (GenBank accession No. EU969033), forward TGAGCGACCAGGACATTG and reverse GAGGGCTTTGTCACTTGGT; *ZmrbohB* (GenBank accession No. EU807966), forward GGCCAGTACTTCGGTGAAACA and reverse ATTACACC AGTGATGCCTTCCA; *ZmrbohC* (GenBank accession No. DQ897930), forward TTCTCTTGCCTGTATGCCGC and reverse CTTTCGTATTCCGCAGCCA; *ZmrbohD* (GenBank accession No. EF364442), forward CCGGCTGCAGACGT TCTT and reverse CCTGATCCGTGATCTTCGAAA; and *ZmACTIN* (GenBank accession No. J01238), forward GTTTCCTGGGATTGCCGAT and reverse TCTG CTGCTGAAAAGTGCTGAG. To standardize the results, the amplification of *ZmACTIN* was determined and used as the internal standard. The data were normalized to the amplification of a maize *ZmACTIN* gene.

Antibody production and immunoprecipitation kinase activity assay

A peptide for ZmCCaMK-C (GDITEPGKLDEVFD) corresponding to the C-terminus of ZmCCaMK was synthesized and conjugated to keyhole limpet hemocyanin. The ZmCCaMK polyclonal antibody was raised in rabbits and purified by affinity chromatography. Protein was extracted from maize leaves as described previously (Ma et al. 2012). Protein content was determined according to the method of Bradford (1976) with bovine serum albumin (BSA) as standard. For immunocomplex kinase assay, protein extract (100 μg) was incubated with anti-ZmCCaMK antibody (7.5 μg) in an immunoprecipitation buffer as described previously (Ma et al. 2012). Kinase activity in the immunocomplex was incubated in reaction buffer [25 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM dithiothreitol (DTT), 2.5 mM $CaCl_2$, 2 μM CaM, 1 mg ml^{-1} myelin basic protein (MBP) with 200 nM ATP plus 1 μCi of [γ-^{32}P]ATP (3,000 Ci mM^{-1}) for 30 min. An equal volume of SDS sample buffer was added to stop the reaction. The reaction mix was boiled at 100°C for 5 min and resolved by SDS–PAGE. The unincorporated [γ-^{32}P]ATP was removed by washing with 5% trichloroacetic acid (w/v)/1% sodium pyrophosphate (w/v) at least three times. The gel was dried onto Whatman 3 MM paper and exposed to Kodak XAR-5 film. Relative activation levels of ZmCCaMK protein were quantitated by Quantity One software (Bio-Rad Laboratories Inc.).

Western blot assay

Proteins were extracted from protoplasts transfected with ubi-*ZmCCaMK*-YFP (yellow fluorescent protein; empty vector as control) or dsRNAs (H_2O as

control), and 20 μg of total protein was subjected to SDS–PAGE. Western blot analysis was performed as described by Sambrook and Russell (2001). Anti-ZmCCaMK antibody was used to detect the ZmCCaMK protein.

Antioxidant enzyme assay

The detached plants and protoplasts were homogenized in 0.6 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 12,000 × *g* for 30 min at 4°C and the supernatant was immediately used for the subsequent antioxidant enzyme assays. The total activities of antioxidant enzymes were determined as previously described (Zhu et al. 2013). Total SOD activity was assayed by monitoring the inhibition of photochemical reduction of nitroblue tetrazolium. One unit of SOD activity was defined as the amount of enzyme that was required to cause 50% inhibition of the reduction of nitroblue tetrazolium, as monitored at 560 nm. Total APX activity was measured by monitoring the decrease in absorbance at 290 nm as ascorbate was oxidized.

Histochemical detection of H_2O_2

H_2O_2 was visually detected in the leaves of plants by using DAB as substrate (Orozco-Cardenas and Ryan 1999).

H_2O_2 detection by confocal laser scanning microscopy

H_2O_2 production in protoplasts was monitored using the H_2O_2-sensitive fluorescent probe 2,7-dichlorofluorescein diacetate (H_2DCF-DA; Molecular Probes) using the method described by Bright et al. (2006). Images acquired were analyzed using Leica IMAGE software. Data are presented as mean pixel intensities.

Measurement of cytosolic calcium concentration ($[Ca^{2+}]i$)

For measurement of $[Ca^{2+}]i$, the maize mesophyll protoplasts were loaded with the Ca^{2+}-sensitive fluorescent dye Fluo-3/AM ester (Molecular Probes) and observed by LSCM according to the method described by Zhang et al. (1998). Fluo-3/AM was added from a stock solution of 1 mM Fluo-3/AM in dimethylsulfoxide. After incubation at 4°C for 1 h in the dark, the protoplasts were washed twice with an isotonic solution and incubated at 25°C for 1 h in the dark. The incubation solution contained 20 μM Fluo-3/AM ester, 0.5 M mannitol, 4 mM MES (pH 5.7) and 20 mM KCl. Fluorescent probes were excited with a 488 nm laser, and emission fluorescence was filtered by a 515 nm filter to eliminate the autofluorescence of Chl. Pictures were taken by scanning three times each for 30 s, and then the fluorescence intensities of these pictures were measured by fluorescence microscopy after establishing a stable baseline.

In vitro synthesis of dsRNA

DNA templates were produced by PCR using primers containing the T7 promoter sequence (5′-TTAATACGACTCACTATAGGGAGG-3′) on both the 5′ and 3′ ends. The primers used to amplify DNA of *ZmCCaMK* were: forward CAAGCCCGAGAA CTGCC and reverse TGGCAGCCGAGACATCC. The PCR amplification consisted of initial denaturation at 94°C for 3 min, and then 35 cycles of 94°C for 20 s, 60°C for 15 s and 72°C for 20 s, and a final extension at 72°C for 2 min. dsRNA of *ZmCCaMK* was synthesized in vitro using the RiboMAX™ Large Scale RNA Production System-T7 (Promega) according to the manufacturer's instructions. The purity and concentration of synthesized dsRNA were checked by 2% agarose gel electrophoresis and spectrophotometry.

Protoplast preparation and transfection with constructs or dsRNAs

Protoplast isolation and transfection with constructs or dsRNAs were based on the protocol for maize mesophyll protoplasts provided online by J. Sheen's laboratory (http://genetics.mgh.harvard.edu/sheenweb) with minor modifications.

For transfection, 1 ml of maize protoplasts (usually 5×10^5 cells ml^{-1}) were transfected with 150 μg of ubi-*ZmCCaMK*-YFP or ubi-*ZmCCaMK*-mCherry fusion constructs (empty vector as control) or 100 μg of dsRNAs (H_2O as control) using a PEG–calcium-mediated method. The transfected protoplasts were then incubated in incubation solution overnight in the dark at 25°C . After that, protoplasts were collected and used for further analysis.

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Disclosures

The authors have no conflicts of interest to declare.

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