Plant Physiology and Biochemistry 185 (2022) 325–335

Contents lists available at ScienceDirect

Plant Physiology and Biochemistry

journal homepage: www.elsevier.com/locate/plaphy

ZmBSK1 positively regulates BR-induced H_2O_2 production via NADPH oxidase and functions in oxidative stress tolerance in maize

Lei Liu [a], Yanchao Sun [a,b], Meijing Zhang [a], Ruixiang Liu [a], Xiaming Wu [a], Yanping Chen [a,*], Jianhua Yuan [a,**]

[a] Provincial Key Laboratory of Agrobiology, Institute of Food Crops, Jiangsu Academy of Agricultural Sciences, Nanjing, 210014, Jiangsu, China
[b] College of Life Sciences, Nanjing Agricultural University, Nanjing, 210095, Jiangsu, China

ARTICLE INFO

Keywords:
Brassinosteroid
H_2O_2
Maize
NADPH oxidase
Oxidative stress
ZmBSK1

ABSTRACT

Brassinosteroid (BR) has been indicated to induce the production of hydrogen peroxide (H_2O_2) in plants in response to various environmental stimuli. However, it remains largely unknown how BR induces H_2O_2 production. In this study, we found that BR treatment significantly raised the kinase activity of maize (*Zea mays* L.) brassinosteroid-signaling kinase 1 (ZmBSK1) using the immunoprecipitation kinase assay. ZmBSK1 could modulate the gene expressions and activities of nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (EC 1.6.3.1) to modulate BR-induced H_2O_2 production. BR could enhance the interaction between ZmBSK1 and maize calcium/calmodulin-dependent protein kinase (ZmCCaMK), a previously identified substrate of ZmBSK1. The BR-induced phosphorylation and kinase activity of ZmCCaMK are dependent on ZmBSK1. Moreover, we showed that ZmBSK1 regulated the NADPH oxidase gene expression and activity via directly phosphorylating ZmCCaMK. Genetic analysis suggested that ZmBSK1-ZmCCaMK module strengthened plant tolerance to oxidative stress induced by exogenous application of H_2O_2 through improving the activities of antioxidant defense enzyme and alleviating the malondialdehyde (MDA) accumulation and electrolyte leakage rate. In conclusion, these findings provide the new insights of ZmBSK1 functioning in BR-induced H_2O_2 production and the theoretical supports for breeding stress-tolerant crops.

1. Introduction

Brassinosteroid (BR) is classified as a kind of plant steroid hormones and is recognized to be involved in regulating diverse physiological processes in plants (Choudhary et al., 2012; Gudesblat and Russinova, 2011; Jiroutova et al., 2018). Besides that, BR is also known to decline the impacts of various environmental stresses on plants (Nolan et al., 2020; Wang, 2012; Xia et al., 2009; Zhang et al., 2011). Hydrogen peroxide (H_2O_2), nitric oxide, and calcium acting as the signaling molecules, can be rapidly upregulated by BR and function in mediating stress response (Li et al., 2016; Yan et al., 2015; Zhang et al., 2010). Thus, it is necessary for us to clarify the mechanisms by which BR induces the production of signaling molecules.

In plants, superoxide anion (O_2^-) is mainly produced by nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (also named as respiratory burst oxidase homologue, rboh), and superoxide dismutase (SOD)-mediated O_2^- dismutation is considered as the major source of H_2O_2 production (Baxter et al., 2014). NADPH oxidase is the main cellular source of reactive oxygen species (ROS) and is reported to function in regulating diverse biological process. For instance, AtrbohH and AtrbohJ play an important role in the growth of pollen tube and maintaining the integrity of cell wall (Boisson-Dernier et al., 2013; Jimenez-Quesada et al., 2019; Kaya et al., 2014; Lassig et al., 2014). AtrbohD and AtrbohF regulate abscisic acid-mediated stomatal closure (Torres et al., 2006; Wang et al., 2020). In addition, rbohs are also involved in plant response to defense and abiotic stress (Kaur et al., 2014; Suzuki et al., 2011). AtrbohD and AtrbohF participate in increasing the tolerance to salt stress (Jiang et al., 2012; Ma et al., 2012b) and defense against pathogens (Kwak et al., 2003; Torres et al., 2006; Wang et al., 2020). BR could obviously upregulate NADPH oxidase gene expression and enzyme activity, thereby improving plant resistance to abiotic stresses (Xia et al., 2009; Yan et al., 2015; Zhang

* Corresponding author.
** Corresponding author.
E-mail addresses: chenyp@jaas.ac.cn (Y. Chen), yuanjh1123@163.com (J. Yuan).

https://doi.org/10.1016/j.plaphy.2022.06.011
Received 28 March 2022; Received in revised form 27 May 2022; Accepted 10 June 2022
Available online 14 June 2022

et al., 2010). Nevertheless, how BR stimulates NADPH oxidase remains to be determined.

Brassinosteroid-signaling kinase (BSK) is a member of receptor-like cytoplasmic kinases subfamily XII (Kim and Wang, 2010). Numerous studies have revealed that BSKs play vital roles in BR signaling pathway, plant immunity as well as process of growth (Majhi et al., 2019; Nolan et al., 2020; Su et al., 2021; Zhang et al., 2016). Genetic studies indicate that AtBSK1, AtBSK2, AtBSK5 and *Oryza sativa* BSK3 (OsBSK3) positively regulated BR signaling (Tang et al., 2008; Zhang et al., 2016). AtBSK3, acting as the scaffold protein, is identified to regulate BR signaling pathway and plant growth and development mediated by BR (Ren et al., 2019). Additionally, AtBSK1, AtBSK5, AtBSK7 and AtBSK8 interacting with an immune receptor flagellin sensitive 2 to function in pattern-triggered immunity initiated by flg22 recognition (Majhi et al., 2019, 2021; Shi et al., 2013). Moreover, BSKs have been investigated to play a crucial role in abiotic stress response. In *Arabidopsis*, AtBSK5 has been studied to be required for drought and salt stresses tolerance (Li et al., 2012). Recently, we found that *Zea mays* BSK1 (ZmBSK1)-mediated phosphorylation of maize calcium/calmodulin-dependent protein kinase (ZmCCaMK) plays significant roles in modulating maize tolerance to drought stress (Liu et al., 2021).

In maize, ZmCCaMK is essential for H_2O_2 production induced by BR (Yan et al., 2015). Therefore, we hypothesize that ZmBSK1 may also participate in that process. Here, we tested this hypothesis and reported that ZmBSK1 regulated the NADPH oxidase gene expression and enzyme activity through phosphorylating ZmCCaMK to mediate BR-induced H_2O_2 production. We also showed that ZmBSK1 phosphorylating ZmCCaMK positively regulated plant tolerance to oxidative stress.

2. Materials and methods

2.1. Plant materials and BR treatment

The sterilized seeds of maize (*Zea mays* L.) inbred line B73 and tobacco (*Nicotiana benthamiana* L.) were cultured into sterile nutrient soil at normal growth conditions (28 °C/25 °C, 16 h/8 h, light/dark). After 10 days, the roots of maize seedlings were cut off and the seedlings were putted in distilled water to eliminate injury for 2 h, then the seedlings were transferred into distilled water containing 50 nM BR (Liu et al., 2019) for indicated times. After BR treatment, the second leaves of seedlings were collected and were stored in a −80 °C freezer.

2.2. Immunoprecipitation (IP) kinase assay

Total proteins of maize leaves were obtained as mentioned by Ma et al. (2012a). As described in Liu et al. (2021), total proteins incubated with either anti-ZmBSK1 (10 μg, Abmart) or anti-ZmCCaMK antibody (10 μg, Abmart) in IP buffer. The kinase activity of immunoprecipitated ZmBSK1 or ZmCCaMK was detected using a gel kinase assay, and myelin basic protein (MBP, Sigma-Aldrich) acted as a general phosphorylation substrate. The dried-gel and phosphor imager (Typhoon 9410, Amersham Biosciences) were used to perform the autoradiography assay to detect the signal.

2.3. Western blot assay

According to the description by Liu et al. (2021), extracted proteins were divided by 12% SDS-PAGE. The gel was transferred to the polyvinylidene fluoride (PVDF) membrane (Merck millipore), which was then blocked with PBST solution containing 5% skimmed milk powder at 25 °C for 2 h. The membrane was incubated with primary antibodies at these dilutions: anti-ZmCCaMK antibody (1:1000), anti-ZmBSK1 antibody (1:1000), anti-actin antibody (1:5000, Biodragon). For analysis of the phosphorylation of Ser67-ZmCCaMK, the membrane was blocked with blocking solution containing TBST solution and 5% BSA (Solarbio) at 25 °C for 2 h. The membrane incubated with anti-pSer67-ZmCCaMK antibody (1:5000, GenScript). The specific anti-pSer67-ZmCCaMK antibody was prepared as described previously (Liu et al., 2021). Horseradish peroxidase-conjugated anti-rabbit or anti-mouse antibodies (Abmart) served as the secondary antibody. A CCD camera (Tanon 5200 Multi) was used to detect the chemi-fluorescent signals in the membrane. Quantification was performed using ImageJ software (version 1.51).

2.4. Firefly luciferase complementation imaging (LCI) assay

The open reading frame (ORF) without stop codon of *ZmCCaMK* and *ZmBSK1* were integrated into *35S:pCAMBIA1300-cLUC* and *35S:pCAMBIA1300-nLUC* vectors, respectively. 4-week-old tobacco leaves were infected with *Agrobacterium* strain GV3101 carried *cLUC-ZmCCaMK* or *cLUC* and *ZmBSK1-nLUC* or *nLUC*. LCI assay was conducted as mentioned by Chen et al. (2008). After 3 days, the infected leaves were evenly sprayed with 1 mM D-luciferin (Sigma-Aldrich) and maintained for 20 min. The LUC signals were then recorded by a CCD camera. For BR treatment, after 3 days of infection, the leaves were collected and the abaxial side of leaves were evenly sprayed with 50 nM BR for 30 min to detect LUC signals.

2.5. Coimmunoprecipitation (Co-IP) assay

Based on the previous report (Liu et al., 2021), total protein extracts were obtained from maize leaves using lysis buffer and incubated with anti-ZmCCaMK antibody (10 μg) bound to protein A/G agarose in IP buffer at 4 °C for 8 h. IP buffer was used to wash the agarose for three times, followed by boiling in 1x SDS loading buffer. After centrifugation, the supernatant was divided by 12% SDS-PAGE and were detected via western blotting with anti-ZmBSK1 antibody (1:1000).

2.6. Site-directed mutagenesis assay

The mutated *ZmCCaMK* was obtained using the Site-Directed Mutagenesis Kit (SBS Genetech) followed by the manufacturer's protocol. The primers used in mutagenesis were listed in Table S1. After mutagenesis, Sanger sequencing was used to confirm the mutated sequence of *ZmCCaMK*.

2.7. Maize transformation

For overexpression transgenic lines, the ORF of *ZmBSK1*, *ZmCCaMK* and its mutant forms were integrated into *Ubi:pCUN-NHF* vector. For knockdown transgenic lines, hairpin structure containing a specific RNA interference (RNAi) fragment of *ZmBSK1* and a 400 bp *GUS* intron was also integrated into *Ubi:pCUN-NHF* vector. B73 line acted as a plant receptor. The maize transformation mediated by *Agrobacterium* infection was conducted according to the descriptions of Liu et al. (2015). The positive transformants were screened with 75 mg L^{-1} Basta (Sangon Biotech) and further identified by PCR. Homozygous T_2 lines were obtained by selfing for further study.

2.8. Total RNA extraction and quantitative real-time PCR (qRT-PCR) assay

Total RNA were extracted from samples using RNAiso kit (TaKaRa) followed by the method of Yan et al. (2021). 5xAll-In-One MasterMix kit (abm) was used to synthesize the cDNA. Gene expression levels were determined by qRT-PCR using CFX96 Touch (Bio-Rad) with EvaGreen 2x qPCR MasterMix (abm) followed by the manufacturer's protocol. The specific primers were listed in Table S1. The transcript levels were normalized against that of *ZmActin2*.

2.9. Detection of H_2O_2 production and NADPH oxidase activity

H_2O_2 production in plants were determined via 3,3-diaminobenzidine (DAB) staining as mentioned by Liu et al. (2022). The quantitative analysis of H_2O_2 was performed using H_2O_2 Detection Kit (Leagene) followed by the manufacturer's protocol. The NADPH oxidase activities were measured by Plant NADPH ELISA Kit (MEIMIAN) followed by the manufacturer's protocol.

2.10. Phenotype analysis and physiological indicators measurements

For phenotype analysis, 10-day-old maize seedlings exposed to 0 mM or 100 mM H_2O_2 for 9 or 10 days. The survival rate was counted after recovering for 7 days. For physiological indicators measurements, 10-day-old maize seedlings exposed to 0 mM or 100 mM H_2O_2 for 3 days, the total activities of catalase (CAT), malondialdehyde (MDA) content and electrolyte leakage rate were detected as mentioned previously (Zhu et al., 2016).

2.11. Statistical analysis

The SPSS (v16.0) software was used to conduct statistical analysis. Statistical significance was verified by one-way or two-way ANOVA corrected with Duncan's multiple range test. Differences are regarded as significant at $P < 0.05$.

2.12. Accession number

Sequence information in this study can be discovered in MaizeGDB database based on the following accession numbers: *ZmBSK1*, Zm00001d048345; *ZmCCaMK*, Zm00001d052944; *ZmrbohA*, Zm00001d042961; *ZmrbohB*, Zm00001d043543; *ZmrbohC*, Zm00001d038762; *ZmActin2*, Zm00001d013873.

3. Results

3.1. BR induces the kinase activity of ZmBSK1

BR signaling pathway is well known to play a significant role in responses of plant to environmental stresses, and BSK1 is a key downstream component in that pathway (Gruszka, 2018; Kim et al., 2009). Here, to examine the impacts of BR on the kinase activity of ZmBSK1 in maize, maize seedlings were subjected to 50 nm BR for various times. The kinase activity of ZmBSK1 was measured through an immunoprecipitation kinase assay using the specific anti-ZmBSK1 antibody (Liu et al., 2021), and MBP acted as a phosphorylation substrate. Our results showed that BR rapidly raised the activity of ZmBSK1, and the activity of ZmBSK1 reached the maximum at 30 min. Meanwhile, BR did not influence the protein levels of ZmBSK1 (Fig. 1). The results indicate that BR positively regulates ZmBSK1 in maize.

3.2. ZmBSK1 affects BR-induced H_2O_2 production

A previous study reported that BR treatment could induce H_2O_2 production in maize leaves (Zhang et al., 2010). To determine whether ZmBSK1 is involved in that process, two independent transgenic lines of *ZmBSK1*-overexpressing (OE-*ZmBSK1*) and two independent transgenic lines of *ZmBSK1*-knockdown (RNAi-*ZmBSK1*) were constructed, and the transcript levels of *ZmBSK1* were further confirmed using qRT-PCR assay (Fig. S1). Then, the H_2O_2 content in wild-type (WT) and transgenic maize leaves were detected via 3,3-diaminobenzidine (DAB) staining. As shown in Fig. 2, under control conditions, no obvious difference in H_2O_2 content was observed between WT plants and transgenic lines. However, the H_2O_2 contents in WT plants were much higher than those in OE-*ZmBSK1* lines, while much lower than those in RNAi-*ZmBSK1* lines when exposed to BR treatment. These data indicate that ZmBSK1 is required for BR-induced H_2O_2 production.

3.3. ZmBSK1 is involved in BR-induced NADPH oxidase gene expression and activity

NADPH oxidases have been considered to be the major source of H_2O_2 production in BR signaling (Xia et al., 2009). Here, to further clarify the effects of ZmBSK1 on H_2O_2 production, we examined the NADPH oxidase gene expression and activity in both WT seedlings and transgenic lines with or without BR treatment. As shown in Fig. 3, there was no obvious difference in gene expressions of *ZmrbohA*, *ZmrbohB* and *ZmrbohC* and NADPH oxidase activity between WT plants and transgenic plants under control conditions. However, BR treatment dramatically elevated the gene expressions of *ZmrbohA*, *ZmrbohB* and *ZmrbohC* and NADPH oxidase activity in WT plants. This elevation was further improved in OE-*ZmBSK1* lines whereas was suppressed in RNAi-*ZmBSK1* lines (Fig. 3). These results indicate that BR-induced NADPH oxidase gene expression and activity depend on ZmBSK1.

3.4. BR enhances the association of ZmBSK1 with ZmCCaMK

Our recent study has found that ZmBSK1 interacting with ZmCCaMK positively regulates maize tolerance to drought stress, and ZmCCaMK is also required for BR-induced gene expression of *Zmrbohs* and H_2O_2

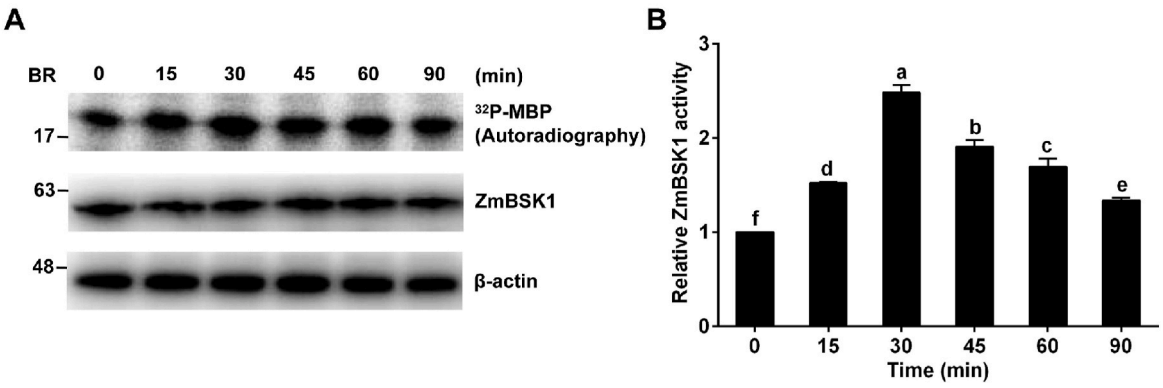

Fig. 1. BR induces the kinase activity of ZmBSK1 in maize. (A) The kinase activity of ZmBSK1 in maize leaves. 10-day-old maize seedlings exposed to 50 nM BR solution for indicated times. The kinase activity of ZmBSK1 was detected via an immunoprecipitation kinase assay, and MBP served as a substrate. The protein levels of ZmBSK1 were determined via western blotting with anti-ZmBSK1 antibody using β-actin as the loading control. The molecular weight (kDa) was displayed on the left. (B) Statistical analysis of ZmBSK1 activity as shown in (A). The kinase activity of ZmBSK1 at 0 min was specified as 1. Data are presented as means ± SD (n = 3). Different letters stand for significant difference at $P < 0.05$.

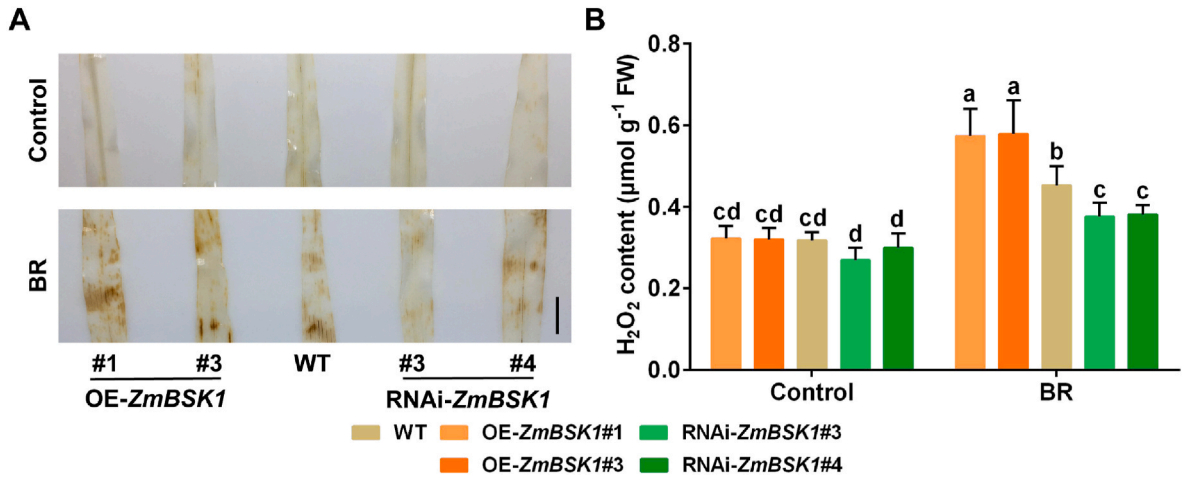

Fig. 2. ZmBSK1 regulates BR-induced H_2O_2 production. (A) DAB staining of H_2O_2 in maize leaves. The leaves were stained with 1 mg mL^{-1} DAB solution for 8 h. Scale bar = 1.5 cm. (B) The content of H_2O_2 in maize plants exposed to BR treatment. In (A–B), WT, OE-*ZmBSK1* and RNAi-*ZmBSK1* transgenic lines exposed to 0 nM or 50 nM BR solution for 45 min. Data are presented as means ± SD (n = 3). Different letters stand for significant difference at $P < 0.05$.

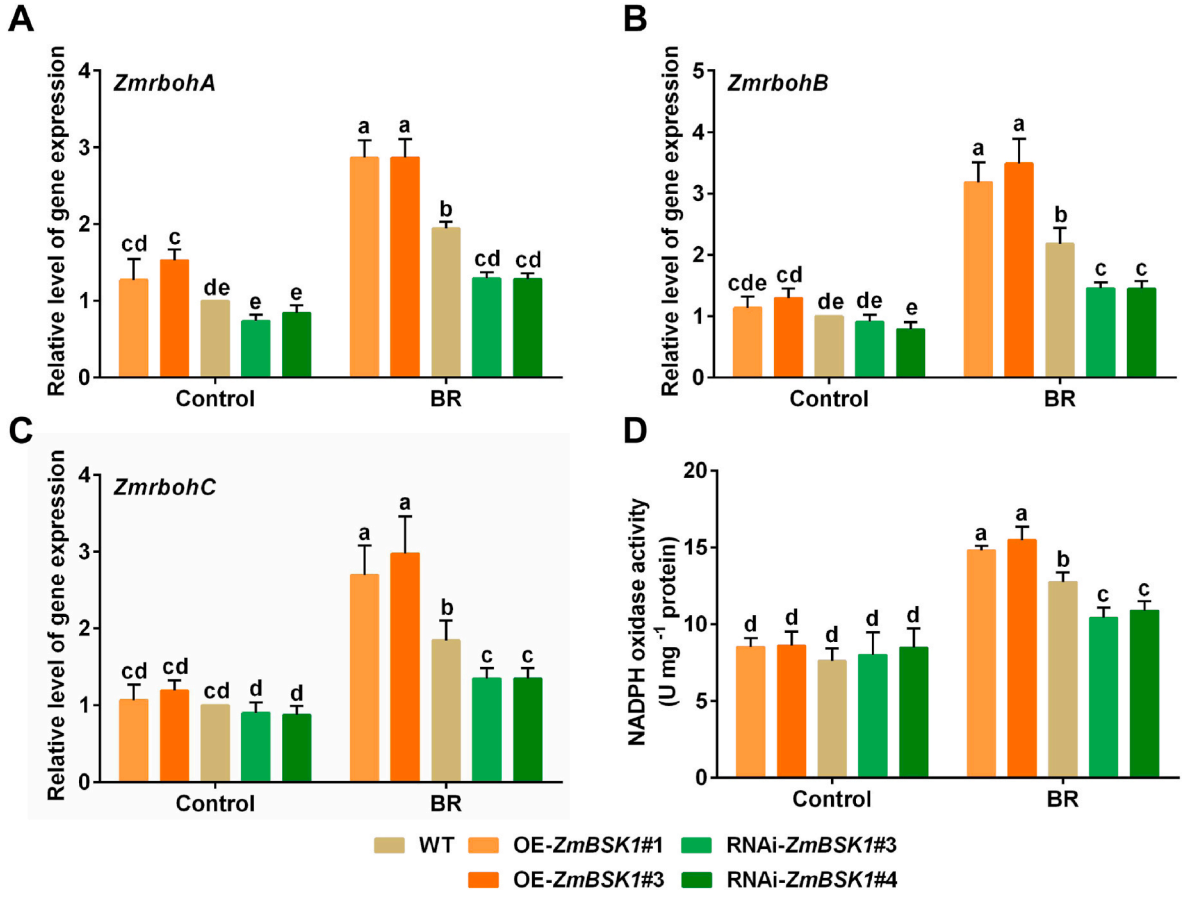

Fig. 3. ZmBSK1 positively regulates BR-induced NADPH oxidase gene expression and activity. (A–C) The gene expression levels of *ZmrbohA* (A), *ZmrbohB* (B) and *ZmrbohC* (C) in maize plants under BR treatment. The gene expression was analyzed by qRT-PCR. *ZmActin2* served as an internal control. (D) NADPH oxidase activity in maize leaves under BR treatment. In (A–D), WT, OE-*ZmBSK1* and RNAi-*ZmBSK1* transgenic lines exposed to 0 nM or 50 nM BR solution for 45 min. Data are presented as means ± SD (n = 3). Different letters stand for significant difference at $P < 0.05$.

production (Liu et al., 2021; Yan et al., 2015). Thus, we hypothesized that the association of ZmBSK1 with ZmCCaMK might play a role in BR-induced H_2O_2 production. To prove this, we first investigated the effect of BR on the interaction between them via luciferase complementation imaging (LCI) assay and coimmunoprecipitation (Co-IP) assay. For LCI assay, no obvious difference was detected in the protein levels of ZmCCaMK and ZmBSK1 in tobacco leaves with or without BR treatment (Fig. 4B), but a stronger fluorescence signal was observed in tobacco leaves after BR treatment (Fig. 4A). To further confirm the effects of BR on their interaction in maize, Co-IP assay was conducted using maize leaves. The protein levels of coimmunoprecipitated ZmBSK1 in maize leaves could be upregulated with BR treatment,

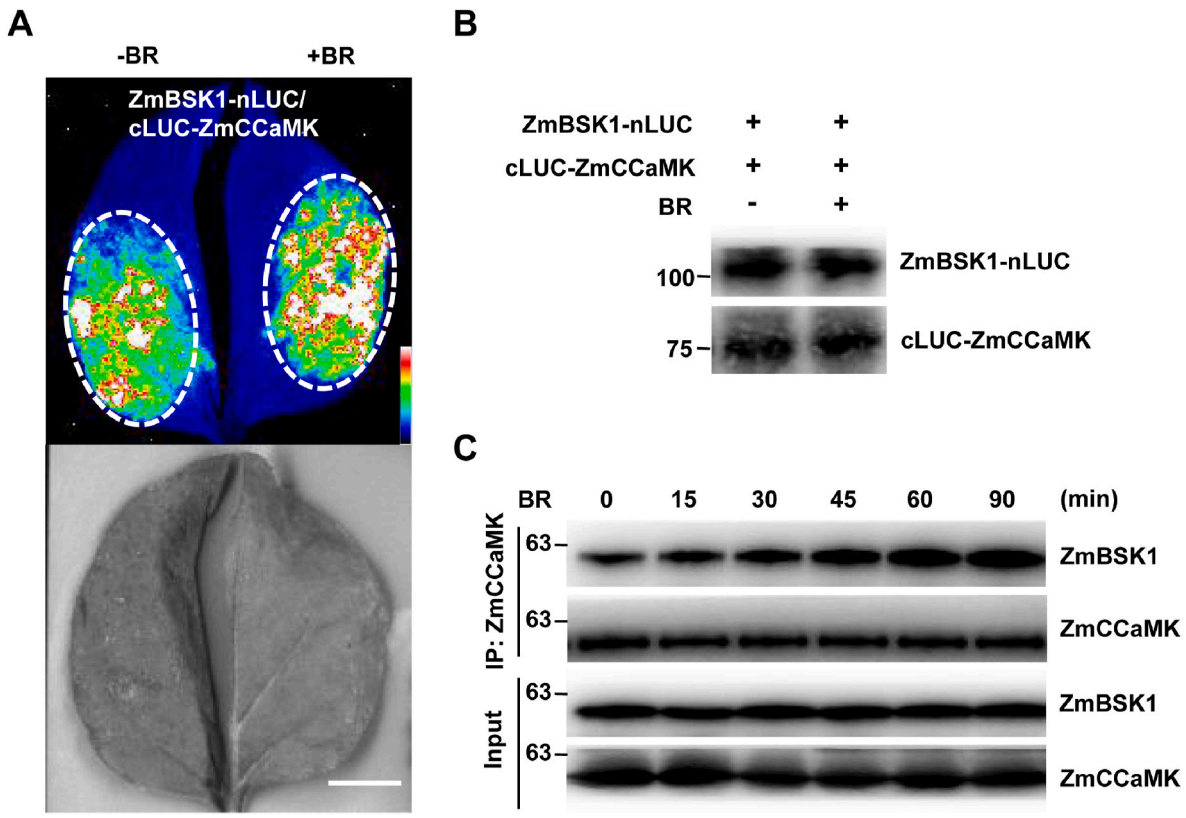

Fig. 4. BR enhances the association of ZmBSK1 with ZmCCaMK. (A) LCI assay. Tobacco leaves were infiltrated with *Agrobacterium* containing recombinant vectors *35S:cLUC-ZmCCaMK* and *35S:ZmBSK1-nLUC*. Image collected from the detached leaves which were subjected to 0 nM or 50 nM BR solution for 30 min. The dashed line indicated the same injection area. Scale bar = 1 cm. (B) Immunoblot analysis of cLUC-ZmCCaMK and ZmBSK1-nLUC in (A). (C) Co-IP assay in maize leaves. 10-day-old seedlings exposed to 50 nM BR solution for indicated times. Anti-ZmCCaMK antibody was applied for immunoprecipitating ZmCCaMK in total protein extracts from leaves. In (B–C), the protein levels of cLUC-ZmCCaMK/ZmCCaMK and ZmBSK1-Nluc/ZmBSK1 were determined via western blotting with anti-ZmCCaMK and anti-ZmBSK1 antibody, respectively. The molecular weight (kDa) was displayed on the left. All experiments had three biological replicates.

peaked at 90 min (Fig. 4C). These data indicate that BR treatment can enhance the association of ZmBSK1 with ZmCCaMK.

3.5. *ZmBSK1 affects BR-induced Ser67 phosphorylation and kinase activity of ZmCCaMK*

We have reported that ZmBSK1 phosphorylated Ser67 in ZmCCaMK both in vivo and in vitro and the phosphorylation site was essential for the kinase activity of ZmCCaMK (Liu et al., 2021). To study the impact of BR on the phosphorylation and activity of ZmCCaMK, WT seedlings were subjected to 50 nM BR, then immunoblotting assay and immunoprecipitation kinase assay were performed. As shown in Fig. 5A–D, both the Ser67 phosphorylation and activity of ZmCCaMK greatly elevated during BR treatment. Furthermore, to explore whether ZmBSK1 functions in modulating BR-induced the phosphorylation in ZmCCaMK and its kinase activity, *ZmBSK1* transgenic lines were used. We found that Ser67 phosphorylation and activity of ZmCCaMK were higher in OE-*ZmBSK1* lines than those in WT seedlings, while lower in RNAi-*ZmBSK1* lines (Fig. 5E–H). When subjected to 50 nM BR, the Ser67 phosphorylation and activity of ZmCCaMK obviously upregulated in WT seedlings, which further improved in OE-*ZmBSK1* lines while suppressed in RNAi-*ZmBSK1* lines. Together, these findings indicate that ZmBSK1 is required for BR-induced improvements in Ser67 phosphorylation levels in ZmCCaMK and its kinase activity.

3.6. *Ser67 phosphorylation in ZmCCaMK promotes BR-induced H_2O_2 production*

We wondered whether Ser67 phosphorylation in ZmCCaMK also affected BR-induced H_2O_2 production. To confirm this, ZmCCaMK^{S67A} nonphosphorylation mutant form and ZmCCaMK^{S67D} phosphomimetic mutant form were generated by substituting Ser67 for Ala and Asp, respectively. Then, *ZmCCaMK*-overexpressing lines (OE-*ZmCCaMK*), *ZmCCaMK*^{S67A}-overexpressing lines (OE-*ZmCCaMK*^{S67A}) and *ZmCCaMK*^{S67D}-overexpressing lines (OE-*ZmCCaMK*^{S67D}) were constructed and further confirmed by qRT-PCR (Fig. S2), followed by being subjected to 50 nM BR treatment. BR treatment obviously increased the expressions of *ZmrbohA*, *ZmrbohB* and *ZmrbohC* and NADPH oxidase activity in OE-*ZmCCaMK* lines, while both of which were further increased in OE-*ZmCCaMK*^{S67D} lines and blocked in OE-*ZmCCaMK*^{S67A} lines (Fig. 6A–D). Next, the H_2O_2 content in WT and transgenic lines was also detected. As shown in Fig. 6E–F, BR treatment could induce the most H_2O_2 production in OE-*ZmCCaMK*^{S67D} lines, while the least in OE-*ZmCCaMK*^{S67A} lines. Our results suggest that Ser67 phosphorylation in ZmCCaMK positively regulates BR-induced H_2O_2 production and NADPH oxidase activity and gene expression.

3.7. *ZmBSK1 enhances oxidative stress tolerance through phosphorylating ZmCCaMK at Ser67*

To further study the biological function of ZmBSK1 in maize, WT plants and *ZmBSK1* transgenic lines exposed to oxidative stress. In the absence of stress conditions, no significant difference was detected in the growth phenotype of WT and transgenic seedlings (Fig. 7A). Under oxidative stress, OE-*ZmBSK1* lines displayed slighter wilt whereas RNAi-*ZmBSK1* lines displayed more serious wilt than WT seedlings (Fig. 7A). After 7 days of recovery, compared with WT plants, OE-*ZmBSK1* lines exhibited higher whereas RNAi-*ZmBSK1* lines exhibited lower survival

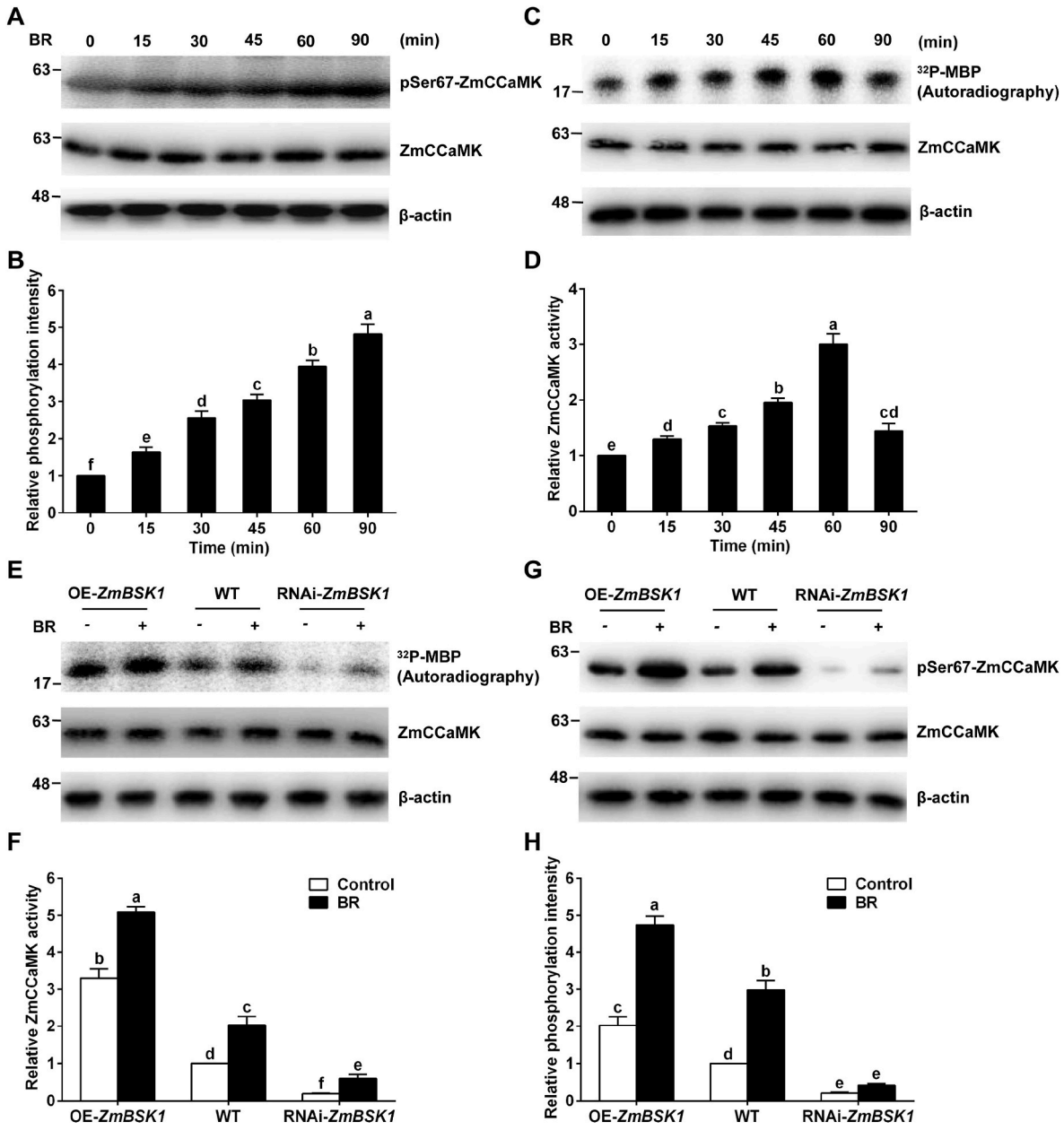

Fig. 5. BR-induced Ser67 phosphorylation in ZmCCaMK and its kinase activity are dependent on ZmBSK1. (A) Ser67 phosphorylation in ZmCCaMK (pSer67-ZmCCaMK) in WT seedlings under BR treatment. (B) Statistical analysis in (A). The degree of pSer67-ZmCCaMK at 0 min was specified as 1. (C) The ZmCCaMK activity in WT seedlings under BR treatment. (D) Statistical analysis in (C). The kinase activity of ZmCCaMK at 0 min was specified as 1. (E) The ZmCCaMK activity of in OE-*ZmBSK1*#1, WT and RNAi-*ZmBSK1*#3 lines. (F) Statistical analysis in (E). The kinase activity of ZmCCaMK in WT in the absence of BR was specified as 1. (G) Ser67 phosphorylation in ZmCCaMK in OE-*ZmBSK1*#1, WT and RNAi-*ZmBSK1*#3 lines. (H) Statistical analysis in (G). The degree of pSer67-ZmCCaMK in WT in the absence of BR was specified as 1. In (A–D), 10-day-old WT seedlings exposed to 50 nM BR solution for indicated times. In (E–H), 10-day-old WT and transgenic seedlings exposed to 0 nM or 50 nM BR solution for 45 min. In (A) and (G), protein extracts from leaves were applied for western blotting with anti-pSer67-ZmCCaMK antibody. In (C) and (E), immunoprecipitation kinase assay was conducted to detect the activity of ZmCCaMK and MBP acted as a substrate. The protein levels of ZmCCaMK were determined via western blotting with anti-ZmCCaMK antibody using β-actin as the loading control. The molecular weight (kDa) was displayed on the left. Data are presented as means ± SD (n = 3). Different letters stand for significant difference at $P < 0.05$.

rate (Fig. 7B). As expected, a similar pattern was discovered in the activity of one antioxidant defense enzyme, catalase (CAT) (Fig. 7C). Moreover, as the indicators of oxidative damage in plants, the MDA content and electrolyte leakage rate also have been determined. Oxidative stress caused obvious improvements in the accumulation of MDA and electrolyte leakage in WT plants when compared with control conditions, which were further intensified in RNAi-*ZmBSK1* lines whereas were declined in OE-*ZmBSK1* lines (Fig. 7D–E), suggesting that ZmBSK1 functions to be a positive regulator in modulating maize seedlings tolerance to oxidative stress.

To further study the role of ZmBSK1-ZmCCaMK module in response to oxidative stress, WT seedlings and *ZmCCaMK* transgenic lines were exposed to oxidative stress. OE-*ZmCCaMK*^{S67D} lines showed the least wilting among these plants and had the highest survival rate under oxidative stress (Fig. 8A–B). And there was no obvious difference in the growth phenotype of WT and transgenic lines under the normal condition. Moreover, OE-*ZmCCaMK*^{S67D} lines had the highest CAT activity (Fig. 8C) and the lowest MDA content (Fig. 8D) and electrolyte leakage rate (Fig. 8E) among these plants. These findings suggest that Ser67 phosphorylation in ZmCCaMK improves plant tolerance to oxidative

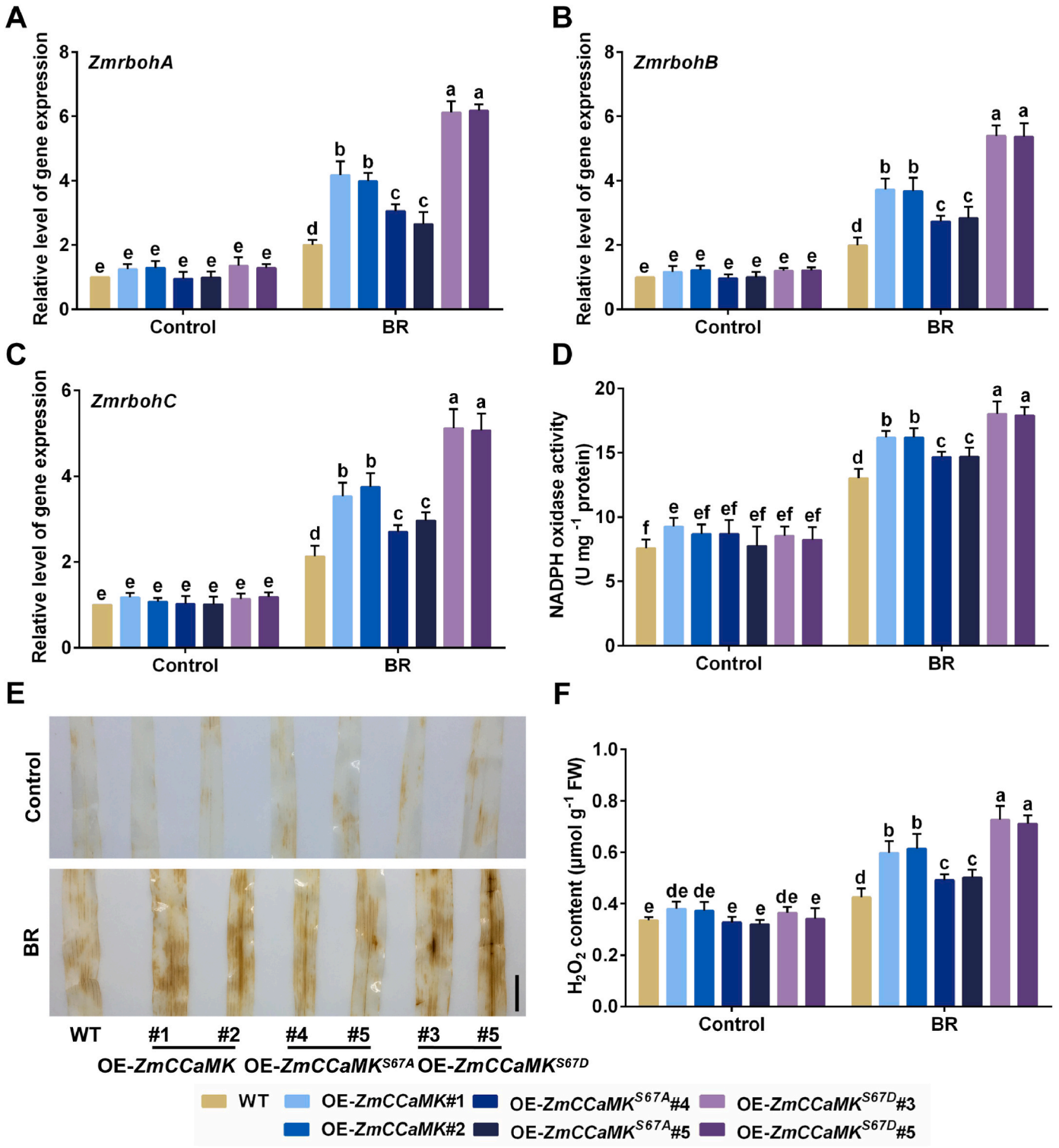

Fig. 6. The effects of Ser67 phosphorylation on NADPH oxidase gene expression and activity and H_2O_2 production. (A–C) The gene expression levels of *ZmrbohA* (A), *ZmrbohB* (B) and *ZmrbohC* (C) in maize plants under BR treatment. The gene expression was analyzed by qRT-PCR. *ZmActin2* served as an internal control. (D) The NADPH oxidase activity in maize leaves under BR treatment. (E) DAB staining of H_2O_2 in maize plants under BR treatment. The leaves were stained with 1 mg mL^{-1} DAB solution for 8 h. Scale bar = 1.5 cm. (F) The H_2O_2 content in maize plants under BR treatment. In (A–F), WT, OE-*ZmCCaMK*, OE-*ZmCCaMK*^{S67A} and OE-*ZmCCaMK*^{S67D} transgenic lines were subjected to 0 nM or 50 nM BR solution for 45 min. Data are presented as means ± SD (n = 3). Different letters stand for significant difference at $P < 0.05$.

stress through regulating the levels of antioxidant defense.

4. Discussion

In plants, BR signal transduction pathway has been extensively studied (Kim and Russinova, 2020). BR perception via brassinosteroid

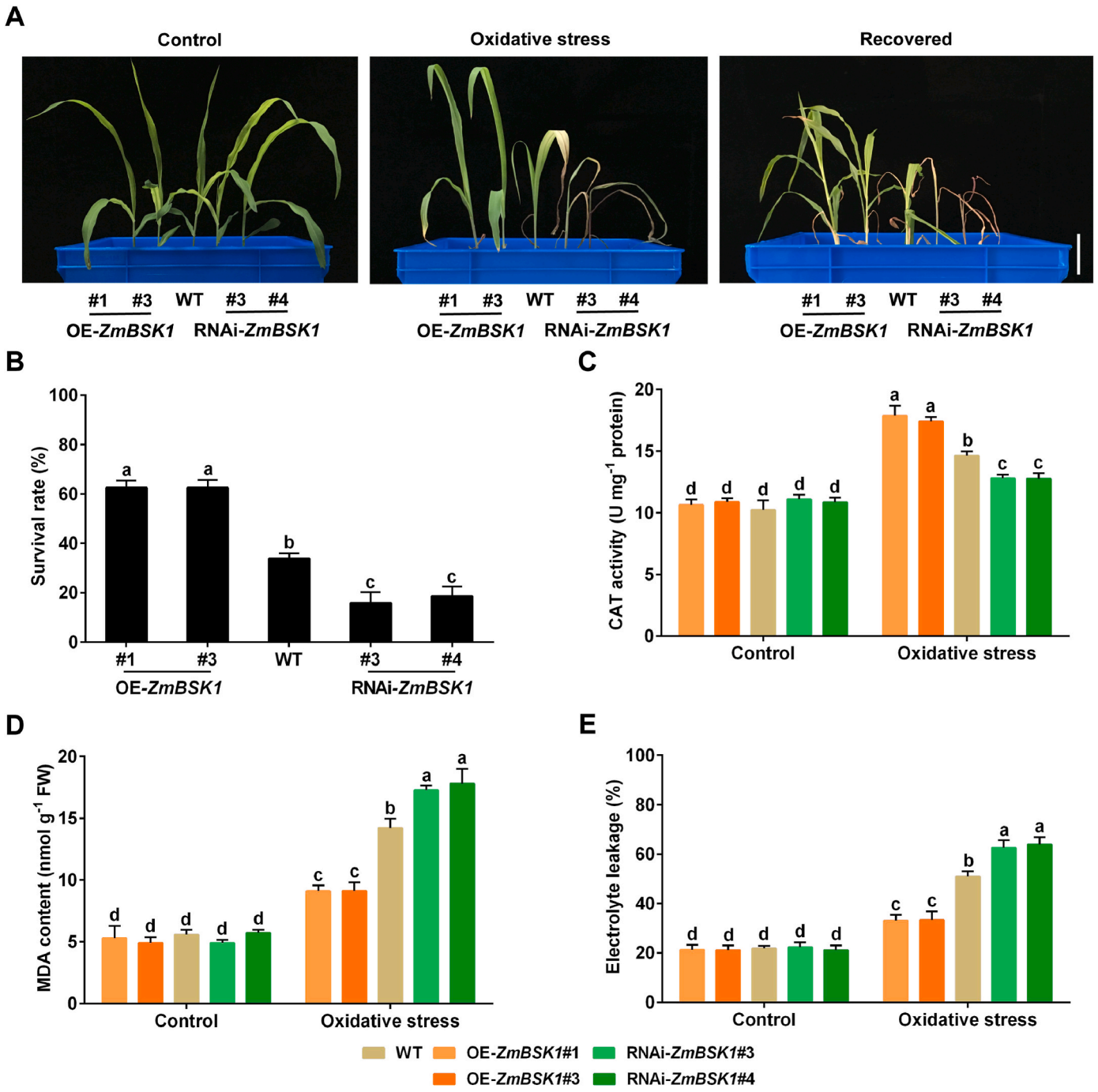

Fig. 7. ZmBSK1 enhances the oxidative stress tolerance in maize. (A) The phenotype of OE-*ZmBSK1*, WT and RNAi-*ZmBSK1* transgenic lines under oxidative stress. 10-day-old seedlings exposed to 100 mM H_2O_2 for 9 days, and then rewatered for 7 days. Scale bar = 5 cm. (B) The survival rate (%) of seedlings in (A) after 7 days of recovery. At least 30 seedlings in each line were applied for survival rate measurement. (C–E) The activity of CAT (C), MDA content (D) and electrolyte leakage rate (E) in maize under oxidative stress. 10-day-old OE-*ZmBSK1*, WT and RNAi-*ZmBSK1* transgenic lines exposed to 100 mM H_2O_2 for 3 days, followed by measuring the activity of CAT, MDA content and electrolyte leakage rate. Data are presented as means ± SD (n = 3). Different letters stand for significant difference at $P < 0.05$.

insensitive 1 (BRI1) and the co-receptor BRI1-associated kinase (BAK1) and then activate BSK1, which promotes a phosphorylation-dephosphorylation cascade reaction to modulate the expressions of BR-responsive gene (Kim and Wang, 2010). BSKs are identified to have many homologous proteins in several species, including 12 members in *Arabidopsis*, 9 members in maize, 5 members in rice (Li et al., 2019; Tang et al., 2008; Wang et al., 2017). The roles of BSKs in BR signaling have been revealed in rice and *Arabidopsis*. For example, AtBSK1, AtBSK2, AtBSK3 and AtBSK5 are considered to be BR-responsive proteins in the primary stage of BR signaling pathway (Tang et al., 2008). AtBSK1 and OsBSK3 are activated by BR and positively regulate BR signaling (Tang et al., 2008; Zhang et al., 2016). Here, our results indicated that BR could upregulate the kinase activity of ZmBSK1, but not affect its protein level (Fig. 1). This implies that BR activates ZmBSK1 in a post-translational modification, which is consistent with the activation mechanism of AtBSK1 in BR signaling pathway (Kim and Wang, 2010).

BR activates metabolic signaling pathway, in which H_2O_2 can be induced as a second messenger to transduce signals to downstream (Xia et al., 2009, 2015; Zhang et al., 2010). However, the detailed

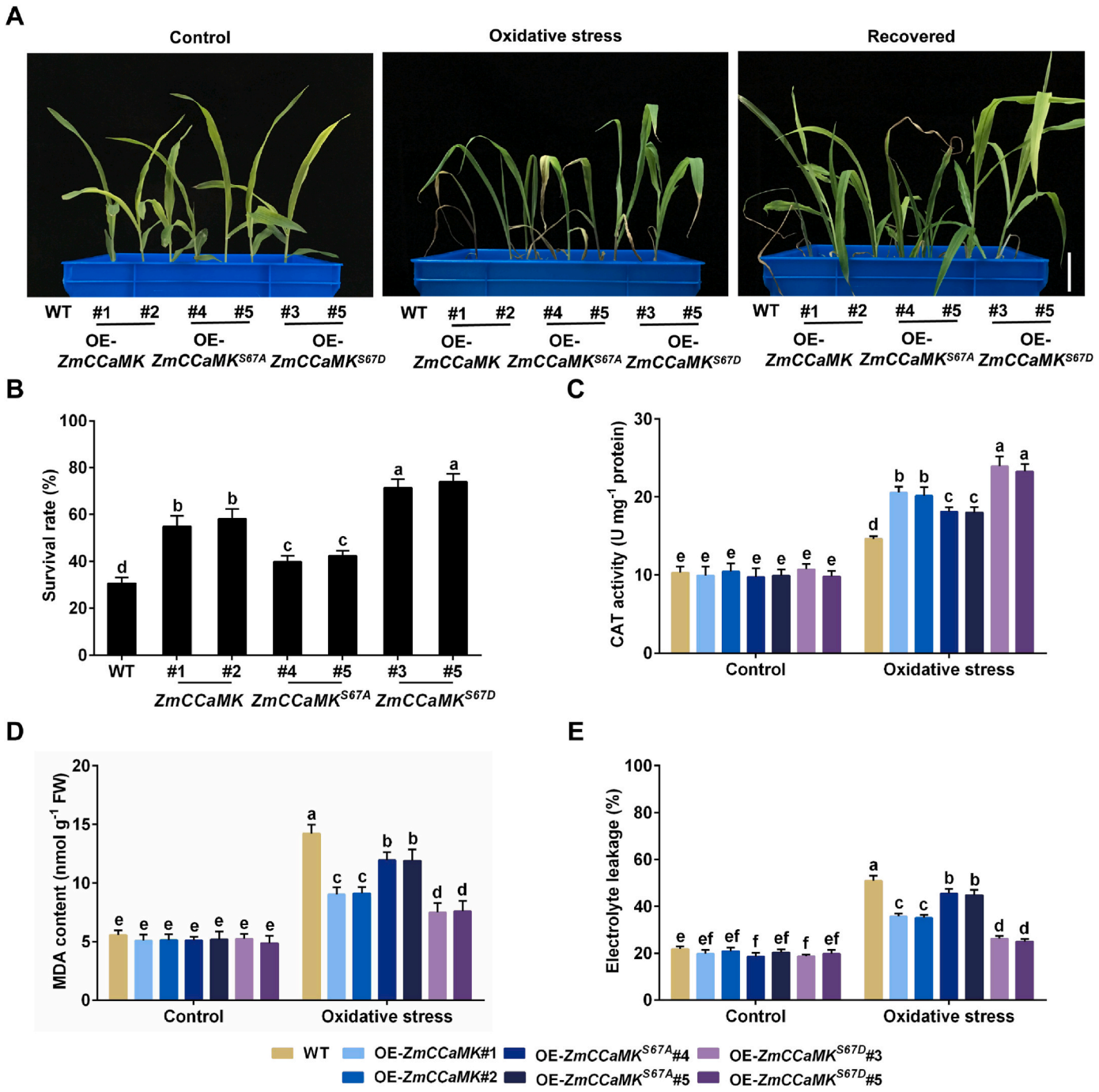

Fig. 8. Ser67 phosphorylation in ZmCCaMK positively regulates oxidative stress tolerance in maize. (A) The phenotype of WT, OE-*ZmCCaMK*, OE-*ZmCCaMK*^{S67A} and OE-*ZmCCaMK*^{S67D} transgenic lines under oxidative stress. 10-day-old seedlings exposed to 100 mM H_2O_2 for 10 days, and then rewatered for 7 days. Scale bar = 5 cm. (B) The survival rate (%) of seedlings in (A) after 7 days of recovery. At least 30 seedlings in each line were applied for survival rate measurement. (C–E) The activity of CAT (C), MDA content (D) and electrolyte leakage rate (E) in maize under oxidative stress. 10-day-old WT, OE-*ZmCCaMK*, OE-*ZmCCaMK*^{S67A} and OE-*ZmCCaMK*^{S67D} transgenic lines were subjected to 100 mM H_2O_2 for 3 days, followed by measuring the activity of CAT, MDA content and electrolyte leakage rate. Data are presented as means ± SD (n = 3). Different letters stand for significant difference at $P < 0.05$.

mechanism by which BR induces H_2O_2 production is poorly understood. In this study, we showed that ZmBSK1 promoted BR-induced H_2O_2 production (Fig. 2). BR-induced H_2O_2 production and accumulation are mainly due to the elevated activity of rbohs (Xia et al., 2009; Zhou et al., 2014). In this process, cytoplasmic Ca^{2+}-activated rbohs can transport electrons across the biological membrane to reduce molecular O_2 to O_2^- in the apoplast, which is recognized as a precursor of H_2O_2, and then O_2^- dismutates spontaneously or catalytically to H_2O_2 via SOD (Baxter et al., 2014). Interestingly, our results have found that ZmBSK1 obviously upregulated the expressions of *ZmrbohA*, *ZmrbohB* and *ZmrbohC* (Fig. 3A–C), implying that it exists the possible regulator(s) between ZmBSK1 and Zmrbohs in BR-induced H_2O_2 production. In addition, ZmBSK1 also significantly affected the total activity of rbohs (Fig. 3D). In plants, the activity of rbohs can be largely regulated in many ways, including protein phosphorylation, the binding of Ca^{2+} to its EF-hand motif in N terminus and the binding of Rac GTPase to its N terminus (Li et al., 2015; Ogasawara et al., 2008). For example, BIK1 directly associates with AtrbohD and phosphorylates its N-terminal domain to

activate AtrbohD (Kadota et al., 2014). A previous report has suggested that the association of the active OsRac1 with the N-terminal of OsrbohB enhances ROS production in rice plants (Wong et al., 2007). Therefore, we cannot exclude that there is also possibility of direct interaction between ZmBSK1 and Zmrbohs.

BSKs function in many processes by interacting with its target proteins, and more and more target proteins were identified. Ren et al. (2019) indicates that BSK3 interacts with BSU1 to activate BR signaling. Some BSK3-interacting receptor-like kinases (RLKs), such as impaired oomycete susceptibility1 (IOS1) and BSK3-interacting RLKs (BSR850), are reported to play an important role in BR-regulated responses (Xu et al., 2014). Recently, we identify a novel target protein of ZmBSK1, ZmCCaMK (Liu et al., 2021). The roles of CCaMKs have been well studied in plant-microbe interactions including symbiosis of arbuscular mycorrhizal and root nodule (Miller et al., 2013; Shimoda et al., 2012; Takeda et al., 2012). Besides, genetic evidence has shown that CCaMK can also regulate plant reaction to abiotic stress. For example, in maize, ZmCCaMK can phosphorylate ZmNAC84 or be phosphorylated by ZmBSK1 to increase plant tolerance to drought (Liu et al., 2021; Zhu et al., 2016). Previous studies find that ZmCCaMK participate in BR-induced H_2O_2 production and is required for BR-mediated increases in expression of *Zmrbohs* gene (Yan et al., 2015). However, these studies do not clarify the function mechanism of ZmCCaMK in that process. Here, we revealed the vital role of ZmBSK1-ZmCCaMK module in BR-induced H_2O_2 production based on the following reasons: first, BR treatment could enhance the interaction between them (Fig. 4); secondly, BR could enhance ZmCCaMK via ZmBSK1-mediated phosphorylation (Fig. 5A–D); thirdly, the ZmBSK1-mediated Ser67 phosphorylation in ZmCCaMK positively regulated BR-induced H_2O_2 production through Zmrbohs (Fig. 5E–H and 6). Though the direct associations between ZmCCaMK and Zmrbohs have not been discovered, a previous report shows that the phosphorylated ZmNAC84 by ZmCCaMK can regulate the gene expression levels of *ZmrbohH* via directly binding to its promoter (Yang et al., 2018), implying that ZmCCaMK may directly modulate the others *Zmrbohs* through diverse transcription factors. Our study not only identify a novel target protein of ZmBSK1 but also further unravel a ZmBSK1-ZmCCaMK module in BR-induced H_2O_2 production.

In past decades, the functions of BSKs have been investigated in detail in plant immunity and BR signaling pathway. However, the function of BSKs in response to abiotic stress is still very unclear. Only AtBSK5 and ZmBSK1 are revealed to participate in response to salt and drought stresses (Li et al., 2012; Liu et al., 2021, 2022). H_2O_2 has been proposed to act as an essential signaling molecule in various stimuli responses (Bright et al., 2006). Since ZmBSK1 is involved in BR-induced H_2O_2 production, it is reasonable to assume that ZmBSK1 may play roles in other abiotic stress response. Indeed, we reported the vital roles of ZmBSK1 in oxidative stress response in this study (Fig. 7). Genetic analysis further explored that ZmBSK1-mediated Ser67 phosphorylation in ZmCCaMK also played a significant role in regulating maize tolerance to oxidative stress (Figs. 5 and 8). Transcriptomic analyses reveal that the expressions of *BSKs* gene were obviously induced by multiple abiotic stresses (including alkali) in many species such as hemp, potato and Kentucky bluegrass (Chen et al., 2019; Jiang et al., 2021; Kang et al., 2021), indicating that the roles of BSKs in adapt to environmental stresses will be discovered in depth in the future.

Based on these data, we propose a work model for ZmBSK1 functioning in BR-induced H_2O_2 production, where BR-enhanced ZmBSK1 phosphorylates ZmCCaMK to produce H_2O_2 and enhances gene expression and activity of Zmrbohs. Moreover, ZmBSK1-ZmCCaMK module also plays an essential role in improving oxidative stress tolerance in maize.

CRediT authorship contributions

Lei Liu: Conceptualization, Investigation, Formal analysis, Writing - Original Draft. Yanchao Sun: Investigation, Formal analysis. Meijing Zhang: Investigation, Resources. Ruixiang Liu: Investigation. Xiaming Wu: Investigation. Yanping Chen: Conceptualization, Writing - Review & Editing, Supervision, Funding acquisition. Jianhua Yuan: Conceptualization, Writing - Review & Editing, Funding acquisition. All authors have read and approved the final version of this manuscript.

Funding

This work was supported by the Jiangsu Province Postdoctoral Science Foundation (2021K406C); the China Agriculture Research System (CARS-02); the Jiangsu Agriculture Science and Technology Innovation Fund (CX(20)1002); and the Natural Science Foundation of Jiangsu Province (BK20191243).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful to Dr. Jingwei Yan (Nanjing Agricultural University) for revising the manuscript.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plaphy.2022.06.011.

References

Baxter, A., Mittler, R., Suzuki, N., 2014. ROS as key players in plant stress signalling. J. Exp. Bot. 65, 1229–1240.
Boisson-Dernier, A., Lituiev, D.S., Nestorova, A., Franck, C.M., Thirugnanarajah, S., Grossniklaus, U., 2013. ANXUR receptor-like kinases coordinate cell wall integrity with growth at the pollen tube tip via NADPH oxidases. PLoS Biol. 11, e1001719.
Bright, J., Desikan, R., Hancock, J.T., Weir, I.S., Neill, S.J., 2006. ABA-induced NO generation and stomatal closure in *Arabidopsis* are dependent on H_2O_2 synthesis. Plant J. 45, 113–122.
Chen, H., Zou, Y., Shang, Y., Lin, H., Wang, Y., Cai, R., Tang, X., Zhou, J.M., 2008. Firefly luciferase complementation imaging assay for protein-protein interactions in plants. Plant Physiol. 146, 368–376.
Chen, Y., Chen, Y., Shi, Z., Jin, Y., Sun, H., Xie, F., Zhang, L., 2019. Biosynthesis and signal transduction of ABA, JA, and BRs in response to drought stress of Kentucky bluegrass. Int. J. Mol. Sci. 20, 1289.
Choudhary, S.P., Yu, J.Q., Yamaguchi-Shinozaki, K., Shinozaki, K., Tran, L.S., 2012. Benefits of brassinosteroid crosstalk. Trends Plant Sci. 17, 594–605.
Gruszka, D., 2018. Crosstalk of the brassinosteroid signalosome with phytohormonal and stress signaling components maintains a balance between the processes of growth and stress tolerance. Int. J. Mol. Sci. 19, 2675.
Gudesblat, G.E., Russinova, E., 2011. Plants grow on brassinosteroids. Curr. Opin. Plant Biol. 14, 530–537.
Jiang, C., Belfield, E.J., Mithani, A., Visscher, A., Ragoussis, J., Mott, R., Smith, J.A.C., Harberd, N.P., 2012. ROS-mediated vascular homeostatic control of root-to-shoot soil Na delivery in *Arabidopsis*. EMBO J. 31, 4359–4370.
Jiang, Y., Sun, Y., Zheng, D., Han, C., Cao, K., Xu, L., Liu, S., Cao, Y., Feng, N., 2021. Physiological and transcriptome analyses for assessing the effects of exogenous uniconazole on drought tolerance in hemp (*Cannabis sativa* L.). Sci. Rep. 11, 14476.
Jimenez-Quesada, M.J., Traverso, J.A., Potocký, M., Žárský, V., Alché, J.d.D., 2019. Generation of superoxide by OeRbohH, a NADPH oxidase activity during olive (*Olea europaea* L.) pollen development and germination. Front. Plant Sci. 10, 1149.
Jiroutova, P., Oklestkova, J., Strnad, M., 2018. Crosstalk between brassinosteroids and ethylene during plant growth and under abiotic stress conditions. Int. J. Mol. Sci. 19, 3283.
Kadota, Y., Sklenar, J., Derbyshire, P., Stransfeld, L., Asai, S., Ntoukakis, V., Jones, J.D., Shirasu, K., Menke, F., Jones, A., 2014. Direct regulation of the NADPH oxidase RBOHD by the PRR-associated kinase BIK1 during plant immunity. Mol. Cell. 54, 43–55.
Kang, Y., Yang, X., Liu, Y., Shi, M., Zhang, W., Fan, Y., Yao, Y., Zhang, J., Qin, S., 2021. Integration of mRNA and miRNA analysis reveals the molecular mechanism of potato (*Solanum tuberosum* L.) response to alkali stress. Int. J. Biol. Macromol. 182, 938–949.
Kaur, G., Sharma, A., Guruprasad, K., Pati, P.K., 2014. Versatile roles of plant NADPH oxidases and emerging concepts. Biotechnol. Adv. 32, 551–563.

Kaya, H., Nakajima, R., Iwano, M., Kanaoka, M.M., Kimura, S., Takeda, S., Kawarazaki, T., Senzaki, E., Hamamura, Y., Higashiyama, T., 2014. Ca^{2+}-activated reactive oxygen species production by *Arabidopsis* RbohH and RbohJ is essential for proper pollen tube tip growth. Plant Cell 26, 1069–1080.
Kim, E.J., Russinova, E., 2020. Brassinosteroid signalling. Curr. Biol. 30, R294–R298.
Kim, T.W., Guan, S., Sun, Y., Deng, Z., Tang, W., Shang, J.X., Sun, Y., Burlingame, A.L., Wang, Z.Y., 2009. Brassinosteroid signal transduction from cell-surface receptor kinases to nuclear transcription factors. Nat. Cell Biol. 11, 1254–1260.
Kim, T.W., Wang, Z.Y., 2010. Brassinosteroid signal transduction from receptor kinases to transcription factors. Annu. Rev. Plant Biol. 61, 681–704.
Kwak, J.M., Mori, I.C., Pei, Z.M., Leonhardt, N., Torres, M.A., Dangl, J.L., Bloom, R.E., Bodde, S., Jones, J.D., Schroeder, J.I., 2003. NADPH oxidase *AtrbohD* and *AtrbohF* genes function in ROS-dependent ABA signaling in *Arabidopsis*. EMBO J. 22, 2623–2633.
Lassig, R., Gutermuth, T., Bey, T.D., Konrad, K.R., Romeis, T., 2014. Pollen tube NAD(P)H oxidases act as a speed control to dampen growth rate oscillations during polarized cell growth. Plant J. 78, 94–106.
Li, M., Ahammed, G.J., Li, C., Bao, X., Yu, J., Huang, C., Yin, H., Zhou, J., 2016. Brassinosteroid ameliorates zinc oxide nanoparticles-induced oxidative stress by improving antioxidant potential and redox homeostasis in tomato seedling. Front. Plant Sci. 7, 615.
Li, X., Zhang, H., Tian, L., Huang, L., Liu, S., Li, D., Song, F., 2015. Tomato SlRbohB, a member of the NADPH oxidase family, is required for disease resistance against *Botrytis cinerea* and tolerance to drought stress. Front. Plant Sci. 6, 463.
Li, Z., Shen, J., Liang, J., 2019. Genome-wide identification, expression profile, and alternative splicing analysis of the brassinosteroid-signaling kinase (BSK) family genes in *Arabidopsis*. Int. J. Mol. Sci. 20, 1138.
Li, Z.Y., Xu, Z.S., He, G.Y., Yang, G.X., Chen, M., Li, L.C., Ma, Y.Z., 2012. A mutation in *Arabidopsis BSK5* encoding a brassinosteroid-signaling kinase protein affects responses to salinity and abscisic acid. Biochem. Biophys. Res. Commun. 426, 522–527.
Liu, L., Sun, Y., Di, P., Cui, Y., Meng, Q., Wu, X., Chen, Y., Yuan, J., 2022. Overexpression of a *Zea mays* brassinosteroid-signaling kinase gene *ZmBSK1* confers salt stress tolerance in maize. Front. Plant Sci. 13, 894710.
Liu, L., Xiang, Y., Yan, J., Di, P., Li, J., Sun, X., Han, G., Ni, L., Jiang, M., Yuan, J., Zhang, A., 2021. BRASSINOSTEROID-SIGNALING KINASE 1 phosphorylating CALCIUM/CALMODULIN-DEPENDENT PROTEIN KINASE functions in drought tolerance in maize. New Phytol. 231, 695–712.
Liu, W., Xiang, Y., Zhang, X., Han, G., Sun, X., Sheng, Y., Yan, J., Scheller, H.V., Zhang, A., 2019. Over-expression of a maize *N*-acetylglutamate kinase gene (*ZmNAGK*) improves drought tolerance in tobacco. Front. Plant Sci. 9, 1902.
Liu, Y.B., Qin, L.J., Han, L.Z., Xiang, Y., Zhao, D.G., 2015. Overexpression of maize *SDD1* (*ZmSDD1*) improves drought resistance in *Zea mays* L. by reducing stomatal density. Plant Cell Tiss. Org. 122, 147–159.
Ma, F., Lu, R., Liu, H., Shi, B., Zhang, J., Tan, M., Zhang, A., Jiang, M., 2012a. Nitric oxide-activated calcium/calmodulin-dependent protein kinase regulates the abscisic acid-induced antioxidant defence in maize. J. Exp. Bot. 63, 4835–4847.
Ma, L., Zhang, H., Sun, L., Jiao, Y., Zhang, G., Miao, C., Hao, F., 2012b. NADPH oxidase AtrbohD and AtrbohF function in ROS-dependent regulation of Na^+/K^+ homeostasis in *Arabidopsis* under salt stress. J. Exp. Bot. 63, 305–317.
Majhi, B.B., Sobol, G., Gachie, S., Sreeramulu, S., Sessa, G., 2021. BRASSINOSTEROID-SIGNALLING KINASES 7 and 8 associate with the FLS2 immune receptor and are required for flg22-induced PTI responses. Mol. Plant Pathol. 22, 786–799.
Majhi, B.B., Sreeramulu, S., Sessa, G., 2019. BRASSINOSTEROID-SIGNALING KINASE5 associates with immune receptors and is required for immune responses. Plant Physiol. 180, 1166–1184.
Miller, J.B., Pratap, A., Miyahara, A., Zhou, L., Bornemann, S., Morris, R.J., Oldroyd, G. E., 2013. Calcium/Calmodulin-dependent protein kinase is negatively and positively regulated by calcium, providing a mechanism for decoding calcium responses during symbiosis signaling. Plant Cell 25, 5053–5066.
Nolan, T.M., Vukasinovic, N., Liu, D., Russinova, E., Yin, Y., 2020. Brassinosteroids: multidimensional regulators of plant growth, development, and stress responses. Plant Cell 32, 295–318.
Ogasawara, Y., Kaya, H., Hiraoka, G., Yumoto, F., Kimura, S., Kadota, Y., Hishinuma, H., Senzaki, E., Yamagoe, S., Nagata, K., 2008. Synergistic activation of the *Arabidopsis* NADPH oxidase AtrbohD by Ca^{2+} and phosphorylation. J. Biol. Chem. 283, 8885–8892.
Ren, H., Willige, B.C., Jaillais, Y., Geng, S., Park, M.Y., Gray, W.M., Chory, J., 2019. BRASSINOSTEROID-SIGNALING KINASE 3, a plasma membrane-associated scaffold protein involved in early brassinosteroid signaling. PLoS Genet. 15, e1007904.
Shi, H., Shen, Q., Qi, Y., Yan, H., Nie, H., Chen, Y., Zhao, T., Katagiri, F., Tang, D., 2013. BR-SIGNALING KINASE1 physically associates with FLAGELLIN SENSING2 and regulates plant innate immunity in *Arabidopsis*. Plant Cell 25, 1143–1157.
Shimoda, Y., Han, L., Yamazaki, T., Suzuki, R., Hayashi, M., Imaizumi-Anraku, H., 2012. Rhizobial and fungal symbioses show different requirements for calmodulin binding to calcium calmodulin-dependent protein kinase in *Lotus japonicus*. Plant Cell 24, 304–321.
Su, B., Zhang, X., Li, L., Abbas, S., Yu, M., Cui, Y., Baluska, F., Hwang, I., Shan, X., Lin, J., 2021. Dynamic spatial reorganization of BSK1 complexes in the plasma membrane underpins signal-specific activation for growth and immunity. Mol. Plant 14, 588–603.
Suzuki, N., Miller, G., Morales, J., Shulaev, V., Torres, M.A., Mittler, R., 2011. Respiratory burst oxidases: the engines of ROS signaling. Curr. Opin. Plant Biol. 14, 691–699.
Takeda, N., Maekawa, T., Hayashi, M., 2012. Nuclear-localized and deregulated calcium- and calmodulin-dependent protein kinase activates rhizobial and mycorrhizal responses in *Lotus japonicus*. Plant Cell 24, 810–822.
Tang, W., Kim, T.W., Oses-Prieto, J.A., Sun, Y., Deng, Z., Zhu, S., Wang, R., Burlingame, A.L., Wang, Z.Y., 2008. BSKs mediate signal transduction from the receptor kinase BRI1 in *Arabidopsis*. Science 321, 557–560.
Torres, M.A., Jones, J.D., Dangl, J.L., 2006. Reactive oxygen species signaling in response to pathogens. Plant Physiol. 141, 373–378.
Wang, J., Shi, H., Zhou, L., Peng, C., Liu, D., Zhou, X., Wu, W., Yin, J., Qin, H., Ma, W., He, M., Li, W., Wang, J., Li, S., Chen, X., 2017. OsBSK1-2, an orthologous of AtBSK1, is involved in rice immunity. Front. Plant Sci. 8, 908.
Wang, R., He, F., Ning, Y., Wang, G.-L., 2020. Fine-tuning of RBOH-mediated ROS signaling in plant immunity. Trends Plant Sci. 25, 1060–1062.
Wang, Z.Y., 2012. Brassinosteroids modulate plant immunity at multiple levels. Proc. Natl. Acad. Sci. U. S. A. 109, 7–8.
Wong, H.L., Pinontoan, R., Hayashi, K., Tabata, R., Yaeno, T., Hasegawa, K., Kojima, C., Yoshioka, H., Iba, K., Kawasaki, T., Shimamoto, K., 2007. Regulation of rice NADPH oxidase by binding of Rac GTPase to its N-terminal extension. Plant Cell 19, 4022–4034.
Xia, X.-J., Zhou, Y.-H., Shi, K., Zhou, J., Foyer, C.H., Yu, J.-Q., 2015. Interplay between reactive oxygen species and hormones in the control of plant development and stress tolerance. J. Exp. Bot. 66, 2839–2856.
Xia, X.J., Wang, Y.J., Zhou, Y.H., Tao, Y., Mao, W.H., Shi, K., Asami, T., Chen, Z., Yu, J. Q., 2009. Reactive oxygen species are involved in brassinosteroid-induced stress tolerance in cucumber. Plant Physiol. 150, 801–814.
Xu, P., Xu, S.-L., Li, Z.-J., Tang, W., Burlingame, A.L., Wang, Z.-Y., 2014. A brassinosteroid-signaling kinase interacts with multiple receptor-like kinases in *Arabidopsis*. Mol. Plant 7, 441.
Yan, J., Guan, L., Sun, Y., Zhu, Y., Liu, L., Lu, R., Jiang, M., Tan, M., Zhang, A., 2015. Calcium and ZmCCaMK are involved in brassinosteroid-induced antioxidant defense in maize leaves. Plant Cell Physiol. 56, 883–896.
Yan, J., Liu, Y., Yang, L., He, H., Huang, Y., Fang, L., Scheller, H.V., Jiang, M., Zhang, A., 2021. Cell wall β-1,4-galactan regulated by the BPC1/BPC2-GALS1 module aggravates salt sensitivity in *Arabidopsis thaliana*. Mol. Plant 14, 411–425.
Yang, Q., Zhang, H.P., Liu, C., Huang, L.P., Zhao, L.L., Zhang, A.Y., 2018. A NAC transcription factor ZmNAC84 affects pollen development through the repression of *ZmRbohH* expression in maize. J. Plant Biol. 61, 366–373.
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_2O_2 in brassinosteroid-induced antioxidant defence in leaves of maize. J. Exp. Bot. 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 Cell Physiol. 52, 181–192.
Zhang, B., Wang, X., Zhao, Z., Wang, R., Huang, X., Zhu, Y., Yuan, L., Wang, Y., Xu, X., Burlingame, A.L., 2016. OsBRI1 activates BR signaling by preventing binding between the TPR and kinase domains of OsBSK3 via phosphorylation. Plant Physiol. 170, 1149–1161.
Zhou, J., Wang, J., Li, X., Xia, X.-J., Zhou, Y.-H., Shi, K., Chen, Z., Yu, J.-Q., 2014. H_2O_2 mediates the crosstalk of brassinosteroid and abscisic acid in tomato responses to heat and oxidative stresses. J. Exp. Bot. 65, 4371–4383.
Zhu, Y., Yan, J., Liu, W., Liu, L., Sheng, Y., Sun, Y., Li, Y., Scheller, H.V., Jiang, M., Hou, X., Ni, L., Zhang, A., 2016. Phosphorylation of a NAC transcription factor by a calcium/calmodulin-dependent protein kinase regulates abscisic acid-induced antioxidant defense in maize. Plant Physiol. 171, 1651–1664.