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OsABA3 is Crucial for Plant Survival and Resistance to Multiple Stresses in Rice

Haoling Ni[1], Wenshi Wu[1], Yanmin Yan[1], Yiyuan Fang[1], Changjian Wang[1], Jiayi Chen[1], Shali Chen[1], Kaini Wang[1], Chunjue Xu[2,3], Xiaoyan Tang[1,2,3*] and Jianxin Wu[1*]

Abstract

Preharvest sprouting (PHS) is a serious problem in rice production as it leads to reductions in grain yield and quality. However, the underlying mechanism of PHS in rice remains unclear. In this study, we identified and characterized a *preharvest sprouting and seedling lethal* (*phssl*) mutant. The heterozygous *phssl/+* mutant exhibited normal plant development, but severe PHS in paddy fields. However, the homozygous *phssl* mutant was seedling lethal. Gene cloning and genetic analysis revealed that a point mutation in *OsABA3* was responsible for the mutant phenotypes. *OsABA3* encodes a molybdenum cofactor (Moco) sulfurase. The activities of the sulfureted Moco-dependent enzymes such as aldehyde oxidase (AO) and xanthine dehydrogenase (XDH) were barely detectable in the *phssl* mutant. As the final step of abscisic acid (ABA) de novo biosynthesis is catalyzed by AO, it indicated that ABA biosynthesis was interrupted in the *phssl* mutant. Exogenous application of ABA almost recovered seed dormancy of the *phssl* mutant. The *knock-out* (*ko*) mutants of *OsABA3* generated by CRISPR-Cas9 assay, were also seedling lethal, and the heterozygous mutants were similar to the *phssl/+* mutant showing reduced seed dormancy and severe PHS in paddy fields. In contrast, the *OsABA3* overexpressing (OE) plants displayed a significant increase in seed dormancy and enhanced plant resistance to PHS. The AO and XDH activities were abolished in the *ko* mutants, whereas they were increased in the OE plants. Notably, the Moco-dependent enzymes including nitrate reductase (NR) and sulfite oxidase (SO) showed reduced activities in the OE plants. Moreover, the OE plants exhibited enhanced resistances to osmotic stress and bacterial blight, and flowered earlier without any reduction in grain yield. Taken together, this study uncovered the crucial functions of OsABA3 in Moco sulfuration, plant development, and stress resistance, and suggested that OsABA3 is a promising target gene for rice breeding.

Keywords Preharvest sprouting, Seed dormancy, *OsABA3*, Molybdenum cofactor, Rice

*Correspondence:
Xiaoyan Tang
txy@frontier-ag.com
Jianxin Wu
wjxin@m.scnu.edu.cn
[1]Guangdong Provincial Key Laboratory of Biotechnology for Plant Development, School of Life Sciences, South China Normal University, Guangzhou 510631, China
[2]Shenzhen Branch, Guangdong Laboratory for Lingnan Modern Agriculture, Agricultural Genomics Institute at Shenzhen, Chinese Academy of Agricultural Sciences, Shenzhen 518120, China
[3]Shenzhen Institute of Molecular Crop Design, Shenzhen 518107, China

Background

When cereal crops mature during a season of high temperature and high humidity, grains may germinate within the mother plant before harvest. This phenomenon is termed as Preharvest sprouting (PHS) (Li et al. 2004; Tai et al. 2021). PHS can cause a severe reduction in grain yield and quality, resulting in substantial economic losses (Bewley et al. 2006).

Abscisic acid (ABA) has been proposed as the primary regulator of PHS by maintaining seed dormancy (Gubler et al. 2005). In higher plants, ABA is derived from carotenoid precursors. Therefore, mutants of the critical carotenoid biosynthetic genes, such as phytoene desaturase, ξ-carotene desaturase, carotenoid isomerase and lycopene β-cyclase, all showed reduced ABA level, and much more severe PHS phenotypes (Fang et al. 2008; Hable et al. 1998; Singh et al. 2003). In the plastid, β-carotene undergoes conversion to 9-cisviolaxanthin and 9-cis-neoxanthin through several steps including hydroxylation, epoxidation and isomerization. These steps are catalyzed by enzymes such as β-carotene hydroxylases, zeaxanthin epoxidase (ZEP), and neoxanthin synthase (Finkelstein 2013; North et al. 2007; Rock and Zeevaart 1991; Sun et al. 1996). The resulting 9-cisviolaxanthin and 9-cis-neoxanthin are cleaved by 9-cis-epoxycarotenoid dioxygenase (NCED) to release xanthoxin (Schwartz et al. 1997b). This is the final plastid-localized step in ABA biosynthesis, and is rate limiting (Finkelstein 2013). In the cytosol, xanthoxin is first converted to abscisic aldehyde by xanthoxin dehydrogenase (XanDH) (Gonzalez-Guzman et al. 2002). Subsequently, abscisic aldehyde is oxidized to ABA catalyzed by abscisic aldehyde oxidase (AAO), a molybdoenzyme (Seo et al. 2000). Loss of function of *ZEP*, *NCED*, *XanDH* or *AAO* resulted in severe PHS phenotypes in various crops (Agrawal et al. 2001; Gonzalez-Guzman et al. 2002; Liu et al. 2019; Schwartz et al. 1997b, 2003).

There are five types of molybdoenzymes in plants, including sulfite oxidase (SO), nitrate reductase (NR), mitochondrial amidoxime reducing component (mARC), aldehyde oxidase (AO), and xanthine dehydrogenase (XDH) (Kaufholdt et al. 2017). SO, NR and mARC require molybdenum cofactor (Moco) for their activity, while AO and XDH require sulfurized Moco (Kaufholdt et al. 2017). The Moco biosynthetic pathway is conserved across eukaryotic kingdoms. In plants, the de novo synthesis of Moco begins with the cyclization of GTP to form a cyclopyranoperin monophosphate (cPMP). This process is catalyzed by Cofactor for Nitrate reductase and Xanthine dehydrogenase (CNX) 2 and CNX3 (Hoff et al. 1995). The resulting cPMP is then sulfurized to form molybdopterin (MPT) by CNX5, CNX6 and CNX7 (Matthies et al. 2004; Suzuki et al. 2006). Finally, the MPT is modified by inserting a molybdenum molecule to form

Moco (Stallmeyer et al. 1995). Moco can be further sulfurized by Moco sulfurase (Bittner et al. 2001). The resulting sulfurized Moco is necessary for ABA biosynthesis. Therefore, defects in Moco biosynthesis reduce ABA level, resulting in reduced seed dormancy and severe PHS phenotypes in crops. For instance, rice mutants of *OsCNX6*, *OsCNX1* as well as maize mutant *ZmCNX1*, *ZmCNX7* exhibited obvious PHS (Liu et al. 2019; Porch et al. 2006; Suzuki et al. 2006, 2015).

In Arabidopsis, Moco sulfurase is encoded by *abscisic acid insensitive 3* (*AtABA3*) (Bittner et al. 2001). The *ataba3* mutants were impaired in converting ABA-aldehyde to ABA, and exhibited typical ABA-deficiency phenotypes including reduced seed dormancy and excessive water loss (Leon-Kloosterziel et al. 1996; Schwartz et al. 1997a). In addition, the *ataba3* mutants were found to be compromised in plant tolerance to freezing, salt, and drought stress (Llorente et al. 2000; Xiong et al. 2001). Heterologous expression of *AtABA3* in crops such as tobacco, cotton, soybean, maize, and rice, have been shown to be significantly improve plant drought tolerance (Li et al. 2013; Lu et al. 2013; Yue et al. 2011, 2012). In response to drought stress, *OsABA3* expression is activated in rice roots (Huang et al. 2009). However, the functions of OsABA3 in rice remain unclear.

In the present study, we revealed that the null mutants of *OsABA3* exhibited severe PHS, impaired Moco sulfuration and seedling lethality. However, overexpression of *OsABA3* in rice significantly increased the activities of sulfurated Moco-dependent enzymes and, improved plant resistance to PHS, osmotic stress, and bacterial blight. In addition, plants overexpressing OsABA3 exhibited an earlier flowering time without any penalty in grain yield. Our findings uncovered the underlying mechanism of *OsABA3* in PHS resistance, and highlighted the functions of *OsABA3* in plant development and stress resistance.

Results

Identification and Characterization of the *phssl* Mutant

To investigate the underlying mechanism of rice PHS, we screened for severe PHS mutants from an EMS mutant library in paddy fields, and identified nine PHS mutants (Hao-ling et al. 2020). One of the mutants later named as *preharvest sprouting and seedling lethal* (*phssl/+*), exhibited normal vegetative and reproductive growth (Fig. 1A), but a severe PHS phenotype (23.60% PHS rate of the mutant plants versus 2.5% of that of the wild type) in paddy fields under high humidity and temperature conditions (Fig. 1B, E). To assess whether seed dormancy is impaired in the *phssl/+* mutant, mature panicles were freshly collected from the *phssl/+* mutant and the wild-type Huanghuazhan (HHZ), and placed on wet filter paper at 30℃ for 2 days. The rate of germinated seeds per
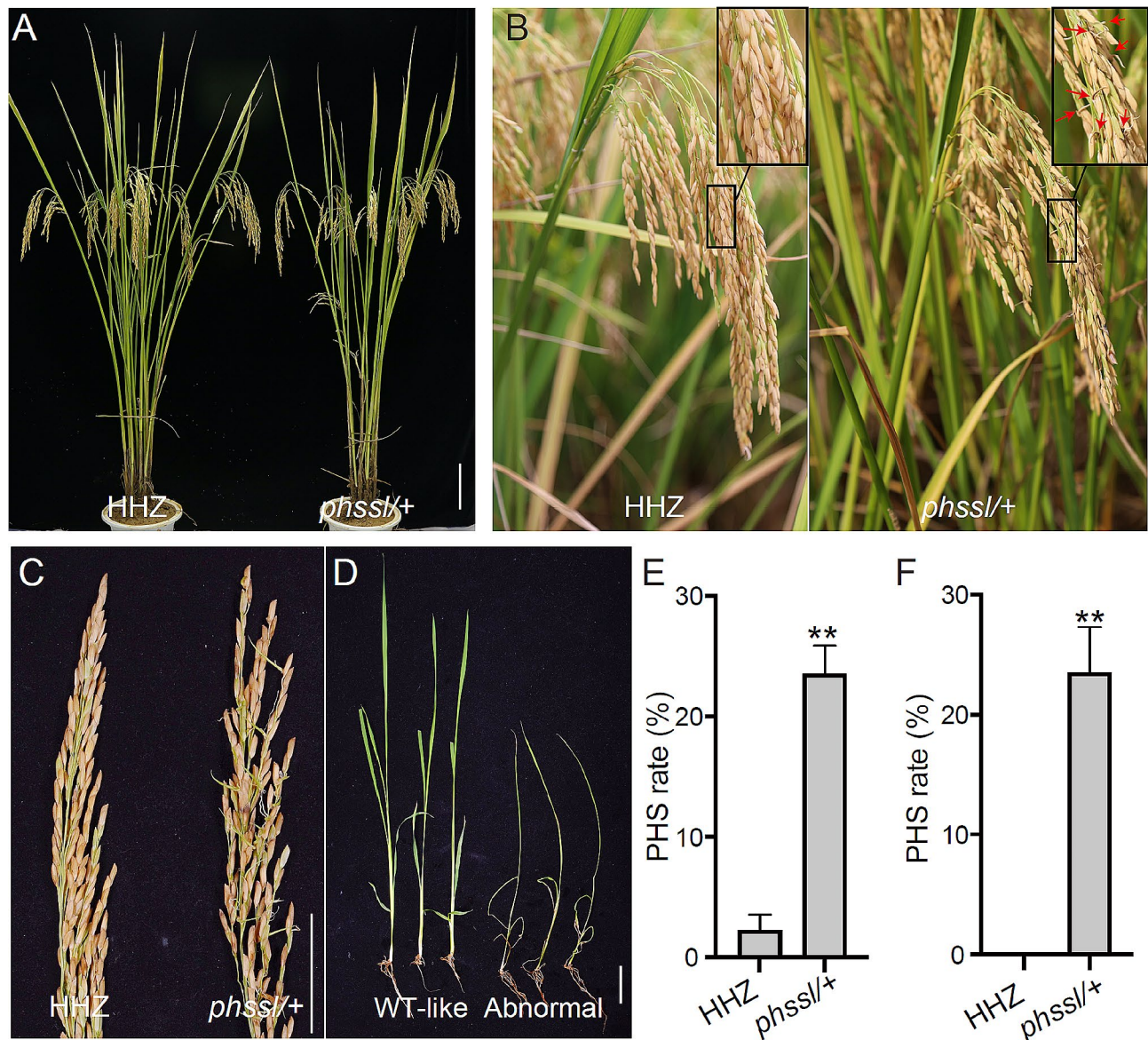
Fig. 1 Phenotypes of the *phssl/+* mutant. (**A**) The wild-type HHZ and *phssl/+* plants. (**B**) Preharvest sprouting (PHS) phenotypes of the *phssl/+* mutant in paddy fields. The magnified views of panicles from the HHZ and *phssl/+* mutant are shown on the top right of the graphs, and germinated seeds are marked with red arrow. (**C**) Germination phenotypes of seeds in freshly collected mature panicles of the HHZ and *phssl/+* mutant after 4 days imbibition in water. (**D**) Phenotypes of the progeny of *phssl/+*. (**E-F**) PHS rates of the HHZ and *phssl/+* mutant in (**B**) and (**C**), respectively. Data represent means ± SD (*n* > 11), student *t*-Test, ** represents *p* < 0.01. Scale bars, 10 cm (**A**), and 4 cm (**C-D**)

panicle of *phssl/+* was 23.58%, which was much higher than that of HHZ (0%) (Fig. 1C, F). Intriguingly, the quickly germinated progeny of *phssl/+* showed twisted and slender leaves, and died at the seedling stage of four to five leaves (Fig. 1D). The statistical analysis of a representative population of *phssl/+* mutant progeny showed that the segregation ratio of dead seedlings to normal seedlings was consistent with the expected ratio of 1:3 for a single recessive mutation (109:334, $\chi^2_{3:1} = 0.037 < \chi^2_{0.05,1} = 3.84$), indicating that the phenotypes of *phssl* were caused by a single recessive gene.

Molecular Cloning of the Responsible Gene for *phssl* Mutant

To identify the candidate gene which was responsible for the phenotypes of *phssl/+*, genomic DNA was extracted from 30 quickly germinated seedlings of *phssl/+* progeny, and bulk-sequencing was performed. The resulting sequence data was analyzed by the simultaneous identification of multiple causal mutations (SIMM) method (Yan et al. 2016). Five Single Nucleotide Polymorphisms (SNPs) were identified as candidate mutation sites. Genetic linkage analysis showed that all plants carrying the homozygous candidate site V were lethal (Table S1),

suggesting that the site V may be the causal mutation. Site V was located in the 14th exon of *LOC_Os06g45860*, and caused a missense mutation from TCC (Ser$_{482}$) to CCC (Pro$_{482}$) (Fig. 2A). *LOC_Os06g45860* is a homolog of Arabidopsis *AtABA3*, which encodes a molybdenum cofactor sulfurase, and was therefore previously named *OsABA3* (Zhang et al. 2019). To confirm that the phenotypes of *phssl* were resulted from the mutation of *OsABA3*, mutants of *OsABA3* were generated by CRISPR-Cas9 assay with two independent targets in *japonica* variety Wuyungeng 7 (WYG) (Fig. 2A). A total of 34 independent T$_0$ mutant lines with various genotypes were obtained. Interestingly, the homozygous *OsABA3* null mutants exhibited phenotypes similar to the homozygous *phssl* mutant, with twisted and slender leaves, and early lethality (Fig. 2B-E). Two transgenic-free heterozygous mutant lines, *ko1/+* and *ko2/+* (Fig. 2D-E), were identified and used for further studies. The homozygous progeny of *ko* mutants also exhibited a seedling lethal phenotype (Fig. S1). In addition, a construct harboring the wild-type CDS of *OsABA3* driven by a ubiquitin promoter was introduced into the *ko1/+* mutant, and the homozygous transgenic plants carrying the homozygous *ko1* allele were identified and named *COM/ko1*. The resulting *COM/ko1* transgenic plants showed normal plant growth and enhanced PHS resistance, effectively complementing the *ko1* mutant phenotypes (Fig. 2F, Fig. S2). Taken together, these data reveled that the mutation of *OsABA3* was responsible for the phenotypes of *phssl*.

Protein Sequence and Phylogenetic Analysis of OsABA3

A typical ABA3 protein contains two conserved domains including the N-terminal NifS-like domain and the ABA3 C-terminal (ABA3-CT) domain (Bittner et al. 2001; Xiong et al. 2001). The NifS-like domain catalyzes the decomposition of L-cysteine to elemental sulfur to form a protein-bound persulfide intermediate (Heidenreich et al. 2005). The ABA3-CT domain may act as a scaffold, facilitating the sulfuration of prebound Moco and mediating the interaction between ABA3 and the target enzymes (Wollers et al. 2008). A homology search of OsABA3 in the public genomic databases Phytozome and NCBI revealed that ABA3 is present in almost all plant species, except for some unicellular algae such as *Micromonas pusilla*, *Chromochloris zofingiensis*, *Ostreococcus lucimarinus* and so on. Phylogenetic analysis showed that ABA3 proteins can be classified into three groups: spermatophytes, chlorophytes, and the group comprising bryophytes and pteridophytes (Fig. S3A). As the plant hormone ABA is critical for drought resistance in land plants, and ABA3 is necessary for ABA de novo biosynthesis (Finkelstein 2013), ABA3 may have played an important role in the plant evolutionary transition from aquatic to terrestrial life. Multiple sequence analysis

of ABA3 homologs from 20 represented plant species revealed that ABA3 proteins are highly conserved, especially in higher plants (Fig. S3B). Several amino acid residues of AtABA3 were reported to be essential for its function. The Cys$_{430}$ residue of AtABA3 was critical for substrate binding and persulfide formation (Heidenreich et al. 2005; Lehrke et al. 2012), and the Arg$_{732}$ residue might be involved in the binding of sulfurated Moco (Wollers et al. 2008). Both of them were highly conserved in ABA3 proteins. Additionally, heterologous expression of *AtABA3* fully rescued the seedling lethal phenotype of *ko1* mutant (Fig. S5), implying that *AtABA3* could mimic the function of *OsABA3* in rice.

Intriguingly, the Ser$_{482}$ residue of OsABA3, which was mutated to proline residue in the *phssl* mutant, is absolutely conserved in ABA3 proteins across species (Fig. S3B). Proline is a peculiar cyclic imino acid. The pyrrolidine ring of proline makes the C-N bond more rigid and less prone to rotation, thus reducing the flexibility of the peptide backbone. Given that the S482P mutation of OsABA3 is lethal for rice plants, it is possible that the S482P mutation of OsABA3 reduces the affinity of OsABA3 for its potential interactors. It has been proposed that Alanine, the simplest chiral amino acid that retains the beta carbon but no other side-chain chemistry, provides flexibility to the peptide backbone (Yu et al. 2012). To test the hypothesis, we introduced a CDS of OsABA3 with the codon change for S482A mutation driven by the maize Ubiquitin promoter into the *ko1/+* mutant. The homozygous transgenic plants carrying homozygous *ko1* allele were identified and named *COM-S482A/ko1*. The *COM-S482A/ko1* plants grew as well as the wild-type plants (Fig. 2F), and exhibited comparable PHS resistance to *COM/ko1* plants (Fig. S2), indicating that OsABA3$_{S482A}$ retains most, if not all, of its normal biological function. These data suggested that appropriate flexibility at residue 482 is required for OsABA3 function.

Gene Expression and Protein Subcellular Localization Analyses of *OsABA3*

To examine the expression pattern of *OsABA3* in rice, total RNA was extracted from seedling shoots, seedling roots, mature plant roots, culm leaf sheaths, leaf blades, young panicles and seeds at different developmental stages, and RT-qPCR assay was performed. The results showed that *OsABA3* was expressed in all tested tissues (Fig. S4A). ABA3 proteins were presumably localized in cytosol (Selles et al. 2022). Consistently, transient expression of OsABA3-GFP in rice protoplasts showed the GFP fluorescence signal in the cytosol (Fig. S4B), indicating that OsABA3 is a cytosolic protein.
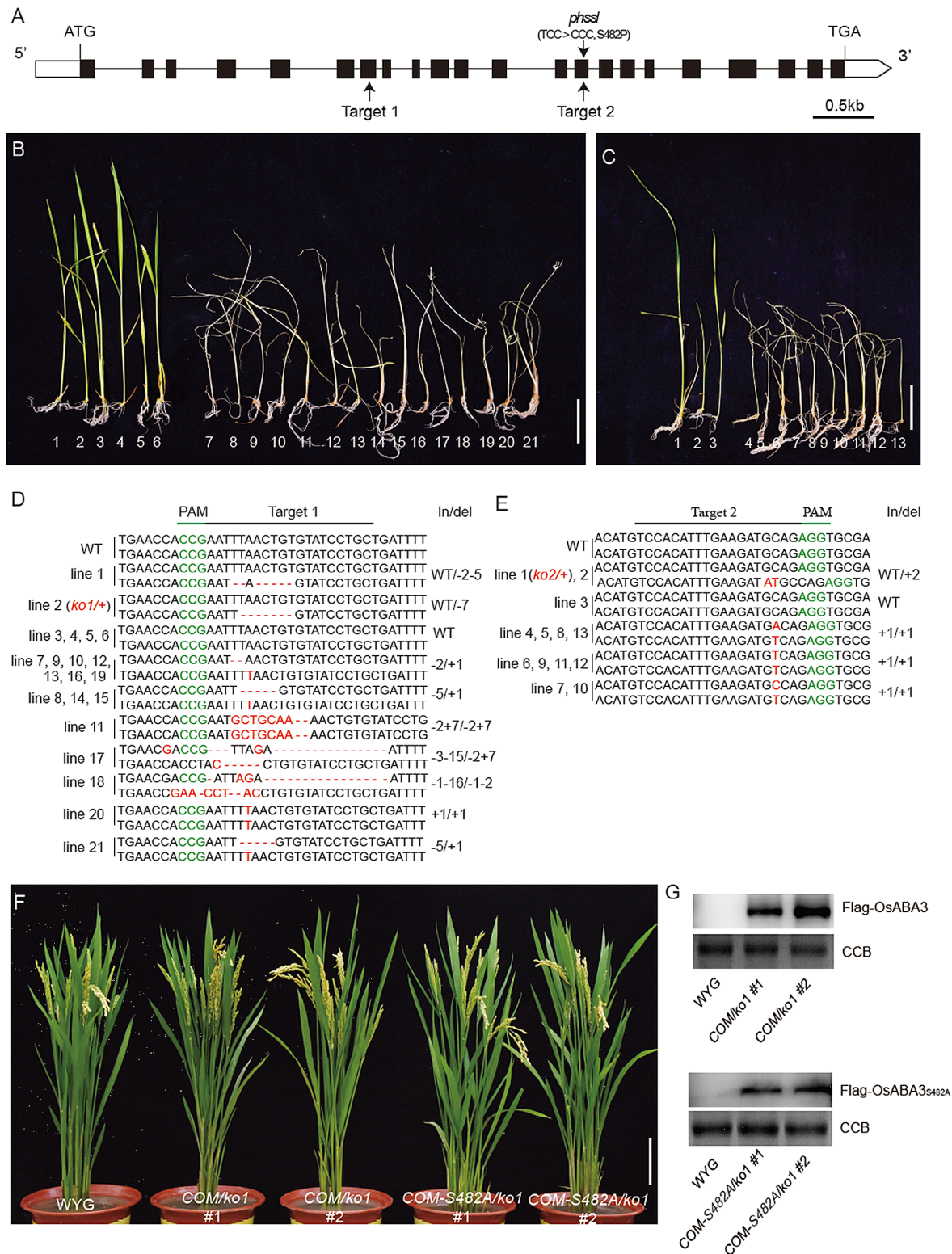
Fig. 2 Characterization of the *knock out* (*ko*) mutants of *OsABA3*, and the transgenic complementation of the null mutant *ko1*. (**A**) The gene structure of *OsABA3* is displayed with the mutation site of *phssl* and target sites of CRISPR-Cas9. (**B**-**C**) The seeding phenotypes of T_0 mutants of *OsABA3* edited by CRISPR-Cas9 at target 1 site (**B**) and target 2 site (**C**). The genotypes of mutants in (**B**) and (**C**) are displayed in (**D**) and (**E**), respectively. (**F**) Transgenic complementation of *ko1* with *OsABA3* or *OsABA3*$_{S482A}$ driven by Ubiquitin promoter. (**G**) The expression of OsABA3 and OsABA3$_{S482A}$ in the corresponding plants analyzed by western blot. Scale bars, 4 cm (**B**-**C**), and 10 cm (**F**)

The Role of OsABA3 in Rice PHS Resistance

Following pollination, the embryo undergoes development, maturation and dormancy. In rice, the embryo enters into dormancy approximately 21 days after pollination (Itoh et al. 2005). A weak seed dormancy is a major cause of PHS. To precisely analyze the seed dormancy in *phssl/+* mutant, the panicles from HHZ and

phssl/+ were harvested at the committed DAP. Seed germination rates were then examined under high humidity conditions for 3 days. The PHS rate of *phssl/+* was much higher than that of HHZ (Fig. 3A, C-F), indicating an impaired seed dormancy in *phssl/+*.

Previous reports showed that *AtABA3* encodes a Moco sulfurase enzyme that is required for the activation of AO
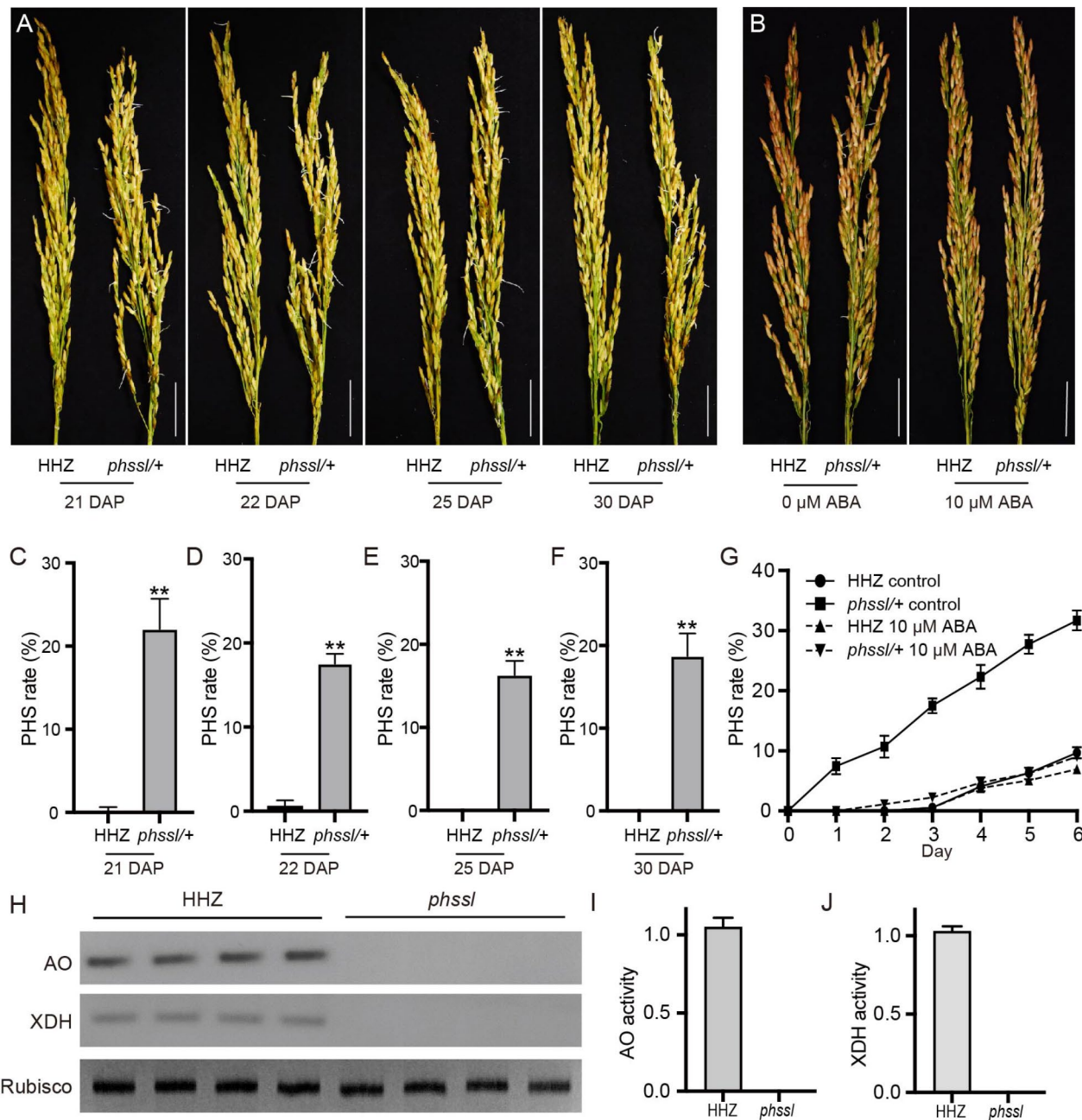

Fig. 3 Analysis of seed dormancy of the *phssl/+* mutant. (**A**, **C-F**) Panicles from HHZ and *phssl/+* mutant were collected at 21, 22, 25 and 30 DAP, and were imbibed in water for 3 days. The PHS phenotypes and rates are shown in (**A**) and (**C-F**), respectively. Data represent means ± SD (*n* = 4), student *t*-Test, ** represents *p* < 0.01. (**B**, **G**) The 22 DAP panicles from HHZ and *phssl/+* mutant were treated with or without 10 μM ABA in water for 6 days. (**B**) The germination phenotypes after 3 days treatment. (**G**) Time course of germination rate for (**B**). (**H**) Comparison of the aldehyde oxidase (AO) and xanthine dehydrogenase (XDH) activities in HHZ and *phssl* mutant. The quantitative activity was calculated relative to the corresponding enzyme activity in wild-type plants (**I-J**). The large subunit of rubisco protein stained by Coomassie brilliant blue was used as a loading control. Scale bars = 3 cm in (**A-B**)

and XDH in Arabidopsis (Bittner et al. 2001; Schwartz et al. 1997a). OsABA3 shares 60% similarity with AtABA3 in protein sequence. Furthermore, the exogenous expression of *AtABA3* in the *ko1* mutant effectively restored its phenotypes (Fig. S5), suggesting a conserved function between OsABA3 and AtABA3. Consistent with this hypothesis, we observed that the activities of AO and XDH were almost abolished in the *phssl* mutant, whereas they were clearly present in WT plants (Fig. 3H-J). Since the AO activity is required for ABA biosynthesis, we tested whether exogenous application of ABA could restore the phenotype of *phssl/+* mutant. The panicles from HHZ and *phssl/+* at 22 DAP were collected and imbibed with or without 10 μM ABA. The results showed that the application of ABA obviously inhibited the seed germination of *phssl/+* mutant (Fig. 3B, G).

During the rice harvest season in June, Guangzhou experiences hot and humid weather, which often leads to occurrences of rice PHS. In the paddy field, the *OsABA3* overexpressing plants OE-1 and OE-2 exhibited more resistance to PHS (Fig. 4A, C), while the heterozygous mutants *ko1/+* and *ko2/+* showed more severe PHS phenotypes (Fig. 4A, C). To assess the impact of *OsABA3* on seed dormancy, seeds on panicles were freshly collected from WYG, *ko1/+*, *ko2/+*, OE-1 and OE-2 at 31–35 DAP, and a germination experiment was carried out. The seeds of *ko* mutants germinated more quickly than that of WYG (Fig. 4B, D), whereas OE-1 and OE-2 exhibited much lower PHS rates than WYG (Fig. 4B, D). These results suggested that *OsABA3* plays a critical in rice seed dormancy and resistance to PHS.

OsABA3 is Involved in Regulating the Activities of Moco-Dependent Enzymes

To further analyze the function of OsABA3 in Moco biosynthesis, we examined the activities of Moco-dependent enzymes in the *ko* and OE plants. AO and XDH require sulfurated Moco for their activity. We observed that the activities of AO and XDH were almost abolished in the *ko1* and *ko2* plants (Fig. 5A-C), while in the *OsABA3/ko1* and *AtABA3/ko1* plants, these activities were fully recovered (Fig. S6). Moreover, both OE-1 and OE-2 plants exhibited increased activities of AO and XDH (Fig. 5D-F). The results indicated that OsABA3 is essential in regulating the level of sulfurated Moco in rice. Unlike AO and XDH, NR and SO require Moco for their activities. Surprisingly, the activities of NR and SO were significantly reduced in the OE-1 and OE-2 plants (Fig. 5G-H), implying a decrease in Moco levels in these plants. Collectively, these results suggested that OsABA3 is vital for maintaining Moco homeostasis, thereby affecting the activities of Moco-dependent enzymes.

Overexpression of *OsABA3* Improves Plant Resistance to Drought and Bacterial Blight

Previous reports have demonstrated that overexpression of *AtABA3* in rice, tobacco, cotton, maize and soybean significantly enhanced plant drought tolerance (Li et al. 2013; Lu et al. 2013; Xiao et al. 2009; Yue et al. 2011, 2012). To assess the role of rice OsABA3 in drought resistance, we first analyzed the performance of the *OsABA3* OE plants under osmotic stress by polyethylene glycol (PEG) treatment. We observed that the survival rates of OsABA3-OE plants were > 55%, much higher than 22% of the wild-type plants after 7 days of recovery (Fig. 6A-B). We next tested the tolerance of *OsABA3* OE plants under drought stress. The seedlings of WT, OE-1 and OE-2 were planted in the same spots, and subjected to drought treatment. After 7 days of water cutoff and 7 days recovery, the seedlings survival rates were calculated. More than 65% seedlings of OE-1 and OE-2 were alive, whereas only 39% WT seedlings remained alive (Fig. 6C-D), indicating that overexpression of *OsABA3* in rice improves plant drought tolerance.

The OE-1 and OE-2 plants appeared normal during vegetative growth. However, during reproductive stage, dark brown lesions were observed in the apical part of older leaves of OE-1 and OE-2 plants (Fig. 6E). Cytological analyzes revealed a reactive oxygen species (ROS) burst and a large number of dead cells were observed in the old leaves of OE plants (Fig. 6F-G). As spontaneous cell death might be associated with plant biotic resistance, we examined the resistance of OE plants to *Xanthomonas oryzae* pv. *oryzae* (*Xoo*) (Fig. 6H-I). The OE plants exhibited much shorter lesions compared to that of wild-type WYG plants. Pathogenesis-related (PR) proteins are very important for plant resistance to pathogen infection. The representative PR genes *PR1a*, *PR1b* and *PR10* were pronouncedly autoactivated in OE plants (Fig. 6J). These results suggested that overexpression of *OsABA3* enhances plant resistance to *Xoo*.

Overexpression of *OsABA3* Promotes Early Flowering But Does Not Impact Grain Yield Under Normal Conditions

To investigate the effects of *OsABA3* overexpression on grain yield in rice, the OE plants and the wild-type WYG were grown in the paddy fields in Guangzhou (23.13 N, 113.26E) under regular care. The agronomic traits were examined. The OE plants showed similar appearances to the wild-type in terms of plant height, number of panicles per plant, grain number per panicle, seed setting rate, grain length, grain width, grain weight and grain yield per plant (Fig. 7A-I), indicating that overexpression of OsABA3 did not affect grain yield under normal conditions. Interestingly, the OE-1 and OE-2 plants flowered 87.6±3.1 and 85.4±1.6 day after sowing (DAS) respectively, which were more than 7 days earlier than WYG
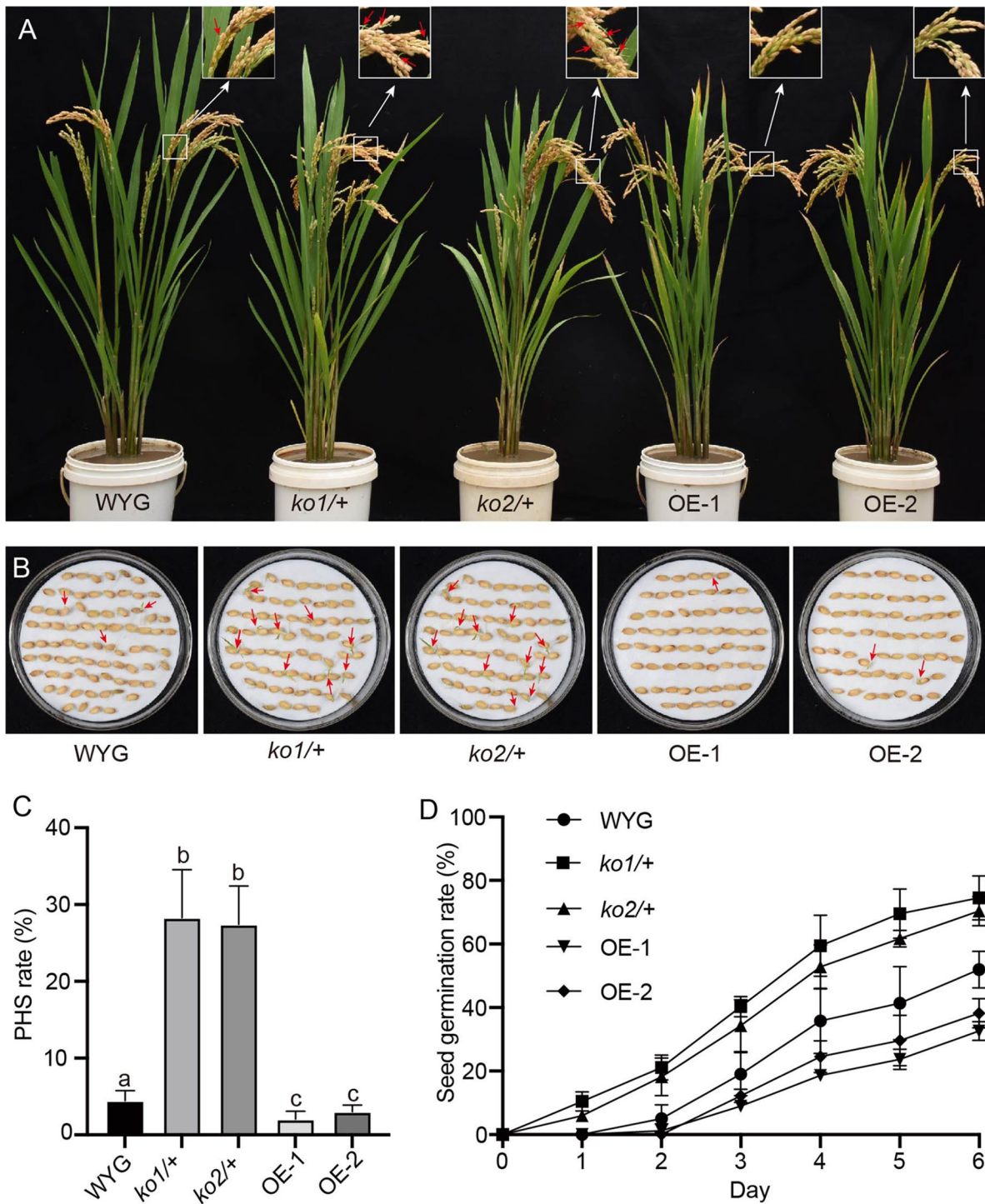
Fig. 4 PHS phenotypes of *ko* mutants and OE plants. (**A**) The wild-type WYG and *ko1/+*, *ko2/+*, OE-1 and OE-2 plants. Panicles with PHS phenotypes are shown in the enlarged views on the top right of the images. (**B**) Germination phenotypes of seeds collected at 31–35 DAP from WYG and *ko1/+*, *ko2/+*, OE-1 and OE-2 plants after 3 days imbibition on the wet filter papers. (**C**) PHS rates for (**A**). Data represent means ± SD (*n* > 8), and significant differences (*p* < 0.05) are indicated by different letters (one-way ANOVA with Bonferroni–Holm post hoc test). (**D**) Time course of seed germination rates for (**B**). Scale bars = 10 cm in (**A**)
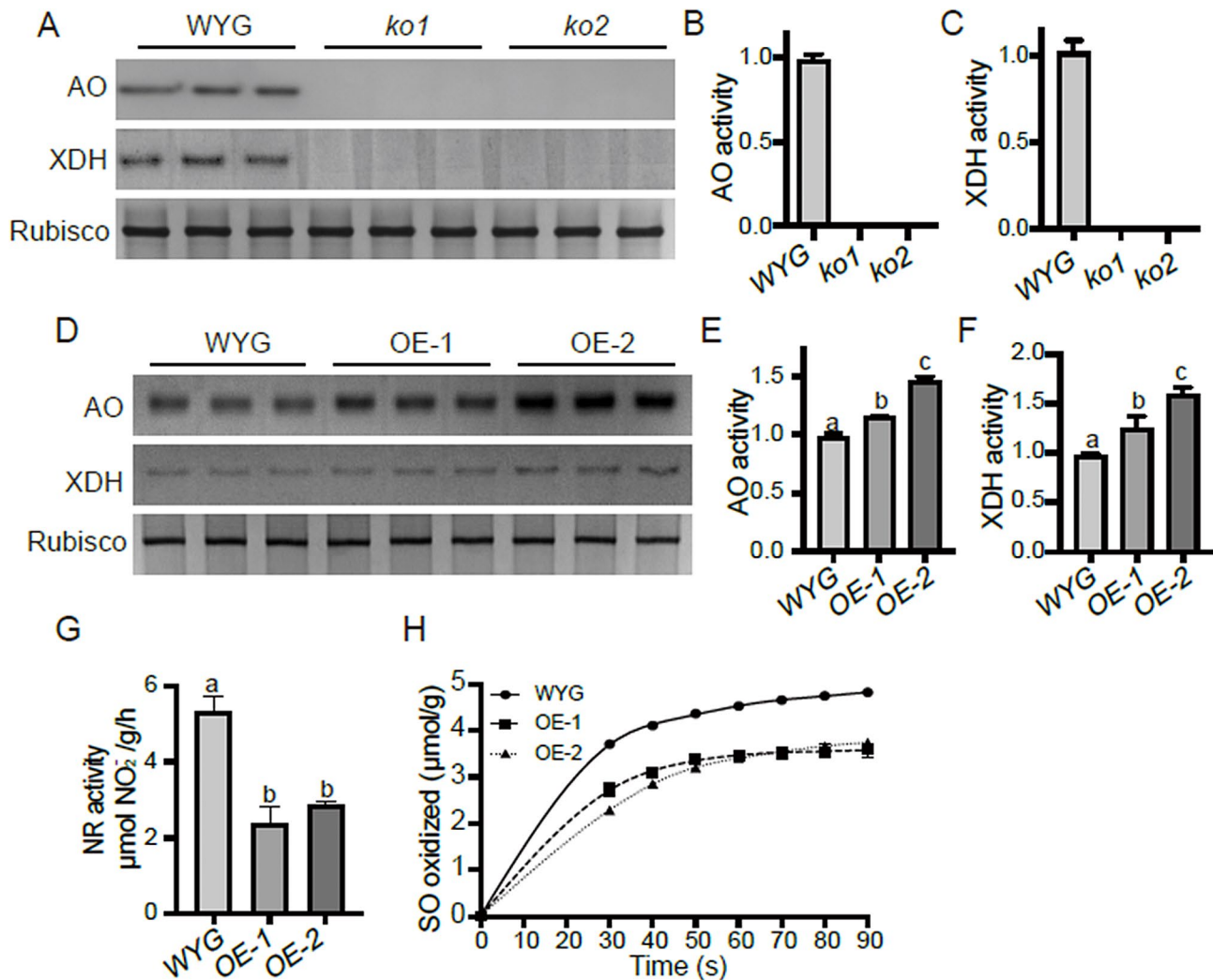
Fig. 5 The activities of Moco-dependent enzymes in the *ko* and OE plants. (**A**-**F**) For analyzes of the AO and XDH activities, native PAGE assays were carried out. The quantitative activity was calculated relative to the corresponding enzyme activity in wild-type plants. The large subunit of rubisco protein stained by Coomassie brilliant blue was used as a loading control. (**A**-**C**) The activities of AO and XDH in the wild-type WYG and *ko* mutants. (**D**-**F**) The activities of AO and XDH in the WYG and OE plants. (**G**-**H**) The activities of nitrate reductase (NR) and sulfite oxidase (SO) in the seedlings of WYG and OE plants, respectively. In (**E**, **F**, **G**), Data represent means $\pm$ SD ($n = 3$), and significant differences ($p < 0.05$) are indicated by different letters (one-way ANOVA with Bonferroni–Holm post hoc test)

(94.9 ± 3.2) under natural short-day conditions (Fig. 7J). We also test the agronomic performances of OE plants in the paddy fields in Shenzhen (22.73 N, 113.95E), and similar results were observed (Fig. S7). Collectively, these finding demonstrated that the OE plants exhibited earlier flowering without any reduction in grain yield.

Discussion

Appropriate seed dormancy is essential for seed crop production. A weak seed dormancy may increase the risk of PHS, especially in the regions with high temperature and humidity (Tai et al. 2021). ABA is considered as the core regulator of seed dormancy. The mutants with impaired ABA biosynthesis exhibited significantly reduced seed dormancy in various plant species (Ali et

al. 2022). The final step in the de novo ABA biosynthesis pathway is catalyzed by AAO, which requires sulfurated Moco for its activity. The molybdenum cofactor sulfurases are encoded by ABA3 family genes in plants (Finkelstein 2013). The *ataba3* mutants were deficient in ABA biosynthesis, displayed pleiotropic phenotypes including loss of seed dormancy, withering under low relative humidity conditions, and susceptibility to cold stress in Arabidopsis (Leon-Kloosterziel et al. 1996; Llorente et al. 2000; Sagi et al. 2002; Xiong et al. 2001). The present study showed that the heterozygous mutants of *OsABA3* exhibited more severe PHS in paddy fields (Figs. 1B and 4A). Biochemical analyzes revealed that the AO activities were barely detected in the homozygous mutants (Figs. 3H and 5A), indicating that ABA biosynthesis
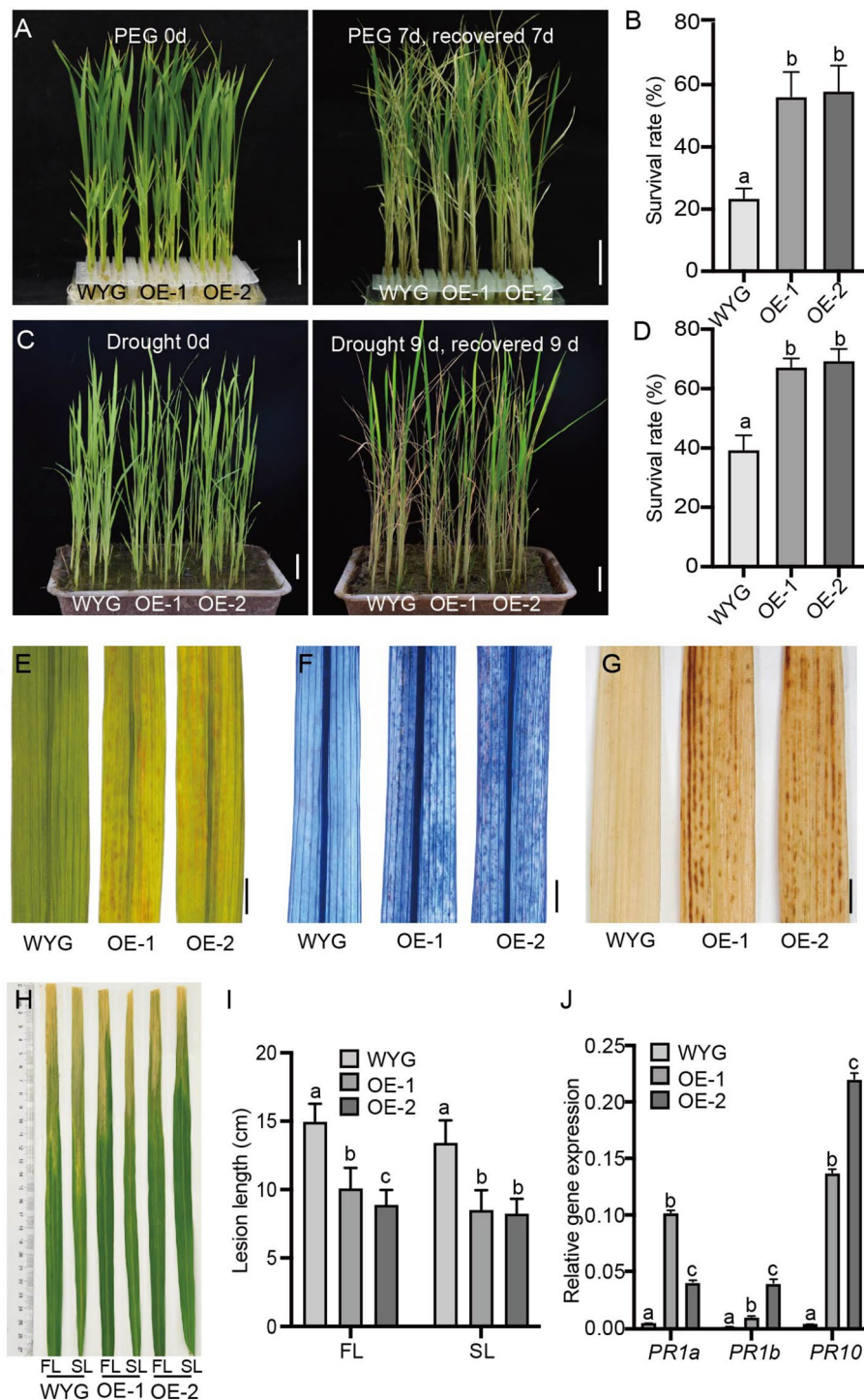
Fig. 6 The phenotypes of OE plants under osmotic and biotic stresses. (**A**) Phenotypes of WYG and OE plants after 20% PEG6000 treatment for 7 days plus 7 days recovery. The seedling survival rates are displayed in (**B**). (**C**) Phenotypes of WYG and OE plants after drought treatment for 9 days plus 9 days recovery. The seedling survival rates are shown in (**D**). (**E-F**) The lesion mimic phenotype of old leaves of OE plants at heading stage. (**E**) The representative leaf sections of OE plants with brown lesions. (**F**) Dead cells in leaves stained by trypan blue. (**G**) H_2O_2 accumulation in leaves detected by DAB staining. (**H**) Phenotypes of WYG and OE plants after inoculation with *Xoo* for 15 days. (**I**) Statistic analysis of the lesion length. FL: the flag leaf; SL: the second leaf. (**J**) The expression of PR genes in the leaves from WYG and OE plants at tillering stage. Gene expression of *PR1a*, *PR1b* and *PR10* analyzed by RT-qPCR. *OsUbq5* was used as an internal control. Data represent means ± SD (**B** and **D**, $N = 3$; I, $n = 15$; J, $n = 4$), and significant differences ($p < 0.05$) are indicated by different letters (one-way ANOVA with Bonferroni–Holm post hoc test). Scale bars, 3 cm (**A**, **C**), and 1 cm (**E-G**)
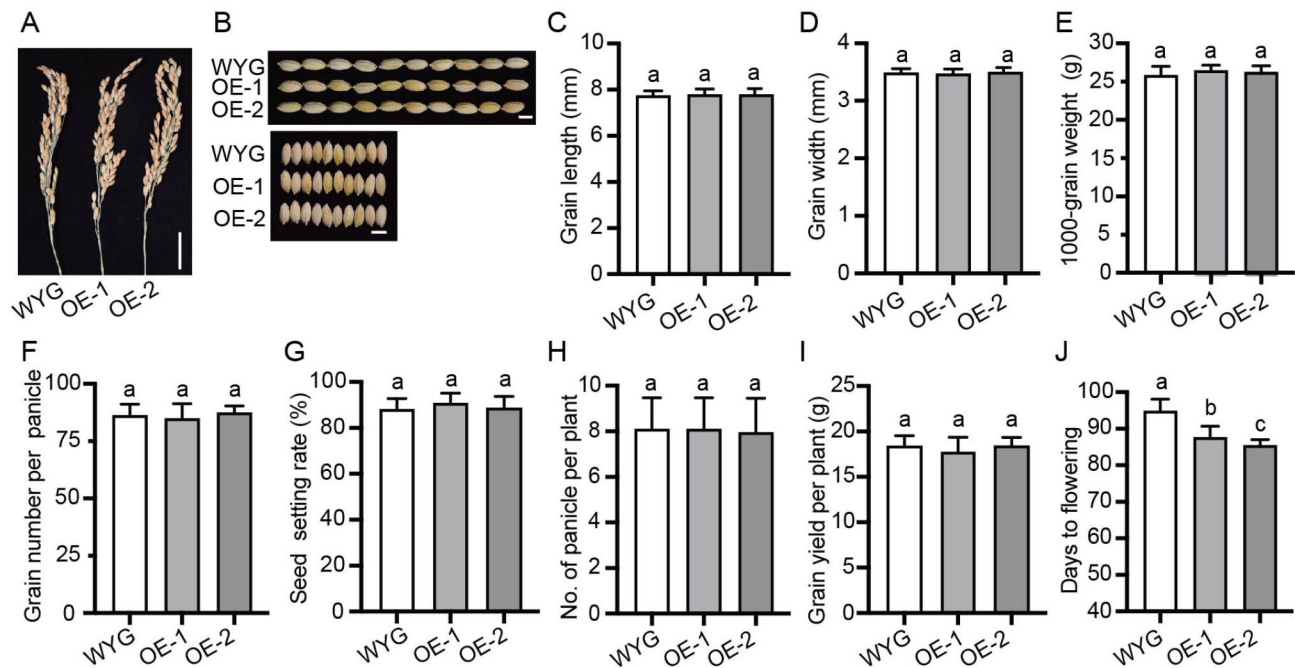
Fig. 7 The performances of OE plants in paddy fields. The wild-type plants (WYG) and OE plants were grown in paddy fields in Guangzhou (23.13 N, 113.26E), China. (**A**) Representative panicles of WYG and OE plants. (**B**) Comparison of the grain size between WYG and OE plants. (**C-I**) Comparison of the agronomic traits corresponding to grain yield between WYG and OE plants. (**C**) Grain length. (**D**) Grain width. (**E**) 1000-grain weight. (**F**) Grain number per panicle. (**G**) Seed setting rate. (**H**) Number of panicles per plant. (**I**) Grain yield per plant. (**J**) Heading date. Data represent means $\pm$ SD (C-I, $n = 20$; J, $n = 48$), and significant differences ($p < 0.05$) are indicated by different letters (one-way ANOVA with Bonferroni–Holm post hoc test). Scale bars, 2 cm (**A**) and 5 mm (**B**)

was interrupted. Furthermore, *OsABA3* overexpression enhanced plant resistance to PHS (Fig. 4A). These findings suggest that *OsABA3* is essential for seed dormancy and PHS resistance in rice.

The ABA present in developing seeds originates from both maternal and zygotic tissues. Maternal ABA and zygote-derived ABA are both critical for seed development (Frey et al. 2004; Karssen et al. 1983; Qin et al. 2021). However, reciprocal crosses of ABA-deficient mutants and wild-type plants have shown that zygote-derived ABA is predominant for the onset of seed dormancy in Arabidopsis (Kanno et al. 2010; Karssen et al. 1983). The more severe PHS phenotype observed in the heterozygous mutants of *OsABA3* indicated that the zygote-derived ABA is also necessary for seed dormancy in rice.

The ABA-deficient mutants typically exhibited severely stunted growth, reduced seed dormancy, and a wilted phenotype in Arabidopsis (Finkelstein 2013). *ABA2* encodes an enzyme that catalyze the production of abscisic aldehyde. The *ataba2* mutants produced seeds with reduced dormancy and much smaller adult plants with a wilty phenotype under desiccating conditions in Arabidopsis (Leon-Kloosterziel et al. 1996; Nambara et al. 1998). Consistently, the *osaba2* mutants showed loss of seed dormancy and sever PHS under wet and high

temperature conditions in rice. However, they also displayed species-specific phenotypes including enhanced growth of leaves and stems, and spontaneous cell death on mature leaves (Liao et al. 2018). The null mutants of *AtABA3* were viable in Arabidopsis (Leon-Kloosterziel et al. 1996; Xiong et al. 2001). However, our present work showed that the null mutants of *OsABA3* were lethal at the seedling stage in rice (Fig. 1D, Fig. S1), indicating ABA3 orthologs may evolved species-specific functions in Arabidopsis and rice. Recently, it was reported that AtABA3 coupled with the sulfur transferase 18 (STR18), represents a new pathway of cytosol sulfur trafficking in Arabidopsis (Selles et al. 2022). Therefore, OsABA3 may have other uncharacterized functions in rice that are essential for rice plant survival.

The activities of molybdoenzymes are coordinated with the levels of Moco or sulfurated Moco in cells. Disruption of the de novo biosynthesis of Moco led a significant decrease in molybdoenzymes activities, including AO, XDH, NR and SO (Liu et al. 2019). *ABA3* encodes a Moco sulfurase, which is required for the formation of sulfurated Moco. In our present work, we found that OsABA3 is critical for maintaining Moco homeostasis in rice. We observed that the activities of AO and XDH were barely detected in the null mutants of *OsABA3* (Figs. 3H and 5A), but were significantly increased in

the OsABA3 overexpressing plants (Fig. 5D). These findings suggest that OsABA3 is indispensable for Moco sulfuration. Interestingly, the NR and SO activities were both decreased in the *OsABA3* overexpressing plants (Fig. 5G-H). Moreover, OsABA3 overexpressing plants exhibited enhanced tolerance to osmotic stress (Fig. 6a-d). It was reported that OsABA3 expression was significantly upregulated in response to drought stress (Huang et al. 2009; Kim et al. 2021). In contrast, the NR activity was significantly decreased in rice seedlings subjected to osmotic stress with a rich nitrate supply (Han et al. 2022). These results take the hypothesis that upregulated *OsABA3* may enhance Moco sulfuration and thus result in an increase of sulfurated Moco and a decrease of Moco content. This alteration may directly affect the activities of Moco-dependent enzymes including AO and NR, and contribute to plant adaptation to drought stress by activating ABA biosynthesis and inhibiting nitrogen assimilation, respectively. However, further experimental evidence is required to test this hypothesis.

ABA is a positive regulator in plant response to drought stress (Wilkinson and Davies 2002). Drought stress dramatically induces ABA biosynthesis that leads to accumulation of ABA, and then promotes stomatal closure to reduce water loss and activates the expression of drought tolerant genes. Overexpression of ABA biosynthesis genes has been shown to effectively improve plant drought tolerance. As ABA3 is a vital enzyme for ABA biosynthesis, lots of studies have been conducted to test the potential use of ABA3 in improving plant drought tolerance in rice, tobacco, cotton, maize and bean (Li et al. 2013; Lu et al. 2013; Xiao et al. 2009; Yue et al. 2011, 2012). All of these studies showed that overexpression of *AtABA3* significantly increased plant fitness under drought stress. It is worth to note that the *AtABA3* overexpressing plants appeared similar to wild-type plants under well-watered conditions. Overexpression of *OsABA3* in rice enhanced plant resistance to bacterial blight (Liu et al. 2022). Here, we showed that the *OsABA3* overexpressing plants displayed enhanced resistance to PHS and bacterial blight (Fig. 6H-I), and early flowering (Fig. 7J). Flowering time, also known as heading date, is critical for rice adaptation and production (Zhou et al. 2020). Earlier flowering leads to a shorter rice growth duration, which facilitates rice production and maximizes the use of field resources. However, early flowering time often results in a reduction in grain yield (Fang et al. 2019). The *OsABA3* overexpressing plants flowered earlier but exhibited normal grain yield under well-watered conditions (Fig. 7, Fig. S7). Collectively, these findings suggested that overexpression of *OsABA3* may be an effective strategy for simultaneously improving multiple agronomic traits in rice. Recently, the CRISPR-Cas9 genome editing assay was successfully employed

to knock-up gene expression in rice by targeting cis elements in the promoter or inducing genomic inversion or duplication (Lu et al. 2021; Tan et al. 2023). Therefore, it is plausible to generate the transgene-free *OsABA3* overexpressing plants by genome editing for rice breeding in the future.

In summary, this study demonstrated that *OsABA3* is critical for Moco biosynthesis and homeostasis, and essential for plant survival and seed dormancy in rice. Notably, overexpression of *OsABA3* significantly improved plant resistance to PHS, osmotic stress and bacterial blight, and promoted plant flowering without any reduction in grain yield, suggesting that OsABA3 is a promising candidate gene for rice breeding.

Materials and methods
Plant Materials
The *phssl* mutant was identified from an ethyl methanesulfonate (EMS) mutant library of the *indica* cv. Huanghuazhan (HHZ). The wild-type *japonica* cv. Wuyungeng 7 (WYG) was used for transgenic analysis. All plants were grown in paddy fields with regular care.

Molecular Cloning of the Mutant Gene of *phssl*
Genomic DNA was extracted from 30 seedlings of the progeny of *phssl/+* plants that had germinated quickly. The resulting DNA was used for bulk-sequencing. The sequence data was analyzed using the simultaneous identification of multiple causal mutations (SIMM) method (Yan et al. 2016). The high resolution melt (HRM) method was employed to examine the mutation sites in the progeny of *phssl/+* plant by PCR with specific primers shown in Table S2 (Lochlainn et al. 2011).

Plasmid Construction and Rice Transformation
The null mutants of *OsABA3* in the WYG background were generated by using the CRISPR/Cas9 method as previously described (Ma et al. 2015), Briefly, two guide RNAs with different target sequences on *OsABA3* were designed and cloned into pYLCRISPR/Cas9. The resulting constructs were introduced to WYG by Agrobacterium-mediated rice transformation (Toki et al. 2006).

To generate *OsABA3* overexpressing plants, the *OsABA3* coding sequence driven by a ubiquitin promoter was cloned into *pCAMBIA1300* using the InFusion HD cloning kit (TaKara, Dalian, China). The final construct was introduced into the Agrobacterium tumefaciens AG10 strain for rice transformation.

To generate the *COM/ko1* and *COM-S482A/ko1* plants, the *pUBQ: OsABA3* and *pUBQ: OsABA3-S482A* expression cassettes were introduced into the progeny of the heterozygous mutant *ko1/+* by Agrobacterium-mediated rice transformation, respectively. The T_2 homozygous transgenic plants carrying the homozygous *ko1* allele

were identified by PCR and HRM genotyping and used for further studies.

Phylogenetic Analysis

The homologs of OsABA3 were identified from the genome database Phytozome (https://phytozome-next.jgi.doe.gov/) and NCBI (https://www.ncbi.nlm.nih.gov/) by using the BlastP method (Goodstein et al. 2012). Sixteen ABA3 proteins from the representative plant species were selected for further analysis. The ABA3 protein sequences from *Volvox carteri* (XP_002950063.1), *Coccomyxa subellipsoidea* (19,457), *Physcomitrium patens* (Pp3c5_23340V3.1), *Sphagnum fallax* (Sphfalx14G015300.1), *Ceratopteris richardii* (Ceric.02G066600.3), Diphasiastrum complanatum (Dicom.12G016300.1), *Selaginella moellendorffii* (173,762), *Manihot esculenta* (Manes.06G173000.1), *Citrus clementina* (Ciclev10004324m), *Gossypium raimondii* (Gorai.002G036800.1), *Arabidopsis thaliana* (AT1G16540.1), *Medicago truncatula* (Medtr4g030930.1), *Solanum lycopersicum* (Solyc07T002813.2), *Ananas comosus* (Aco008369.1), *Oryza sativa* (LOC_Os06g45860.1), *Triticum aestivum* (Traes_7DL_162505318.2), *Brachypodium distachyon* (Bradi1g32350.5), *Panicum hallii* (Pahal.D00546.2), *Zea mays* (Zm00001d036635_P001), and *Sorghum bicolor* (Sobic.010G224000.3) were aligned by using ClustalX with default parameters. The phylogenetic tree was constructed using the Neighbor-Joining algorithm (1,000 replicates) in MEGA11.

RNA Extraction and RT-qPCR Assay

Total RNA was extracted from various rice tissues using the Plant RNA Kit R6827 (Omega Bio-Tek, Santa Clara, CA, USA), and reverse-transcribed using a PrimeScript RT reagent kit (TaKaRa, Dalian, China). RT-qPCR was performed using SYBR Premix Ex Taq II (TaKaRa, Dalian, China) on a Lightcycler instrument (Roche, Germany).

Subcellular Protein Localization

The *OsABA3* coding sequence was amplified from cDNA of WYG using the specific primers and was cloned into *pAN580*. The resulting plasmid *pAN580-OsABA3*, carrying the expression cassette *35 S: OsABA3-EGFP* was introduced into rice protoplasts using a polyethylene glycol (PEG)-mediated transformation assay. The fluorescence signal was examined and photographed by a confocal laser scanning microscope (LSM 800, Zeiss, Germany).

Assays of XDH and AO Activities

The activities of AO and XDH were analyzed as previously described (Liu et al. 2019). Briefly, 0.2 g of fresh seedling shoots or plant leaves per sample were collected and ground to a powder in liquid nitrogen. The powder was thawed in 1 ml of the extraction buffer (0.1 M potassium phosphate pH 7.5, 1 mM EDTA, 10 mM 2-hydroxy-1-ethanethiol, 1% Triton X-100), and centrifuged at 16,000 g for 30 min at 4 ℃. 15 μL of the resulting supernatant was loaded onto 6.5% polyacrylamide gel and subjected to native polyacrylamide gel electrophoresis at 4 ℃ for 4 h (50 V, 20 mA). Following electrophoresis, the gel was equilibrated in 0.1 M sodium pyrophosphate (pH 8.0) and subsequently stained in AO buffer (0.1 M Tris-HCl pH 8.5, 1 mM indole-3-aldehyde, 1 mM thiazolyl blue tetrazolium bromide, 0.1 mM phenazine methosulfate) for 30 min at room temperature in the dark. For XDH assay, the gel was stained in XDH buffer (0.1 M sodium pyrophosphate, pH 8.0, 2 mM hypoxanthine, 1 mM MTT, 0.1 mM phenazine methosulfate) for 30 min (at room temperature in the dark). After destaining with water, the gel was imaged and analyzed using ImageJ software (http://imagej.nih.gov/ij/).

Assays of NR and SO Activities

The NR activity in rice was analyzed following previously described method (Liu et al. 2019). Briefly, 0.2 g of homogenized sample powder was thawed in 2 ml of NR extraction buffer (25 mM phosphate buffer pH 7.5; 5 mM cysteine; 5 mM EDTA) and centrifuged at 16,000 g for 10 min at 4 ℃. Subsequently, 20 μL of the resulting supernatant was mixed with 80 μL of assay buffer (60 μL of 0.2 M KNO_3 phosphate buffer; 20 μL of 2.0 mg/ml NADH) and incubated at 30 ℃ for 30 min. The mixture was subjected to color development by adding 50 μL of stop solution (1% 4-aminobenzene sulfonic acid) and 50 μL of reaction buffer (0.2% 1-naphthylamine) at 30 ℃ for 30 min in the dark. The absorbance of the sample was immediately measured at 540 nm using a spectrophotometer (Enspire, PerkinElmer). NR activity was determined as the conversion of NO_2^- per gram of fresh weight per hour.

For the analysis of SO activity, a homogenized sample of 0.2 g was thawed in 1 ml of SO extraction buffer (0.1 M HEPES, 1 mM EDTA, 5% glycerol, 1 mM Na_2MoO_4, 1 mM phenylmethylsulfonylfluoride, pH 7.3) and centrifuged at 16,000 g at 4 ℃ for 30 min. 20 μL of the supernatant was mixed with 180 μL of assay buffer (20 mM Tris−acetic acid pH 8.0, 0.1 mM EDTA, 395 μM potassium ferricyanide, 400 μM sodium sulphite). The sample's absorbance was then measured at 420 nm using a spectrophotometer (Enspire, PerkinElmer). The reduction of 2 mol ferricyanide was assumed to be caused by 1 mol sulfite. The rate of sulfite conversion was used to measure SO activity (Liu et al. 2019).

Germination Assays

More than three fresh panicles from each genotype were harvested at 31–35 DAP. The panicles were then immersed in plastic boxes with distilled water and incubated at 30 °C in a growth chamber under a 14 h/10 h light/dark regime for 2 days. The germination rate per panicle was calculated.

To analyze the seed dormancy more precisely, homogeneous seeds from each genotype were freshly harvested at 31–35 DAP. The seeds were placed on wet filter paper in Petri dishes with at least three repeats. The number of germinated seeds was counted daily, and germination rates were calculated.

Histochemical Analysis

Leaves exhibiting lesion mimic spots from the *OsABA3* overexpressing plants, and leaves from the same growth stage of the WYG were collected for histochemical analysis. To examine cell death, trypan blue staining was conducted following established procedures (Liao et al. 2018). In brief, the samples were immersed in a lactic acid-phenol-trypan blue solution (2.5 mg/ml trypan blue, 25% (w/v) lactic acid, 23% water-saturated phenol, and 25% glycerol in H_2O), vacuumed for 30 min, and boiled for 2 min, followed by destaining with 30% (w/v) chloral hydrate for several times.

For hydrogen peroxide assay, detached leaves were immersed in the staining solution (0.5 mg/ml 3,3'-diaminobenzidine (DAB)) and vacuumed for 30 min. Subsequently, the leaves were incubated at room temperature overnight, followed by decolorization in 95% ethanol at 80 °C in a water bath.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12284-024-00724-w.

Supplementary Material 1

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