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BZR1-NAC29-NAC31 signaling promotes the sheath blight resistance and tillering in rice

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Abstract

Improving disease resistance is a primary objective in crop breeding. However, the signaling mechanisms governing disease resistance and yield are frequently controlled antagonistically, posing challenges in balancing crop productivity and disease resistance. Prior research has shown that Brassinazole resistant 1 (BZR1) enhances resistance to rice sheath blight (ShB) and increases tillering in rice. Here, we found that BZR1 interacts with NAC29 and NAC31, the primary transcription factors responsible for regulating the formation of the secondary cell wall. The interaction between NAC29 and NAC31 was demonstrated by yeast two-hybrid and bimolecular fluorescence complementation tests. The expression of *NAC29* and *NAC31* increased significantly in response to *Rhizoctonia solani* infection. The susceptibility of the *nac29nac31* double mutant to ShB was increased, but the overexpression of *NAC29* and *NAC31* reduced susceptibility to ShB compared with the single mutants or wild-type plants. Like the *bzr1-D*, a *BZR1* dominant negative mutant, NAC29 and NAC31 overexpressors increased tiller numbers. Co-expression of BZR1 and NAC29 or NAC31 enhanced *cinnamyl alcohol dehydrogenase 8B* (*CAD8B*) expression while inhibiting *teosinte branched 1* (*TB1*) expression to a greater extent than the expression of BZR1 alone. Furthermore, the expression level of *CAD8B* was elevated in *NAC29-OX* and *NAC31-OX* plants, but the expression level of *TB1* was reduced in *NAC29-OX* and *NAC31-OX* plants compared to the wild-type plants. The results showed that BZR1-NAC29-NAC31 improves rice resistance and tillering by controlling the expression of *CAD8B* and *TB1*, respectively. These findings offer valuable targets for breeding that can avoid the trade-off between growth and defense.

Keywords Sheath blight, Transcription factor, Brassinazole resistant 1, Defense, Growth

Background

Rice (*Oryza sativa L.*), the staple food for one-third of the world's population, is essential for global food security. However, its yield is significantly threatened by sheath blight (ShB), a disease caused by *Rhizoctonia solani* Kühn. This disease can reduce rice yields by 10% to 30% and, in severe cases, by up to 50% (Savary et al. 2000). As such, breeding rice cultivars with high resistance to ShB and stable yields is becoming increasingly vital to ensure a secure food supply for the future.

In crop breeding, the use of the majority of disease-resistance genes is restricted by their concomitant fitness costs. WRKY45 has been vital for *Panicle blast 1* (*Pb1*)-mediated rice blast resistance (Inoue et al. 2000); however, overexpression of *WRKY45* results in defects in plant growth, therefore causing a huge loss in yield (Goto et al. 2015; Peng et al. 2021). Blast (caused by *Magnaporthe oryzae*) resistance tests have shown the important

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role of *OsNPR1* in benzothiadiazole (BTH)-induced blast resistance (Sugano et al. 2010). However, the *OsNPR1*-overexpressed lines have also shown the phenotypes with decreases in the seed weight, root system, tiller number, and internode elongation (Li et al. 2016). Considering these results, it can be concluded that pleiotropic functional genes may also influence the balance between plant immunity and yield. Many studies look at genes that could balance the trade-off between immunity and yield in rice. Ideal Plant Architecture 1 (IPA1) promotes the development and growth of rice by binding to the promoter of *WRKY51*, *DEP1*, and *WRKY71* (Wang et al. 2018a, b; He et al. 2021). Rice plants with higher expression levels of IPA1 enhance immunity to *M. oryzae* via an increase of Ser 163 phosphorylation level, and thus the activation of S^{163} phosphorylation of IPA1 promotes transcriptional activation to *WRKY45* after pathogen attack, as well as increases grains per panicle and plant height in a case of dephosphorylating S163 (Wang et al. 2018a, b; Liu et al. 2019). A ubiquitin-conjugating enzyme, OsUBC45, is involved in the endoplasmic reticulum-associated protein degradation system, and overexpression of OsUBC45 improves the yield and resistance to a wide range of diseases in rice. The OsUBC45 promotes the degradation of glycogen synthase kinase 3 (OsGSK3) and aquaporin (OsPIP2), which negatively influence the grain size and PTI, respectively (Wang et al. 2023). However, only a handful of genes have been identified, effectively balancing the trade-off between immunity and yield in rice. Thus, there is a strong need for genes that confer resistance, particularly those that can activate disease resistance without compromising yield.

The architecture and grain yield of cereal plants are significantly influenced by the number of tillers, the angle between the tillers and the leaves, and the height of the plant (Wang et al. 2018a, b). The number of tillers per plant determines the panicle number, a critical element of cereal productivity. In rice, brassinosteroids (BRs) are a group of plant-specific steroidal phytohormones that promote bud outgrowth, thereby enhancing tillering (Bai et al. 2007; Tong et al. 2012; Fang et al. 2020; Yao et al. 2023). Brassinazole resistant 1 (BZR1) is a key transcription factor (TFs) of the BR signaling pathway (Wang et al. 2014). In rice, BR signaling promotes rice tillering by stabilizing BZR1 (Lozano-Durán et al. 2013), ensuing *TB1* suppression by BZR1 (Fang et al. 2020). TB1 suppresses rice tillering by activating *grassy tiller 1* (*GT1*) (Kumar et al. 2021). By controlling the expression of certain genes, BZR1 helps maintain the equilibrium between defensive responses and growth promotion in plants (Tong et al. 2012; Lozano-Durán et al. 2013; Wang et al. 2014; Fang et al. 2020; Kumar et al. 2021). Our previous studies show that BR suppresses ShB resistance (Yuan et al. 2018), while BZR1 increases rice resistance to ShB (Yuan et al. 2022). This suggests that BZR1-mediated ShB resistance is independent of BR signaling. BZR1 directly activates *CAD8B* expression, which enhances rice resistance to ShB^{20}. Furthermore, *bzr1-D* exhibits increased resistance to ShB and a greater number of tillers (Chen et al. 2024). Despite recent progress, the regulatory system responsible for BZR1-mediated ShB resistance and tillering in rice has yet to be fully understood.

In this study, we found that BZR1 interacted with NAC29 and NAC31. NAC29 and NAC31 positively regulated ShB resistance, tillering, and yield. In addition, NAC29 interacted with NAC31 in the nucleus. BZR1 and NAC29/31 formed a TF complex to activate the expression of *CAD8B* to enhance ShB resistance and inactivate the expression of *TB1* to increase rice tiller number. Altogether, our studies discover that the BZR1-NAC29-NAC31 complex regulates not only the immunity but also tillering of rice plants, which provides a beneficial strategy for improving yield and disease resistance in rice.

Results

BZR1 interacts with NAC29 and NAC31

Enhancing immunity is a primary objective in crop breeding, yet the signaling pathways that govern yield and immunity are often controlled antagonistically (Ning et al. 2017). The previous report indicated that BR signaling has a negative effect on rice ShB resistance but a positive effect on tillering (Lozano-Durán et al. 2013). On the other hand, BZR1 has a positive effect on both rice ShB resistance and tillering, which are important traits in breeding (Wang et al. 2014; Yuan et al. 2022). This suggests that BZR1 promotes both yield and ShB resistance without compromising growth and immunity, giving it a significant advantage in rice breeding. To gain a deeper understanding of how BZR1 controls ShB resistance and tillering, the present study conducted a yeast two-hybrid (Y2H) screening to obtain the proteins that interact with BZR1. Out of the identified BZR1 interactors, NAC29 and NAC31 were chosen for further assessment due to their high sensitivity to *R. solani* infection (Yuan et al. 2020). Further RT-qPCR analysis revealed that the *R. solani* infection significantly up-regulated the expression of NAC29 and NAC31 (Additional file 1: Figure S1), indicating a potential role for NAC29 and NAC31 in rice immunity.

The Y2H assay showed that yeast cells co-expressing BD-BZR1 with AD-NAC29 or AD-NAC31 could successfully grow on an SD-TLH medium; in contrast, yeast cells expressing BD-BZR1 with AD could not grow on the selective medium (Fig. 1a, b), indicating that BZR1 interacted with NAC29 or NAC31 in yeast. A BiFC experiment was performed to validate

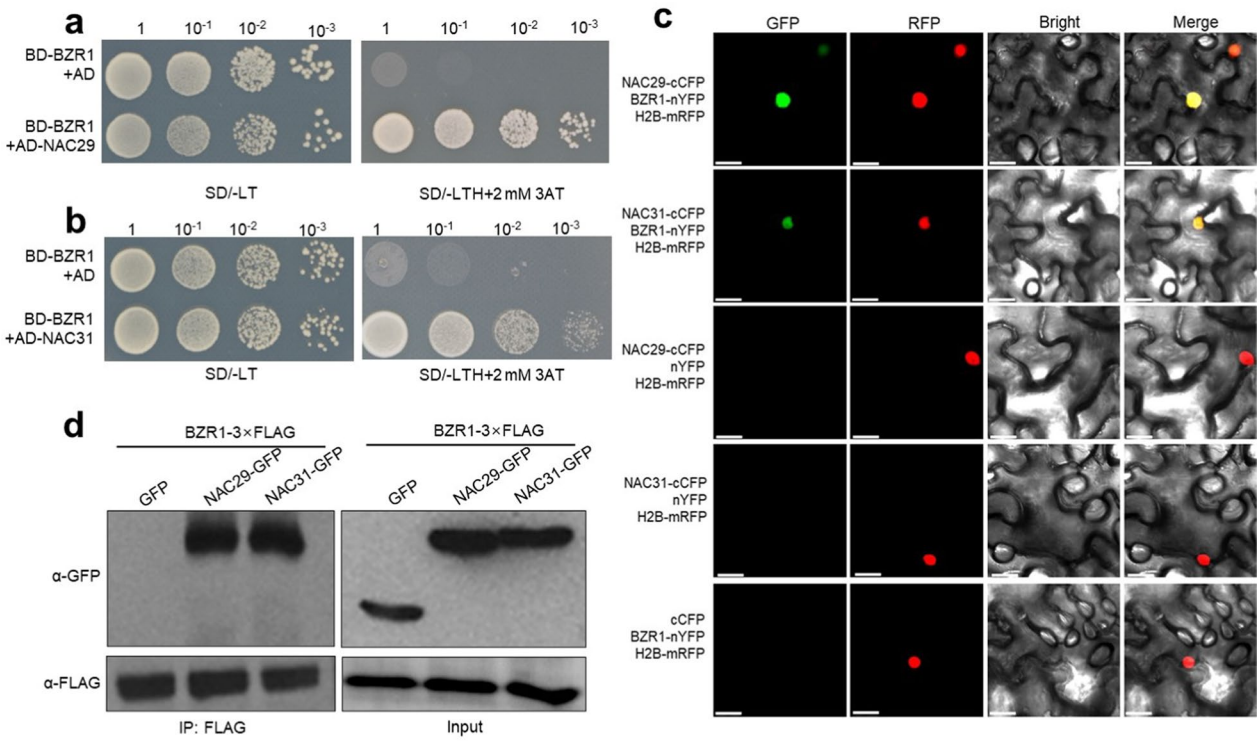

Fig. 1 BZR1 interacts with NAC29 and NAC31. **a, b** Y2H assays showed the interaction between BZR1 and NAC29 or NAC31. The transformants were grown on SD-Leu-Trp (SD-LT) and SD-Leu-Trp-His (SD-LTH) plates containing 2 mM 3-AT, a histidine biosynthesis inhibitor. **c** BiFC assay showed the BZR1 interaction with NAC29 or NAC31 in the nucleus. Co-expression of BZR1-nYFP, NAC29-cCFP or NAC31-cCFP, and H2B-mRFP1 in *N.benthamiana* leaves. GFP is an indicator of protein–protein interactions. Scale bars = 20 μm. **d** CoIP assay showed BZR1 interaction with NAC29 or NAC31. Co-expression of BZR1-3 × FLAG and NAC29-GFP or NAC31-GFP in *N.benthamiana* leaves. Total proteins were extracted and immunoprecipitated with anti-FLAG beads, then subjected to western blot assay with anti-FLAG and anti-GFP antibodies

these interactions. Agrobacterial strains expressing different combinations of proteins were co-infiltrated with the nuclear marker H2B-mRFP1 on the leaves of *Nicotiana benthamiana*. A strong fluorescence signal was detected in the nucleus when NAC29 or NAC31 were co-expressed with BZR1. However, no fluorescence signal was recorded in the leaves where the negative controls were co-expressed (Fig. 1c). Co-immunoprecipitation (Co-IP) experiments demonstrated a connection between BZR1 and the two NACs in *N.benthamiana* leaves, as shown in Fig. 1d. These findings suggest that NAC29 and NAC31 interact with BZR1 in the nucleus.

NAC29 interacts with NAC31

The unrooted phylogenetic tree was constructed using the conserved NAC domain from both rice and previously published NAC genes (Fig. 2a). In rice, 136 NAC domain proteins have been identified and categorized into five groups (I-V), with each subfamily showing significant diversification. The subgroup NACI-1 contains 13 genes in total. Phylogenic analysis showed that NAC29 (Os08g02300.1) and NAC31 (Os08g01330.1) can be subsequently classified into subgroup NACI-1 as indicated by phylogenic analysis (Fig. 2a, Additional file 2: Table S1). The amino acid sequence revealed that NAC29 and NAC31 are highly conserved in the NAC domain, but they contain largely different C-terminal regions

(See figure on next page.)

Fig. 2 NAC29 interacts with NAC31. **a** Phylogenic analysis of rice NAC domain protein sequences. **b** Amino acids alignment of NAC29 and NAC31.**c** BiFC assay showed that NAC29 interacts with NAC31 in the nucleus. Co-expression of NAC29-cCFP and NAC31-nYFP with H2B-mRFP1 in *N. benthamiana* leaves. GFP is an indicator of protein–protein interactions. Scale bars = 20 μm. **d** Y2H assays showed the interaction between NAC29 and NAC31. The transformants were grown on SD-Leu-Trp (SD-LT) and SD-Leu-Trp-His (SD-LTH) plates containing 2 mM 3-AT, a histidine biosynthesis inhibitor

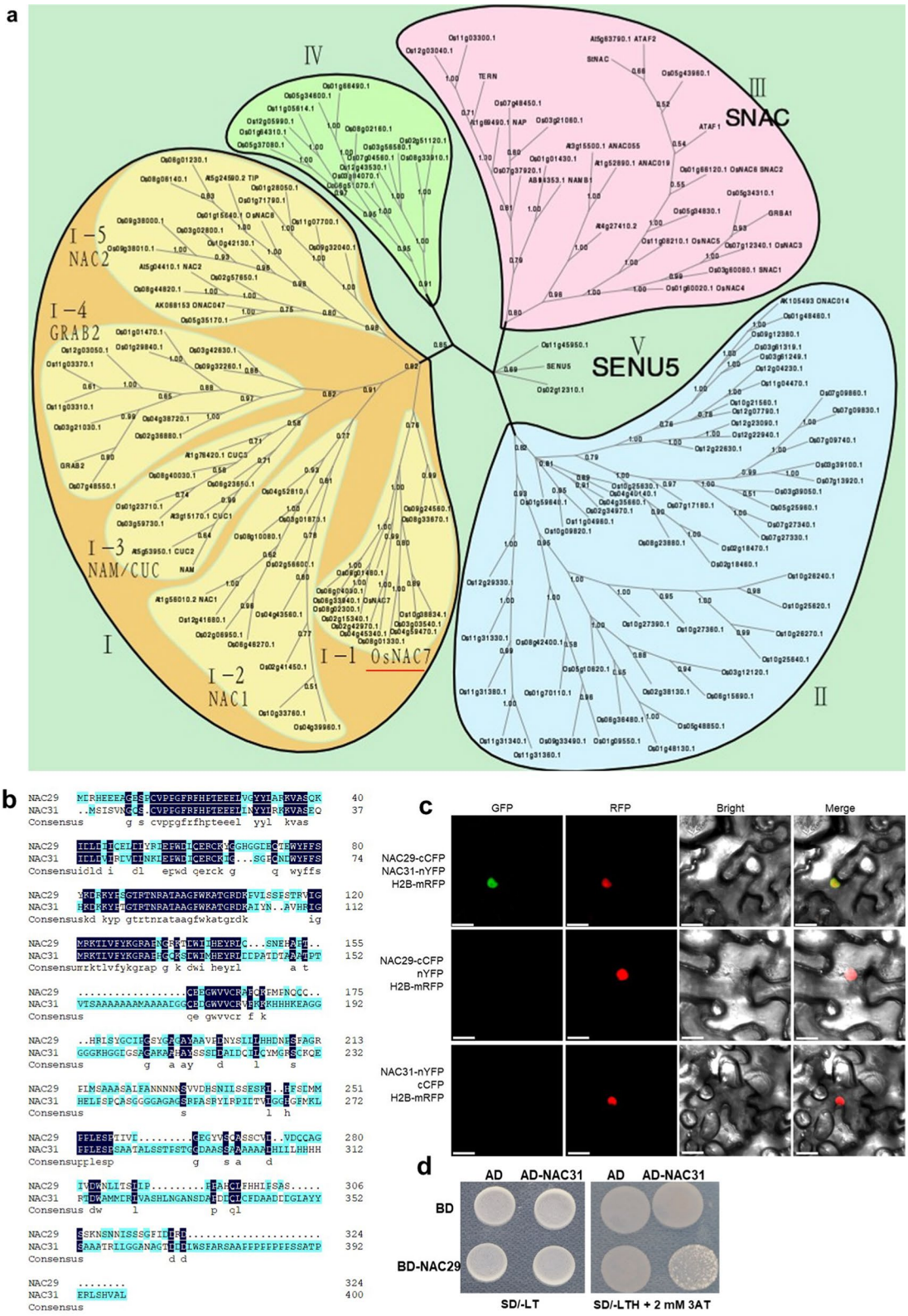

Fig. 2 (See legend on previous page.)

(Fig. 2b). Since both NAC29 and NAC31 are homologous and interact with BZR1, the association between NAC29 and NAC31 was tested. The Y2H assay showed that yeast expressing BD-NAC29 with AD-NAC31 grew on the SD-TLH medium, whereas other negative controls could not grow on the selective medium (Fig. 2c), suggesting that NAC29 interacts with NAC31 in yeast. In the BiFC assay on the leaves of *N. benthamiana*, cells co-expressing NAC29 and NAC31 displayed a strong fluorescence signal in the nucleus, indicated by the co-expressed nuclear marker H2B-mRFP1, whereas the leaves co-expressing the negative controls did not show any fluorescence signal (Fig. 2d). These findings suggested that NAC29 interacts with NAC31 to establish a TF complex in the nucleus.

NAC29 and NAC31 promote rice ShB resistance

To determine if NAC29 and NAC31 play a role in rice resistance to ShB, genome editing via CRISPR/Cas9 was employed to develop *NAC29* and *NAC31* mutant plants (*nac29*, *nac31*, and *nac29nac31* double mutant) in the ZH11 background. Sequencing results revealed that the *nac29* mutant has a 1 bp insertion in the exon region of *NAC29*, whereas the *nac31* mutant has a 1 bp deletion in the exon region of *NAC31* (Fig. 3a, b). The *nac29nac31* double mutant has 1 bp insertion in the exon region of both *NAC29* and *NAC31* (Fig. 3c). The inoculation of rice plants with *R. solani* AG1-IA demonstrated that the *nac29nac31* double mutant was more susceptible to ShB, whereas the *nac29* and *nac31* single mutants did not show a significant difference from the WT plants (Fig. 3d). As shown in Fig. 3e, the lesion length of the *nac29nac31* mutants was longer than that of WT plants, whereas *nac29* and *nac31* did not show any significant difference from WT plants (Fig. 3e).

NAC29 and NAC31-overexpression plants (*NAC29-OX* and *NAC31-OX*) were generated in the ZH11 background. The resistance of NAC29 and NAC31-overexpression lines against ShB was evaluated. Results of RT-qPCR displayed significantly higher expression levels of *NAC29* and *NAC31* in the overexpression plants compared to WT plants (Additional file 1: Figure S2). Upon inoculation with *R. solani*, *NAC29-OX* and *NAC31-OX* plants showed reduced susceptibility to ShB compared with WT plants (Fig. 3f). The lesion length in *NAC29-OX* and *NAC31-OX* plants were significantly shorter than in WT plants (Fig. 3g). These results indicated that both NAC29 and NAC31 positively regulate rice resistance against ShB.

NAC29/31 positively regulates rice tillering and grain size

Remarkably, *NAC29-OX* and *NAC31-OX* plants not only enhance the rice resistance to ShB but also promote the tillering and grain size. Compared to wild-type plants, *nac29nac31* mutant developed fewer tillers, and *NAC29-OX* and *NAC31-OX* plants developed more tillers (Fig. 4a, b). The size and length of the panicles were significantly reduced in the *nac29nac31* mutant, as seen in Fig. 4c and d, but increased in the *NAC29* or *NAC31-OX* plants (Fig. 4c, d). Meanwhile, the size and length of grains produced in *NAC29* and *NAC31-OX* plants were larger than those in WT plants (Fig. 4e, f). Consistently, the grain weight per plant in *NAC29* and *NAC31-OX* plants increased by around 30% compared to that of WT plants, as shown in Fig. 4g. The panicle size, grain numbers per panicle, and grain weight of *nac29nac31* mutants showed a reduction in comparison to those seen in WT plants (Fig. 4c–g). These findings suggest that NAC29 and NAC31 have a favorable role in controlling tillering and grain size.

BZR1-NAC29/31 directly regualtes *CAD8B* and *TB1*

BR signaling promotes rice tillering by stabilizing BZR1 and subsequent *TB1* suppression by BZR1 (Li et al. 2016). Given the interaction between NAC29 and NAC31 with BZR1, tests were conducted to determine the expression levels of *TB1* in *NAC29-OX* and *NAC31-OX* plants. The RT-qPCR results indicated a significant reduction in *TB1* expression in *NAC29-OX* and *NAC31-OX* plants compared to WT plants (Fig. 5a), suggesting that NAC29 and NAC31 promote rice tillering by down-regulating the expression of *TB1*. It has been found that BZR1 positively regulates rice resistance to ShB by directly binding to the *CAD8B* promoter. The *CAD8B* encodes a key enzyme involved in lignin biosynthesis, which is crucial for strengthening rice resistance to ShB (Yuan et al. 2018; Li et al. 2019; Yuan et al. 2022). Therefore, the expression level of *CAD8B* was investigated, and RT-qPCR results showed that the expression level of *CAD8B* was higher in *NAC29-OX* and *NAC31-OX* plants compared to WT plants (Fig. 5b). These findings suggest that NAC29 and NAC31 may enhance rice resistance to ShB and tillering by activating *CAD8B* and repressing *TB1* expression.

Furthermore, the effects of the BZR1-NAC29/NAC31 interaction on the expression levels of *CAD8B* and *TB1* were investigated. Transactivation analysis showed that *CAD8B* levels in cells expressing BZR1 were significantly enhanced by the additional co-expression of NAC29 or NAC31 (Fig. 5c, d). In contrast, the levels of *TB1* in cells expressing BZR1 were reduced when NAC29 or NAC31 were co-expressed (Fig. 5e, f). These findings indicate that OsBZR1 interacts with NAC29 and NAC31 to enhance the expression of *CAD8B* during rice defense and suppress the expression of *TB1* during rice tillering.

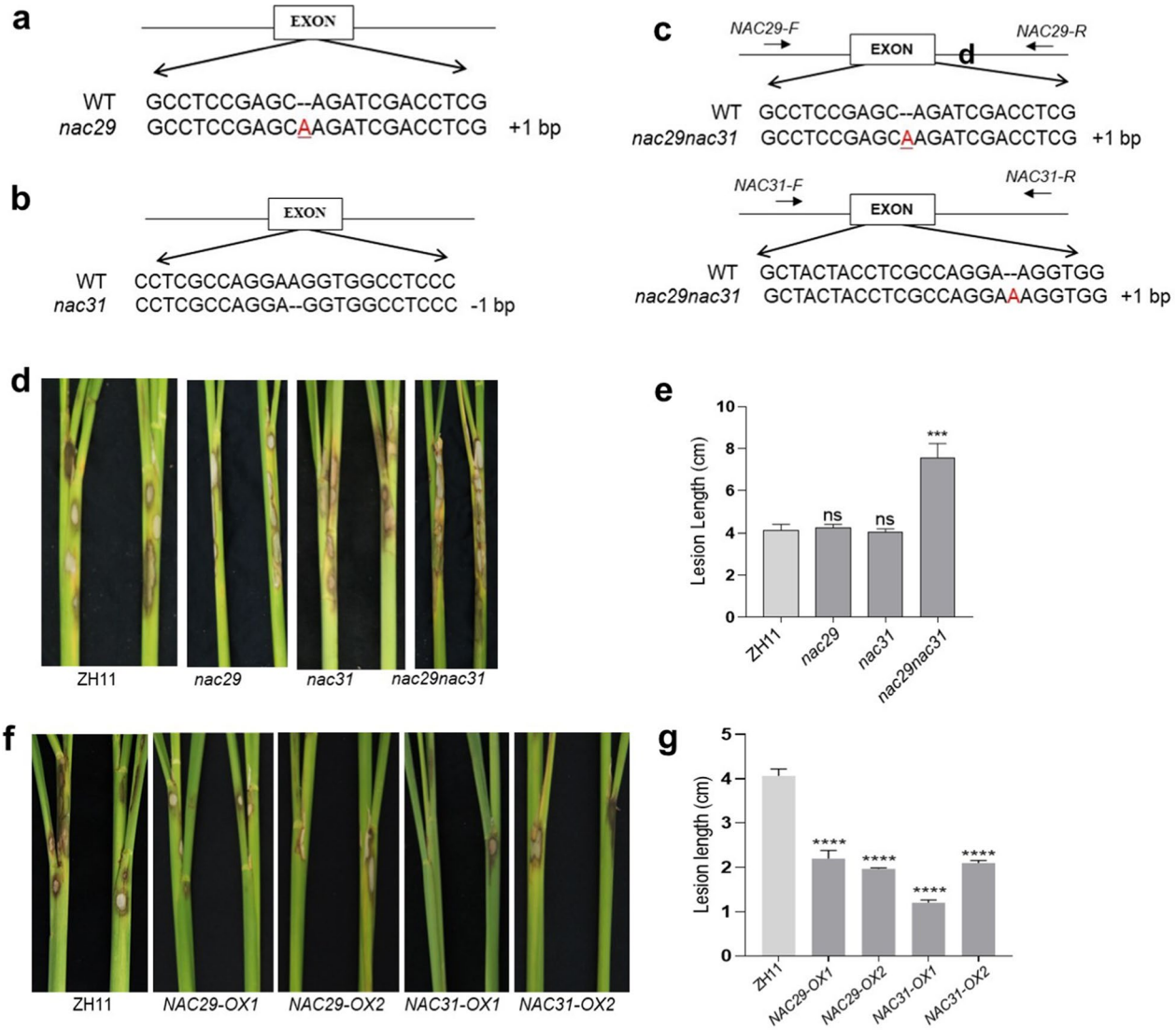

Fig. 3 NAC29 and NAC31 positively regulate rice resistance to ShB. **a, b, c** The target sequence alignment in *nac29*, *nac31*, and *nac29nac31*. The bases in red represent the changed bases. An 'A' was inserted in *nac29* (**a**), an 'A' was deleted in *nac31* (**b**), and *nac29nac31* double mutant has 1 bp insertion in *nac29* and *nac31* (**c**). **d** Disease symptoms of wild-type and mutant plants inoculated with *R. solani*. **e** Average lesion length was measured. Statistically significant differences are indicated by asterisks. **f** Disease symptoms of wild-type and overexpressor plants inoculated with *R. solani*. **g** Average lesion length was calculated. Statistically significant differences are indicated by asterisks (*** $P < 0.001$,.**** $P < 0.0001$, one-way ANOVA with Tukey's significant difference test)

Discussion

Rice is the primary cereal crop, and enhancing its productivity is vital for ensuring worldwide food security. Plant breeding aims to achieve high crop output and immunity to diseases. However, it is often seen that immunity comes at the cost of reduced yield (Wang et al. 2021; Zhang et al. 2023). Previously, we found that *bzr1-D* exhibited conspicuous attributes conducive to multi-tillering and increased resistance to ShB. Furthermore, BZR1 has a favorable role in regulating rice resistance to ShB through a pathway independent of BR. Nevertheless, the precise mechanism by which BZR1 mediates resistance to ShB and promotes tillering requires further explanation. The present investigation revealed that BZR1 interacts with NAC29 and NAC31, and together, they regulate the expression of the defense-related gene *CAD8B* and the tiller-related gene *TB1*, thereby controlling both the defense and tillering in rice (Fig. 6).

As a class of TFs unique to plants, NAC proteins serve a variety of biological purposes. Increasing data indicate that NAC TFs play an important role in plant response to abiotic stresses, encompassing developmental processes,

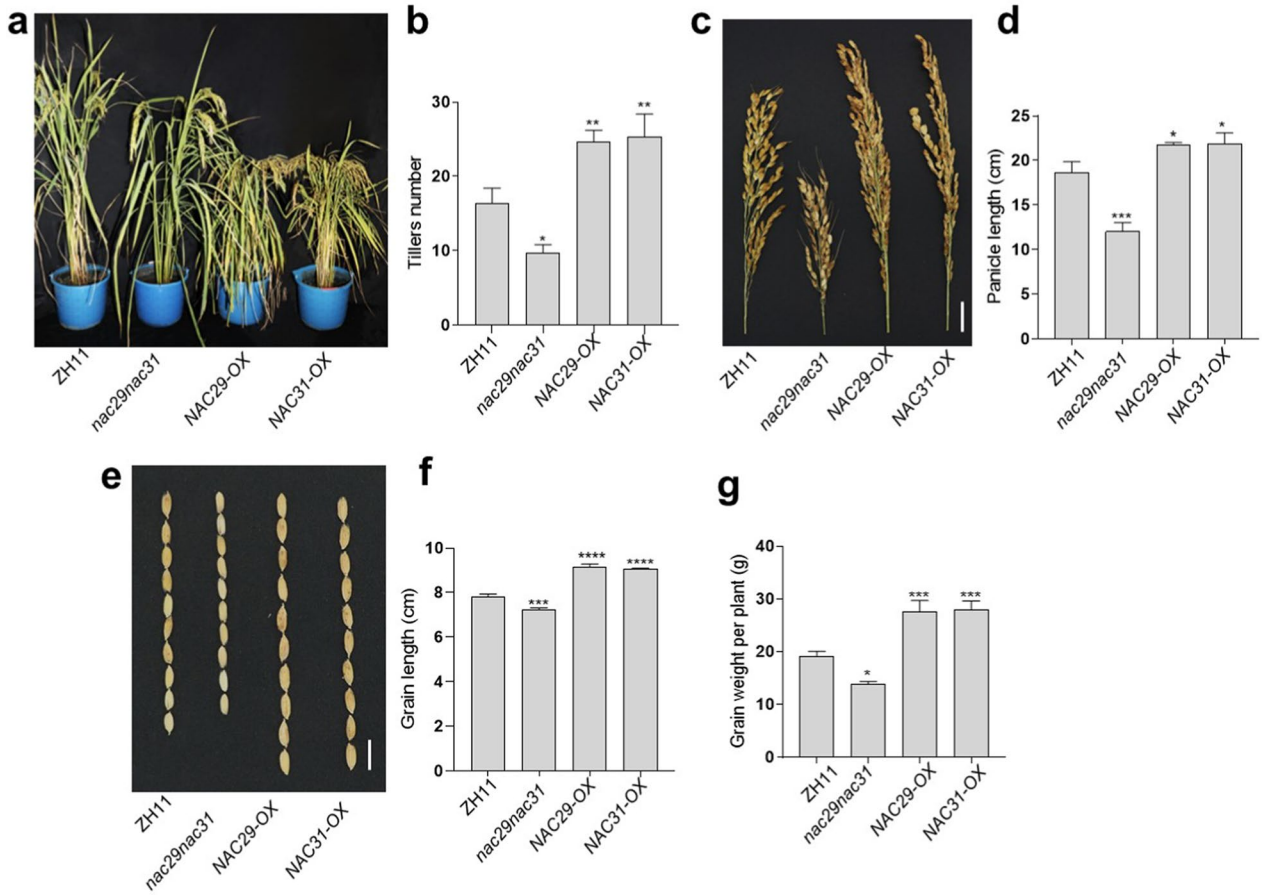

Fig. 4 NAC29 and NAC31 promote rice tillering and grain size. **a** Comparison of phenotypes of wild-type, *nac29nac31*, and *NAC29-OX*, *NAC31-OX* plants. **b** Quantification of tiller number of the plants in **a**. **c** Images showing panicles. **d** Bar graph showing panicle length of wild-type, *nac29nac31*, *NAC29-OX*, and *NAC31-OX* plants. Bar = 1 cm. **e** Mature grain of wild-type, *nac29nac31*, *NAC29-OX*, and *NAC31-OX* plants. Bar = 1 cm. **f** Quantification of grain length of the plants in **e**. **g** The grain yield per plant of wild-type, *nac29nac31*, *NAC29-OX*, and *NAC31-OX* plants. Statistically significant differences are indicated by asterisks ($^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$, one-way ANOVA with Tukey's significant difference test)

drought, salinity, cold, submergence, and immune responses (Xie et al. 1999, 2020; He et al. 2005; Selth et al. 2005; Hu et al. 2006; Nakashima et al. 2007; Jeong et al. 2010; Nuruzzaman et al. 2010, 2012). OsNAC6 participated in regulating defense responses against *M. grisea* (Nakashima et al. 2007). Infection of rice seedlings by rice stripe virus and rice tungro spherical virus triggers the activation of six *OsNAC* genes (Nuruzzaman et al. 2010). Present findings revealed that rice NAC29 and NAC31 play a crucial role in promoting resistance to ShB. The overexpression of NAC29 or NAC31 enhances rice resistance to ShB (Fig. 3f, g). In contrast, the *nac29nac31* double mutant reduces the resistance of rice to ShB (Fig. 3d, e). The study also found that *nac29* and *nac31* mutants showed no significant difference from WT plants in their response to ShB. Both NAC29 and NAC31 interact with BZR1 (Fig. 1), and NAC29 also interacts with NAC31 (Fig. 2). These interactions indicate that NAC29 and NAC31 display functional redundancy in ShB resistance. Importantly, it was observed that NAC29/31 promotes rice yield. Overexpression of NAC29/31 resulted in increased rice tillering, panicle size, panicle length, grain size, grain length, and grain weight per planta (Fig. 4). Our results provide clues for engineering crop varieties by overexpressing NAC29 or NAC31 that can enhance disease resistance without any significant yield penalty and may even increase yield.

Previous studies demonstrated that BZR1 binds to the promoter of *CAD8B* to activate its expression, resulting in enhanced rice resistance to ShB (Wang et al.2014). In the present research, plants overexpressing *NAC29* and *NAC31* showed increased *CAD8B* expression compared to WT plants (Fig. 5a). In transactivation assays, the expression level of CAD8B was higher when BZR1 was co-expressed with NAC29 or NAC31 compared to BZR1 alone (Fig. 5b), indicating

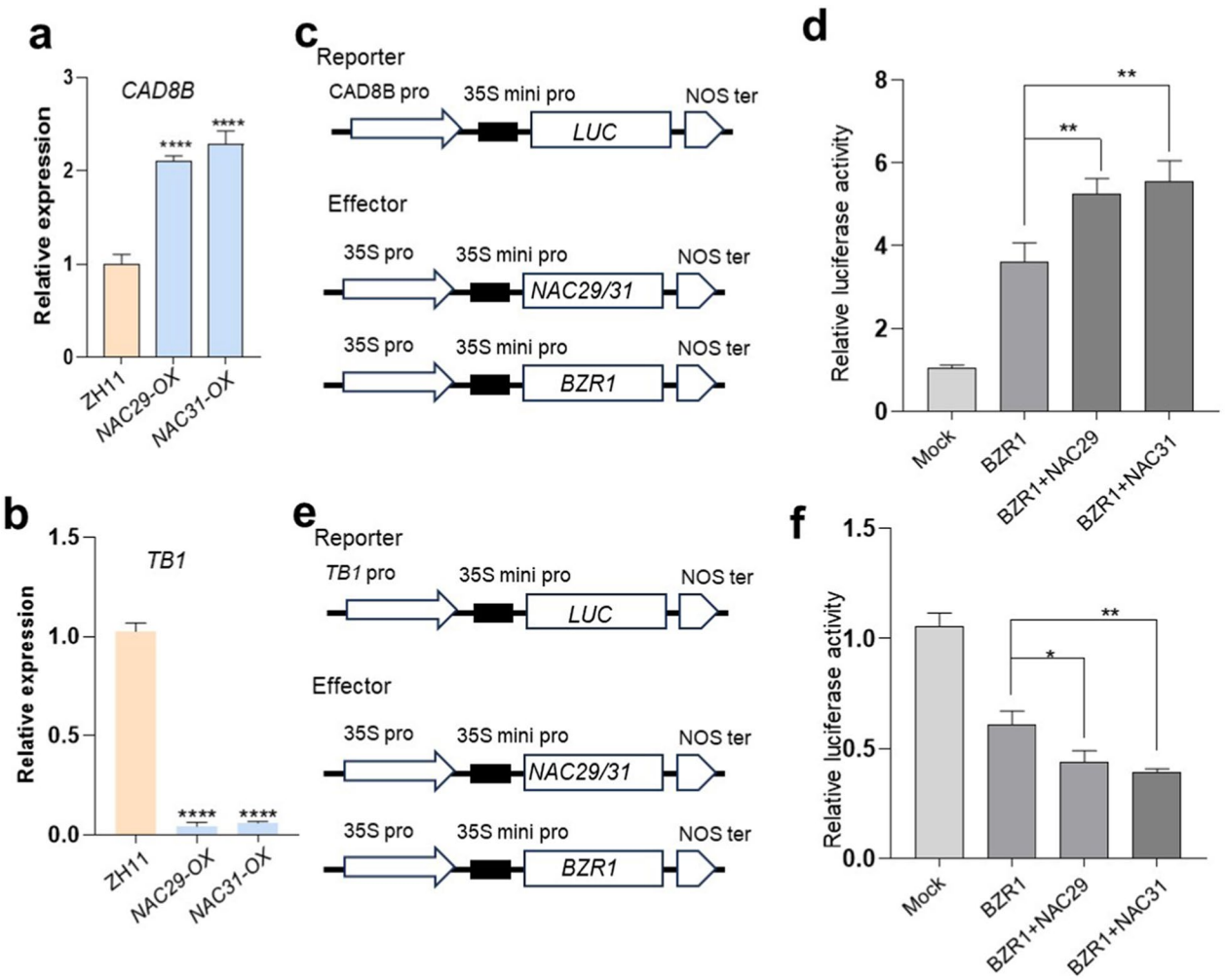

Fig. 5 Co-expression of BZR1 and NAC29/NAC31 enhances *CADB8* and inhibits *TB1* expression. **a** Expression levels of *CAD8B* in WT, *NAC29-OX*, and *NAC31-OX* plants. **b** Expression levels of *TB1* in WT, *NAC29-OX*, and *NAC31-OX* plants. **c** Diagrams of the effector and reporter constructs used in **d**. **d** The transactivation activity was monitored by assaying the luciferase activities, showing that both BZR1 and NAC29/31 activate *CAD8B* expression in an additive manner. **e** Diagrams of the effector and reporter constructs used in **f**. **f** The transactivation activity was monitored by assaying the luciferase activities, showing that both BZR1 and NAC29/31 inhibit *TB1* expression in an additive manner. Statistically significant differences are indicated by asterisks ($^{*}P<0.05$, $^{**}P<0.01$, $^{****}P<0.0001$, one-way ANOVA with Tukey's significant difference test)

that the BZR1-NAC29/31 complex positively regulates *CAD8B*. The BZR1 also interacts with the promoter of *TB1* to suppress its expression and enhance tillering (Wang et al.2014). Plants overexpressing NAC29 and NAC31 showed a significant decrease in *TB1* expression compared with wildtype (Fig. 5d). In transactivation assay, the expression level of *TB1* was decreased when BZR1 co-expressed with NAC29 or NAC31 compared to that expressing BZR1 alone (Fig. 5f), indicating that BZR1-NAC29/31 complex negatively regulates *TB1*. Here, the interaction between BZR1 and NAC29/31 indicated that BZR1-NAC29/31 signaling plays a favorable role in enhancing rice resistance to ShB by activating the transcription of *CAD8B*. Likewise, this signaling pathway enhances rice tillering by repressing the transcription of *TB1* (Fig. 6). NAC29 and NAC31 specifically bind to the SNBE motif inside their target genes (Huang et al. 2015). Analysis of the promoter sequence identified two SNBE motifs in the *CAD8B* promoter and three SNBE elements in the *TB1* promoter (Additional file 1: Figure S3). Yeast one-hybrid (Y1H) assays demonstrated that NAC31 could bind to the P2 region, but not the P1 region, of the *CAD8B* and *TB1* promoters, respectively (Additional file 1: Figure S3). However, NAC29 did not show significant binding to either promoter (data not shown).

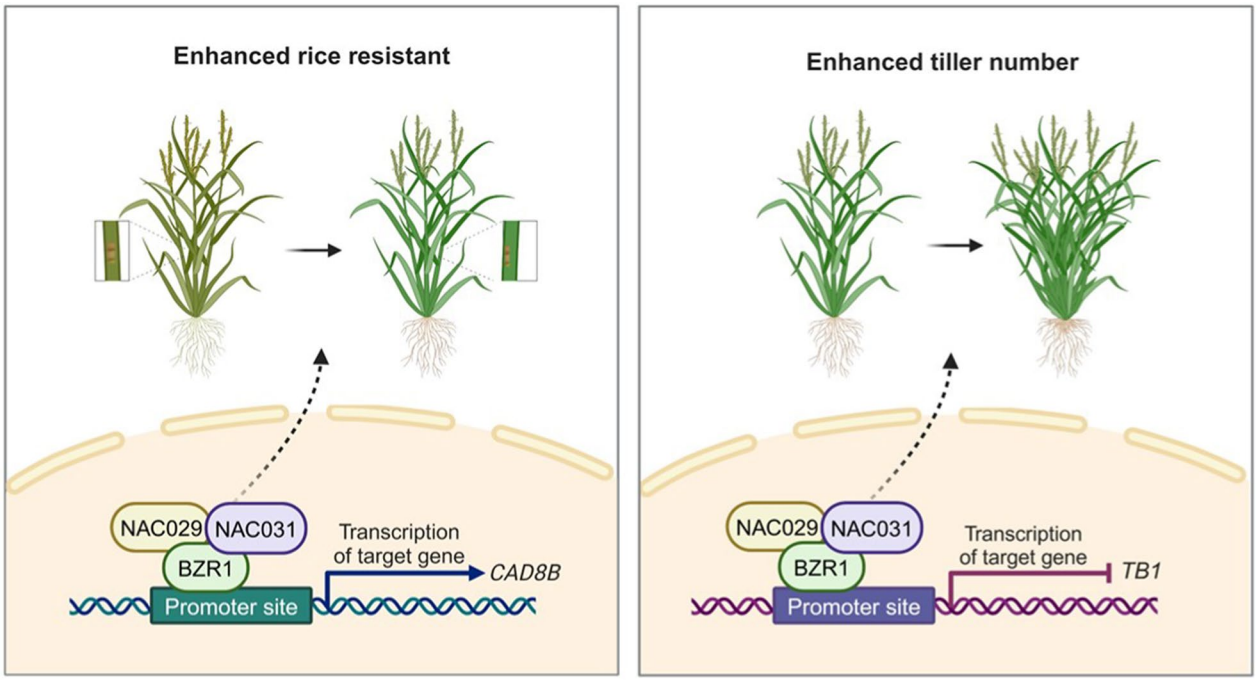

Fig. 6 Schematic representation of the hypothesized function of the BZR1-NAC29-NAC31 during *R. solani* infection. NAC29 interacts with NAC31, and both NACs interact with BZR1 in the nucleus. BZR1 and NAC29/31 form a TF complex to activate the expression of *CAD8B* to enhance ShB resistance (left) and to inactive the expression of *TB1* to increase rice tillers (right)

This result suggests that NAC31 may directly bind to the *CAD8B* and *TB1* promoters to regulate their expression.

Activation of the BR signal triggers tillering and grain size. However, *bzr1-D* showed no significant difference in grain size. The NAC29-NAC31 signal promotes grain size, which differs from the action of BZR1, suggesting that NAC29-NAC31 commonly regulates ShB resistance and tillering together with BZR1, but not grain development. Here, we identified that NAC29-NAC31 signaling regulates lignin biosynthesis by activating *CAD8B*. In addition, previous studies demonstrated that NAC29-NAC31 activates cellulose synthetase genes via MYB61 (Huang et al.2015). These findings indicate that NAC29-NAC31 signaling likely activates multiple aspects of cell wall biosynthesis to enhance physical resistance against pathogens.

Conclusions

This study reveals that BZR1 plays a role in regulating ShB resistance and tillering through its interaction with NAC29/31. Furthermore, NAC29/31 promotes both yield and disease resistance in rice, making it a promising gene for enhancing these traits in rice cultivation.

Methods

Plant growth conditions and *R. solani* inoculation

All rice (*O. sativa* L.) plants were grown in the greenhouse at 23 °C–30 °C, 40%–60% relative humidity (RH), and a 12 h light/12 h dark photoperiod. Wood veneer was cut into 0.5 cm × 1 cm rectangles of uniform size, sterilized, and placed in a circle on the potato dextrose agar (PDA) plate inoculated with *R. solani* and incubated at 30℃ for 48–72 h in an incubator, so that the mycelia grew evenly on each piece of wood veneer. Rice tillers with similar growth status on healthy rice plants were selected and cultivated for 2 months. Complete leaves of the four leaf age and position at the base of the stem were selected, and the pieces of wood veneer covered with mycelia were placed on the inner side of the leaf sheath. An appropriate amount of sterile water was sprayed, and plastic film was wrapped around the sheaths to ensure humidity and prevent the wood veneer from falling off. The disease incidence was recorded and photographed after approximately 7–10 days. Each group of experiments was repeated at least three times.

Generation of DNA constructs and transgenic plants

The *NAC29* or *NAC31* open reading frame (ORF) sequences were amplified and cloned into pCAMBIA1302 binary vector to generate *NAC29-GFP* or

NAC31-GFP overexpression transgenic plants. The *NAC29* and *NAC31* mutants were obtained using the CRISPR/Cas9 genome editing system, and the genetic background of all mutants and overexpression plants was Zhonghua11 (ZH11).

RNA extraction and real-time quantitative PCR (RT-qPCR) analysis

Total RNA was extracted from rice samples using RNAiso Plus reagent (TaKaRa, Dalian, China). RNA was reverse transcribed into cDNA using the TaKaRa Reverse Transcription Kit according to the instructions. Before performing qRT-PCR, the cDNA was diluted 30-fold and then quantitatively detected by a real-time fluorescence quantitative PCR. qRT-PCR assays were performed using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) using the QuantStudio 1 instrument (ABI, Palo Alto, CA, USA). Rice ubiquitin was used as an internal reference gene. A two-step method with a melting curve was used for the test, and three technical replicates, as well as three biological replicates, were performed for each sample. The primers used are listed in (Additional file 2: Table S2).

Yeast two-hybrid assay (Y2H)

To test the interaction between NAC29 or NAC31 and BZR1, the Gal4 DNA-binding domain (BD) was N-terminally fused to BZR1, while NAC29 or NAC31 ORFs were cloned into the pGAD424 vector. The different pair was further transformed in the yeast strain PJ69-4A (Clontech, http://www.clontech.com/). Yeast cells were grown on SD/Trp−/Leu- and SD/Trp−/Leu-His- plates. The primers used are listed in (Additional file 2: Table S2).

Bimolecular fluorescence complementation (BiFC) assay

The BiFC assays were performed as previously reported (Kim et al. 2009). The coding sequences of *NAC29*, *NAC31*, and *BZR1* were amplified and cloned into the BiFC vectors pXNGW and pXCGW to create the *BZR1-nYFP*, *NAC29-cCFP*, and *NAC31-cCFP* fusion-DNA constructs. All constructs were transformed into the *A. tumefaciens* strain EHA105. The interaction pairs were mixed with P19 and the nucleus marker H2B-mRFP1 in a 1:1:1:1 ratio to infiltrate the *Nicotiana benthamiana* leaves. Fluorescence data were collected on the confocal microscope after 48 h of infiltration. The primers used are listed in (Additional file 2: Table S2).

Co-immunoprecipitation (Co-IP)

The coding sequences of *NAC29* and *NAC31* were amplified and cloned into the pEH19 vector, and *BZR1* was amplified and cloned into the pGD-3×FLAG vector to create the *NAC29-GFP*, *NAC31-GFP*, and BZR13×FLAG fusion constructs.

Total proteins were extracted from *N. benthamiana* leaf tissues that were ground in liquid nitrogen and homogenized in 2 ml/g extraction buffer [10% (v/v) glycerol, 25 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaC, 2% (w/v) PVPP, 10 mM DTT, 1×protease inhibitor cocktail (Sigma-Aldrich), and 0.1% (v/v) Triton X-100 (Sigma-Aldrich)]. After centrifugation at 3000 *g* for 10 min at 4 °C and filtration with a 0.45-μm filter, the clarified lysate was incubated with 4% BSA-pre-blocked anti-FLAG M2 agarose beads (Sigma-Aldrich) for 3 h, and the complex was washed three times with IP buffer [10% (v/v) glycerol, 25 mM Tris–HCl (pH 7.5), 1 mM EDTA, 150 mM NaCl, and 0.1% (v/v) Triton X-100]. The immunoprecipitates were denatured and subjected to immunoblotting using corresponding antibodies.

Western blot analysis

Total proteins were extracted from infiltrated *N. benthamiana* leaves using 2×sodium dodecyl sulfate (SDS) sample buffer [100 mM Tris (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, and 0.2% (w/v) bromophenol blue]. Proteins were separated with SDS poly-acrylamide gel electrophoresis. Western blots were performed with the anti-FLAG and anti-GFP antibodies (EASYBIO).

Transactivation assay

The transactivation assays were performed as previously reported (Yuan et al. 2022) with minor modifications. The agrobacterial strains bearing individual effector plasmids (*p35S::BZR1*, *p35S::NAC29*, *p35S::NAC31*) and the reporter (*pCAD8B*, *pTB1*), as well as an internal control plasmid (*p35S::LUC*), were co-transformed into *N. benthamiana* leaves. A luciferase assay was performed using a Luciferase Assay Kit (Promega) The primers used for constructing vectors for the transient assay are listed in (Additional file 2: Table S2).

Abbreviations

BiFC	Bimolecular fluorescence complementation
Co-IP	Co-immunoprecipitation
Dpi	Days post inoculation
GFP	Green fluorescent protein
Hpi	Hours post infiltration
ShB	Sheath blight
Y2H	Yeast two-hybrid assay
YFP	Yellow fluorescent protein

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s42483-025-00321-9.

Additional file 1: Figure S1. Detection of expression levels of *NAC29* and *NAC31* after *R. solani* infection at 0, 24, 48, and 72 h post-inoculationusing quantitative real-time PCRanalysis. **Figure S2.** Expression levels of *NAC29*

and *NAC31* in *NAC29* and *NAC31* overexpression lines compared with WT were determined using RT-qPCR. **Figure S3.** NAC31 binds *CAD8B* and *TB1* prompter.

Additional file 2: Table S1. Accession number used in this study. **Table S2.** Primers used in this study.

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Author contributions
QS and YX designed the research; QS, YZ, and LL performed the experiments; ZL, JZ, QZ, HD, XT, and XZ analyzed the data; QS and YX wrote the paper. All authors read and approved the final manuscript.

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Availability of data and materials
The data and materials that support the findings of this study are available from the corresponding author upon request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare that they have no competing interests.

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