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NAC transcription factor ONAC066 positively regulates disease resistance by suppressing the ABA signaling pathway in rice

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Abstract

Key message **This is the first time to dissect the mechanism of NACs-mediated disease resistance in plants using metabolomic approach and discover the involvement of ABA signaling pathway in NACs-mediated disease resistance.**

Abstract NAC transcription factors have been validated as important regulators in stress responses, but their molecular mechanisms in plant disease resistance are still largely unknown. Here we report that the NAC gene *ONAC066* (*LOC_Os01g09550*) is significantly activated by rice blast infection. *ONAC066* is ubiquitously expressed and this protein is localized in the nucleus. Overexpression of *ONAC066* quantitatively enhances resistance to blast disease and bacterial blight in rice. The transcript levels of *PR* genes are also dramatically induced in *ONAC066* overexpressing plants. Exogenous abscisic acid (ABA) strongly activates the transcription of *ONAC066* in rice. Further analysis shows that overexpression of *ONAC066* remarkably suppresses the expression of ABA-related genes, whereas there are no obvious differences for salicylic acid (SA) and jasmonic acid (JA)-related genes between wild-type and *ONAC066* overexpressing plants. Consistently, lower endogenous ABA levels are identified in *ONAC066* overexpressing plants compared with wild-type plants before and after blast inoculation, while no significant differences are observed for the SA and JA levels. Yeast one-hybrid assays demonstrate that *ONAC066* directly binds to the promoters of *LIP9* and *NCED4* to modulate their expression. Moreover, the metabolomic study reveals that the *ONAC066* overexpressing plants accumulated higher contents of soluble sugars and amino acids both before and after pathogen attack, when compared to wild-type plants. Taken together, our results suggest that *ONAC066* positively regulates rice resistance to blast and bacterial blight, and *ONAC066* exerts its functions on disease resistance by modulating of ABA signaling pathway, sugars and amino acids accumulation in rice.

Keywords NAC · Disease resistance · ABA · Soluble sugar · Amino acid · Rice

Introduction

As sessile organisms, plants are frequently under the attack by various fungus, bacterial and viruses. To survive, sophisticated mechanisms have been evolved in plants to cope with these adverse stresses (Sharma et al. 2013). Upon exposure to the pathogen attack, numerous genes are induced at the

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transcriptional level. Some of these induced genes can protect plant cells from pathogen-induced damage by the generation of pivotal metabolic proteins, while the others function as regulatory proteins that modulate the genes for signal transduction in defense response pathways (Nakashima et al. 2007; Song et al. 2011). Typical examples of such regulatory proteins are transcription factors, including NACs, bZIPs, MYBs and WRKYs, which have been demonstrated to play pivotal roles in plant disease resistance by modulating the transcription of downstream defense-related genes (Nakashima et al. 2009; Song et al. 2011; Puranik et al. 2012; Jiang et al. 2017).

NAC (NAM, ATAF1/2, and CUC2) is one of the largest families of transcription factors that are specifically expressed in plants (Puranik et al. 2012). They have a highly conserved DNA-binding domain in the N-terminal but a usually diversified transcriptional regulating domain in the C-terminal, and this remarkable diversification across plants reflects their numerous functions (Yokotani et al. 2014; Fang et al. 2015). Thus far, NAC proteins have been validated to regulate a series of biological processes, including the development of embryos and flowers, leaf senescence, cell division, hormone signaling and the synthesis of cell wall (Takada et al. 2001; Olsen et al. 2005; Guo and Gan 2006; Zhong et al. 2006; Kim et al. 2016). In addition, they also act as master regulators in regulating plant response to both biotic and abiotic stresses (Olsen et al. 2005; Puranik et al. 2012; Nuruzzaman et al. 2013). For instance, *ATAF1* negatively regulates defense against necrotrophic fungal and bacterial pathogens but positively regulates resistance against non-host pathogen *Blumeria graminis* f. sp. *hordei* in *Arabidopsis* (Jensen et al. 2008; Wang et al. 2009). Overexpressing of *GsNAC019* exhibited enhanced tolerance to alkaline stress in *Arabidopsis* (Cao et al. 2017a) and overexpression of *TaRNAC1* caused enhanced drought tolerance in wheat root (Chen et al. 2017).

The rice genome encodes 151 NAC transcription factors (Nuruzzaman et al. 2010). However, so far, only 6 *NAC* genes (*OsNAC6, ONAC122, ONAC131, OsNAC111, OsNAC4* and *RIMI*) have been validated to be involved in defense responses against pathogen attack (Nakashima et al. 2007; Kaneda et al. 2009; Yoshii et al. 2009; Sun et al. 2013; Yokotani et al. 2014). *ONAC122, ONAC131* and *OsNAC111* positively regulate plant resistance to *Magnaporthe oryzae* (Sun et al. 2013; Yokotani et al. 2014), whereas silencing of *RIMI* confers resistance to *Rice dwarf virus (*Yoshii et al. 2009*)*. The functions of other rice *NAC* genes in disease resistance are still unknown. Moreover, few have been reported about the detailed mechanisms of NAC genes in response to plant disease.

In this study, we show that the expression of *ONAC066* (*LOC_Os03g56580*) is strongly induced by *M. oryzae* infection. Overexpression of *ONAC066* causes enhanced resistance to rice blast and bacterial blight disease in rice. Further analysis reveals that *ONAC066* regulates rice responses to pathogen infection through modulating the abscisic acid (ABA) signaling pathways. Moreover, we also explore the primary metabolomes in the wild-type *Nipponbare* and *ONAC066* overexpressing plants using a GC–MS based metabolomic approach. The results show that *ONAC066*-mediated disease resistance is also associated with the regulation of sugar and amino acids metabolism in rice.

Materials and methods

Plant materials and pathogens

The japonica rice *Nipponbare* and the blast-resistant line BC10 (Liu et al. 2004) were used in this study. The blast isolate (*M. oryzae*) GD08-T13 and Chinese *Xanthomonas oryzae pv. Oryzae* (*Xoo*) race 4 isolate were used for rice blast and bacterial blight inoculation, respectively.

Hormone treatments

Phytohormone treatments were performed using the same method as described in Liu et al. (2016a). Briefly, rice seedlings at the three- to four-leaf stage were sprayed with different hormone solutions, each at a concentration of 100 μM. Plants sprayed with water were used as the controls. Seedling samples were collected at 3 h, 6 h, 12 h and 24 h after treatment. The experiments were repeated twice.

Pathogen inoculation

Spraying and punch methods were used for blast inoculation. *M. oryzae* isolate GD08-T13 inoculum was prepared as described by Beltenev et al. (2007). The revived isolate GD08-T13 was cultured on prune agar plates (three pieces prune, 5 g lactose, 1 g yeast extract, and 20 g agar bar in 1 L, pH of 6–6.5, autoclaved) for 7 days at room temperature, and mycelial growth was scraped with sterilized spatula and exposed to fluorescent light for 4–5 days to induce sporulation. Spores were collected and suspended in water to reach a concentration of 1×10^6 mL^{-1}. For the spraying inoculation, rice seedlings at the three- to four-leaf stage were inoculated with this isolate by the spraying method (Liu et al. 2016a). The inoculated plants were firstly incubated at 25 °C in dark for 24 h, and then followed by a 12-h photoperiod. Sampling for RNA extraction was conducted at 6 h, 12 h, 24 h and 48 h after inoculation. Disease was assessed 6 days after inoculation by measuring the DLA. For punch inoculation, leaves of *Nipponbare* and *ONAC066* overexpressing plants at the tillering stage were inoculated with GD08-T13 using

the punch method described by Ding et al. (2012). Lesion size was determined 10 days after inoculation. For determination of in planta sporulation after punch inoculation, leaf strips containing a lesion spot were excised and submerged in 100 µL of distilled water in a 1.5 mL microcentrifuge tube. After the suspension was vigorously mixed, spores were counted with a microscope (Ding et al. 2012).

To evaluate bacterial blight resistance, Chinese *Xoo* race 4 isolate was grown on nutrient yeast sucrose broth for 72 h at 30 °C, and then re-suspended in sterile water at an optical density (OD600) of 0.5. Plants were inoculated with the isolate at the booting stage by the leaf-clipping method (Kauffman et al. 1973). Lesion length (centimeters) was measured after 14 days post inoculation (dpi).

Subcellular localization of ONAC066 protein

The CDS sequence of ONAC066 was amplified using primers ONAC066-GFP-F/R (Supplemental Table 1) and the fragment was inserted into the pGFP1 vector to generate the fusion protein pGFP1–ONAC066. The empty pGFP1 and pGFP1–ONAC066 plasmids (1 µg) were transformed into rice stem protoplasts as described by Zhang et al. (2011), or introduced into onion epidermal cells as described by Liu et al. (2016b), respectively. The GFP signals were detected by a laser confocal microscopy (Zeiss LSM710, Germany) after 24 h incubation at 25 °C.

Vector construction and plant transformation

We amplified the full-length CDS of *ONAC066* from BC10 using primers ONAC066-OE-F/R (Supplemental Table 1) and subsequently the product was cloned into the pOX vector which has an ubiquitin promoter to generate the *ONAC066* overexpressing plants. The promoter region of *ONAC066* (1330 bp) was amplified from BC10 using primers ONAC066-GUS-F/R (Supplemental Table 1), and then the fragment was inserted into the pcambia1381Z vector. The constructed plasmids were transformed into the wild-type *Nipponbare* plants using the method as described by Toki et al. (2006).

Cis-elements analysis of the promoter and the promoter–GUS reporter assay

We downloaded the promoter sequence (about 1330 bp) of *ONAC066* from MSU Rice Genome Annotation Project, and then the sequence was scanned by PlantCARE for cis-elements analysis (Lescot et al. 2002).

The GUS reporter assay was conducted using the same method according to our previous report (Liu et al. 2016a).

Real-time PCR analysis

Total RNA was extracted from rice seedlings using Eastep Super total RNA extraction kit (Promega Biotech Co., Ltd, USA). The methods for real-time PCR have been described previously (Liu et al. 2016a). Three plants were used for each replicate. The primers used in the present study are shown in Supplemental Table 1.

Metabolic profiling by GC–MS

The primary metabolites in rice leaves were extracted following the procedures described in detail in the previous study (Salem et al. 2016). Four biological replicates were performed for each treatment, and 12 plants were used for each biological replicate. The dried extract from the lower polar phase was derivatized with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (MSTFA), and further analyzed by GC–MS (7890A-5975C, Agilent) following the protocol described previously (Lisec et al. 2006). LECO-Fiehn Rtx5 database and the NIST library were used for peak identification after the pretreatment of GC–MS raw data by Chroma TOF 4.3X software (LECO Corporation) (Kind et al. 2009). The resulting three-dimensional data involving the peak number, sample name, and normalized peak area were fed to the SIMCA software package (V14, Umetrics AB, Umea, Sweden) for multivariate statistical analyses. To refine this statistical analysis for significantly changed metabolites, the metabolites with $|P(corr)| \geq 0.5$ and a P-value < 0.05 were considered different between the two comparison groups. P-value was obtained by one way ANOVA (Kind et al. 2009).

Quantification of endogenous hormones and their precursors

The hormone was extracted and quantified using the same method as described previously (Liu et al. 2016b). The optimized MS/MS conditions that used for quantification of ABA, violaxanthin, SA and jasmonic acid (JA) were listed in Supplemental Table 2.

Seed germination assays

The seeds of wild-type *Nipponbare* and *ONAC066* overexpressing plants were sterilized with 75% ethanol and 2.5% NaClO and pre-germinated on 1/2 MS medium for 2 days. Then, the identically sprouted seeds were transplanted on fresh 1/2 MS medium supplemented with ABA

or without supplementation of ABA (control). 7 days later, the lengths of the shoot and primary roots were measured.

Yeast one-hybrid assays

The yeast one-hybrid assays were conducted using the same method as described in our previous study (Liu et al. 2016a). The coding sequence of *ONAC066* was inserted into the pGADT7 vector while the 1.0 kb promoters of *LEA3, LIP9, Rab16A, ABI2, NCED3* and *NCED4* were inserted into the pAbAi vector, respectively. The experiments were performed according to the manufacturer's protocol (Clontech Yeast Protocol Handbook; BD Biosciences Clontech). P53 is used as the positive control.

Results

Expression of *ONAC066* is induced by blast inoculation

In our previous study to identify genes that were regulated by leaf blast infection using microarray in a blast resistant line *BC10* (Liu et al. 2004), we identified that the transcription of *ONAC066* was significantly induced during leaf blast inoculation (data not shown). To further confirm this result in the present study, we performed quantitative RT-PCR to analyze the expression of *ONAC066* in *Nipponbare* seedlings after inoculation with blast isolate GD08-T13. We identified that the expression of *ONAC066* was induced by *M. oryzae* infection and the level remained high until 48 h after inoculation (Fig. 1a), suggesting that *ONAC066* might play an important role in rice against blast pathogen attack.

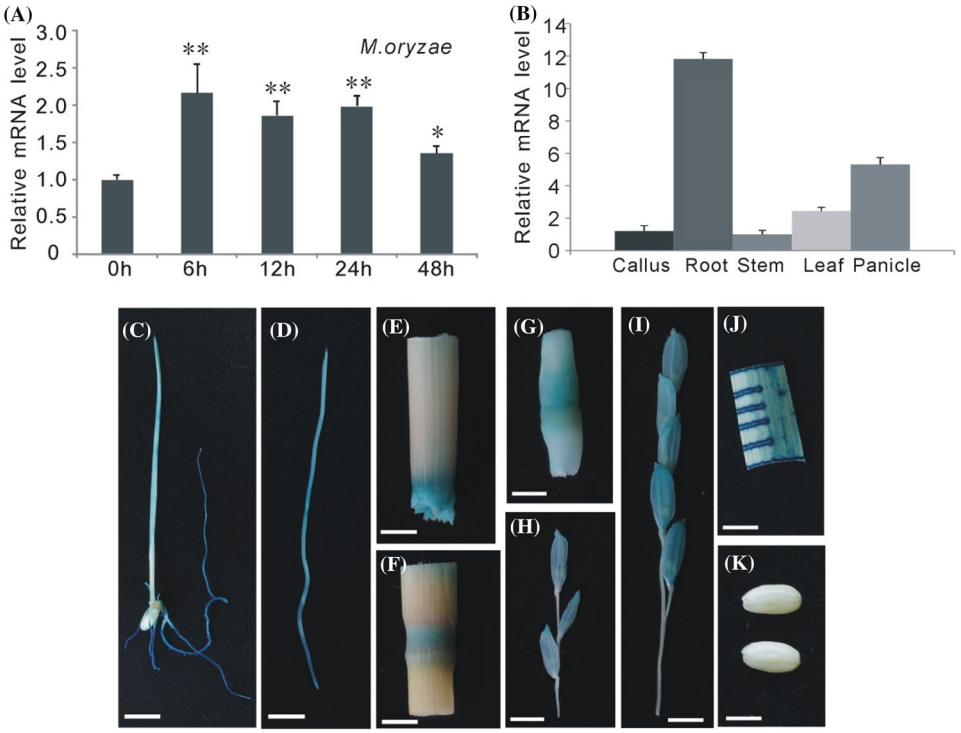


Fig. 1 The expression patterns of *ONAC066* to rice blast infection, and in different rice tissues. **a** The transcription of *ONAC066* was induced by blast inoculation. Values are means ± standard error (SE) from three biological replicates. The asterisks represent significant differences relative to 0 h treatment (*t* test, $^{**}P < 0.01$ and $^{*}P < 0.05$). **b** Relative expression levels of *ONAC066* in different tissues of *Nipponbare* plants by real-time PCR. Values are means ± SE from three biological replicates. **c**–**k** *GUS* staining analysis of *ONAC66* in different tissues of *Nipponbare*. **c** 1-week old seedling; **d** root of 1-week old seedling; **e** the first node at the booting stage; **f** the second node at the booting stage; **g** the third node at the booting stage; **h** panicle at the booting stage; **i** panicle at the initial heading stage; **j** leaf at the booting stage; **k** mature seeds. **c**–**j** Bar = 1 cm. **k** Bar = 0.5 cm

ONAC066 is highly expressed in leaves and panicles

To investigate the spatial expression of *ONAC066* in rice plants, we analyzed the expression pattern of *ONAC066* in various tissues of rice by quantitative RT-PCR. As shown in Fig. 1b, *ONAC066* was expressed in all rice tissues examined, but the expression was relatively higher in roots, leaves and panicles. To further confirm this result, transgenic rice plants were generated using the GUS reporter gene, which was driven by the *ONAC066* promoter (approximately 1.4 kb upstream of the translation start site). GUS signal was detected in the root and shoot, node, panicles at the booting stage and heading stage, and leaves at the booting stage (Fig. 1c–k), agreeing well with the results from qRT-PCR.

Nuclear localization of ONAC066 protein

To determine the subcellular localization of ONAC066 protein, the coding region of ONAC066 was fused to green fluorescence protein (GFP) which was under the control of the CaMV 35S promoter. The transient expression analysis showed that the GFP signal was localized in the nucleus of rice protoplast cells transfected with GFP-ONAC066 protein, whereas the control cells (transformed with the empty vector) exhibited ubiquitous GFP signal (Fig. 2a). We also assayed the localization using onion epidermal cells and similar results were obtained, indicating that ONAC066 is a nucleus localized protein (Fig. 2b).

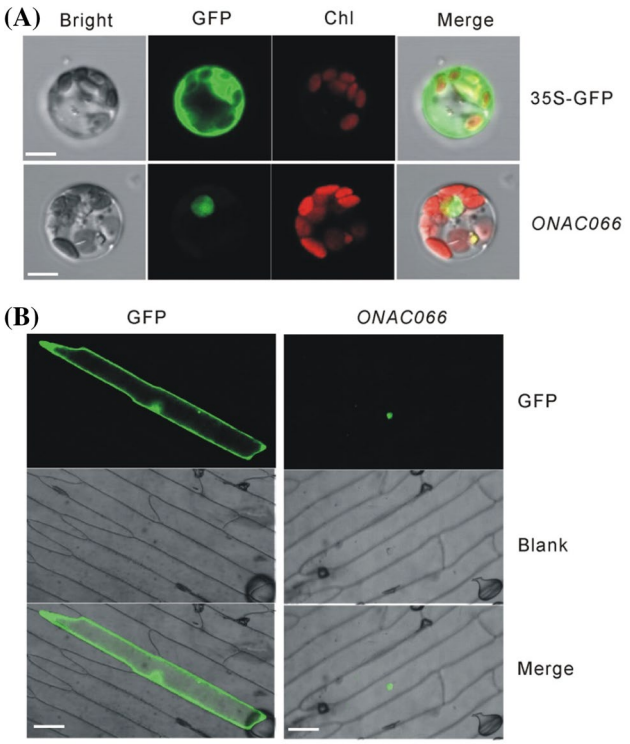


Fig. 2 Nuclear localization of ONAC066. **a** pGFP1 and pGFP1–ONAC066 fusion proteins were transiently expressed in rice protoplasts. Bar = 2 μm. **b** pGFP1 and pGFP1–ONAC066 fusion proteins were transiently expressed in onion epidermal cells. Bar = 2 μm

Overexpression of *ONAC066* quantitatively enhanced resistance to blast and bacterial blight in rice

To explore the potential functions of *ONAC066* in rice disease resistance, transgenic plants overexpressing *ONAC066* (OX-ONAC066) were generated by using the maize ubiquitin promoter. The plant heights of OX-ONAC066 plants were remarkably higher than wild-type *Nipponbare* (NPB) plants. However, the tiller numbers of OX-ONAC066 plants were significantly reduced, and as a result the grain weights per plant were also significantly lower in the OX-ONAC066 plants compared with the weights in the *Nipponbare* plants (Supplemental Table 3). Two independent homozygous lines (OX-2 and OX-3) were selected for disease evaluation (Supplemental Fig. 1). Real-time PCR analysis showed that the transcript level of *ONAC066* was significantly increased in these transgenic lines (Fig. 3a). We firstly inoculated the 2-week-old OX-ONAC066 plants with blast isolate GD08-T13, which showed strong virulence on wild-type *Nipponbare* plants, using the spray-inoculation method. The OX-ONAC066 plants showed significantly enhanced resistance to blast disease ($P < 0.01$), with the diseased areas ranging from 29.8 to 32.2%, compared with 61.3% for wild-type *Nipponbare* plants (Fig. 3b, c). To confirm this result, the OX-ONAC066 plants were inoculated with the same isolate by punch method and a smaller lesion size was identified in the OX-ONAC066 plants relative to the wild-type *Nipponbare* plants (Fig. 3d, e). The spores in the infected OX-ONAC066 leaves were also fewer than that in the wild-type *Nipponbare* leaves (Fig. 3f). Moreover, the OX-ONAC066 plants also exhibited remarkably increased resistance to bacterial blight ($P < 0.05$), with the lesion length ranging from 10.27 to 10.77 cm for the transgenic plants versus 14.21 cm for wild-type *Nipponbare* plants (Fig. 4a, b). The growth rate of *Xoo* on the transgenic plants was also much slower ($P < 0.05$) than that on the wild-type *Nipponbare* plants at 12 and 16 days after inoculation (Fig. 4c).

Since Yokotani et al. (2014) reported that *OsNAC111* could enhance rice blast resistance through activation of *PR2* (acidic pathogenesis-related protein 2) and *PR8*, we also investigated if *ONAC066* enhanced blast resistance through activation of the PR genes. The expression patterns of nine *PR* genes were analyzed in the OX-ONAC066 and wild-type *Nipponbare* plants both before and blast infection. The results showed that pathogen inoculation strongly induced the transcription of *PR1a, PR2, PR3, PR4, PR5, PR5-1, PR8* and *PR10* both in the wild-type *Nipponbare*

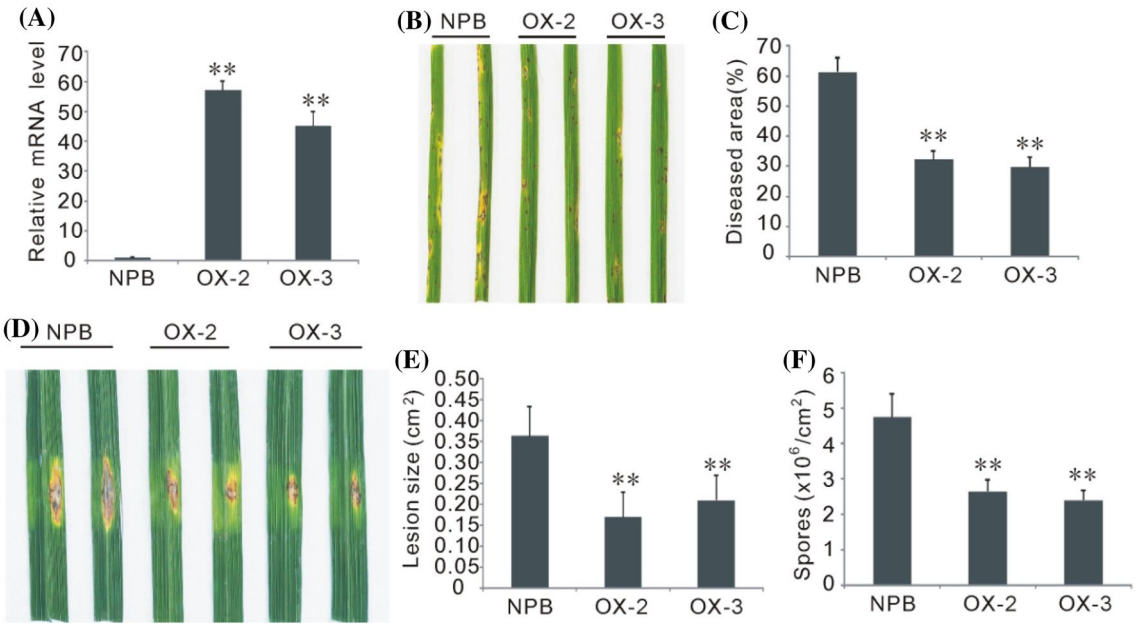


Fig. 3 Overexpressing *ONAC066* causes enhanced resistance to rice blast. Asterisks represent significant differences relative to wild-type *Nipponbare* (NPB) plants (Student's *t* test, $^{**}P < 0.01$). **a** Expression analysis of *ONAC066* overexpressing (OX-ONAC066) plants by real-time PCR. Values are mean ± SE from three replicates. **b** The OX-ONAC066 plants produced less diseased lesions compared to the *Nipponbare* plants after inoculation with GD08-T13 using the spraying method. **c** Relative diseased area in the OX-ONAC066 and *Nipponbare* plants after blast inoculation. Values are means ± SE from 30 biological replicates. **d** The OX-ONAC066 plants produced smaller diseased lesions compared to the *Nipponbare* plants after inoculation with GD08-T13 using punch method. **e** Relative lesion size in the OX-ONAC066 and *Nipponbare* plants after blast infection. Values are means ± SE from at least ten biological replicates. **f** The infection ratio of the OX-ONAC066 and *Nipponbare* plants after punch inoculation. Values are means ± SE from at least ten biological replicates

and OX-ONAC066 plants (Fig. 5). Nevertheless, the transcripts of the nine genes were significantly higher ($P < 0.05$) in OX-ONAC066 plants compared with *Nipponbare* plants both before and after *M. oryzae* inoculation. Taken together, these results suggest that *ONAC066* positively regulates the responses of rice plants to both blast and bacterial blight diseases.

ONAC066 reduces the expression of genes involved in ABA signaling pathway

To dissect the potential mechanism of *ONAC066* in regulation of disease resistance in rice, the promoter sequence of *ONAC066* was subjected for *cis*-elements analysis firstly. Two TC-rich repeats involved in defense and stress responsiveness were identified (Supplemental Table 4), further supporting the regulatory effect of *ONAC066* on disease response. Besides, we also observed three ABA-responsive elements (ABREs), two salicylic acid (SA)-responsive elements (TCA element) and two methyl jasmonic acid (MeJA)-responsive elements (TGACG-motif) (Supplemental Table 4). These hormone response elements in the promoter region of ONAC066 imply that ONAC066-mediated disease resistance may involve regulation of these hormone signaling pathways in rice. To confirm this inference, we treated the wild-type *Nipponbare* plants with exogenous ABA, SA and JA at the three- to four-leaf stage, respectively, and then analyzed the transcription of *ONAC066* at different time points after hormone treatment using qRT-PCR. The expression levels of *ONAC066* were remarkably increased at 6, 12 and 24 h after ABA treatment in *Nipponbare* plants, whereas its expression level showed no obvious change under SA or JA treatment (Supplemental Fig. 2).

Moreover, experiments were also conducted to analyze the expression patterns of several well-known stress-related genes that are involved in ABA, JA or SA signaling pathways. The ABA-related genes include three ABA responsive genes (*LEA3, Rab16A* and *LIP9*), an ABA signaling gene (*ABI2*) and two ABA biosynthesis genes (*NCED3* and *NCED4*) (Liu et al. 2012; Chen et al. 2015). *LOX* and *AOS2* are involved in the biosynthesis of JA, while *PAL1, ICS1, NH1* and *PAD4* are four genes that related to the SA signaling pathway (Deng et al. 2012). Under normal conditions, significantly lower transcription levels of the six ABA-related genes were observed in OX-ONAC066 plants compared with the wild-type *Nipponbare* plants (Fig. 6a). Pathogen inoculation significantly reduced the transcription of the six genes in both *Nipponbare* and transgenic plants, while these six genes also exhibited lower expression levels in OX-ONAC066 plants relative to *Nipponbare* plants

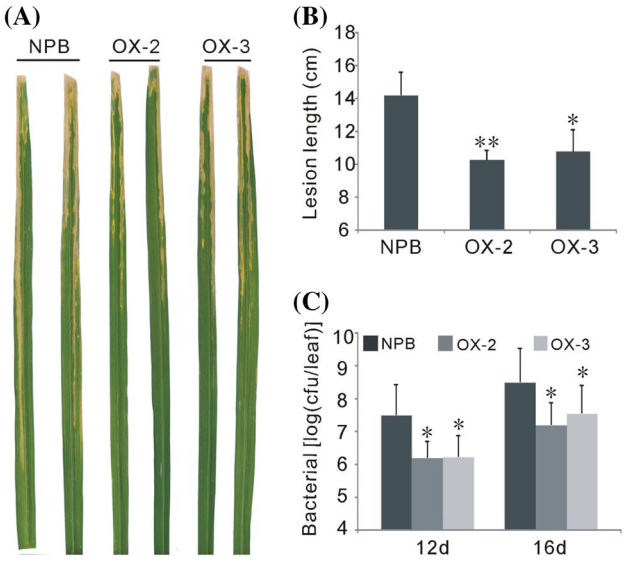


Fig. 4 The phenotypes of *ONAC066* overexpressing (OX-ONAC066) plants to bacterial blight disease. The asterisks represent significant differences relative to wild-type *Nipponbare* (NPB) plants (*t* test, $^{**}P < 0.01$ and $^{*}P < 0.05$). **a** Overexpression of *ONAC066* enhanced resistance to bacterial blight. **b** Lesion length in the OX-ONAC066 and *Nipponbare* plants after *Xoo* inoculation. Values are mean ± SE from 16 biological replicates. **c** The growth rates of *Xoo* race 4 in the leaves of OX-ONAC066 and *Nipponbare* plants. Three leaves 12 or 16 days after inoculation were collected for determination of bacterial populations by counting colony-forming units (cfu). Two independent biological experiments were performed, resulting in similar results. Values are means ± SE from three biological replicates

after pathogen infection (Fig. 6a). However, the expression levels of the genes involved in JA or SA signaling pathways showed no obvious differences between *Nipponbare* plants and OX-ONAC066 plants before and after blast inoculation even though the transcripts of *LOX, AOS2, ICS1, NHI* and *PAD4* were remarkably up-regulated by pathogen infection (Fig. 6b). These results together indicate that the disease resistance conferred by *ONAC066* is associated with the ABA signaling pathway but not the JA or SA signaling pathways.

ONAC066 functions dependent on the ABA signaling pathway

To further understand the relationship between *ONAC066* and the ABA signaling pathway, we quantified the contents of the endogenous ABA in the seedlings of wild-type *Nipponbare* and OX-ONAC066 plants before and after *M. oryzae* infection. Blast inoculation significantly reduced the endogenous level of ABA in both *Nipponbare* and OX-ONAC066 plants (Fig. 7). Nevertheless, the endogenous ABA concentration was significantly lower ($P < 0.05$) in transgenic (OX-ONAC066) plants than that in *Nipponbare* plants both before and after pathogen inoculation (Fig. 7a). In consistent with the results of gene expression analysis, endogenous JA and SA levels showed no significant differences between *Nipponbare* and OX-ONAC066 plants though blast inoculation remarkably induced the accumulation of

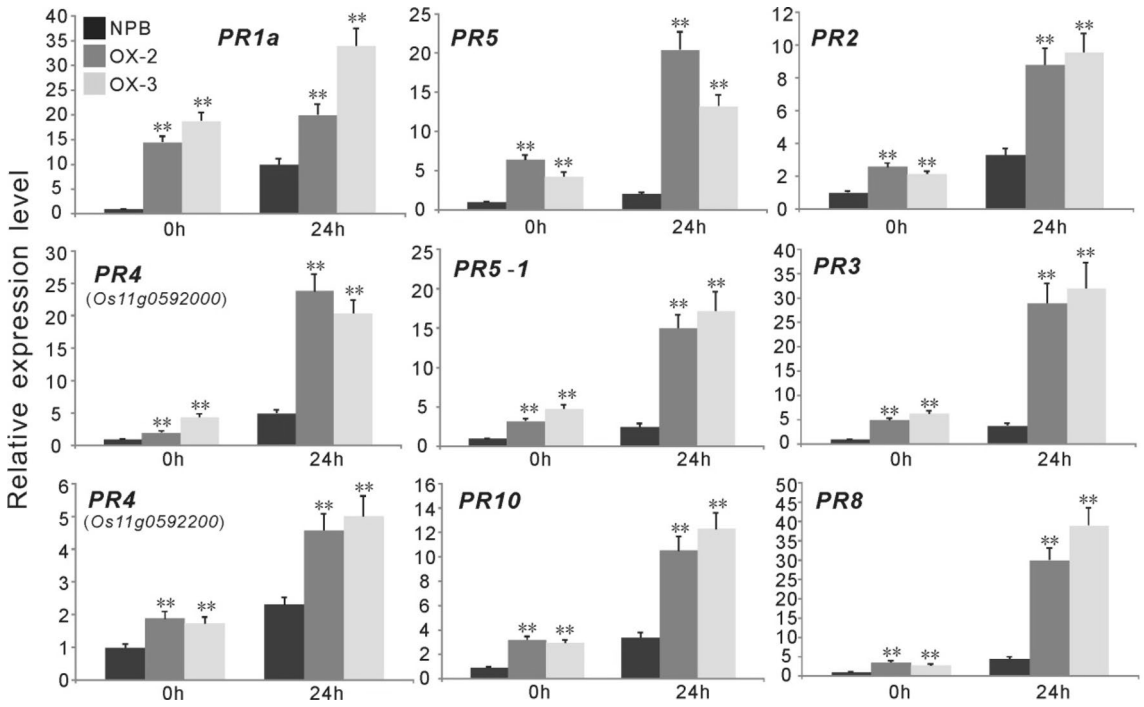


Fig. 5 Overexpression of *ONAC066* activates the transcription of *PR* genes before (0 h) and after (24 h) blast inoculation. Values are mean ± SE from three biological replicates. The asterisks represent significant differences relative to *Nipponbare* plants (*t* test, $^{**}P < 0.01$)

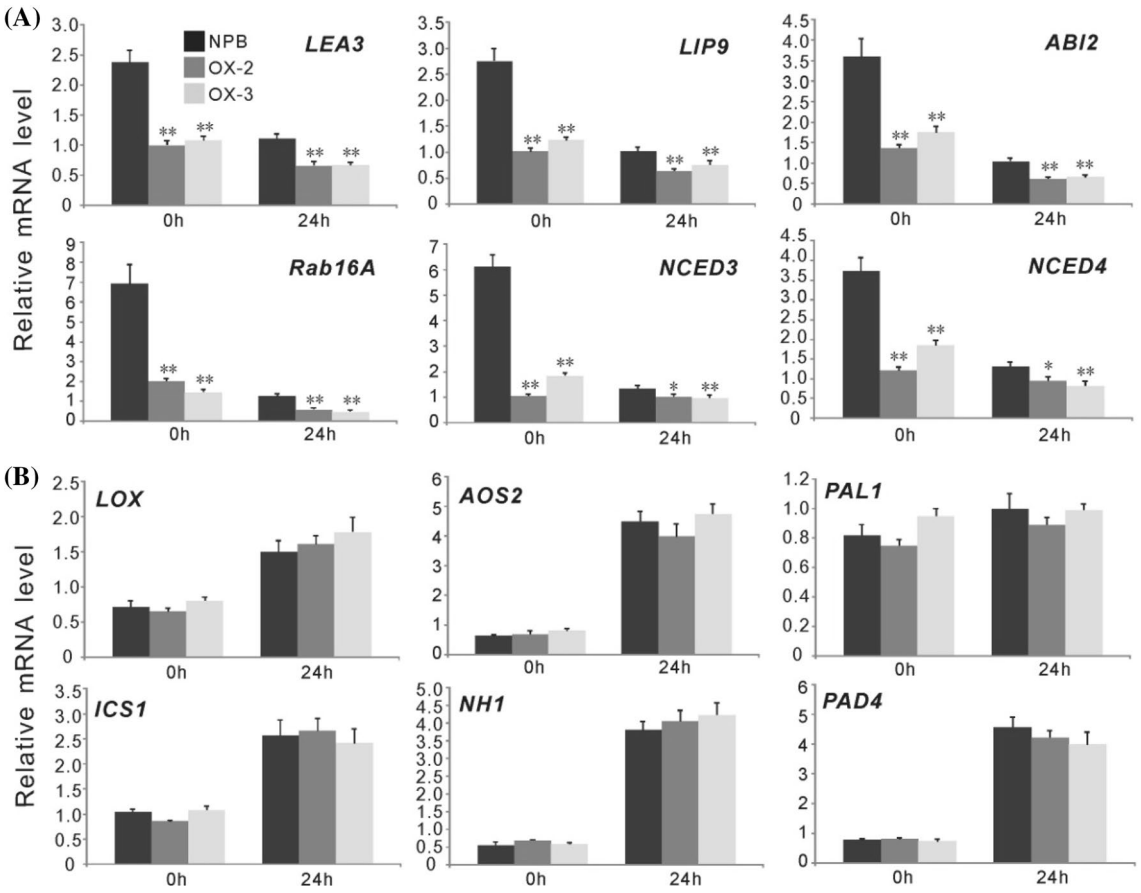


Fig. 6 *ONAC066* reduces the expression of genes involved in ABA signaling pathway. Values are mean ± SE from nine biological replicates. Asterisks represent significant differences relative to *Nipponbare* plants (*t* test, $^{**}P < 0.01$ and $^{*}P < 0.05$). **a** The expression patterns of genes involved in ABA signaling pathway in OX-ONAC066 and *Nipponbare* plants before (0 h) and after blast (24 h) inoculation. **b** The expression patterns of genes involved in SA or JA signaling pathway in OX-ONAC066 and *Nipponbare* plants before and after pathogen attack

SA in both these plants (Fig. 7). These results further suggest that *ONAC066*-mediated defense response is at least partially dependent on the ABA signaling pathway in rice.

To further confirm the interactions between *ONAC066* and the ABA signaling pathway, we quantified the endogenous level of violaxanthin, the precursor of ABA (Neuman et al. 2014), in the seedlings of the same plants used for measuring the concentration of hormones before and after *M. oryzae* inoculation. Similar to ABA, the endogenous level of violaxanthin was significantly reduced in both *Nipponbare* and OX-ONAC066 plants, but the violaxanthin level was remarkably lower in transgenic plants than in *Nipponbare* plants before and after blast infection (Fig. 7a). A yeast one-hybrid assays were also performed to determine that whether *ONAC066* can directly bind to the promoters of the six ABA-related genes. We identified the direct interactions between *ONAC066* and the *LIP9* and *NCED4* promoters (Fig. 7b), whereas no direct binding were observed between *ONAC066* and the promoters of *LEA3, Rab16A, ABI2*, and *NCED3* (data not shown). Importantly, the seed germination assays showed that the shoot and root length of the OX-ONAC066 seeds were more sensitive to ABA treatments than the wild-type *Nipponbare* seeds (Supplemental Fig. 3). Taken together, these results indicate that *ONAC066*-mediated defense response is indeed involved in the ABA signaling pathway in rice.

Metabolic changes are different between wild-type and transgenic plants

To further compare the metabolic responses between wild-type *Nipponbare* and *ONAC066* overexpressing plants before and after *M. oryzae* infection, metabolomic studies were conducted in the leaves of *Nipponbare* (NPB) and *ONAC066* overexpressing plants (the transgenic line, OX-2) both before (0 h) and after 24-h (24 h) blast inoculation. Totally, 84 metabolite peaks were detected in the rice leaf by gas chromatography tandem mass spectrometry (GC–MS),

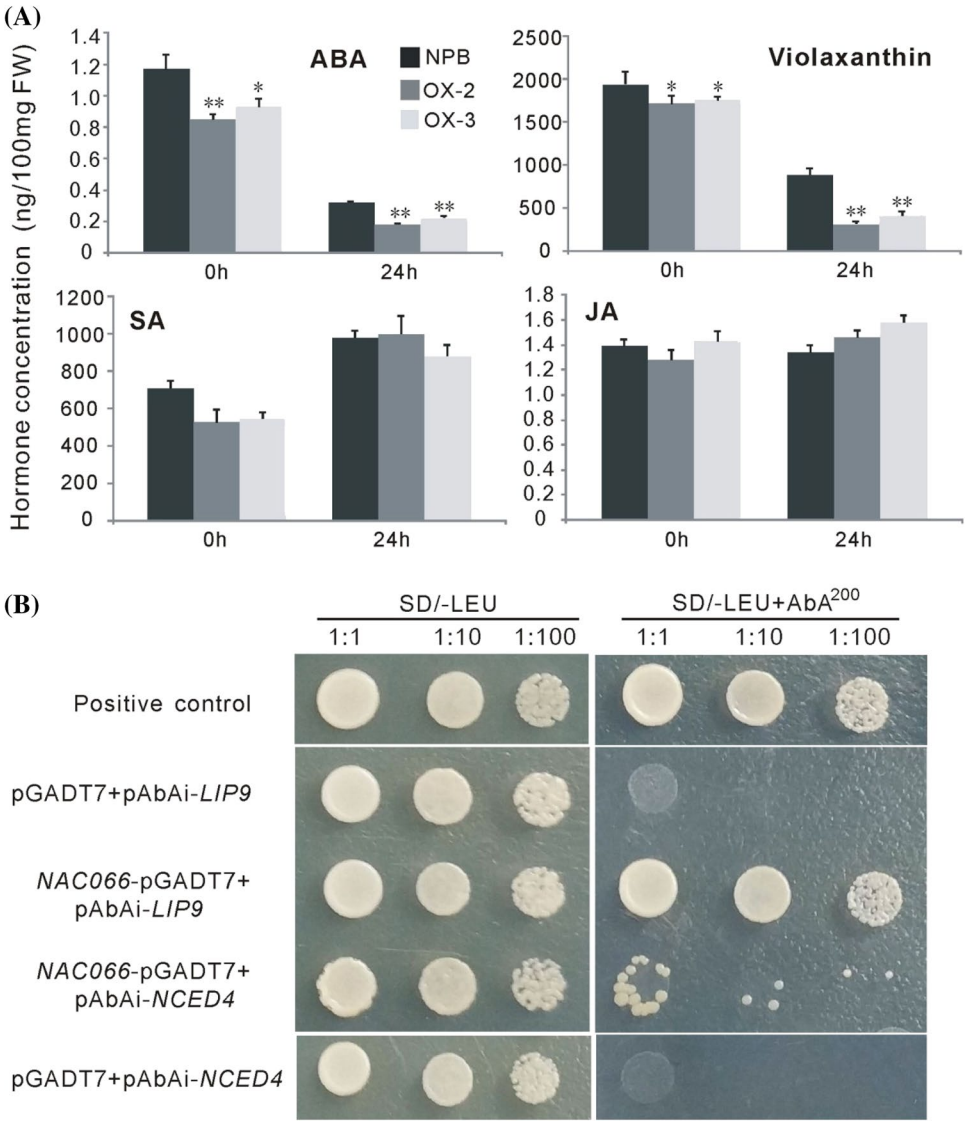


Fig. 7 *ONAC066* functions dependent on the ABA signaling pathway. **a** The endogenous levels of ABA, violaxanthin, SA and JA in the wild-type *Nipponbare* and OX-ONAC066 plants before (0 h) and after blast (24 h) inoculation. The values are the mean of 12 biological replicates. Asterisks represent significant differences relative to *Nipponbare* plants before and after blast inoculation (*t* test, $^{**}P < 0.01$ and $^{*}P < 0.05$). *FW* fresh weight. **b** Yeast one-hybrid assays indicate the direct interactions between *ONAC066* and the promoters of *LIP9* and *NCED4*. P53 is the positive control

and 51 peaks (representing 48 metabolites) were identified based on their authentic standards, 33 peaks were annotated by matching the MS spectra to those in the NIST11 library (Supplemental Table 5). Retention times and levels of these compounds are provided in Supplemental Table 5. Among the 48 identified metabolites, 11 were identified as sugar-related compounds, 17 were amino acid-related compounds, 5 were free fatty acids, 5 were tricarboxylic acid (TCA) cycle intermediates, and 10 were other compounds. Most were primary metabolites.

The relative intensities of the 84 metabolite peaks were normalized based on the intensity of an internal standard, and used to perform multivariate statistical analyses. Orthogonal partial least squares-discriminant analysis (OPLS-DA) with supervised pattern recognition was conducted to generate an overview of the metabolic pattern of the four samples. The results showed that separation was

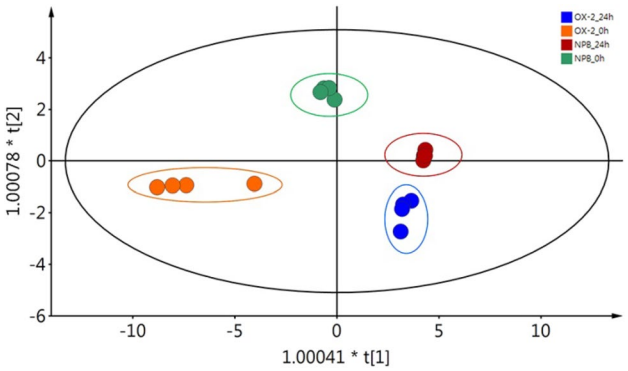
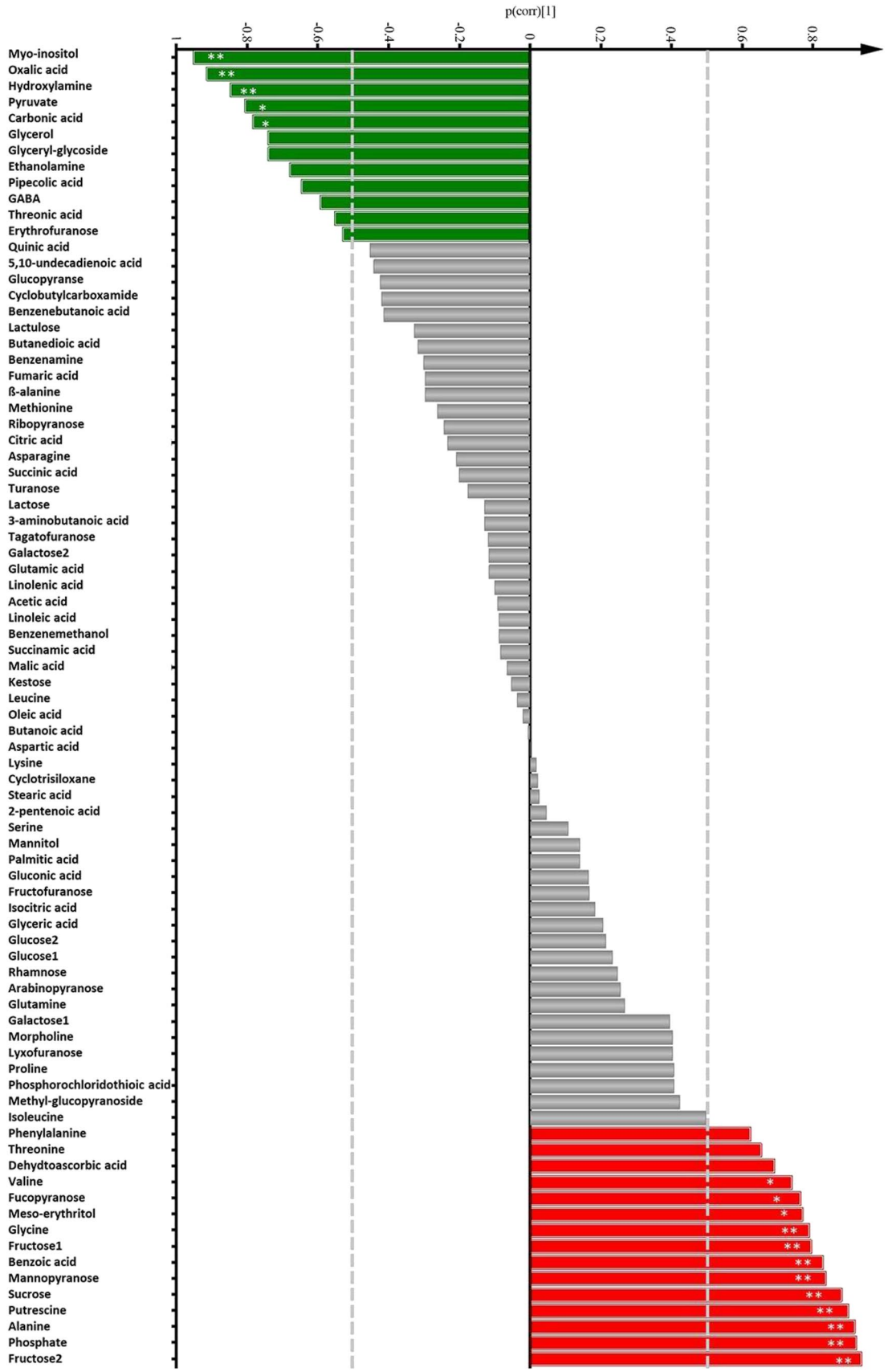


Fig. 8 The score plot generated from GC–MS data using the cross-validated OPLS-DA model, demonstrating the dynamic metabolome change for OX-ONAC066 (OX-2) and *Nipponbare* (NPB) plants both before (0 h) and after (24 h) leaf blast inoculation


p(corr)[1]
-1
-0.8
-0.6
-0.4
-0.2
0
0.2
0.4
0.6
0.8
Myo-inositol
Oxalic acid
Hydroxylamine
Pyruvate
Carbonic acid
Glycerol
Glyceryl-glycoside
Ethanolamine
Pipecolic acid
GABA
Threonic acid
Erythrofuranose
Quinic acid
5,10-undecadienoic acid
Glucopyranse
Cyclobutylcarboxamide
Benzenebutanoic acid
Lactulose
Butanedioic acid
Benzenamine
Fumaric acid
ß-alanine
Methionine
Ribopyranose
Citric acid
Asparagine
Succinic acid
Turanose
Lactose
3-aminobutanoic acid
Tagatofuranose
Galactose2
Glutamic acid
Linolenic acid
Acetic acid
Linoleic acid
Benzenemethanol
Succinamic acid
Malic acid
Kestose
Leucine
Oleic acid
Butanoic acid
Aspartic acid
Lysine
Cyclotrisiloxane
Stearic acid
2-pentenoic acid
Serine
Mannitol
Palmitic acid
Gluconic acid
Fructofuranose
Isocitric acid
Glyceric acid
Glucose2
Glucose1
Rhamnose
Arabinopyranose
Glutamine
Galactose1
Morpholine
Lyxofuranose
Proline
Phosphorochloridothioic acid
Methyl-glucopyranoside
Isoleucine
Phenylalanine
Threonine
Dehydtoascorbic acid
Valine
Fucopyranose
Meso-erythritol
Glycine
Fructose1
Benzoic acid
Mannopyranose
Sucrose
Putrescine
Alanine
Phosphate
Fructose2

◂**Fig. 9** The p (corr)-plot analysis of all metabolites detected by GC–MS in the OX-ONAC066 plants (OX-2) and *Nipponbare* (NPB) plants after 24 h leaf blast infection. Red column represents up-regulation and green column represents down-regulation with the threshold |P(corr)| > 0.5. Asterisks represent significant differences relative to *Nipponbare* plants (*t* test, $^{**}P < 0.01$, $^{*}P < 0.05$)

more evident between the NPB_0h and OX-2_0h groups, suggesting more metabolic differences between NPB and OX-2 rice leaves before infection (Fig. 8). Pairwise comparisons of the leaf metabolome using the OPLS-DA model were performed to find metabolites that differed significantly in the two comparison groups. The OPLS-DA score plots showed distinct differences among metabolomes (Supplemental Figs. 4–7). Parameters of OPLS-DA models were used to evaluate model fitting and the predictive ability of the model. All models were found to have a satisfactory fit with good predictive power values: all R_2Y and Q^2 values were higher than 0.9, and the *P*-values of all CV-ANOVA were < 0.05 (Supplemental Table 6).

ONAC066 induces the accumulation of soluble sugars and amino acids

P(corr)-plot analyses allowed us to identify metabolites that were up- or down-regulated between the pair-wise comparisons. For each pairwise comparison, 63 metabolites were remarkably different between NPB_0h and NPB_24h (Supplemental Fig. 8); 61 metabolites were remarkably different between OX-0h and OX-2_24h (Supplemental Fig. 9); 39 metabolites were remarkably different between NPB_0h and OX-0h (Supplemental Fig. 10); and 17 metabolites were remarkably different between NPB_24h and OX-2_24h (Fig. 9), using univariate statistical analyses based on the Student's *t* test.

We identified that the concentrations of many kinds of sugars were significantly down-regulated after pathogen inoculation in both the wild-type *Nipponbare* and OX-ONAC066 plants (Supplemental Figs. 8, 9). However, the contents of several sugars, such as sucrose, glucose, fructose, mannopyranose, and fucopyranose, were remarkably increased in OX-ONAC066 plants relative to *Nipponbare* plants either before or after blast infection (Fig. 9; Supplemental Fig. 10). Furthermore, the contents of amino acids were significantly up-regulated in both *Nipponbare* and OX-ONAC066 plants after pathogen infection (Supplemental Figs. 8, 9). The concentrations of many amino acids, including valine, glycine, leucine, proline, and alanine, were also much higher in OX-ONAC066 plants compared to *Nipponbare* plants either before or after pathogen inoculation (Fig. 9; Supplemental Fig. 10). Adversely, the level of oxalic acid was dramatically down-regulated in OX-ONAC066 plants compared with *Nipponbare* plants both before and after blast inoculation (Fig. 9; Supplemental Fig. 10). These results together suggest the important roles of sugars, amino acids and oxalic acid in *ONAC066*-mediated disease resistance.

Discussion

Although several NAC transcription factors have been demonstrated to play crucial roles in biotic stress response in rice (Nakashima et al. 2007; Kaneda et al. 2009; Yoshii et al. 2009; Sun et al. 2013; Yokotani et al. 2014), the regulatory roles of other family members in disease resistance are still largely unknown. Besides, the regulatory mechanisms of NAC genes in rice defense response were not well understood. In this study, we have shown that the transcription of *ONAC066* was strongly induced by blast infection and *ONAC066* overexpressing transgenic rice plants exhibited quantitatively enhanced resistance to blast and bacterial blight resistance. We also revealed that *ONAC066* influenced the metabolisms of sugars and amino acids in normal condition or after pathogen inoculation in rice. To our knowledge, this is the first report to dissecting the mechanism of NACs-mediated disease resistance using metabolomic approach.

ONAC066 exerts its functions on disease resistance through modulating the transcription of stress-related genes

As transcription factors, previous reports have indicated that NAC genes can directly bind to the promoters of downstream target genes to regulate their expression in plants (Hu et al. 2006; Shen et al. 2017). In this study, we have shown that the transcription of nine defense-related *PR* genes were significantly induced in *ONAC066* overexpression plants relative to *Nipponbare* plants both before and after pathogen inoculation whereas the expression of ABA pathway genes (*LEA3, LIP9, Rab16A* and so on) were remarkably reduced in *ONAC066* overexpressing plants relative to *Nipponbare* plants before blast infection. These data strongly suggest that *ONAC066* exerts its functions on disease resistance through modulating the transcription of well-known stress-related genes in rice. Since Yokotani et al. (2014) reported that *OsNAC111* directly activates the promoters of *PR2* and *PR8* to induce their expression by *M. oryzae* infection, and Shen et al. (2017) demonstrated that *LEA3* and *Rab16A* are the direct targets of *OsNAC2* in the rice response to drought and salt stresses, we deduced that the stress-related genes in our study were also likely to be the direct targets of *ONAC066*. To verify this inference, a series of yeast one-hybrid assays were conducted. Six ABA-related genes (*LEA3, LIP9, Rab16A, ABI2, NCED3* and *NCED4*) and seven PR genes (*PR1a, PR10, PR4, PR5, PR2, PR3* and *PR8*) were chosen

to perform this experiment. As expected, we identified the interactions between *ONAC066* and the promoters of *LIP9* and *NCED4*, indicating the direct regulation of *LIP9* and *NCED4* by *ONAC066*. The direct binding of *ONAC066* to PR gene promoters was not found (data not shown).

ONAC066 confers disease resistance by regulating the ABA-dependent pathway

Generally, it is considered that the responses of plant to pathogens are modulated either by the SA signaling pathway or the JA signaling pathway (Bari and Jones 2009). However, in this study, we show that the disease resistance conferred by *ONAC066* is dependent on the ABA signaling pathway but not the SA or JA signaling pathways. The transcripts of stress-related genes, *LEA3, LIP9, Rab16A, ABI2, NCED3* and *NCED4*, which are involved in the ABA-dependent pathway, were remarkably reduced in OX-ONAC066 plants compared with wild-type *Nipponbare* plants either before or after blast infection. Yeast one-hybrid assays confirmed that *ONAC066* could bind directly to the promoters of *LIP9* and *NCED4* to regulate their transcription. Besides, the expression level of *ONAC066* was strongly induced by exogenous ABA, and the OX-ONAC066 plants were more sensitive to ABA treatments than wild-type *Nipponbare* plants. Moreover, we also identified that the endogenous ABA and violaxanthin levels were significantly lower in *ONAC066* overexpressing plants compared with *Nipponbare* plants after pathogen infection. In contrast, the expression of *ONAC066* was not influenced by exogenous SA and JA, and the endogenous levels of these two hormones also exhibited no obvious changes between *Nipponbare* and *ONAC066* overexpressing plants before and after pathogen attack. These results together suggest that *ONAC066* confers disease resistance by regulating the ABA-dependent pathway. This is consistent with the previous report that ABA plays a negative role in rice-*M. oryzae* interactions (Jiang et al. 2010; Ulferts et al. 2015). Recently, several studies suggested that the balance between ABA and SA signaling pathways is an important determinant for the outcome of plant–pathogen interactions, and ABA-mediated susceptibility to pathogen infection may partially depend on the suppression of the SA signaling pathway (Jiang et al. 2010; Xu et al. 2013; Gao et al. 2016). Our results here also indicate that ONAC066-mediated disease resistance involves regulation of the ABA signaling pathway and ONAC066 alleviates ABA-mediated disease susceptibility.

Roles of soluble sugars in *ONAC066*-mediated defense response

Recently, more and more evidence has shown the important roles of sugars in plant disease resistance (Gómez-Ariza et al. 2007; Qian et al. 2015; Machado et al. 2015; Moore et al. 2015). For instance, a higher level of sucrose was accumulated in the leaves of *PRms* overexpressing rice plants that exhibited enhanced blast resistance, and pre-treatment of wild-type rice plants with exogenous sucrose causes enhanced resistance to *M. oryzae* (Gómez-Ariza et al. 2007). In the present study, higher concentrations of sucrose and fructose were also identified in resistance-enhanced *ONAC066* overexpressing plants relative to *Nipponbare* plants both before and after *M. oryzae* inoculation. These results together suggest the positive roles of sugars in regulating plant response to blast disease in rice. However, we also found that the levels of most sugars (sucrose, fructose, and others) were dramatically reduced after 24 h blast infection in both *Nipponbare* and *ONAC066* overexpressing plants. Similar results were also observed by Parker et al. (2009). In their experiments, the levels of sucrose, glucose and fructose were significantly increased upon the appearance of visible blast lesions in rice plants. Our result may reflect the status of sugar contents at the early stage during *M. oryzae* infection.

The potential roles of amino acids in plant disease resistance

Amino acids are well-known for their central roles in regulating plant growth and development, including protein synthesis, nitrogen metabolism and energy supply (Jespersen et al. 2017). In recent years, emerging evidences have shown that amino acids were also involved in the plant response to various stresses (Parker et al. 2009; Yun et al. 2013; Cao et al. 2017b; Jespersen et al. 2017). For example, increased accumulation of glycine, serine and other amino acids were associated with improved tolerance to heat or drought stresses in creeping bentgrass (Jespersen et al. 2017), and glycine treatment caused enhanced cold tolerance by improving leaf photosynthesis in rice (Cao et al. 2017b). In this study, the metabolic analyses revealed the significant accumulation of many amino acids in both the wild-type and ONAC066 overexpressing plants after blast inoculation, consistent with the previous reports that increases in amino acids were identified in biotrophic pathogen-challenged leaves (Solomon et al. 2003; Parker et al. 2009). Notably, higher levels of glycine, alanine and valine were identified in ONAC066 overexpressing plants compared with wild-type plants after pathogen inoculation, suggesting their important roles in *ONAC066*-mediated disease resistance. Just as expected, glycine is the necessary component for the synthesis of glutathione, which has been well-documented for its role in the plant defense response (Noctor et al. 1997). However, less information was available on how alanine and valine function in plant disease resistance. Further research will be needed to address these questions.

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Author contributions QL conducted the quantitative qRT-PCR assay, transgenic functional confirmation and chemical treatment experiments, drafting the manuscript and proposal writing. SY conducted the metabolic study, drafting the manuscript and proposal writing. WH performed the quantification of endogenous ABA. JY evaluated the blast and *Xoo* resistance. JD, SZ, JZ, TY and XM participated in RNA extraction and quantitative qRT-PCR assays. XZ and BL conceived of the study, drafted proposal and corrected manuscript.

Compliance with ethical standards

Conflict of interest The authors declare no conflict of interest.

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