

The *OsOX02*, *OsOX03* and *OsOX04* positively regulate panicle blast resistance in rice

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Original article

Keywords: Rice (*Oryza sativa* L.), Panicle blast, OXO (Oxalate oxidase)

Posted Date: December 31st, 2020

DOI: <https://doi.org/10.21203/rs.3.rs-137340/v1>

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Version of Record: A version of this preprint was published at Rice on June 5th, 2021. See the published version at <https://doi.org/10.1186/s12284-021-00494-9>.

Abstract

Background

Although panicle blast is more destructive to yield loss than leaf blast in rice, the cloned genes that function in panicle blast resistance is still very limited and the molecular mechanisms underlying panicle blast resistance remain largely unknown.

Results

In the present study, we have confirmed that the three *OXO* genes, *OsOXO2*, *OsOXO3* and *OsOXO4* from a blast-resistant cultivar BC10 function in panicle blast resistance in rice. The expression of *OsOXO2*, *OsOXO3* and *OsOXO4* were induced by panicle blast inoculation. Subcellular localization analysis revealed that the three *OXO* genes are all localized in the cell wall. Simultaneous silencing of *OsOXO2*, *OsOXO3* and *OsOXO4* decreased rice resistance to panicle blast, whereas the *OsOXO2*, *OsOXO3* and *OsOXO4* overexpression rice plants showed enhanced panicle blast resistance. More H₂O₂ and higher expression levels of *PR* genes were observed in the overexpressing plants than in the control plants, while the gene silencing plants exhibited less H₂O₂ and lower expression levels of *PR* genes compared to the control plants. Moreover, phytohormone treatment and the gene expression analysis showed that panicle blast resistance mediated by the three *OXO* genes was associated with the induction of JA and ABA signaling pathways but suppression of SA signaling pathway.

Conclusion

OsOXO2, *OsOXO3* and *OsOXO4* positively regulate panicle blast resistance in rice. The *OXO* genes could modulate the accumulation of H₂O₂ and expression levels of *PR* gene in plants. Moreover, the *OXO* genes mediated panicle blast resistance is associated with the activation of JA and ABA signaling pathways but suppression of the SA signaling pathway.

Background

Rice (*Oryza sativa* L.) is a major food crop for more than half of the world's population. The demand is increasing with growing population. However, rice production is facing many challenges. Rice blast disease, caused by *Magnaporthe oryzae* (*M. oryzae*) is one of the most destructive diseases in rice plant, causing 10% – 30% of yield loss every year (Skamnioti and Gurr, 2009). Use of resistant variety is considered to be the most economical and environment-friendly approach to solve this problem (Hu et al., 2008). Rice blast can be classified into leaf blast and panicle blast based on the infected parts, and generally panicle blast is more destructive in terms of yield loss (Sirithunya et al., 2002; Zhuang et al., 2002; Liu et al., 2016a; Liu et al., 2016b). Recently, more and more studies have shown that the correlations between leaf blast and panicle blast are not always positive, and there exists different

regulation mechanisms between leaf blast resistance and panicle blast resistance (Zhuang et al., 2002; Liu et al., 2016b; Fang et al., 2019). But, nowadays, almost all the work in rice blast is focused on leaf blast, and few genes for panicle blast resistance have been cloned (Liu et al., 2016a; Liu et al., 2016b; Inoue et al., 2017). Besides, our knowledge on the mechanism of panicle blast resistance is still very limited. Since blast disease may occur in different developmental stages and the blast pathogen may infect different parts (leaf or panicle) in rice, it is necessary for effective disease control to identify the genes associated with panicle blast resistance and understand their regulatory mechanisms.

Oxalate oxidases (OXOs) which belong to the germin protein family have been demonstrated to play important roles in various environmental stresses in plants (Hu et al., 2003; Dong et al., 2008; Wan et al., 2009; Partridge-Telenko et al., 2011; Karmakar et al., 2016). OXO catalyzes the degradation of oxalic acid to produce hydrogen peroxide (H_2O_2) and carbon dioxide. H_2O_2 generated from the reaction can not only contribute to the cell wall modification, but also functions as a secondary messenger to activate the hypersensitive response, the phytoalexin biosynthetic pathways as well as the expression of pathogenesis-related (PR) genes in plants (Hammond-Kosack et al., 1994; Lamb and Dixon, 1997; Carter et al., 1998). Owing to the function of H_2O_2 , OXO genes were speculated to play a key role in plant disease resistance. Indeed, many studies have indicated the involvement of OXOs in plant basal host resistance. For example, overexpressing a wheat OXO gene (*gf-2.8*) resulted in the induction of defense proteins and increased resistance to *Sclerotinia sclerotiorum* in sunflower (Hu et al., 2003). Transgenic oilseed rape plants overexpressing a wheat OXO gene exhibited enhanced resistance to *Sclerotinia sclerotiorum* (Dong et al., 2008). Recently, Yang et al. (2019) reported that overexpressing a wheat OXO gene (GenBank No M21962.1) also showed enhanced resistance to *sclerotinia stemrot* in *Glycine max* (Yang et al., 2019).

In rice plants, four OXO genes (*OsOXO1–OsOXO4*) with >90% nucleotide sequence identity are identified on chromosome 3. They form a tandemly duplicated cluster and co-localize with a blast disease resistance QTL (Ramalingam et al. 2003; Wu et al. 2004). Existing studies have shown that *OsOXO4* was expressed during rice-*M. oryzae* infection in leaf blast, and the expression of *OsOXO4* increased earlier in blast-resistant cultivar Moroberekan than blast-susceptible cultivar Vandana (Carrillo et al., 2009). Moreover, overexpressing *OsOXO4* driven by the green tissue-specific promoter and co-expression of *OsCHI11* and *OsOXO4* all showed increased resistance to sheath blight pathogen in rice (Molla et al., 2013; Karmakar et al., 2016). Although the four OXO genes (*OsOXO1–OsOXO4*) have been demonstrated to co-localize with a blast resistant QTL, their specific regulation roles in rice blast resistance have not been reported so far.

In the present study, we identified that the transcription levels of *OsOXO2*, *OsOXO3* and *OsOXO4* were significantly induced by panicle blast inoculation. To confirm their functions in panicle blast resistance, we performed sub-cellular localization, spatio-temporal expression and transgenic analysis. Our results showed that *OsOXO2*, *OsOXO3* and *OsOXO4* cloned from a blast-resistant line BC10 function as positive regulators of panicle blast resistance in rice. In addition, their regulatory mechanisms of panicle blast resistance were also investigated in this study.

Results

The *OXO* genes are localized in the cell wall and exhibit different temporal and spatial expression patterns in rice

In our previous study, we have performed a microarray analysis using a blast resistant line BC10 (Liu et al. 2004) and discovered that the expression levels of the three *OXO* genes, *OsOXO2*, *OsOXO3* and *OsOXO4*, were strongly induced by panicle blast inoculation within a period of 48 h (Table S2). To further confirm these results, we analyzed the expression patterns of the three *OXO* genes in panicles in BC10 plants after blast infection using real-time PCR in this study. The results showed that the expression of *OsOXO2* in infected panicles was induced dramatically at 6 h, 12 h and 24 h, and peaked at 12 h for about three folds compared to 0 h treatment. The expression levels of *OsOXO3* increased significantly at 6 h, 12 h and 24 h, and peaked at 48 h after pathogen inoculation. Similarly, the transcription level of *OsOXO4* was also induced at all time points after pathogen inoculation (Fig. 1A). These results suggest the important roles of the three *OXO* genes in regulating panicle blast resistance in rice.

To analyze the sub-cellular localization of the three *OXO* genes, we fused the coding region of them with the red fluorescent protein (mcherry) fragment under the control of the cauliflower mosaic virus 35S promoter and expressed the fusion proteins into onion epidermal cells, respectively. Laser confocal microscopy showed that the red fluorescent signal in the mcherry control vector was distributed both in the nucleus and the cytoplasm, whereas the red fluorescent signal in the *OsOXO2*-mcherry, *OsOXO3*-mcherry, *OsOXO4*-mcherry vectors were distributed only in the cell wall of the transfected onion epidermal cells (Fig. 1B).

To investigate the temporal and spatial expression patterns of the three *OXO* genes in rice plants, we analyzed the transcription of them in various tissues of *Nipponbare* by quantitative real-time PCR using EF1 α as the internal control. As shown in Fig. 1C, the expression of *OsOXO2* was almost hard to be detected in all rice tissues examined. *OsOXO3* expressed in callus, leaf and panicle, with the highest expression level in callus, but its background expression level is relatively low. Moreover, *OsOXO4* expressed in all rice tissues examined, with a relatively higher expression levels in stem, leaf and panicle. To further confirm the high expression level of *OsOXO4* in panicles, we generated transgenic *Nipponbare* plants in which the expression of a β -glucuronidase (GUS) was driven by the promoter of *OsOXO4*. Strong GUS signal was detected in panicles at the booting stage and heading stage (Fig. 1D), agreeing well with the result from qRT-PCR.

Overexpression of *OsOXO2*, *OsOXO3* and *OsOXO4* enhances panicle blast resistance in rice

To confirm the function of the three *OXO* genes in panicle blast resistance in rice, we generated the *OsOXO2*, *OsOXO3* and *OsOXO4* overexpressing plants, designated as OE*OXO2*, OE*OXO3* and OE*OXO4*, respectively using *Nipponbare* which was susceptible to blast isolate GD08-T13. The transgenic plants showed no obvious differences in non-target traits compared to *Nipponbare* plants and were fertile. Three independent homozygous lines of each *OXO* gene were used for panicle blast resistance evaluation. Real-

time PCR analysis showed that the transcript levels of the three *OXO* genes were significantly increased in their corresponding transgenic lines (Fig. 2A). Cotton-wrapping inoculation showed that the infected main axis length was 82.1% for *Nipponbare* plants and 83.2% for the transformed empty vector control (PHQSN) plants, but 31.46–35.25% for the *OXO* overexpressing plants (Table 1, Fig. 2B). The enhanced panicle blast resistance was correlated with the increased expression of *OXO* genes in all transgenic plants (Fig. 2C). However, there were no significant difference in diseased leaf area between the *OXO* overexpressing plants and the control plants (PHQSN) after blast inoculation (Table S3).

Table 1
The infected main axis length of control (PHQSN) and *OXO* overexpressing plants after inoculation.

Name	Total unumber	Infected main axis length (%)	<i>P</i> -value
PHQSN	12	83.231 ± 8.36	
Nip	7	82.1 ± 7.66	0.3924
OEOX02(OE2)	15	35 ± 7.07	1.2362E-09
OEOX02(OE3)	15	32.66 ± 5.62	1.13E-14
OEOX02(OE5)	14	32.85 ± 5.78	5.77868E-14
OEOX03(OE1)	12	31.46 ± 5.45	2.05E-14
OEOX03(OE2)	13	34.15 ± 6.22	1.75E-14
OEOX03(OE6)	13	33.46 ± 6.88	1.14298E-12
OEOX04(OE3)	12	32.36 ± 5.78	1.85E-13
OEOX04(OE4)	15	35.25 ± 5.32	2.03E-14
OEOX04(OE6)	17	34.11 ± 6.90	1.62594E-14

OsOX02, OsOX03 and OsOX04 silencing rice plants are more susceptible to panicle blast

To further confirm the function of *OXO* genes in panicle blast resistance, we generated the *OXO* silencing plants in *Nipponbare* using an RNAi vector containing a 327 bp homologous coding sequence among *OsOX02*, *OsOX03* and *OsOX04*. We obtained 13 silenced transgenic lines, which showed no differences in the non-target traits compared to the wild type *Nipponbare* and were fertile. Quantitative RT-PCR analysis showed that both the expression levels of *OsOX03* and *OsOX04* remarkably decreased compared to the control plants (PHQSN). The expression level of *OsOX02* was too low to be detected in the silencing plants (Fig. 3A). The *OXO* silencing plants exhibited reduced resistance to panicle blast, with higher percentages of infected main axis length in both T₀ and T₂ generation plants when compared to the control plants (Table 2, Fig. 3B). The reduced expression levels of *OsOX03* and *OsOX04* were correlated with increased percentages of infected main axis length in the silencing plants (Fig. 3C). However, there were no significant difference in diseased leaf area between the *OXO* silencing plants and

the control plants (PHQSN) after blast inoculation (Table S4). The results from gene overexpressing and silencing experiments suggest that *OsOXO2*, *OsOXO3* and *OsOXO4* positively regulate panicle blast resistance in rice.

Table 2
The infected main axis length of control (PHQSN) and OXO silencing plants after inoculation

Name	Total number	Infected main axis length(%)	P-value
PHQSN	10	38.21 ± 2.73	
Nip	7	37.14 ± 4.18	0.412172464
RNAi (T0 generation)	13	72.5 ± 9.57	5.39314E-05
RNAi (3 - 2, T2 generation)	17	75.29 ± 10.27	1.23875E-07
RNAi (7 - 1, T2 generation)	11	73.63 ± 12.86	1.56343E-05

***OsOXO2*, *OsOXO3* and *OsOXO4* modulate the expression of defense-related genes**

It is well-known that *PR* genes play important role in plant defense responses (Kaur et al. 2017). Previous study reported that the expression levels of *PR* genes significantly increased in *OsERF83* overexpressing plants which showed enhanced rice blast resistance (Tezuka et al., 2018). In this study, the transcriptions of several *PR* genes were analyzed in the *OXO* transgenic plants and control plants (PHQSN). The results showed that the expression levels of *PR2*, *PR3*, *PR5* and *PR5-1* were all up-regulated in *OE**OXO3* and *OE**OXO4* plants, while the transcriptions of *PR8* were up-regulated in *OE**OXO2*, *OE**OXO3* and *OE**OXO4* plants compared to the control plants. However, the induced expression of *PR10* was only identified in *OE**OXO3* plants. Moreover, all the six *PR* genes were down-regulated in *OXO* silencing plants compared to the control plants, suggesting that the three *OXO* genes-mediated disease resistance is partially associated with the expression of *PR* genes in rice (Fig. 4).

The *OsOXO2*, *OsOXO3* and *OsOXO4* influence the endogenous levels of H₂O₂ in the transgenic plants

Previous studies have shown that higher levels of H₂O₂ contribute to *OXO* genes mediated fungal resistance in plants (Lamb and Dixon 1997;Carter et al., 1998;Wan et al.,2009). To confirm if the three *OXO* genes mediated panicle blast resistance is associated with the altered endogenous levels of H₂O₂, we firstly measured the H₂O₂ concentrations in the panicles of control (PHQSN) and transgenic plants using the xylenol orange method (Kim and Hwang, 2014). The results showed that the H₂O₂ concentrations were significantly higher in the *OXO* overexpressing plants, but were lower in the *OXO* silencing plants when compared with the control plants (Fig. 5A). Similar results were also observed using the DAB staining method (Thordal-Christensen et al., 1997). Brown staining was observed in the leaves of both control and *OXO* overexpressing plants, but the staining was stronger in the *OXO* overexpressing plants than in control plants. No visible staining was observed in the leaves of *OXO* silencing plants (Fig. 5B).

The three *OXO* genes mediated panicle blast resistance is associated with activation of the JA and ABA signaling pathways but suppression of the SA signaling pathway

To dissect the potential mechanisms of *OXO* genes-mediated panicle blast resistance in rice, we firstly analyzed the cis-elements in the promoters of *OsOXO2*, *OsOXO3* and *OsOXO4*, respectively. One ethylene (ET)-responsive element (ERE), two TC-rich repeats involved in defense and stress responses and one salicylic acid (SA)-responsive element (TCA-element) were found in the promoter of *OsOXO2*. Three abscisic acid (ABA)-responsive elements (ABREs), two methyl jasmonic acid (MeJA)-responsive elements (CGTCA-motif and TGACG-motif) and one TC-rich repeats were identified in the promoters of both *OsOXO3* and *OsOXO4* (Table S5). The existence of TC-rich repeats further supported the regulatory roles of *OsOXO2*, *OsOXO3* and *OsOXO4* in disease resistance. Meanwhile, the presence of the hormone response elements in the promoter region implies that the expression of the three *OXO* genes could be regulated by these hormones. To confirm this inference, we treated the wild-type *Nipponbare* plants with exogenous ABA, SA, ET and JA at the three leaf stage, respectively. The expression levels of the three *OXO* genes were analyzed both before and after hormone treatments using qRT-PCR. Unfortunately, the expression of *OsOXO2* could not be detected. The expression of *OsOXO3* and *OsOXO4* were significantly induced by ABA, SA and JA treatment (Fig. 6). Transcription of *OsOXO3* was significantly induced but the transcription of *OsOXO4* was remarkably reduced by ET treatment (Fig. 6). These observations indicated that ABA, SA and JA may act upstream of *OsOXO3* and *OsOXO4* to induce their expression.

To further validate the relationship between the three *OXO* genes and hormone signaling pathways, we analyzed the expression patterns of several well-known stress-related genes that were involved in ABA, JA or SA signaling pathways, including *ICS1* and *NH1* which were related to the SA signaling pathway (Deng et al. 2012), *LOX2* and *AOS2* which were related to the JA signaling pathway (Deng et al. 2012; Liu et al. 2016) and ABA signaling pathway related genes *LEA3*, *NCED3*, *NCED4*, and *Rab16A* (Liu et al. 2012; Chen et al. 2015). Compared with the control plants, the transcription levels of *ICS1* and *NH1* were significantly decreased in OE*OXO2*, OE*OXO3* and OE*OXO4* plants. In contrast, the transcription level of *LOX2* was increased in OE*OXO3* and OE*OXO4* plants, while the transcription of *AOS2* was induced in all OE*OXO2*, OE*OXO3* and OE*OXO4* plants. The expression levels of *LEA3* and *NCED3* were significantly higher in OE*OXO3* and OE*OXO4* plants than in control plants, while the transcription levels of another two ABA-dependent pathway related genes *NCED4* and *Rab16A* were significantly higher in all OE*OXO2*, OE*OXO3* and OE*OXO4* plants than in control plants (Fig. 7). Just contrary to the results of overexpressing plants, the expression of *ICS1* and *NH1* were significantly up-regulated in the *OXO* silencing plants compared to the control plants, and the expression levels of *LOX2*, *AOS2*, *LEA3*, *NCED3*, *NCED4* and *Rab16A* were remarkably lower in the *OXO* silencing plants than in the control plants (Fig. 7). These results together suggest that the disease resistance conferred by *OsOXO2*, *OsOXO3* and *OsOXO4* is associated with activation of JA and ABA signaling pathways but suppression of SA signaling pathway.

Discussion

OsOXO2, OsOXO3 and OsOXO4 positively regulate panicle blast resistance in rice

Since oxalate oxidases were first isolated and characterized in barley and wheat, they have been reported to play important roles in disease resistance in many plant species (Hu et al., 2003; Livingstone et al., 2005; Welch et al., 2007; Dong et al., 2008; Walz et al., 2008; Barman et al., 2015; Yang et al., 2019). The four *OXO* genes in cluster on chromosome 3 have been reported to be co-localized with a QTL for rice blast resistance (Carrillo et al., 2009). However, the other study showed that the transgenic plants overexpressing *OsOXO1* or *OsOXO4* of Zhonghua11 did not show improved resistance to rice blast disease (Zhang et al., 2013). To further confirm the actual functions of the three *OXO* genes on blast resistance, we used BC10, a strong blast-resistant line (Liu et al. 2004) instead of Zhonghua11, a blast susceptible variety in the study conducted by Zhang et al. (2013) for cloning of the *OXO* genes in the present study. We identified that the expression of *OsOXO2*, *OsOXO3* and *OsOXO4* were induced by panicle blast infection in blast resistant line BC10 in microarray experiments and real-time PCR assays. All the overexpressing plants showed enhanced panicle blast resistance as manifested by the lower percent infected main axis length when compared to the control plants. In contrast, the silencing plants exhibited decreased panicle blast resistance with higher percent infected main axis length than the control plants. However, there were no significant difference in diseased leaf area between the transgenic plants (overexpressing and silencing) and the control plants (Table S3 and Table S4). From these results, we can conclude that the three *OXO* genes (*OsOXO2*, *OsOXO3* and *OsOXO4*) function as positive regulators to modulate panicle blast resistance in rice. These results are different from the previous study (Zhang et al., 2013). We believed that the conflict could be attributed to the use of different materials for cloning of the three *OXO* genes. To confirm this inference, we compared the sequences of the protein coding regions of the three *OXO* genes between BC-10 and Zhonghua11 (Fig. S1). The results revealed that there were several SNPs which lead to the changes of translating proteins. The changes of translating proteins could result in difference in blast resistance. The fact that the three *OXO* genes exert different functions in leaf blast resistance and panicle blast resistance provides a new evidence for the difference between leaf blast resistance and panicle blast resistance in rice.

The panicle blast resistance conferred by OsOXO2, OsOXO3 and OsOXO4 is associated with the accumulation of H₂O₂ and the expression of PR genes

The enzymes encoded by *OXO* genes degrade oxalate acid to generate CO₂ and H₂O₂, the latter has been proved to play a key role in plant disease resistance through cell wall modification by cross linking of plant cell wall proteins in papillae at the infection sites (Olson and Varner 1993; Wei et al., 1998; Liu et al., 2016; Xu et al., 2018; Li et al., 2019; Pei et al., 2019). In the present study, higher H₂O₂ concentration was observed in the *OXO* overexpressing plants whereas lower H₂O₂ content was observed in the *OXO* silencing plants than in the control plants. These results together with the fact that the three *OXO* proteins all localize in the cell wall, we inferred that the modified disease resistance in the transgenic plants might be partially attributed to the changed endogenous H₂O₂ level, which then influence the reinforcement of cell wall. In addition to its direct function in cell wall modification, H₂O₂ also function as a secondary

messenger to activate *PR* gene expression in plants (Hammond-Kosack et al., 1994; Carter et al., 1998). Here, we also identified that the expression levels of several *PR* genes were significantly up-regulated in *OXO* overexpressing plants (which harbors more H₂O₂) when compared to the control plants. This is consistent with the previous report that the sunflower plants overexpressing a wheat *OXO* gene could regulate a number of defense-related genes after pathogen infection (Hu et al., 2003). However, it should be noted that the expression patterns of the *PR* genes were different among different *OXO* gene overexpressing plants. For instance, the induction of *PR2* was identified in the OE*OXO3* and OE*OXO4* plants but not in the OE*OXO2* plants, and the induction of *PR10* was only observed in the OE*OXO3* plants. These results implied that though *OsOXO2*, *OsOXO3* and *OsOXO4* all positively regulate panicle blast resistance in rice, their regulatory mechanisms may be different at least to some extent.

The important roles of hormone signaling pathways in *OsOXO2*, *OsOXO3* and *OsOXO4* mediated panicle blast resistance in rice

Phytohormones are the well-known endogenous signal molecules that function in diverse biological processes including plant defense responses. Each hormone generates and transmits a distinct defense signal and the crosstalk between different hormones has been considered as a universal defense response employed by many plant species (Robert-Seilaniantz et al., 2011; Yang et al., 2013; Huot et al., 2014; Takatsuji and Jiang, 2014; Yang et al., 2019). In this study, the crosstalks between JA, SA and ABA were identified for the panicle blast resistance conferred by *OsOXO2*, *OsOXO3* and *OsOXO4*. We discovered that there were JA, SA and ABA response elements in the promoter region of the three *OXO* genes, and the expression levels of *OsOXO3* and *OsOXO4* were significantly induced by exogenous JA, SA and ABA. Furthermore, our results showed that the transcription of *LOX2* and *AOS2* which are involved in JA biosynthesis, *LEA3*, *NCED3*, *NCED4* and *Rab16A* which are involved in the ABA signaling pathway were remarkably up-regulated in *OXO* gene overexpressing plants compared to the control plants. Reversely, the expression of *ICS1* and *NH1* which are involved in the SA signaling pathway were remarkably reduced in the overexpressing plants compared to the control plants. The expression patterns of these stress-related genes in the *OXO* silencing plants were just opposite to the results of the overexpressing plants. Together, these results suggest that *OsOXO2*, *OsOXO3* and *OsOXO4* mediated panicle blast resistance is associated with activation of the JA and ABA signaling pathways but suppression of the SA signaling pathway.

In general, JA and SA are in most cases antagonized in regulating plant disease resistance (Robert-Seilaniantz et al., 2011). Our results here also indicated the antagonized roles between JA and SA in rice blast resistance. ABA has been well known for its regulatory roles in abiotic stress response and plant development. However, recently, more and more studies have discovered the important roles of ABA in regulating plant biotic stresses (Nambara et al., 2005; Ton et al., 2009; Jiang et al., 2010; Jiang et al., 2013; Liu et al., 2018). For instance, ABA has been identified to positively regulate plant resistance to *Alternaria brassicicola* and *Plectospharella cucumerina* in Arabidopsis (Nambara et al., 2005; Ton et al., 2009). Furthermore, our previous study also demonstrated that the transcription factor *ONAC066* mediated leaf blast resistance is involved in suppression of ABA signaling pathway, indicating the

negative role of ABA in leaf blast disease (Liu et al., 2018). Nevertheless, in this study, we found that the panicle blast resistance conferred by *OXO* genes is associated with the activation of ABA signaling pathway, suggesting the positive role of ABA in panicle blast disease. The opposite regulatory roles of ABA may also partially explain the differential mechanisms between leaf blast resistance and panicle blast resistance.

Conclusion

In conclusion, we have confirmed that *OsOXO2*, *OsOXO3* and *OsOXO4* from blast-resistant line BC10 positively regulate panicle blast resistance in rice in the present study. The *OXO* genes could modulate the accumulation of H₂O₂ and expression levels of *PR* genes in plants. Moreover, the *OXO* genes mediated panicle blast resistance is associated with the activation of JA and ABA signaling pathways but suppression of the SA signaling pathway. However, there are still some issues to be elucidated in the future study. For example, are there any additive effects among the three *OXO* genes (*OsOXO2*, *OsOXO3* and *OsOXO4*). What are the common and different mechanisms of the three *OXO* genes in panicle blast resistance? Further studies are needed to address these issues.

Methods

Vector Construction and Rice Transformation

For overexpression vectors construction, the coding region sequences of *OsOXO2*, *OsOXO3* and *OsOXO4* were amplified from the blast-resistant line BC10 using the primers in Supplemental Table 1. The RNAi vector was generated by cloning a homologous sequence among *OsOXO2*, *OsOXO3* and *OsOXO4* using the primers in Supplemental Table 1. The resulting products were cloned into pEASY-T1 (TransGen) vector and verified by sequencing. The entry clones for overexpressing plants were then inserted into PHQSN (modified from pCAMBIA1390) which harbors a CaMV35S promoter. The clone for RNAi plants was constructed into pRNAi-Ubi, which was suitable for generation of hairpin-RNA constructs. For *P_{OsOXO4}-GUS* construction, ~2.0-kb fragment was amplified from the upstream of *OsOXO4* in BC10 genomic DNA using specific primers (Table S1). Then the fragments were sub-cloned into pCAMBIA1381Z. All the positive plasmids and control vectors were electroporated into *Agrobacterium tumefaciens* EHA105 and then introduced into calli of the cultivar *Nipponbare* via *Agrobacterium*-mediated genetic transformation.

Total RNA extraction and Real-time quantification of mRNAs

Total RNA was extracted with Trizol reagent (Invitrogen) and purified with NucleoSpin RNA Clean-up (MACHEREYNAGEL) according to the manufacturers' instructions. RNA quality and quantity were assessed by formaldehyde denaturing agarose gel electrophoresis and spectrophotometry (Nanodrop-1000), respectively. The purified total RNA was reverse-transcribed using the Primescript™ RT reagent kit (Takara) to generate cDNA and real-time PCR was carried out using SYBR ExTaq™ (Takara). *EF1a* gene

was chosen as a reference gene. Gene expression was quantified by the comparative CT method. Experiments were performed in triplicate, and the results were presented by their means \pm standard derivation (SD). Gene-specific primers used were listed in Supplemental Table 1.

GUS staining analysis

We analyzed GUS activity in transgenic panicles by histochemical staining with 5-bromo-4-chloro-3-indolyl-b-Dglucuronicacid (X-Gluc) as described previously (Liu et al. 2016a). Briefly, we incubated the transgenic panicles overnight at 37°C in staining buffer (100 mM sodium phosphate [pH 7.0], 10 mM EDTA, 0.5 mM $K_4Fe(CN)_6$, 0.5 mM $K_3Fe(CN)_6$, 0.1% [v/v] Triton X-100 and 1 mM X-Gluc) and then decolorized in 100% ethanol before photographed.

Sub-cellular localization analysis

We amplified the protein coding region of *OsOXO2*, *OsOXO3* and *OsOXO4* from BC10 using the primers in Supplemental Table 1 and cloned them into the pGY1-mcherry vector to generate the OXO-mcherry fusion proteins, respectively. The plasmids of *OsOXO2*-mcherry, *OsOXO3*-mcherry, *OsOXO4*-mcherry and empty pGY1-mcherry were extracted using UNIQ-50 Column Plasmid Max-Preps Kit (Sango). For the transient expression assay, 1 μ g of every plasmid DNA was introduced into onion epidermal cells using the PDS-1000/He particle delivery system (BioRad, Hercules, CA, USA). After 24 h incubation at 28°C without light, onion epidermal cells were observed and photographed under a laser confocal microscopy (Zeiss LSM710, Germany).

Cis-elements analysis of the promoter

We downloaded approximately 1500 bp sequences upstream of *OsOXO2*, *OsOXO3* and *OsOXO4* from MSU Rice Genome Annotation Project (<http://rice.plantbiology.msu.edu/>), respectively. The sequences were scanned by PLACE (Lescot et al., 2002) (<http://www.dna.affrc.go.jp/PLACE/>) for cis-acting element analysis.

Phytohormone treatments

Hormone treatments were performed using the same method as our previous study (Liu et al., 2016b). Mature seeds of *Nipponbare* were soaked in distilled water for 2 days and pre-germinated for 2 days at 30°C without light. Germinated seeds were placed in a salver for incubation in a growth chamber at 28°C, 70% relative humidity and 12 h photoperiod. When the seedlings grown to three-leaf stage, they were sprinkled with different plant hormone solution and distilled water (control), respectively. The concentration of each hormone solution was 100 μ M. Sampling for RNA extraction was conducted at 0 h before treatment and 3 h, 6 h, 12 h, 24 h after treatment. The experiments were repeated twice.

Evaluation of disease resistance

We got T₀ transgenic plants by vector construction and rice transformation. T₁ and T₂ segregating progeny germinated from T₀ transgenic seeds were grown in soil in greenhouse. *M. oryzae* isolate GD08-T13 inoculum was used for blast resistance evaluation and was prepared as described by Beltenev et al. (2007). For leaf blast inoculation, leaves of transgenic plants and the empty-vector control (PHQSN) plants at the tillering stage were inoculated with GD08-T13 using the punch method as described by Ding et al. (2012). The inoculated plants were sprayed with water for 2-3 min every 2 h to maintain the humidity. Disease was assessed 10 days after inoculation by measuring the lesion size of the leaves. For panicle blast inoculation, cotton-wrapping inoculation method was used as described by Liu et al. (2016b). Briefly, we wrapped the upper-middle part of a panicle by cotton in 1-2 days after heading, and injected 2 ml spore suspension of 1×10^6 spores/ml of GD08-T13 into the cotton and then wrapped the cotton with foil. Each inoculated panicle was sprayed with water for 2-3 min every 2 h to maintain the humidity. Disease was assessed at 3 weeks after inoculation by calculating the percentage of infected main axis length (infected main axis length/main axis length of the inoculated panicle). Each treatment was repeated twice.

The 3, 3'-diaminobenzidine (DAB) staining and measurement of H₂O₂

The DAB staining of H₂O₂ was conducted according to the previously reported method (Thordal-Christensen et al., 1997; Kim and Hwang, 2014). DAB was dissolved by sterile ddH₂O and reduced PH to 3.8 with HCl to get the 1 mg/ml DAB staining solution. Similar leaves of the transgenic and PHQSN plants were selected and immersed into the DAB staining solution immediately. After 3 h incubation in a growth chamber at 28°C with relative humidity of 60% and light intensity of 50 $\mu\text{mol m}^{-2}\text{s}^{-1}$, the leaves were decolorized in a water-bath at 80-90°C for 1 h. Then, the decolorized leaves were photographed.

The measurement of H₂O₂ was conducted using the hydrogen peroxide assay kit (Beyotime Institute of Biotechnology, China) as described in our previous study (Liu et al., 2016). The similar panicles of the transgenic and PHQSN plants were collected and grinded with liquid nitrogen. The 200 mL lysis buffer solution was added to the 10 mg panicle dry powder and blended fully. The supernatant was collected by centrifuging at 12,000g for 5 min. Then, the 50 μL of the supernatants and 100 μL of test solutions were transferred to the test-tubes immediately and the mixed solution was kept for 30 min at room temperature. H₂O₂ concentration was monitored by measuring the absorbance at 560 nm using a Thermo Scientific Multiskan Spectrum (Thermo, USA).

Abbreviations

OXO: Oxalate oxidases; PR: pathogenesis-related; JA: jasmonic acid; ABA: abscisic acid; ET: ethylene ; SA: salicylic acid; GUS: β -glucuronidase; QTL: Quantitative trait loci; ICS1: *Isochorismate synthase 1*; NH1: *Nonexpressor of PR1*; LOX2: *Lipoxygenase 2*; AOS2: *Allene oxide synthase 2*; LEA3: *Late embryogenesis*

abundant protein gene 3; NCED3/4: 9-cis-epoxycarotenoid dioxygenase 3/4; Rab16A: ABA-responsive rice gene.

Declarations

Ethics approval and consent to participate

No applicable.

Consent for publication

No applicable.

Availability of data and material

The datasets supporting the conclusions of this article are provided within the article and its additional files.

Competing interests

The authors declare that they have no competing interests.

Funding

This work was jointly supported by grants from the National Key Research and Development Project (2018YFD0200302), the Innovation Team Project of Guangdong Modern Agricultural Industrial System (2019KJ106, 2020KJ106), the Natural Science Foundation of Guangdong Province (2018A0303130310), and the Special fund for scientific innovation strategy-construction of high level Academy of Agriculture Science (R2020PY-JX001).

Authors' contribution

JF D and L Z conducted the experiments, performed data analysis and wrote the manuscript. AQ F and XY Z conducted the disease resistance analysis. SH Z, H F, L C, JL Z, TF Y, W Y, YM M, and J W participated in material development, sample preparation and data analysis. Q L and B L conceived and designed the experiment, drafted proposals and corrected the manuscript. All authors read and approved the final manuscript.

Acknowledgments

We thank Professor Jan Leach in Colorado State University and Professor Hei Leung at International Rice Research Institute for their very helpful discussions and suggestions during this study.

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Additional Files

Additional file 1: Figure S1 Sequence alignments of *OsOXO2*, *OsOXO3* and *OsOXO4* between Zhonghua 11 and the blast-resistant line BC10. The red bases indicate the changed bases of the *OXO* genes in BC10 compared to Zhonghua 11 .

Additional file 2: Table S1. Primers used for vector construction and real-time PCR analysis. **Additional file 3: Table S2.** Microarray data of *OXO* genes after panicle blast inoculation. The value is \log_2 ratio;

Additional file 4: Table S3. The diseased leaf area of control (PHQSN) and *OXO* overexpressing plants after inoculation;

Additional file 5: Table S4. The diseased leaf area of control (PHQSN) and *OXO* silencing plants after inoculation.

Additional file 6: Table S5. The cis-elements identified in the promoters (1500bp upstream from the transcriptional starting site) of *OXO* genes;

Figures

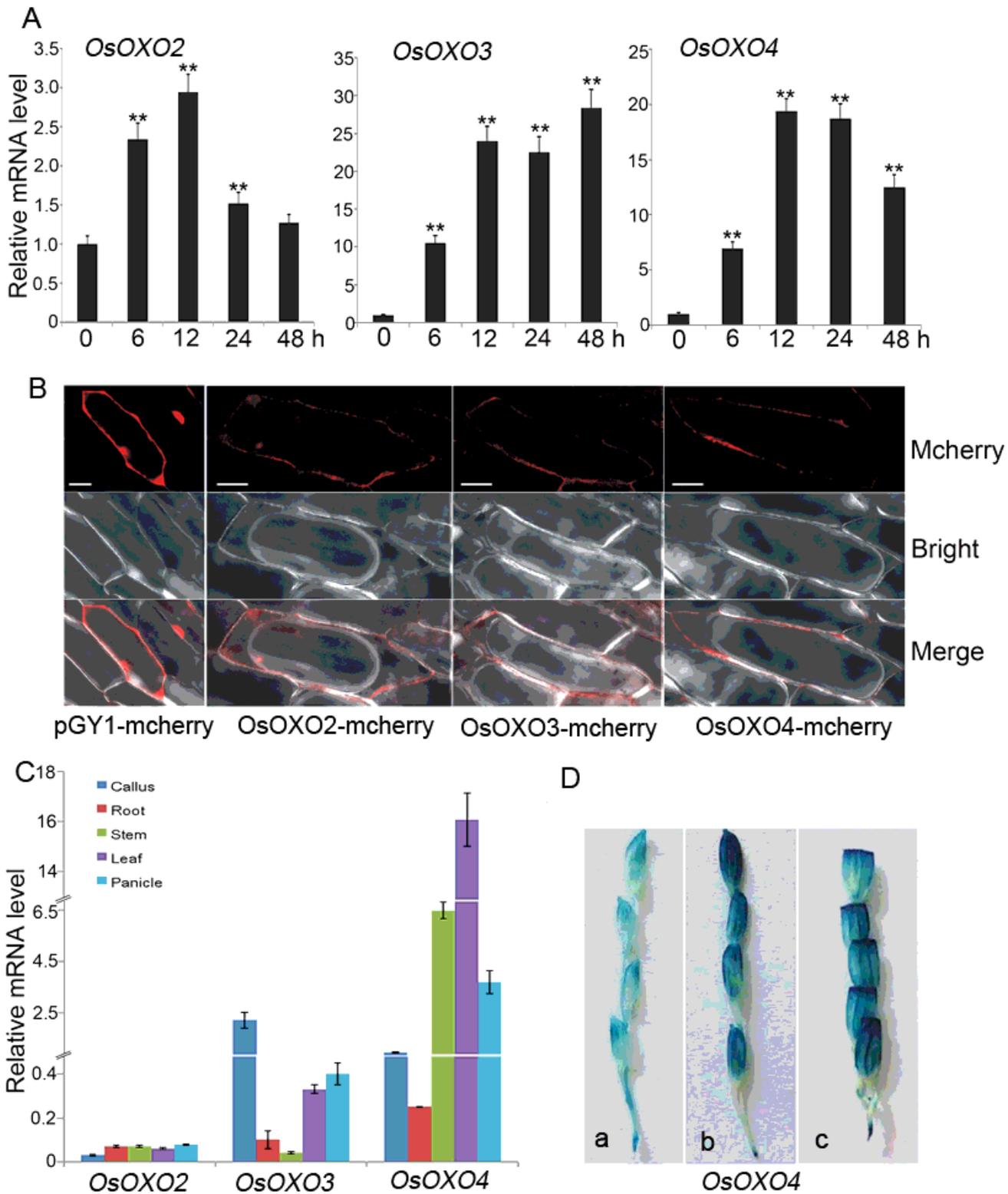


Figure 1

The temporal and spatial expression patterns of OsOXO2, OsOXO3 and OsOXO4 and their subcellular localizations. A. The expression of OsOXO2, OsOXO3 and OsOXO4 during panicle blast infection as assessed by quantitative RT-PCR at 0 h before inoculation and 6 h, 12 h, 24 h, 48 h after inoculation. Error bars indicate the standard deviation (SD) from three biological replicates and ** indicates a statistically significant difference compared with 0 h treatment (t test, $P < 0.01$). B. Sub-cellular localization of

OsOXO2, OsOXO3 and OsOXO4. The bar of pGY1-mcherry was 2 μ m and the others were 4 μ m. C. Expression analysis of OsOXO2, OsOXO3 and OsOXO4 in different rice tissues by quantitative RT-PCR. Error bars indicate the standard deviation (SD) from three biological replicates. D. GUS activity of OsOXO4 in the panicles of Nipponbare. a, panicle at the early booting stage; b, panicle at the booting stage; c, panicle at the initial heading stage.

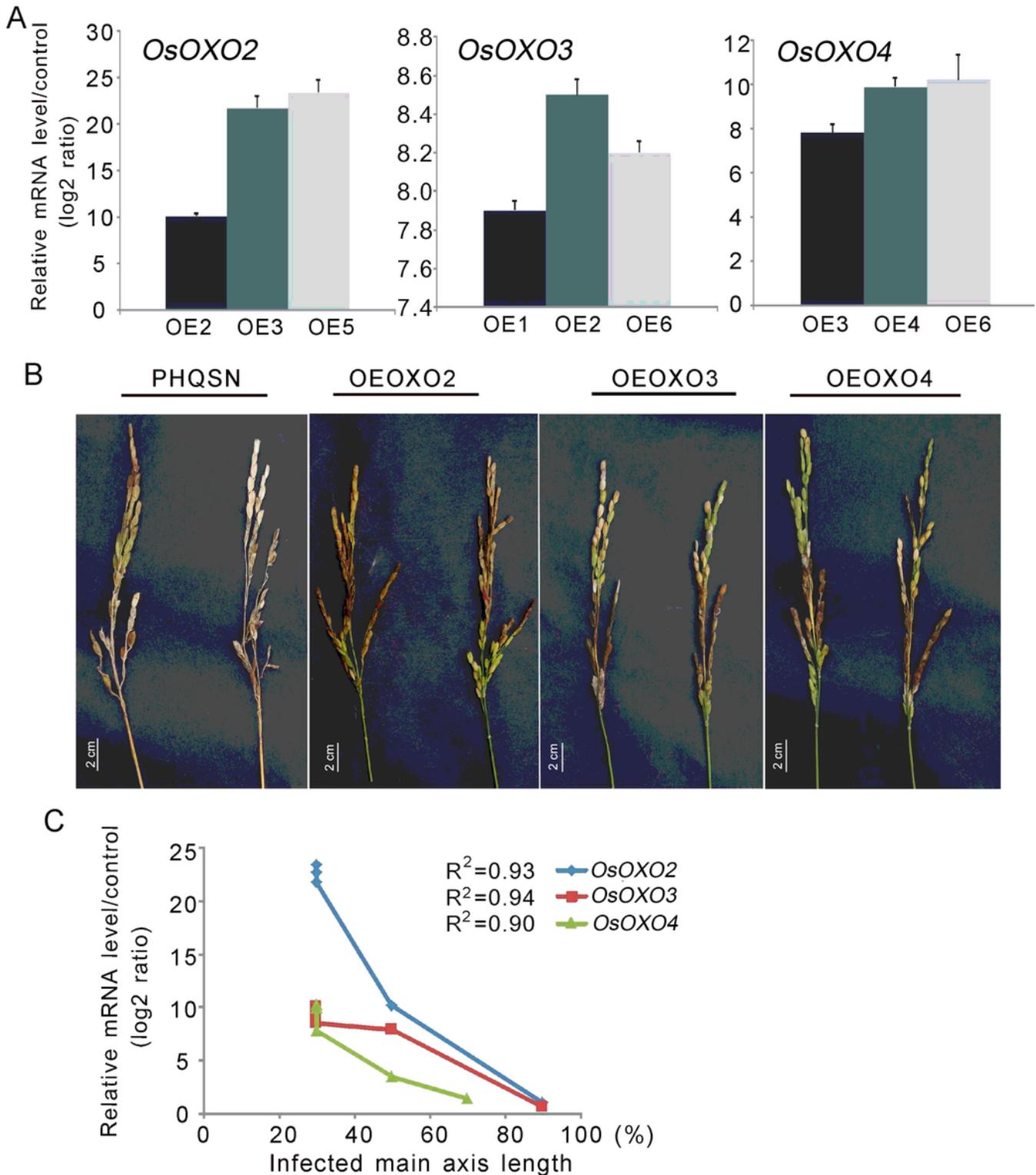


Figure 2

The disease phenotypes of the OXO gene overexpressing plants before and during panicle blast infection. OE0X02 indicates the plants overexpressing OsOXO2, OE0X03 indicates the plants overexpressing OsOXO3 and OE0X04 indicates the plants overexpressing OsOXO4. A. Transcription analysis of OsOXO2, OsOXO3 and OsOXO4 in their corresponding overexpressing plants. Error bars indicate the SD from three biological replicates. B. Disease phenotypes of OE0X02, OE0X03, OE0X04, and the empty vector control (PHQSN) plants at the heading stage after inoculation with *M. oryzae* using the cotton-wrapping method. C. Correlation analysis between enhanced resistance and the expression levels of OsOXO2, OsOXO3 and OsOXO4 in the overexpressing plants after panicle blast inoculation. The correlation coefficient is calculated by linear regression.

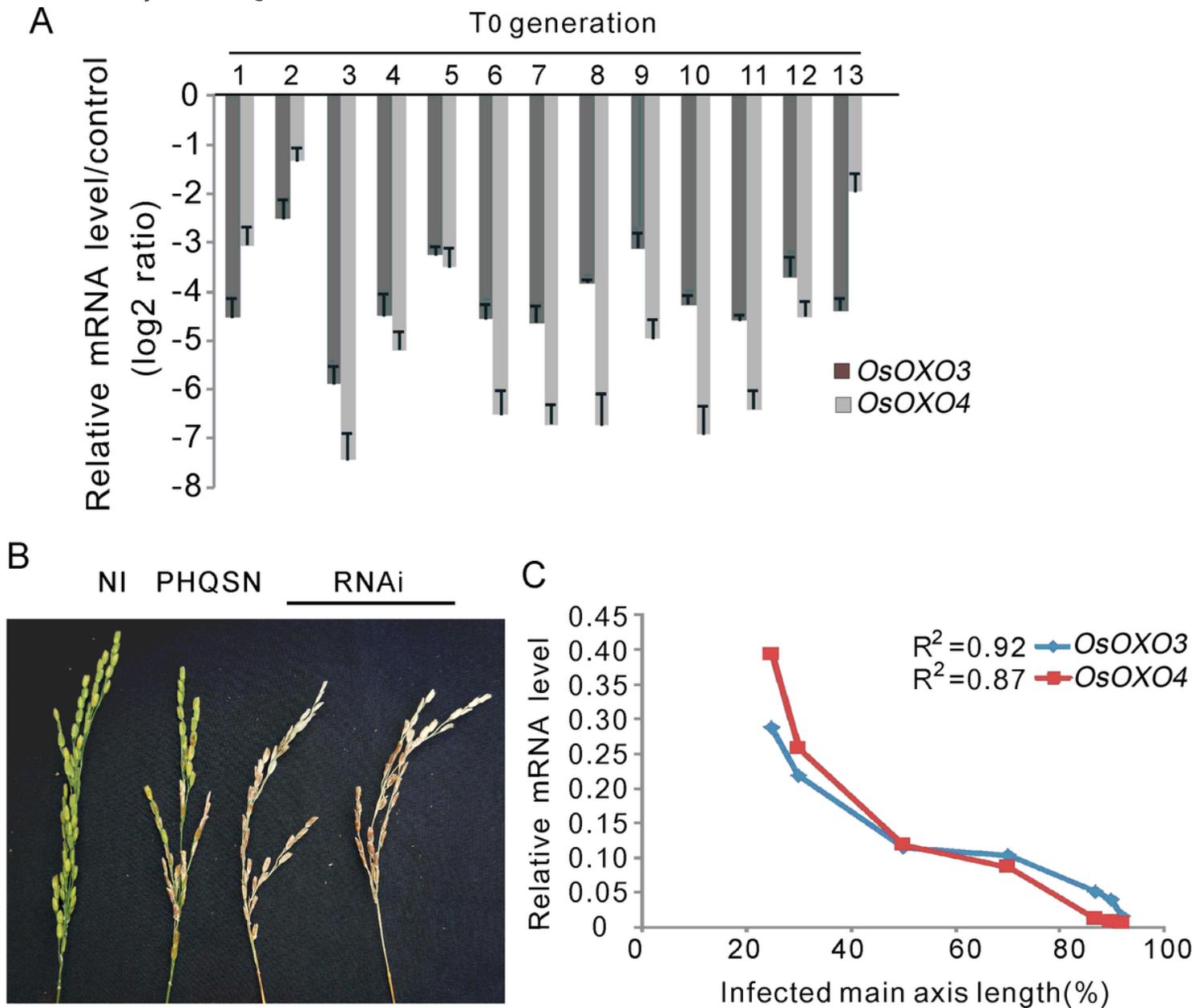


Figure 3

The disease phenotypes of OXO silencing (RNAi) plants to panicle blast infection. A. Transcription analysis of OsOXO3 and OsOXO4 in the RNAi plants by quantitative RT-PCR. B. Disease phenotypes of

the RNAi and control (PHQSN) plants at the heading stage after inoculation with *M. oryzae* using the cotton-wrapping method. NI indicates the non-inoculation panicle. C. Correlation analysis between decreased resistance and the expression levels of OsOXO3 and OsOXO4 in the RNAi plants after panicle blast inoculation. The correlation coefficient is calculated by linear regression.

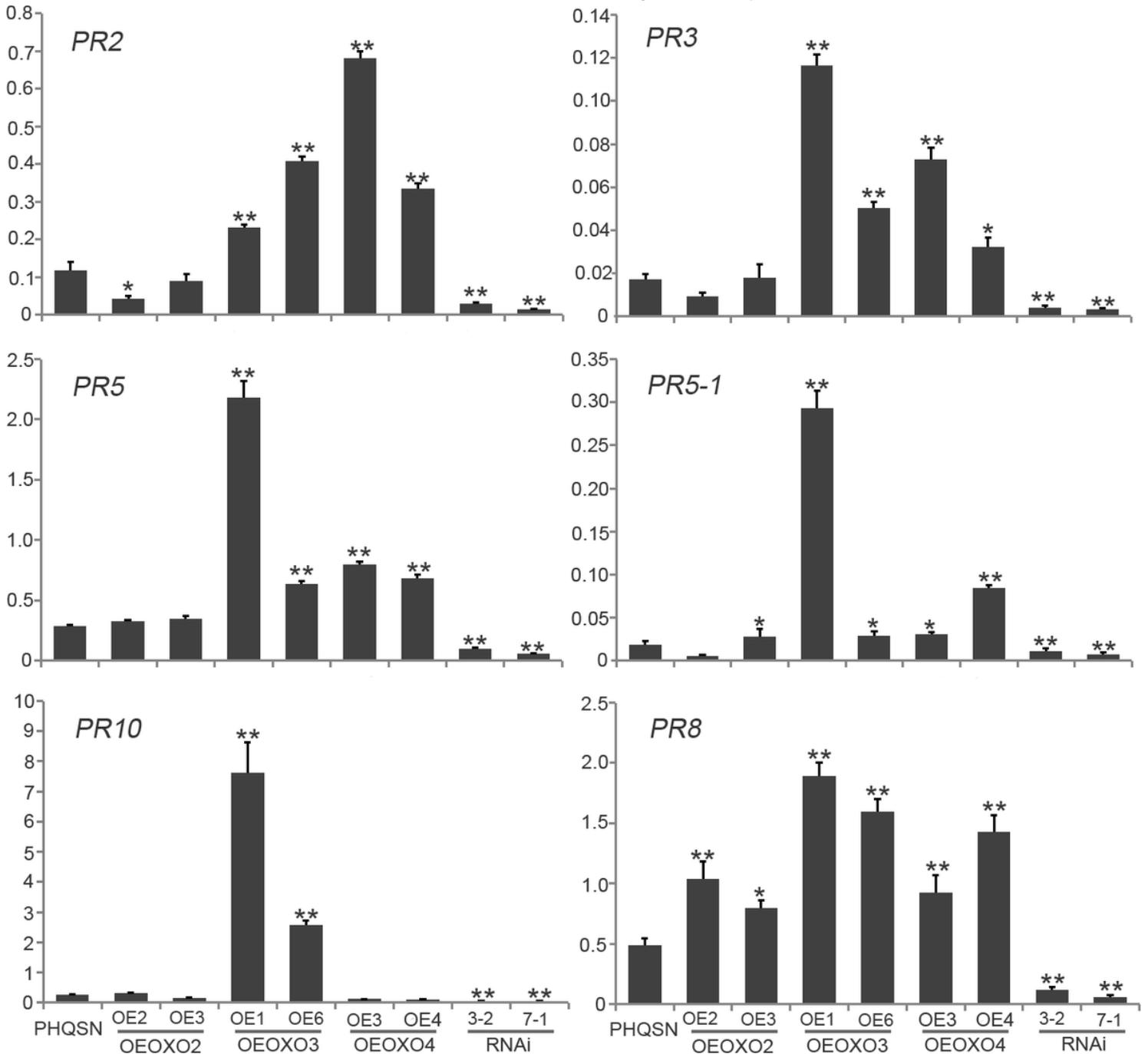


Figure 4

Expression analysis of pathogenesis-related (PR) genes in the OXO transgenic plants and control plants by quantitative RT-PCR. Error bars indicate the SD from three biological replicates and asterisks indicate statistically significant differences compared to the control plants (t test, **P < 0.01; *P < 0.05).

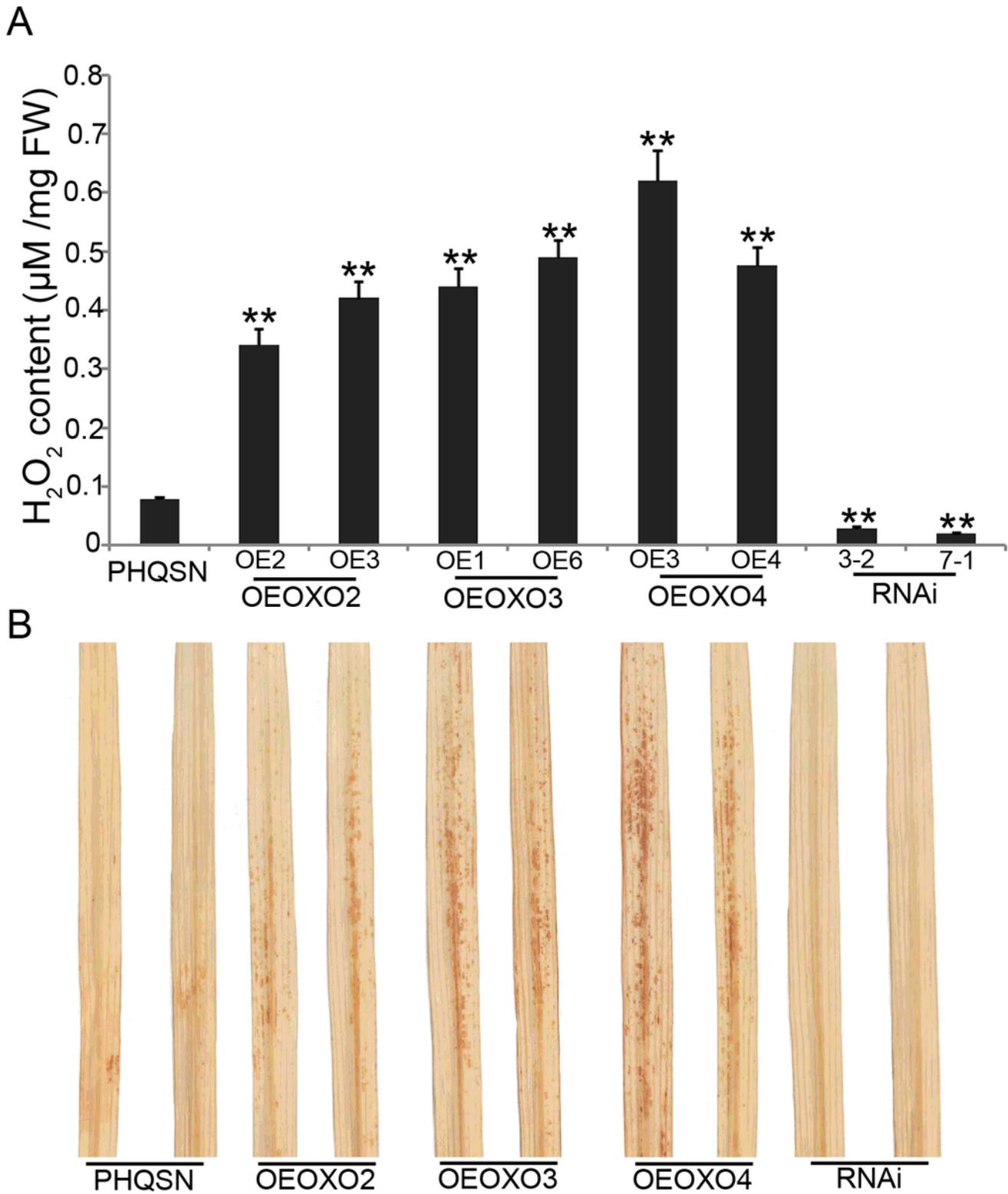


Figure 5

Hydrogen peroxide (H₂O₂) contents in the OXO transgenic plants and control plants. A. H₂O₂ contents in the panicles of PHQSN, OEEXO2, OEEXO3 OEEXO4 and RNAi plants. B. DAB staining of the leaves for PHQSN, OEEXO2, OEEXO3 OEEXO4 and RNAi plants. Error bars indicate the SD of at least ten biological replicates and ** indicates significant differences between the transgenic and control plants (t test, P < 0.01). FW means fresh weight.

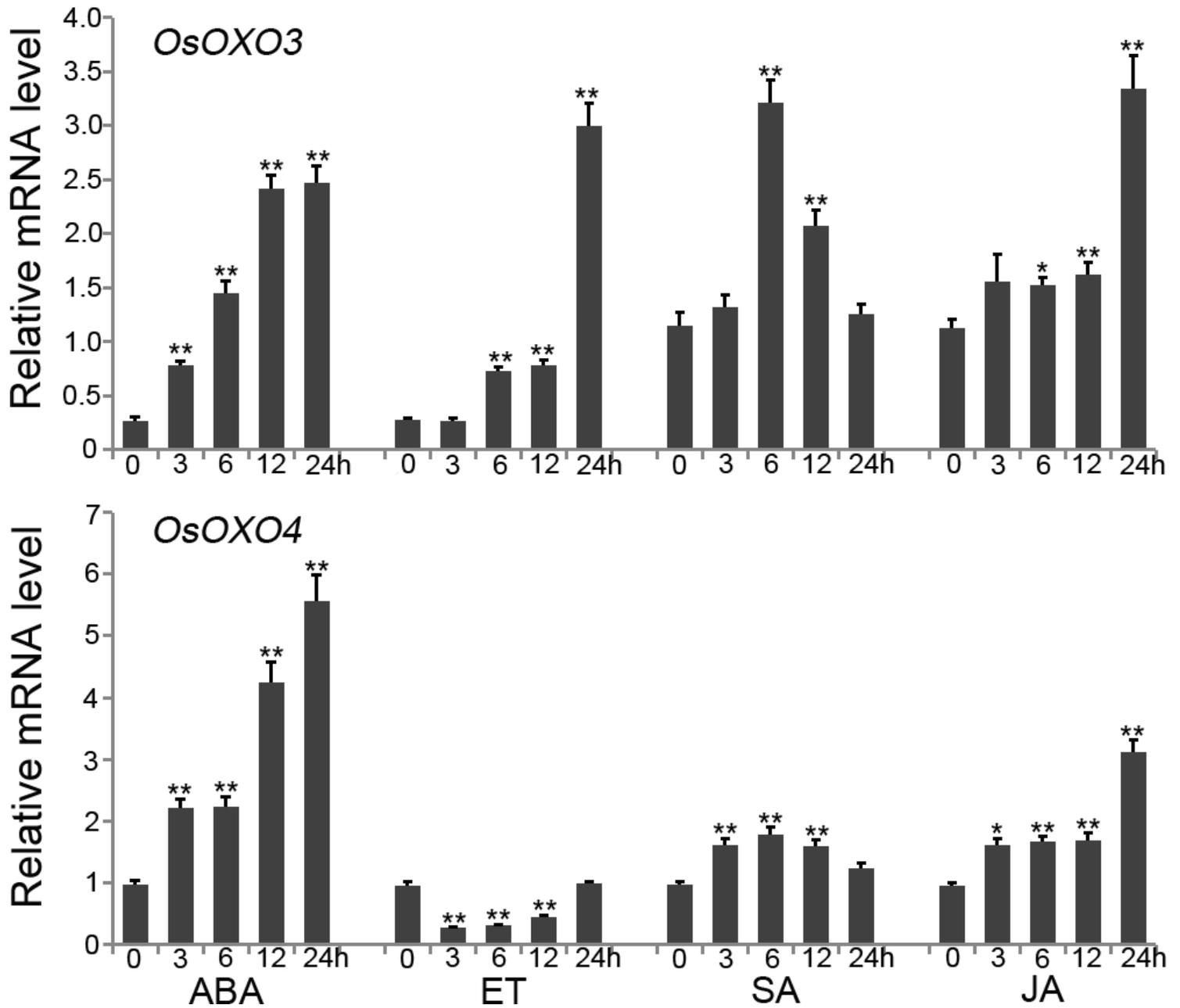


Figure 6

Time-course transcription analysis of OsOXO3 and OsOXO4 after abscisic acid (ABA), ethylene (ET), salicylic acid (SA), jasmonic acid (JA) treatments by quantitative RT-PCR. Error bars indicate the SD from three biological replicates and asterisks indicate statistically significant differences compared with water treatment (t test, **P < 0.01 and *P < 0.05).

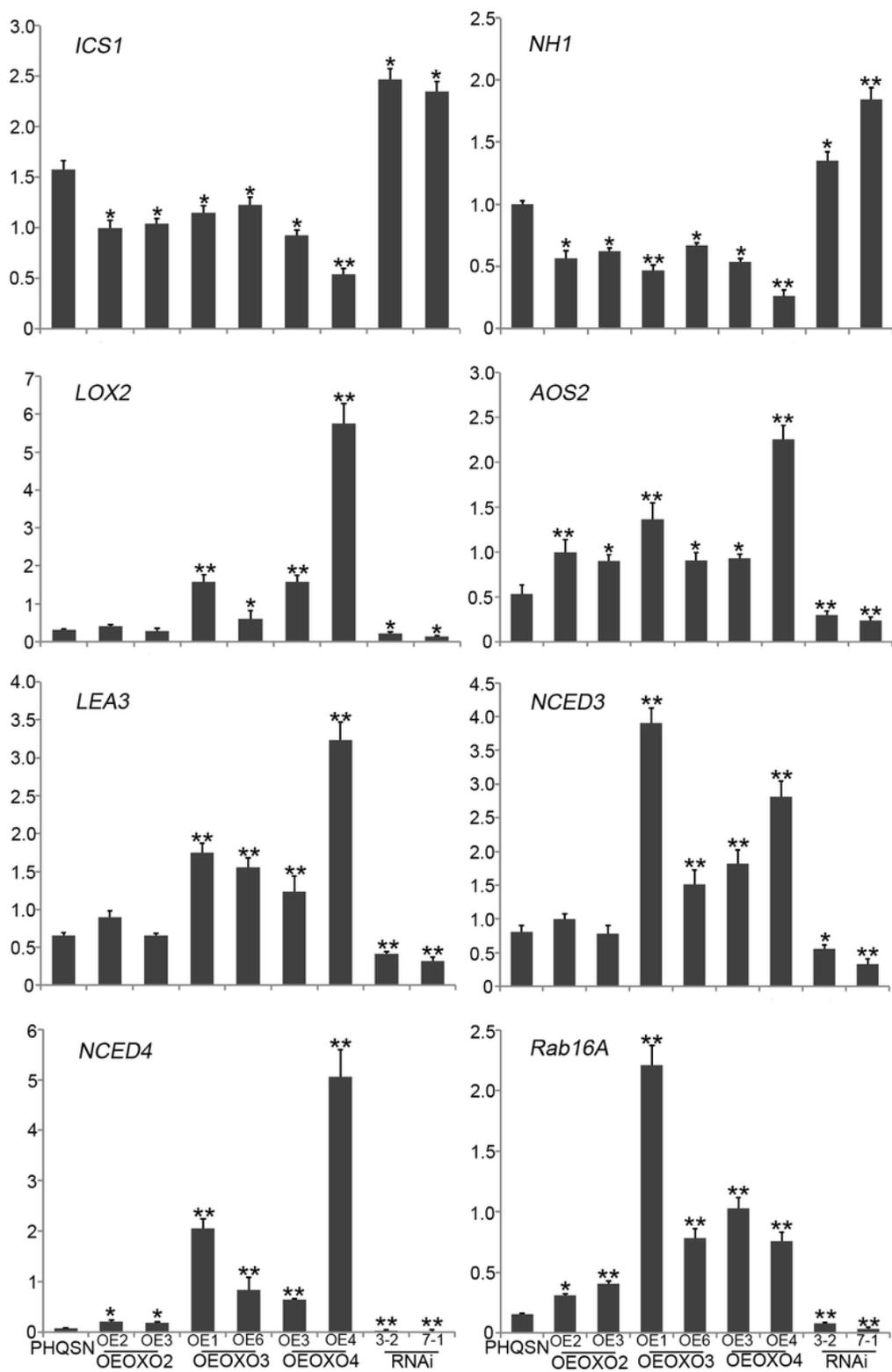


Figure 7

The expression of plant hormone related genes in the OXO transgenic plants and control plants by quantitative RT-PCR. Error bars indicate the SD from three biological replicates and asterisks indicate statistically significant differences compared to the control plants (t test, **P < 0.01 and *P < 0.05).

Supplementary Files

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