AGC Kinase OsOxi1 Positively Regulates Basal Resistance through Suppression of OsPti1a-Mediated Negative Regulation

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OsPti1a, a functional ortholog of tomato SlPti1, negatively regulates both basal resistance and R-gene-mediated resistance in rice. To investigate the molecular function of OsPti1a in defense responses, we searched for components interacting with OsPti1a using a yeast two-hybrid system. One of the interacting proteins is a Ser/Thr kinase that directly phosphorylates OsPti1a in vitro. This protein belongs to the AGC kinase family and is highly similar to AtOxi1, which is induced in response to a wide range of reactive oxygen species (ROS)-generating stimuli in Arabidopsis. Thus, it was designated OsOxi1. OsOxi1 was transiently phosphorylated in response to ROS and chitin elicitor. Both OsOxi1-overexpressing transgenic lines and the ospti1a mutant were highly sensitive to ROS treatment, indicating that OsOxi1 and OsPti1a are involved in ROS-mediated signaling in opposing ways. OsOxi1 is specifically expressed at infection sites where ROS are produced after inoculation with a blast fungus, Magnaporthe oryzae. Overexpression of OsOxi1 enhanced basal resistance to the blast fungus, indicating that OsOxi1 positively regulates disease resistance. OsOxi1 phosphorylates Thr-233 of OsPti1a and a point mutation of Thr-233 enhanced disease susceptibility to a bacterial pathogen Xanthomonas oryzae pv. oryzae (Xoo), suggesting that the phosphorylation of OsPti1a by OsOxi1 is essential for basal resistance to Xoo. Taken together, our data suggest that OsOxi1 positively regulates defense responses through the phosphorylation of OsPti1a, causing the release from an OsPti1a-dependent inhibition of the responses.

Keywords: AGC kinase • Phosphorylation • Rice • Basal resistance • Pto-interacting protein • ROS.

Abbreviations: BAP, Bacterial Alkaline phosphatase; DAB, 3,3′-diaminobenzidine tetrahydrochloride; DTT, dithiothreitol; ETI, effector-triggered immunity; GST, glutathione S-transferase; GUS, β-glucuronidase; HR, hypersensitive responses; HRP, horseradish peroxidase; MAP kinase, mitogen-activated protein kinase; NB, Nipponbare; Oxi1, oxidative signal inducible 1; PAMP, pathogen-associated molecular pattern; PIF, Pdk1-interacting fragment; PRR, pattern recognition receptor; PR, pathogenesis-related; PTI, PAMP-triggered immunity; Pti1, Pto-interacting protein 1; RBOH, respiratory burst oxidase homolog; ROS, reactive oxygen species; RT–PCR, reverse transcription–PCR; WT, wild type; Xoo, Xanthomonas oryzae pv. oryzae.

Nucleotide sequences OsOxi1 (AK067266), OsPti1a (AK104870), OsPti1b (AK066231), SlPti1 (U28007), OsPR1b (U89895), OsActin1 (AK100267) and AtOxi1 (At3g25250) have been submitted to the GenBank/EMBL data libraries.

Introduction

Plants have evolved two types of defense systems against pathogens. Recognition of pathogen-associated molecular patterns (PAMPs) by pattern recognition receptors (PRRs) triggers a basal resistance including callose deposition (Kim et al. 2005), the production of reactive oxygen species (ROS) (Nurnberger et al. 2004, Asai et al. 2008), and the induction of pathogenesis-related (PR) genes (Zippel 2008). These plant innate immune responses are collectively referred to as PAMP-triggered immunity (PTI). The second type of defense is based on plant resistance (R) gene products that recognize effector proteins that promote pathogen virulence, and is called effector-triggered immunity (ETI) (Jones and Dangl 2006). ETI is often accompanied by rapid ion fluxes, ROS accumulation, expression of PR genes and programmed cell death at the infection site, collectively known as hypersensitive responses (HR; Torres et al. 2006). Thus, plant immune responses are complex and consist of the overlapping outputs of PTI and ETI (Tao et al. 2003, Navarro et al. 2004, Tsuda et al. 2008). Recently, the rice resistance gene Xa21 was reported to function as a pattern recognition receptor to recognize a PAMP, Xa21 (activator of Xa21-mediated immunity) of Xanthomonas oryzae pv. oryzae (Xoo).
(Lee et al. 2009). This report suggested that PAMPs and avirulence genes on one hand and PRRs and R genes on the other cannot be strictly separated based on currently accepted definitions. Rather, PTI and ETI appear to share signaling components for their activation. However, the molecular mechanisms are still obscure.

In an effort to isolate rice mutants in which shared components of PTI and ETI are affected, we have isolated the ospti1a mutant from rice insertional mutant lines induced by an endogenous retrotransposon Tos17 (Takahashi et al. 2007). The ospti1a null mutant shows a lesion-mimic phenotype. Accompanying lesion formation, the mutant exhibits a series of defense responses, such as the expression of defense genes, accumulation of phytoalexin and enhanced disease resistance to a compatible race of rice blast fungus. Furthermore, lines overexpressing OsPti1a show enhanced disease susceptibility to an incompatible race of rice blast and a compatible race of Xoo. These observations indicate that OsPti1a negatively regulates PTI as well as ETI in rice (Takahashi et al. 2007). However, little is known about the regulatory mechanisms of disease resistance mediated by OsPti1a.

Tomato Pti1 (SlPti1) complements the ospti1a mutant phenotype, demonstrating that the two Pti1 proteins, SlPti1 and OsPti1a, are functionally equivalent (Takahashi et al. 2007). SlPti1 was originally identified as a component acting downstream of the tomato R-gene, Pto (Zhou et al. 1995). Pto encodes a Ser/Thr kinase and confers resistance to bacterial speck disease (Martin et al. 1993). SlPti1 is a secondary Ser/Thr kinase, and the Pto–Pti1 interaction was proposed to be an initial step toward elicitation of HR (Sessa et al. 2000). SlPti1 was suggested to positively regulate defense signaling downstream of Pto (Zhou et al. 1995, Sessa et al. 2000). However, pathways of SlPti1-mediated signaling are still obscure, because no loss-of-function analysis has been carried out in tomato.

Three Arabidopsis homologs, AtPti1-1, AtPti1-2 and AtPti1-3, have been isolated as interacting partners of AtOxi1 (oxidative signal inducible 1), which belongs to the AGC kinase VIIIb family (Anthony et al. 2006). Ten members of the AtPti1 gene family have been identified in the NCBI database by homology searches, and all share a highly conserved kinase domain. In Arabidopsis protoplasts, AtPti1-2 was shown to be activated in response to various stimuli, such as phosphatidic acid, xylanase, flg22 and H2O2. AtOxi1 is a downstream element of ROS signaling in Arabidopsis (Rentel et al. 2004), since ROS treatment induces its expression and the corresponding kinase activity. The oxi1 mutant shows enhanced susceptibility to the oomycete pathogen Hyaloperonospora arabidopsis. Additionally, AtOxi1 is required for full activation of the mitogen-activated protein kinases (MPKs) MPK3 and MPK6 after treatment with H2O2 or elicitor. Although an AtOxi1–AtPti1-2 interaction has been demonstrated by biochemical approaches (Anthony et al. 2006), the underlying biological function is poorly understood.

To identify OsPti1a-mediated defense signaling events that control both PTI and ETI, we searched for a potential interaction partner of OsPti1a. We showed that OsOxi1 directly phosphorylates OsPti1a in vitro. Moreover, OsOxi1 is regulated by ROS signaling and positively regulates disease resistance in rice. Additionally, we showed that residue Thr-233 of OsPti1a, a major phosphorylation target for OsOxi1, has an important role in the activation of PTI.

**Results**

**Isolation and characterization of an AGC kinase, OsOxi1, interacting with OsPti1a**

To investigate the molecular function of OsPti1a, we screened for proteins interacting with OsPti1a using a yeast two-hybrid system, and identified 70 candidates. We excluded pseudo-clones and confirmed interaction between the candidates and OsPti1a by MEL1 reporter assays and complementation of auxotrophy when bait and prey were exchanged. One of the candidates encodes a Ser/Thr protein kinase that shared extensive similarity with AtOxi1 (52% similarity at the amino acid level); we designated this protein OsOxi1 (Fig. 1A). AtOxi1 and OsOxi1 belong to the AGC kinase subfamily VIIIb (Fig. 1B). The OsOxi1 protein consists of 462 amino acid residues, and its deduced Mr was 49 kDa. A 90 amino acid insertion is found within the activation loop signature motif that is characteristic of AGC VIII subfamily kinases (Fig. 1A). We reported previously that SlPti1 as well as OsPt1b, a rice paralog of OsPti1a, complemented the ospt1a mutant phenotype, indicating that these Pti1 proteins, SlPti1, OsPti1a and OsPt1b, are functionally homologous in different plant species (Takahashi et al. 2007). Therefore, we examined whether SlPti1 and OsPt1b interact with OsOxi1 in a yeast two-hybrid assay. We found that OsOxi1 interacted not only with OsPti1a, but also with OsPt1b and SlPti1 (Fig. 1C).

An in vitro pull-down experiment confirmed that the interaction between OsPti1a and OsOxi1 was direct (Fig. 1D). Furthermore, we attempted to determine whether OsOxi1 interacts with OsPti1a in planta. Because the detection of OsOxi1–OsPti1a interaction in rice cultured cells was not successful, we used the Agrobacterium-mediated transient expression assay (Agroinfiltration) in Nicotiana benthamiana. HA-epitope-tagged OsPti1a co-immunoprecipitated with the FLAG-OsOxi1 protein (Fig. 1E), showing that OsOxi1 and OsPti1a interact in planta.

**OsPti1a is a target for phosphorylation by OsOxi1**

To determine whether OsOxi1 is a functional protein kinase, we applied an in vitro autophosphorylation assay and found that OsOxi1 showed autophosphorylation activity (Fig. 2A). The Lys-56 to Asn (K56N) mutation, located in the putative ATP-binding site of OsOxi1, abolished this activity (Fig. 2A). Ser-283 (S283) in OsOxi1 is the predicted Ser/Thr phosphate acceptor site of Pdk1 (3-phosphoinositide-dependent protein kinase 1), a kinase acting upstream of AGC kinases (Bogre et al. 2003, Devarenne et al. 2006). The S283 to Ala (S283A) mutation
Fig. 1 Comparison of OsOxi1 and OsOxi1-related proteins, and interaction with OsPti1. (A) Alignment of the predicted amino acid sequences of OsOxi1 and AtOxi1. The protein kinase domains are marked with orange lines, while the blue line highlights the AGC kinase C-terminal motif and the red line indicates the PIF (Pdk1-interacting fragment) motif. The gray arrow marks the position of the 90 amino acid insertion into the PK domain. Red arrowheads indicate Lys-56 in the ATP-binding domain and Ser-283, a phosphorylation site and predicted Pdk1 target. (B) Reconstruction of phylogenetic relationships based on the amino acid sequences of the kinase domain of OsOxi1 and AGC kinase VIIIb family members from several plant species. (C) OsOxi1 interacts with OsPti1a, OsPti1b and SIPti1 in a yeast two-hybrid assay. pBD-OsPti1a and pAD-GaLA-2.1 or pBD-OsPti1b and pAD-GaLA-2.1 or pBD-SIPti1 and pAD-GaLA-2.1 served as negative controls. (D) Pull-down assay for GST-fusion OsPti1a and His6-tagged OsOxi1. The pulled-down fractions were analyzed by SDS–PAGE followed by immunoblotting using anti-His6 and anti-GST antibodies. (E) Co-immunoprecipitation of FLAG-OsOxi1 with OsPti1a-3XHA in vivo. All constructs were agroinfiltrated into N. benthamiana in the combinations shown on top. The immunoprecipitation (IP) obtained with HA beads was subjected to anti-FLAG immunoblotting.
drastically decreased the autophosphorylation activity of OsOxi1. On the other hand, the S283 to Asp (S283D) mutation, which mimics the constitutively phosphorylated condition, greatly increased the autophosphorylation activity (Fig. 2A). This result suggests that OsOxi1 possesses another phosphorylation site that may be autophosphorylated by the activated OsOxi1. Thus, the phosphorylation of S283 is a key event for full OsOxi1 activation. Next, we investigated whether OsOxi1 interacts with OsPti1a as its substrate by conducting an in vitro cross-phosphorylation assay. When OsOxi1 was incubated with the inactive OsPti1aK96N protein, phosphorylation of OsPti1aK96N was observed (Fig. 2B); however, OsPti1a did not phosphorylate OsOxi1K56N as a substrate, suggesting that OsOxi1 functions upstream of OsPti1a.

OsOxi1 responds to ROS and chitin elicitor
AtOxi1 senses the signal of ROS, produced in response to various stimuli, and transmits the signal to downstream components such as MAP kinases (Rentel et al. 2004). To investigate whether OsOxi1 is involved in ROS signaling as an ortholog of AtOxi1, we performed RNA blot analysis of a rice cell culture line responding to H$_2$O$_2$. The expression of OsOxi1 was transiently, but significantly, induced at 1 h after H$_2$O$_2$ treatment (Fig. 3A). To analyze the post-transcriptional regulation of OsOxi1 by ROS signal, we constructed HA-streptII-tagged OsOxi1 and expressed it in wild-type (WT) ‘Nipponbare’ (NB) cells. We immunoprecipitated the OsOxi1 protein from H$_2$O$_2$-treated and control cells. The mobility of OsOxi1 protein in polyacrylamide gels was changed within 10 min after H$_2$O$_2$ treatment (Fig. 3B lower panel). For further evaluation of the phosphorylation state of OsOxi1 in vivo, we employed a new technique for separating phosphorylated isoforms, Phos-tag gels (Kinosita et al. 2006). The Phos-tag is a phosphate-binding compound that, when incorporated into polyacrylamide gels, can result in an exaggerated mobility shift for phosphorylated proteins (Kinosita et al. 2006). When OsOxi1 proteins were run on Phos-tag gels, a slowly migrating OsOxi1 band was observed 10 min after H$_2$O$_2$ treatment, and this band was eliminated by alkaline phosphatase treatment (Fig. 3B upper panel). These results indicate that OsOxi1 is phosphorylated in response to H$_2$O$_2$. We also showed that the phosphorylation of OsOxi1 was induced as early as 5 min after H$_2$O$_2$ treatment (Supplementary Fig. S1). Therefore, the ROS stimulus triggers transcriptional regulation and post-translational modification of OsOxi1. In rice cell culture, ROS are rapidly generated in response to chitin oligosaccharide (chitin elicitor), which is a common component of fungal cell walls (Yamaguchi et al. 2005). To investigate whether OsOxi1 responds to this PAMP, we analyzed the phosphorylation status of OsOxi1 following exposure to chitin elicitor in rice cultured cells. The Phos-tag assay showed that phosphorylation of OsOxi1 was induced 5 min after chitin elicitor treatment, and that OsOxi1 remained in the phosphorylated state for 20 min (Fig. 3C). This indicates that OsOxi1 is involved in PAMP signaling in rice. We concluded that OsOxi1 probably contributes to the activation of PTI in rice.

OsOxi1-overexpressing lines and the ospti1a mutant are highly sensitive to H$_2$O$_2$
We overexpressed full-length OsOxi1 and OsOxi1S283D under the control of the maize ubiquitin promoter in the NB background (lines called OsOxi1-OE and OsOxi1S283D-OE, respectively). The cultured cells were made from seeds of the T1 generation of the vector control, OsOxi1-OE and OsOxi1S283D-OE lines. First, we examined whether overexpression of OsOxi1 affects cell death induced by H$_2$O$_2$ treatment. In cultured cells of OsOxi1-OE and OsOxi1S283D-OE, cell death was strongly induced by H$_2$O$_2$ after 24 h compared with a vector-control line (Fig. 3D), indicating that sensitivity to H$_2$O$_2$ is enhanced in OsOxi1-OE and OsOxi1S283D-OE. The responses to H$_2$O$_2$ of OsOxi1-OE and OsOxi1S283D-OE cultured cells were similar, perhaps suggesting that the S283D mutation was insufficient for fully activating OsOxi1 in vivo. Intriguingly, H$_2$O$_2$-induced cell death was greatly increased in the ospti1a mutant compared with the WT. We confirmed that the expression of OsPti1a cDNA in the ospti1a mutant complemented the mutant phenotype (Supplementary Fig. S2), indicating that the high H$_2$O$_2$ sensitivity of the ospti1a mutant was caused by the lack of OsPti1a.
OsOxi1–OsPti1a interaction regulates rice defense

A

![Image 1](https://academic.oup.com/pcp/article-abstract/51/10/1731/1829570)

B

![Image 2](https://academic.oup.com/pcp/article-abstract/51/10/1731/1829570)

C

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D

![Image 4](https://academic.oup.com/pcp/article-abstract/51/10/1731/1829570)

**Fig. 3** OsOxi1 responds to H$_2$O$_2$ and chitin elicitor, and OsOxi1-overexpression lines and the ospti1a mutant are sensitive to H$_2$O$_2$

To analyze OsOxi1 expression patterns in response to pathogen attack, incompatible and compatible races of the blast fungus were inoculated on leaves of OsOxi1$_{pro}$:GUS plants. GUS activity was unambiguous adjacent to the HR at 7 d post-inoculation (dpi) with the incompatible race of rice blast fungus (Fig. 4G).

**OsOxi1 expression is tightly regulated and localized to the area of the HR and disease symptoms**

To learn more about the regulation of OsOxi1 in defense responses, we analyzed the expression pattern of OsOxi1 using RT-PCR (Supplementary Fig. S3A). These transgenic lines exhibited a phenotype identical to the ospti1a mutant phenotypes (Supplementary Fig. S3B), suggesting that OsOxi1 functions either upstream of OsPti1a or in an independent pathway.

These data suggest that OsOxi1 and OsPti1a regulate ROS signaling in opposite ways.

For epistatic analysis, we introduced a full-length cDNA of OsAtOxi1 under the control of the maize ubiquitin promoter into a homozygous ospti1a mutant. We obtained three independent transgenic lines and confirmed gene expression of OsOxi1 using RT–PCR (Supplementary Fig. S3A). These transgenic lines exhibited a phenotype identical to the ospti1a mutant phenotypes (Supplementary Fig. S3B), suggesting that OsOxi1 functions either upstream of OsPti1a or in an independent pathway.
leaves regardless of the fungal race applied, probably suggesting that OsPti1a exerts a constitutive negative control of the defense responses (Fig. 4G). These results suggest that the expression of OsOxi1 was induced by the accumulation of H$_2$O$_2$, that results from pathogen infection, and that the expression of OsOxi1 regulates OsPti1a-mediated signaling in the area of pathogen attack.

**OsOxi1 positively regulates disease resistance**

In light of the results presented so far, we hypothesized that OsOxi1 regulates disease resistance upstream of OsPti1a. To test this hypothesis, OsOxi1-OE and OsOxi1$^{S283D}$-OE plants were inoculated with the compatible race of rice blast fungus. OsOxi1-OE and OsOxi1$^{S283D}$-OE plants showed a decreased number of susceptible-type lesions compared with NB and vector-control plants at 7 dpi (Fig. 5A). Transgene expression in OsOxi1-OE and OsOxi1$^{S283D}$-OE in the T1 generation was confirmed using reverse transcription (RT)–PCR (Fig. 5B). To evaluate disease severity in these transgenic plants, we compared total lesion area per leaf (Fig. 5C). Lesion areas in OsOxi1-OE and OsOxi1$^{S283D}$-OE plants were significantly smaller than in WT and vector-control plants. These results indicate that OsOxi1 positively regulates basal resistance to rice blast fungus. To investigate whether OsOxi1-OE and OsOxi1$^{S283D}$-OE plants also possess enhanced disease resistance to other compatible pathogens, we inoculated them with the compatible race of the bacterial blight pathogen, *Xoo* (Fig. 5D). Transgene expression in OsOxi1-OE and OsOxi1$^{S283D}$-OE individuals of the T1 generation was confirmed using RT–PCR (Fig. 5E). Significant differences in resistance to *Xoo* between NB and OsOxi1-OE plants were not detected, probably due to the strong virulence of *Xoo*. On the other hand, OsOxi1$^{S283D}$-OE plants showed decreased *Xoo*-induced lesion sizes compared with NB and the vector control (Fig. 5F). Collectively, our data support the notion that OsOxi1 is involved in the regulation of basal resistance in rice.

**OsOxi1 phosphorylates residue Thr-233 of OsPti1a**

Thr-233 (T233) of SlPti1 is required for its physical interaction with Pto and is phosphorylated by Pto in tomato (Sessa et al. 2000). On the other hand, Lys-96 (K96) of OsPti1a is part of the ATP-binding site and is required for autophosphorylation (Takahashi et al. 2007). To examine whether T233 of OsPti1a is required for the interaction with OsOxi1, we replaced T233 with alanine (T233A) by site-directed mutagenesis with or without additional mutation in the ATP-binding site (K96N) and performed a yeast two-hybrid assay using constructs containing these mutations (OsPti1a$^{K96N}$, OsPti1a$^{T233A}$ and OsPti1a$^{K96N/T233A}$). OsOxi1 interacted with all of these OsPti1a mutants (Fig. 6A), indicating that T233 in OsPti1a is not essential for the interaction with OsOxi1. Additionally, an in vitro phosphorylation assay showed that the K96N and T233A mutants abolished the autophosphorylation activity of OsPti1a (Fig. 6B). Next, we performed a cross-phosphorylation assay between the OsPti1a mutant proteins and the OsOxi1 protein.
OsOxi1–OsPti1a interaction regulates rice defense

OsOxi1 WT and OsOxi1K96N were strongly phosphorylated by OsOxi1, whereas the phosphorylation of OsPti1aT233A and OsPti1aK96N/T233A were markedly decreased (Fig. 6C), indicating that T233 of OsPti1a is a major phosphorylation site for OsOxi1.

To investigate the effect of OsPti1a phosphorylation by OsOxi1, we constructed vectors carrying the OsPti1aK96N cDNA under the control of the OsActin1 promoter (Act pro :OsPti1aK96N) or OsPti1aT233A cDNA and OsPti1aK96N/T233A cDNA under the control of the cauliflower mosaic virus 35S promoter (35S:OsPti1aT233A and 35S:OsPti1aK96N/T233A). These constructs were introduced into the homozygous ospitila mutant. We obtained four to six independent transgenic plants and used their T1 generations for the following analyses. First, we checked lesion formation and the disease resistance of these transgenic plants. Act pro :OsPti1aK96N transgenic plants did not show a lesion-mimic phenotype and were as healthy as the WT (Fig. 6D). 35S:OsPti1aT233A and 35S:OsPti1aK96N/T233A transgenic plants showed mild dwarf phenotypes as compared with 35S:OsPti1a WT transgenic plants. Interestingly, the spontaneous lesion formation due to the ospitila mutation was abolished in all three lines (Fig. 6D). In these lines, OsPti1a accumulation was confirmed by protein blot analysis using an OsPti1a antibody (Fig. 6E). Consistent with the suppression of lesion formation, RNA blot analysis revealed that the expression of PR1a was reduced in the Act pro :OsPti1aK96N, 35S:OsPti1aT233A and 35S:OsPti1aK96N/T233A lines (Fig. 6F). Act pro :OsPti1aK96N transgenic plants had a completely complemented phenotype, although these plants showed lower levels of OsPti1a expression than the WT. We conclude that the presence of OsPti1a is sufficient for the suppression of lesion formation and disease resistance regardless of its phosphorylation state.

Phosphorylation of residue Thr-233 of OsPti1a is required for disease resistance

To determine the functional significance of the phosphorylation state of OsPti1a T233 in defense responses, we performed an inoculation test with the compatible race of rice blast fungus, whereas the WT and the vector control developed disease symptoms on the leaves (Takahashi et al. 2007; Fig. 7A). The three transgenic lines exhibited disease symptoms to a similar degree to the OsPti1a WT-complemented line and there was no observable enhancement of susceptibility (Fig. 7A, B). Protein blot analysis confirmed OsPti1a accumulation in these lines (Fig. 7C). These results demonstrate that accumulation of all OsPti1a mutant proteins complemented OsActin gene as a control. (F) Lesion length in plants of the T1 generation of OsOxi1–OE, OsOxi1T233D–OE, vector control and WT. Results shown are means ± SE from three independent experiments (n > 12). The asterisk indicates significant difference from the WT and vector control (Tukey–Kramer test, P < 0.05).

Fig. 5 OsOxi1–OE and OsOxi1T233D–OE plants exhibit enhanced disease resistance to compatible pathogens. (A) Three-week-old transgenic plants carrying pUbciOsOxi1, pUBciOsOxi1T233D or an empty vector were inoculated with a compatible race of rice blast fungus by spraying with an aqueous spore suspension (6.5 x 10^6 spores ml^-1). The leaves were photographed at 7 dpi. (B) Gene expression in OsOxi1–OE and OsOxi1T233D–OE plants. RT–PCR analysis was performed on total RNA isolated from T1 progeny of the transgenic lines. RT–PCR of the actin gene was used as a control. (C) Degree of disease symptoms (lesion sizes) in plants of the T1 generation of OsOxi1–OE, OsOxi1T233D–OE, vector control and WT 7 d after inoculation. L4, L17, L3 and L8 represent independent transgenic lines. Results from three independent experiments are presented as means ± SD (n = 15). (D) Representative leaves showing the extent of lesion development 21 d after inoculation with Xoo (race 1) in leaves of OsOxi1–OE, OsOxi1T233D–OE, vector control and WT. (E) Gene expression in OsOxi1–OE and OsOxi1T233D–OE plants. RT–PCR analysis was performed on total RNA isolated from T1 progeny of the transgenic lines. RT–PCR of the
Fig. 6 Functioning of mutant OsPti1a protein in the ospti1a background. (A) OsOxi1 interacts with OsPti1aWT, OsPti1aK96N, OsPti1aT233A and OsPti1aK96N/T233A in a yeast two-hybrid assay. Yeast growth on the SM−Leu, −Trp and −His medium. Yeast strain AH109 was co-transformed with the indicated pBD and pAD plasmid constructs. The positive control was pBD-WT and pAD-WT, and the negative control was pBD-GAL4-cam and pAD-GAL4-2.1. In (B) and (C), the top panel represents the kinase assay (phosphorimage) and the bottom panel is the assay input (Coomassie-stained gel). (B) Effects of mutations of the Lys-96 and Thr-233 residues in OsPti1a on autophosphorylation activity. (C) Analysis of cross-phosphorylation assays between OsOxi1 protein and mutated Pti1a proteins. Kinase activity of GST–OsOxi1 protein was assayed with GST–OsPti1aWT and GST–OsPti1aT233A and GST–OsPti1aK96N proteins as substrates. (D) Complementation of the ospti1a mutation with OsPti1a WT, OsPti1aK96N, and OsPti1aT233A cDNA. cDNA of the OsPti1aK96N gene under the control of the OsActin promoter and the ospti1a homozygous mutant. (E) Total protein was extracted from transgenic rice carrying the 35S:OsPti1aWT, Actpro:OsPti1aK96N, 35S:OsPti1aT233A, and 35S:OsPti1aK96N/T233A constructs or an empty vector. The minus sign indicates the untransformed ospti1a mutant. (F) Total RNA was extracted from the WT vector control and the pti1a homozygous mutant with the introduced constructs 35S:OsPti1aWT, Actpro:OsPti1aK96N, 35S:OsPti1aT233A and 35S:OsPti1aK96N/T233A (T1 generation). Numbers indicate independent lines used in this assay. Ten micrometers of total RNA was hybridized with radiolabeled probes as indicated. rRNA served as a loading control.

Discussion

OsOxi1 positively regulates basal resistance via ROS signaling

ROS are involved in the regulation of plant responses to biotic and abiotic stresses (Overmyer et al. 2003) and are important in the functioning of the ospti1a phenotype, including enhanced resistance to rice blast. Thus, the effect of mutations in OsPti1a on the compatible interaction may be too small to be detectable due to the strong virulence of this fungus. Therefore, to evaluate whether the T233 phosphorylation affects basal resistance, we carried out an inoculation test with a compatible race of Xoo in the transgenic lines. We used the T1 generation of the transgenic lines in the ospti1a background for inoculation with Xoo after examining OsPti1a protein levels by immunoblotting (Fig. 7E). Xoo-induced lesions were 1.5-fold longer in leaves of the 35S:OsPti1aWT transgenic line than in the vector control (Fig. 7D, F). Because the amount of OsPti1a in 35S:OsPti1aWT transgenic lines was higher than in WT and the vector control, this result is consistent with the previous finding that the suppression of basal resistance depends on increased OsPti1a levels. In contrast, Xoo-induced lesions were significantly longer in the 35S:OsPti1aK96N and 35S:OsPti1aK96N/T233A transgenic lines than in the vector control. The accumulation of Pti1a protein was lower in 35S:OsPti1aT233A and 35S:OsPti1aK96N/T233A than in 35S:OsPti1aWT plants. Nevertheless, Xoo-induced lesions in the 35S:OsPti1aT233A and 35S:OsPti1aK96N/T233A lines were significantly longer than in 35S:OsPti1aWT plants. These results indicate that T233 of OsPti1a has a role in basal resistance to compatible pathogens. There was no difference in resistance between Actpro:OsPti1aK96N transgenic lines and the vector control (Fig. 7D, F), indicating that the phosphorylation activity of OsPti1a is not important for basal resistance in rice.
OsOxi1–OsPti1a interaction regulates rice defense

for the induction of disease resistance (Yoshioka et al. 2009). NADPH oxidases and respiratory burst oxidase homologs (RBOHs) have been isolated from Arabidopsis, rice, potato, tomato and N. benthamiana (Torres et al. 2002, Yoshioka et al. 2003, Sagi et al. 2004, Kobayashi et al. 2006, Takahashi et al. 2007, Wong et al. 2007). Loss of RBOH function suppresses ROS production in the defense response, and prevents disease resistance to pathogens (Torres et al. 2002, Yoshioka et al. 2003). However, the signaling pathway downstream of ROS is still unclear. In this study, we demonstrated that OsOxi1 is tightly regulated by ROS transcriptionally and post-translationally (Fig. 3A–C, Supplementary Fig. S1). Further, OsOxi1 is phosphorylated by chitin elicitor treatment (Fig. 3C). In Arabidopsis, AtOxi1 is regulated by 3-phosphoinositide-dependent protein kinase1 (AtPdk1) through its phosphorylation in response to PAMPs (Anthony et al. 2006). This result suggests that OsOxi1 is regulated in a ROS-dependent and independent manner. The expression of OsOxi1 was induced at the infection site where H$_2$O$_2$ accumulates (Fig. 4G, H). Therefore, we conclude that OsOxi1 is induced locally in response to ROS production. These results imply that the phosphorylation of OsOxi1 in response to ROS and elicitor is an immediate–early response in disease resistance, and that the induction of OsOxi1 expression is a delayed response that serves to further restrict or slow the pathogen infection. Since expression of OsPti1a is observed in whole leaves (Fig. 4G), OsPti1a constitutively and negatively regulates disease resistance in leaves. Consequently, we suggest that the OsPti1a-mediated negative regulation of defense signaling is suppressed by OsOxi1 in response to the ROS signal. This idea is supported by enhanced resistance, first, to the compatible blast pathogen in OsOxi1-overexpressing transgenic plants, and second, to a compatible race of rice blast in OsOxi1S283D-overexpressing plants (Fig. 5). Notably, the Arabidopsis oxi1 mutant shows enhanced susceptibility to the compatible pathogen H. arabidopsis (Rentel et al. 2004). Taken together, these results indicate that OsOxi1 and AtOxi1 positively regulate basal resistance in the context of ROS signaling.

The present study also suggests some functional diversification of Oxi1 proteins between rice and Arabidopsis. AtOxi1-overexpressing Arabidopsis plants exhibit increased susceptibility to H. arabidopsis and Pseudomonas syringae (Petersen et al. 2009), whereas OsOxi1-overexpressing transgenic rice showed enhanced resistance. Based on this finding, it was proposed that the level of AtOxi1 protein is crucial and modulation of the protein level causes increased susceptibility to the

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**Fig. 7** Effects of mutations of OsPti1a on compatible pathogen infection. (A) Three-week-old transgenic plants carrying 35S:OsPti1a WT, Act$_{pro}$:OsPti1a$^{K69N}$, 35S:OsPti1a$^{T233A}$ and 35S:OsPti1a$^{K69N/T233A}$ (T1 generation) in the ospti1a mutant background or WT plants were inoculated with a compatible race of the rice blast fungus by spraying with an aqueous spore suspension containing $1.5 \times 10^5$ spores per ml. The leaves were photographed at 10 dpi. (B) Degree of disease symptoms (relative lesion area) in transgenic plants carrying 35S:OsPti1a WT, Act$_{pro}$:OsPti1a$^{K69N}$, 35S:OsPti1a$^{T233A}$ and 35S:OsPti1a$^{K69N/T233A}$ (T1 generation) in the ospti1a mutant background or WT plants at 10 dpi. Results from three independent experiments are presented as means $\pm$ SD ($n=4–8$). (C) Accumulation of OsPti1a protein was detected by protein blots using the anti-OsPti1a antibody. (D) Representative leaves showing the extent of lesion development at 21 dpi with Xoo (race 1) in leaves of transgenic plants (T1 generation) in the ospti1a background (35S:OsPti1a WT, Act$_{pro}$:OsPti1a$^{K69N}$, 35S:OsPti1a$^{T233A}$ and 35S:OsPti1a$^{K69N/T233A}$) and WT. (E) Lesion length measurements for the T1 generation of each transgenic line. Results shown are means $\pm$ SE from three independent experiments ($n=12$). Columns with the same lower case letters do not differ from each other at $P<0.05$ in the Tukey–Kramer test. (F) OsPti1a in total protein was detected with anti-OsPti1a antibody.
pathogens (Petersen et al. 2009). The Arabidopsis axi1 mutant showed enhanced susceptibility to the biotrophic pathogens *H. arabidopsis* and *P. syringae* (Rentel et al. 2004, Petersen et al. 2009) but not to the necrotrophic *Botrytis cinerea*, suggesting that AtOxi1 is not required for the activation of defense responses to necrotrophic pathogens (Petersen et al. 2009). Our results show that OsOxi1 regulates the defense against the hemibiotrophic pathogen, rice blast fungus (Fig. 5A, C) and against the biotrophic pathogen *Xoo* (Fig. 5D, E). It will be interesting to examine whether OsOxi1 is required for the activation of defense responses to necrotrophic pathogens in rice.

AtOxi1 is required for both R-gene-mediated and basal resistance (Petersen et al. 2009). To see whether OsOxi1 also positively regulates R-gene-mediated resistance, a loss-of-function analysis of OsOxi1 is essential. Unfortunately, we were unable to obtain knock-out or even knock-down lines, although a number of *OsOxi1*-RNA interference lines were produced. It seems likely, though, that OsOxi1 participates in R-gene-mediated resistance as well as basal resistance, because ROS are important for both types of defense responses in plants.

**Phosphorylation of OsPti1a positively regulates basal resistance**

We investigated the role of the phosphorylation of OsPti1a by OsOxi1 in disease resistance. The K96N mutation in OsPti1a abolished its autophosphorylation activity, but had no effect on its phosphorylation by OsOxi1 in vitro (Fig. 6B, C). Act*<sup>35S:OsPti1a<sup>K96N</sup> transgenic plants in the *ospti1a* mutant background showed normal growth, and unchanged disease susceptibility to a compatible race of rice blast fungus (Figs. 6D, 7A, B). Thus, the kinase activity of OsPti1a is not required for its negative regulatory function. On the other hand, the residue T233 of OsPti1a is required not only for autophosphorylation activity, but also for phosphorylation mediated by OsOxi1 (Fig. 6B, C). 35S:OsPti1a*<sup>T233A</sup> transgenic plants in the *ospti1a* background showed enhanced susceptibility to *Xoo* (Fig. 7D, E), indicating that the phosphorylation of OsPti1a T233 is important for the activation of basal resistance to pathogens.

On the basis of our observations, we propose a model for defense signaling in rice involving OsPti1a and OsOxi1 (Fig. 8). When OsOxi1 is activated by ROS produced in response to pathogen infection, it phosphorylates residue T233 of OsPti1a, resulting in the induction of defense responses by the suppression of the negative regulatory function of OsPti1a. T233 of SlPti1, which is the major phosphorylation site for Pto, is conserved at the corresponding position in both OsPti1a (T233) and AtPti1-2 (T238) (Sessa et al. 2000, Anthony et al. 2006, Takahashi et al. 2007). This fact suggests that the regulation by phosphorylation of a Thr residue in Pti1 proteins is important for Pti1 function. In Arabidopsis, AtPti1-2 activates oxidative stress-inducible gene expression in the presence of AtOxi1, suggesting that AtPti1-2 may activate downstream components of ROS signaling by AtOxi1-mediated phosphorylation (Anthony et al. 2006).

**Mechanism of OsPti1a function in defense responses**

All mutant Pti1a proteins tested complemented the lesion-mimic phenotype and PR1b gene expression in the *ospti1a* mutant background (Fig. 6D, F). This suggests that OsPti1a negatively regulates disease resistance without phosphorylation or kinase activity in rice. Based on our results, we hypothesize that the dephosphorylated state of OsPti1a is necessary for the suppression of defense responses. This hypothesis implies that the negative regulatory function of OsPti1a is suppressed by its phosphorylation in response to pathogen infection. Similar types of regulation have been reported in other cases of defense-related signaling. For example, Arabidopsis RPM1-interacting protein 4 (RIN4), a negative regulator of basal resistance, is suppressed by its phosphorylation through interaction with *P. syringae* type III effectors AvrRpm1 and AvrB, resulting in the activation of their cognate R protein, RPM1 (Mackey et al. 2002, Axtell and Staskawicz, 2003, Kim et al. 2005). In addition, we previously reported that the silencing of OsRAR1 cancels the *ospti1a* mutant phenotype (Takahashi et al. 2007). A complex involving OsRAR1 functions as a positive regulator of basal resistance to blast fungus and bacterial blight in rice, and is involved in ROS production (Thao et al. 2007). Based on these observations, it appears possible that OsPti1a phosphorylation by OsOxi1 through OsRAR1-mediated signaling might be required for the activation of defense responses. To further understand the molecular mechanisms of this activation, it will be crucial to identify the
relationship between the OsRAR1 complex and the OsOxi1–OsPti1a cascade.

**Materials and Methods**

**Plant and pathogen materials**

*Oryza sativa* L. cv. ’Nipponbare’ was used as the WT. Rice cells were suspension-cultured at 25°C in liquid N6D medium and subcultured in fresh medium every 7d. Cells at 5d after subculture were used for experiments. Chitin elicitor (N-acetylmuramoyl-

L-alanine N-acetylglucosaminyl-N-acetylglucosamine) was prepared by re-N-acetylation of the chitosan oligosaccharide [kindly supplied by Yaizu Suisan Kagaku Industrial Co. Ltd (Shizuoka, Japan)] and used at a final concentration of 1 μg/ml. Plant growth conditions and pathogen infection conditions were described previously (Takahashi et al. 2007). NB carries the blast resistance genes *Pish*. Two strains of *Magnaporthe oryzae* pv. *oryzae*, Kytu89-246 (MAFF101506; race 003.0) as a compatible and Kytu77-07A (avrPish; Race 102.0) as an incompatible race were used. Bacterial blight inoculation experiments were performed with the Japanese *Xoo* race 1 using a scissors-dip method (Kauffman et al. 1973).

**Yeast two-hybrid assay**

cDNA libraries from rice seedling and callus were constructed using the HybriZAP-2.1 lambda vector (Stratagene, La Jolla, CA, USA). The libraries were converted to the pAD-GAL4-2.1 plasmid form by mass excision following the manufacturer’s instructions. Yeast strain AH109 (Clontech, Mountain View, CA, USA) was transformed with the *pAD-GAL4-2.1* reporter plasmid. The yeast colonies carrying the possible interacting proteins were screened by complementation of plasmid libraries. The yeast expressing the OsPti1a-GAL4 binding-domain fusion protein was co-introduced with rice cDNA libraries. The interaction between proteins of interest was confirmed by their expression in the yeast colonies carrying the possible interacting proteins. The interaction between proteins of interest was confirmed by their expression in the yeast colonies carrying the possible interacting proteins.

**OsOxi1 cDNA cloning**

The sequence of the 1389-bp OsOxi1 cDNA fragment was used to search the Rice Annotation Project Database (RAP-DB; http://rapdb.lab.nig.ac.jp) for a full-length OsOxi1. The OsOxi1 cDNA found was cloned into pENTR/D-TOPO (Invitrogen, Carlsbad, CA, USA) and confirmed by sequencing. Site-directed mutagenesis was carried out using PCR primers. The OsOxi1 mutagenized forms (K56N, S283A, K56N/S283A and S283D) were cloned into pENTR/D-TOPO as well as pENTR/SD/D-TOPO.

**Sequence analysis**

Multiple sequence alignments were produced with a Web-based version of ClustalW (http://clustalw.ddbj.nig.ac.jp/top-j.html) using default settings (matrix = blosum; GAPOPEN = 0, GAPEXT = 0, GAPDIST = 8 and MAXDIV = 40). The phylogenetic tree was calculated using the neighbor-joining method and bootstrap analysis (1000 replicates) using PHYLIP via the same website and visualized with NJ plot (http://pbil.univ-lyon1.fr/software/njplot.html).

**Protein expression, in vitro binding assay and in vitro kinase assay**

Site-directed mutagenesis was carried out using PCR primers, and PCR products were cloned into pENTR/SD/D-TOPO (Invitrogen). OsPti1a and OsPti1a<sup>K56N</sup> cDNAs were previously cloned into pENTR/SD/D-TOPO (Takahashi et al. 2007). Recombination to pDEST15 or pDEST17 was performed with the LR Clonase II enzyme mix. GST fusion proteins were expressed and purified from *Escherichia coli* BL21-AL cells (Invitrogen) according to the manufacturer’s protocol (GE Healthcare, Buckinghamshire, UK). His<sub>6</sub>-tagged proteins were expressed and purified according to the manufacturer’s protocol (GE Healthcare). Purified proteins were desalted on PD-10 columns containing Sephadex<sup>™</sup> G-25M (GE Healthcare).

**In vitro binding assay**

Purified proteins were mixed in the binding buffer [50 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM dithiothreitol (DTT), 10 mM MgCl<sub>2</sub>, 0.2% Triton X-100]. Glutathione–Sepharose 4B (GE Healthcare) for precipitating GST-tagged fusion proteins was added and the mixture was incubated for 2 h at 4°C. Beads were washed, and proteins were eluted and analyzed by SDS–PAGE. GST–OsPtia1a or His–OsOxi1 was visualized by anti-GST horseradish peroxidase (HRP) conjugate antibody (GE Healthcare) or anti-His antibody (GE Healthcare), respectively. The blots were incubated with primary antibodies using the following dilutions: GST–HRP conjugate antibody (1:5000); anti-His antibody (1:5000). Detection was performed using ECL Plus Western Blotting Detection Reagents (GE Healthcare).

**In vitro phosphorylation assay**

The in vitro phosphorylation assays were performed as described previously (Zhou et al. 1995) with the following minor modifications. Purified His–OsOxi1 proteins (OsOxi1<sup>WT</sup>, OsOxi1<sup>K56N</sup>, OsOxi1<sup>S283A</sup>, OsOxi1<sup>K56N/S283A</sup> or OsOxi1<sup>S283D</sup>) or purified GST–OsPtia1a proteins (OsPtia1a, OsPtia1a<sup>K56N</sup>, OsPtia1a<sup>T233A</sup> or OsPtia1a<sup>K56N/T233A</sup>) were incubated with kinase buffer (50 mM Tris–HCl pH 7.5), 100 mM NaCl, 1 mM DTT, 10 mM MgCl<sub>2</sub>, 20 µM ATP and 2.5 µCi of [γ-<sup>32</sup>P]ATP. Assays were done in a kinase reaction mixture (25 µl) including purified proteins, and reactions were incubated at room temperature for 30 min. The samples were subsequently analyzed using 10% SDS–PAGE gels and visualized by Coomassie Brilliant Blue R250 staining followed by autoradiography using BAS-2500 (FUJIFILM, Tokyo, Japan).

To test whether OsOxi1 phosphorylates OsPtia1a in vitro, a phosphorylation assay was performed using His–OsOxi1 proteins and GST–OsPtia1a<sup>K56N</sup> protein as substrate, or GST–OsPtia1a protein and His–OsOxi1<sup>K56N</sup> protein as substrate. Reactions and detection were as described above. Cross-phosphorylation
assays were done in a kinase reaction mixture (25 µl) that contained 50 mM Tris–HCl pH 7.5, 150 mM NaCl, 1 mM DTT, 10 mM MgCl₂, 20 mM ATP, 2.5 µCi of [γ-32P]ATP, purified His–OsOxi1 protein and purified OsPti1a proteins (OsPti1a, OsPti1aK96N, OsPti1aT233A or OsPti1aK96N/T233A). Reactions and detection were as described above.

Agroinfiltration of N. benthamiana

Agroinfiltration of N. benthamiana was performed as described previously (Kobayashi et al. 2006) with modifications. OsPti1a and OsOxi1 were cloned into pE12Ω-MCS vector (Takahatake et al. 2007). Agrobacterium tumefaciens strain EHA105 carrying the cDNA of OsPti1a, OsOxi1 and control were used to infiltrate 5-week-old N. benthamiana leaves. Agrobacterium culture was resuspended in a buffer containing 10 mM MgCl₂, 10 mM MES pH 5.6 and 150 µM acetylsyringone at optical density at 600 nm of 0.3, incubated at room temperature for 2–3 h, and infiltrated into the fourth and fifth leaves of N. benthamiana using a needleless 1 ml syringe. Two days after inoculation, total proteins were extracted by grinding the N. benthamiana leaves in liquid nitrogen and extraction buffer [50 mM Tris–HCl pH 7.5, 50 mM NaCl, 5 mM EDTA, 2 mM DTT, 0.2% (w/v) Triton X-100, 10% (v/v) glycerol and Complete Protein Inhibitor Cocktail Tablets (Roche, Mannheim, Germany)]. Cell debris was removed by centrifugation at 15 000 × g at 4°C for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 20 µl of anti-FLAG M2–agarose affinity gel (Sigma, St. Louis, MO, USA) for 20 min.

Protein extraction, immunoprecipitation and protein gel blot analysis

Proteins were extracted by grinding the rice suspension cell cultures in liquid nitrogen and extraction buffer [50 mM Tris–HCl pH 7.5, 50 mM NaCl, 1 mM MgCl₂, 5 mM DTT, 10% glycerol, 0.5% Triton X-100 and complete protein inhibitor cocktail tablets (Roche)]. Cell debris was removed by centrifugation at 14 000 × g for 30 min. Protein concentrations were determined using the Bradford Protein Reagent (Bio-Rad, Hercules, CA, USA) with bovine serum albumin as standard. For immunoprecipitation, the extract (1 ml) containing 5 mg of total proteins was incubated with 30 µl of Strep-Tactin beads (IBA GmbH, Goettingen, Germany) at 4°C for 2 h with gentle rocking. The matrix was washed five times with the extraction buffer, resuspended in 30 µl of 2× SDS sample buffer, boiled for 5 min and 20 µl aliquots were loaded on SDS–PAGE gels. To detect phosphorylated OsOxi1 by SDS–PAGE, we used Phos-tag AAL-107 (NARD Institute Ltd, Amagasaki, Japan). Phosphorylated OsOxi1 was separated on 8% (w/v) SDS–PAGE gels containing 40 µM Phos-tag. For dephosphorylation, the matrix was prepared in 50 µl of 1× alkaline phosphatase buffer, incubated with 2 units of alkaline phosphatase (E. coli C75) (Takara, Kyoto, Japan) for 2 h. After the reaction, the matrix was resuspended in 10 µl of 5× SDS sample buffer, boiled for 5 min and 20 µl aliquots were loaded on SDS–PAGE gels. The blots were incubated with the HA antibody (Covance, Berkeley, CA, USA) as primary antibodies using the following dilutions: HA antibody (1: 5000). Detection was as described above.

OsOxi1pro::GUS, OsPti1a pro::GUS and Actpro::GUS reporter gene constructs, and GUS staining

An 1824 bp fragment containing the promoter of OsOxi1, a 4168 bp fragment containing the promoter of OsPti1a and an 1491 bp fragment containing the promoter of OsActin1 were amplified from genomic DNA by PCR and cloned into pENTR/D-TOPO. The following primers were used: OsOxi1 promoter (5′-CACCAAGGAGGAGAAGAATTGGAGGGCA-3′ and 5′-GGCTGGCGAAGGACC-3′), OsPti1a promoter (5′-CACCAAAAGGAGGAGAATTTGGAGGGCA-3′ and 5′-GAGCTGTTGTGGTACCAGCGGTGAACCTA-3‘), OsActin1 promoter (5′-CACCCTCGAGGTCATTCATATGCTTG-3′ and 5′-GGATGATAACTTCGATGTTATTC-3′). These vectors introduced the construct into NB. As a positive control, OsActin1 promoter::GUS fusion gene (Actpro::GUS) was used (Sentoku et al. 2000). We obtained three independent transgenic lines for each construct and used their T1 generation for subsequent studies. Plant tissues were stained for GUS activity by submersion in staining solution [modified from Sasaki et al. (2007): 1 mM 5-bromo-4-chloro-3-indoly-β-D-glucuronide (X-gluc), 50 mM potassium phosphate buffer pH 7.0, 5% (v/v) methanol, 10 µg/ml cycloheximide and 5 mM diethiothreitol and vacuum infiltrated to 80 kPa for 1 h. Samples were incubated at 37°C for 1 d after which the reaction was stopped by the addition of 70% ethanol.

DAB staining

To visualize ROS in situ, DAB staining was performed as described by Torres et al. (2002) with modifications. The inoculated leaves were detached. Leaf wax was removed with acetone treatment and the leaves washed in water. The detached leaves were incubated in 1 mg/ml DAB solution for 8 h, followed by fixation in a solution of 3:1 ethanol/ acetic acid.
Determination of cell death using Evans Blue

Evans Blue staining was performed as described elsewhere (Kurusu et al. 2005).

Rice transformation

To overexpress cDNA fragments, we constructed the Ti-plasmid vectors pEASY-Ubi_pro and pEASY-Act_pro. These vectors were then fused to the maize (Zea mays) Ubi promoter or the OsActin1 promoter with the Gateway System in pPZP2Ha3(+). cDNAs of OsOxi1 and OsPti1aK96N were cloned into pEASY-Ubi_pro using LR Clonase II enzyme mix (Invitrogen). For the construction, pEASY-Ubi_pro–HA-streplII–OsOxi1, the HA-streplII epitope (Witte et al. 2004), was inserted into pGEM32Z(+) (Promega, Madison, WI, USA). The cDNA fragment of full-length OsOxi1 was introduced into pEASY-Ubi_pro as described above. cDNA fragments of the OsPti1a mutation (T233A and K96N/T233A) were generated by PCR and cloned into pENTR/D-TOPO. HA-streplII–OsOxi1 was introduced into pEASY-Ubi_pro as above. RNA extraction, RT–PCR and RNA blot analysis

Total RNA was extracted from rice tissues by ISOGEN (NipponGene, Tokyo, Japan). Total RNA was digested by DNase treatment using an RNeasy Mini Kit (Qiagen, Valencia, CA, USA). One microgram of total RNA was used as template to synthesize first-strand cDNA by ReverTra Ace RT–PCR kit (TOYOBO, Osaka, Japan). Primers for RT–PCR analyses are OsOxi1–RT–FW (5′-GGAGGCGGCGGCGCAAGAAGACGGGA-3′) and OsOxi1–RT–RV (5′-CCGCATGGAATCGCAA TCAGTG-3′) for OsOxi1, and OsAct1–FW (5′-GCCATCTTGC ATCAGCAG-3′) and OsAct1–RV (5′-GATCGCAGCGAT CGATC-3′) for rice Actin. For semi-quantitative RT–PCR amplification, 1 µg of first-strand cDNA corresponding to the product from 25 ng of total RNA was used as the template for PCR. The PCR product was subjected to agarose gel electrophoresis and visualized by staining with ethidium bromide. RNA blot analysis was performed as previously described (Takahashi et al. 2007).

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