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Rice Rab11 is required for JA-mediated defense signaling

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ABSTRACT

Rab proteins play an essential role in regulating vesicular transport in eukaryotic cells. Previously, we characterized OsRab11, which in concert with OsGAP1 and OsGDI3 regulates vesicular trafficking from the trans-Golgi network (TGN) to the plasma membrane or vacuole. To further elucidate the physiological function of OsRab11 in plants, we performed yeast two-hybrid screens using OsRab11 as bait. OsOPR8 was isolated and shown to interact with OsRab11. A co-immunoprecipitation assay confirmed this interaction. The green fluorescent protein-OsOPR8 fusion product was targeted to the cytoplasm and peroxisomes of protoplasts from *Arabidopsis thaliana*. OsOPR8 exhibited NADPH-dependent reduction activity when 2-cyclohexen-1-one (CyHE) and 12-oxo-phytodienoic acid (OPDA) were supplied as possible substrates. Interestingly, NADPH oxidation by OsOPR8 was increased when wild-type OsRab11 or the constitutively active form of OsRab11 (Q78L) were included in the reaction mix, but not when the dominant negative form of OsRab11 (S28N) was included. OsRab11 was expressed broadly in plants and both OsRab11 and OsOPR8 were induced by jasmonic acid (JA) and elicitor treatments. Overexpressed OsRab11 transgenic plants showed resistance to pathogens through induced expression of JA-responsive genes. In conclusion, OsRab11 may be required for JA-mediated defense signaling by activating the reducing activity of OsOPR8.

1. Introduction

The Rab protein family is the largest member of the Ras superfamily of monomeric G proteins, also referred to as small ATPases [1]. Members of this family were first identified in yeast, and since then, approximately 70 have been identified in mammals. Rab proteins have also been identified and characterized in plants. Like their counterparts in yeast and mammalian cells, they are essential in intracellular vesicular trafficking [2]. The importance of Rab proteins' regulation of membrane trafficking is indicated by their diverse effects on plant development, including regulation of root hair development in legumes [10] and pollen development in Arabidopsis [3].

Small GTP-binding proteins have also been implicated in defense [4] and stress signaling pathways [5]. For example, in salt-stressed tomato plants, expression of Rab11b was repressed and expression of Rab2 was induced, as shown by microarray analysis of total RNA [6]. Similarly, transgenic tobacco plants that overexpressed *Prosopis juliflora* Rab7 were resistant to high-salt stress [7] and ectopic expression of OsRab7 enhanced tolerance of peanut plants to several abiotic stresses.

Jasmonic acid (JA) is the terminal product of the octadecanoid pathway, in which 12-oxo-phytodienoate-10,11-reductase (OPR) catalyzes NADPH-dependent reduction of 12-oxo-phytodienoic acid to JA [8]. Therefore, OPR is a key enzyme in JA accumulation in response to environmental stress [9]. OPR genes have been identified in a number of plant species [10–13]. Their expression is tissue specific and induced by a variety of abiotic and biotic stresses, including wounding, infection, and signaling molecules [14–16].

In a previous study, we characterized a Rab protein in rice (*Oryza sativa*), OsRab11, with respect to its role in membrane trafficking [7,28]. Here, we have further characterized this protein and one of its targets, OsOPR8, with particular emphasis on their possible role in plant defense. The jasmonic acid signaling pathway is a critical component of plant responses to stress [17], including infection, and therefore, was an integral part of our investigation.

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2. Materials and methods

2.1. Yeast two-hybrid screening

A Hybrid Hunter Version D kit (Invitrogen, Carlsbad, CA) was used for this assay. *OsRab11* cDNA fragments were ligated into the pHybLexZeo plasmid, which contains a zeocin selection marker. The prey cDNA library was constructed into the pYESTrp2 vector containing the B42-activation domain. Interactions between fusion proteins were investigated by cotransforming appropriate plasmids into the yeast reporter strain L40. Transformed yeast cells harboring both plasmids were selected on synthetic complete (SC) medium lacking tryptophan (Trp), uracil (Ura), lysine (Lys) and histidine (His) (SC-Trp, Ura, Lys, His), and SC-Trp, Ura, Lys as a control. Histidine positive colonies were further tested for β-galactosidase (*lacZ*) activity, according to the manufacturer's protocol (Invitrogen). The growth of blue colonies in histidine-deficient medium indicates a positive interaction. Positive yeast colonies were isolated and identified. Plasmids from these clones were recovered, and the presence of the insert sequences was reconfirmed by sequencing. The sequences were subjected to BLAST analysis using GenBank database.

2.2. Co-immunoprecipitation assay

For the Co-IP assay, protein extracts were obtained from the transformed L40 cells containing various *LexA-DB* and *B42-V5-AD* constructs by using co-IP buffer (PBS buffer containing protease inhibitors, 0.5% Nonidet P-40, and 1% Triton X-100). Protein A beads (40 μl) were incubated with 0.5 μg of anti-LexA-DB antibody and 200 μg of protein extracts for 3 h at 4 °C. After washing five times with co-IP buffer, the washed samples were subjected to SDS–PAGE, and then, immunoprecipitated with anti-LexA-DB or anti-V5-AD antibody was done.

2.3. Purification of OsRab11 and OsOPR8 fusion proteins

For bacterial expression, the full-length OsRab11 was fused to the C-terminus of GST. The *GST-OsRab11* fusion construct was generated by digesting the full-length *OsRab11* in pBluescript SK- with *Bam* HI/*Eco* RI, and inserting the excised fragment into the corresponding site of the GST expression vector pGEX-2T (Amersham, Buckinghamshire, UK). The *OsOPR8* cDNA in pBluescript SK- was also digested with *Eco* RI/*Xho* I and then ligated to pET28a vector to generate 6xHis-tagged *OsOPR8* construct. After then, the resulting constructs were introduced into *Escherichia coli* strain BL21 (pLysS), the GST-fused and 6xHis-tagged proteins were expressed, and purified by using glutathione-agarose beads and Ni-NTA beads according to the manufacturer's instructions (Amersham Buckinghamshire, UK).

2.4. Assay of enzymatic activity

NADPH-dependent α/β-barrel oxidoreductase activity of OsOPR8 was measure by the method previously described [18] with minor modification. The activity of recombinant OsOPR8 protein was determined spectrophotometrically by monitoring the reduction of absorbance at 366 nm concomitant with the disappearance of NADPH. The reaction was performed at 25 °C in a 200 μl reaction mixture consisting of 50 mM potassium-phosphate buffer (pH 7.5), 0.4 mM NADPH, 0.4 mM substrates and 2 μg OsOPR8 or OsRab11 recombinant protein.

2.4.1. Transient expression assay

OsOPR8 cDNA was fused upstream of the GFP cDNA under the control of the CaMV 35S promoter (pUC::GFP). The construct (*GFP-OsOPR8*) was co-transformed into Arabidopsis protoplasts along with the cyan fluorescent protein cDNA fused to a peroxisome marker (AtPTS2-CFP) [19]. Transient expression of GFP- and CFP-fused constructs in *Arabidopsis* protoplasts was performed according to the method described by Heo et al. [20]. Briefly, recombinant plasmids were introduced by polyethylene glycol (PEG)-mediated transformation into *Arabidopsis* protoplasts that had been prepared from leaf tissues. Expression of the fusion constructs was monitored after transformation, and observed by fluorescence microscopy (Olympus AX70 TR, Olympus).

2.5. Plant materials and treatments

To investigate the inducible expression of OsRab11 and OsOPR8 genes, mature seeds were sterilized and grown on MS agar medium at 22 °C in 16 h light/8 h dark. After 2 weeks of growth, the seedlings were subjected to various treatments, including hormones and elicitor. For hormone treatments, solutions of 250 μM JA and 5 mM SA were separately sprayed on seedlings. For rice blast fungal elicitor treatment, a conidial suspension (1×10^5 conidia/mL) of rice blast fungus (race KJ401) was sprayed on seedling using an air sprayer.

2.5.1. RT-PCR and qRT-PCR analyses

For RT-PCR analysis, tota1 RNAs were extracted using Trizol reagent according to the manufacturer's protocol (Invitrogen) and then, cleaned up again using Qiagen column (QIAGEN). Isolated RNAs were treated with RNase-free DNase (Sigma) to evade genomic DNA contamination. The cDNA was synthesized from RNA treated with Moloney murine leukemia virus (MMLV) reverse transcrip-tase RNaseH and an oligo(dT)18 primer (Promega). The RT-PCR reactions were repeated three times. For qRT-PCR analysis, total RNA extraction was performed as described above, and the isolated RNA was treated with RNase-free DNase (Sigma) to exclude genomic DNA contamination. The cDNA was synthesized from treatment of RNA with MMLV reverse transcriptase RNaseH and an oligo(dT)18 primer (Promega). Quantitative real-time PCR was performed using the Power SYBR Green PCR Master Mix (Applied Biosystems) by a Real Time PCR System machine (Bio-RAD, California, USA), following the manufacturer's protocols. Relative quantitative values were calculated by normalization to rice actin and Arabidopsis actin as internal controls.

2.6. Generation of overexpressed-OsRab11 transgenic plants

The *OsRab11* cDNA was amplified and subcloned into the pGEM-T vector (promega) followed by ligation into vector pCAMBIA1300PT. The construct was transformed into wild-type plants (Col-0) via *Agrobacterium*-mediated transformation [21]. Transgenic lines were selected on selective antibiotic marker-containing medium, and confirmed by genotyping PCR.

3. Results

3.1. OsRab11 interacts with OsOPR8

In our previous study, OsRab11 was characterized as a positive regulator of vesicular trafficking in rice [20,22]. Here, we wished further characterize its physiology and began this undertaking by identifying proteins that interact with OsRab11. As a first step, a yeast two-hybrid library screening was carried out using OsRab11

as bait. One of the proteins recovered from the screen was 12-oxo-phytodienoic acid reductase 8 (OsOPR8).

Next, full-length OsOPR8 fused to an activation domain was co-transformed with full-length OsRab11 fused to the DNA-binding protein LexA in yeast. Consistent with our contention that these two proteins interact, the full-length OsRab11 interacted with the full-length OsOPR8 in yeast (Fig. 1A). This result was further confirmed by a co-immunoprecipitation assay between LexA-DB-OsRab11 and V5-AD-OsOPR8 (Fig. 1B). V5-AD-OsOPR8 precipitated with LexA-DB-OsRab11. Co-transformations of plasmids expressing the OsRab11 and OsOPR8 fused to the same extremity of the split-YFP fragments (e.g., OsRab11-YFP^N/OsOPR8-YFP^C) allowed the formation of a bimolecular fluorescence complementation (BiFC) complex in the cytosol (Fig. 1C), indicating that OsOPR8 is indeed a target protein of OsRab11.

3.2. OsOPR8 is localized in the cytosol and peroxisome

3.2.1. We studied the subcellular localization of the OsOPR8 protein

Its subcellular location was tentatively predicted with a program on the PSORT WWW serve. This tentative prediction localized OsOPR8 to the cytoplasm and peroxisome (Supplemental Fig. 1). The OsOPR8, OsOPR7, and AtOPR3 proteins all have a consensus signal sequence that consists of three amino-acid residues at the C-terminus, and is consistent with targeting to the peroxisomes (Fig. 2A) [23]. To confirm the subcellular localization, free green fluorescent protein (GFP) and GFP fused to OsOPR8 (GFP-OsOPR8) were introduced into Arabidopsis protoplasts separately and the GFP signal was observed by fluorescence microscopy. As shown in Fig. 2B, free GFP (the control) was distributed in the cytosol and nucleus, but GFP-OsOPR8 exhibited the cytosol and punctate staining patterns consistent with localization to the peroxisome, as predicted by the program. These two localization patterns of GFP-OsOPR8 in protoplasts were represented with similar ratio. Next, we examined the exact localization of GFP-OsOPR8 by co-transformation of *GFP-OsOPR8* with *AtPTS2-CFP*, a peroxisome marker [19] in *Arabidopsis* protoplasts. The green and cyan punctate staining patterns from GFP-OsOPR8 and AtPTS2-CFP overlapped exactly, providing additional evidence that OsOPR8 localizes to the cytosol and peroxisome in plant cells.

3.3. OsRab11 enhances NADPH oxidation by OsOPR8

The OsOPR protein family has significant homology to other flavoproteins such as morphinone reductase and the old yellow enzymes of *Saccharomyces cerevisiae* [24]. These members of the α/β-barrel flavin oxidoreductase family, including OPR, catalyze the NADPH-dependent reduction of α/β-unsaturated aldehydes or ketones [11]. Based on this, one would predict that OsOPR8 has NADPH-dependent reduction activity. The reductase activity of the recombinant OsOPR8 was measured with 2-cyclohexen-1-one (CyHE), 12-oxo-phytodienoic acid (OPDA), or ABA supplied as

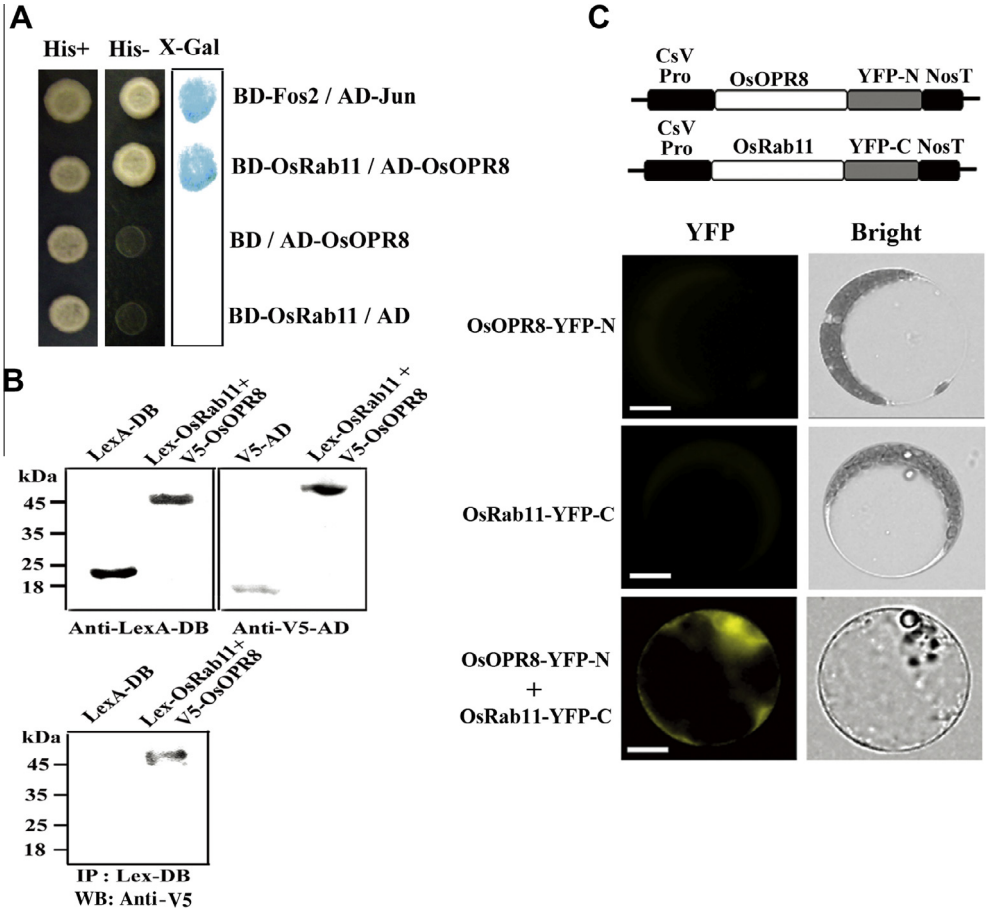

Fig. 1. OsRab11 interacts with OsOPR8. (A) A yeast two-hybrid assay. Each co-transformants was grown on synthetic medium with (His+) or without histidine (His−) and β-galactosidase assay was carried out on filter paper containing X-Gal solution. Fos2/Jun was used as a positive control, BD vector/AD-OsOPR8 and BD-OsRab11/AD vector were used as negative controls. (B) Co-immunoprecipitation assay. Protein extract obtained from the indicated strains were subjected to co-immunoprecipitation using the anti-LexA-BD antibody. The immune precipitates were separated by 12% SDS–PAGE, transferred to polyvinylidene difluoride membranes, and blotted with anti-V5 antibody. (C) BiFC assay. Both BiFC constructs were co-transfected into Arabidopsis protoplasts and observed by fluorescent microscope. Bar 10 um.

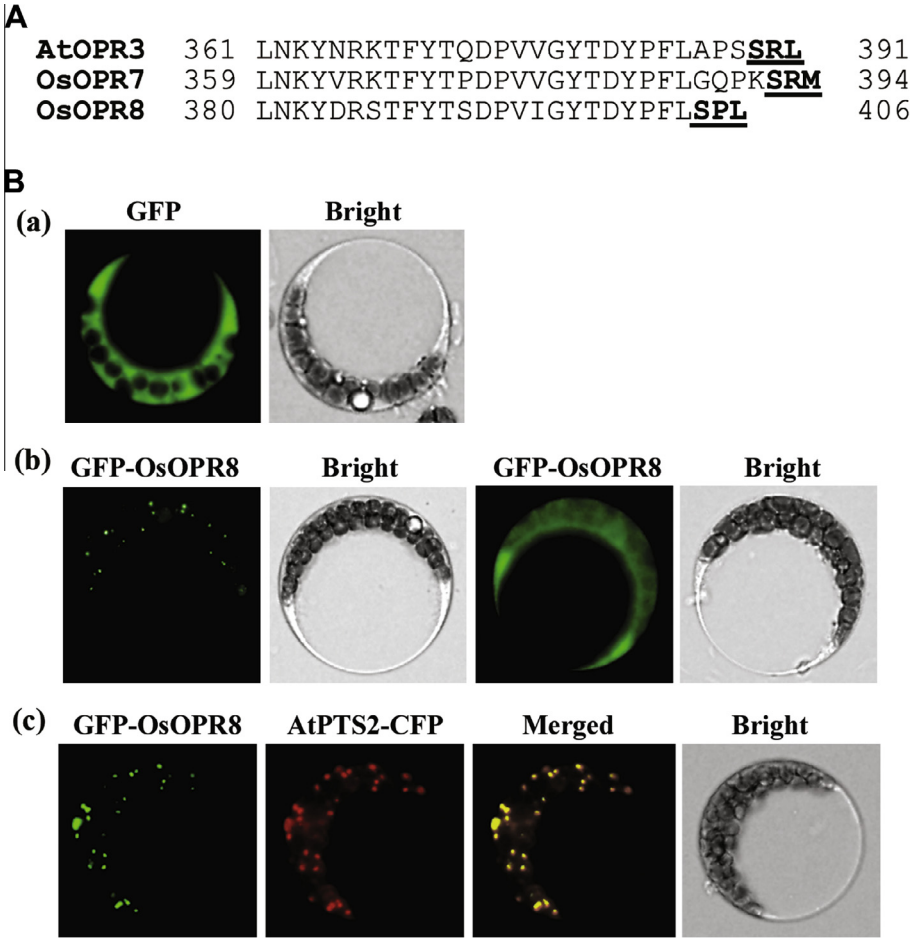

Fig. 2. Subcellular localization of OsOPR8. (A) Amino acid alignment of the C-terminal regions of OsOPR8 with AtOPR3 and OsOPR7. Three amino acids from the C-terminal ends (underline) represent a putative peroxisomal targeting signal. Bar, 10 um. (B) *Arabidopsis* protoplasts were individually transformed with GFP (a) or GFP-*OsOPR8* (b) alone, and GFP fluorescence was examined. (c) Cotransformation of GFP-OsOPR8 with *AtPTS2*-CFP in *Arabidopsis* protoplasts. Bar, 10 um.

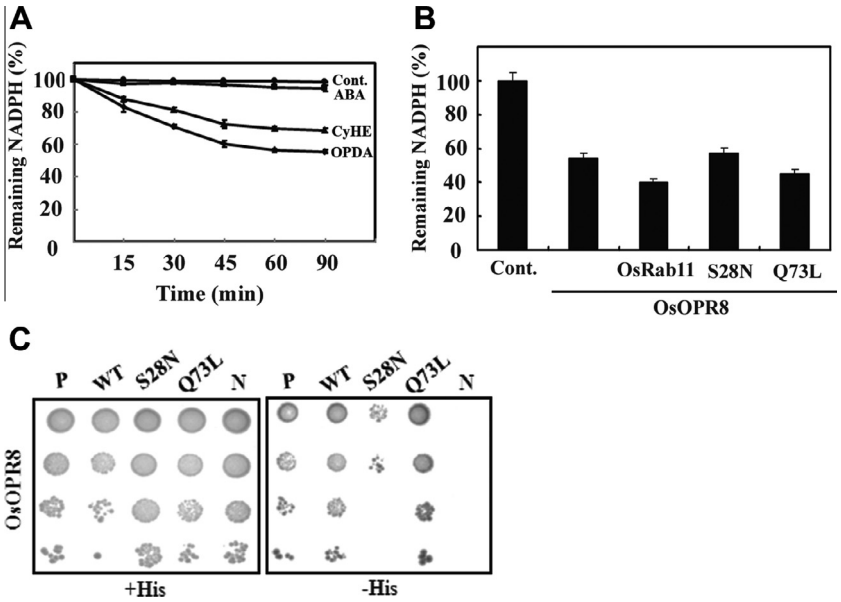

Fig. 3. NADPH-dependent α/β-barrel oxidoreductase activity of OsOPR8 protein. (A) NADPH-dependent-reductase activity. Activity was measured by the consumption of NADPH. The values for the remaining NADPH were obtained spectrophotometrically with each substrate, OPDA, CyHE or ABA and without substrate as control. (B) The effect of OsRab11 in OsOPR8 activity. Reactions were performed as described in (A) in the presence of substrate, OPDA and OsRab11 proteins. (C) Physical interaction test between OsRab11 mutants and OsOPR8. The growth of co-transformants was observed on synthetic medium with (His+) or without histidine (His−). P and N indicate a positive and negative control, respectively, as described in Fig. 1A.

possible substrates. As shown in Fig. 3A and consistent with a previous report [31], NADPH was oxidized when OPDA or CyHE were substrates, indicating that the OsOPR8 protein has NADPH-dependent activity.

We next asked if OsRab11 enhances the NADPH-dependent reductase activity of OsOPR8. When OsRab11 protein and OsOPR8 were both present in the reaction mix, oxidation of NADPH was greater than when only OsOPR8 was included (Fig. 3B). The effects of the dominant negative (S28N) and the constitutively active (Q73L) forms of OsRab11 protein on NADPH oxidation by OsOPR8 were measured. Both OsRab11 Q73L and wild-type OsRab11 enhanced NADPH oxidation compared to the control without any Rab protein, but OsRab11 S28N had little effect on NADPH oxidation.

A yeast two-hybrid assay was performed to assess the interaction between the OsRab11 protein forms used in the previous assay and OsOPR8. As shown in Fig. 3C, the interaction between OsRab11 S28N and OsOPR8 was much weaker than interaction between either the wild-type OsRab11 or the constitutively active OsRab11 Q73L constitutively active and OsOPR8. The correlation of interaction with OsOPR8 and enhanced reductase activity strengthens our conclusion that OsRab11 regulates OsOPR8 and enhances its NADPH-dependent reductase activity.

3.3.1. Transgenic Arabidopsis overexpressing OsRab11 shows a pathogen-resistant phenotype

The physiological role of OsRab11 in the stress response, specifically the response to infection, was next investigated. The expression pattern of *OsRab11* in rice was determined with reverse transcription PCR analysis of total RNAs isolated from various tissues of rice at different stages of development. *OsRab11* was widely expressed in all the tissues, but was weakly expressed in seedlings and leaves (Fig. 4A), indicating that it is not tissue specific. Also, similar to the response of *OsOPR8*, *OsRab11* was induced by JA and elicitor treatment but not induced by SA treatment (Fig. 4B). Next, transgenic plants overproducing OsRab11 were generated. Full-length *OsRab11* cDNA was fused with the cauliflower mosaic virus (CaMV) ^{35}S promoter and transformed into *Arabidopsis*. The expression level of OsRab11 in the transgenic lines was confirmed by RT-PCR (Supplemental Fig. 2) and two representative overexpressing OsRab11 lines (lines 6-1 and 14-1) were chosen for the next experiments. The transgenic and control plants (Col) were inoculated with *Pseudomonas syringae* pv. tomato DC3000 (*Pst*DC3000) by infiltration with the bacteria. Growth of the pathogen in the plant was measured three days later. As shown in Fig 4C, the transgenic plants exhibited elevated resistance to the pathogen *P. syringae* compared to control plants (Fig. 4C).

The expression levels of JA- or SA-regulated genes in non-transgenic and transgenic plants were assessed. In the absence of exogenous JA or wounding, expression of the jasmonate-responsive genes [25,26] *VSP*, *AOS*, *LOXII*, *DHS1*, and *PDF1.2* was elevated in the overexpressing transgenic plants compared to the control plants. However, SA-responsive genes [25], such as *PR1* and *PR5*, were not induced in these plants. This result is reminiscent of the gene expression pattern induced by exogenous jasmonate treatment. The results with the overproducing OsRab11 plants suggest that OsRab11 plays an important role in plant resistance to pathogens through regulating the expression of jasmonate-responsive genes.

4. Discussion

Rab proteins regulate intracellular vesicular trafficking [27], affect plant development [10,11], and participate in defense [4] and stress signaling pathways [5]. In this report, we further characterize a rice Rab protein, OsRab11, with particular emphasis on its role in the defense response. *Rab11* was expressed throughout the plant.

A yeast two-hybrid screen was used to identify OsRab11 target proteins and led to the isolation of OsOPR8, which may be involved

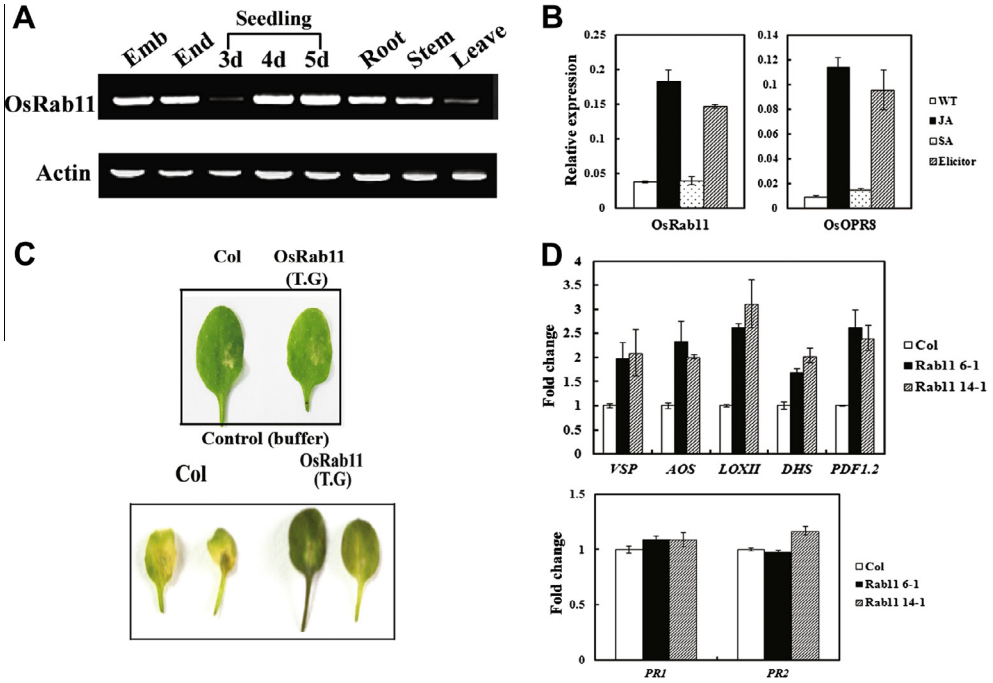

Fig. 4. Overexpressed-OsRab11 *Arabidopsis* transgenic plants exhibit pathogen resistance. (A) Expression pattern of OsRab11 mRNAs in various tissues of rice was confirmed by RT-PCR. The expression of the *Actin* gene was used as an internal control. (B) Expression of pattern of OsRab11 and OsOPR8 mRNAs in response to stimuli. (C) Resistance of the transgenic Arabidopsis against a bacterial pathogen, *P. syringae*. Inoculated plants were grown further for 3 day and inoculated leaves were observed between Col and transgenic plants.

in the JA biosynthesis pathway. *OsOPR8* mRNA was moderately or highly expressed in most tissues except the stem [28]. Over-expressing OsRab11 *Arabidopsis* transgenic plants clearly exhibited enhanced resistance to the pathogen *P. syringae* (Fig. 4C), providing evidence for the involvement of OsRab11 in defense signaling. In the transgenic plants, expression of various jasmonate-responsive genes was elevated in the absence of jasmonate treatment and wounding, whereas there was no elevated expression of SA-responsive genes (Fig. 4D). This suggests that OsRab11 plays an important role in JA-mediated defense signaling.

Several lines of evidence suggest that OsRab11 acts with OsOPR8 to positively regulate JA-mediated signaling through activation of the JA biosynthetic pathway. First, we conclude that OsOPR8 is a target protein of OsRab11. OsOPR8, like OsOPR7, is localized in the cytoplasm and peroxisome (Fig. 2B) [38]; the latter is the site of reduction of OPDA to JA [29] in the JA biosynthetic pathway and an important organelle in JA biosynthesis. OsOPR7 is a regulator of JA biosynthesis [38]. OsOPR8 has the NADPH-dependent reduction activity of α/β-unsaturated aldehydes or ketones like CyHe and OPDA (Fig. 3A) and therefore has the functional capacity to reduce OPDA. Finally, NADPH oxidation by the recombinant OsOPR8 protein was enhanced when either OsRab11 or the constitutively active, GTP-bound form OsRab11 Q78Lwas added to the reaction mix (Fig. 3B), while the dominant negative and GDP-bound form OsRab11 S28N did not increase the activity of OsOPR8 (Fig. 3B). It is possible that OsRab11 cycling between the GTP- and GDP-bound states is required for enhanced OsOPR8 activity in JA biosynthesis.

We suggest that OsRab11 and OsOPR8 function together in JA-mediated defense signaling. OsOPR8 is a direct target of OsRab11. It is likely that OsRab11 is required for activation of NADPH-dependent OPDA reduction activity of OsOPR8 in JA biosynthesis. More studies will be needed to characterize in detail the physiological relationship between OsRab11 and OsOPR8 in rice. Knock-out mutants of both the rice *OsRab11* and *OsOPR8* genes would be useful in determining if the biosynthesis of endogenous JA in rice depends on the activity of OsOPR8 and help of OsRab11.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.04.014.

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