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The germin-like protein OsGLP2-1 enhances resistance to fungal blast and bacterial blight in rice

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Abstract

Key message This is the first report that GLP gene (*OsGLP2-1*) is involved in panicle blast and bacterial blight resistance in rice. In addition to its resistance to blast and bacterial blight, *OsGLP2-1* has also been reported to co-localize with a QTLs for sheath blight resistance in rice. These suggest that the disease resistance provided by *OsGLP2-1* is quantitative and broad spectrum. Its good resistance to these major diseases in rice makes it to be a promising target in rice breeding.

Abstract Rice (*Oryza sativa*) blast caused by *Magnaporthe oryzae* and bacterial blight caused by *Xanthomonas oryzae pv. oryzae* are the two most destructive rice diseases worldwide.

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Germin-like protein (GLP) gene family is one of the important defense gene families which have been reported to be involved in disease resistance in plants. Although GLP proteins have been demonstrated to positively regulate leaf blast resistance in rice, their involvement in resistance to panicle blast and bacterial blight, has not been reported. In this study, we reported that one of the rice GLP genes, *OsGLP2-1*, was significantly induced by blast fungus. Overexpression of *OsGLP2-1* quantitatively enhanced resistance to leaf blast, panicle blast and bacterial blight. The temporal and spatial expression analysis revealed that *OsGLP2-1* is highly expressed in leaves and panicles and sub-localized in the cell wall. Compared with empty vector transformed (control) plants, the *OsGLP2-1* overexpressing plants exhibited higher levels of H_2O_2 both before and after pathogen inoculation. Moreover, *OsGLP2-1* was significantly induced by jasmonic acid (JA). Overexpression of *OsGLP2-1* induced three well-characterized defense-related genes which are associated in JA-dependent pathway after pathogen infection. Higher endogenous level of JA was also identified in *OsGLP2-1* overexpressing plants than in control plants both before and after pathogen inoculation. Together, these results suggest that *OsGLP2-1* functions as a positive regulator to modulate disease resistance. Its good quantitative resistance to the two major diseases in rice makes it to be a promising target in rice breeding.

Introduction

Rice (*Oryza sativa*) blast caused by *Magnaporthe oryzae* and bacterial blight caused by *Xanthomonas oryzae pv. oryzae* are two of the most destructive rice diseases leading to

severe yield losses in rice production worldwide (Hu et al. 2008; Liu et al. 2014). Thus far, though many approaches, such as chemical controls, biological controls, cultivation and disease forecasting, have been widely applied to control these diseases, the most economical and environmentally friendly method is still the application of host resistance (Hu et al. 2008). The host resistance in plants was usually classified into two categories: qualitative (complete) resistance and quantitative (partial) resistance (Kou and Wang 2010; Fu et al. 2011). In most cases, qualitative resistance mediated by major disease resistance (R) genes is specific to pathogen race and is lifetime limited due to the strong selection pressure and the rapid evolution of the pathogen (McDonald and Linde 2002). On the contrary, quantitative resistance conferred by quantitative trait loci (QTLs) is presumably race non-specific and considered to be more durable (Roumen 1994). Thus, there is increasing interest in the development of variety with quantitative and race non-specific resistance in plants (Hayashi et al. 2010).

For the past decades, considerable progress has been made in the study of rice host resistance for blast and bacterial blight. So far, more than 100 R genes for blast resistance and 37 R genes for bacterial blight resistance have been identified and mapped, 23 R genes for blast resistance and 6 R genes for bacterial blight resistance have been cloned (Liu et al. 2014). Marker-assisted selection (MAS) has been successfully used to improve qualitative blast resistance in rice (Jia et al. 2009; Roychowdhury et al. 2012). Furthermore, more than 300 QTLs for quantitative blast resistance (Ballini et al. 2008) and more than 50 QTLs for quantitative bacterial blight resistance (Li et al. 2006) have been identified. However, although quantitative disease resistance is the preferred strategy for sustainable control of plant disease and numerous QTLs have been identified, there are rare successful cases in MAS for quantitative disease resistance. This issue is attributed to the complex genetic control of quantitative resistance and unknown functional genes underlying resistance QTLs. Therefore, isolation of the genes underlying resistance QTLs is the key for effective use of quantitative resistance for disease control.

Map-based cloning has been commonly used for isolation of R genes. However, this strategy is difficult to be used for isolation of the genes for quantitative disease resistance due to their polygenic nature and smaller effect of each QTL (Hu et al. 2008). Therefore, a new strategy should be employed. Recently, increasing evidences have demonstrated the close correlation between defense responsive (DR) genes and quantitative disease resistance in plants. DR genes are predicted to function in plant disease resistance in a quantitative manner, and frequently recognized based on their increased expression pattern during plant defense response (Ramalingam et al. 2003; Hu et al. 2008). A large number of DR genes have been identified in rice and

mapping of these DR genes on molecular linkage maps has indicated that some of the genes co-localize with disease resistance QTLs (Ramalingam et al. 2003; Liu et al. 2004; Wu et al. 2004; Fu et al. 2009; Fukuoka et al. 2009). Our previous research indicated that the five DR genes which encode putative germin-like protein (GLP), dehydrin, PR-1, chitinase, and 14-3-3 protein, accounted for 30.0, 23.0, 15.8, 6.7, and 5.5 % of blast diseased leaf area (DLA) variation, respectively and co-localized with resistance QTL identified by interval mapping (Liu et al. 2004). These findings imply that some of the DR genes could be the candidates underlying disease resistance QTLs. Using this strategy, Hu et al. (2008) have characterized four candidate genes and shown that they could influence the disease phenotypes in the interaction with *Xoo* or *M. oryzae* (Hu et al. 2008), suggesting that the candidate DR gene approach is a good strategy for isolation of disease resistance QTLs and characterization of the contributed DR genes will enhance our understanding of the mechanisms underlying quantitative disease resistance.

GLP gene family is one of the important defense gene families and has been considered to play an important role in several aspects of plant development or stress tolerance (Knecht et al. 2010). It has been considered that GLP genes can function as a cofactor for reinforcement of the cell wall by cross-linking of plant cell wall proteins at the infection site through the production of H_2O_2 due to their OXO or SOD activity (Olson and Varner 1993; Wei et al. 1998). Its product H_2O_2 can also acts as a signal molecule to induce a range of defense responses in a direct or indirect manner (Lane 1994; Zhou et al. 1998; Knecht et al. 2010). Thus, GLPs are expected to play important roles in disease resistance in plants. Indeed, nowadays, more and more evidence have indicated that GLPs are involved in plant basal host resistance (Lane 2002), including the observation that expression of several GLPs are highly induced by pathogen attack, or application of disease resistance-associated chemicals such as hydrogen peroxide (H_2O_2), salicylic acid (SA), and ethylene (Lou and Baldwin 2006; Zimmermann et al. 2006; Godfrey et al. 2007; Manosalva et al. 2009; Himmelbach et al. 2010). Transgenic plants ectopically expressing GLPs have provided direct evidence for their defense role in basal resistance. For example, transient overexpression of *TaGLP4* and *HvGLP4* enhanced resistance against *B. graminis* in wheat and barley, whereas transient silencing the two genes reduced basal resistance in both cereals (Christensen et al. 2004). Similarly, silencing of a GLP gene in *Nicotiana attenuata* improved the performance of native herbivores (Lou and Baldwin 2006).

In rice plants, GLP protein family has many members which are scattered on chr 1, 2, 3, 4, 5, 8, 9, 11 and 12, respectively (Carrillo et al. 2009). Our previous report has indicated that the GLPs on chr 8 were involved in leaf blast resistance (Liu et al. 2004; Manosalva et al. 2009). Moreover,

siRNA-mediated gene silencing of *OsGLP1* (*Os08g35760*) also exhibited increased susceptibility to fungal diseases (Banerjee and Maiti 2010). These results suggest that rice GLP proteins may play important roles in rice disease resistance. However, the functions of the other GLP family members on rice disease resistance are still unknown. Leaf blast and panicle blast are different types of blast disease in rice. In terms of yield loss, panicle blast is more destructive compared to leaf blast (Liu et al. 2016). Inconsistent results between leaf blast resistance and panicle blast resistance were observed in rice production (Zhuang et al. 2002), implying that there may be different mechanisms between leaf and panicle blast resistance. Although the panicle blast resistance is emphasized, the effect of GLPs on panicle blast has never investigated.

Four GLP genes were located on chromosome 2, and they are *Os02g0491600, Os02g0491700, Os02g0491800* and *Os02g0532500*, respectively. In the present study, we identify that the member, *Os02g0532500*, was significantly induced by panicle blast infection and leaf blast infection. We designated this gene as *OsGLP2-1*. To confirm its function on leaf and panicle blast resistance and look insight into its mechanism on blast resistance, we have performed spatio-temporal expression and subcellular localization analysis. Transgenic plants were developed to confirm its functions on blast resistance and bacterial blight resistance as well. The putative molecular mechanisms of *OsGLP2-1* were also investigated in this study.

Materials and methods

Plant materials

Rice cultivars, *Nipponbare* (*ssp. japonica*) and the resistant advanced backcross line *BC10* (*ssp. indica*) were used in this study. All the plants were grown in soil in a greenhouse.

Chemicals treatments

The experiments were performed in wild-type *Nipponbare* plants using the same method as indicated previously with minor modifications (Liu et al. 2016). Mature seeds of *Nipponbare* were incubated at 49 °C for 4 days prior to germination, and then seeds were soaked in distilled water for 2 days in room temperature. Next, the seeds were embedded in wet towel for another 2 days in 32 °C for germination. Germinated seeds were then placed in gauze and transferred to salver for incubation in a growth chamber at 26 °C, 75 % relative humidity, 16,000 Lux and a 16 h/light and 8 h/dark interval. Rice seedlings at three- to four-leaf stage were sprinkled onto leaves with different plant hormone solutions in a concentration of 100 µM. For hormone treatment on the

panicle tissue at the heading stage, the same cotton-wrapping inoculation method was used as for panicle blast inoculation. All the treatment experiments were repeated thrice.

Subcellular localization of GLP protein

The protein coding region of *OSGLP2-1* was amplified from *BC10* using the following primers. Forward primer, 5′-ATGGCGCATCGTCGTCGTT-3′; reverse primer, 5′-ATCCTCCATCTCCGCCTTCTT-3′. Then the product was cloned into the pGY1-mcherry vector to generate the GLP-mcherry fusion gene. The empty pGY1-mcherry plasmid was used as the control. For the transient expression assay, 1 µg of plasmid DNA was introduced into onion epidermal cells using the PDS-1000/He particle delivery system (Bio-Rad, Hercules, CA, USA). After 24 h incubation in the dark, onion epidermal cells were observed under a laser confocal microscopy (Zeiss LSM710, Germany).

Gene transcription analysis

Plant tissues were grinded with liquid nitrogen and total RNA was extracted using RNAiso Plus (Takara, Japan). Then, 1 µg total RNA was reverse-transcribed to cDNA using the primescript™ RT reagent kit (Takara, Japan). Real-time PCR was performed using SYBR Premix ExTaq™ (Takara, Japan) according to the manufacturer's instructions. EF1α gene was used as an internal control. The resulting melting curves were analyzed to ensure specificity of product detection. Each Experiment was performed in triplicate, and the result was represented by the mean ± standard derivation (SD). The Gene-specific primers that were used are listed in Supplemental Table 1.

Vector constructs and rice transformation

For constructing the *OsGLP2-1* overexpressing (*OXGLP2-1*) plants, the cDNA sequence of *OsGLP2-1* was amplified from a rice blast resistant cultivar *BC10* by RT-PCR using the following primers. Forward primer, 5′-AGTGACAGAACGAGCGTAGAAT-3′; reverse primer, 5′-TCACTAACGGGGAGTAACCTAA-3′. The cDNA product was then inserted into pHQSN (modified from pCAMBIA1390) which harbors a CaMV*35S* promoter, and the constructed vector was electroporated into *Agrobacterium tumefaciens EHA105*. Rice transformation was performed as described with minor modifications (Toki et al. 2006).

Southern blot analysis

Genomic DNA was isolated from 1 g of 2-week-old rice seedlings using the CTAB method. After digested overnight by EcoR1, DNA samples (5–10 µg) were separated by

electrophoresis on 0.8 % agarose gel, followed by transferring to Hybond nylon membrane (Amersham, GE Healthcare Limited, Buckinghamshire, UK). Labeling of hygromycin probe and detection of the signals were carried out using the DIG high prime DNA labeling and detection starter kit 1 (Cat. No: 11745832910; Roche Diagnostics, Basel, Switzerland) according to the manufacturer's protocol.

Pathogen inoculations

The control and transgenic plants were grown in soil in greenhouse. Leaf blast and panicle blast inoculations were conducted using the same method as described previously with some modifications (Liu et al. 2016). Concisely, seedlings at three- to four-leaf stage were inoculated by spraying with spore suspension of 1×10^6 spores/ml of a *Magnaporthe oryzae* isolate GD08-T13. Disease was assessed 6 days after inoculation by measuring the DLA. For panicle blast inoculation, the upper-middle part of a panicle was wrapped by cotton in 1 to 2 days after heading and 2 ml spore suspension of 1×10^6 spores/ml of GD08-T13 was injected into the cotton and the cotton was then wrapped by foil. Each inoculated panicles were sprayed with water for 2–3 min every 2 h to maintain the humidity. Disease was assessed in

3 weeks after inoculation by measuring the percent infected main axis length. Each treatment was repeated twice.

To evaluate bacterial blight disease, plants were inoculated with *X. oryzaepv. Oryzae* Chinese *Xoo* race 4 at the booting stage by the leaf-clipping method (Kauffman et al. 1973). Disease was assessed by measuring the percent lesion length (diseased leaf length/total leaf length) at 2 weeks after inoculation.

Measuring the level of H_2O_2 and detection of SOD enzyme activity

The concentration of H_2O_2 was measured using the hydrogen peroxide assay kit (Beyotime Institute of Biotechnology, China). The leaves of control and *OXGLP2-1* plants at three- to four-leaf stage both before and after blast inoculation were collected and grinded with liquid nitrogen. Then 200 mL of the lysis buffer solution was added to per 10 mg dry powder. After mixing well, the supernatants were collected by centrifuging at 12,000g for 5 min. Next, 50 μL of the supernatants and 100 μL of test solutions were immediately added to the test-tubes and placed at room temperature for 30 min. H_2O_2 production was monitored by measuring the absorbance at 560 nm using a Thermo Scientific Multiskan Spectrum (Thermo, USA).

The same samples that used for H_2O_2 quantification were used for SOD activity analysis. Total SOD activity was measured with the WST-8 method using the detection kit according to the manual (Cat. NO: S0101, Beyotime Institute of Biotechnology, China).

***Cis*-elements analysis for the promoter**

About 1500 bp sequence upstream of *OsGLP2-1* was downloaded from MSU Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/) for *cis*-elements analysis. The sequence was scanned by PLACE (http://www.dna.affrc.go.jp/PLACE/) which is a database of nucleotide sequence motifs found in plant cis-acting regulatory DNA elements.

Jasmonic acid (JA) quantification by liquid chromatography tandem mass spectrometry (LC-MS)

The JA was extracted from the rice leaf according to Pan et al. (2010), then 10 μL of each JA sample solution was injected onto a C18 column (AQUITY UPLC BEH 130, 1.7 μm, 2.1 by 100 mm, Waters) at a flow rate of 0.1 mL/min, and the column was maintained at 30 °C. The JA sample solution was separated by reversed phase ultra-fast LC (Shimadzu, Kyoto) with a multi-step linear gradient elution using solution A (water with 5 mM ammonium formate and 0.1 % formic acid) and solution B (Methanol, with 5 mM ammonium formate and 0.1 % formic acid) over 30 min, as follows: 30 % solution B at 0–2 min, 30–100 % solution B at 2–20 min; 100 % solution B
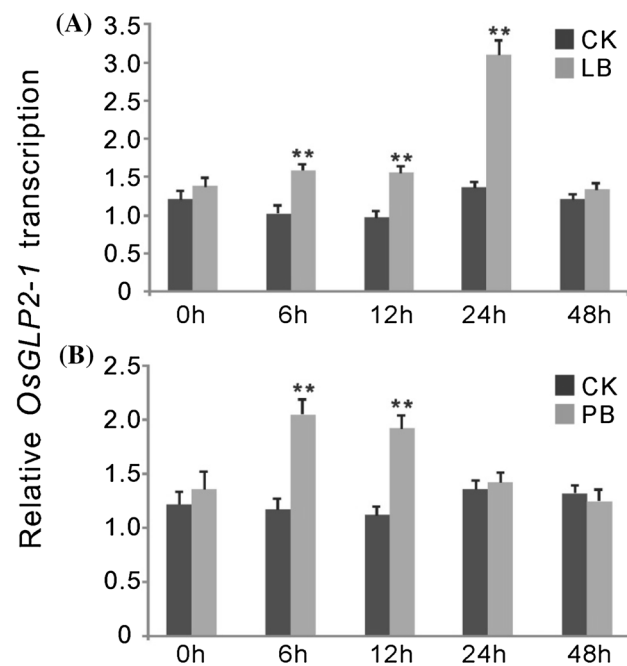

Fig. 1 Expression pattern of the *OsGLP2-1* gene after inoculation with blast disease as assessed by Quantitative RT-PCR; CK = water treatment, LB = leaf blast inoculation and PB = panicle blast inoculation. 0, 6, 12, 24 and 48 h indicate the time after inoculation. *Error bars* indicate the standard deviation (SD) from three biological replicates, and *asterisks* indicate statistically significant differences compared with water treatment (*t* test, **P < 0.01). **a** Expression pattern of the *OsGLP2-1* gene after inoculation with leaf blast. **b** Expression pattern of the *OsGLP2-1* gene after inoculation with panicle blast

Fig. 2 Tissue expression of *OsGLP2-1* in rice plants and subcellular localization of this protein. **a** Quantitative RT-PCR analysis of *OsGLP2-1* transcription in different tissues of *Nipponbare* plants. *Error bars* indicate the SD from three biological replicates. **b** Laser confocal microscopy images deriving from mcherry of onion epidermal cells transiently expressing mcherry or mcherry–*OsGLP2-1* fusion protein. *Bar* 2 μm. *Arrow* indicates the nucleus. Cell plasmolysis was induced by a 30 % sucrose solution for 7 min before observation
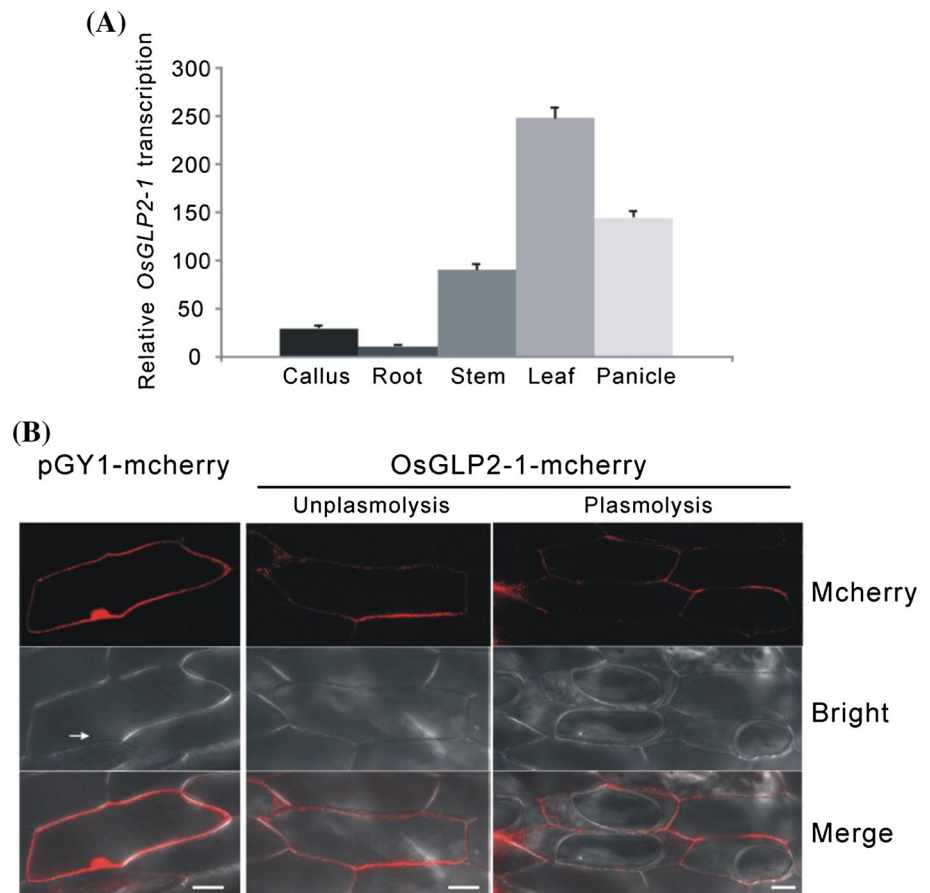
at 20–22 min, 100–30 % solution B at 22–25 min; and 30 % solution B at 25–30 min. The eluate was then introduced into the electrospray ion source of a tandem triple quadrupole MS analyser (API4000, AB SCIEX, Foster City, CA), and the JA compound was quantified in multiple reaction monitoring (MRM) mode using optimized MS/MS conditions, which were listed in Supplemental Table 2. The MS conditions were as follows: source, Turbo IonSpray; ion polarity, negative; IonSpray voltage, −4500 V; source temperature, 550 °C; gas, nitrogen; curtain gas, 30 psi; nebulizing gas (GS1), 55 psi; Collision gas (GS2), 55 psi; scan type, MRM; Q1 resolution: unit; Q3 resolution: unit. The Analyst 1.5.2 software (AB SCIEX, Foster City, CA) was used to control the instrument and to acquire and process all of the MS data.

Results

Transcription of *OsGLP2-1* is induced after rice blast infection

Our previous microarray-assisted gene expression profiling of a blast resistant advanced backcross line (*BC10*) inoculated with blast isolate revealed that the GLP gene, *Os02g0532500*, designated as *OsGLP2-1* was significantly induced by both leaf blast infection and panicle blast infection within a period of 48 h (data not shown). In the present study, real-time PCR was adopted to confirm this result. Our result showed that the transcription level of *OsGLP2-1* was significantly increased at 6 and 12 h both in the leaf blast inoculation and panicle blast inoculation, whereas it returned to the basal level at 24 h during the panicle blast inoculation and remained highly induced in leaf blast inoculation (Fig. 1). These results suggest that *OsGLP2-1* is responsive to leaf and panicle blast infection and may play an important role in rice blast disease resistance.

OsGLP2-1 is highly expressed in leaves and seedlings, and the protein is localized in the cell wall

To understand the biological functions of the *OsGLP2-1* gene in rice, we examined its temporal and spatial transcription in different rice tissues using the rice EF1α gene as the internal control. We identified that *OsGLP2-1* was expressed throughout the entire life cycle of rice plant and in almost all rice tissues examined, with a relatively higher expression levels in leaves and panicles (Fig. 2a). To determine the subcellular localization of *OsGLP2-1* in rice cells, we fused the coding region of *OsGLP2-1* with the red fluorescent protein (mcherry) fragment under the control of the cauliflower
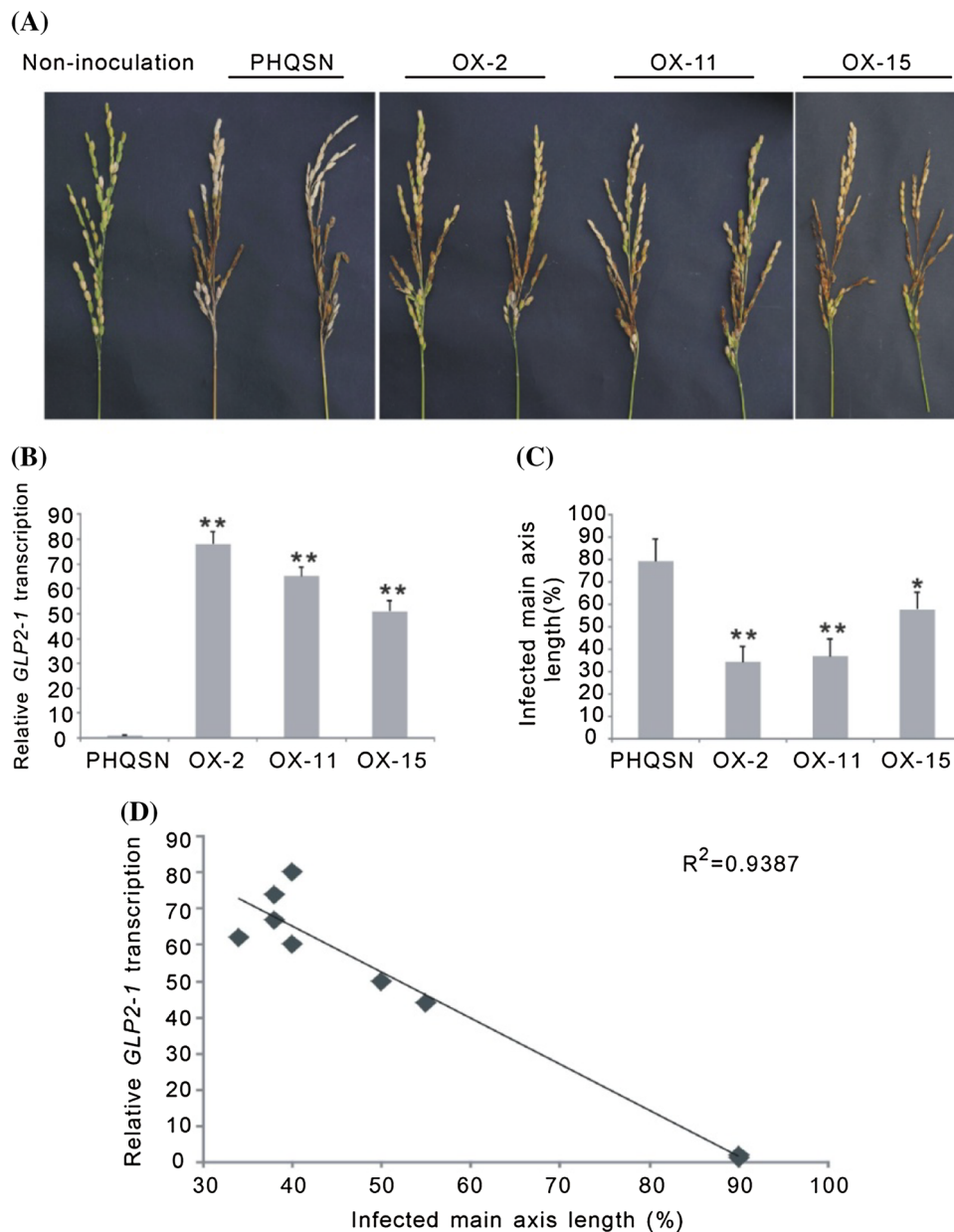
(A)

(B)

(C)

(D)

Fig. 3 Phenotypes of the *OsGLP2-1* overexpressing (*OXGLP2-1*) plants to panicle blast infection. OX-2, OX-11 and OX-15 are three independent homozygous transgenic lines of *OXGLP2-1* in T$_2$ generation. **a** Disease phenotypes of the *OXGLP2-1* plants and PHQSN (control) plants after inoculation with *M. oryzae* isolate GD08-T13 using the cotton-wrapping method at the heading stage. **b** Transcription analysis of the *OXGLP2-1* plants by quantitative RT-PCR. *Error bars* indicate the SD from three biological replicates, and *asterisks* indicate statistically significant differences compared with control (PHQSN)

plants (*t* test, **$P < 0.01$). **c** Relative diseased main axis length in the *OXGLP2-1* plants and control plants after panicle blast infection. *Error bars* indicate the SD from at least ten biological replicates, and asterisks indicate statistically significant differences compared with control plants (*t* test, **$P < 0.01$; *$P < 0.05$). **d** The enhanced resistance co-segregated with the increased level of *OsGLP2-1* in transgenic plants (T$_2$ generation) after panicle blast inoculation. The correlation coefficient was calculated by linear regression

mosaic virus 35S promoter and expressed it transiently in onion epidermal cells via the particle bombardment method (Shaner et al. 2004). Laser confocal microscopy showed that the red fluorescent signal was localized in the cell wall of the transfected onion epidermal cells, whereas the red fluorescent signal in the mcherry control vector was distributed both in the nucleus and the cytoplasm (Fig. 2b).

Overexpression of *OsGLP2-1* in transgenic rice quantitatively enhances resistance to blast disease

To elucidate the putative function of *OsGLP2-1* on rice blast disease, transgenic rice plants constitutively overexpressing *OsGLP2-1* were produced in *Nipponbare*, which was highly susceptible to blast isolate GD08-T13 in both leaf
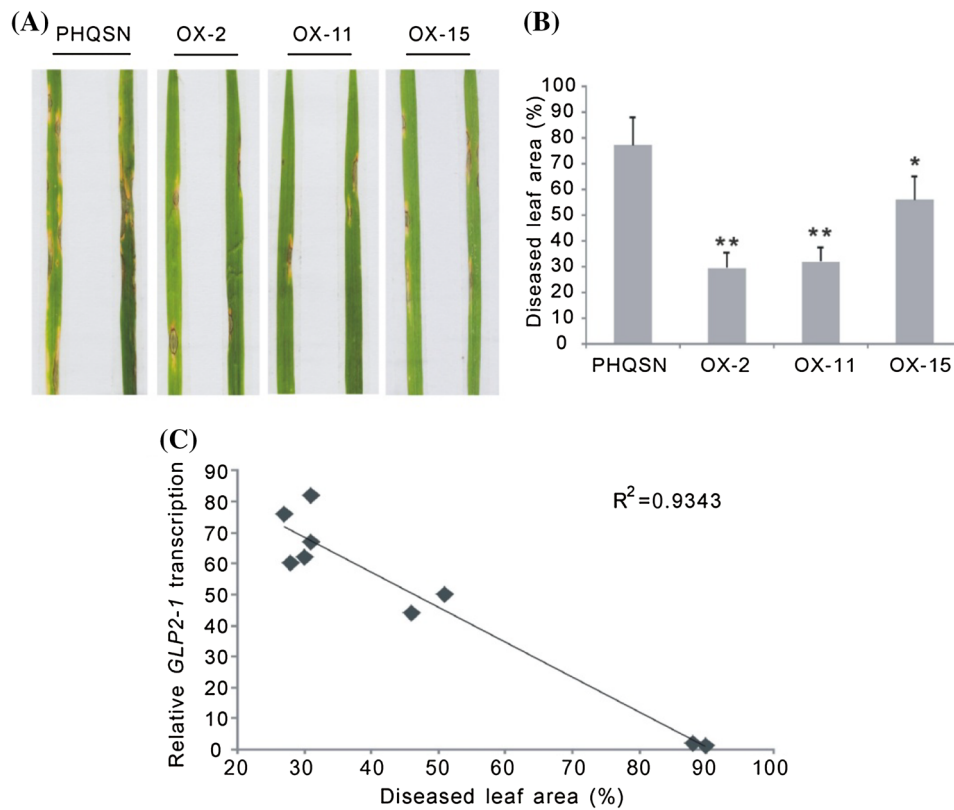
Fig. 4 Phenotypes of the *OsGLP2-1* overexpressing (*OXGLP2-1*) plants to leaf blast infection. **a** Disease phenotypes of the *OXGLP2-1* plants (T$_2$ generation) and control plants after spray inoculation with *M. oryzae* isolate GD08-T13 for 7 days at the three- to four-leaf stage. **b** Relative diseased leaf area (DLA) in the *OXGLP2-1* plants and control plants after leaf blast inoculation. *Error bars* indicate the SD from at least ten biological replicates, and *asterisks* indicate statistically significant differences compared with control plants (*t* test, *$P < 0.05$, **$P < 0.01$). **c** The enhanced resistance co-segregated with the increased level of *OsGLP2-1* in transgenic plants (T$_2$ generation) after leaf blast inoculation. The correlation coefficient was calculated by linear regression

and panicle blast. Eighteen transgenic lines were obtained. The transgenic plants (*OXGLP2-1*) showed no morphological changes and were fertile. We used the resistance gene hygromycin to screen for putative overexpressing lines (Supplemental Fig. 1) and southern hybridization was used to confirm *OsGLP2-1* transgene integration in rice genome (Supplemental Fig. 2). The evaluation of blast resistance showed that the T$_0$ plants showed significantly enhanced resistance to panicle blast, with lower percentage of infected main axis length compared with wild type (Supplemental Fig. 3). The real-time PCR experiment indicated that the enhanced resistance was associated with the increased expression of *OsGLP2-1* ($R^2 = 0.8115$; Supplemental Fig. 1). To validate that the overexpression of *OsGLP2-1* accounts for the increased resistance of the transgenic plants, three independent homozygous lines (OX-2, OX-11, OX-15; T$_2$ generation) were further analyzed individually for their resistance to GD08-T13 and the *OsGLP2-1* expression levels (Fig. 3b). Our result showed that the enhanced resistance could be stably inherited in these plants, with the infected main axis length ranging from 34.3 to 57.8 % (average of 43 %) in the transgenic plants, compared with 79.4 %

for the empty vector transformed plants (PHQSN) (Fig. 3a, c). The transcript level analysis showed that the enhanced resistance was associated with overexpression of *OsGLP2-1* in these transgenic lines ($R^2 = 0.9387$; Fig. 3d).

The three transgenic lines (OX-2, OX-11, OX-15) were also subjected to blast inoculation at the three- to four-leaf stage. The DLA ranged from 29.61 to 56.02 % (average of 39.23 %) in transgenic plants, compared with 77.35 % for PHQSN control plants (Fig. 4a, b). The enhanced resistance of the plants correlated with over-presented expression of *OsGLP2-1* ($R^2 = 0.9343$; Fig. 4c; Supplemental Table 3). Taken together, these results showed that overexpression of *OsGLP2-1* quantitatively enhances resistance to both leaf blast and panicle blast.

Overexpression of *OsGLP2-1* in transgenic rice quantitatively enhanced resistance to bacterial blight

Bacterial blight caused by *Xanthomonas oryzae pv. oryzae (Xoo)* is another devastating rice disease worldwide. Previous research has indicated that fungal blast resistance and bacterial blight resistance might share common pathways
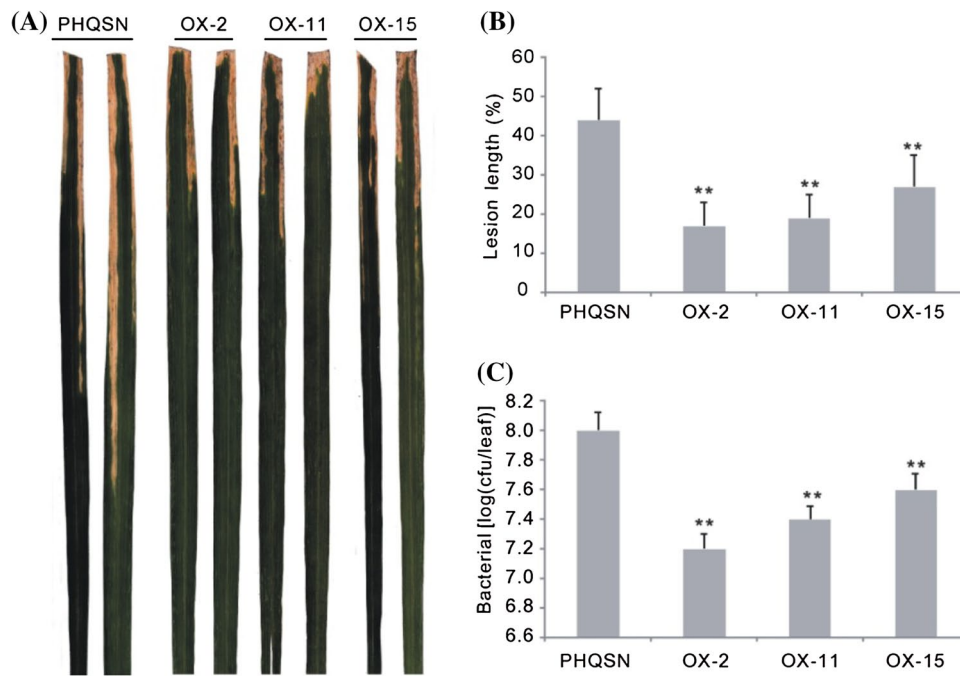
Fig. 5 Phenotypes of the *OXGLP2-1* plants to bacterial blight infection. **a** Disease phenotypes of the *OXGLP2-1* plants and control plants after *Xoo* inoculation. **b** Relative lesion length in the *OXGLP2-1* plants and control plants after *Xoo* inoculation. *Error bars* indicate the SD from at least ten biological replicates, and *asterisks* indicate statistically significant differences compared with control plants (*t* test, ***P* < 0.01). **c** Growth of *Xoo* race 4 in leaves of *OXGLP2-1* plants and control plants. Bacterial populations were determined from three leaves 16 days after inoculation by counting colony-forming units (cfu). Similar results were obtained in two independent biological experiments. *Error bars* indicate the SD from six biological replicates, and *asterisks* indicate statistically significant differences compared with control plants (*t* test, ***P* < 0.01)

to some extent (Hu et al. 2008). To see if *OsGLP2-1* gene also functions on bacterial blight, we evaluated the bacterial blight resistance of the *OXGLP2-1* (*OsGLP2-1* overexpressing) plants by inoculation with an isolate from Chinese *Xoo* race 4. Our results showed that the *OXGLP2-1* plants showed significantly enhanced resistance to bacterial blight, with lesion length ranging from 17 to 27 % (average of 21 %), compared with 44 % for PHQSN control plants (Fig. 5a, b). The growth rate of bacteria in *OXGLP2-1* plants was 2.4- to 3.2-fold lower than that in control plants (Fig. 5c). These results suggest that overexpression of *OsGLP2-1* can also quantitatively enhances resistance to bacterial blight.

Overexpression of *OsGLP2-1* results in accumulation of H_2O_2 in transgenic rice

GLPs have been proposed to exhibit superoxide dismutase (SOD) activity leading to hydrogen peroxide (H_2O_2) production, which is often correlated with plant defense responses (Lane 1994; Christensen et al. 2004; Laloi et al. 2004; Zimmermann et al. 2006). To confirm if the enhanced disease resistance by *OsGLP2-1* is due to the accumulation of H_2O_2, we determined the H_2O_2 content using the xylenol orange method (Kim and Hwang 2014). An accumulation of H_2O_2 was identified at 48 h after pathogen inoculation

both in the control and *OXGLP2-1* plants (Fig. 6). However, the H_2O_2 content was significantly higher in *OXGLP2-1* plants than in control plants both before and after pathogen inoculation (Fig. 6a). These results suggest that overexpression of *OsGLP2-1* results in H_2O_2 accumulation in *OXGLP2-1* plants. To further validate that if the generation of H_2O_2 in the transgenic plants was due to the SOD activity of *OsGLP2-1*, the SOD enzyme activity was analyzed in the leaves of the same plants that used for H_2O_2 quantification. As shown in Fig. 6b, the SOD activity was higher in *OsGLP2-1* overexpressing plants than in control plants both before and after blast inoculation, indicating that *OsGLP2-1* harbors SOD activity.

OsGLP2-1 modulates the transcription of defense-related genes that are involved in JA dependent pathway in rice

To identify the regulatory components and understand the mechanism of *OsGLP2-1* in regulation of disease resistance in rice, about 1500 bp promoter sequences from the translational start site of *OsGLP2-1* were analyzed for *cis*-elements. We observed three GCCCOREs (Supplemental Table 4), which has been shown to function as a jasmonic acid (JA) responsive element that is necessary for
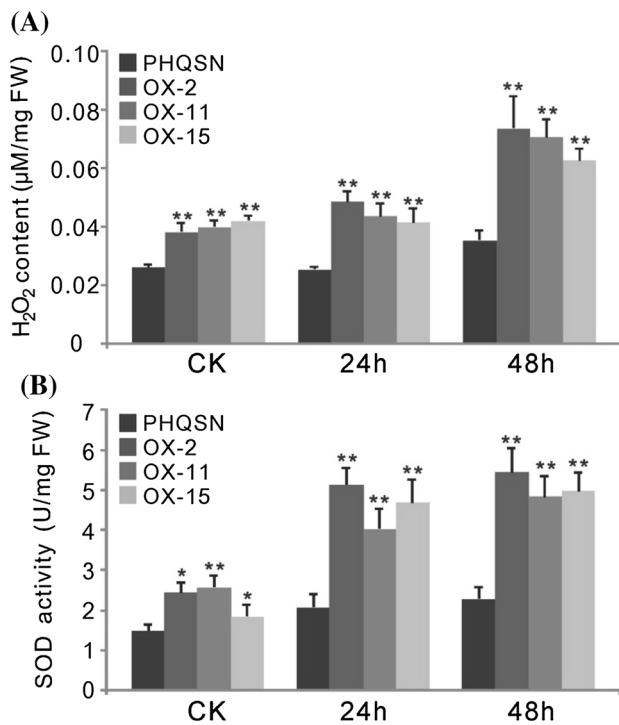
Fig. 6 Analysis of the hydrogen peroxide (H_2O_2) content and SOD activity in the leaves of *OXGLP2-1* plants and control plants. *Error bars* indicate the SD from at least ten biological replicates, and *asterisks* indicate significant differences between transgenic and control plants before blast inoculation (CK) and at 24 and 48 h after inoculation with *M. oryzae* (t test, **$P < 0.01$ and *$P < 0.05$). *FW* fresh weight. **a** The H_2O_2 content in the leaves of *OXGLP2-1* plants and control plants. **b** The SOD activity in the leaves of *OXGLP2-1* plants and control plants

the regulation of transcription by JA (Brown et al. 2003). These response elements of JA in the promoter region of *OsGLP2-1* imply that this hormone may act upstream of *OsGLP2-1* to modulate its activity and then ultimately regulate rice blast resistance. To confirm this, the wild-type *Nipponbare* plants were treated with exogenous JA at the seedling stage and heading stage respectively. The expression levels of *OsGLP2-1* at 3, 6, 12 and 24 h after treatment were analyzed using RT-PCR. The results indicated that the transcription levels of *OsGLP2-1* were increased at the four time points after JA treatment in *Nipponbare* both at the leaf and panicle tissue (Fig. 7). These observations indicate that JA may act upstream of *OsGLP2-1* to induce its transcription.

To further validate if *OsGLP2-1* mediated disease resistance is involved in JA signaling pathway, we analyzed the expression patterns of some well-characterized defense-related genes which are involved in JA-dependent pathway, including the lipoxygenases (*LOX1* and *LOX11*), an allene oxide synthase 2 (*AOS2*) gene and a pathogenesis-related [PR] gene *PR10* (Deng et al. 2012; Liu et al. 2016; Mei et al. 2006). The pathogen infection

strongly induced the expression of *LOX1, AOS2* and *PR10* in both the control (PHQSN) and *OsGLP2-1* overexpressing plants both in the seedling and panicle tissue but the expression levels of *LOX1, AOS2* and *PR10* were significantly higher in *OXGLP2-1* plants than in control plants after inoculation (Fig. 8a; Supplemental Fig. 4). Only significant difference was identified for *LOX1* between the control and *OXGLP2-1* plants at the seedling stage before pathogen inoculation (Fig. 8a), and there was no obvious difference in expression level of *LOX11* between transgenic plants and control plants either before or after pathogen inoculation (data not shown). These results suggest that *OsGLP2-1* mediated disease resistance is involved in the JA signaling pathway.

OsGLP2-1 influences the accumulation of JA

To gain further insight into the relationship between *OsGLP2-1* and the JA signaling pathway, we quantified the concentrations of the endogenous JA in the leaves of control and *OXGLP2-1* plants both before and after inoculation with blast isolate GD08-T13. The endogenous level of JA was significantly induced by pathogen inoculation in both the control and *OXGLP2-1* plants, but the endogenous JA level was significantly higher ($P < 0.01$) in *OXGLP2-1* plants than that in control plants both before and after pathogen inoculation (Fig. 8b). These results further indicate that *OsGLP2-1* mediated disease resistance is indeed associated with the JA signaling pathway.

Discussion

OsGLP2-1 quantitatively enhances disease resistance in rice and the resistance is broad spectrum

GLPs encoded by a heterogeneous group of genes present in many land plants including monocots, dicots, gymnosperms and moss (Davidson et al. 2009). Many *GLP* genes have been reported to play important roles in disease resistance in plants (Christensen et al. 2004; Lou and Baldwin 2006; Zimmermann et al. 2006; Godfrey et al. 2007; Manosalva et al. 2009; Himmelbach et al. 2010). In rice, there are 41 GLPs that are scattered on different chromosomes, respectively (Carrillo et al. 2009). However, only the GLPs on chr 8 have been confirmed their functions on leaf blast resistance and sheath blight resistance (Manosalva et al. 2009). In this study, we have demonstrated that one member of GLPs on chr 2, *OsGLP2-1*, was significantly induced by leaf blast and panicle blast infection (Fig. 1). By overexpressing *OsGLP2-1*, we were able to generate enhanced resistance to leaf blast, panicle blast and bacterial blight in rice (Figs. 3, 4, 5). These results demonstrate that *OsGLP2-1* functions as
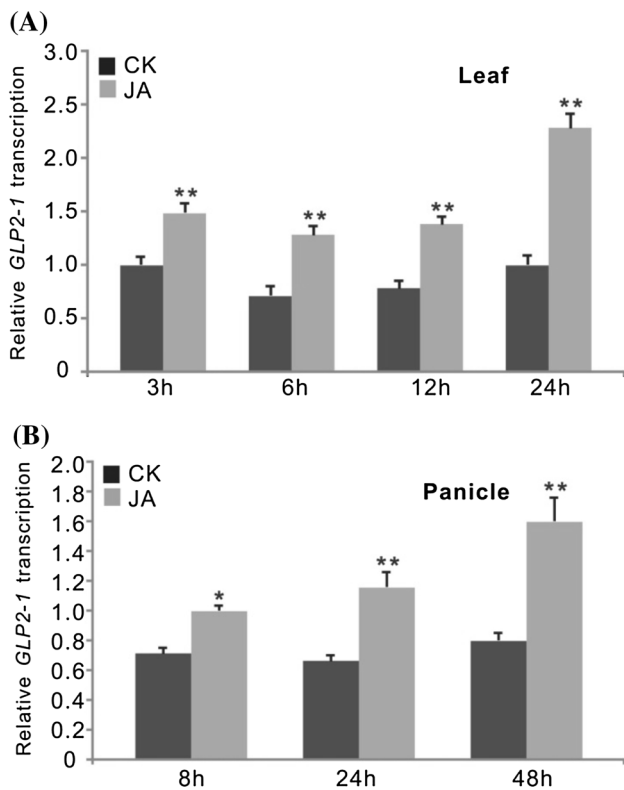
(A)

(B)

Fig. 7 Expression analysis of *OsGLP2-1* after JA (jasmonic acid) treatment in *Nipponbare* plants. *Error bars* indicate the SD from three biological replicates, and *asterisks* indicate statistically significant differences compared with water treatment (*t* test, **$P < 0.01$; *$P < 0.05$). **a** Time-course transcription analysis of *OsGLP2-1* after JA and water (CK) treatments by quantitative RT-PCR analysis at the seedling stage in *Nipponbare* plants. **b** Time-course transcription analysis of *OsGLP2-1* after JA and water treatments by quantitative RT-PCR analysis at the heading stage in *OXGLP2-1* plants

a positive regulator in blast and bacterial blight resistance in rice. To our knowledge, this study provides the first evidence that *GLP* genes are also involved in panicle blast and bacterial blight resistance. Furthermore, our results showed that the enhanced disease resistance correlated with increasing transcription levels of this gene (Figs. 3, 4). The *OsGLP2-1* overexpressing rice plants displayed less DLA and lower percent infected main axis length during leaf and panicle blast infection, and lower percent lesion length and fewer spores during bacterial blight infection compared with the control plants (Figs. 4, 5), exhibiting the nature of quantitative resistance. In addition to its resistance to blast and bacterial blight, *OsGLP2-1* has been reported to co-localize with a QTLs for sheath blight resistance in rice (Davidson et al. 2009). Taken together, these results suggest that the disease resistance provided by *OsGLP2-1* is quantitative and broad spectrum. These characteristics make *OsGLP2-1* to be a good candidate in rice improvement for disease resistance in rice plants.

The broad spectrum of disease resistance of *OsGLP2-1* is associated with the generation of H_2O_2 and the gene expression patterns

H_2O_2 has been proved to kill pathogens directly and also plays a key role in reinforcement of the cell wall by cross-linking of plant cell wall proteins in papillae at the infection sites (Olson and Varner 1993; Wei et al. 1998). The previous study has demonstrated that the enhanced resistance by over-expressing *BvGLP-1* in *Arabidopsis* was correlated with the elevated H_2O_2 content (Knecht et al. 2010). Moreover, the enhanced resistance of *GF14e* silenced plants was attributed to the higher expression of peroxidase gene (*POX22.3*), and accumulation of reactive oxygen species (ROS), such as H_2O_2 (Manosalva et al. 2011). Based on these results, a possible explanation for the function of *OsGLP2-1* on disease resistance in rice could be the generation of H_2O_2 through its enzymatic activity (OxO or SOD) in plant cells. Indeed, in the present study, accumulation of H_2O_2 was observed in the *OsGLP2-1* overexpressing plants duing to its SOD activity (Fig. 6), and our sub-cellular localization experiment also indicated that *OsGLP2-1* localized in the cell wall (Fig. 2b). Furthermore, we also observed a drastic reduction in the amount of spores of *Xoo* in the infected leaves of the *OsGLP2-1* overexpressing plants compared to the control plants (Fig. 5c), indicating the strong inhibition of the bacterial propagation in transgenic rice leaves. From these results, it can be deduced that the accumulated H_2O_2 may contribute to the reinforcement of cell wall to protect the cell from pathogen penetration and may also act as a toxin to prevent pathogen propagation. Moreover, the expression pattern analysis revealed that *OsGLP2-1* was expressed throughout the life cycle of the rice plants and in almost all rice tissues examined (Fig. 2a). The expression pattern of *OsGLP2-1* agrees with its resistance to both leaf blast and panicle blast. Together, we can conclude that the broad spectrum of disease resistance conferred by *OsGLP2-1* is associated with the generation of H_2O_2 and its expression pattern.

OsGLP2-1 mediated disease resistance is involved in JA signaling pathway

JA and SA are the well-known endogenous signal molecules that involved in the regulation of resistance against different pathogens. In the present study, the *cis*-element analysis revealed that there were JA response elements in the promoter region of *OsGLP2-1* (Supplemental Table 4) and the expression of *OsGLP2-1* was induced by JA treatment in *Nipponbare* plants (Fig. 7). In contrast, the transcription of *OsGLP2-1* exhibited no obvious difference when subjected to exogenous SA treatment in *Nipponbare* plants
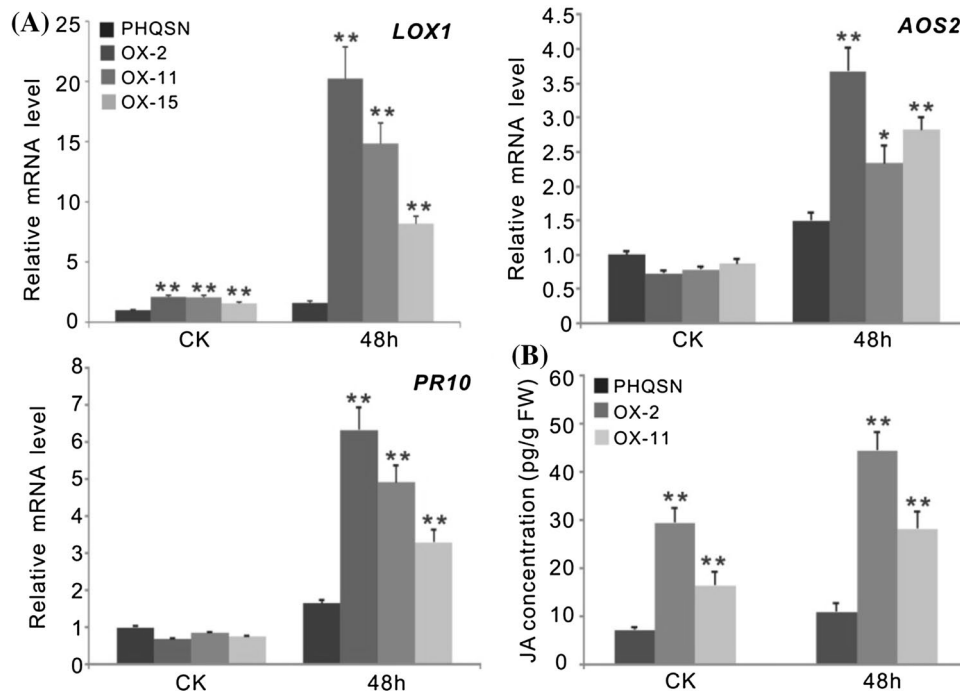
Fig. 8 *OsGLP2-1* mediated disease resistance is involved in the JA signaling pathway. *Asterisks* indicate significant differences between transgenic and control plants before blast inoculation (CK) and at 48 h after inoculation with *M. oryzae* (t test, **$P < 0.01$; *$P < 0.05$).

Expression data are the means of three replicates ± SD. **a** Overexpressing of *OsGLP2-1* influences the expression of a set of defense-related genes at the seedling stage. **b** Overexpressing of *OsGLP2-1* leads to the accumulation of endogenous JA

(Supplemental Fig. 5). These results imply that *OsGLP2-1* mediated disease resistance may be regulated by JA but not SA. Furthermore, our results showed that the expression levels of the defense-related genes, *LOX1, AOS2* and *PR10* which are involved in JA-dependent pathway, were significantly higher in *OsGLP2-1* transgenic plants than in control plants after pathogen infection, which results in a more active JA signaling pathway in *OsGLP2-1* over-expressing plants (Fig. 8). Contrastingly, the expression levels of *NH1* and *PAL1*, which are associated with SA-dependent pathway, were not significantly different between transgenic plants and control plants after pathogen infection (Supplemental Fig. 6). These observations are consistent with the results from hormone treatments, suggesting that *OsGLP2-1* mediated disease resistance is involved in JA signaling pathway but not the SA-dependent pathway.

Generally, plant resistance to necrotrophic pathogens is frequently regulated by the JA-dependent pathway, whereas resistance to biotrophic and hemibiotrophic pathogens is frequently controlled by the SA-dependent pathway (Bari and Jones 2009). Blast fungus is a hemibiotrophic pathogen and *Xoo* is a biotrophic pathogen. Thus, we expect that *OsGLP2-1* mediated resistance to blast and bacterial blight is modulated by the SA-dependent pathway. However, the results presented here suggest that *OsGLP2-1* mediated resistance to blast and bacterial blight was involved in the activation of JA-dependent pathway instead of SA-dependent pathway.

In summary, in this study, we have confirmed the functions of *OsGLP2-1* on disease resistance through differential expression and transgenic method and investigated its possible regulatory mechanism. Our results suggest that *OsGLP2-1* positively regulate leaf blast, panicle blast and bacterial blight resistance in rice. *OsGLP2-1* mediated disease resistance is partially due to the accumulation of H_2O_2, and is also associated with activation of the JA-dependent pathway. However, because there are numerous GLP genes in rice, we do not know whether other GLP proteins (in addition to GLPs on chr 8 and *OsGLP2-1*) are involved in plant resistance to *M. oryzae* and *Xoo* in rice. Furthermore, we still do not know whether there are transcription factors acting upstream of *OsGLP2-1* and how *OsGLP2-1* coordinates with the other GLP genes to contribute to disease resistance in rice. Further study is still needed to address these issues.

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