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Rice Germin-Like Proteins: Allelic Diversity and Relationships to Early Stress Responses

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Abstract Germin-like protein (GLP) markers were associated with quantitative trait loci (QTL) for resistance to the rice blast pathogen, *Magnaporthe oryzae* in multiple rice (*Oryza sativa*) mapping populations. Twelve paralogous *OsGLP* gene family members are located within the physical QTL region on chromosome 8, and gene silencing studies suggest that they contribute collectively to the resistance phenotype. We compared sequence and expression profiles of *OsGLP* alleles in two resistant and two susceptible parental rice lines to find functional polymorphisms that correlated with the resistant phenotype. Based on coding and promoter sequences, the genes belong to two germin subfamily groups (GER3 and GER4). *OsGLP* members from both subfamilies were constitutively expressed and developmentally regulated in all cultivars. Transient induction above constitutive levels was observed for some *OsGLP*s, especially GER4 subfamily members, at early time points after *M. oryzae* infection and mechanical wounding. Varying 5′ regulatory regions and differential expression of some family members between resistant and susceptible cultivars corresponded with differential hydrogen peroxide (H_2O_2) accumulation after the same stimuli. OsGLP of both GER subfamilies localized to the plant cell wall. The protein location and early gene induction suggest that *OsGLPs* protect rice leaves at early stages of infection before fungal penetration and subsequent ingress. Our data suggest that regulation of *OsGLP* genes defines resistant versus susceptible phenotypes.

Keywords Rice · Germin-like proteins · *Magnaporthe oryzae* · Quantitative trait loci · Disease resistance

Introduction

Rice blast, caused by *Magnaporthe oryzae*, is one of the most destructive rice (*Oryza sativa*) diseases (Willocquet et al. 2004). Genetic resistance controlled by single genes has been widely used to control rice blast, but the resistance is often short-lived due to changes in the fungal population structure (Ballini et al. 2008). Disease resistance governed by quantitative trait loci (QTL), or many genes with small effects, however, is predicted to be durable partially because it exhibits no specificity to a given pathogen group or race and, in some cases, confers resistance to multiple types of pathogens (Leung 2008; Poland et al. 2009). There is good evidence that QTL might control broad-spectrum genetic resistance in some rice populations. A germin-like protein (GLP) gene marker that was associated with a QTL on rice chromosome (chr) 8 conferred resistance against rice blast in multiple rice mapping populations across diverse environments (Ramalingam et al. 2003; Liu et al. 2004; Wu et al. 2004). Twelve paralogous *OsGLP* genes were identified within the physical QTL region (Manosalva et al. 2009). Members of the *OsGLP* family were shown to contribute

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collectively to disease resistance because silencing of several genes confers susceptibility to two distinct fungal pathogens, *M. oryzae* and *Rhizoctonia solani*, the sheath blight pathogen (Manosalva et al. 2009). The susceptible phenotypes of the *OsGLP*-silenced mutants confirm roles for some gene family members, particularly those in the GER4 subfamily, in broad-spectrum disease resistance (Manosalva et al. 2009).

The rice chr 8 *OsGLP*s belong to two subfamilies of germins, the GER3 and GER4 (Davidson et al. 2009; Manosalva et al. 2009). These subfamilies were also described in barley (*Hordeum vulgare*), wheat (*Triticum aestivum*), grapevine (*Vitus viniferous*), and *Arabidopsis thaliana* (Schweizer et al. 1999; Membre et al. 2000; Zimmermann et al. 2006; Godfrey et al. 2007). GLP genes in these subfamilies are developmentally regulated and constitutively expressed in multiple tissue types including leaves, roots, and flowers (Dunwell et al. 2008; Davidson et al. 2009). Many of these genes are induced by fungal and bacterial infections, and some produce proteins with superoxide dismutase (SOD) activity (Schweizer et al. 1999; Christensen et al. 2004; Godfrey et al. 2007; Davidson et al. 2009). GLP gene induction was observed in rice after infection with *M. oryzae* (Davidson et al. 2009; Manosalva et al. 2009), in barley and wheat leaves after infection with the powdery mildew pathogen (Wei et al. 1998; Christensen et al. 2004; Zimmermann et al. 2006), in barley spikelets after infection with *Fusarium graminearum* (Federico et al. 2006), and in grapevine leaves and fruit after infection with *Erysiphe necator* (Godfrey et al. 2007). Transcripts of wheat and barley GER3 and GER4 genes accumulated predominantly in epidermal cells compared to mesophyll cells as early as 6 h post-inoculation (hpi; Wei et al. 1998; Schweizer et al. 1999; Zimmermann et al. 2006). Interestingly, *HvGER4* members were also induced by exogenous application of hydrogen peroxide (H_2O_2) and the non-host soybean fungus, *Phakopspora pachyrhizi* (Zimmermann et al. 2006). Consistent with gene silencing results in rice (Manosalva et al. 2009), transient silencing of barley *HvGER4d* resulted in hypersusceptibility to *Blumeria graminis,* although silencing of *HvGER3a* did not (Zimmermann et al. 2006). Genes in both subfamilies show induction by pathogen infection; however, gene silencing of only GER4 subfamily members results in increased susceptibility.

The proposed mechanism by which GLPs provide broad-spectrum resistance relates to their SOD activity and localization in plant cell walls (Wei et al. 1998; Membre et al. 2000; Christensen et al. 2004). GLP enzymes with SOD activity convert the superoxide anion into H_2O_2 (Woo et al. 2000). Hydrogen peroxide is a player in early plant defense responses and has potential roles in cell wall reinforcement and papillae formation (Wojtaszek 1997; Schweizer et al. 1999), as a signaling molecule for downstream defense responses (Grant et al. 2000; Gechev et al. 2006), and as a toxic defense compound against invading microbes (Shetty et al. 2008). Cell wall localization of GLPs was documented in barley and wheat (Wei et al. 1998; Schweizer et al. 1999), but has not been studied in rice.

We have shown that *OsGLPs* in the GER4 subfamily function in non-specific resistance to two fungal pathogens (Manosalva et al. 2009). The molecular basis for allelic differences in the involvement of specific *OsGLP*s in QTL-based resistance, however, is still unknown. The goals of this study were to understand the differences among cultivar-specific *OsGLP* alleles and to functionally link sequence polymorphisms observed in QTL mapping to differential defense responses. Differences between alleles underlying QTL can vary from single nucleotide substitutions to large insertions or deletions between parental cultivars (Paran and Zamir 2003). Some differences may affect coding sequences, while others may occur in upstream regulatory regions and influence gene expression. We predicted defense-related *OsGLP* family members by comparing the putative promoter regions of *OsGLP*s from the two reference rice genomes, cv. Nipponbare (ssp. *japonica*; Yuan et al. 2005) and cv. 93-11 (ssp. *indica*; Zhao et al. 2004). Then, *OsGLP* alleles of two rice blast resistant cultivars containing the effective QTL on chr 8 (both ssp. *indica*) and two susceptible rice cultivars without the chr 8 QTL (both ssp. *japonica*) were studied for coding sequence polymorphisms and differential gene expression both developmentally and after biotic and abiotic stresses. Furthermore, we confirm the subcellular localization of GER3 and GER4 subfamily members and study H_2O_2 peroxide accumulation in QTL-based resistant and susceptible interactions with *M. oryzae*. This information can guide breeding efforts to accumulate desirable alleles from resistant donors into locally adapted cultivars.

Results

A germin-like protein gene family is located within the chr 8 QTL region

Twelve germin-like protein genes were predicted in a 2.8-Mb region within the proposed rice blast QTL region on rice chr 8 (Fig. 1). Eleven family members are clustered in a short 80-kb section from approximately 5.18 to 5.26 Mb, and the 12th is located downstream at approximately 7.99 Mb. Six *OsGLP* were sequenced and described previously (Table 1). Alignments of gene and protein sequences from the Nipponbare reference genome (MSU Pseudomolecules, Build 4.0; Yuan et al. 2005) indicate that the chr 8 *OsGLP* are highly similar to each other (data not shown). Pairwise comparisons of genomic sequences range in identity from 47% to 88%, and corresponding comparisons of inferred amino acid sequences range from 70% to 98%. The gene

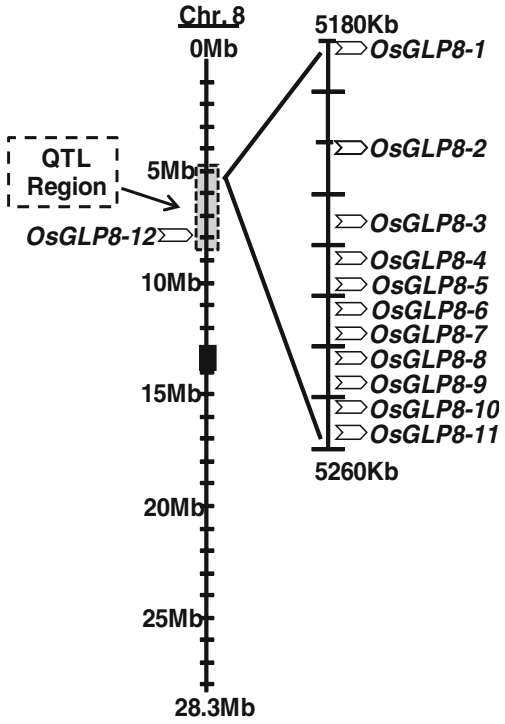

Fig. 1 Distribution of *OsGLP* family members within the proposed rice blast disease resistance QTL region (*shaded box*) on chromosome 8 of *O. sativa*. Gene predictions were based on MSU Rice Pseudomolecules, Build 4.0 (Yuan et al. 2005). The chromosome and inset are represented as 1-Mb and 10-kb increments, respectively. The centromeric region is shown as a *black rectangle*, and relative positions of the 12 *OsGLP* genes are shown as *white arrows*.

family members are predicted to be transcribed from the positive strand with the exception of *OsGLP8-12*. All putative proteins have predicted N-terminal signal sequences of 22–25 amino acids and are predicted to be extracellular.

Phylogenetic reconstruction of *OsGLP* alleles among five rice cultivars including those from the reference cv. Nipponbare shows that the chr 8 *OsGLP* cluster contains genes in two germin subfamily groups, GER3 and GER4 (Electronic supplementary materials (ESM) Fig. 1; Davidson et al. 2009; Manosalva et al. 2009). The GER3 subfamily includes *OsGLP8-1, 8-2, 8-3,* and *8-12.* The GER4 subfamily includes seven members, *OsGLP8-5* to *8-11;* these are tandemly arranged as a cluster within 30 kb (Fig. 1) and have pairwise amino acid identities in the range of 90–98%.

Motifs in putative 5′ regulatory regions are shared among *OsGLP* subfamily members

Bioinformatic comparisons of putative *OsGLP* promoter sequences from the two rice reference genomes, cv. Nipponbare (ssp. *japonica*) and cv. 93-11 (ssp. *indica*) revealed an 858-bp insertion sequence in the *indica* version of *OsGLP8-6* (Fig. 2). The insertion fragment is located 590 bp upstream from the initiation codon (Fig. 2a). The two resistant *indica* cultivars, IR64 and SHZ-2, contained the insertion, while the susceptible cultivars Azucena and LTH, like the *japonica* Nipponbare, did not (Fig. 2b).

Shared motif analysis was performed using the 12 putative *OsGLP* promoter sequences from the Nipponbare reference genome sequence. The insertion sequence in the *indica* version of the putative promoter of *OsGLP8-6* was included in the training set (N=13) to look for additional *cis*-elements. The 15 most statistically overrepresented motifs among the 13 sequences were identified by the MEME algorithm. Each of the 15 motifs was identified in many *OsGLP* members (ESM Table 1). Motif sequences were analyzed for the presence of known plant *cis*-elements in the PLACE database (496 entries), and totals were summarized for each putative regulatory sequence (Table 2). With the exception of the TATA box, only those with multiple copies were counted. Sequence degeneracy was observed across

Table 1 Previously Described *OsGLP* Genes on Rice Chromosome 8

Previous name	GenBank acc. no.[a]	Locus ID[b]	Gene description	New gene name
GER3[c]	AF032973	LOC_Os08g08960	cDNA from panicle at ripening	*OsGLP8-2*
GLP16[e]	AF042489	LOC_Os08g08960	cDNA from immature seed	*OsGLP8-2*
GER2[c]	AF032972	LOC_Os08g08970	cDNA from etiolated shoot	*OsGLP8-3*
GER1[c]	AF032971	LOC_Os08g08980	cDNA from etiolated shoot	*OsGLP8-4*
GER6[c]	AF032976	LOC_Os08g09010	cDNA from green shoot	*OsGLP8-7*
RGLP2[d]	DQ414400	LOC_Os08g09060	promoter region from indica	*OsGLP8-10*
RGLP2[e]	AF141879	LOC_Os08g09060	cDNA from rice root	*OsGLP8-10*
RGLP1[e]	AF141880	LOC_Os08g09080	cDNA from rice root	*OsGLP8-11*

[a] GenBank gene sequence database (http://www.ncbi.nlm.nih.gov)

[b] MSU Rice Genome Annotation Database (http://rice.plantbiology.msu.edu/)

[c] Membre and Bernier (1998)

[d] Mahmood et al. (2007)

[e] Unpublished

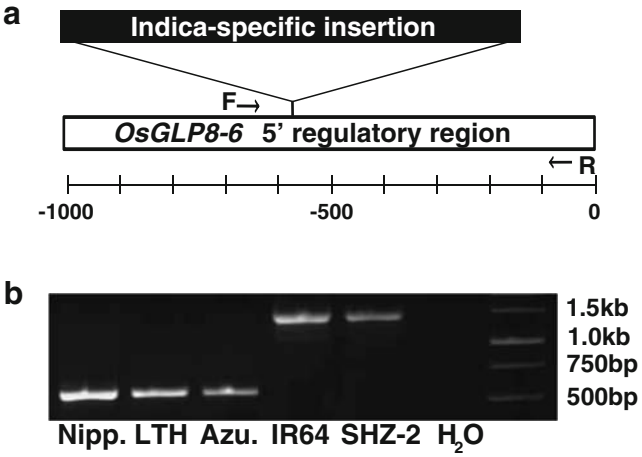

Fig. 2 Allelic alternatives of the 5′ regulatory region in the *OsGLP8-6* gene. **a** An 858-bp insertion was identified in the putative 5′ regulatory region of *OsGLP8-6* of the 93-11 (ssp. *indica*) reference genome and was not found in the Nipponbare (ssp. *japonica*) reference genome. Primers designed upstream of the initiation codon amplified the insertion. **b** Predicted size products (626 bp) were amplified from the *japonica* cultivars, Nipponbare, LTH and Azucena and larger sized fragments (1,478 bp) were observed from the *indica* cultivars, IR64 and SHZ-2.

motif sequence alignments especially in W-BOX and TATA box motifs (ESM Table 1).These are not the only putative *cis*-elements in the 5′ regulatory regions of *OsGLP* genes, but rather, they represent motifs that are present in multiple copies and are shared among family members.

Known plant-associated *cis*-elements were identified within overrepresented motifs (Table 2). TATA box sequences (TATATAA; Grace et al. 2004) which are present just upstream of initiation codons were identified between 77 and 152 bp upstream from the ATG for the 12 *OsGLP* genes, but not in the *OsGLP8-6* insertion sequence. The W-BOX motif [(T)TGAC(Y)], a *cis*-element associated with rapid induction by wounding or fungal elicitors (Eulgem et al. 1999; Nishiuchi et al. 2004), was present in multiple copies in all of the putative 5′ regulatory regions and the *OsGLP8-6* insertion sequence. More W-BOX elements were discovered in GER4 family members compared to GER3. A pollen-specific *cis*-element (GTGA; Rogers et al. 2001) and an endosperm-specific element (AAAG; Yanagisawa and Schmidt 1999) were observed in all but two *OsGLP* regulatory regions, and higher copy numbers were also present in GER4 compared with GER3.

OsGLP alleles show sequence diversity among rice cultivars

Gene sequences of all 12 *OsGLP* alleles from two resistant cultivars with the chr 8 QTL and two susceptible cultivars without the QTL were analyzed for sequence variation. Single nucleotide polymorphisms (SNPs) were present in one or more cultivars for all genes (Table 3). SNPs predicted to result in non-synonymous codon changes were observed in one or more cultivars for all genes except *OsGLP8-6* (Table 3). There were no nucleotide and/or codon substitutions predicted to change protein function that correlated with resistant and susceptible phenotypes. Two *OsGLP* alleles from the most resistant cultivar, SHZ-2, contained SNPs conferring premature stops. A 1-bp deletion in *OsGLP8-1* caused a frameshift mutation, and a nucleotide substitution in *OsGLP8-11* resulted in a premature stop codon.

Expression of *OsGLP* genes is developmentally regulated

To examine the constitutive and developmental expression of *OsGLP* genes, expression was monitored using gene-specific primers for all 12 genes in three positions of non-stressed leaves in three cultivars: Azucena, IR64, and SHZ-2 (Table 4). Transcripts for *OsGLP8-1*, *8-2,* and 8-*10* were

Table 2 Numbers of Plant *cis*-Elements in Statistically Overrepresented Motifs Among *OsGLP* Gene Family 5′ Regulatory Regions

cis-Element	*OsGLP8* promoters													
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	6 insert.[a]	Functional association
AAAG	1	2	0	1	2	3	3	3	3	3	0	1	1	Endosperm-specific Yanagisawa and Schmidt (1999)
GTGA	1	1	1	0	1	2	3	3	3	2	1	0	1	Pollen-specific (Rogers et al. 2001)
TATATAA	1	1	1	1	1	1	1	1	1	1	1	1	0	Gene transcription (Grace et al. 2004)
(T)TGACY	2	1	2	2	1	3	3	4	4	3	2	2	2	Early plant defense/wounding (Eulgem et al. 1999; Nishiuchi et al. 2004)

Motifs: the 15 most overrepresented motifs (size-2–12 bp) among training set sequences (n=13) were recognized by the MEME algorithm (Bailey et al. 2006). Known plant associated *cis*-elements in motif sequences were identified using PLACE signal scan (Higo et al. 1999). The numbers of *cis*-elements that are present in multiple copies are presented in the table

OsGLP gene family 5′ regulatory regions: 1,000-bp sequences upstream of ATG initiation codons for *OsGLP8-1* to *8-12* were extracted from the Nipponbare reference genome sequence, MSU Pseudomolecules, V.4

[a] The indica-specific *OsGLP8-6* insertion sequence (858 bp) was cloned and sequenced from cultivars IR64 and SHZ-2 and was included in the motif search training set

Table 3 Nucleotide (SNP) and aa Substitutions Among *OsGLP* Alleles from Four Rice Cultivars

OsGLP	MSU locus ID	Rice cultivars					
		Azucena		IR64		SHZ-2	
		SNPs	aa subs	SNPs	aa subs	SNPs	aa subs
8-1	LOC_Os08g08920	3	1	4	2	1[c]	–
8-2	LOC_Os08g08960	1	0	3	1[a]	2	0
8-3	LOC_Os08g08970	0	0	5	1	4	2
8-4	LOC_Os08g08980	1	1	2	1	5	1
8-5	LOC_Os08g08990	1	0	7	1[b]	3	0
8-6	LOC_Os08g09000	0	0	2	0	0	0
8-7	LOC_Os08g09010	0	0	3	2	2	1
8-8	LOC_Os08g09020	0	0	11	5	3	2
8-9	LOC_Os08g09040	0	0	2	1	0	0
8-10	LOC_Os08g09060	2	0	4	2	0	0
8-11	LOC_Os08g09080	0	0	0	0	18[d]	–
8-12	LOC_Os08g13440	2	2	5	1	4	1

All reported nucleotide and amino acid substitutions are in reference to allele sequences from the temperate japonica cultivar, LTH

[a] 3-bp deletion/1 amino acid deletion

[b] 3-bp insertion/ 1 amino acid insertion

[c] 1-bp deletion / frameshift mutation/premature termination codon

[d] Nucleotide substitution / premature termination codon

not detected in any of the three leaf positions tested in any cultivar. The two resistant cultivars showed constitutive expression of *OsGLP8-3* in all leaf positions, while the susceptible variety showed no expression. Conversely, *OsGLP8-9* was expressed only in Azucena and not in IR64 and SHZ-2. Some *OsGLP* family members exhibited developmental regulation of gene expression among leaf positions.

Table 4 Developmental Expression of *OsGLP* Genes in Leaves of 21-Day-Old Rice Plants

Gene	Leaf position[a]								
	Azucena (-QTL)			IR64 (+QTL)			SHZ-2 (+QTL)		
	1	2	3	1	2	3	1	2	3
OsGLP8-1	–	–	–	–	–	–	–	–	–
OsGLP8-2	–	–	–	–	–	–	–	–	–
OsGLP8–3	-	–	–	+	+	+	+	+	+
OsGLP8-4	+	+	–	+	+	+	+	–	+
OsGLP8-5	+	+	+	+	+	+	+	+	+
OsGLP8-6	–	+	–	–	–	+	+	–	+
OsGLP8-7	+	+	+	+	+	+	+	+	+
OsGLP8-8	–	+	–	–	–	–	–	–	+
OsGLP8-9	+	+	–	–	–	–	–	–	–
OsGLP8-10	–	–	–	–	–	–	–	–	–
OsGLP8-11	+	+	+	+	+	+	–	–	–
OsGLP8-12	+	+	+	+	+	+	+	+	+

Developmental expression: (+) indicates presence of band in RT-PCR after 35 cycles

[a] Three leaf positions include youngest emerging leaf (*1*), second youngest, expanded leaf (*2*), and third youngest, mature leaf (*3*)

OsGLP genes are transiently induced in response to *M. oryzae* and wounding

To determine if differential expression of *OsGLP* family members correlates with disease phenotypes, gene expression was tested by RT-PCR on a time course (0, 12, 24, and 48 hpi) after *M. oryzae* infection (Fig. 3). As observed in developmental studies, transcripts of *OsGLP8-1* and 8-*2* were not detected in any cultivar under any treatment or time point. Transcripts of GER3 subfamily members, *OsGLP8-3* and *OsGLP8-4,* were observed in untreated tissue in SHZ-2 and IR64, respectively, but not in the other cultivars (Fig. 3). *OsGLP8-12* was more highly expressed in untreated tissue of Azucena and LTH compared to IR64 and SHZ-2. Transient induction of *OsGLP8-12* was observed at 12 and 24 hpi in three of four cultivars.

GER4 subfamily genes were clearly induced in response to *M. oryzae* infection in all cultivars (Fig. 3). *OsGLP8-5* and *8-7* were constitutively expressed and transiently induced by *M. oryzae* infection in all cultivars. Low baseline levels of *OsGLP8-8, 8-9*, and *8-10* were present at 0 h, and all were transiently induced at 12 and 24 hpi. Basal transcript levels among cultivars differed for *OsGLP8-11*, but elevated expression was observed at 12 and 24 hpi. As predicted, transcripts of *OsGLP8-11* were not observed from SHZ-2 likely because of the nonsense mutation in the gene sequence. SHZ-2 plants had a higher constitutive level of *OsGLP8-6* transcripts compared to other cultivars (Figs. 3 and 4). This gene was induced at 12 and 24 h in all lines, but the relative induction levels were greater in resistant compared to susceptible cultivars.

Overall, *OsGLP* expression between resistant and susceptible alleles differs at two time points: 0 h and 48 hpi (Figs. 3 and 4). The resistant cultivars constitutively

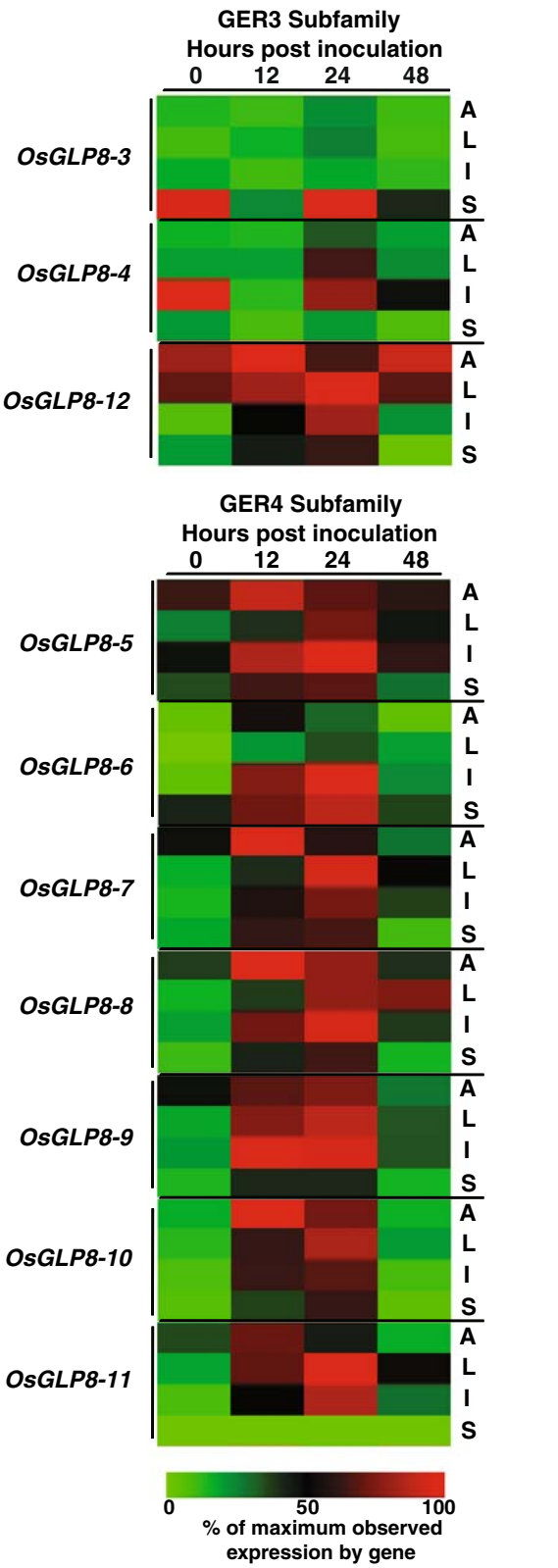

Fig. 3 *OsGLP* genes are induced by rice blast infection. Gene expression patterns of *OsGLP* genes after challenge with *M. oryzae*. Three-week-old plants of two susceptible, Azucena (*A*) and LTH (*L*), and two resistant, IR64 (*I*) and SHZ-2 (*S*), rice cultivars were challenged with the virulent *M. oryzae* isolate P06-6 and sampled at 0, 12, 24, and 48 hpi. *OsGLP* transcript levels were assayed with gene-specific primers by RT-PCR. The average band intensities of three biological replications (n=3) were normalized to the highest intensity per gene and are shown in the heat maps. *OsGLP* genes in both GER3 and GER4 subfamilies were induced by *M. oryzae* infection. Transcripts of *OsGLP8-11* were not observed in the most resistant cultivar, SHZ-2.

express different combinations of *OsGLP* genes compared to the susceptible. For the GER3 subfamily, the indica varieties utilize *OsGLP8-3* and *OsGLP8-4*, while the japonicas express *OsGLP8-12*. The most resistant cultivar, SHZ-2, is the only one to constitutively express *OsGLP8-6* and also has the fewest number of genes induced at 48 hpi.

Most of the *OsGLP* genes were also induced by the mock spray treatment (ESM Fig. 2), although to lower levels than after *M. oryzae* inoculation. To test whether induction of *OsGLP* genes by the mock treatment is consistent with response to wounding, a subset of *OsGLP* genes was tested by RT-PCR in leaf tissue after pressurized water spray and mechanical wounding (Fig. 5). Of the five genes tested, all were induced by both treatments, except for *OsGLP8-11* which was not observed from SHZ-2. Overall, these results show that all *OsGLP* genes are induced by biotic and abiotic stimuli and that the timing and levels of induction vary among the different family members.

OsGLP proteins are localized in the plant cell wall

Fusion proteins of OsGLP8-6 and OsGLP8-12 with C′-terminal green fluorescent proteins (GFP) were visualized by transient expression in the heterologous plant, *N. benthamiana* (Fig. 6a). OsGLP-GFP proteins were co-expressed with a golgi-specific marker, G-rk CD3-967 (Nelson et al. 2007), to compare subcellular localizations. Both OsGLP-GFP fusion proteins showed similar punctate patterns to each other. In some cases, they associated with subcellular organelles at cell perimeters and co-localized with golgi-specific markers (Fig. 6a, merged images). The punctate, fluorescent patterns observed for OsGLP fusion proteins, however, were usually not located with the golgi markers and when viewed in multiple planes appeared to be associated with the cell surfaces (Fig. 6a, green channel and merged images).

Denatured monomer subunits of OsGLP fusion proteins were observed by immunoblot at the expected size of 55 kDa (OsGLP predicted size, 24 kDa) compared to the control GFP protein of 26 kDa (Fig. 6b). Two bands were observed for OsGLP8-12+C′GFP proteins, suggesting multiple isoforms of this protein.

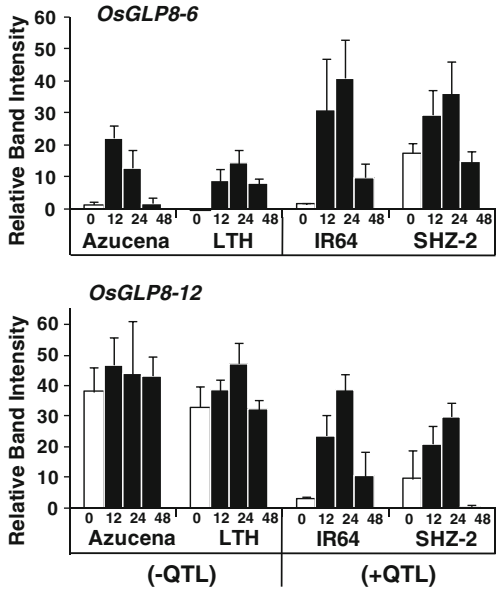

Fig. 4 Differential expression between resistant and susceptible cultivars was observed for two *OsGLP* genes. **a** *OsGLP8-6* is upregulated in resistant cultivars. SHZ-2, a highly resistant cultivar, showed higher constitutive expression (0 hpi, *white bars*) than the other cultivars. Both resistant (+QTL) cultivars had higher relative induction by *M. oryzae* infection (*black bars*) compared to susceptible (-QTL). **b** *OsGLP8-12* is upregulated in susceptible cultivars. There is high constitutive expression of *OsGLP8-12* in Azucena and LTH (-QTL) compared to IR64 and SHZ-2 (+QTL). The resistant cultivars show peak induction of this gene at 24 hpi with *M. oryzae*. *OsGLP* transcript levels were assayed with gene-specific primers by RT-PCR, and the average band intensities of three biological replications (n=3) with standard errors are shown.

Resistant varieties produce H_2O_2 in early responses to rice blast infection

H_2O_2 was visualized in leaf tissue after rice blast inoculation by oxidation of 3,3′-diaminobenzidine (DAB; Fig. 7a). The SHZ-2 leaves showed a few DAB-stained spots as early as 12 hpi with *M. oryzae*, while the LTH leaves did not. Leaves of SHZ-2 also showed a higher number of DAB-stained spots at 24 and 48 hpi compared to LTH. Mock-inoculated plants sprayed with gelatin solution displayed fewer and fainter spots than the *M. oryzae*-inoculated leaves, suggesting that infection by *M. oryzae* induces H_2O_2 production.

To test for H_2O_2 production after abiotic stimulation, leaves of SHZ-2 were subjected to a gelatin spray treatment and mechanical wounding with a needle (ESM Fig. 3). H_2O_2 accumulated in faint spots after the water spray compared to the untreated, control leaf. There was also H_2O_2 accumulation in areas directly surrounding the needle wounds at 24 h post-treatment. These results show that rice leaves produce H_2O_2 after a variety of stimuli, including wounding and pathogen infection.

Discussion

We previously demonstrated that as more chr 8 *OsGLP* genes are silenced in a transgenic plant, the plants become more susceptible to *M. oryzae* and *R. solani* (Manosalva et al. 2009). This suggested that several of the 12 *OsGLP* genes within the QTL region contribute collectively to broad-spectrum disease resistance. Our objectives were to identify functional polymorphism(s) in *OsGLP* alleles among rice blast resistant cultivars with the effective chr 8 QTL compared to susceptible cultivars without the chr 8 QTL. The resulting story is that of duplicated genes in two germin subfamilies with complex, cultivar-specific expression patterns. Biotic stress-inducible expression confirms that multiple *OsGLP*s are pathogenesis-related proteins in rice as in other crop species (van Loon et al. 2006). The early temporal induction of *OsGLPs* and the location in the plant cell wall suggest that they play roles in early layers of plant defenses. The earlier expression of some GLP at higher levels in resistant versus susceptible cultivars corresponds with differences in early physiological defense responses such as H_2O_2 accumulation. We hypothesize that earlier and higher induction of certain *OsGLP* genes provides the main advantage to resistant cultivars as opposed to loss of function mutations in coding sequences of susceptible cultivars.

The 12 *OsGLP* genes within the QTL region (Fig. 1) represent two germin subfamilies, GER3 and GER4 (ESM Fig. 1). Members of these groups have been identified from several taxa (Davidson et al. 2009; Manosalva et al. 2009). Phylogenetic analyses indicate that both subfamilies were

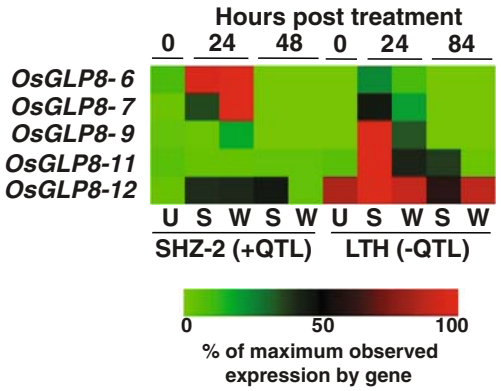

Fig. 5 *OsGLP* genes are differentially induced by mechanical wounding. Induction of selected *OsGLP* genes after pressure spray (*S*) and mechanical wounding (*W*) compared to untreated controls (*U*) in rice cultivars SHZ-2 and LTH. *OsGLP* transcript levels were assayed with gene-specific primers by RT-PCR, and average band intensities of three biological replications (n=3) were normalized to the highest intensity per gene and are shown in the heat maps. Multiple *OsGLP* genes were induced by both treatments in the two cultivars. Transcripts of *OsGLP8-11* were not observed in the most resistant cultivar, SHZ-2.

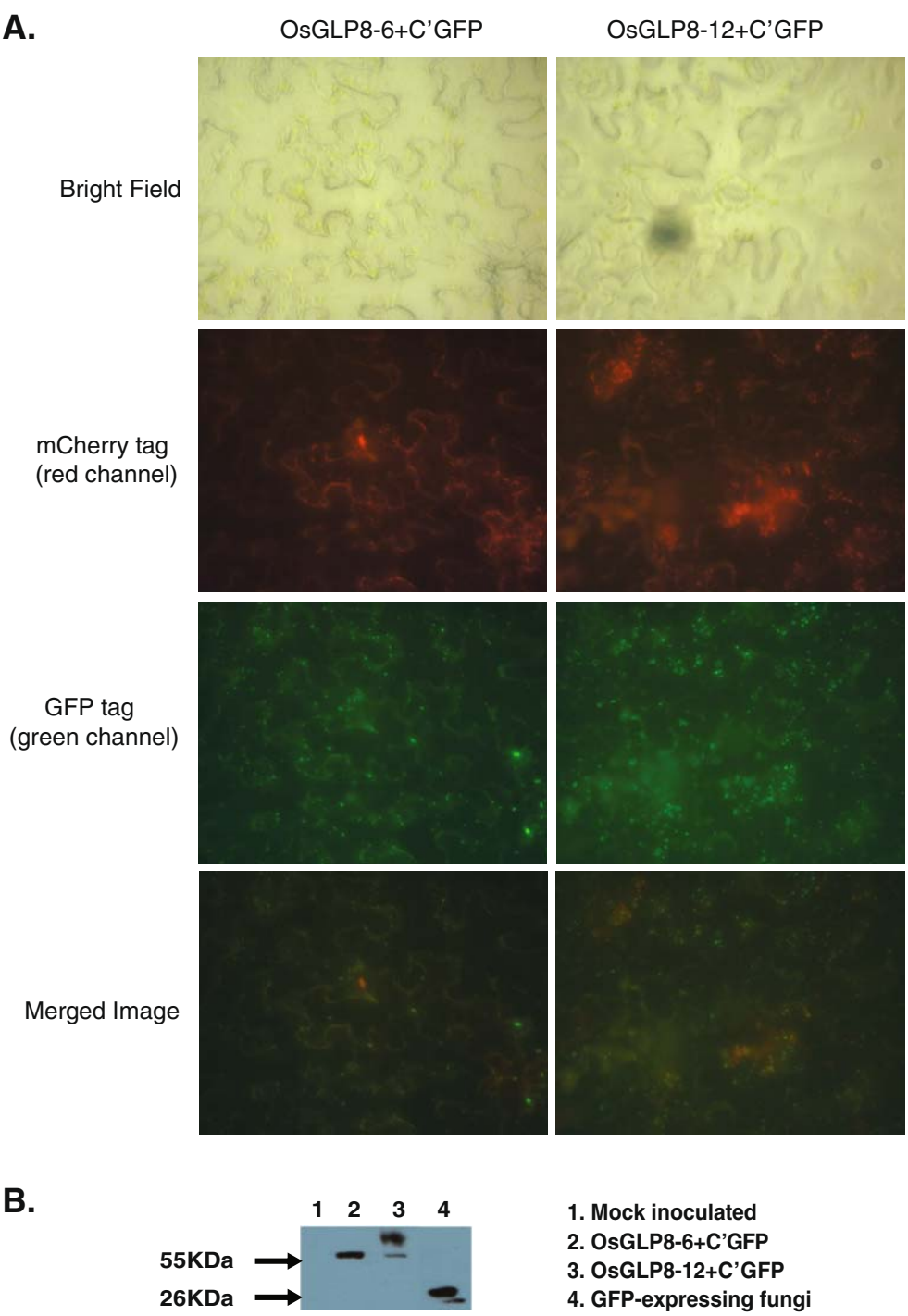

Fig. 6 Subcellular localization of OsGLP8-6 and OsGLP8-12. **a** Each GLP protein was fused with a C′GFP tag and co-expressed with a golgi-specific marker protein with an mCherry tag (Nelson et al. 2007) in *N. benthamiana* via *A. tumefaciens* transformation. Epidermal peels were examined by fluorescence microscopy 4 days post-inoculation with *A. tumefaciens* strains containing the constructs and images are shown at ×400 magnification. Both GLP proteins show bright punctate patterns in green channel images. In some cases, GLP proteins co-localized with golgi marker proteins at cell margins (merged images). However, GLP proteins also showed distinct localizations in several planes of view across cell surfaces. **b** Fusion proteins were detected by immunoblot with anti-GFP antibodies. Both show expected sizes around 56 kDa compared to GFP expressed in *M. oryzae* at 26 kDa (Kankanala et al. 2007). OsGLP12+C′GFP showed two bands indicating multiple isoforms.

derived from a common ancestor and that gene duplication events occurred after separation of subfamily progenitors. The most recent duplication events occurred in the GER4 subfamily based on their close physical proximity to one another and similarities in gene and putative regulatory sequences (Fig. 1 and Table 2 and ESM Table 1). Allelic diversity occurs among *OsGLP* genes within a single cultivar and among cultivars for a given *OsGLP* gene. Therefore, the potential number of allelic combinations of *OsGLP*s present among rice germplasm is very high.

We analyzed the variation in coding sequences among the alleles from the various cultivars. Most sequence variations correlated to subspecies pedigrees (*japonica* vs. *indica*), so we focused on mutations that putatively cause major effect functional changes. Of all putative amino acid (aa) substitutions and insertion/deletions identified (Table 3), none

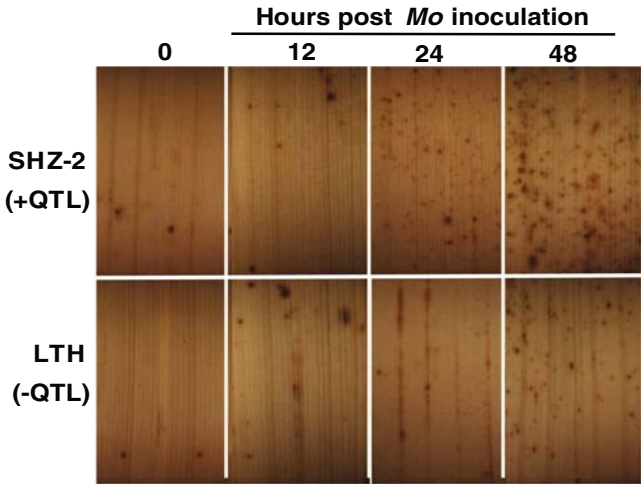

Fig. 7 Hydrogen peroxide (H_2O_2) accumulation in leaf tissue of resistant and susceptible cultivars after inoculation with *M. oryzae* isolate P06-6. H_2O_2 was visualized using 3,3′-diaminobenzidine (*DAB*); oxidation of DAB by H_2O_2 results in a dark brown color (*dark spots* on image). Twenty-one-day-old plants of highly resistant cultivar, SHZ-2, and susceptible cultivar, LTH, were spray-inoculated with *M. oryzae*, and leaves were collected at 0, 12, 24, and 48 hpi. Leaves of SHZ-2 showed a higher number of DAB stained spots at 24 and 48 hpi compared to LTH.

were predicted in conserved functional domains such as active sites or N-terminal signal sequences. The most resistant cultivar, SHZ-2, had nonsense mutations in two genes (*OsGLP8-1* and *OsGLP8-11*), consistent with lack of detectable transcripts. These mutations were not present in the moderately resistant cultivar, IR64, and gene silencing of *OsGLP8-11* conferred some loss of resistance in the cultivar, Kitaake (Manosalva et al. 2009). It is unlikely that the loss of these genes provides an advantage to SHZ-2.

Analyses of putative 5′ regulatory regions suggested that GER4 subfamily members are most important for defense (Table 2), which is consistent with gene silencing studies (Manosalva et al. 2009). Gene expression profiling shows that many *OsGLP* of the GER4 subfamily are induced after challenge with *M. oryzae* in all cultivars, although one GER3 (*OsGLP8-12)* is also induced (Figs. 2 and 3). In barley, *HvGER4* transcripts accumulated rapidly, as early as 3 hpi, and were correlated with the formation of papilla, structures associated with defense responses (Wei et al. 1998; Zimmermann et al. 2006). We also observed induction of *OsGLP* early in defense responses (12 hpi, Fig. 3), and this induction preceded the penetration of the cell wall by *M. oryzae* that occurs between 18 and 24 hpi (Kankanala et al. 2007; Mosquera et al. 2009). The genes also showed sustained induction through 48 hpi, indicating that they remain present throughout pathogenesis.

Many *OsGLP* were also induced by the mock spray treatment and mechanical wounding (Fig. 5 and ESM Fig. 2). The presence of multiple W-BOX transcription factor binding site motifs in *OsGLP* promoters is consistent with the induction by wounding. WRKY transcription factors are rapidly induced by pathogen elicitors and in response to wounding and physically bind to W-BOX motifs (Eulgem et al. 1999; Nishiuchi et al. 2004). The GER4 subfamily member 5′ regulatory regions contained the most W-BOX motifs, consistent with their induction by wounding and *M. oryzae*. Some GER4 genes were also shown to be induced by inoculation with the bacterial blight pathogen *Xanthomonas oryzae* pv. *oryzae* in genome wide expression profiling experiments (Nobuta et al. 2007; Davidson et al. 2009).

Two obvious differences were observed between *OsGLP* expression patterns in resistant and susceptible plants. The first includes constitutive expression and transient induction of *OsGLP8-6*, a GER4 subfamily member (Fig. 4). The coding region of this gene was highly conserved across all four cultivars, suggesting that it may be functionally important (Table 3). The promoter insertion sequences identified in the two indica cultivars likely influence the differential expression patterns, especially with the addition of two W-BOX motifs to the existing three copies. WRKY proteins bind cooperatively to clusters of W-BOX motifs (Eulgem 2006). Thus, we hypothesize that enhanced expression of *OsGLP8-6* in resistant cultivars is conferred through this alternative promoter. The second difference was observed in expression of *OsGLP8-12* which was more highly expressed in susceptible versus resistant cultivars (Figs. 3 and 4). *OsGLP8-12* is a member of the GER3 subfamily and was not detected as important for disease resistance in rice or barley (Zimmermann et al. 2006; Manosalva et al. 2009). In fact, transient silencing of *HvGER3* resulted in enhanced resistance to *B. graminis* (Zimmermann et al. 2006). Scrutiny of genome-wide rice small RNA libraries suggests that there is opposite regulation of GER3 and GER4 genes (Davidson et al. 2009). Alternatively, there could be transcriptional suppression of GER4 genes through feedback inhibition. A reasonable hypothesis is that *OsGLP8-12* expression suppresses GER4 family members, which results in a constitutive disadvantage for the susceptible cultivars.

OsGLP genes also show developmental regulation in leaves (Table 4) and were detected in cDNA libraries from panicles, shoots, seeds, and roots (Table 1; Membre and Bernier 1998). The presence of developmentally related *cis*-elements in *OsGLP* promoters is consistent with these observations (Table 2; Yanagisawa and Schmidt 1999; Rogers et al. 2001). The detection of GLPs of rice and other species in plant cell walls is also consistent with their roles as mediators of cell wall expansion (Membre et al. 2000) and a role in cell wall reinforcement after stress induction (Vallelian-Bindschedler et al. 1998).

Highly related orthologs of *OsGLP* have SOD activity that converts unstable and transient superoxide anions

into H_2O (Woo et al. 2000; Christensen et al. 2004; Godfrey et al. 2007). Superoxide is produced quickly in the apoplast following recognition of pathogen elicitors by NAPDH oxidases and peroxidases (Wojtaszek 1997). In addition, *M. oryzae* requires fungal-produced superoxide for successful penetration of plant cuticles (Egan et al. 2007). If rice *OsGLP* also exhibit SOD activity in the cell wall as predicted from studies of orthologous proteins (Wei et al. 1998; Christensen et al. 2004; Godfrey et al. 2007), then they likely produce some or all of the H_2O_2 produced after stress stimuli which may impede fungal penetration. Indeed, we observed H_2O_2 accumulation after the same stimuli that induced *OsGLP* genes (Figs. 3 and 6). The earlier accumulation of H_2O_2 after infection with *M. oryzae* in the resistant cultivar, SHZ-2, compared to the susceptible, LTH, indicates that timing of the defense response is key to the resistance strategy. This is supported by observations of collective *OsGLP* expression in which SHZ-2 has the earliest transient gene induction after *M. oryzae* infection and the fewest genes induced at 48 hpi.

The evidence presented here, as well as that from other monocot and dicot species, provide insights into the contributions of *GLP* to disease resistance QTL and the complexity of the gene family member regulation. Our data show that *OsGLP* genes, particularly GER4 subfamily members, are induced by pathogens in all cultivars but that temporal expression patterns differed. Corresponding with differential *OsGLP* expression among cultivars were variations in the 5′ regulatory regions and differential accumulation of H_2O_2 after pathogen infection. Taken together, we conclude that *OsGLP* play roles in early defense responses and that early induction may be beneficial for protecting plants at or near fungal penetration sites. With respect to breeding strategies, *OsGLP*s may be effectively utilized by selecting for the resistant donor GER4 gene cluster including regulatory regions. This identification of genes contributing to broad-spectrum disease resistance facilitates the introgression of donor alleles into adapted cultivars.

Materials and methods

Plant materials

Rice cultivars, Azucena (ssp. *japonica*), Lijiangxin-tuanheigu (LTH; ssp. *japonica*), IR64 (ssp. *indica*), and Sanhuangzhan 2 (SHZ-2; ssp. *indica*) were grown in a Bacto soilless media (Michigan Peat Co., Houston, TX) for 21 days and fertilized once a week with 5 g/L NH_4SO_4. Plants were grown with a photoperiod of 16-h light/8-h dark in a growth chamber and alternating day/night temperatures of 28/26°C and 80% RH.

Cloning of *OsGLP* alleles

The 12 *OsGLP* were PCR-amplified from genomic DNA of four cultivars (Azucena, LTH, IR64, and SHZ-2) and cloned into pGEM-T (Promega, Madison, WI) using primers and annealing temperature described in Manosalva et al. (2009). Genes were sequenced from plasmids at a minimum of 4× coverage. Protein predictions were performed using FGENESH (http://linux1.softberry.com/berry.phtml?topic=fgenes_plus&group=programs&subgroup=gfs).

5′ regulatory region motif analysis

Putative regulatory sequences that are 5′ to *OsGLP* genes (1 peroxidases upstream from initiation codon) for the 12 gene family members were extracted from the reference *japonica* genome sequence (cv. Nipponbare; MSU Pseudomolecules, Build 4.0; Yuan et al. 2005) using a custom perl script. Similar regions in the reference *indica* genome sequence (cv. 93-11, Zhao et al. 2004) were extracted through blastn searches. The putative 5′ regulatory sequences for the 12 *OsGLP* genes and the *OsGLP8-6* insertion sequence (858 bp, only present in the *indica* sequence) were analyzed as a training set (N=13) for statistically overrepresented sequence motifs using the MEME algorithm (Bailey et al. 2006; ESM Table 1; motif size = 2–11 bp, 15 motifs, +/− strand, $p < 0.001$). The 15 most significant motif sequences were searched against the PLACE database of 496 known plant-associated *cis*-elements using PLACE Signal Scan (Higo et al. 1999). *cis*-elements within motif sequences were counted for all putative regulatory sequences in the training set, and those with multiple copies were summarized (Table 2).

Because sequence differences were observed in the 5′ regulatory regions of *OsGLP8-6* between the two reference genomes (*japonica* vs. *indica*), the regions were amplified from each of the four cultivars with primers designed 639 and 85 bp upstream from start codon (Fig. 4b, c; GLP6proF2, 5′ CGTTCAATTTTCTAAGCCAGATTGTG 3′ and oxo6ProR 5′ CTTCCCATCAGAGAAAGATAGCAG 3′). The sequences were cloned into pGEM-T (Promega) and sequenced at 4× coverage.

Fungal growth conditions, inoculation/wounding methods

Plants were inoculated with Philippine *M. oryzae* isolate PO6-6 that exhibits broad virulence to rice genotypes and is routinely used for the evaluation of quantitative resistance to blast (Wang et al. 1994; Liu et al. 2004; Manosalva et al. 2009). Cultures were grown on oatmeal agar media under constant light at 26°C for 21 days. Plants were spray-inoculated with 5×10^5 spores per milliliter suspended in a gelatin solution at 20 psi using an artist's air brush (Valent

et al. 1986). Mock inoculation consisted of a spray with gelatin solution.

For the wounding experiments, LTH and SHZ-2 were grown for 21 days. The two youngest fully expanded leaves were pierced with a needle at 1-cm intervals from the tip downward for 8 cm.

Plant harvest, RNA isolation and RT-PCR

To monitor developmental expression of the *OsGLP* genes, tissue was harvested from the three most fully expanded leaves from six 21-day-old plants per cultivar; six leaves from each position were pooled per cultivar. For expression experiments after *M. oryzae* inoculation, tissue was harvested by combining the three most fully expanded leaves pooled from two plants per cultivar. In the wounding experiments, the two youngest, wounded leaves were pooled from two plants per cultivar.

Samples were ground in liquid nitrogen, and total RNA was isolated using Trizol (Invitrogen, Carlsbad, CA). RNA was treated with one unit of DNase (Promega) per micrograms total RNA. Single-strand cDNA was synthesized from 2 μg of total RNA using Superscript III reverse transcriptase (RT; Invitrogen) and 50 pmol oligod$T_{(20)}$ primer. Gene-specific primers (10 pmol of each primer) were used in RT-PCR for 35 cycles at the appropriate annealing temperature (Manosalva et al. 2009). Total RNA was measured with a spectrophotometer, and *18s rRNA* or *EF1*-alpha were used as reference genes (Kim et al. 2003) to ensure equal RNA loading among samples.

Expression profiling by RT-PCR was performed from leaf tissue of three independent plant inoculation/wounding experiments. PCR products were fractionated on 0.8% agarose gels (*w*/*v*) and stained with ethidium bromide. Stained gels were visualized and digitally photographed using the Syngene Gene Genius Bioimaging System, and gel band intensities were quantified using Gene Tools Gel Analysis software (Syngene, Frederick, MD). Band intensity values were normalized by subtracting the signal of the negative control in a given gel. Intensities for each gene/treatment/cultivar combination were averaged over biological replicates (n=3).

Construct design, transient protein expression, GFP microscopy, and immunodetection

OsGLP8-6 and *OsGLP8-12* genes from cultivar SHZ-2 were amplified from p-GEM-T plasmids using the primers (GLP6_prot_F, 5′ CACCATGGCTTCACCCTCTTCCCT 3′, GLP6_prot_R, 5′ GTAGTGATTGTTCTCCCAGAAC 3′, GLP12_prot_F, 5′CACCATGGCCTCCTCTTCCT TATTTC 3′, and GLP12_prot_R, 5′ GTAGTTGTTCTCC CAGAACTGAG 3′) and were ligated into the entry vectors (pENTR-D TOPO; Invitrogen). Positive entry clones were digested into two fragments using *Mlu*I and were purified using sodium acetate precipitation. Plasmid fragments containing GLP genes were recombined into the GFP protein fusion plasmid, pEarleyGate103 (Earley et al. 2006) using LR Clonase (Invitrogen). After amplification in *Escherichia coli*, purified plasmids were transformed into *Agrobacterium tumefaciens* strain EHA105. An *A. tumefaciens* strain containing a plasmid encoding a golgi-specific protein marker with an mCherry tag called G-rk CD3-967 (Nelson et al. 2007).

Transformed *A. tumefaciens* strains containing one of three constructs (OsGLP6+C′GFP, OsGLP12+C′GFP, and G-rk CD3-967) were grown in 20 ml cultures according to (Sparkes et al. 2006). For co-expression experiments, two *A. tumefaciens* strains containing single constructs were mixed and infiltrated into leaves of *Nicotiana benthamiana*. Epidermal peels of transformed *N. benthamiana* leaves were imaged at ×400 magnification with a compound epifluorescent microscope in bright field, green channel (509 nm) to observe GFP fluorescence and the red channel (610 nm) to observe mCherry fluorescence. Images were merged using Metamorph 6.1 imaging software (Downingtown, PA).

GFP fusion proteins were visualized by immunodetection. *N. benthamiana* leaves were ground in Laemmli buffer (BioRad, Hercules, CA) 4 days post-inoculation with *A. tumefaciens* containing the constructs (OsGLP6+C′ GFP, OsGLP12+C′GFP). Crude protein extracts were boiled for 5 min and separated on 12% SDS-PAGE gels. Proteins were transferred to a nylon membrane and were probed with anti-GFP and anti-rabbit antibodies. Antibodies were detected with West Pico Chemi-luminescent Substrate (Thermo Scientific, Waltham, MA).

Hydrogen peroxide (H_2O_2) staining

H_2O_2 accumulation was visualized in leaf tissue using DAB as described (Thordal-Christensen et al. 1997). Twenty-one-day-old plants of cultivars LTH and SHZ-2 were spray-inoculated with *M. oryzae* P06-6. Leaves were collected at three time points after inoculation and then stained with DAB. Leaves from SHZ-2 were subjected to a mock spray treatment or mechanical wounding with a needle, were harvested 24 h post-treatment, and then stained with DAB. Leaves were cleared of chlorophyll in 95% ethanol and digital images were taken with a dissecting microscope.

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