The rice *GRH2* and *GRH4* activate various defense responses to the green rice leafhopper and confer strong insect resistance

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Received April 20, 2015; accepted June 15, 2015 (Edited by K. Yano)

Abstract Rice *GRH2* confers enhanced resistance to green rice leafhoppers (GRH), *Nephotettix cincticeps* Uhler. A pyramided line carrying *GRH2* and *GRH4* (TGRH29) showed strong resistance to GRH insects compared with a *GRH2* near-isogenic line (TGRH11), although *GRH4* alone did not confer any resistance to GRH. To explore the effects of *GRH2* and *GRH4* on GRH resistance, we investigated the transcriptional response of rice plants to GRH infestation using DNA microarray analysis. The expression of a large number of genes encoding pathogenesis-related proteins, lipoxygenases, terpene synthase (TPS) and WRKY transcription factor, was upregulated in response to GRH infestation in TGRH11 and TGRH29 compared with control plants. Quantitative RT-PCR revealed that expression of *JAmyb* and *TPS* was more strongly and more rapidly upregulated in TGRH29 compared with TGRH11 after GRH infestation. These results suggest that TGRH29 plants can more rapidly and strongly activate the defense response compared with plants carrying *GRH2* alone. Furthermore, sesquiterpenes were emitted from TGRH29 plants in response to attack by GRH. The strong induction of sesquiterpene production in the TGRH29 line was correlated with the transcript levels of *TPS* genes. Our results suggest that *GRH2* and *GRH4* activate various defense responses and confer strong GRH insect resistance.

Key words: Defense signaling, green rice leafhopper, insect resistance, rice, sesquiterpene, upregulated genes.

The green rice leafhopper (GRH), *Nephotettix cincticeps* Uhler, is a major insect pest of cultivated rice in temperate Asia. GRH is closely related to the green leafhopper (GLH) *Nephotettix virescens* Distant, which transmits rice tungro disease. Rice tungro disease causes major constraints in rice production in southeast Asia (Hull 1996). GRH penetrates plant tissues using stylets and ingests sap from both the phloem and xylem of susceptible rice cultivars (Kawabe 1985), causing considerable losses in rice yield. In addition, GRH also causes severe damage to rice plants indirectly by transmitting several viral and phytoplasmal diseases, such as rice dwarf disease and transitory yellowing disease (Nakasuji and Nomura 1968; Shinkai 1962).

Plants have evolved immunity mechanisms to protect themselves against pests, such as bacteria, fungi, viruses and insects (Bernoux et al. 2011; Chisholm et al. 2006; Lukasik and Takken 2009). Resistance (*R*) genes play a central role in plant disease resistance. Plant disease resistance can be triggered by recognition of specific

pathogen effectors by *R* proteins. The recognition often generates a hypersensitive response (HR), which restricts cell death to the pathogen infection site and prevents further expansion of the pathogens. A number of *R* genes have been cloned from many plant species (Bernoux et al. 2011; Chisholm et al. 2006; Dangl and Jones 2001; Goggin 2007; Lukasik and Takken 2009). However, three *R* genes against sucking insects have been cloned to date. These genes encode nucleotide binding and leucine-rich repeat (NB-LRR) proteins. *Mi-1* confers resistance to both root-knot nematodes (*Meloidogyne incognita*) and potato aphids (*Macrosiphum euphorbiae*) (Vos et al. 1998), and *Bph14* and *Bph26* confer resistance to brown planthoppers (BPH; *Nilaparvata lugens*) in rice (Du et al. 2009; Tamura et al. 2014).

Plants activate different defense responses in response to pest species. Insects can be classified into 2 groups: chewing insects and sucking insects. For chewing insects, jasmonic acid (JA) is a ubiquitous signal that activates defense responses (Howe and Jander 2008;

Abbreviations: BPH, Brown planthopper; GRH, Green rice leafhopper; JA, Jasmonic acid; PR, Pathogenesis-related; SA, Salicylic acid; T65, Taichung 65; TPS, Terpene synthase.

This article can be found at <http://www.jspcmb.jp/>

Published online July 18, 2015

Thompson and Goggin 2006). In fact, the disruption of rice *lipoxygenase* (*OsHI-LOX*) and *CORONATINE INSENSITIVE1* (*OsCOI1*) increases the susceptibility of rice plants to striped stem borer (SSB, *Chilo suppressalis*) and rice leaf folder (LF, *Cnaphalocrocis medinalis*) (Ye et al. 2012; Zhou et al. 2009). In contrast, little is known about the molecular mechanisms underlying sucking insect resistance. Salicylic acid (SA) levels in wild-type plants are increased in response to BPH infestation, whereas no significant difference in JA levels is found between BPH-infested and non-infested rice plants (Tong et al. 2012; Ye et al. 2012). SA synthesis-related genes are expressed more strongly and rapidly in *Bph14*-containing plants than wild-type plants. Therefore, *Bph14* is thought to activate an SA signaling pathway (Du et al. 2009). Suppressed expression of *OsHI-LOX* confers enhanced resistance to BPH, whereas the BPH susceptibility of *OsCOI1* RNAi plants and the GRH susceptibility of the constitutively active JA signaling mutant *rim1* are similar to that of wild-type plants (Ye et al. 2012; Yoshii et al. 2009, 2010; Zhou et al. 2009). These results suggest that rice resistance to BPH and GRH does not depend on JA-mediated defense signaling.

In studies examining plant defense mechanisms against aphids, several components that function downstream of the R gene *Mi-1* have been identified. Silencing the mitogen-activated protein kinases (MAPKs) *LeMPK2* and *LeMPK1* or silencing *LeMPK3* and the MAPK kinase *LeMKK2* resulted in the attenuation of *Mi-1* mediated aphid resistance (Li et al. 2006). The co-chaperone SGT1 and the heat shock protein Hsp90-1 are required for *Mi-1* mediated resistance against root-knot nematodes (RKN; *Meloidogyne* spp.) and potato aphids (*Macrosiphum euphorbiae*) (Bhattarai et al. 2007). Virus-induced gene silencing of the transcription factors *SlWRKY72a* and *SlWRKY72b* in tomato resulted in a reduction of *Mi-1*-mediated resistance as well as basal defense against RKN and potato aphids (Bhattarai et al. 2010). The tomato receptor-like kinase *SISERK1* is required for *Mi-1*-mediated resistance to potato aphids (Mantelin et al. 2011). Thus, pathogen defense-related genes are involved in *Mi-1*-mediated aphid resistance.

Some *indica* varieties exhibit resistance against GRH or GLH (Fujita et al. 2006; Ghani and Khush 1988). Genetic analyses using molecular markers have identified 6 loci and a quantitative trait locus (QTL) with a minor effect on GRH resistance (Fujita et al. 2010). However, none of these GRH resistance genes has been cloned to date. *GRH2* and *GRH4* are found on chromosomes 11 and 3 of Lepe duma and DV85, respectively (Fukuta et al. 1998; Kadowaki et al. 2003). Two near-isogenic lines (NILs) and a pyramided line (PYL) were developed by introducing *GRH2* and *GRH4* (Fujita et al. 2006, 2010). TGRH11 has a substituted

segment including *GRH2* on chromosome 11 from DV85. The size of the substituted segment is less than 9.3 Mbp. There are no other substituted segments in the Taichung 65 (T65) genetic background. TGRH16 has a substituted segment including *GRH4* (0.6 to 10.3 Mbp) on chromosome 3 from DV85. TGRH16 also carries substituted segments on chromosome 2 (with a size of less than 5.1 Mbp) and on chromosome 9 (with a size of less than 7.9 Mbp). TGRH29 has substituted segments including *GRH2* and *GRH4*. TGRH11 exhibits enhanced resistance to GRH insects: the nymph mortality on *GRH2*-NIL is about 75% at 3 days after infestation (Fujita et al. 2010). *GRH2/GRH4*-PYL showed higher nymph mortality than *GRH2*-NIL (Fujita et al. 2006). Regarding the defense response against insects, the signal transduction pathways that are activated by R genes after pest recognition are largely unknown. To explore the molecular mechanisms that underlie *GRH2/GRH4*-mediated insect resistance, we investigated the transcriptional response of rice plants during infestation. We found that the expression of *proteinase inhibitors*, *lipoxygenases* and *terpene synthase* (TPS) genes were upregulated in response to GRH infestation in resistant lines. Furthermore, the transcript levels of TPS genes were correlated with the strong induction of sesquiterpenes in the TGRH29 line. Thus, *GRH2* and *GRH4* activated various defense responses and conferred strong GRH resistance.

Materials and methods

Evaluation of GRH resistance

Rice (*Oryza sativa*) plants and insects were grown at 25°C under a 16-h light/8-h dark photoperiod. The GRH resistance test was performed as described previously (Fujita et al. 2010). Briefly, seedlings at the second-leaf stage were infested with 7 to 10 first- or second-instar nymphs in test tubes. GRH resistance was evaluated by calculating the survival rates of GRH. For the honeydew assay, a plastic dish was placed at low position on the plant stem, and filter paper was placed in the dish. An inverted plastic cup was placed onto the filter paper. Five adult GRH insects were introduced into each plastic cup. After 24 h, the filter papers were treated with 0.1% ninhydrin in acetone solution and dried at 55°C for 30 min.

Oligo DNA microarray analysis

For gene expression analysis, rice plants were grown in a growth chamber at 25°C under a 16-h light/8-h dark photoperiod. Seedlings at the second-leaf stage were infested with 10 to 15 first- or second-instar nymphs in test tubes. Shoot samples were collected at 0 h or 30 h after GRH infestation. For RNA extraction, three independent plant materials were pooled to produce a single sample. Rice total RNA was extracted using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany), and 16 RNA samples were prepared for

the microarray experiment with two biological replicates. The RNA integrity was evaluated on an Agilent 2100 bioanalyzer using RNA nanochips. The RNA quantity was determined on a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies). For microarray analysis, the probe and arrays were designed using eArray version 4.5 (supplied by Agilent Technologies), and 43494 probes were selected for this custom array. Four sets of the 43494 probes (4×44 K microarray formats) were blotted on a glass slide (25×75 mm). Cyanine 3 (Cy3)- or cyanine 5 (Cy5)-labeled complementary RNA (cRNA) samples were synthesized from 850 ng of total RNA using a Quick Amp Labeling Kit (Agilent Technologies) according to the manufacturer's instructions. GRH-infested and uninfested samples were labeled with Cy3 and Cy5, respectively. The hybridization solution was prepared with 825 ng each of Cy3- and Cy5-labeled cRNA preparations using an In Situ Hybridization Kit Plus (Agilent Technologies). Hybridization and washing of the microarray slides were performed according to the manufacturer's protocols. After washing, slide image files were produced using a DNA microarray scanner (G2505B; Agilent Technologies). The signal intensities of Cy3 and Cy5 were extracted from the image files and normalized in each array by Feature Extraction version 9.5 (Agilent Technologies). The signal intensities of all samples were transformed into log₂-based numbers and normalized according to the quantile method by EXPANDER version 5.0 (Shamir et al. 2005). Gene expression analyses were conducted based on the normalized signal intensities using Excel. The median value of the normalized signal intensities of Cy3 and Cy5 of all the probes are calculated, the resulted value was 6.53. The microarray data were deposited in the Gene Expression Omnibus Database (accession no. GSE46409).

Gene expression analysis

RNA isolation and quantitative reverse transcription PCR (qRT-PCR) assays were performed as described previously (Asano et al. 2012). Rice total RNA was extracted from shoots using an RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The isolated RNA was treated with TURBO DNase (Applied Biosystems/Ambion, Austin, TX, USA). cDNA synthesis was performed using an iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA, USA). qRT-PCR analysis was performed using the iCycler iQ System (Bio-Rad, Hercules, CA, USA) with iQ SYBR Green Supermix (Bio-Rad, Hercules, CA, USA) in a total volume of 25 μ l. The reactions were performed in biological triplicates using RNA samples extracted from 3 independent samples of plant material. The expression values were normalized to the *ubiquitin-conjugating enzyme* (*UBC*) gene (Jain et al. 2006). The primers that were used for qRT-PCR are listed in Supplementary Table S1.

Analysis of volatiles in rice

Seven-day-old seedlings were planted in graduated cylinders and infested with adult GRH. After 24 h of infestation, the volatiles released from the plants were captured using a solid-

phase microextraction (SPME) fiber coated with 100 μ m polydimethylsiloxane (PDMS) (Red, Supelco, Bellefonte, PA, USA). Volatile capturing was conducted for 30 min, and the volatiles were immediately analyzed by GC-MS (Agilent 7890A GC system interfaced with a JEOL JMS-T100GC time-of-flight mass spectrometer) equipped with an INNOWax column (30 m×0.25 mm i.d.×0.25 μ m film thickness; Agilent Technologies). Helium was used as the carrier gas at a flow rate of 1 ml/min. The injection temperature was set to 200°C (splitless mode). The GC oven temperature was set at 50°C for 2 min. The temperature was then a ramped to 240°C at 5°C/min (1-min hold). The mass spectrometer was set to electron ionization (EI) mode at 70 eV and 200°C. Mass spectra were recorded with a scanning range of 30 to 400 mass-to-charge ratio (*m/z*), and the mass spectral data were deposited in the NIST mass spectral library.

Results

GRH2 confers enhanced resistance to GRH

An *indica* cultivar, DV85, carrying *GRH2* and *GRH4* shows resistance to GRH (Fujita et al. 2010). *GRH2*-NIL (TGRH11), *GRH4*-NIL (TGRH16) and *GRH2/GRH4*-PYL (TGRH29) were developed by introducing *GRH2* and *GRH4* from DV85 (Fujita et al. 2006, 2010). To investigate the effects of *GRH2* and *GRH4* on GRH resistance, we examined the survival rate of GRH on TGRH11, TGRH16 and TGRH29 from 1–4 days after GRH infestation. Two days after infestation, most of the GRH on T65 and TGRH16 survived, whereas most of the GRH on TGRH29 died. The survival rate of GRH on TGRH11 gradually decreased, and 2/3 of the GRH died at 4 days after infestation (Figure 1A). Seven days after infestation, T65 and TGRH16 developed damage symptoms, such as leaf wilting and yellowing. In contrast, TGRH11 and TGRH29 showed significantly less damage compared with T65 and TGRH16 (Figure 1B). Thus, TGRH11 and TGRH29 confer apparent resistance to GRH. Although TGRH16 showed no GRH resistance, TGRH29 had greater resistance to GRH than TGRH11 due to the synergistic effect of *GRH4*.

We subsequently evaluated the feeding activity of GRH insects on the plants by visualizing honeydew deposited on filter paper with ninhydrin reagent. The intensity of stained honeydew was correlated with the levels of GRH resistance of the plants. Intense staining was observed on T65 and TGRH16 lines after 24 h of infestation. The staining was significantly less intense on TGRH29 and TGRH11 lines compared with T65 and TGRH16 lines (Figure 1C, D). This result suggests that *GRH2* confers resistance to GRH by reducing GRH feeding.

Identification of GRH-responsive genes in rice

To investigate the response of the rice plants to GRH infestation, we performed DNA microarray analysis

Figure 1. Effects of *GRH2* and *GRH4* on GRH resistance. (A) Survival rate of GRH. GRH insects were placed on 7-day-old rice seedlings. Error bars indicate the standard error of the mean (SEM) ($n=9$ or 10). Asterisks indicate a significant difference at $p<0.05$. (B) Phenotypes of rice plants at 7 days after GRH infestation. (C) Honeydew assay. The size of the honeydew and the intensity of the honeydew color indicate the GRH feeding activity. The honeydew area was evaluated after 24 h of GRH infestation. (D) Honeydew area. The area of honeydew color was estimated by ImageJ. Error bars indicate SEM ($n=7$). Asterisks indicate a significant difference at $p<0.05$.

Figure 2. Summary of microarray analysis. (A) The number of GRH-responsive genes. Genes that were 2-fold upregulated or downregulated by GRH infestation (30 h post-infestation) were selected from the microarray data. (B) The number of upregulated genes. Gene expression levels of respective NIL lines were compared with that of T65 at 0 h or 30 h after GRH infestation. Genes that were 2-fold elevated in respective NIL lines were shown.

using a 60-mer oligonucleotide microarray (platform no. GPL7252, available at NCBI GEO). We extracted RNAs from the shoots of the respective rice lines at 0 h or 30 h post-infestation. Transcriptional changes in the respective rice lines were detected by comparison between GRH-infested and non-infested plants. We excluded spots with low signal intensity ($<$ median) and extracted genes that were 2-fold upregulated or downregulated from the microarray data. During GRH infestation at the seedling stage, the expression levels

of 751 and 976 genes were increased and decreased, respectively in the TGRH29 line. The number of GRH-responsive genes is shown in Figure 2. Significantly, the expression levels of fourteen genes such as *transferase family protein* (Os12g27254), *terpene synthase* (Os08g07100 and Os08g07080), *lipoxygenase*, *OsRCI-1* (Os12g37260), *ABC transporter* (Os08g43120), *SAM-dependent carboxyl methyltransferase* (Os06g13390 and Os02g48770), *Bowman-Birk type bran trypsin inhibitor* (Os01g03320), *crocetin dialdehyde* (Os08g28240),

lipase/acylhydrolase (Os07g39740), *hypothetical protein* (Os01g40950), *phosphorylase domain containing protein* (Os05g13970), *late embryogenesis abundant protein, group 3* (Os05g46480) and *cinnamoyl CoA reductase* (Os01g18120) were greatly increased (>100-fold) in the TGRH29 line (Supplementary Table S3).

In contrast, only *probenazole-induced protein* (PBZ1) was significantly upregulated (>100-fold) in the T65 line (data not shown). Among the 322 upregulated genes in both TGRH29 and T65, 25 genes overlap with genes induced by JA treatment (GEO accession no. GPL6864) and 54 genes overlap with genes induced by benzothiadiazole (BTH) treatment (Shimono et al. 2007). In contrast, among the 429 remaining upregulated genes in TGRH29, 30 and 21 genes showed altered the expression level in response to treatment with JA and BTH, respectively. Furthermore, the expression levels of JA synthesis- or signaling-related genes such as *allene oxide synthase* (AOS), *12-oxophytodienoate reductase* (OPR7), *4-coumarate: CoA ligase* (PCL), *acyl-coenzyme A oxidase* (ACX) and *COI1* were comparable between GRH-infested TGRH29 and GRH-infested T65 plants (data not shown). The expression of *NPR1 homolog 1* (*NH1*, a key regulator of SA-mediated systemic acquired resistance) (Yuan et al. 2007) and the pathogenesis-related (PR) gene *PR1b* (Os01g28450) was induced in response to GRH infestation in T65 but not in TGRH29.

Identification of up-regulated genes in GRH-resistant lines

To identify the upregulated genes in TGRH11, TGRH16 and TGRH29, we compared the gene expression levels between NILs/PYL and control plants (T65). The expression levels of a few genes were higher in TGRH11, TGRH16 and TGRH29 compared with T65 before GRH infestation (Figure 2B). In contrast, the expression levels of 127 and 202 genes were higher in TGRH11 and TGRH29, respectively, compared with their levels in T65 at 30 h post-infestation. These results suggest that *GRH2* induces the defense response by recognizing GRH invasion. To explore the defense mechanism of TGRH29, we attempted to identify the upregulated genes in this line. However, the significantly upregulated genes, such as *SAM-dependent carboxyl methyltransferase* (>90-fold) and *TPS* (>150-fold) were excluded by the abovementioned filtering (data not shown). To prevent the exclusion of important genes during the filtering process, we first selected the spots with signal intensities > median only in infested TGRH29, and we subsequently selected the genes with a 2-fold/4-fold increase or decrease in spot intensity when comparing NILs/PYL and T65. The intensities of 1504 genes were increased by 2-fold or more in infested TGRH29 compared with their levels in infested T65. Moreover, the expression levels of 497 genes were ≥ 4 -fold

increased in TGRH29. In contrast, the expression levels of 909 and 158 genes were 2-fold and 4-fold decreased, respectively, in TGRH29. These include genes encoding PR proteins, proteinase inhibitors, transcription factors and metabolism-related genes (Supplementary Table S2). Among the 497 highly expressed genes in TGRH29, the expression of 232 genes was increased in response to GRH infestation in TGRH29 but not in T65, and expression levels of 115 genes ≥ 2 -fold increased in TGRH29 compared with TGRH11. Among the 158 downregulated genes in TGRH29, the expression of 43 genes was decreased in response to GRH infestation in TGRH29.

Among the defense-related genes with a 4-fold or greater intensity ratio at 30 h after GRH infestation, the expression levels of *PR6* genes were especially upregulated in response to GRH infestation in TGRH29. Furthermore, 8 *Bowman-Birk type bran trypsin inhibitors*, 2 *wound-induced proteinase inhibitors* and 9 putative *proteinase inhibitors* were significantly upregulated in TGRH29 compared with T65 (Figure 3, Supplementary Tables S2, S3). Thus, several types of proteinase inhibitor genes were highly expressed in the TGRH29 line.

Several metabolism-related genes were also upregulated in the GRH-resistant line and, most of these genes belonged to the cytochrome P450 family. The expression levels of *lipoxygenase* genes (Os12g37260, Os12g37350 and Os03g49350) were found to be significantly upregulated in response to GRH infestation in the TGRH29 line, whereas the expression of one gene (Os08g39840) was significantly downregulated in response to GRH infestation (Figure 3, Supplementary Table S2, S3). In addition, 4 *TPS* genes were highly upregulated in response to GRH infestation in TGRH29 (Figure 4, Supplementary Table S2, S3). Other highly expressed genes encoded SAM-dependent carboxyl methyltransferase, polyphenol oxidase, O-methyltransferase, aminotransferase, phenylalanine ammonia-lyase, lipase and 2OG-Fe(II) oxygenase superfamily proteins (Figure 4, Supplementary Table S3). The transcriptional repressor protein genes *OsJAZ5*, *OsJAZ8*, *OsJAZ9* and *OsJAZ11* were significantly upregulated in the TGRH29 line during GRH infestation. Eight WRKY transcription factor genes were upregulated in TGRH29, whereas the expression levels of 2 WRKY genes were decreased in TGRH29 compared with T65. Five MYB transcription factor genes were upregulated in TGRH29, whereas one MYB gene was downregulated (Figure 4).

To assess the validity of the microarray data and to examine gene expression over time, we investigated the expression of 3 *TPS* genes, *transferase family protein* (Os12g27254), *OsRCI-1*, *OsJAZ8*, *JAmyb*, *PR6* (*subtilisin-chymotrypsin inhibitor*, Os12g36210) and *OsCCR24* at 0, 6 and 30 h after GRH infestation by qRT-PCR. The

Figure 3. The expression of defense-related genes. The left column indicates the differences in gene expression levels between T65 and NIL lines. The right column shows the transcriptional response to GRH infestation in the respective rice lines. Bowman: *Bowman-Birk type bran trypsin inhibitor*; Win: *Similar to Barwin*.

results indicated that all 9 genes were reproducibly upregulated by GRH infestation in TGRH11 and TGRH29 (Figure 5). The expression of 2 *TPS* genes (*Os04g27670* and *Os08g07080*), *transferase family protein*, *OsRCI-1*, *PR6* and *OsCCR24* was upregulated by GRH infestation in TGRH29, reaching peak expression at 30 h. The expression levels of *OsJAZ8*, *JAmyb* and *TPS2* (*Os02g02930*) in TGRH29 were greater than their levels in T65, and these genes reached maximum expression levels 6 h after GRH infestation. Furthermore, the expression levels of *PR6*, *JAmyb*, *OsCCR24* and *TPS* genes (*Os04g27670* and *Os08g07080*) were higher in TGRH29 than in TGRH11 (Figure 5).

Figure 4. Changes in gene expression during GRH infestation. The left column indicates the differences in gene expression levels between T65 and NIL lines. The right column shows the expression changes induced by GRH infestation in the respective rice lines. LOX: *lipoxygenase*; PPO: *polyphenol oxidase*; SAM: *SAM-dependent carboxyl methyltransferase*; OMT: *O-methyltransferase*; PAL: *phenylalanine ammonia-lyase*.

GRH infestation induced the emission of plant terpenes

Rice *TPS* genes were among the most highly upregulated genes in response to GRH infestation in TGRH29 (Supplementary Table S2). *TPS* genes constitute a large multigene family consisting of 40 genes in *Arabidopsis thaliana* (Aubourg et al. 2002) and were involved in production of monoterpenes, diterpenes and sesquiterpenes (Lee and Chappell 2008). Rice *TPS* genes showed a high level of sequence similarity with sorghum and maize sesquiterpene synthase genes.

Figure 5. Transcriptional response to GRH infestation. Total RNA was extracted from the shoots and subjected to quantitative RT-PCR analysis. The expression level of each gene relative to the expression level of *UBC* at each time point is indicated. Error bars indicate the SEM of 3 independent experiments.

Figure 6. Total ion chromatograph of volatiles emitted in response to GRH infestation. Volatiles were collected after no treatment (control) or after 24 h of GRH infestation and analyzed by GC-MS. The peaks were identified as zingiberene (peak 1), β -bisabolene (peak 2) and β -sesquiphellandrene (peak 3).

Herbivore-induced plant volatiles are thought to be involved in direct/indirect defenses against insects. To determine whether the production of volatiles is induced by GRH infestation, we identified the volatiles emitted from TGRH29 plants using gas chromatography-mass spectrometry (GC-MS). Peaks were detected in samples from GRH-infested TGRH29 plants, whereas no significant peaks were found in GRH-infested T65 plants or TGRH29 plants that were not infested with GRH. GC-MS revealed strong induction of sesquiterpenes such as

zingiberene, β -sesquiphellandrene and β -bisabolene in GRH-infested TGRH29 plants (Figure 6).

Discussion

R genes play a central role in the plant defense system. Only 3 insect *R* genes have been cloned to date, whereas a number of *R* genes conferring resistance against pathogens have been cloned from many plant species (Du et al. 2009; Tamura et al. 2014; Vos et al. 1998). Significantly, the *R*-gene-mediated defense mechanism against BPH and GRH is poorly understood. In this study, we investigated the effects of *GRH2* and *GRH4* on GRH resistance. TGRH11 carrying *GRH2* exhibits increased resistance to GRH insects, whereas the susceptibility of TGRH16 carrying *GRH4* is comparable to that of T65. Furthermore, TGRH29 carrying *GRH2* and *GRH4* shows strong insect resistance. The feeding activity of GRH insects on TGRH29 was dramatically reduced (Figure 1). These results suggest that the combination of *GRH2* and *GRH4* confers strong resistance to GRH by reducing GRH feeding.

Elucidation of the insect resistance response of rice plants is important for the production of GRH-resistant rice plants. Gene expression profiles in response to BPH infestation have been analyzed by several groups; however the mechanism of BPH resistance remains unclear (Wang et al. 2008, 2012). In our microarray analyses, the gene expression levels of most JA/SA synthesis- or signaling-related genes in resistant lines

were similar to their levels in T65. These results suggest that JA and SA do not play an important role in GRH resistance. More genes were upregulated in infested TGRH29 compared with T65 than in uninfested TGRH29 compared with T65, suggesting that *GRH2* confers increased resistance to GRH. Our results showed that the expression levels of several types of genes, including *PR* genes, proteinase inhibitors, transcription factors and metabolism-related genes, are increased in response to GRH infestation, and the expression levels are higher in TGRH29 than in T65, suggesting that these genes are involved in GRH resistance.

Sesquiterpenoids are involved in either direct or indirect defenses to insects (Huffaker et al. 2011; Schnee et al. 2006). Rice *TPS* genes (*Os04g01810*, *Os04g27340*, *Os04g27670*, *Os08g07080* and *Os08g07100*) were upregulated in response to GRH infestation in shoots of TGRH29 plants. Maize sesquiterpene synthases, which are closely related to rice *TPS*s, reportedly function in the production of sesquiterpenes. In fact, highly induced expression of rice *TPS* genes was associated with the strong induction of sesquiterpenes (including zingiberene, β -sesquiphellandrene and β -bisabolene) in the GRH-infested TGRH29 plants. These results suggest that GRH infestation induces the production of sesquiterpenes in the resistant line TGRH29. The accumulation of pathogen-induced sesquiterpenoid phytoalexins has a direct positive relationship with the transcript levels of *Tps6* and *Tps11* (Huffaker et al. 2011), and plants in which these genes are silenced exhibit increased susceptibility to *Ustilago maydis* (van der Linde et al. 2011). Further studies using the loss-of-function lines in which the expression of *TPS* genes is suppressed are needed to elucidate the biological role of rice *TPS* genes in GRH resistance.

Oxylipins encompass a large family of oxidized fatty acids and play pivotal roles as signaling molecules and protective compounds in plant responses to biotic stress. LOX has a central role in the oxylipin biosynthesis pathway (Blee 2002; Farmer et al. 2003; Nalam et al. 2012). The expression level of *OsRCI-1* (*Os12g37260*) was higher in GRH-infested shoots of TGRH29 compared with T65. Furthermore, the expression of *Os3g49350* was upregulated in response to GRH infestation in the shoots of TGRH29. In contrast, the expression of *OsLOX-RLL* (*Os08g39840*) was downregulated in response to GRH infestation in TGRH29. These transcription of these *LOX* genes was differentially regulated by the GRH2/GRH4-mediated defense response, although *OsRCI-1* and *OsLOX-RLL* encode 13-LOX, which incorporates oxygen at the C-13 position of the fatty acid to yield a 13-fatty acid hydroperoxide (Ohta et al. 1992, Peng et al. 1994, Schaffrath et al. 2000). The expression of *OsRCI-1* is induced in response to treatment with 2,6-dichloroisonicotinic acid (INA), JA, BTH and

probenazol (Schaffrath et al. 2000). The BTH response of *OsRCI-1* appeared unaffected by the knockdown of either *WRKY45* (*OsWRKY71*) or *NH1*, suggesting that the upregulation of *OsRCI-1* depends on another regulator (Shimono et al. 2007). This result agrees with our data that *NH1* and *PR1b* transcripts are increased in the susceptible line T65, whereas neither gene responded to GRH infestation in TGRH29. Suppression of *OsHI-LOX* (*OsLOX-RLL*) expression confers enhanced resistance to BPH (Zhou et al. 2009). In our microarray analyses, the expression of *OsLOX-RLL* was downregulated in response to GRH infestation in TGRH29. Thus, suppression of *OsLOX-RLL* expression may be associated with plant resistance to GRH. The functional role of *LOX*s in GRH resistance is unclear, although the modulation of *LOX* expression is thought to be involved in plant resistance.

TGRH29 exhibited a higher level of GRH resistance compared with TGRH11 (Figure 1). It is noteworthy that TGRH29 has strong insect resistance and defends itself against GRH insects via an inhibition of GRH feeding activity. The results of microarray analyses indicate that a large number of genes were upregulated in response to GRH infestation in TGRH29 compared with TGRH11 (Figure 2B). Furthermore, the expression levels of *TPS* (*Os04g27670*), *PR6* (*Os12g36210*) and *OsCCR24* were higher in TGRH29 than in TGRH11 at 30 h after GRH infestation. Transcripts of *JAmyb* and *TPS* (*Os08g07080*) accumulated to higher levels in TGRH29 than in TGRH11 at 6 h after GRH infestation. These results suggest that TGRH29 plants carrying both *GRH2* and *GRH4* can more rapidly and strongly activate their defense responses compared with plants expressing *GRH2* alone. In TGRH29, *GRH2* is thought to play an important role in GRH resistance and may be involved in the recognition of GRH invasion and the induction of the defense response because expression of *GRH4* alone did not provide any resistance to GRH. However, the presence of *GRH2* alone is not sufficient to protect rice plants against GRH. The GRH resistance of rice plants carrying *GRH2* is broken down by virulent biotypes under experimental conditions in the laboratory. In contrast, a virulent biotype that is effective against rice plants that carry *GRH2* and *GRH4* from Lepe duma and C203-1 could not be identified by continuously rearing the GRH insects on the resistant rice lines (Hirae et al. 2007). Due to the presence of both *GRH2* and *GRH4*, TGRH29 plants rapidly recognize GRH invasion and subsequently activate various defense-related genes; eventually, these plants not only show strong GRH resistance but also prevent the appearance of a virulent biotype. Thus, *GRH2* and *GRH4* have a synergistic function in plant defense and confer strong GRH resistance.

Acknowledgements

We thank Setsuko Kimura and Aeni Hosaka for technical assistance. This work was supported by a grant from the Ministry of Agriculture, Forestry and Fisheries for Global-Scale Issue International Research Network Projects.

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