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The non-host pathogen *Puccinia triticina* elicits an active transcriptional response in rice

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Abstract Rice (*Oryza sativa L.*) is not susceptible to rust fungi, including the wheat leaf rust fungus *Puccinia triticina*. Upon inoculation with *P. triticina* spores, infection hyphae and appressoria were observed on the leaf surfaces of the rice cultivar Nipponbare. The cultivar responded to the inoculation with brown discoloration of the local tissue and fragmentation of rust infection hyphae and appressoria. A microarray gene-expression analysis of the host transcriptional response was performed 24 h after inoculation, revealing rice genes that were up- or down-regulated following the interaction. In particular, the loci represented by five probe sets (Os.55776.1. S1_x_at, Os.55647.1. A1_at, Os.55776.1. S1_at, OsAffx.10944.1. S1_x_at, and OsAffx.10944.1. S1_at) displayed the highest increase in gene expression compared to the control inoculation. The probe sets included members of the receptor-like kinase family (RLK) that occurs within a cluster of RLK genes on chromosome 1. Other RLK genes, within the RLK gene cluster and at another location, also showed increases in gene expression after *P. triticina* inoculation. The RLK genes varied in response to challenges with different rust strains or when challenged with several non-rust wheat pathogens that are also non-pathogenic to rice. The results indicate that rice has an active transcriptional and possible defense priming reaction in response to *P. triticina* and other non-host fungal pathogens.

Keywords Non-host resistance · *Oryza sativa* · *Puccinia triticina* · Defense pathway · Microarray · Receptor-like kinase

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Introduction

The ability of all individuals of a plant species to resist infection by a pathogenic species is known as non-host resistance (NHR). NHR reflects host adaptations for resistance against an otherwise pathogenic species or the absence of specific virulence mechanisms within the pathogenic species to infect and colonize a given species (Zellerhoff et al. 2010; Nurnberger and Lipka

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2005; Heath 2000). The study of NHR may reveal adaptations of non-host plants to specific pathogens, here referred to as non-adapted pathogens, which can be exploited for resistance that is both durable over time and broadly effective against most or all isolates of a pathogenic species (Ayliffe et al. 2011a, 2011b; Zipfel 2008). In *Arabidopsis*, studies of NHR to a variety of fungal pathogens have led to the identification of pre-invasion and post-invasion factors, including the proteins PEN1, PEN2, and PEN3 (Collins et al. 2003; Lipka et al. 2005; Stein et al. 2006). However, the PEN proteins are only three components of more complex NHR mechanisms that prevent penetration and growth as *pen* mutants of *Arabidopsis* remain resistant to infection by the barley powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* (*Bgh*). Other host mutations combined with *pen* mutations further reduce NHR. *Arabidopsis* plants with the *pen2*, *pad4* (phytoalexin-deficient 4) and *sag101* (senescence-associated gene 101) mutations, for example, allow full *Bgh* haustoria development (Lipka et al. 2005). The *pen2* and *pen3* mutants, alone, allow increased haustoria formation by *Phytophthora infestans*. However, *pen2* mutants are less susceptible to *Plectosphaerella cucumerina*, a necrotrophic pathogen of *Arabidopsis*, and *pen3* mutants are more susceptible to *P. cucumerina* (Lipka et al. 2005; Stein et al. 2006). Only *pen2* affected pre-invasion by the rice blast pathogen *Magnaporthe oryzae*, and *agb1* and *pmr5* mutations further inhibited NHR to blast disease (Maeda et al. 2009). Therefore, genes affecting NHR do not fit into a single pathway or process and diverse processes might contribute to NHR in any given host-pathogen combination. These findings are consistent with early models in which NHR was proposed to consist of multiple barriers operating in parallel to limit pathogen colonization (Stam et al. 2014; Senthil-Kumar and Mysore 2013; Fan and Doerner 2012; Heath 2000).

One approach to understanding NHR involves transcriptome profiling following the challenge of plants with non-adapted pathogens. Non-adapted pathogens are species or strains of a species that have infect particular host species but are not virulent on the host in question. A comparative study presented the transcriptional responses of barley cultivar (*Hordeum vulgare subsp. Vulgare 'Ingrid'*) to three pairs of adapted and non-adapted pathogens causing powdery mildew, rust, and blast, respectively (Zellerhoff et al. 2010). During the interactions of barley with the different host or non-adapted pathogens, similar patterns of overrepresented and underrepresented functional gene categories were found. Powdery mildew- and blast-induced differences in transcript abundance between host and non-adapted interactions were correlated with differences between a near-isogenic pair of barley lines carrying either the *Mlo* wild-type allele or the recessive *mlo5* allele for resistance to powdery mildew. The results indicated that NHR and basal host defense of barley are functionally related (Zellerhoff et al. 2010). The results also indicated that NHR responses to different fungal pathogens was associated with a more robust regulation of complex but largely non-overlapping sets of pathogen-responsive genes and pathways (Zellerhoff et al. 2010).

Rust fungi are highly specialized obligate parasites that infect many plant species, including members of the grass family with the noted exception of rice (Kolmer et al. 2009). Rice, therefore, may represent a good model species to investigate NHR to rust fungi. A variety of rice defense compounds have been shown to be induced in response to rust fungi in various experimental situations (Giraldo and Valent 2013). In an analysis of the infection of *Arabidopsis*, wheat leaf rust pathogen *P. triticina* rarely penetrated the leaf epidermis (Shafiei et al. 2007). Infection of a series of well-characterized defense mutants showed no altered infection phenotype. However, the attempted infection induced the formation of reactive oxygen species and salicylic acid, in addition to the induction of several defense related genes (Shafiei et al. 2007). Prats et al. (2007) indicated that the non-host reaction between the respective non-adapted wheat and barley leaf rust strains resulted in a rapid programmed cell death in the early stages of infection on the respective nonhost species (Prats et al. 2007). Cereal rusts can initiate infection and interact with rice leaves. Cytological analysis of the interaction between rice and the wheat stem rust pathogen (*Puccinia graminis*) identified the production of callose deposition, reactive oxygen species and autofluorescent material surrounding some infection sites (Ayliffe et al. 2011a, 2011b). Several rice mutants exhibit compromised NHR to *P. striiformis* f. sp. *tritici* (*Pst*) compared with the wild-type Nipponbare (Yang et al. 2014). These observations showed an active host response with features similar to typical R gene-mediated resistance response. However, the molecular mechanisms of NHR against rust fungi remain unclear.

Puccinia triticina is a rust fungal pathogen of wheat. The pathogen initiates an infection in wheat with the

germination of urediniospores on the leaf surface, forms an infection peg under anappressorium, penetrates the stoma, and forms vesicles in the substomatal chamber. Hyphae proliferate from the substomatal chamber into surrounding mesophyll tissue, and initial haustorial mother cells are formed. The goal of this study was to investigate the transcriptional response of rice plants challenged with *P. triticina* and several additional other non-adapted fungal pathogens. Ultimately, the specific genes that are regulated in response to non-adapted fungal challenge should prove useful in characterization of the immunity of rice to rust diseases.

Materials and methods

Plant materials

Rice cultivar Nipponbare was used in this study. Seeds were soaked in water overnight and germinated in a plastic container filled with two layers of filter paper that were soaked with a thin layer of water at 30 °C for 5 days. Water was changed twice daily during the 5-day germination period. Seedlings were transplanted into eight-inch pots, with 10 seedlings per pot, and kept in growth chambers at 25 °C with a daily photoperiod of 14 h light and 10 h dark.

Inoculum preparation and procedure

Wheat leaf rust strain PNMQ was used in the microarray analysis. Three additional strains, BBBM, PRTU55 and MCDSB, were also used in follow-up quantitative real-time PCR (qRT-PCR) measurements. Urediniospores of *P. triticina* were suspended in Soltrol oil (Chevron Phillips Chemical Company, Woodlands, TX) at 50 mg/ml, and 18-day-old Nipponbare seedlings were pressure-sprayed with the fungal spore suspensions (5 ml/24 plants) with an atomizer (De Vilbiss Co., Somerset, PA), Soltrol oil alone was used as a mock control. The experiments were performed with three biological replications, each replication was kept in a separate tray, and 10 rice seedlings were used for each treatment. After spray-inoculation, inoculated plants were air dried for 10 min to allow spores to adhere to the leaves. Inoculated plants were kept in a dark mist chamber with 100 % humidity at 23 °C for 24 h and then transferred to a normal growth chamber at 25 °C with a 14-h light period. Leaves were harvested as follows: at 3, 6, 12, 18, 24, 48, 72, 96 h, and 1, 2, 3, 4, and 5 weeks post-inoculation for histological observations; at 24 h for microarray; and at 3, 6, 9, 12, and 24 h for qRT-PCR experiments. For spore heat-inactivation experiment, BBBM and MCDSB spores were treated in 70 °C for 20 min, and inoculated with live spores as described above. Gene expression levels on selected RLK genes were measured by qRT-PCR. Heat-killed spores and live spores were also compared for germination in 2 % water agar for 14 days.

For wheat leaf blotch (*Septoria tritici*) inoculation, fungal spores from 7- to 10 day-old cultures on V8 juice agar (150 ml of V8 juice, 3 g of $CaCo_3$, 15 g of agar, and 850 ml of distilled water) were streaked onto fresh V8 agar in petri dishes at room temperature (22 ± 2 °C) and cultured for 7 days under light. Spores were harvested by blending a single agar plate in 150 ml of distilled water with a commercial blender and filtered through two layers of cheesecloth. Spores were counted with a hemacytometer and suspensions were adjusted to 4×10^5 spores/ml with distilled water. Eighteen-day old Nipponbare seedlings were spray-inoculated with the fungal spore suspensions (10 ml / 24 plants). Inoculated plants were air dried for about 30 min, and seedlings were stored in a moist chamber (25 ± 5 °C) at 100 % humidity for 72 h and then returned to the growth chamber. The leaf tissues were collected at 3, 6, 9, 12, and 24 h for further analyses.

For wheat tan spot inoculation, a strain of race 1 of *Pyrenophora tritici-repentis* was used. Spores were produced by placing 0.5 cm^2 mycelial plugs of *P. tritici-repentis* from one-fourth-strength potato dextrose agar (1/4 PDA) in the center of plates of V-8 agar. Plates were incubated in the dark at 23 °C for 5 days until the colony reached ~5 cm in diameter. Mycelia were pushed in contact with the agar media using a sterile glass rod, and plates were incubated in the light (~30 $\mu E\ s^{-1}\ m^{-2}$) at 23 °C for 12 h to produce conidiophores and then in the dark at 16 °C for 12 h to produce conidia. Spore suspensions were quantified using a hemacytometer and adjusted with distilled water to 20,000 spores/ml. The spore suspension (10 ml/24 plants) was pressure-sprayed, and plants were air-dried and placed into a mist chamber to maintain continual leaf wetness for 48 h at 20 to 28 °C. After the mist treatment, plants were transferred to the growth chamber (20 to 28 °C). The leaf tissues were collected at 3, 6, 9, 12, and 24 h for further analysis.

Histology

Rice leaves were detached from inoculated plants at the designated time points and treated with a mixture of 95 % ethanol and acetic acid (1:1 mixed) for 24 h to remove chlorophyll, followed by treatment with 70 % ethanol until they became transparent. The leaves were then stained with a 0.8 mM trypan blue staining solution or 0.1 % calcofluor white M2R (Amresco, Solon, OH, USA) for 5–10 min, washed with distilled water, and viewed under differential interference contrast optics and UV light, respectively. Fluorescence was observed by epifluorescence microscopy.

Specimen preparation for scanning electron microscopy

Harvested leaves were cut into 3 mm squares and fixed in 4 % glutaraldehyde in 0.05 M phosphate buffer (pH 6.8) for 24 h at 4 °C, washed twice in the buffer, then dehydrated in a graded ethanol series. Specimens were critical-point dried with carbon dioxide as a transition fluid and were mounted on copper stubs, sputter-coated with gold-palladium. Materials were examined with a JEOL-6360 scanning electron microscope (Japan Electronics, Tokyo, Japan) operating at 10–15 kV.

RNA isolation and quality controls

Total RNA was extracted using TRIzol Reagent (Invitrogen/ThermoFisher, Carlsbad, CA) according to manufacturer's instructions. Isolated RNA was further purified with Qiagen (Valencia, CA) RNeasy column. The concentration of each sample was measured using a NanoDrop ND-1000 Spectrophotometer (NanoDrop/ThermoFisher, Wilmington, DE). RNA samples with 260/280 ratios of 1.9–2.1 and 260/230 ratios greater than 2.0 were used for microarray and qRT-PCR analyses. The integrity of the RNA samples was also assessed using an Agilent Technologies Bioanalyzer 2100 (Santa Clara, CA) prior to microarray analysis.

Microarray hybridization

Five micrograms of total RNA were used for first and second strand cDNA syntheses followed by cRNA amplification and labeling using the Affymetrix (Santa Clara, CA) One-Cycle Target Labeling and Control Reagent and following the manufacturer's instructions. Fifteen micrograms of cRNA was fragmented and the quality of the fragmented RNA was analyzed using the Agilent Bioanalyzer 2100 to ensure that the cRNA fragments were in the desired size range prior to microarray chip hybridization. Affymetrix GeneChip Rice Genome Arrays containing 57,381 probe sets representing 49,824 unique transcripts were used for the experiment. Microarray chip hybridization, washing, staining, and scanning were carried out with the Affymetrix GeneChip GCS3000 Instrument System according to the manufacturer's instructions. The quality of the microarray hybridization was monitored in terms of scaling factors, signal-to-noise ratio, and overall signal intensity.

Microarray data analysis

The average signal intensity of each chip was scaled to 500 in the Affymetrix GCOS software and exported into GeneSpring GX (Agilent Technologies) for further analysis. Each chip was pre-normalized to the 50th percentile and normalized using a "per gene normalization to median" algorithm, in which the signal of each gene in a given sample is divided by the median signal of the same gene in all samples. Microarray data were deposited in the NCBI gene expression omnibus (http://www.ncbi.nlm.nih.gov/geo/) under accession numbers GSM761678-GSM761683.

Three biological replications for the treatment and the control were analyzed individually for the effects of the different inoculations on gene expression. Only the probe sets that had an overall correlation coefficient of greater than 0.95 within the three biological replications and were present in two out of three replicated chips within the same treatment groups (3 chips for the treatment and 3 for mock control) as determined by Student's t-test ($P < 0.05$) were selected for further analysis. The preselected probe sets were subjected to a one-way analysis of variance (ANOVA) test to generate a significantly ($P \leq 0.05$) and differentially (≥2-fold) expressed gene list. False discovery rate was calculated as Q-value using Benjamini-Hochberg method (Benjamini and Hochberg 1995). The average coefficients of variation (CV) was compared for the top nineteen shared and most highly and stably expressed genes as determined by Wang et al. (2010).

Initial annotations of the probe sets on the arrays were performed using Affymetrix online tool NetAffx (http://www.affymetrix.com), the TIGR rice annotation project (ftp://occams.dfci.harvard.edu/pub/bio/tgi/software/), the Rice Functional Genomic Express Database (http://signal.salk.edu/cgi-bin/RiceGE), and

the HarvEST tool generated by University of California at Riverside (http://harvest.ucr.edu/). Selected sequences were used to search for homologous sequences in the GenBank nr-nt and EST-other databases through both nucleotide (blastn) and predicted protein (tblastx) algorithms.

Real-time reverse transcription PCR (qRT-PCR) analysis

Differential expression of representative genes from the microarray data analysis was analyzed by qRT-PCR analysis. Primers were designed using Beacon Designer 7.2 Software (PREMIER Biosoft International, Palo Alto, CA). At least one primer of a pair was designed from a strongly expressed individual probe selected from 11 probes within the probe set in the Rice Genome Array (Table 4). First-strand cDNA was synthesized by reverse transcribing 2 μg of total RNA in a final reaction volume of 20 μl using a SuperScript™ III Reverse Transcriptase kit (Invitrogen, Carlsbad, CA). A minimum of two independent biological replicates of each sample and three technical replicates of each biological replicate were subjected to qRT-PCR analysis. The expression level of each gene in different RNA samples was normalized with the reference gene, rice elongation factor 2 (EF2: Os02g32030), which selected from available microarray data as the internal control. Gene expression levels of the treated samples relative to controls were calculated using the delta-delta Ct method (Liu and Saint 2002).

Results

Wheat leaf rust spores germinate and form appressoria on rice leaves

Light, autofluorescent, epifluorescent and scanning electron microscopy were used to follow the fate of rust spores on rice leaves after spray-inoculation with spores of the *P. triticina* strain PNMQ. Spore germination, appressoria formation, and substomatal cavity invasion were observed within 24 h (Fig. 1a, b and c). Accumulation of hydrogen peroxide (H_2O_2) was subsequently observed in the substomatal cavity at 2 days post inoculation (dpi) (Fig. 1d), and accumulation extended to the cells near the appressoria at 4 dpi (Fig. 1e). At 7 dpi H_2O_2 accumulated in the area surrounding the initial site of penetration (Fig. 1f), and autofluorescent material was observed at the tissue in contact with the appressoria and the surrounding vascular tissue (Fig. 1g). Upon clearing the tissue, a brown callose-like material was observed surrounding the invaded tissue (Fig. 1h, i), and fragmented infection hyphae and appressoria were evidenced after trypan blue staining (Fig. 1h, i). No substomatal vesicles, haustorial mother cells, or haustoria were observed.

Microarray analysis reveals an active transcriptional response in rice to rust infection

Transcriptional responses of rice cultivar Nipponbare, upon challenging with the wheat leaf rust isolate PNMQ, were measured by microarray hybridization. Scaling factors for three biological replications of treated and mock controls ranged from 1.77 to 3.19 with an average of 2.34. Noise ratios were from 1.41 to 1.63 and averaged 1.53. All pairwise correlation coefficients were greater than 0.90 (Soltrol oil, 0.92, 0.91, 0.95; leaf rust, 0.97, 0.93, 0.92). The six treatments had an average of 23,642 ± 2259 probe sets that passed the statistical filter of $p < 0.05$. A total of 31,030 probe sets were detected as present in all treatments combined. The average coefficients of variation (CV) for the top nineteen shared and most highly expressed genes was 14 % compared to the average CV of 16 % in the study by Wang et al. (2010) (Table 1).

Forty-nine probe sets that were present in at least two of the three arrays within the same treatment were detected as being up-regulated by three-fold or greater. The top 23 probe sets represented 20 putative genes due to several annotation ambiguities (Table 2). Five of the top 10 up-regulated probe sets corresponded to two adjacent annotated loci, Os01g06882 and Os01g06876, both of which reside in a cluster of putative receptor-like kinase (RLK) genes on chromosome 1 (Fig. 2). Both Os01g06882 and Os01g06876 have multiple cDNA matches (Table 2). The highly induced probe set that corresponded to the hypothetical protein Os08g31950 matched the cDNA clone CT835941 (Table 2). Another probe set corresponded to Os07g44140, which belongs to the large cytochrome P450 family, and another set corresponds to Os01g62950 is a homolog of RAB11C, a putative Ras-related GTP-binding protein (Ren et al. 1996). Other highly induced genes corresponded to a WD domain/G-beta repeat protein, which is typically involved in signal transduction and transcriptional

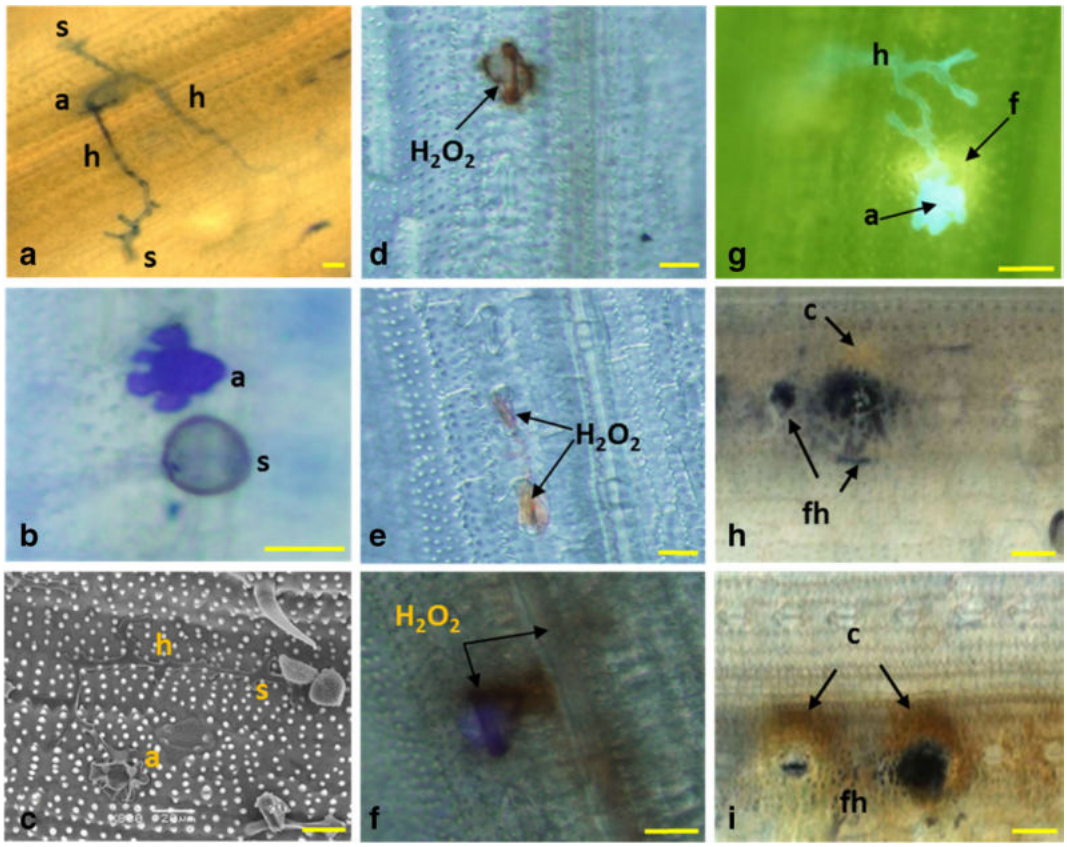

Fig. 1 Leaves of the rice cultivar Nipponbare were inoculated with *P. triticina*, and the infection process was observed under fluorescent and electron scanning microscopes. A and B: Germinated *Pt* spores and appressoria over the rice stoma at 24 hpi. Leaf tissues were stained with aniline blue and observed by epifluorescence microscopy. C: Germinated *Pt* spores and appressoria over the rice stoma at 24 hpi examined by scanning electron microscope. D to E: Detection of hydrogen peroxide (H_2O_2) at infection sites. Staining at appressoria (arrows) at 2 dpi (D) and extension to the cells in the surrounding tissue at 4 dpi (E). H_2O_2 accumulated in the area surrounding the initial site of penetration (F), and autofluorescence was also observed around the tissue at 7 dpi (G). Leaf tissues were stained with 3, 3-diaminobenzidine and calcofluor white M2R and observed under Nomarski interference optic and UV light respectively. h: infection hyphae; f: rice leaf autofluoresence; H and I: callose, fragmented infection hyphae and appressoria were observed surrounding the invaded tissue, Leaf tissues were stained with trypan blue and observed under epifluorescence microscope. s: fungal spore; a: appressoria; fh: fragmented fungal infection hyphae and appressoria; and c: host callose deposition. Yellow bar in the lower right corner in each panel indicates a scale of 20 μm

regulation (Neer et al. 1994); a blue copper-binding protein, often involved in stress responses (Ruan et al. 2011); an NAC (for NAM, ATAF1/2, and CUC2) transcription factor, which is potentially involved in JA-mediated defense responses (Bu et al. 2008); an MDR-like ABC transporter (Jasinski et al. 2003); and two additional RLK genes (Afzal et al. 2008). Of the 10 most down-regulated genes (Table 3), Os01g03340 and Os01g03320 correspond to members of a seven-gene cluster of Bowman Birk trypsin inhibitors on chromosome 1 (Qu et al. 2003). Genes for homologs of the WRKY transcription factor (Wu et al. 2005) and a SHR5 receptor-like kinase (Vinagre et al. 2006) were also among the most down-regulated genes (Table 3).

Analysis of gene expression by qRT-PCR following challenge with four strains of *P. triticina*

Additional *P. triticina* race isolates, which are representative race isolates maintained at KSU, were used to determine if gene induction was a general response to *P. triticina* or specific to a specific strain of the pathogen using a few select genes derived, in part, from the microarray results. Primer pairs (Table 4) were used for genes corresponding to Os01g06876 and Os01g06882, which were induced in the inoculation with PNMQ, and four additional RLK-like genes, Os01g06520 and Os01g06790, which occur in the same RLK 218 kb region on chromosome 1 but were not

Table 1 Comparison of nineteen stable and highly expressed genes in rice

Probe set ID	*Mean signal*	*Signal SD**	*CV**(%) Present Wang study* et al		*Annotation*
Os.12602.1. S1_at	21,452	1817	8	15	Triosephosphate isomerase
Os.4705.1. S1_at	16,015	1460	9	17	Protein elongation factor
Os.7897.1. S1_at	26,279	2662	10	16	Endothelial differentiation factor
Os.12237.1. S1_a_at	29,280	3071	10	11	Expressed protein
Os.11479.1. S1_at	14,377	1538	11	19	Ubiquitin-conjugating enzyme
Os.22660.3. S1_x_at	23,044	2575	11	17	Polyubiquitin
Os.10931.1. S1_at	27,090	3106	11	14	ADP-ribosylation factor
Os.4746.1. S1_a_at	18,735	2214	12	21	60S ribosomal protein L31
Os.8152.1. S1_at	16,591	1996	12	20	GTP-binding nuclear protein
Os.10152.1. S1_at	22,515	2974	13	12	Profilin
Os.4157.1. S1_at	21,276	3264	15	16	Peptidyl-propyl *cis-trans* isomerase
Os.7945.1. S1_at	25,160	4027	16	16	Protein translation factor SUI1
Os.12178.1. S1_at	12,409	2003	16	21	Peptidyl-propyl isomerase
Os.28425.1. S1_x_at	27,357	4690	17	9	Gly-rich RNA-binding protein
Os.12168.2. S1_s_at	27,985	4989	18	17	GAPDH
Os.46231.1. S1_a_at	24,726	4451	18	15	Gly-rich RNA-binding protein
Os.10139.1. S1_s_at	18,809	3530	19	16	Ubiquitin monomer
Os.12749.1. S1_s_at	12,085	2309	19	21	Actin-depolymerizing factor
Os.318.1. S1_at	12,824	2468	19	19	Translation initiation factor
Average			14	16	

**SD* Standard deviation

***CV* Coefficient of variation

induced in the microarray experiment, and Os01g06836 and Os01g06870, which were not represented in the microarray and also reside in the same RLK cluster (Fig. 2). Both Os01g06876 and Os01g06882 were highly induced following inoculations with MCDSB and PRTU55 to a moderate level by strain PNMQ, while isolate BBBM provoked a minimal induction for Os01g06876 and Os01g6882 as measured by qRT-PCR (Fig. 3). Neither genes for Os01g06520 and Os01g06790 were found to be induced following the inoculations with any of the four *P. triticina* isolates (Fig. 3). Genes Os01g06836 and Os01g06870 were induced after inoculation with three of the four *P. triticina* isolates (Fig. 3). Os03g50810, which was found to be induced 4.9-fold in microarray analysis (Table 2) and annotated as an RLK-related gene, was induced to a lesser extent by qRT-PCR. Os01g53920 and Os08g28710 were induced by microarray analysis and qRT-PCR (Fig. 3). In general, rust isolates MCDSB and PRTU55 elicited higher levels of gene expression in comparison to PNMQ. Isolate BBBM provoked the lowest average responses for all loci in comparison to the other isolates (Fig. 3). Gene expression were especially pronounced to high levels by inoculation with strain MCDSB, ranging from 4-fold to greater than 60-fold compared with the mock control; this strain was therefore selected for use in subsequent experiments.

Gene expression profiles of selected RLK genes were measured by qRT-PCR at 3, 6, 9, 12, and 24 h post-inoculation with *P. triticina* strain MCDSB (Fig. 4). The expression levels for Os01g06520, Os01g06790, and Os01g56330 were not induced over the 24 h period (Fig. 4). Loci for Os01g06836, Os01g06876, Os01g06882, and Os03g50810 were induced by 9 h and achieved even higher expression levels at 24 h. The expression levels of Os01g06870 and Os08g28710 continued to increase over time. The expression of Os01g53920 was also increased over time, and a high expression level was maintained from 9 to 24 h.

Table 2 Top twenty up-regulated rice genes after challenge with leaf rust strain PNMQ

Probe set ID	*Gene*[a]	*Gene annotation*	*Fold L/Ck*[b]	*P L/Ck*[c]	*Q FDR*[d]	*cDNA Accession*
Os.55776.1. S1_x_at	Os01g06876	Receptor-like kinase	16	0.01	0.03	AK108785.1
Os.55647.1. A1_at	Os01g06882	Hypothetical protein	15	0.01	0.03	AK108484.1
Os.55776.1. S1_at	Os01g06876	Receptor-like kinase	15	0.03	0.04	AK108785.1
OsAffx.10944.1. S1_x_at	Os01g06882	Hypothetical protein	10.7	0.01	0.03	AK108484.1
Os.13954.1. S1_x_at	Os08g31950	Hypothetical protein	10.1	0.04	0.04	CT835941
OsAffx.10944.1. S1_at	Os01g06882	Hypothetical protein	6.7	0.02	0.03	AK108484.1
Os.34462.1. S1_at	Os01g62950	GTP-binding, RAB11C	6.6	0.05	0.05	AK071303.1
Os.8741.1. S1_at	Os03g12820	Hypothetical protein	6.0	0.03	0.04	AK102303.1
Os.9067.1. S1_at	Os07g44140	Cytochrome P450	5.4	0.03	0.04	AK121733.1
Os.27279.1. A1_at	Os03g26870	WD domain	5.2	0.00	0.00	AK072984.1
Os.7393.1. S1_at	Os07g02200	Blue copper-binding protein-like	5.1	0.02	0.03	AK073121.1
Os.4149.2. S1_x_at	Os03g50810	Protein kinase	4.9	0.01	0.03	AK062249.1
Os.14971.1. S1_at	Os08g40850	mit. Energy transfer	4.8	0.03	0.04	AK063556.1
Os.55314.1. S1_at	Os03g38350	GDSL-like lipase	4.8	0.02	0.03	AK107849.1
Os.6450.1. S1_at	Os07g09190	1-deoxy-D-xylulose 5-phosphate synthase	4.5	0.05	0.05	AK100909.1
Os.50910.2. S1_x_at	Os04g45160	KDEL motif protein	4.5	0.03	0.04	AK059412.1
Os.10099.1. S1_at	Os03g13870	Expressed protein	4.5	0.02	0.03	AK065146.1
Os.53795.1. S1_at	Os03g08840	Zinc finger protein	4.4	0.04	0.04	AK100103.1
Os.37865.1. S1_at	Os02g40700	Hypothetical protein	4.4	0.03	0.04	AK058853.1
Os.34471.1. S1_at	Os01g64310	NAC transcription factor	4.3	0.00	0.00	AK071274.1
Os.14210.1. S1_at	Os10g36360	Hypothetical protein	4.2	0.05	0.05	BI805326
Os.11800.1. S1_s_at	Os01g50100	ABC transporter	4.2	0.03	0.04	AK108373.1
Os.53435.1. S1_at	Os06g49890	Coiled-coil domain	4.1	0.02	0.03	AK108484.1

[a] Probe set ID matched gene locus in rice genome database

[b] Ratios (fold-change) of the expression level in *P. triticina*-inoculated treatments relative to the mock control

[c] Student's t-test and a value of $p < 0.05$ were used to determine significant differences between treated samples and controls

[d] Q-value calculated as false discovery rate (FDR) using Benjamini-Hochberg method

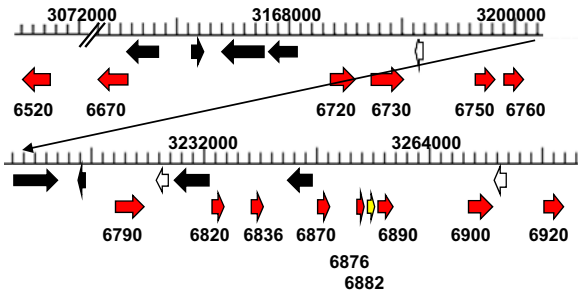

Fig. 2 RLK gene location and organization in a 218 kb region of rice chromosome 1 (GenBank accession # AP003209). Arrows indicate gene orientations. Tentative RLK genes are shown in red. Os01g06882 is annotated as a single gene, but may be part of a larger ORF and is indicated in yellow. Solid and open arrows indicate other genes in this region. The scale is in kb. "Os01g0" is omitted from each locus, e.g., 6870 represents Os01g06870

Effect of rice circadian rhythm on gene expression

To determine if the expression levels of the selected genes were subject to circadian fluctuations, uninoculated rice leaves were harvested four times during the day: 2 h and 10 h after light, and 2 h and 8 h after dark in a 14 h light and 10 h dark photoperiod setting, and leaf mRNA expression was analyzed by qRT-PCR. The results indicate that the expression levels of all the RLK genes changed slightly with the circadian fluctuations of rice (note the scale difference from Fig. 3). These genes have higher expression levels in light than in the dark; however, the degree of change is no more than 1.8-fold (Fig. 5). Compared with the differentially expressed RLK genes inoculated with *P. triticina*, those caused by circadian rhythm are rather small.

Table 3 Top ten down-regulated genes in rice 24 h after challenging with wheat leaf rust strain PNMQ

Probe Set ID	*Gene*[a]	*Gene annotation*	*Fold Ck2/L*[b]	*P Ck2/L*[c]	*Q FDR*[d]	*cDNA Accession*
Os.4653.1. S1_at	Os01g03340	Bowman Birk trypsin inhibitor	5	0.05	0.05	AK067257
Os.19803.5. S1_at	Os01g05900	Histone H_2B	4	0.02	0.03	CF291303
Os.428.1. S1_at	Os03g55770	Hypothetical rice protein	4	0.02	0.03	AB054528
Os.27011.1. A1_s_at	Os01g04550	Receptor kinase PR_5K	4	0.03	0.04	CB633485
Os.12713.1. S2_a_at	Os01g03320	Bowman Birk trypsin inhibitor	4	0.05	0.05	AK060800
Os.5629.1. S1_at	Os08g01170	GCN_5-related N-acetyltransferase	3	0.00	0.00	AK068156
Os.9046.1. S1_x_at	Os10g22980	WRKY transcription factor	3	0.05	0.05	AK121005
Os.27084.1. A1_a_at	Os04g52640	SHR_5-receptor-like kinase	3	0.04	0.04	AK112032
Os.24307.1. S1_at	Os10g39150	Thylakoid phosphoprotein	3	0.02	0.03	AK066849
Os.7137.2. S1_x_at	Os01g66980	Hypothetical protein	3	0.04	0.04	NM_190253

[a] Probe set ID matched gene locus in rice genome database

[b] Ratios (fold-change) of the expression in the mock control relative to *P. triticina*-inoculated treatments

[c] Student's t-tests ($p < 0.05$) were used to determine significant differences between treated samples and controls

[d] Q-value calculated as false discovery rate (FDR) using Benjamini-Hochberg method

Effect of killed rust spores on RLK gene expression

Inoculations were performed with live spores and heat-killed spores to determine if the transcriptional response could be triggered by contact with spores alone. Live spores from two rust isolates (BBBM and MCDSB) were compared with heat-killed spores. No spore germination in the heat-treated spore inoculations were observed for the 14 days of incubation, while spore germination rates for unheated spores were 83 % for BBBM and 84 % for MCDSB. The results showed that all responsive RLK genes (except three unresponsive genes: Os01g06520, Os01g06790, and Os01g56330) were expressed to much higher levels with live spores compared to heat-treated spores (Fig. 6).

Expression of RLK genes in response to other non-adapted fungi

To assess responses to other non-adapted fungal species, the expression of the *P. triticina*-responsive RLK genes were evaluated in Nipponbare after inoculation with

Table 4 Primer sequences and amplicon sizes used for amplifying selected genes by qRT-PCR

Gene locus ID	*Sequence length*	*Product length*	*Forward primer (5'-3')*	*Reverse primer (5'-3')*
Os01g06836	1563	172	GCCATTATCACGGACTTATC	GGTTTCCATTCACATCTATTG
Os01g06870	1041	142	CACATACCGTCCTCATTTGC	AGCCTGATTCCGATCCTTG
Os01g06876	1080	128	GCTCAACATCTCCAAGTG	CCAGACTGCCAATATGAAG
Os01g06882	950	99	AACATAACAGGCTTTCCG	TGTCCATGTGCTGTCTAG
Os01g06520	839	133	GGCATCCTTGGACTTCCTCAC	TGGACGACCGCACAAACC
Os01g53920	780	163	CTCCAGACCCTGTTTCTC	TGTTCTCCACGGTTAAGC
Os01g56330	729	118	TTTATGGACCTGGAAGTCAGAGAG	CAGCGGTAGAGTACAATACAAAGC
Os01g06790	980	152	CAGTCGGTGTTCAGTTTC	CTGCTGCTACTCACATTG
Os03g50810	960	103	TGACGGAAGCAGAACATC	TACAAGGCAGCACATAGC
Os08g28710	900	87	CTGTCCAACATCCTGCTC	CGTACACTCTACCTCGTC
Os02g32030 (EF2*)	2532	126	CTGCTTTCGTTGTCGTATTTCA	CAGGTTCCACATCCGTTACA

**EF2* Rice Elongation Factor 2

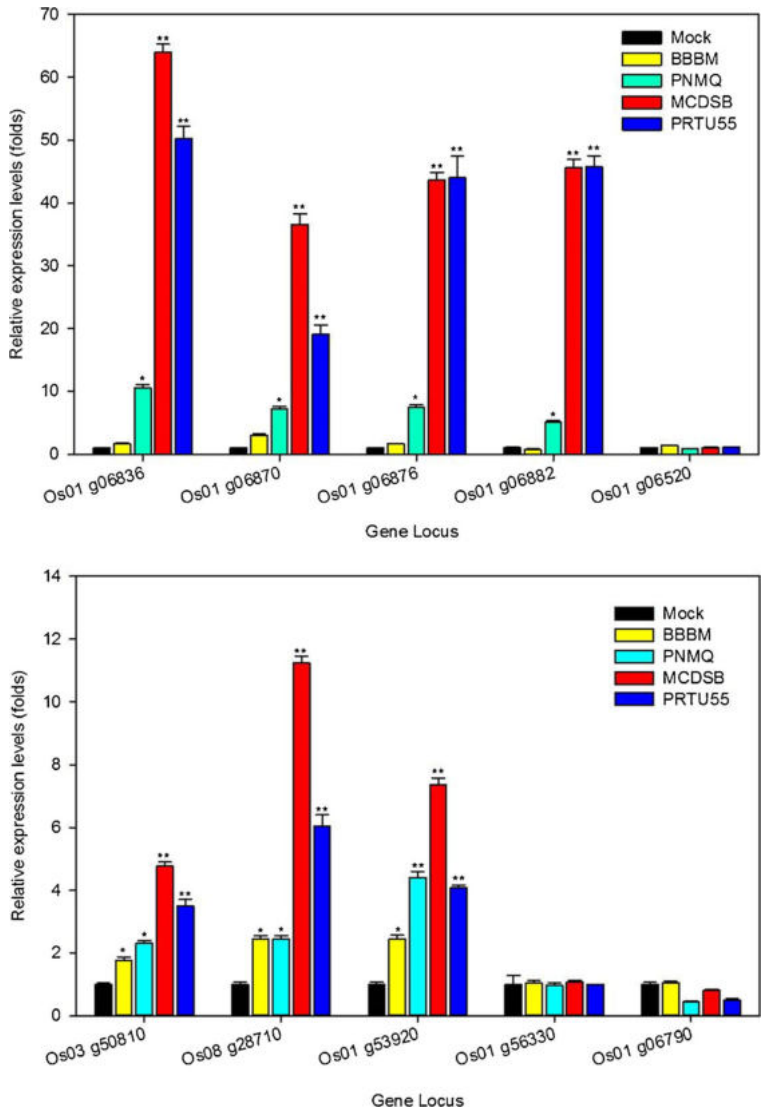

Fig. 3 Ten RLK gene responses to four different races of wheat leaf rust 24 h post inoculation. Gene expression level in fold-change was measured by qRT-PCR 24 h after spray-inoculation. The expression levels of the *RLK* genes were normalized to the rice (*Oryza sativa* L.) Elongation Factor 2 gene (EF_2) as the internal control. Data are expressed as the mean ± SE with three replications. Asterisks indicate a significant difference between mock and treated samples at $*p < 0.05$ or $**p < 0.01$ by *t*-test

causal isolates of wheat tan spot (*Pyrenophora tritici-repentis*) and wheat leaf blotch (*Septoria tritici*). Four genes, Os01g06836, Os01g06870, Os01g06876 and Os01g53920, were induced to a range of 5.9- to 9.8-fold by tan spot (Fig. 7). Seven genes, Os01g06836, Os01g06870, Os01g06876, Os01g53920, Os08g28710, Os03g50810, and Os01g56330, were induced by *S. tritici* with fold-change ranging from 4.2- to 120.3 (Fig. 7).

Annotation of the RLK gene cluster

Os01g06876 and Os01g06882 were both up-regulated by the *P. triticina* challenge and are located adjacent to each other on chromosome 1. The original annotations of Os01g06876 and Os01g06882 are supported by the apparent full-length cDNA clones AK108484 and AK108785, respectively. Clone AK108484 contains a coding sequence of 669 bp, which is unusually short for an RLK gene. An additional BLAST search identified the cDNA clone NM_001048631, which is 1080 bp long and encompasses the entire sequence of Os01g06876. BLAST search against the non-redundant protein database with Os01g06876 identified proteins (BAG98523 and EAZ10649) that are identical both in amino acid sequence and protein length to the conceptually translated protein from Os01g06876. A BLAST search of Os01g06882 also indicates relatedness to EAZ10649,

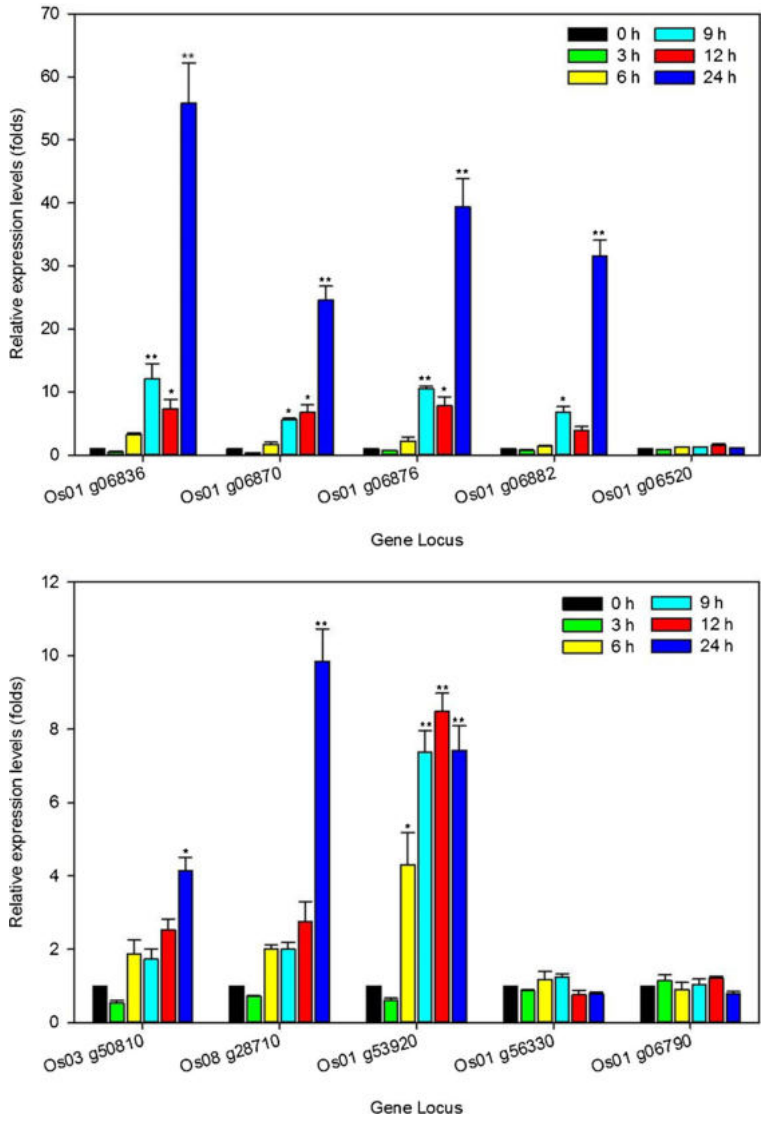

Fig. 4 Expression of ten rice RLK genes after challenge with *P. triticina* strain MCDSB at five time points post-inoculation. Rice leaves were spray-inoculated and harvested at 3, 6, 9, 12 and 24 h post-inoculation. The expression levels of the genes were normalized to that of the rice (*Oryza sativa* L.) Elongation Factor 2 gene (EF2) as the internal control. Values are in fold-change against mock control. Data are expressed as the mean ± SD of three replicates

which is a predicted LRR-containing protein of 847 aa. At the same time, no full length cDNA has been reported that spans both Os01g06876 and Os01g06882. The two loci, therefore, may represent a single gene. An effort to clone and sequence the conjunction of Os01g06876 and Os01g06882 from challenged rice mRNA was unsuccessful (data not shown).

Discussion

NHR is not a well-understood phenomenon and may hold great potential for designing durable mechanisms for resistance against major plant pathogens (Stam et al. 2014; Senthil-Kumar and Mysore 2013; Fan and Doerner 2012). In addition to pre-formed barriers, induced mechanisms of resistance have been shown to play roles in NHR (Lipka et al. 2005; Hao et al. 2011). NHR may the manifestation of multiple layers of host adaptation to some pathogens, including combinations of pathogen-associated molecular pattern (PAMP)-triggered immunity and effector-triggered immunity (Jones and Dangl 2006). Regardless of the components, relatively few genes mediating this phenomenon have been isolated (Ayliffe et al. 2011b). Our observation indicated that rice cultivar Nipponbare responded to rust infection by expression of a relatively limited set of genes. Furthermore, varying levels of expression were elicited

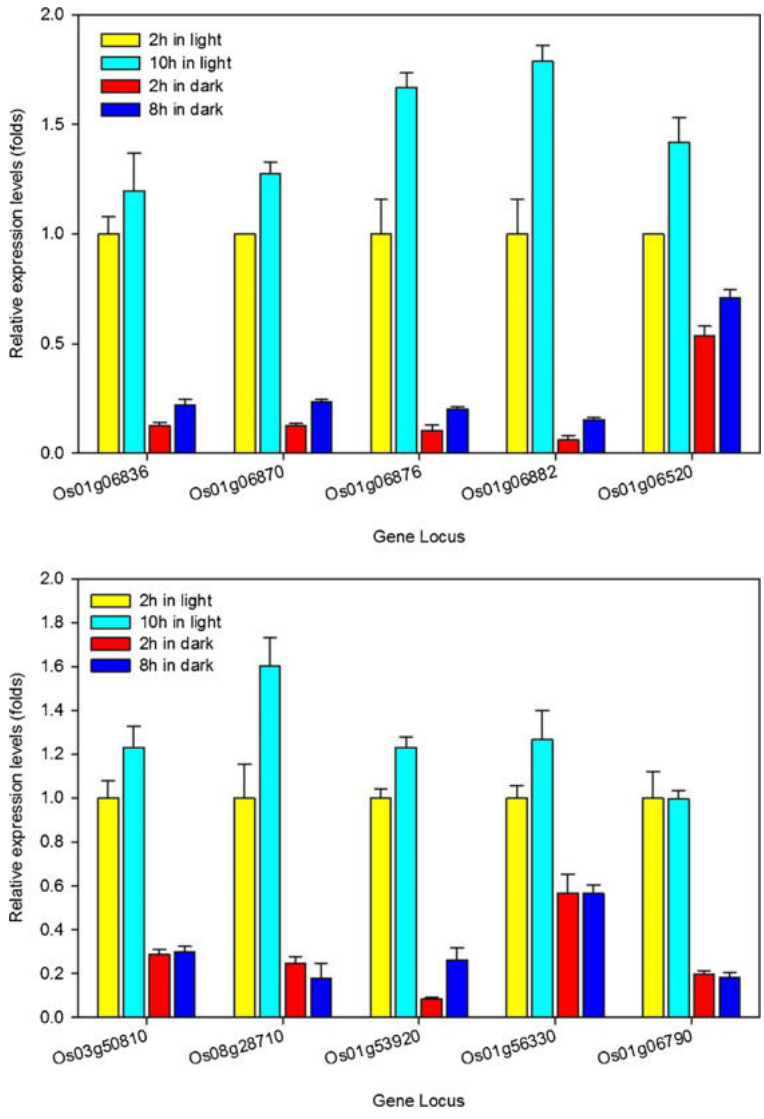

Fig. 5 Expression levels of ten RLK genes were monitored by qRT-PCR at four time points during one day. The photoperiods of the growth chamber were set to 14 h light and 10 h dark. The expression levels of the RLK genes were normalized to rice (*Oryza sativa* L.) EF2 was used as the internal control. Data are expressed as the mean ± SD with three replications

by different strains, representing different races, of *P. triticina*. At the same time, induction of the same responsive genes was observed following inoculation with two additional non-adapted fungal pathogens. The annotations of most highly up-regulated genes are consistent with a robust transcriptional response to non-adapted fungal challenge.

Blockage at the leaf penetration is a prevalent form of NHR to rust fungi (Yang et al. 2014; Li et al. 2012; Ayliffe et al. 2011a, 2011b). In this study, the wheat leaf rust germinated and penetrated the leaf stomata, forming both infection hyphae and appressoria. Accumulation of H_2O_2 in the epidermal cells around the appressorium indicates an induced defense response to *P. triticina,* similar to observations in rice–*Puccinia striiformis* f. sp. *tritici* (*Pst*) interactions (Yang et al. 2014) and pepper–*Blumeria graminis* f. sp. *tritici* interactions (Hao et al. 2011). All infections by *P. triticina* isolates failed in the sense that invasive hyphae were never observed and the infection process was halted. After time, hyphal staining revealed short hyphal segments and reduced appressoria-like structures, which we interpreted as fragmented fungal tissue, surrounded by callose-like materials. The fact that the hyphae growth within the plant to the point of appressoria formation and then terminate indicates an active response to the invasion of the host.

The transcriptome analysis indicated 736 probe sets were differentially expressed. More than half ($n = 422$) of

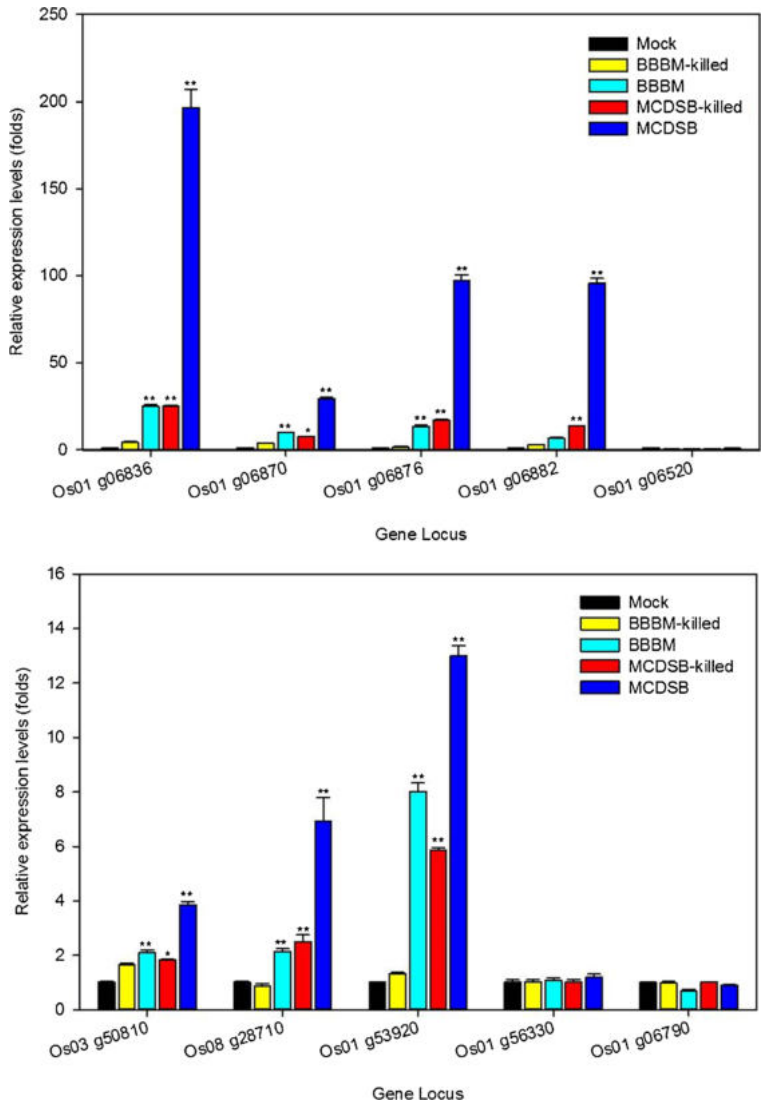

Fig. 6 Comparison of the RLK gene expression patterns of rice inoculated with killed leaf rust spores and fresh leaf rust spores 24 h post inoculation. Gene expression level in fold-change was measured by qRT-PCR 24 h after spray-inoculation. The expression levels of the *RLK* genes were normalized to rice (*Oryza sativa* L.) EF2 was used as the internal control. Data are expressed as the mean ± SE with three replications. Asterisks indicate a significant difference between mock and treated samples at $*p < 0.05$ or $**p < 0.01$ by *t*-test

them are annotated with unknown functions. Among the annotated genes, 78 % ($n = 212$) are predicted to encode receptor kinases, DNA or nucleotide binding proteins, two-component signal transduction components, membrane proteins, ion binding or transport components, and enzymes that involve in catalytic activities. Among the top twenty-three up-regulated probe sets five represented two adjacent loci on chromosome 1, namely, Os01g06882 and Os01g06876. Os01g06876 and Os01g06882 up-regulation was not related to circadian rhythm. Os01g06876 is annotated as a putative RLK and sits within a genomic region rich in RLK-related genes. Some evidence exists that Os01g06876 and Os01g06882 are, in fact, a single locus. Although we could not validate this conjecture, the locus may be improperly expressed or spliced in the cultivar Nipponbare. Two additional predicted RLK-related loci (Os01g06836 and Osg06870) in the gene cluster were also up-regulated during rust infection. Therefore, a number of the genes within the 218 kb cluster on chromosome 1 may represent genes of a response regulon that is triggered by non-adapted fungal pathogens. RLKs play various roles in plant cells such as hormone signaling, meristem development, cell differentiation, pollen recognition, and plant resistance to bacterial and fungal infections (Shiu and Bleecker 2003; Godiard et al. 2003; Llorente et al. 2005). Previous studies have demonstrated that RLK genes are up-regulated by a number of elicitors, including challenge by pathogens (Du and Chen 2000; Rayapuram et al. 2012). The annotation and whether these RLKs are directly or

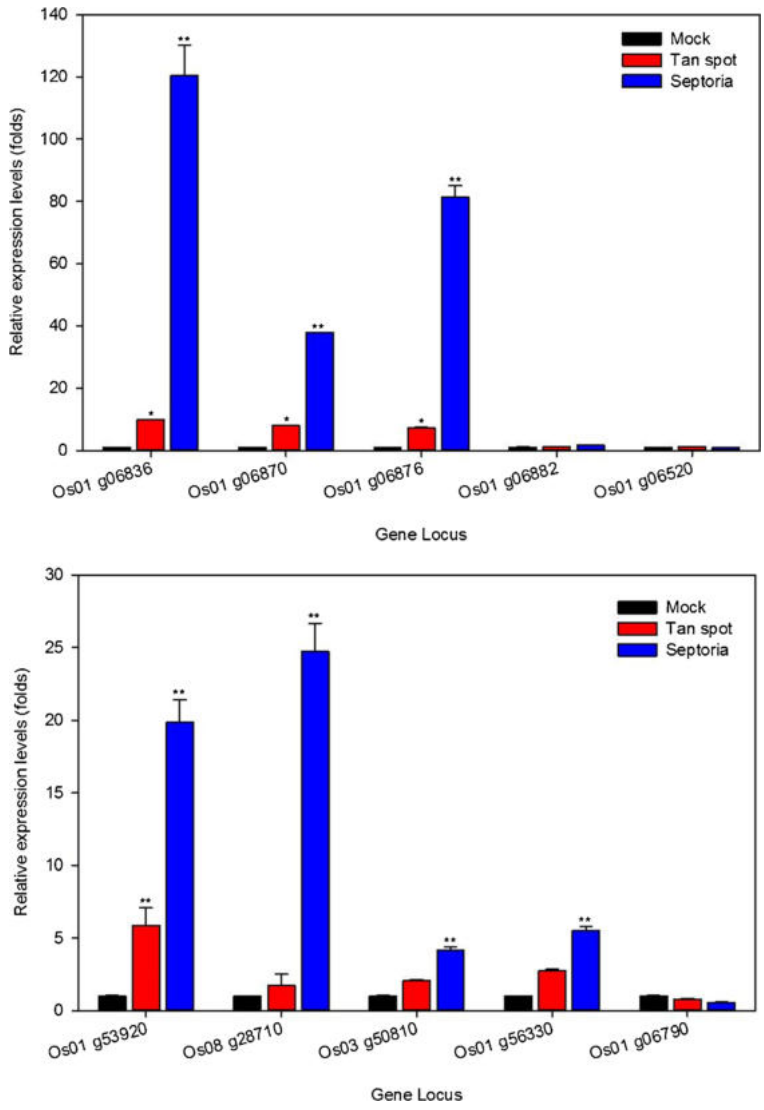

Fig. 7 Ten RLK gene responses to wheat pathogens tan spot *(Pyrenophora tritici-repentis)* and wheat *Septoria tritici* 24 h post inoculation. Gene expression level in fold-change was measured by qRT-PCR 24 h after spray-inoculation. The expression levels of the *RLK* genes were normalized to rice (*Oryza sativa* L.) EF2 was the internal control. Data are expressed as the mean ± SE with three replications. Asterisks indicate a significant difference between mock and treated samples at $*p < 0.05$ or $**p < 0.01$ by *t*-test

indirectly involved in the reaction to *P. triticina* strains needs further investigation.

One up-regulated transcript encodes a putative Ras-related GTP-binding protein RAB11C, which belongs to the Rab family in the Ras superfamily. Proteins of this family have been recognized as molecular switches and the central regulators involved in signal transduction and biotic defenses (Valster et al. 2000). In *Nicotiana benthamiana*, a Ras-related GTP-binding protein (ARF1) has been identified to be involved in NHR (Coemans et al. 2008). Over-expression of rice Ras-related GTP-binding protein (RARF1) induced pathogenesis-related genes and pathogen resistance in tobacco plants (Lee et al. 2003). A strong over-representation of up-regulated genes belonging to the NAC transcription factor family has been described in association with NHR. NAC transcription factors are proposed to function in both abiotic and biotic stress adaptation as well as in developmental processes (Olsen et al. 2005). A barley NAC transcript termed HvNAC6 confers barley basal resistance to *Bgh* (Jensen et al. 2007). Growing evidence points to the fact that some plant NAC genes, such as *Arabidopsis* VND6 and VND7, are transcriptional switches for plant metaxylem and protoxylem vessel formation and contribute to the composition of the secondary cell wall (Kubo et al. 2005). In this study, a rice NAC gene (Os01g64310) was significantly induced by *P. triticina*.

Another highly induced gene encodes a putative GDSL-like lipase/acylhydrolase, a family of membrane proteins involved in the breakdown of lipids, fatty acids and isoprenoids that has been found to respond to plant biotic stresses (Kottapalli et al. 2006). *Arabidopsis* GDSL lipase-like 1 (GLIP1) plays an important role in plant immunity, eliciting both local and systemic resistance in plants (Kwon et al. 2009). GDSL-like lipase/acylhydrolase is related to lipid metabolism and the response to plant biotic stresses. A putative cytochrome P450 gene was highly induced after the challenge with *P. triticina.* The cytochrome P450 gene *PAD3* has been confirmed to play a key role in Arabidopsis NHR to *Alternaria brassicicola. PAD3* is required for the biosynthesis of camalexin (an indole phytoalexin) in Arabidopsis (Zhou et al. 1999). A chili pepper putative cytochrome P450 gene, *CYP83B1*, has been shown to confer NHR in *Arabidopsis* to the oomycete pathogen *Phytophthora infestans* (Huitema et al. 2003). Cytochrome P450 genes might direct the synthesis of antimicrobial phytoalexin compounds that play key roles in host and nonhost responses (Mikkelsen et al. 2003; Kim et al. 2006).

The ATP-binding cassette protein (ABC) transporter was also elevated by inoculation with *P. triticina*. Plant ABC transporters have been demonstrated to participate in detoxification processes, chlorophyll biosynthesis, Fe/S clusters formation, stomatal movement, and possibly ion flux (Jasinski et al. 2003; Martinoia et al. 2002). Many ABC transporters (especially PDR-like ABC transporters), such as *PEN3, NtPDR1* and *LR34 (Yr18)*, are involved in the secretion of secondary metabolites that play key roles in plant basal resistance to pathogens (Stein et al. 2006; Sasabe et al. 2002; Krattinger et al. 2009; Jasinski et al. 2001). ABC transporters might help to scavenge toxic secondary metabolites and maintain the balance of ion flux.

Other highly induced transcripts included the followings: a WD-domain/G-beta repeat protein and a blue copper-binding protein, which might be involved in the stress response; 1-deoxy-D-xylulose 5-phosphate, which is related to energy transfer and metabolism; and the putative DTA2 that is related to amino acid metabolism. Several amino acid biosynthetic proteins are implicated in tryptophan biosynthesis, which was identified previously as potentially important in barley for defense-related signaling or synthesis of antifungal compounds (Bednarek et al. 2009). The mitochondrial coiled-coil domain-containing protein 90 A precursor and a mitochondrial carrier protein were also identified and have functions related to protein transport and turnover in mitochondria. Two of the 20 top up-regulated rice genes in our study were weakly induced by the host pathogen *Magnaporthe grisea* (Mosquera et al. 2009). Os03g26870 coding for a WD domain was induced to 5.2 fold by *P. triticina* and 1.7 fold by *M. grisea*. Another locus, Os03g08840, as annotated to code for a zinc finger protein, was upregulated to 4.4 fold by the wheat leaf rust, and 2.2 fold by the rice blast fungus.

An interesting result of the research was the induction of multiple RLK genes within a cluster of genes on chromosome 1, which may reflect a defense priming response. Indeed, two of the genes were among the most highly induced genes. At the same time, the selected RLK genes responded differently to the different wheat leaf rust strains. Strains MCDSB and PRTU55 induced several RLK genes to much higher levels than those induced by BBBM and PNMQ. Different levels of gene expression were also observed when rice plants were challenged with two additional non-adapted pathogens for wheat tan spot and wheat leaf blotch, respectively. Variations of rice plants may indicate that elicitation may be due to variations in types or amounts of elicitors in the respective pathogens. The RLK proteins are an ancient class of proteins and present in a variety of different plant species (Wolfe et al. 1989). RLKs may play a role in NHR specifically to rust pathogens and, in general, to a variety of non-adapted fungal pathogens. Mutational studies will help address any critical roles played by these genes in NHR.

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