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Suppression subtractive hybridization and microarray analysis reveal differentially expressed genes in the *Lr39/41*-mediated wheat resistance to *Puccinia triticina*

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Abstract Wheat leaf rust caused by *Puccinia triticina* (*Pt*) is one of the most severe fungal diseases threatening the global wheat production. The use of leaf rust resistance (*Lr*) genes in wheat breeding programs is the major solution to solve this issue. Wheat isogenic line carrying the *Lr39/41* gene has shown a moderate to high resistance to most of the *Pt* pathotypes detected in China. In the present study, a typical hypersensitive response (HR) was observed using microscopy in leaves of the *Lr39/41* isogenic line inoculated with the avirulent *Pt* pathotype THTT from 48 h-post inoculation. Two *Lr39/41* resistance-associated suppression subtractive hybridization (SSH) libraries with a total of 6000 clones were established. Microarray hybridizations were performed on all obtained SSH clones using RNAs extracted from leaves of the *Pt*-inoculated and non-inoculated *Lr39/41* isogenic lines, and leaves of the *Pt*-inoculated and non-inoculated Thatcher susceptible lines. Differentially expressed clones were analyzed by significance analysis of microarrays (SAM), followed by further sequencing. A total of 36 *Lr39/41*-resistance-related differentially expressed genes (DEGs) were identified, many of which had been previously reported to be involved in the plant defense response. The expression levels of eight selected DEGs during different stages of the *Lr39/41*-mediated resistance were further quantified by a qRT-PCR assay. Several pathogenesis-related (*PR*) and HR-related genes seem to be crucial for the *Lr39/41*-mediated resistance. In general, a brief profile of DEGs associated with the *Lr39/41*-mediated wheat resistance to *Pt* was drafted.

Keywords Wheat · Leaf rust · Suppression subtractive hybridization · Microarray · Differentially expressed genes

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Introduction

In their cropping environments, plants are continuously threatened by various pathogens. The first layer of plant innate immune response is achieved by the recognition of pathogen-associated molecular patterns (PAMPs) based on plasma membrane-localized pattern recognition receptors (PRRs). The PAMPs-triggered immunity (PTI) leads to the occurrence of various events that prevent pathogens from further invasion, including transcriptional induction of genes encoding pathogenesis-related (PR) proteins, burst of reactive oxygen species

(ROS), deposition of callose at the cell wall, and signal transduction by mitogen-activated protein kinases (MAPKs) (Fritig et al. 1998; Jones and Dangl 2006; Newman et al. 2014).

The hypersensitive response (HR) triggered by the recognition of a pathogen avirulent (Avr) effector by a plant resistance (R) protein has been widely considered as the second layer of plant innate immune response. Currently, most of the cloned plant *R* genes have been found to encode nucleotide-binding site-leucine-rich repeat (NBS-LRR) proteins (Michelmore et al. 2013). Rapid accumulations of ROS and auto-florescent phenolic compound seem to be the earliest events in the HR (Kamoun et al. 1999; Wang et al. 2007). Then, a large number of programmed cell death (PCD)-related genes, including PCD regulators and protein caspases, are successively activated (Wang et al. 2012; Tang et al. 2015). In addition, such effector-triggered immunity (ETI) can also induce the systemic acquired resistance (SAR), an inducible form of plant defense conferring broad-spectrum immunity to secondary infection beyond the initial infection site. In Arabidopsis, SAR involves the generation of mobile signals, accumulation of salicylic acid (SA) hormone, and transcriptional activation of *PR* genes (Fu and Dong 2013).

Wheat leaf rust caused by *Puccinia triticina* (*Pt*) is one of the most severe fungal diseases threatening the global wheat production. Typically, the yield loss caused by wheat leaf rust can reach as high as 40% under favorable conditions. In China, several destructive epidemics of wheat leaf rust occurred in the 1970s, which had especially resulted in dramatic yield losses in the Yellow-Huai areas. Due to the trends of global warming and high-density wheat cultivation, leaf rust has expanded its area of infection to most of the wheat-planting regions in China. Recent epidemics of wheat leaf rust in China were reported in 2012 (Zhou et al. 2013a). Currently, the utilization of wheat leaf rust resistance (*Lr*) genes in breeding programs is still the most effective way to control this disease.

So far, more than 70 *Lr* genes have been designated, based on which two distinct types of resistance conferred by different *Lr* genes have been identified: seedling resistance and adult-plant resistance (APR). According to the gene-for-gene theory, seedling resistance is conferred by a single dominant *R* gene controlling the mentioned HR or ETI. Most of the cloned *Lr* genes in seedling resistance encode NBS-LRR proteins, including *Lr1*, *Lr10*, and *Lr21* (Feuillet et al. 2003; Huang et al. 2003; Cloutier et al. 2007). On the other hand, APR is normally controlled by quantitative trait loci (QTL), and some of the *APR* genes have displayed the durable resistance to multiple pathogens, including wheat leaf rust (*Lr*), yellow rust (*Yr*), stem rust (*Sr*), and powdery mildew (*Pm*). At present, only three *APR* genes have been cloned in wheat, which are *Lr34*/*Yr18*/*Pm38* encoding an ABC transporter, *Yr36* encoding a kinase-START protein, and *Lr67*/*Yr46* encoding a hexose transporter (Fu et al. 2009; Krattinger et al. 2009; Moore et al. 2015).

Until now, several transcriptome studies have been carried out to investigate the mechanism of wheat resistance to various fungal diseases. For instance, the genes associated with *Lr28*-mediated wheat resistance to *Pt* have been identified by the Serial Analysis of Gene Expression (SAGE) (Singh et al. 2017). Resistance responses in wheat upon infections by stripe rust, powdery mildew, and Fusarium head blight were investigated by large-scale transcriptomic comparisons (Xiao et al. 2013; Zhang et al. 2014). Both SSH library construction and transcriptome sequencing were conducted to understand the mechanism of APR in an elite wheat germplasm "Xingzi 9104" (Huang et al. 2013; Hao et al. 2016). The common genes identified from these related studies may be valuable genetic resources for the improvement of broad-spectrum resistance in wheat to various pathogens.

Previously designated seedling resistance genes *Lr39* and *Lr41* were transferred from *Aegilops tauschii* into common wheat and reported as the same or closely linked genes on chromosome 2DS, with an approximate distance of 1.9 cM to the SSR marker *Xgdm35* (Singh et al. 2004). In a more recent investigation, wheat isogenic line carrying the *Lr39/41* gene was found to have moderate to high resistance to most of the *Pt* pathotypes detected in China (Li et al. 2010). However, the *Lr39/41* gene has not been cloned yet, and the transcriptional changes associated with the *Lr39/41*-mediated resistance remain largely unknown. In the current study, a typical HR was observed by microscopy in leaves of the *Lr39/41* isogenic line inoculated with the avirulent *Pt* pathotype THTT from 48 h post-inoculation (hpi). The differentially expressed genes (DEGs) involved in the *Lr39/41*-mediated resistance were initially revealed by constructing SSH libraries and subsequent microarray hybridization. Using a qRT-PCR assay, the expression profiles of eight selected *Lr39/41* resistance-related DEGs were validated.

Materials and methods

Plant materials, *Pt* inoculation, histological observation, and RNA extraction

Seedling plants of wheat isogenic line carrying the *Lr39/41* gene in a Thatcher background and Thatcher susceptible line were maintained and inoculated with the *Pt* pathotype THTT following the procedures and conditions described earlier (Zhou et al. 2013b). Seedlings inoculated with sterile water were used as a control, and disease symptoms were scored at 10 days post-inoculation (dpi).

For histological observation, wheat leaves from the *Lr39/41* isogenic and Thatcher susceptible lines were collected at 24, 48, and 120 hpi with *Pt* pathotype THTT. The samples were bleached using ethanol and acetic acid. Then, the rust fungal structures were specifically stained using Calcofluor White (Sigma, St. Louis, MO, USA) as previously described (Wang et al. 2014). The hyphal structure and accumulation of phenolic autofluorescence at the infection sites were observed and photographed by an Olympus DP70/DP30BW microscope camera (excitation filter, 485 nm; dichromic mirror, 510 nm; barrier filter, 520 nm).

Plant samples were collected for RNA isolation at 0 and 48 hpi for SSH libraries construction and further microarray hybridization. RNA samples were harvested at 0, 1, 2, 5, and 8 dpi for qRT-PCR assay. Four independent biological replicates were set at each time point. Besides, all samples were rapidly frozen in liquid nitrogen and stored at −80 °C.

Construction of SSH libraries

The frozen samples collected at 48 hpi were grinded in liquid nitrogen. Total RNA was extracted using an RNA Extraction Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Subsequently, mRNA was obtained by purification of total RNA from each sample using a ployATract mRNA Purification Kit (Clontech, Mountain View, CA, USA). Two SSH libraries were constructed using a BD PCR-selectTM cDNA Subtraction Kit (Clontech). In detail, SSH library "JD" was constructed using mRNA samples from *Pt*-inoculated *Lr39/41* isogenic line as the tester (being enriched) and from non-inoculated *Lr39/41* isogenic line as the driver (being suppressed). Another SSH library "BJ" was constructed with mRNA samples from non-inoculated *Lr39/41* isogenic line as the tester and from non-inoculated Thatcher susceptible line as the driver. cDNAs synthesized from the tester and the driver were digested with *Rsa*I, extracted by phenol/chloroform, precipitated by ethanol, and finally re-suspended in water. Initially, the tester cDNA was split into two pools that were then ligated with different adaptors. Two rounds of hybridization and PCR amplification were carried out to normalize and enrich the differentially expressed cDNA. The 1st PCR analysis was performed in 27 cycles (94 °C for 15 s; 66 °C for 30 s; and 72 °C for 90 s), whereas 12 cycles (94 °C for 30 s; 68 °C for 30 s; 72 °C for 90 s) were performed in the 2nd PCR. The PCR products were purified and cloned into the pGEM T-easy vector (Promega) and transformed into *Escherichia coli* DH5a competent cells.

Microarray hybridization

A total of 6000 clones from these two SSH libraries were used as PCR templates. Inserts were amplified using the nested PCR primers 1 and 2R (Clontech, Table S1) that flanked each end of the insert region. A 50-μL PCR mixture containing 37.0 μL of sterile water, 5.0 μL of 10 × PCR buffer, 10 μL of 10 mM dNTP mix, 2.0 μL of each primer (10 μM), 1.0 μL of 50 × Advantage cDNA polymerase mix, and 1 μL of *E. coli* culture were prepared. The following PCR conditions were used: an initial denaturation at 94 °C for 25 s, followed by 12 cycles of 94 °C for 10 s, 66 °C for 30 s, and 72 °C for 90 s, and then a final extension was realized at 72 °C for 10 min. The PCR products were precipitated with anhydrous ethanol and subsequently re-suspended in 40 μL of sterile water. PCR products of 23 clones from each of these two SSH libraries were run on 1% agarose gel to check the size of inserts.

Then, PCR products were precipitated one more time with 100 μL of anhydrous ethanol and re-suspended in 15 μL of 50% dimethyl sulphoxide (DMSO) to a final concentration of 0.1–0.5 μg/μL. All the samples were spotted in three replicates onto amino-silane glass slides by the SmartArrayerTM Arrayer (CapitalBio, Beijing, China). Eight sequences derived from intergenic regions of yeast genome, showing no significant homology to any existing wheat sequences in GenBank (https://www.ncbi.nlm.nih.gov/), were spotted multiple times onto the slides as exogenous controls. In the following steps, wheat total RNA was spiked with a mixture of yeast exogenous control RNAs to verify the semi-quantitative

microarray result. The slides were baked for 1 h at 80 °C and dried at room temperature. Prior to hybridization, the slides were rehydrated over 65 °C water for 10 s, briefly dried on a 100 °C heating block for 5 s, cross-linked by 250 mJ/cm^2 UV light irradiation, washed with 0.5% SDS for 15 min at room temperature, dipped in anhydrous ethanol for 30 s, and spin-dried at 1000 g for 2 min.

RNA samples extracted from leaves of the *Pt*-inoculated and non-inoculated *Lr39/41* isogenic lines, and from leaves of the *Pt*-inoculated and non-inoculated Thatcher susceptible lines, respectively, were used for the Cy5/Cy3-labeled microarray hybridizations (Guo et al. 2005). Briefly, cDNA was synthesized using 10 μg of total RNA extracted from each sample with a cDNA Synthesis Kit (Takara), which was then purified using a PCR Purification Kit (QIAGEN). Subsequently, cRNA was synthesized using a T7 RiboMAX Express Large-scale RNA Production System (Promega, Madison, WI, USA) and purified with a RNeasy Mini Kit (QIAGEN). cDNA was fluorescently labeled with Klenow enzyme after reverse transcription with Superscript II Reverse Transcriptase (Invitrogen, Waltham, MA, USA) and purified with a PCR Purification Kit (QIAGEN). Later, equal amounts of labeled control and test samples were mixed into a hybridization solution (3 × SSC solution, 0.2% sodium dodecyl sulfate, 25% formamide, and 5 × Denhart's solution) based on the efficiency of Cy5-dCTP or Cy3-dCTP incorporation. The hybridization solution was denatured at 95 °C for 3 min prior to loading onto the PCR product-immobilized slides. Then, the slides were hybridized at 42 °C overnight and washed with two washing solutions (2 × SSC solution at 42 °C for 5 min and 0.2% SSC solution at room temperature for 5 min) consecutively. Each hybridization was comprised of two biological replicates and was performed in duplicate by dye swap.

Microarray data analysis

Microarray slides were scanned using a confocal laser scanner LuxScan 10 K-A (CapitalBio) and the resulting images were analyzed using the GenePix Pro v4.0 software. Spots with the signal pixels less than 50% of the local background value were removed. The ratio values were normalized according to the spatial and intensity-dependent (LOWESS) normalization method (Yang et al. 2002). Clones with less than eight data sets were removed. Differentially expressed clones were identified using the significance analysis of microarrays (SAM) method (Tusher et al. 2001). SAM plot was generated for each microarray assay. Mean and standard error (SE) of the fold change, SAM score, and local false discovery rate (FDR) for each clone were calculated. Clones with FDR less than 0.01 and a fold change greater than 1.5 were defined as significantly upregulated, whereas clones with FDR less than 0.01 and a fold change less than 0.5 were considered as downregulated.

Sequencing and annotations of differentially expressed clones

A total of 386 clones remarkably induced or suppressed in the microarray assay were further sequenced using T7/SP6 primers derived from the pGEM T-easy vector (Promega). All sequences were annotated with both the GenBank non-redundant protein database (Blastx, http://blast.ncbi.nlm.nih.gov) and URGI wheat genome database (Blastn, https://urgi.versailles.inra.fr). The redundant clones were removed. Snapshots of comparative expression changes were presented using MAPMAN software, which were achieved by assigning DEGs to the "biotic stress" pathway based on "Taes_AFFY_0709" mapping file from *Triticum aestivum* (Thimm et al. 2004; Barbierato et al. 2016). Additionally, sequences of all the DEGs were annotated with Gene Ontology (GO, http://amigo1.geneontology.org/cgi-bin/amigo/blast.cgi).

qRT-PCR assay

RNAs for qRT-PCR assay were extracted using a RNA Extraction Kit (QIAGEN) in accordance with the manufacturer's instructions, and the first-strand cDNA was synthesized using a Reverse Transcription Kit (Takara). The primers utilized for qRT-PCR were listed in Table S1. Wheat *Actin* gene (*TaActin*, GenBank accession AB181991.1) was used as an internal reference for qRT-PCR analyses (Paolacci et al. 2009). The amplification efficiency of each pair of primers was calculated by preliminary qRT-PCR assay using six 2-fold diluted cDNA samples (1:1, 1:2, 1:4, 1:8, 1:16, and 1:32). Dissociation curves at temperatures ranging from 60 °C to 94 °C were generated to ensure that a single and specific product was amplified. The gene expression was quantified by the threshold values (Ct) generated from the Roche LightCycler 96 based on the Delta Ct method (Wang et al. 2014, 2016).

Data availability The datasets during and/or analysed during the current study are available from the corresponding author on reasonable request.

Results

HR was observed in leaves of the *Lr39/41* wheat isogenic line inoculated with the avirulent *Pt* pathotype THTT

In our previous research, wheat isogenic line carrying the *Lr39/41* gene showed a moderate to high resistance to most of the *Pt* pathotypes detected in China, including FCST, TCGT, FGSQ, FHST, PHSS, FHGS, THTT, and FCTT. Particularly, THTT was the most prevalent pathotype in China, which had displayed high virulence to many of the designated *Lr* genes (Li et al. 2010). After inoculating wheat seedlings with the *Pt* pathotype THTT, necrotic spots phenotype (Infection Type; or 1) was observed on leaves of the *Lr39/41* isogenic line, whereas heavy rust pustules (Infection Type 4) were observed on leaves of the Thatcher susceptible line (Fig. 1a).

To observe the histological changes associated with the *Lr39/41*-mediated resistance, *Pt*-inoculated leaves from both the *Lr39/41* isogenic and the Thatcher susceptible lines were collected at 24, 48, and 120 hpi, respectively. After successful penetration of the germ tube and formation of the stomatal vesicles, the production of *Pt* hypha was blocked by the HR generated in leaves of the *Lr39/41* isogenic line from 48 hpi. In comparison, *Pt* hypha extended, and haustoria were formed in leaves of the Thatcher susceptible line (Fig. 1b).

SSH libraries construction and *Lr39/41* resistance-associated DEGs

Two *Lr39/41* resistance-associated SSH libraries were established. The first one, containing 3456 clones, was constructed using mRNA samples from the *Pt*-inoculated *Lr39/41* isogenic line as the tester and the non-inoculated *Lr39/41* isogenic line as the driver. The second, with 2544 clones, was developed using mRNA samples from the non-inoculated *Lr39/41* isogenic line as the tester and the non-inoculated Thatcher susceptible line as the driver. Twenty-three clones randomly selected from each of the SSH libraries were amplified by PCR with an average size of the inserts of 350 bp (Fig. S1).

A total of 6000 clones from these two SSH libraries were further used for microarray hybridization. RNA samples extracted from leaves of the *Pt*-inoculated (*Pt* pathotype THTT at 48 hpi) and non-inoculated *Lr39/41* isogenic lines, as well as from leaves of the *Pt*-inoculated and non-inoculated Thatcher susceptible lines, were employed for Cy5/Cy3-labelled microarray assays, respectively. Through conducting SAM analysis, clones with "FDR < 0.01 and a fold change > 1.5" were defined as significantly upregulated, whereas clones with "FDR < 0.01 and a fold change < 0.5" as downregulated (Fig. 2). All these differentially expressed clones were further sequenced and gene annotations were performed using both GenBank non-redundant protein database and URGI wheat genome database. A total of 34 upregulated and 2 downregulated DEGs were identified through comparing the *Pt*-inoculated *Lr39/41* isogenic line with the non-inoculated one as "*Lr39/41* resistance-related DEGs" (Table 1), whereas 11 upregulated and 18 downregulated DEGs by comparing the *Pt*-inoculated Thatcher susceptible line with the non-inoculated one as "Thatcher susceptible-related DEGs" (Table 2). The DEGs shared among different groups are presented in Fig. S2.

Pathway analysis and GO category for *Lr39/41* resistance-related DEGs

A total of 15 *Lr39/41* resistance-related DEGs were annotated in the "biotic stress" pathway using the MAPMAN software (Fig. 3a and Table S2). On the other hand, 10 Thatcher susceptible-related DEGs were annotated (Fig. S3a and Table S3). All the DEGs were categorized according to their GO annotations into functional groups of three main categories (Fig. 3b and Fig. S3b). For the molecular function category, the majority of *Lr39/41* resistance-related DEGs were annotated with "catalytic activity" (69.4%, Fig. 3b), whereas most of the Thatcher susceptible-related DEGs were predicted to be involved in "binding" (62.1%, Fig. S3b). In terms of the biological process category, a much higher ratio of *Lr39/41* resistance-related DEGs were annotated with "response to stimulus" (55.6%, Fig. 3b) than that of Thatcher susceptible-related DEGs (37.9%, Fig. S3b).

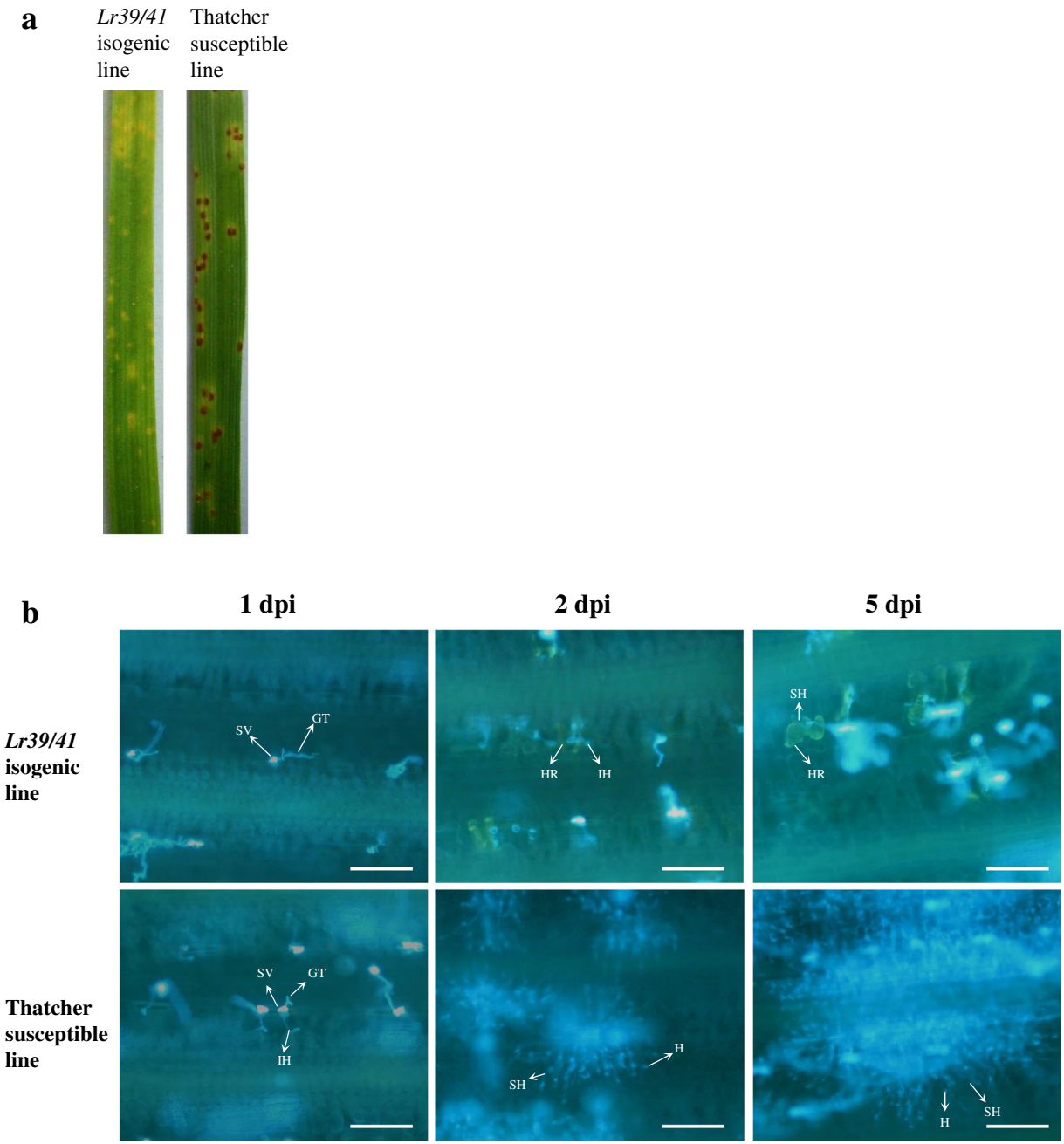


Fig. 1 HR was observed in leaves of the *Lr39/41* wheat isogenic line inoculated with the avirulent *Pt* pathotype THTT. **a** Wheat isogenic line carrying the *Lr39/41* gene had displayed a moderate to high resistance to *Pt* pathotype THTT, which was identified as the most prevalent pathotype in China. Necrotic spots phenotype (Infection Type; or 1) was observed on leaves of the *Lr39/41* isogenic line, whereas heavy rust pustules (infection type 4) were observed on leaves of the Thatcher susceptible line at 10 dpi. **b** To observe the histological changes associated with the *Lr39/41*-mediated resistance, samples from both the *Lr39/41* isogenic and the Thatcher susceptible lines were collected at 24, 48, and 120 hpi. Fungal structures were specifically stained using Calcofluor White. After successful penetration of the germ tube and formation of the stomatal vesicles, the production of *Pt* hypha was blocked by the HR generated in leaves of the *Lr39/41* isogenic line from 48 to 120 hpi, whereas the *Pt* hypha extended, and mature haustoria were formed in leaves of the Thatcher susceptible line. GT, germ tube; SV, substomatal vesicle; IH, initial hypha; SH, secondary hypha; H, haustoria; and HR, hypersensitive response. Scale bars = 100 μm

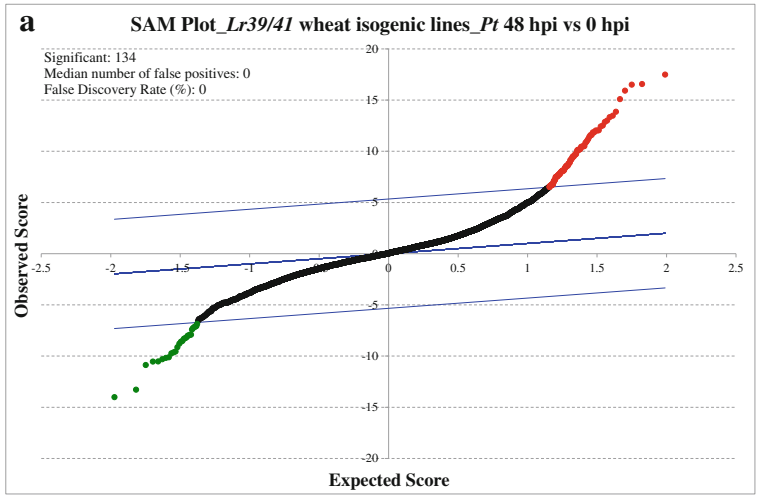


Fig. 2 Differentially-expressed clones in the microarray assay. A total of 6000 SSH clones were used for microarray hybridization. Each hybridization was comprised of two biological replicates and was performed in duplicate by dye swap. Differentially expressed clones were analyzed according to SAM. Mean and SE of the fold change, SAM score, and FDR for each clone were calculated. Clones with "FDR < 0.01 and a fold change > 1.5" were defined as significantly upregulated, whereas clones with "FDR < 0.01 and a fold change < 0.5" as downregulated. **a** SAM plot for the distribution of all the SSH clones in the microarray using RNA samples from the *Pt*-inoculated and non-inoculated *Lr39/41* isogenic lines. A total of 134 differentially expressed clones were identified. **b** SAM plot for the distribution of all the SSH clones in the microarray using RNA samples from the *Pt*-inoculated and non-inoculated Thatcher susceptible lines. A total of 160 differentially-expressed clones were identified

Validation of microarray data by a qRT-PCR assay

The expression levels of eight DEGs with GO annotation of "response to stimulus" were further measured by a qRT-PCR assay. RNA samples from leaves of the *Lr39/41* isogenic line inoculated with the *Pt* pathotype THTT were collected at 0, 1, 2, 5, and 8 dpi. Mock inoculation with sterilized water served as a control. From 2 to 8 dpi, the expression levels of a wheat gene encoding pathogenesis-related protein 1 (TaPR1, GenBank accession FJ815169.1) in the *Pt*-inoculated *Lr39/41* isogenic line were relatively higher than those in the mock inoculation (Fig. 4a), which indicates that a basal plant resistance response has been activated.

During the *Lr39/41*-mediated resistance, the expression levels of five DEGs showed upregulation, including wheat genes encoding for lipid transfer protein 4 (TaLTP4, homolog of PR14, Fig. 4b), from 2 to 8 dpi; inactive purple acid phosphatase 27 (TaBCI6, also known as barley chemical induced gene 6, Fig. 4c), at 1 dpi; thaumatin-like protein 8 (TaTLP8, homolog of PR5, Fig. 4d), from 1 to 2 dpi; endochitinase (TaChit, homolog of PR3, Fig. 4e), at 2 and 8 dpi; and wheat induced resistance 1 (TaWIR1, Fig. 4f), at 8 dpi. In contrast, three DEGs were suppressed during the *Lr39/41*-mediated resistance containing wheat genes encoding for stress responsive protein (TaSRP, Fig. 4g) and zinc finger CCCH domain-containing protein 19 (TaZF19, Fig. 4h), at 2 and 8 dpi, respectively; and Phospho-2-dehydro-3-deoxyheptonate aldolase 2 (TaPADD2, Fig. 4i), at 5 dpi.

Discussion

Currently, most of the cloned genes in wheat for seedling resistance to leaf rust, including *Lr1*, *Lr10*, and *Lr21*, have

Table 1 *Lr39/41*-resistance-related DEGs in the microarray assay

Lr39/41 resistance upregulated clones	Fold Mean	Fold SE	SAM Score	Local FDR%	URGI accession	Annotation
JD1.2A3	2.88	0.24	9.57	0.00	457075_5DS	Cyanate hydratase
JD5.4E11	2.37	0.12	12.99	0.04	327885_4BS	Glutathione-S-transferase
BJ1.3D4	2.33	0.17	6.44	0.00	063094_1DL	Heat responsive transcription factor
JD6.2C4	2.20	0.13	7.20	0.00	158815_2DL	ABC transporter B family member 29
JD1.1D1	2.19	0.11	10.91	0.07	158753_2DL	Proline-rich protein
JD3.4F12	2.18	0.08	16.51	0.00	290729_4AL	Prosaposin-like
JD9.2G5	2.15	0.12	9.21	0.00	093624_2AL	Alternative oxidase
JD1.2A10	2.11	0.17	4.58	0.00	289983_4AL	Thaumatin-like protein 8 (TaTLP8, homolog of PR5)
JD1.4A4	1.97	0.15	8.10	0.00	272354_3DS	Sphingosine-1-phosphatelyase
JD1.1A11	1.96	0.11	6.62	0.00	112185_2AS	NAD-dependent malic enzyme
JD6.1E11	1.93	0.11	10.15	0.02	526988_6DL	Transcription factor EMB1444-like
JD6.4C1	1.90	0.13	9.65	0.00	393328_5AS	Homocysteine methyltransferase
JD7.1F9	1.90	0.16	8.59	0.00	576902_7BL	Mitogen-activated protein kinase 5
JD8.2C2	1.89	0.10	5.32	0.00	526363_6DL	Zinc finger CCCH domain-containing protein 19 (TaZF19)
BJ2.4G2	1.89	0.16	4.34	0.39	250162_3DL	Carbonic anhydrase
JD4.1C2	1.84	0.12	5.77	0.00	320374_4BL	Inactive purple acid phosphatase 27 Barley chemical induced gene 6 (TaBCI6)
JD8.3G10	1.83	0.10	11.84	0.07	423213_5BS	Wheat Induced Resistance 1 (TaWIR1)
BJ7.1E12	1.82	0.12	4.90	0.00	131434_2BL	Naringenin,2-oxoglutarate 3-dioxygenase
JD1.4G4	1.79	0.15	5.79	0.00	435654_5DL	60S ribosomal protein L17–1
JD4.1E2	1.78	0.13	5.92	0.00	177445_2DS	Phospho-2-dehydro-3-deoxyheptonate aldolase 2 (TaPDDA2)
BJ1.2C8	1.78	0.09	5.16	0.00	621670_7DS	60S ribosomal protein L9
JD6.2D3	1.76	0.11	7.53	0.00	231826_3B	Lipid transfer protein 4 (TaLTP4, homolog of PR14)
BJ2.3F2	1.73	0.10	4.90	0.00	378813_5AL	1-aminocyclopropane-1-carboxylate oxidase 1-like
JD2.1C4	1.72	0.08	6.63	0.00	577069_7BL	Phytoene synthase 7B
JD8.4C4	1.72	0.11	6.35	0.00	328187_4BS	Protein disulfide isomerase
JD7.3G1	1.69	0.19	6.45	0.00	406660_5BL	Wall-associated receptor kinase 3
JD7.3H2	1.68	0.11	6.34	0.00	194056_3AL	Cyclin-dependent kinase C-2
JD9.4F3	1.67	0.09	7.77	0.00	424380_5BS	Adenine phosphoribosyltransferase 1
BJ4.1C8	1.66	0.08	5.77	0.00	145980_2BS	Stress responsive protein (TaSRP)
JD6.1A10	1.66	0.08	7.70	0.00	049430_1BS	Mannosyl-oligosaccharide 1,2-alpha-mannosidase
JD7.1E8	1.63	0.11	7.29	0.00	159104_2DL	Endochitinase (TaChit, homolog of PR3)
JD8.2F9	1.63	0.10	9.13	0.00	036335_1BL	Phospholipase
JD9.3C2	1.60	0.11	4.52	0.00	433916_5DL	Stress-associated protein 1
JD8.2A7	1.55	0.06	8.09	0.00	061461_1DL	Xyloglucan endotransglycosylase/ hydrolase protein 8
Lr39/41 resistance downregulated clones	**Fold Mean**	**Fold SE**	**SAM Score**	**Local FDR%**	**URGI accession**	**Annotation**
JD5.1A2	0.30	0.06	−10.12	0.03	526363_6DL	Zinc finger CCCH domain-containing protein 19 (TaZF19)
JD9.1D9	0.37	0.05	−10.87	0.07	623443_7DS	Detoxification 34-like

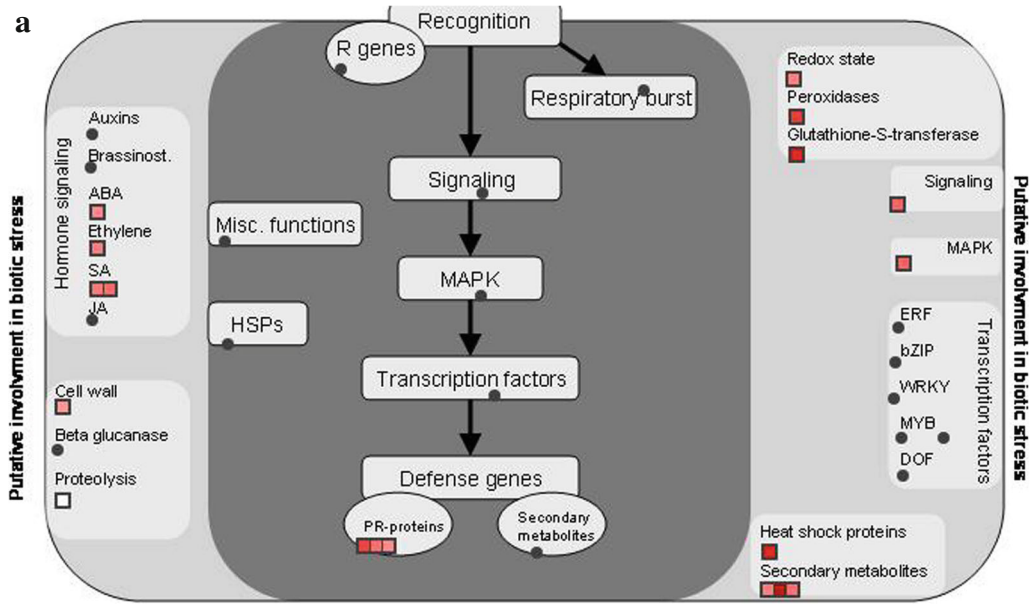
Table 2 Thatcher-susceptible-related DEGs in the microarray assay

Thatcher susceptible upregulated clones	Fold Mean	Fold SE	SAM Score	Local FDR%	URGI accession	Annotation
JD8.3E11	3.21	0.26	4.09	0.00	404374_5BL	Chlorophyll a-b binding protein
JD1.1A9	2.53	0.24	3.77	0.00	404397_5BL	Acyl carrier protein 3
JD1.3E7	2.32	0.21	3.37	0.00	093624_2AL	Alternative oxidase
BJ1.2B2	2.08	0.14	6.61	0.00	453540_5DL	Photosystem II CP43 chlorophyll apoprotein
BJ4.3A3	1.95	0.08	7.16	0.00	224943_3B	Ankyrin repeat-containing protein
BJ6.1A5	1.81	0.08	6.18	0.00	474572_6AL	Succinyl-CoA ligase
BJ1.1F10	1.79	0.15	3.24	0.00	576997_7BL	50S ribosomal protein
JD6.4E1	1.73	0.09	3.87	0.00	036335_1BL	Phospholipase
JD8.4C4	1.58	0.06	5.36	0.00	328187_4BS	Protein disulfide isomerase
JD9.3C2	1.54	0.10	3.62	0.00	433916_5DL	Stress-associated protein 1
JD1.1C2	1.54	0.12	5.81	0.00	374928_5AL	Glucose-6-phosphate 1-epimerase
Thatcher susceptible downregulated clones	Fold Mean	Fold SE	SAM Score	Local FDR%	URGI accession	Annotation
JD2.2H1	0.23	0.08	−7.65	0.03	526363_6DL	Zinc finger CCCH domain-containing protein 19 (TaZF19)
JD6.2B7	0.28	0.07	−8.82	0.10	378245_5AL	3-dehydroquinate synthase
JD4.4H4	0.33	0.11	−4.23	0.00	320374_4BL	Inactive purple acid phosphatase 27 Barley chemical induced gene 6 (TaBCI6)
BJ2.3A3	0.34	0.11	−3.79	0.00	249716_3DL	Calreticulin
JD7.2F4	0.37	0.08	−5.95	0.00	322700_4BL	Eukaryotic translation initiation factor
JD9.1D9	0.38	0.08	−6.48	0.00	623443_7DS	Detoxification 34-like
BJ7.1B4	0.39	0.08	−5.14	0.00	160530_2DL	E3 ubiquitin-protein ligase
JD1.3D5	0.41	0.07	−6.30	0.00	404374_5BL	Chlorophyll a-b binding protein
JD2.3F3	0.41	0.07	−6.96	0.00	471142_6AL	Cinnamyl alcohol dehydrogenase
JD7.4G5	0.42	0.10	−3.87	0.00	328917_4BS	Protein phosphatase inhibitor
BJ4.2C12	0.42	0.06	−6.59	0.00	433940_5DL	Lectin-domain containing receptor kinase
JD6.1D10	0.44	0.08	−5.17	0.00	342856_4DL	U2 small nuclear ribonucleoprotein
JD2.3D3	0.44	0.07	−6.24	0.00	061096_1DL	Dehydration-induced 19
JD9.1G2	0.45	0.06	−6.52	0.00	515602_6BS	DNA topoisomerase 1
JD8.1F1	0.47	0.08	−5.44	0.00	061867_1DL	ElF1 superfamily transcriptions factor
BJ2.1H7	0.48	0.05	−7.46	0.00	375034_5AL	CCG-binding protein
JD8.3B7	0.48	0.08	−5.52	0.00	112552_2AS	Cytokinin-O-glucosyltransferase
JD8.1F7	0.50	0.09	−3.66	0.00	456564_5DS	5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase

been recognized to encode proteins with the NBS-LRR structure and function through a cell death-mediated pathway (Feuillet et al. 2003; Huang et al. 2003; Cloutier et al. 2007; Michelmore et al. 2013). Based on the HR observed during the *Lr39/41*-mediated resistance in the present study (Fig. 1), we speculate that the *Lr39/41* gene also encodes a NBS-LRR protein. However, no DEGs encoding such type of protein were detected in our SSH and microarray assays. Nevertheless, several HR-related and *PR* genes identified in the current study seem to be crucial for the *Lr39/41*-mediated resistance.

HR during the *Lr39/41*-mediated resistance

Accumulations of ROS and phenolic-based secondary metabolites are common features of the HR during incompatible interaction between wheat and rust (Moldenhauer et al. 2006; Wang et al. 2007).



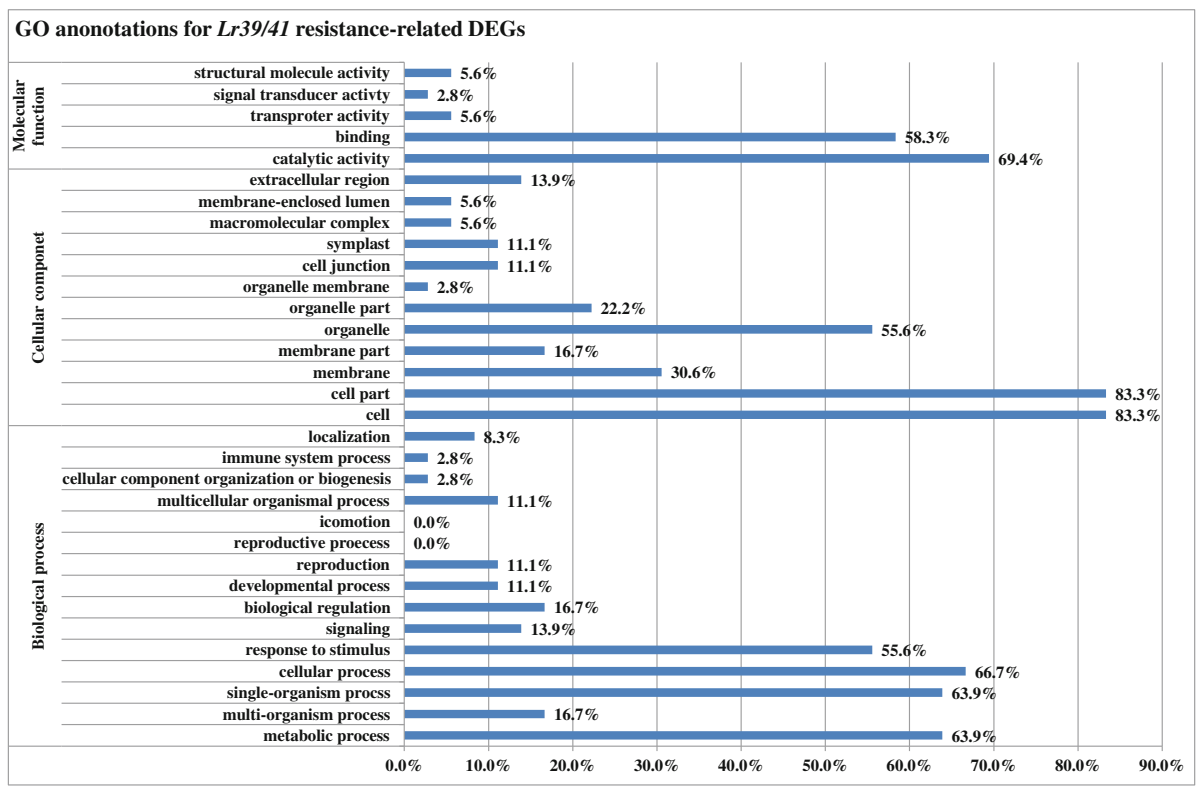


Fig. 3 Pathway analysis and GO annotations for all the *Lr39/41* resistance-related DEGs. **a** A total of 15 *Lr39/41* resistance-related DEGs were annotated in the "biotic stress" pathway using MAPMAN software (detailed information was available in Table S2). **b** All the *Lr39/41* resistance-related DEGs were categorized based on their GO annotations into 32 functional groups of three main categories, respectively: biological process, cellular component, and molecular function. The y-axis indicates the percentage of genes in a category

Researchers have concentrated on discovering HR-related genes involved in this biological process (Tang et al. 2015). In the current study, at least four DEGs have been reported to be involved in the HR triggered by the *Lr39/41* resistance gene, including *TaZF19*, *TaWIR1*, *TaSRP*, and *TaPADD2*.

In our qRT-PCR assay, the expression levels of *TaZF19* gene were suppressed at 2 and 8 dpi (Fig. 4h). A previous study showed that the overexpression of its ortholog, *GhZFP1* from *Gossypium hirsutum*, in transgenic tobacco plants enhanced plant tolerance to salt stress and resistance to *Rhizoctonia solani*. Moreover, lesions generated by *Rhizoctonia solani* in the *GhZFP1*-OE transgenic tobacco plants were much smaller in size and lower in density than those in the wild type ones (Guo et al. 2009). Consequently, we speculate that this gene may function as a negative regulator of the *Lr39/41*-mediated HR.

TaWIR1 and its ortholog in barley have been well-characterized during the resistance of *Triticeae* crops to various fungal pathogens, including *Magnaporthe oryzae*, *Blumeria graminis*, and *Giberella zeae* (Diethelm et al. 2011; Douchkov et al. 2011; Tufan et al. 2012). It was reported to be co-segregated with the resistance QTL, contributing only to the post-penetration resistance. Recent transcriptome analysis demonstrated that *TaWIR1* was also involved in wheat resistance to stripe rust at the adult-plant stage (Hao et al. 2016). Induction of *TaWIR1* at 8 dpi observed in our qRT-PCR assay (Fig. 4f) indicates a possible involvement of this gene during the late stage of the *Lr39/41*-mediated resistance.

Regarding *TaSRP*, its rice ortholog showed induction by abscisic acid (ABA) and salt stress (Moons et al. 1995, 1997). Although the role of ABA in plant defense

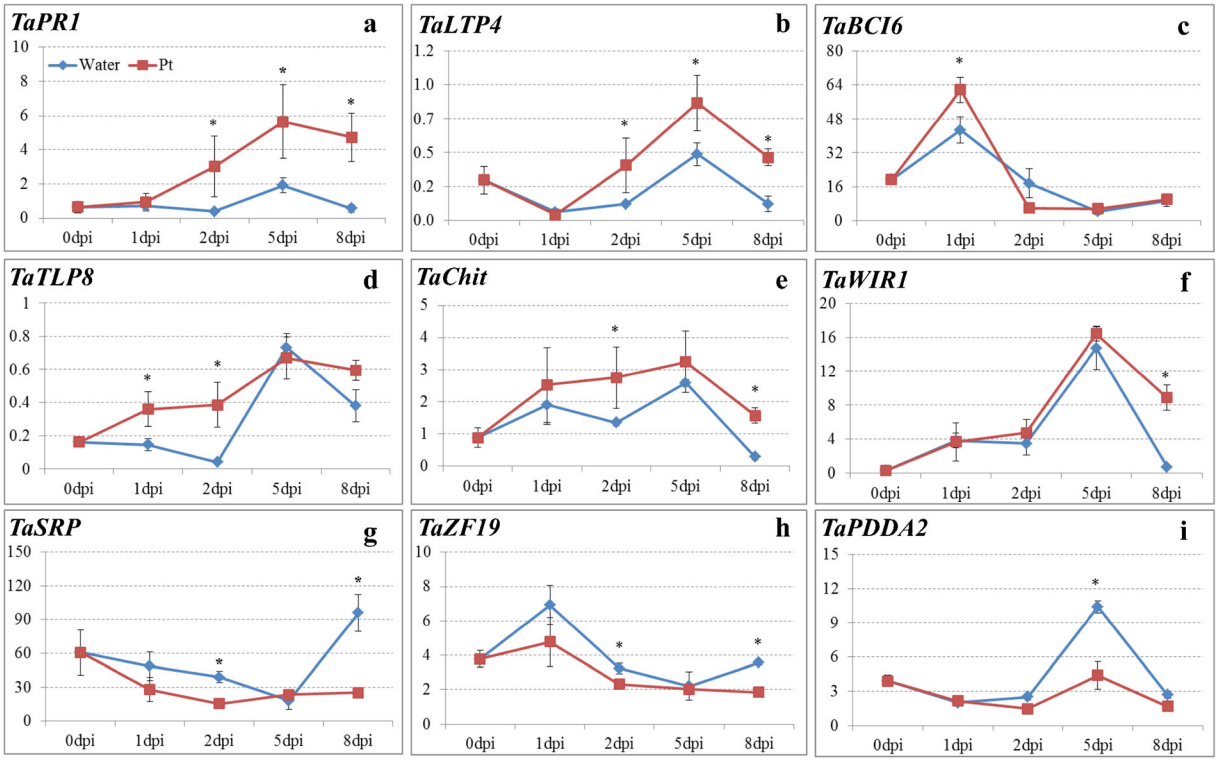


Fig. 4 Validation of DEGs by a qRT-PCR assay. The transcript levels of eight selected DEGs during the *Lr39/41*-mediated wheat resistance to *Pt* pathotype THTT at 0, 1, 2, 5, and 8 dpi were monitored by a qRT-PCR assay. Mock inoculation with sterilized water served as a control. The transcript levels for all genes were expressed as linearized fold-*TaActin* levels, which were calculated according to the formula $2^{(\text{ACTIN CT} - \text{TARGET CT})}$. **a** *TaPR1* was used as a positive control. **b-f** The expression levels of *TaLTP4*, *TaBCI6*, *TaTLP8*, *TaChit*, and *TaWIR1* showed significant upregulation during the *Lr39/41*-mediated resistance to *Pt* pathotype THTT, which were consistent with the microarray results. **g-i** The expression levels of *TaSRP*, *TaZF19*, and *TaPADD2* presented significant downregulation. Data were expressed as mean ± SE from four biological replicates. The asterisk indicated a significant difference ($p < 0.05$) between the mock and *Pt*-inoculated samples upon *t*-test

had been intensively studied during the last decade (Adie et al. 2007; Lim et al. 2015; Vos et al. 2015), there is no specific report on its involvement in wheat resistance to rust. Further studies on *TaSRP* may provide an initial clue for understanding the role of ABA in the *Lr39/41*-mediated resistance.

We have observed an inconsistent expression profile of *TaPADD2* between our microarray (upregulation, Table 1) and qRT-PCR assays (downregulation at 5 dpi, Fig. 4i). In a previous study, its homolog was reported to be involved in wheat resistance to *Magnaporthe oryzae* by mediating the shikimate pathway during the production of phenolic-based secondary metabolites (Tufan et al. 2009). The elucidation of the molecular function of *TaPDDA2* in the *Lr39/41*-mediated wheat resistance to *Pt* requires further investigation.

Involvement of the SA pathway-related genes during the *Lr39/41*-mediated resistance

Four DEGs or their orthologs in other plant species, including *TaLTP4*, *TaBCI6*, *TaChit*, and *TaTLP8*, were previously reported to be involved in SA-related plant defense response (Caruso et al. 1999; Beßer et al. 2000; Wang et al. 2010; Li et al. 2015). *PR* genes are normally considered to be the downstream responsive genes of both the PTI and the SA-mediated SAR. Several *PR* genes were recently reported to be responsible for the *Sr13*-mediated high temperature resistance in wheat to stem rust (Zhang et al. 2017). In a previous transcriptome analysis, *PR* genes, including those encoding thaumatin-like protein (PR5) and lipid transfer protein (PR14), were highly induced during *Fhb1*-mediated wheat resistance to *Fusarium* head blight (Xiao et al. 2013). The constant upregulation of *TaLTP4*, *TaTLP8*, and *TaChit* genes observed in our qRT-PCR assay (Fig. 4b, d, and e) suggests that these *PR* genes may be functional downstream of the *Lr39/41*-mediated resistance.

TaBCI6 belongs to a group of genes sensitive to SA (or its orthologs BTH and DCINA) treatment in *Triticeae* crops (Beßer et al. 2000). BTH treatment in wheat protects the crops effectively against powdery mildew and leaf rust by affecting multiple steps in the life cycle of the pathogens (Görlach et al. 1996; Hafez et al. 2014). In our microarray analysis, we identified *TaBCI6* in both *Lr39/41*-upregulated and Thatcher-downregulated DEGs (Tables 1 and 2). Along with its significant induction at 1 dpi in the qRT-PCR assay (Fig. 4c), we speculate an involvement of this gene in the basal defense response during the *Lr39/41*-mediated resistance.

The endogenous SA level during plant defense response is largely dependent on the severity of pathogen-triggered cell death (Fu and Dong 2013). In a recent study, endogenous SA levels during *YrSu*-mediated wheat resistance to yellow rust were measured using high performance liquid chromatography-mass spectroscopy (HPLC-MS), and a significant elevation of SA accompanying with the HR was observed at 48 hpi (Wang et al. 2017). Therefore, we speculate that SA and SA-mediated pathway may play key roles in the *Lr39/41*-mediated resistance.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Human and animal rights and informed consent No Human Participants and/or Animals are involved in this research. Informed consent is not applied.

Ethical approval The authors declare that they have followed the guidelines of Committee on Publication Ethics (COPE) and obeyed all the Ethical Standards requested by EJPP.

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