

RESEARCH PAPER

RLP1.1*, a novel wheat receptor-like protein gene, is involved in the defence response against *Puccinia striiformis* f. sp. *tritici

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Abstract

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is one of the most serious diseases of wheat; therefore, exploring effective resistance-related genes is critical for breeding and studying resistance mechanisms. However, only a few stripe rust resistance genes and defence-related genes have been cloned. Moreover, transgenic wheat with enhanced stripe rust resistance has rarely been reported. Receptor-like proteins (RLPs) are known to be involved in defence and developmental pathways. In this research, a novel *RLP* gene *TaRLP1.1* was characterized as an important stripe rust defence gene. *TaRLP1.1* was screened by GeneChip and was found to be induced by *Pst* specifically in the resistant variety. Knock down of *TaRLP1.1* in the stripe rust-resistant plants resulted in increased susceptibility to *Pst*, and phenolic autofluorogen accumulation at the pathogen–host interaction sites, usually correlated with the hypersensitive response, was decreased dramatically. However, when the *TaRLP1.1* gene was transformed into the susceptible wheat variety Yangmai158, the transgenic plants showed highly increased resistance to *Pst*, and the hypersensitive response was enhanced at the infection sites. Meanwhile, the expression of pathogenesis-related genes decreased in the *TaRLP1.1*-silenced plants and increased in the *TaRLP1.1*-overexpressing plants. Thus, it was proposed that *TaRLP1.1* greatly contributed to the hypersensitive response during the pathogen–host interaction. Along with the functional analysis, an evolutionary study of the *TaRLP1* family was performed. Characterization of *TaRLP1.1* may facilitate breeding for stripe rust resistance and better understanding of the evolution of the *RLP* genes in wheat.

Key words: Disease resistance, hypersensitive response, receptor-like protein, stripe rust, transgenic, virus-induced gene silencing.

Introduction

Stripe rust, caused by *Puccinia striiformis* f. sp. *tritici* (*Pst*), is one of the most destructive diseases of wheat in the world, and China is the largest endemic area (Kang *et al.*, 2010). Utilization of disease-resistant varieties has proved to be the safest, most economical, and effective method to control

stripe rust, and resistance gene cloning is vital for the development of resistant varieties. However, a big challenge for resistance breeding is that *Pst* isolates evolved very rapidly, leading to breakdown of widely used resistance genes—especially in China where most new destructive epidemics

of *Pst* have originated. *Yr10* (Laroche *et al.*, 2000), *Yr18* (Krattinger *et al.*, 2009), and *Yr36* (Fu *et al.*, 2009) are the only stripe rust resistance genes cloned to date. Using the virus-inducing gene silencing (VIGS) approach, *TaRLK-R1*, *-R2*, and *-R3* (Zhou *et al.*, 2007), *TaHsp90.2* and *TaHsp90.3* (G.F. Wang *et al.*, 2011), *TaDAD2* (X.J. Wang *et al.*, 2011), *TaMCA4* (Wang *et al.*, 2012), and *TaRab7* (Liu *et al.*, 2012) have been characterized as the genes required for stripe rust resistance. However, no stripe rust resistance-improved transgenic wheat has been developed by using these genes, so their effect in breeding remains unknown. The stripe rust resistance gene *Yr26* located on chromosome 1B was introduced from *Triticum turgitum* cv. γ -80 (Ma *et al.*, 2001), and has been widely used as the main gene resource in China since 2003. *Yr26* is a broad-spectrum resistance gene, but, more importantly, *Yr26* shows potential durable resistance. Thus cloning the genes related to stripe rust resistance from *Yr26*-containing wheat should help in understanding the mechanism of its resistance and in developing varieties resistant to stripe rust.

Receptor-like proteins (RLPs) are cell surface receptors which contain an extracellular leucine-rich repeat (LRR) domain, a single transmembrane domain, and a small cytoplasmic tail (Kruijt *et al.*, 2005). Several *RLP* genes are known to be involved in plant–pathogen interactions. The first identified *RLP* gene was tomato *Cf-9* that mediated resistance against *Cladosporium fulvum* (Jones *et al.*, 1994), and several other *Cf* resistance genes have also been characterized to be *RLP* genes (Dixon *et al.*, 1996, 1998). In tomato, two *Ve* genes involved in plant resistance against vascular wilt and two *LeEix* genes encoding receptors for the fungal elicitor were also reported to encode RLPs (Kawchuk *et al.*, 2001; Ron and Avni, 2004). In apple, *HcrVf2* conferred resistance to the scab fungus *Venturia inaequalis* (Belfanti *et al.*, 2004). In *Arabidopsis*, three *RLP* genes were found to be involved in the resistance response, namely *AtRLP52* (Ramonell *et al.*, 2005), *AtRLP30* (Wang *et al.*, 2008), and *snc2-1D* (Zhang *et al.*, 2010). Apart from defence against pathogens, *RLP* genes also play significant roles in plant development. In *Arabidopsis*, *TWO MANY MOUTHS (TMM)* was shown to be involved in stomatal patterning (Nadeau and Sack, 2002), while *CLAVATA2 (CLV2)* and its orthologue *FASCINATED EAR2* in maize were shown to be involved in meristem development (Jeong *et al.*, 1999; Taguchi-Shiobara *et al.*, 2001). Some research was also carried out to elucidate the mechanism of RLP-mediated resistance, such as the *Cf*-mediated hypersensitive response (HR) in tomato (Stulemeijer *et al.*, 2007), and the *Vel*-mediated signals in tomato and in *Arabidopsis* (Fradin *et al.*, 2011).

Due to the availability of genome sequences, many *RLP* genes have been identified from model species: there are 57 predicted *RLP* genes distributed in 33 loci in *Arabidopsis*, and 90 *RLP* genes distributed in 38 loci in rice (Wang *et al.*, 2008). However, despite being an important food crop, there have been few investigations of *RLP* genes in wheat. Identification of *RLP* genes is necessary for extensive study and better understanding of their biological functions in wheat. In the present study, an *RLP* gene, *TaRLP1.1*, was cloned

and characterized as an important gene involved in stripe rust resistance. *TaRLP1.1* has a huge potential role in future genetic engineering for development of varieties resistant to stripe rust. Along with *TaRLP1.1*, a large *TaRLP1* family was identified, which may facilitate better understanding of the evolution of *RLP* genes in wheat.

Materials and methods

Plant materials, pathogen inoculation, and chemical treatments

Triticum aestivum L. cv. 92R137, Yuanfeng175, Neimai9, and R236 with the stripe rust resistance gene *Yr26*, and cv. Yangmai158 susceptible to stripe rust were used in this study. R236 and Yangmai158 are near-isogenic lines. The seedlings were maintained and inoculated with *Pst* CYR32, the predominant isolate in China (X.J. Wang *et al.*, 2011). Leaves were harvested at 0, 12, 24, 36, 48, and 72 h post-inoculation (hpi) for RNA isolation. For chemical treatments, 3-week-old seedlings of cv. 92R137 were sprayed with 5 mM salicylic acid (SA), 100 μ M methyl jasmonate (MeJA), 200 μ M ethylene (ET), or 7 mM hydrogen peroxide (H₂O₂) in 0.05% Tween-20 solution, and with 0.05% Tween-20 used as control. Leaves were harvested at 0, 0.5, 2, 6, 12, and 24 hpi for RNA isolation. Three independent biological replications were performed.

GeneChip microarray analysis

The wheat GeneChip (part no. 900515, Affymetrix) was chosen to screen the *Pst* resistance-responsive genes in the *Yr26*-containing materials. Leaves of 2-week-old seedlings of resistant varieties 92R137 and R236 and susceptible Yangmai158 were collected at 0, 12, and 36 hpi with *Pst* CYR32, respectively. In this study, the microarray was used only to screen some genes for the downstream functional analysis, so the hybridization of each sample was not repeated. However, two resistant lines were used to increase the credibility of the screened positive genes, and the near-isogenic lines were used to reduce the interference of the genetic background. The transcript profile of each sample was obtained using genechip hybridization conducted in the National Engineering Center for Biochip in Shanghai, China, according to the Affymetrix technical manual. Six separate comparisons (categories) were performed to identify the differentially expressed genes between different samples, namely 'R236-12h versus R236-0h', 'R236-36h versus R236-0h', '92R137-12h versus Yangmai158-12h', 'R236-12h versus Yangmai158-12h', '92R137-36h versus Yangmai158-36h', and 'R236-36h versus Yangmai158-36h'. For each category, probe sets were filtered for a fold change threshold of >2.0 and a parametric two-way analysis of variance (ANOVA) ($P < 0.001$) with false discovery rate (FDR) multiple testing correction at 5% was applied to identify statistically significant differentially expressed probe sets. Only when the signal ratio value was >2 was the corresponding probe regarded as up-regulated.

Semi-quantitative RT-PCR and real-time PCR analysis

A 2 μ g aliquot of total RNA was used to synthesize the first-strand cDNA by the AMV reverse transcriptase. To evaluate the effectiveness of the gene silencing by VIGS, the expression of *TaRLP1.1* was analysed by real-time quantitative reverse transcription-PCR (RT-PCR) with a pair of primers (*TaRLP1.1*-RT, Supplementary Table S1 available at JXB online) specific to *TaRLP1.1*, using the 18S rRNA (*18rRNA*-RT, Supplementary Table S1) as the internal control. The reagents were from Takara (Japan), and the reaction was performed in the ABI 7500fast (ABI, USA). The responses of the *TaRLP1.1* gene to pathogen and chemical treatments, the expression of three *PR* genes (Supplementary Table S1) in the gene-silenced and over-expressing plants, and the expression of *TaRLP1.1* in the transgenic

plants were analysed by semi-quantitative RT-PCR, using the tubulin gene (*Tubulin*-RT, Supplementary Table S1) as the internal control. PCR was performed in 25 µl of reaction mixture. The program used was as follows: 3 min at 94 °C, followed by 27 cycles at 94 °C for 30 s, 55–60 °C for 30 s, and 72 °C for 40 s, and then retained at 10 °C.

Isolation of cDNA and genomic sequences of *TaRLP1*

The fragment of *TaRLP1.1*, a homologue of *Pst*-up-regulated probe Ta.22666.2.S1_at in cv 92R137 and R236, was obtained by RT-PCR using the primers (*Ta.22666.2.S1*-EST, Supplementary Table S1 at JXB online) designed according to the probe. Rapid amplification of cDNA ends (RACE) was then conducted to obtain the full-length cDNA of *TaRLP1.1* from cv. 92R137 inoculated for 36 h using the SMART™ RACE cDNA Amplification Kit (Clontech). Primers for 5'-RACE and 3'-RACE (*TaRLP*-RACE, Supplementary Table S1) were designed according to the fragment of *TaRLP1.1*. To obtain the full-length *TaRLP1.1*, RT-PCR was performed with a pair of primers (*TaRLP1.1*-FL, Supplementary Table S1) designed based on the sequence of the 5' and 3' cDNA ends. The homologues of *TaRLP1.1* in cv. Neimai9 and Yuanfeng175 were also amplified using the above primers. The genomic sequence of *TaRLP1.1*, and of its homologues in cv. 92R137 and Yangmai158, was obtained with primers (*TaRLP1.1*-ORF, Supplementary Table S1) designed based on the full-length cDNA of *TaRLP1.1*.

Sequence analysis and phylogenetic tree construction

The cDNA sequence was analysed using the software of the DNAMAN, BLAST, and ORFFinder. Protein sequences were analysed using PROSITE Scan (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_prosite.html) for prediction of the conserved domains and motifs, SignalP (<http://www.cbs.dtu.dk/services/SignalP/>) for prediction of the signal peptide, and TMpred (http://www.ch.embnet.org/software/TMPRED_form.html) for prediction of the transmembrane region. The MEGA 4.0 software was used for construction of the phylogenetic tree.

Subcellular localization of *TaRLP1.1*

The cDNA fragments containing the *TaRLP1.1* open reading frame (ORF) were amplified by PCR with the specific primers containing *NcoI* and *SacI* enzyme sites (*TaRLP1.1*-SL, Supplementary Table S1 at JXB online), and then inserted to the 5' end of the green fluorescent protein (GFP) coding region in the *pCaMV35S:GFP* vector. The plasmid DNAs of *pCaMV35S:TaRLP1.1-GFP* and *pCaMV35S:GFP* were transformed into onion epidermal cells by particle bombardment as described (Liu *et al.*, 2012), respectively. The GFP signal was detected using a confocal microscope (Leica, Germany).

Plasmid construction and functional analysis of *TaRLP1.1* by VIGS

VIGS mediated by *Barley stripe mosaic virus* (BSMV) (Zhou *et al.*, 2007) in 92R137 was adopted to investigate the potential involvement of *TaRLP1.1* in the HR to *Pst*. A 186bp cDNA fragment covering both part of the coding region and part of the 3'-untranslated region (UTR) was amplified with primers (*TaRLP1.1*-VIGS, Supplementary Table S1 at JXB online), and then was inserted in reverse orientation into the *BSMV:γ* vector to form the recombinant vector *BSMV:TaRLP1.1as*. The second fully expanded leaves of the seedlings were infected with virus *BSMV:TaRLP1.1as*, using *BSMV:TaPDSas* and *BSMV:γ* as the controls. Three independent sets of inoculations were performed, with a total of 72 seedlings inoculated for each BSMV virus. For the control, 24 seedlings were mock-inoculated with 1× GPK buffer. At 10–12 d after virus inoculation, the fourth leaves were further treated with *Pst* CYR32. At 15 d after pathogen inoculation, when extensive fungal growth was

visible in Yangmai158, the infection types of stripe rust were examined in the gene-silenced leaves of 92R137. The fourth leaves were also sampled at 72 and 120 hpi with *Pst* for histological observation and gene silencing efficiency analysis.

Plasmids construction and wheat transformation

The ORF of *TaRLP1.1* was amplified using the primers containing *Bam*HI and *Sac*I sites (*TaRLP1.1*-WT, Supplementary Table S1 at JXB online), and was then inserted into the plant expression vector *pBI220* under the control of the 35S promoter to form the recombinant vector *pBI220:TaRLP1.1*. Because there is no selective marker gene available in the vector *pBI220*, another plant expression vector *pAHC25*, containing the β-glucuronidase gene (*GUS*) and the herbicide tolerance gene (*Bar*) driven by the ubiquitin (*Ubi*) promoter, was used as the co-transformation vector. The *pBI220:TaRLP1.1*- and *pAHC25*-co-transformed wheat plants were produced by particle bombardment of calli cultured from immature embryos of susceptible Yangmai158 as described (Xing *et al.*, 2008). For screening the *TaRLP1.1*-transformed positive transgenic plants from the 238 regenerated plants, the root tips of 20 T₁ seeds from each T₀ plant were first stained with X-gluc (Xing *et al.*, 2008), and the target *TaRLP1.1* was then detected by PCR using the DNA of the corresponding T₀ plant whose T₁ seeds showed *GUS* activity. The target gene was also detected in the T₁ generation of the positive T₀ plants. To avoid interference from the endogenous homologous genes in Yangmai158, the forward primer (35S-F, Supplementary Table S1) was located in the 35S promoter, and the reverse primer (*TaRLP1.1*-R, Supplementary Table S1) was located in the target *TaRLP1.1* gene.

Evaluation of stripe rust resistance of transgenic plants

Stripe rust resistance of T₁ transgenic plants was evaluated via inoculation with urediniospores of *Pst* CYR32 using cv. Yangmai158 and 92R137 as the controls at the two-leaf stage as described by X.J. Wang *et al.* (2011). About 30 seedlings of the T₁ generation from each positive T₀ line were inoculated with CYR32 and kept in a dew chamber at 10 °C for 24 h in darkness, and then grown in a growth chamber at 16/8 h light/dark with diurnal temperatures gradually changing from 4 °C at 02:00 h to 20 °C at 14:00 h. Infection type data were recorded 18 d after inoculation based on the 0–9 scale described by X.J. Wang *et al.* (2011). Infection types of 0–3 were considered resistant, while those >5 were considered susceptible.

Histological observations of fungal growth and host response

Wheat leaves infected with BSMV were sampled at 72 and 120 hpi with *Pst* and stained as described (X.J. Wang *et al.*, 2011). Bleached leaf segments were examined with an Olympus BX-51 microscope (Olympus, Japan) for infection sites, and only those sites where substomatal vesicles had formed in the stomata were considered to be successfully penetrated. Autofluorescence of the mesophyll cells in the infected sites was measured as phenolic autofluorogen accumulation area by epifluorescence microscopy (excitation filter, 485 nm; dichromic mirror, 510 nm; and barrier filter, 520 nm). The autofluorescence areas, hyphal length, and hyphal branches were calculated by DP-BSW software, and the final data of each index were the mean of at least 60 infection sites for each of the five randomly selected leaf segments per treatment. Standard deviations and a paired sample *t*-test for statistical analysis were performed with SAS software.

Results

Screening for *Pst*-responsive genes in Yr26-containing wheat

To screen the *Pst* resistance-related genes in wheat containing the stripe rust resistance gene *Yr26*, microarrays were

used to obtain the transcriptomes of seven samples, including two resistant varieties 92R137 and R236, and one susceptible cv. Yangmai158, with or without *Pst* inoculation as described (X.J. Wang *et al.*, 2011). The data of six differentially expressed probes (2-fold up- and down-regulated and P -value ≤ 0.001) were consequently produced. Bioinformatics analysis showed that in total 35 probes were shared by all of the above six data groups, with 17 up-regulated and 18 down-regulated; thus 35 probes showed a differentially expressed pattern not only between *Pst*-inoculated samples of resistant and susceptible lines, but also between *Pst*-inoculated and uninoculated samples of the resistant lines. Among the 17 probes, Ta.22666.2.S1 and TaAffx.55699.1.S1_{at} were two putative *RLP* genes (Supplementary Table S2 at *JXB* online). Although there was no homology between Ta.22666.2.S1 and TaAffx.55699.1.S1, these two probes showed similarity to the different parts of the same genes in GenBank, indicating that they represented the same *RLP* gene. *RLP* genes have been reported to be involved in the defence responses to different pathogens; however, no *RLP* has been identified in wheat, so the screened *RLP* gene was selected as of interest for investigation of its function in stripe rust resistance.

Cloning and structural analysis of the TaRLP1.1 gene from stripe rust-resistant variety 92R137

The homologue of Ta.22666.2.S1_{at} in cv. 92R137 inoculated with CYR32 was obtained by RT-PCR using the primers (Ta.22666.2.S1-EST, Supplementary Table S1 at *JXB* online) designed according to the probe Ta.22666.2.S1_{at}. The amplified fragment shared 99% similarity with Ta.22666.2.S1_{at}, so the homologue in cv. 92R137 was designated as *TaRLP1.1*, since it showed high homology with other *RLP* genes. The full-length sequence of *TaRLP1.1* was further obtained by RACE from cv. 92R137, which was then verified by RT-PCR using the primers (*TaRLP1.1*-FL, Supplementary Table S1) designed based on the 5'- and 3'-UTR. The full length of the *TaRLP1.1* was 1273 bp, which contained a putative 801 bp ORF coding for 266 amino acids.

The predicted primary structure of TaRLP1.1 comprised several conserved domains which have been identified in other RLPs (Supplementary Fig. S1A, B at *JXB* online). Domain 'a' is a signal peptide, a hydrophobic N-terminus found in TaRLP1.1, which may target the protein to the cytoplasmic membrane. Domain 'b' is an LRR region with three imperfect copies of LRR; each one is a 23–25 amino acid consensus sequence [xLxxLxLSxNxLSGxIP] associated with protein–protein interactions (Jones and Jones, 1997). The presence of a glycine within the LRR is considered as the characteristic of extracytoplasmic proteins with the ability to recognize the extracellular pathogen ligands (Jones *et al.*, 1994). As frequently observed in membrane-spanning proteins, a hydrophobic sequence with a predicted α -helix secondary structure (domain c, containing GxxxG-type motifs) is the putative transmembrane region, following a negatively charged extracytoplasmic domain (domain d) and followed by a positively charged cytoplasmic tail. In the short cytoplasmic tail domain, there is a 'Yxx φ ' signal sequence, where

' φ ' is an amino acid with a hydrophobic side chain, and this motif has been shown to stimulate receptor-mediated endocytosis and degradation of mammalian cell surface receptors (Ron and Avni, 2004; Kruijft, *et al.*, 2005).

Identification and chromosome location of different members of the TaRLP1 family in resistant cv. 92R137 and susceptible cv. Yangmai158

To determine whether *TaRLP1.1* belonged to a gene family and whether there was a difference in *TaRLP1.1* between the resistant variety 92R137 and susceptible cv. Yangmai158, PCR was conducted using primers (*TaRLP1.1*-ORF, Supplementary Table S1 at *JXB* online) to identify the homologues of *TaRLP1.1* in the DNA of both materials. In cv. 92R137, in addition to *TaRLP1.1*, another nine *TaRLP1* genes of different sizes were identified; and in cv. Yangmai158, eight *TaRLP1* genes of different sizes were identified (Supplementary Table S3). Sequence analysis showed that *TaRLP1*₇₃₈, *TaRLP1*₈₀₀₋₁, *TaRLP1*₈₀₁₋₂, *TaRLP1*₈₀₄₋₁, and *TaRLP1*₈₁₃ existed in both cv. 92R137 and Yangmai158; however, other genes were identified to be specific to each cultivar.

The specific primers were designed based on the sequence of the above genes (Supplementary Table S1 at *JXB* online) and were used for gene chromosome location in cv. Chinese Spring-derived nulli-tetrasomic lines. *TaRLP1.1* was located to chromosome 3D, *TaRLP1*₈₀₄₋₁ to 3B, and *TaRLP1*₈₁₃ to 3A (Supplementary Fig. S2). The result indicated that the *TaRLP1* gene family may be located in the homoeologous group 3 chromosomes.

Phylogenetic analysis of TaRLP1 family members

The result of the multisequence alignment and phylogenetic analysis of the *RLP* genes from common wheat suggested that *TaRLP1* is a rapidly evolving gene family due to single nucleotide variation, deletion or insertion, and recombination (Supplementary Fig. S3 at *JXB* online) (Dixon *et al.*, 1998). For example, compared with *TaRLP1*₈₀₁, *TaRLP1*₈₀₀ was produced by one missing nucleotide, *TaRLP1*₈₀₄ was the outcome of a three-nucleotide (TCA) insertion into *TaRLP1*₈₀₁, and *TaRLP1*₈₁₃ was the product of a nine-nucleotide insertion (CCTATCC and TAA) into *TaRLP1*₈₀₄. Gene duplication occurred on *TaRLP1*₈₀₁ and *TaRLP1*₈₀₄, resulting in more members of both types of genes in wheat (Supplementary Fig. S4).

Recombination between the homologous regions of different members of one gene cluster is a common event that produces new genes. In this study, *TaRLP1*₈₀₀₋₂ was the product of recombination between *TaRLP1*₇₃₈ and *TaRLP1*₈₀₄₋₄: the first half of *TaRLP1*₈₀₀₋₂ was identical to *TaRLP1*₇₃₈, and the second half was identical to *TaRLP1*₈₀₄₋₄.

Polymorphism of the DNA sequence leads directly to the variation in the putative protein sequences (Supplementary Fig. S5 at *JXB* online). Compared with TaRLP1.1, TaRLP1₈₀₀₋₁ and TaRLP1₈₀₀₋₂ are two truncated proteins due to one nucleotide missing and pre-stop in the reading

frame; while in *TaRLP1*₇₃₈ there were 22 amino acids missing. Although many variations were detected, the effect on gene function needs to be elucidated.

As to the homology of *TaRLP1.1* with other putative RLPs, *TaRLP1.1* showed the highest similarity (>70% identity) to its homologues from other monocots (Supplementary Fig. S6A at *JXB* online); however, it showed the lowest sequence similarity to *Arabidopsis* *AtRLP44* (38% identity) (Supplementary Fig. S6B).

Expression analysis of the *TaRLP1* family in the resistant cv. 92R137 and susceptible cv. Yangmai158

To determine how many *TaRLP1* genes were expressed in both the resistant and susceptible plants, the primers used to amplify the homologous gene from 92R137 and Yangmai158 were also used to amplify the expressed genes from the cDNA of the cv. 92R137 and Yangmai158. There were 22 positive clones from the cv. 92R137 sequenced, and three *TaRLP1* genes were found to be expressed: *TaRLP1.1* (19 of 22), *TaRLP1*₈₀₀₋₁ (two of 22), and *TaRLP1*₈₁₃ (one of 22). Twenty positive clones from cv. Yangmai158 were sequenced, and two *RLP* genes, *TaRLP1*₈₀₀₋₁ (19 of 20) and *TaRLP1*₈₀₁₋₂ (one of 20), were detected as expressed. In two other resistant cultivars, Neimai 9 and Yuanfeng 175, *TaRLP1.1* was also detected as the predominant transcript as in cv. 92R137. Sequence analysis showed that *TaRLP1*₈₀₀₋₁ produced a truncated 57 amino acid protein due to a missing nucleotide compared with *TaRLP1.1*. *TaRLP1*₈₀₁₋₂ also produced a 266 amino acid protein; however, the similarity with *TaRLP1.1* was only 88%. *TaRLP1*₈₁₃ produced a 270 amino acid protein, and similarity with *TaRLP1.1* was 81% (Supplementary Fig. S5 at *JXB* online).

TaRLP1.1 was the dominant expressed gene specifically in the *Yr26*-containing cv. 92R137, Neimai 9, and Yuanfeng 175; thus, it was proposed that *TaRLP1.1* may play an important role in the *Yr26*-mediated resistance pathway.

Responses of *TaRLP1.1* to *Pst* infection and chemical stress treatments

Because *TaRLP1.1* was the dominant transcript in cv. 92R137 and another two *Yr26*-containing materials based on expression analysis, to examine further whether the expression of *TaRLP1.1* was really induced by *Pst*, semi-quantitative RT-PCR analysis was conducted using primers (*TaRLP1.1*-RT, Supplementary Table S1 at *JXB* online) specific to *TaRLP1.1*. It was found that *TaRLP1.1* was significantly up-regulated in cv. 92R137 at 12 hpi with *Pst*, and reached a peak at 36 hpi (Fig. 1A). In another two *Yr26*-containing cultivars, Neimai 9 and Yuanfeng175, *TaRLP1.1* was also induced by CYR32 and reached a peak at 72 and 36 hpi, respectively. However, no transcript was detected in susceptible Yangmai158. Therefore, *TaRLP1.1* may play an important role in resistance to stripe rust fungus, and perhaps take part in the signal transduction mediated by *Yr26*.

Seedlings of cv. 92R137 were treated with exogenous chemicals—SA (5 mM), MeJA (100 μ M), ET (200 μ M), and H₂O₂

(7 mM)—for analysis of the responses of *TaRLP1.1* by semi-quantitative RT-PCR. The *TaRLP1.1* was substantially up-regulated by both SA and H₂O₂ treatments; however, there was no response to the MeJA and ET (Fig. 1B).

Subcellular localization of *TaRLP1.1*-GFP fusion protein

The subcellular localization of *TaRLP1.1* was studied using the constructed fused protein *TaRLP1.1*-GFP driven by the CaMV 35S promoter by the transient expression system in onion epidermal cells. The result showed that the green signal produced by GFP protein alone was uniformly distributed throughout the cell; however, the green signal produced by the fused protein *TaRLP1.1*-GFP was clearly localized only to the cytomembrane (Supplementary Fig. S7 at *JXB* online). The subcellular localization indicated that *TaRLP1.1* was localized to the membrane as predicted by its transmembrane 'GxxxG' motif and *TaRLP1.1* may act as a receptor to receive the stimuli from outside of the cell in the resistance pathway.

Silencing of *TaRLP1.1* compromised resistance to *Pst* and suppressed expression of *PR* genes in cv. 92R137

To evaluate the function of *TaRLP1.1*, a BSMV-based VIGS approach (Zhou *et al.*, 2007) was used to silence the genes in the stripe rust resistance cv. 92R137. Fifteen days after *BSMV:TaPDSas* inoculation, leaf bleaching was observed due to PDS gene silencing, indicating that the VIGS system for gene functional analysis was successfully silencing the target gene (Fig. 2A). The efficiency of VIGS was analysed by quantitative RT-PCR using the fourth leaves of wheat seedlings, and expression levels of *TaRLP1.1* were reduced by 81% and 69% in leaves challenged with *BSMV:TaRLP1.1as* at 72 and 120 hpi, respectively, compared with the control inoculated with *BSMV: γ* (Fig. 2B). At 2 weeks after *Pst* isolate CYR32 inoculation, a robust HR was observed on leaves infected with *BSMV: γ* , or mock-inoculated only with GPK buffer. However, fungal sporulation emerged only on fourth leaves previously infected with *BSMV:TaRLP1.1as* (Fig. 2C).

In the incompatible host-pathogen interaction, autofluorescence due to the accumulation of phenylpropanoid compounds is common, and usually correlated with the HR. Because the incompatible interaction of 92R137 and *Pst* CYR32 was accompanied by a sharp HR, an investigation was carried out to determine what would happen to the HR or phenolic autofluorogen accumulation when the compatible interaction formed in the *TaRLP1.1*-silenced 92R137. Detailed histological changes were also observed at 72h and 120h after *Pst* CYR32 inoculation of the *BSMV:TaRLP1.1as*-, *BSMV: γ* -, and mock-infected plants, and four parameters were measured: proportion of the infection sites producing phenolic autofluorogen accumulation, autofluorescence area, hyphal length, and hyphal branches (Fig. 3). In the control plants infected with *BSMV: γ* or mock infected, a large proportion of infection sites producing phenolic autofluorogen accumulation could be detected

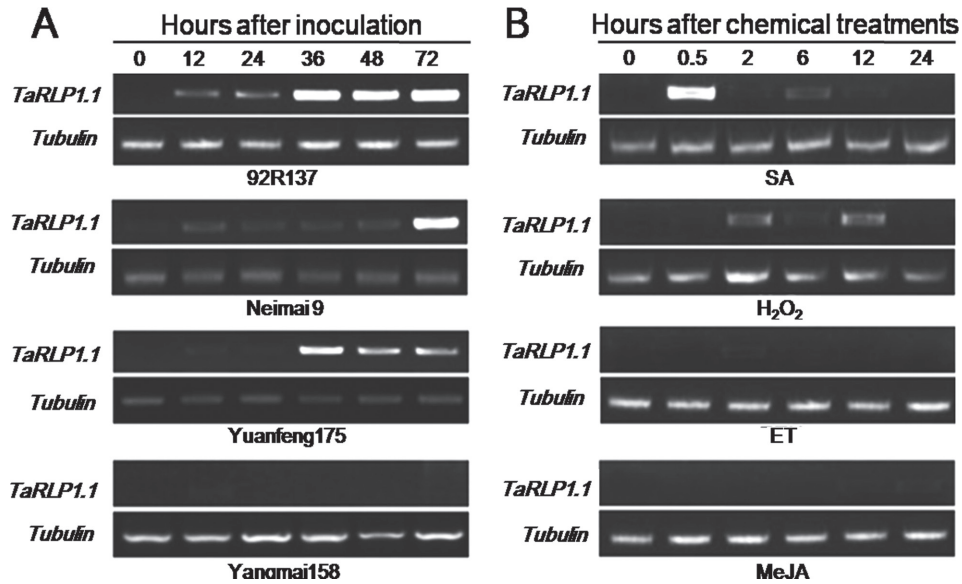


Fig. 1. Evaluation of expression changes of *TaRLP1.1* in response to biotic (stripe rust infection), and chemical treatments (SA, ET, MeJA, and H₂O₂) stresses in wheat seedlings by semi-quantitative RT-PCR. The *tubulin* gene was used as the internal control. (A) *TaRLP1.1* was up-regulated by *Pst* CYR32 in resistant wheat (cv. 92R137, Neimai 9, and Yuanfeng 175), while it was not induced in susceptible cv. Yangmai158. (B) *TaRLP1.1* was induced by SA and H₂O₂, but not by ET and MeJA.

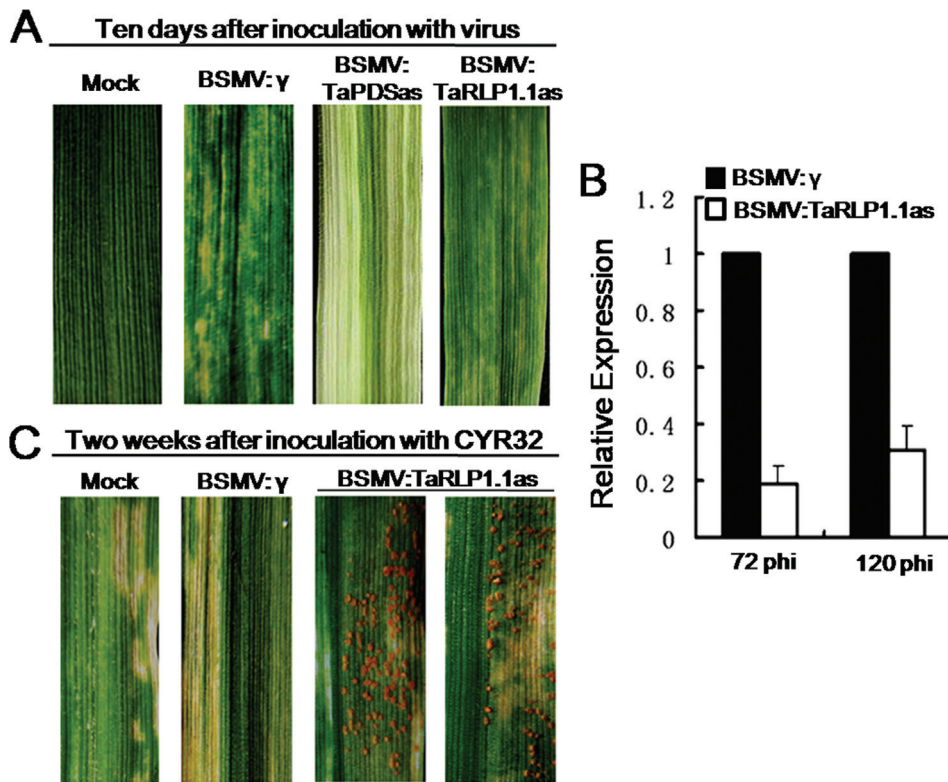


Fig. 2. Functional characterization of *TaRLP1.1* by the BSMV-based VIGS approach. (A) Successful virus infection. Mild chlorotic mosaic symptoms were observed on the fourth leaves inoculated with *BSMV:γ* or *BSMV:TaRLP1.1as* at 10 days post-inoculation (dpi). Photobleaching displayed on leaves infected with *BSMV:TaPDSas* at 15 dpi. Typical photographs were taken at 15 d after virus inoculation. (B) The expression level of *TaRLP1.1* was decreased dramatically at both 72h and 120h after CYR32 inoculation in the *BSMV:TaRLP1.1as*-infected plants. (C) The resistance to *Pst* CYR32 was compromised in the *BSMV:TaRLP1.1as*-infected plants, and the rust sori were observed in the *TaRLP1.1*-silenced leaves. The photographs were taken 2 weeks after fungal inoculation.

(Fig. 3A), and a large autofluorescence area could be observed at 72 h [Fig. 4A (a1, b1)] or 120 h [Fig. 4B (a1, b1)] after *Pst* CYR32 inoculation (Fig. 3B). In test plants infected with

BSMV:TaRLP1.1as, the proportion of infection sites producing phenolic autofluorogen accumulation and the autofluorescence area were significantly ($P < 0.001$) lower than

for control leaves at 72 h [Figs 3A, B, 4A (c1)] and 120 h [Figs 3A, 4B, B (c1)] after CYR32 inoculation, and hyphal length was significantly ($P < 0.001$) longer than for the control [Figs 3C, 4A (c), 4B (c)]. However, there was no significant difference ($P < 0.05$) in hyphal branching between control and test plants (Fig. 3D). These results indicated that knocking down *TaRLP1.1* decreased the autofluorescence areas generated at infection sites, and the HR was compromised.

The loss of resistance usually correlated with the decreased expression of some defence-related genes. Some pathogenesis-related genes were tested in the *TaRLP1.1*-silenced plants and controls. Expression levels of *TaPR1*, *TaPR2*, and *TaPR5* were decreased in *TaRLP1.1* knocked-down leaves at 72 or 120 hpi, in contrast to *BSMV:γ*-infected control (Supplementary Fig. S8 at JXB online). *PR1*, *PR2*, and *PR5* are usually considered to be marker genes of the SA pathway, so their decreased expression in *TaRLP1.1*-silenced plants implied some damage to the SA pathway.

Overexpression of *TaRLP1.1* enhanced the stripe rust resistance of susceptible Yangmai158

The function of *TaRLP1.1* was further studied by developing *TaRLP1.1*-overexpressing transgenic plants. Susceptible cv. Yangmai158 is a near-isogenic line of *Yr26*-containing

resistant cv. R236, and cv. Yangmai158 lacks *TaRLP1.1*. Additionally, cv. Yangmai158 is a good variety for tissue culture, and so *TaRLP1.1* was transformed into cv. Yangmai158 by particle bombardment in this study. The *pBI220:TaRLP1.1* was co-transformed with the *GUS* gene-containing vector *pAHC-25*. *GUS* staining using the root tips of the T_1 generation seeds and target gene detection in the T_0 generation was used to identify positive transgenic plants—22 T_0 plants were determined to be positive transgenic plants (Supplementary Fig. S9A, B at JXB online). Expression analysis in the T_1 generation showed that *TaRLP1.1* was successfully overexpressed in the transgenic plants (Supplementary Fig. S9C).

Evaluation of the resistance to *Pst* CYR32 was performed using the T_1 plants of each positive T_0 plant. The second leaves of plants were inoculated with *Pst* isolate CYR32, and 2 weeks later robust hypersensitive cell death was observed—the transgenic plants showed high resistance to CYR32 (Fig. 5A). Because of heterozygosity of *TaRLP1.1* in the T_0 generation, the *TaRLP1.1* was segregated in T_1 generation seedlings, and only those plants transformed with *TaRLP1.1* showed increased resistance with resistance of grade 2–4, while the recipient Yangmai158 showed the highest level of susceptibility of grade 9 (Fig. 5B; Supplementary Fig. S10) at JXB online.

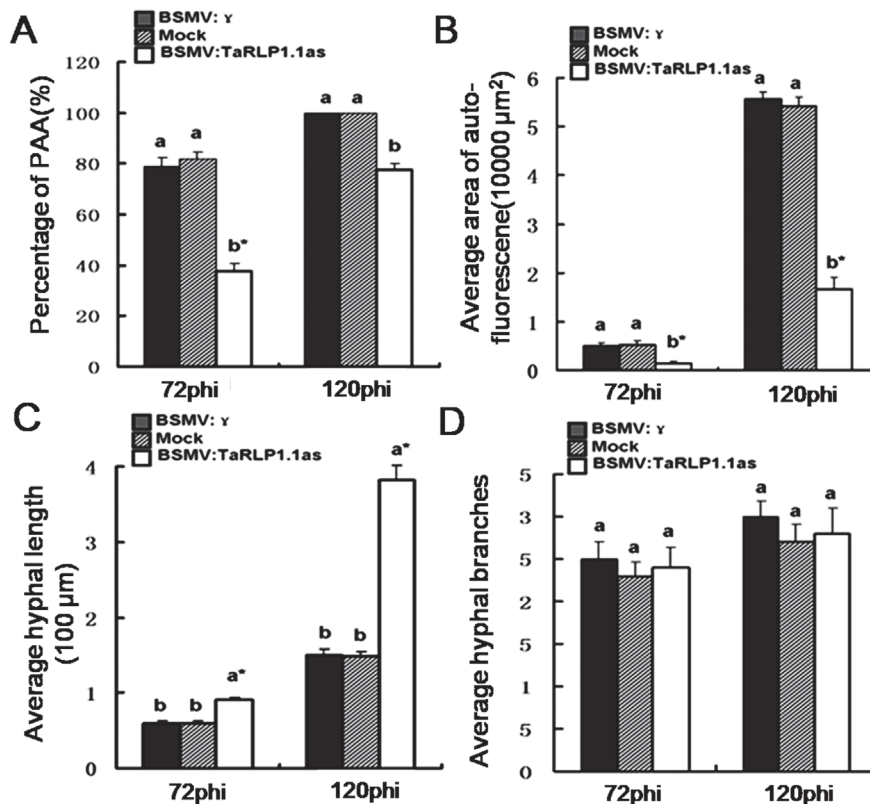


Fig. 3. Quantitative comparisons of the percentage of the infection sites producing phenolic autofluorogen accumulation, average area of autofluorescence, and average linear length of hyphae at 72 and 120 hpi after CYR32 inoculation on leaves pre-infected with *BSMV:TaRLP1.1as* or *BSMV:γ*, or mock infected. The data from each treatment were calculated from at least 60 infection sites on each seedling. Statistical significance was determined using ANOVA and *t*-test. Mean values followed by different letters are significantly different from each other (b* indicates $P < 0.001$ and b indicates $P < 0.05$). (A) Percentage of infection sites producing phenolic autofluorogen accumulation. (B) Average area of autofluorescence in the mesophyll cells. (C) Average hyphal length from the base of substomatal vesicles to hyphal tips. (D) Average number of hyphal branches.

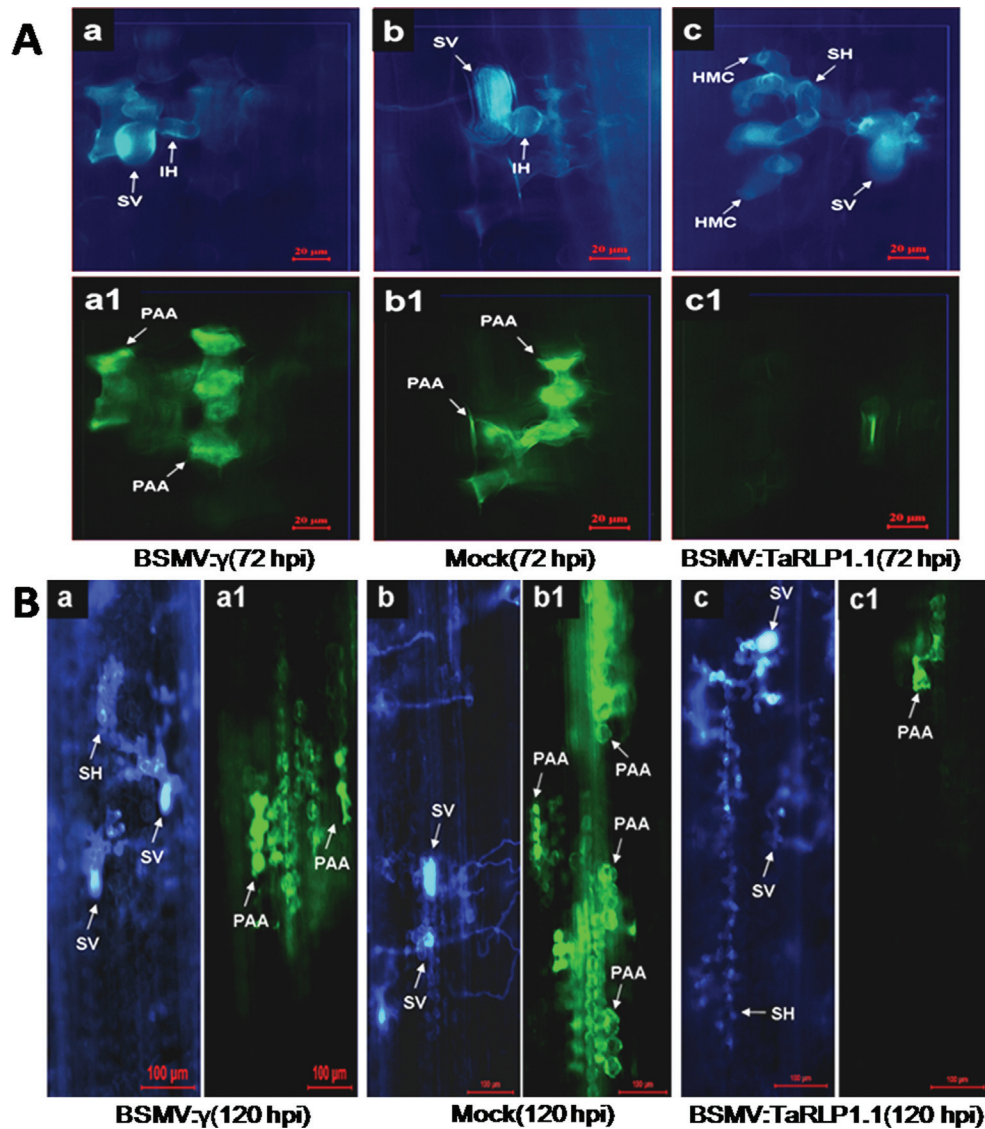


Fig. 4. Histological observation in wheat cv. 92R137 at 72 hpi (A) and 120 hpi (B) after *Pst* CYR32 inoculation in the *BSMV:γ*- (a, a1), mock- (b, b1), and *BSMV:TaRLP1.1as*- (c, c1) pre-infected leaves. The same infection site was observed by white fluorescence microscopy for fungal growth (a, b, c) and epifluorescence microscopy for hypersensitive cell death (a1, b1, c1). (A) The spreading of secondary hyphae was suppressed (a, b), and mesophyll cells at the infection sites showed strong phenolic autofluorogen accumulation (a1, b1). However, secondary hyphae were formed (c) and autofluorescence was not obvious (c1) in the *BSMV:TaRLP1.1as*-infected leaves. Bars=20 μm . (B) Secondary hyphae were longer in the *BSMV:TaRLP1.1as*-infected leaves (c) than in the *BSMV:γ*- (a) and mock- (b) infected leaves. However, the area of autofluorescence was smaller in the *BSMV:TaRLP1.1as*-infected leaves (c1) than in the *BSMV:γ*- (a1) and mock- (b1) infected leaves. Bars=100 μm . SV, substomatal vesicle; IH, infection hypha; HMC, haustorial mother cell; SH, secondary hyphae; PAA, phenolic autofluorogen accumulation.

To determine whether the HR contributed to increased resistance in the *TaRLP1.1* transgenic plants, the proportion of infection sites producing phenolic autofluorogen accumulation, the autofluorescence area, and the hyphal length at the infection site were investigated by histological observations at 120 h after CYR32 inoculation. A higher percentage of infection sites producing phenolic autofluorogen accumulation (73.8%), a larger area of autofluorescence (40 798.8 μm^2), and shorter hyphae (147.6 μm) at infection sites were observed in transgenic plants compared with corresponding values of 59.5%, 11 754.2 μm^2 , and 348.2 μm in Yangmai158 (Fig. 6A, B). It is proposed that the increased resistance of transgenic plants was closely related to the HR.

In addition, *TaPR1*, *TaPR2*, and *TaPR5* were induced in transgenic wheat leaves challenged by CYR32 at 72 and 120 hpi (Supplementary Fig. S11 at JXB online). These results suggest that *TaRLP1.1* played a positive role in plant defence reactions and enhanced resistance to the *Pst* fungus.

Discussion

Plants are exposed to diverse pathogen infection, and various responses are activated to deal with such attacks. Among the complex defence networks, the signal pathways mediated by *RLP* genes have recently been found

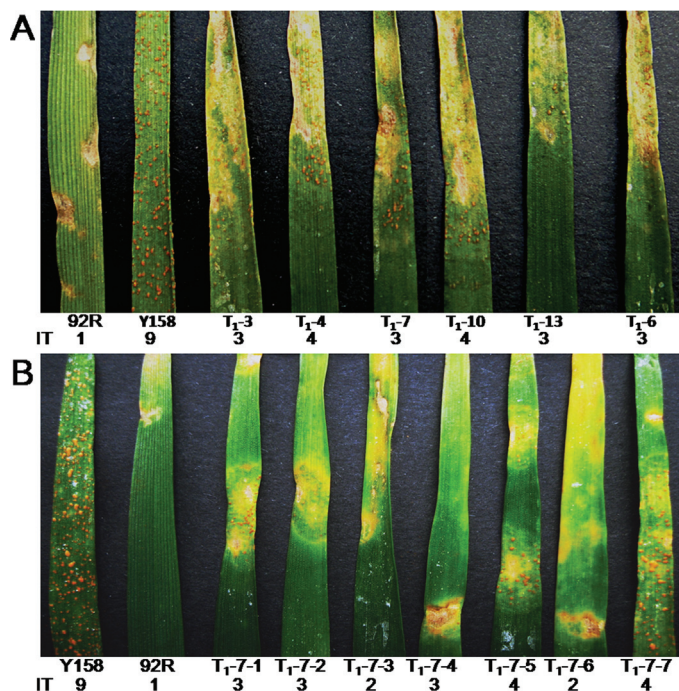


Fig. 5. Stripe rust resistance evaluation of *TaRLP1.1*-transformed plants. (A) T_1 transgenic plants from different T_0 plants showed a varied level of resistance to *Pst* CYR32. (B) T_1 transgenic plants from the T_0 -7 showed a high level of resistance to *Pst* CYR32. IT, infection type; 92R, cv. 92R137; Y158, cv. Yangmai158.

to be required in the response to pathogen attacks in *Arabidopsis* and tomato. However, no *RLP* genes have been studied in monocots. Wheat is one of the world's most important crops and is adversely affected by *Pst*. In this study, a *TaRLP1.1* gene was isolated from a stripe rust-resistant variety. The putative protein structure of *TaRLP1.1* indicated that *TaRLP1.1* was a typical *RLP*, and strong evidence suggested that *TaRLP1.1*, a member of the *RLP* gene family, was involved in the defence response to stripe rust.

The role of TaRLP1.1 in stripe rust resistance of wheat

TaRLP1.1 was induced in all four tested and genetically diverse *Yr26*-containing resistant wheat varieties after *Pst* inoculation, but not in susceptible cv. Yangmai158. Furthermore, a gene silencing experiment indicated that *TaRLP1.1* was required for the full resistance to *Pst*, while the complementary experiment showed that overexpression of *TaRLP1.1* could dramatically improve resistance. Histological analysis implied that it had important roles in HR regulation and activation of *PR* genes. The above results indicate that *TaRLP1.1* was involved in the stripe rust resistance.

SA, JA, and ET are signalling molecules to regulate complex defence responses by inducing *PR* genes, and the SA signal pathway is generally correlated with biotrophic pathogen defences and HR (Spoel *et al.*, 2008). In the present study, strong HR could be induced, and the *PR* genes (*PR1*, *PR2*,

and *PR5*—the marker genes of SA) were up-regulated in the *Yr26*-containing wheat after the biotrophic *Pst* infection; thus the SA pathway probably participated in defence. *TaRLP1.1* expression was dominantly induced by exogenous SA; meanwhile, the extent of HR and the expression of *PR1*, *PR2*, and *PR5* changed along with the expression abundance of *TaRLP1.1*. This suggests that *TaRLP1.1* participated in the SA-mediated signalling pathway in the wheat–stripe rust interactions.

The *Cf* type *RLP* genes were characterized as disease resistance genes (Jones *et al.*, 1994; Dixon *et al.*, 1996, 1998). In the present study, the silencing of *TaRLP1.1* resulted in decreased resistance of cv. 92R137; however, the level of susceptibility was not as serious as that of the susceptible cv. Yangmai158. Conversely, overexpression of *TaRLP1.1* increased the resistance of cv. Yangmai158, but not to a level as high as that of cv. 92R137. Thus, gene expression analysis and functional evaluation indicated that *TaRLP1.1* was a potential downstream factor involved in the *Yr26*-mediated signal pathway. In addition to the signal pathway containing *TaRLP1.1*, there are probably other branches of signal pathways contributing to full resistance to *Pst* mediated by *Yr26*.

CYR32 is the most frequently identified isolate of *Pst*, accounting for a frequency of 29.6% throughout China. More importantly, with broad virulence and high fitness, CYR32 will probably cause widespread epidemics of stripe rust in the near future (Kang *et al.*, 2010). The identification of *TaRLP1.1* as a positive regulator of the HR and an enhancer of resistance to *Pst* CYR32 may provide molecular targets for the development of varieties resistant to stripe rust. The spectrum of resistance will be determined in the higher generations of *TaRLP1.1* transgenic plants using more *Pst* isolates.

Protein–protein interaction cascades are crucial for signalling pathways. Like other resistance proteins, the LRR domain of *RLPs* is widely involved in disease resistance by binding to pathogen elicitors or other proteins in the plants to activate downstream signals (Krujij *et al.*, 2005). The 'GxxxG' conserved motif in the transmembrane regions of *RLP* genes is likely to be involved in protein–protein interactions, as found in the study of the *Cf-RLP* genes, and this protein interaction plays a critical role in *RLP* signal transduction from the extracellular to the cytoplasmic space (Stulemeijer *et al.*, 2007). The binding protein and the mechanism of the resistance mediated by *TaRLP1.1* remain to be elucidated. Thus, transgenic plants of *TaRLP1.1* fused with a Myc-tag in the C-terminus are being produced to facilitate the search for the interacting proteins of *TaRLP1.1*.

The evolution of TaRLP1 genes in wheat

The disease resistance genes are usually distributed on chromosomes as clusters, and this has been widely shown concerning the *NBS-LRR* and some *RLK* resistance genes. For *RLP* resistance genes, a similar manner of gene organization was identified in several cases; for example, the *Cf-2* gene cluster in *Lycopersicon* (now *Solanum*) *pimpinellifolium* included three *Hcr2* genes (Dixon *et al.*, 1996), and the *Cf-5* gene cluster in *Lycopersicon esculentum* (now *Solanum lycopersicum*) var.

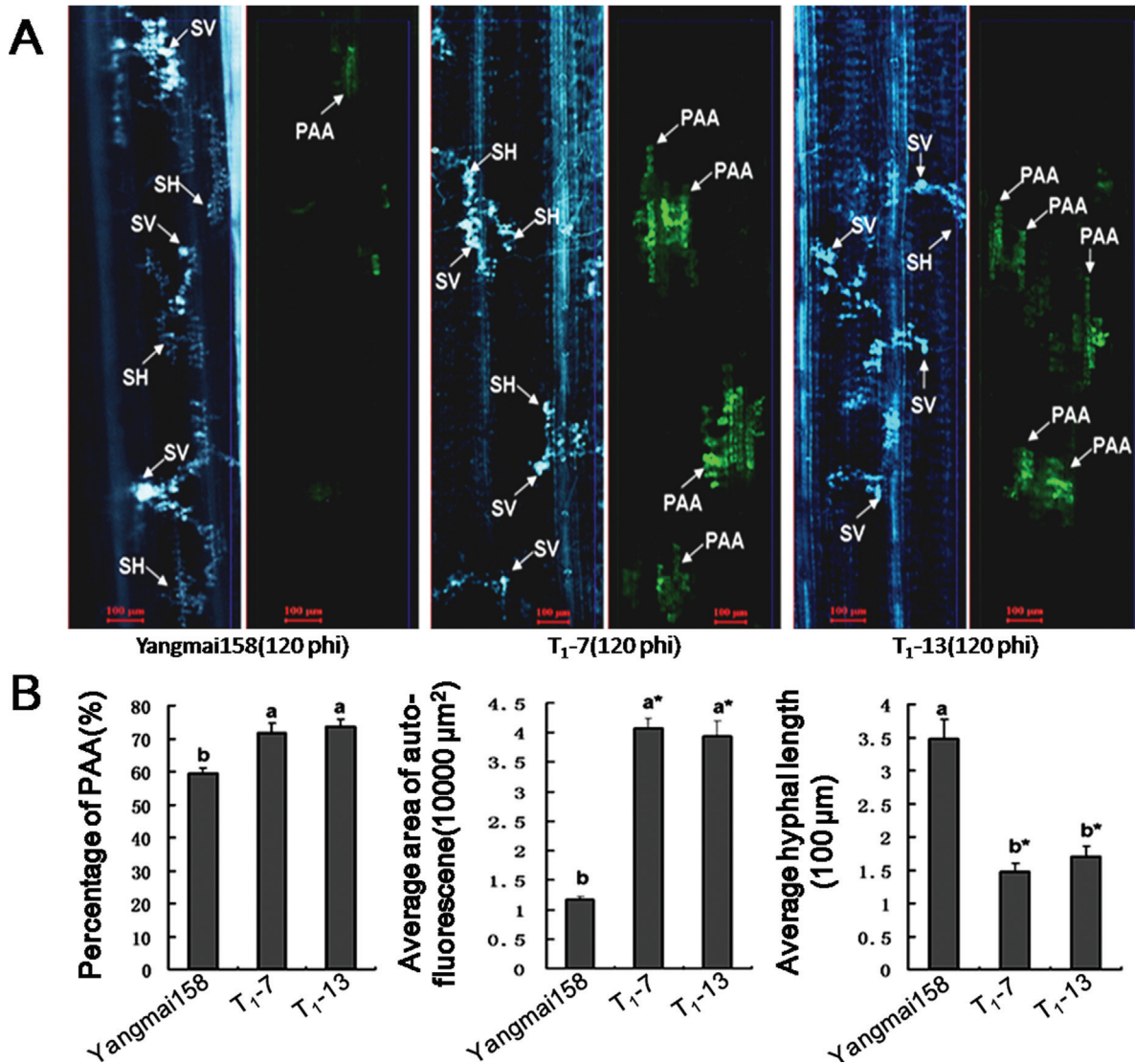


Fig. 6. Histological observation of the hyphal development and phenolic autofluorescence accumulation at the infection sites between *Pst* CYR32 and *TaRLP1.1*-overexpressing transgenic plants at 120 hpi using the susceptible cv. Yangmai158 as the control. (A) In cv. Yangmai158, the hyphae elongated normally, and there was almost no autofluorescence at the infection site. However, in transgenic plants, the fungal growth was slower, and autofluorescence was obvious at the infection sites. The same infection site was observed by white fluorescence or epifluorescence microscopy. Bars=100 μm. SV, substomatal vesicle; SH, secondary hyphae; PAA, phenolic autofluorescence accumulation. (B) Quantitative comparisons of the percentage of infection sites producing phenolic autofluorescence accumulation, the average area of autofluorescence, and average linear length of hyphae emerging on the leaves of infection sites. Means (\pm SE) were calculated using the measurements from six seedlings, and at least 30 infection sites for each seedling. Significance was determined according to the paired sample *t*-test method (b* indicates $P < 0.001$ and b indicates $P < 0.05$).

cerasiforme included four members (Dixon *et al.*, 1998). In *Arabidopsis*, 57 *RLP* genes were distributed on 33 loci, and in rice 90 *RLP* genes were distributed on 38 loci (Wang *et al.*, 2008). In the present study, 10 members of *TaRLP1* in resistant cv. 92R137 and eight members in susceptible cv. Yangmai158 were identified, and it was proposed that the *TaRLP1* family clustered on the homoeologous group 3 chromosomes.

To adapt to the quick evolution of the pathogen, disease resistance genes often undergo dramatic evolution, such as duplication, deletion/insertion, and recombination, to produce new members to recognize the new pathogens. The gene

structure and function analysis of *Cf* genes showed that gene duplication and recombination played an important role in the evolution of *RLP* genes. In some cases, the duplicated gene preserved the function of the original genes (Dixon *et al.*, 1996). In *L. pimpinellifolium*, *Hcr9* is the product of recombination between *Hcr9-9D* and *Cf-9* (Kruijt *et al.*, 2004). In the *TaRLP1* family, there were identical members shared by the two materials; however, there were also some members specific to the individual materials. Gene duplication, deletion/insertion, and recombination were identified in the gene evolution of the *TaRLP1* family.

Gene clusters are considered to be the outcome of co-evolution of host and pathogen, and different members of a gene cluster may be aimed at different virulent races (Bhullar *et al.*, 2009). In a gene cluster, some members confer resistance to available pathogen races, while others have no identified resistance function and represent a reservoir to deal with new emerging races or producing new genes (Kruijt *et al.*, 2004). For example, among the four members in the *Cf-5* family of cherry tomato, *Hcr2-5C* is the only determinant of Avr5 recognition (Dixon *et al.*, 1998). In this study, a number of *TaRLP1* members were identified in wheat, but only two and three were expressed in cv. Yangmai158 and 92R137 under the tested conditions, respectively. It will be interesting to study the expression pattern of the other *TaRLP1* genes, and their response to stimuli.

In conclusion, strong evidence in the present study suggested that *TaRLP1.1* plays an important role in the stripe rust defence response in the monocot wheat. Furthermore, the rapid evolution of the *TaRLP1* family was revealed. This work supplied a good gene resource for genetic engineering in wheat breeding.

Supplementary data

Supplementary data are available at *JXB* online.

Figure S1. The primary structure and conserved domains of TaRLP1.1.

Figure S2. Chromosomal location of *TaRLP1.1*, *TaRLP1804-1*, and *TaRLP1813* with specific primers using the nullitetrasonic (NT) lines derived from cv. Chinese Spring.

Figure S3. Phylogenetic analysis of the DNA sequence of *TaRLP1* genes from common wheat using MEGA 4.0.

Figure S4. The multisequence alignment of the identified RLP genes from the stripe rust-resistant wheat cv. 92R137 and susceptible cv. Yangmai158.

Figure S5. The multisequence alignment of the deduced proteins of the identified RLP genes from the stripe rust-resistant wheat cv. 92R137 and susceptible cv. Yangmai158.

Figure S6. Sequence alignment and phylogenetic analysis of TaRLP1.1 and other putative RLP proteins using DNAMAN.

Figure S7. Subcellular localization of TaRLP1.1 protein.

Figure S8. Expression analysis by semi-quantitative RT-PCR of three pathogenesis-related genes, *TaPR1*, *TaPR2*, and *TaPR5*, at both 72 and 120 h after CYR32 inoculation in the *BSMV:TaRLP1.1as*-infected plants and the BSMV-infected control plants.

Figure S9. Identification of positive transgenic plants by GUS staining, gene detection, and gene expression analysis.

Figure S10. Positive *TaRLP1.1* transgenic plants showed robust hypersensitive response to *Pst* 15 d after inoculation.

Figure S11. Expression analysis by semi-quantitative RT-PCR of three pathogenesis-related genes *TaPR1*, *TaPR2*, and *TaPR5*, at both 72 h and 120 h after CYR32 inoculation in *TaRLP1.1*-overexpressing transgenic plants, the *Yr26*-containing cv. 92R137 and the susceptible recipient Yangmai158.

Table S1. Designed primers.

Table S2. The fold up-regulation of Ta.22666.2.S1_at and TaAffx.55699.1.S1_at between the different samples of tests and the controls.

Table S3. The genes identified in resistant cv. 92R137 and susceptible cv. Yangmai158.

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