Two members of TaRLK family confer powdery mildew resistance in common wheat

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Abstract

Background: Powdery mildew, caused by Blumeria graminearum f.sp. tritici (Bgt), is one of the most severe fungal diseases of wheat. The exploration and utilization of new gene resources is the most effective approach for the powdery mildew control.

Results: We report the cloning and functional analysis of two wheat LRR-RLKs from T. aestivum c.v. Prins-T. timopheevii introgression line IGV1-465, named TaRLK1 and TaRLK2, which play positive roles in regulating powdery mildew resistance in wheat. The two LRR-RLKs contain an ORF of 3,045 nucleotides, encoding a peptide of 1014 amino acids, with seven amino acids difference. Their predicted proteins possess a signal peptide, several LRRs, a trans-membrane domain, and a Ser/Thr protein kinase domain. In response to Bgt infection, the TaRLK1/2 expression is up-regulated in a developmental-stage-dependent manner. Single-cell transient over-expression and gene-silencing assays indicate that both genes positively regulate the resistance to mixed Bgt inoculums. Transgenic lines over-expressing TaRLK1 or TaRLK2 in a moderate powdery mildew susceptible wheat variety Yangmai 158 led to significantly enhanced powdery mildew resistance. Exogenous applied salicylic acid (SA) or hydrogen peroxide (H2O2) induced the expression of both genes, and H2O2 had a higher accumulation at the Bgt penetration sites in RLK over-expression transgenic plants, suggesting a possible involvement of SA and altered ROS homeostasis in the defense response to Bgt infection. The two LRR-RLKs are located in the long arm of wheat chromosome 2B, in which the powdery mildew resistance gene Pm6 is located, but in different regions.

Conclusions: Two members of TaRLK family were cloned from IGV1-465. TaRLK1 and TaRLK2 contribute to powdery mildew resistance of wheat, providing new resistance gene resources for wheat breeding.

Keywords: Triticum aestivum L, Powdery mildew, Receptor-like kinase, Transgenic wheat

Background

Upon the detection of pathogen, plants activate innate immune system to defend pathogen attack. Receptor-like kinase (RLK) membrane proteins serve as pattern recognition receptors (PRRs) and play essential roles in detecting pathogen-associated molecular patterns (PAMPs). They initiate basal and broad-spectrum defense, known as pattern triggered immunity (PTI). RLKs have been identified in many plant species and have been implicated in regulating the processes of plant growth, development, and responses to biotic and/or abiotic stresses. Most of the RLKs identified as being involved in plant defense are of the LRR-RLK class including the rice Xa21 protein and the Arabidopsis Flagellin Sensitive 2 (FLS2) and bacterial translation elongation factor EF-Tu receptor (EFR). Recent identification in rice of a lysine-motif (LysM) receptor kinase involved in the recognition of the fungal elicitor chitin [1] and a lectin receptor kinase (LecRK) involved disease resistance indicates that other RLK classes may also play...
important or overlapping roles in plant defense and pathogen recognition [2]. FLS2 and EFR act as PRRs to detect PAMPs and trigger immune responses in A. thaliana. The chitin elicitor receptor kinase 1 of Arabidopsis (AtCERK1) directly binds chitin through its lysine motif (LysM)-containing ectodomain to activate immune responses [3]. The rice gene Xa21, which codes for an LRR-RLK with 23 extracellular LRR repeats of 24 amino acids each and an intracellular serine/threonine kinase domain, confers race-specific resistance to Xoo (Xanthomonas oryzae pv oryzae) [4]. Xa21 is developmentally controlled: juvenile rice plants challenged with Xoo are less resistant than older plants [5]. Xa3/Xa26 also encodes an LRR-RLK, but does not appear to be developmentally regulated, as both juvenile and adult plants exhibit resistance against Xoo [6]. The tomato resistance gene Cf, which encodes a protein containing extracellular LRR domains but lack the cytoplasmic protein kinase domain, also confer a race-specific resistance to Cladosporium fulvum [7]. Up to now, only a few RLKs have been functionally identified, which is even more so in common wheat (Triticum aestivum L.).

Wheat powdery mildew, caused by a biotrophic fungus Blumeria graminis f.sp. tritici (Bgt) is one of the most serious diseases of common wheat. Breeding and utilizing wheat varieties with powdery mildew resistance are widely-accepted strategies for the disease control. To date, among the 45 identified powdery mildew resistance genes [8], only Pm3 together with its multiple alleles, Pm38, Pm8 and a key member of Pm21 have been cloned [9, 10]. Pm3 is located on the short arm of wheat chromosome 1A [11] and 15 functional alleles have been identified at this locus (Pm3a to Pm3g, Pm3k to Pm3r). Further results reveal that the Pm3 alleles confer race-specific resistance to different subsets of Bgt races [9, 12, 13]. Recent research show that the rye Pm8 is an ortholog of Pm3, which suppresses the Pm8-mediated powdery mildew resistance in lines containing Pm8 [14]. The Pm21 gene is located on the chromosome 6VS of Haynaldia villosa, which is a diploid wheat relative. Cao et al. reported that a key member of Pm21 encoding a putative serine and threonine protein kinase conferred broad-spectrum resistance to powdery mildew in wheat [9, 12, 13].

A series of Triticum aestivum c.v. Prins- Triticum timopheevii (2n = 4x = 28, genome AAGG) introgression lines with different introgressed 2G chromosome fragment sizes have been developed and characterized [15–18]. The powdery mildew resistance gene Pm6, whose effects depend on developmental stages, is located on the long arm of chromosome 2G. The developed introgression lines all show powdery mildew resistance, especially at their adult stages, and have been widely used in wheat breeding programs. However, the genetic mechanism for powdery mildew resistance of these introgression lines is still not clear. In the present study, two members from the LRR-RLK cluster were cloned from T. aestivum c.v. Prins- T. timopheevii introgression line IGV1-465. The two genes both exhibit a developmentally dependent expression manner in response to Bgt infection. Their transient and stable transformation improved the powdery mildew resistance of the susceptible wheat variety Yangmai 158, while knockdown of the genes by transient gene silencing compromised the resistance level of the resistant lines, suggesting that both genes are involved in powdery mildew resistance in wheat.

**Methods**

**Plant materials**

The powdery mildew susceptible Swedish common wheat variety Prins, powdery mildew resistant T. timopheevii, and nine T. aestivum (2n = 42, genome AABBDD)-T. timopheevii introgression lines (IGV1-465, IGV1-448, IGV1-458, IGV1-463, IGV1-464, IGV1-465, IGV1-466, IGV1-468, and IGV1-474) were kindly provided by Dr. J. Mackey, Swedish Agricultural University, Uppsala, Sweden. The sizes of introgressed 2G fragments in the above nine introgression lines have been characterized using molecular markers [17]. Three “Chinese Spring” (CS) nulli-tetrasomic lines for homoeologous group 2 were introduced from Wheat Genetics and Genomics Resources Center (WGGRC), Kansas State University, USA, and used to determine the chromosome location of TaRLK1/2 genes. IGV1-465 and Prins were used for gene cloning of TaRLK1/2 and its homologs, and for single-cell transient over-expression and gene-silencing, respectively. Common wheat variety Yangmai 158, which is moderately susceptible to powdery mildew, was used as the receptor of genetic transformation. A highly powdery mildew susceptible wheat variety, Sumai 3, was used for the production of fresh Bgt inoculums.

**Bgt isolates and inoculation**

The naturally occurring Bgt population was collected from the field in Nanjing (Lat 31°14’ N to 32°37’ N, Lng 118°22’ E to119°14’ E), Jiangsu province, China, which was permitted for research. The inoculums were increased on Sumai 3 plants under a spore-proof greenhouse conditions prior to setting up the disease evaluation experiment. Inoculation was accomplished by gently shaking conidia from leaves of infected Sumai 3 plants, which was grown at 20 °C with 16 h daylight and 80 % relative humidity, onto the foliage of the tested lines.

**Rapid Amplified cDNA End (RACE)**

The SMART™ RACE cDNA Amplification Kit (Clontech, America) was used to clone the full-length cDNA of different members of the LRR-RLK gene family from the cDNA mixture of IGV1-465 at 1, 6, 12, 24, 36, 48 and 72 hours after inoculation (hai) according to the manufacturer’s instructions. Primers for 5′-RACE (LRR-RLK-5′ out and LRR-RLK-5′ inner) and 3′-RACE (LRR-RLK-3′ out and LRR-RLK-3′ inner)
are described in Additional file 1: Table S1. Two pairs of primers (LRR-RLK1-QC-F/R and LRR-RLK2-QC-F/R) were designed based on the sequence of the 5′and 3′DNA ends and used to obtain the full length cDNA of TaRLK1 and TaRLK2 (Additional file 1: Table S1). The PCR products were cloned into the pGEM-T Easy Vector (Takara, Japan) and sequenced by BGI (China).

Sequence analysis, domain prediction, and phylogenetic analysis
Signal peptide, transmembrane and kinase domains were predicted using the SMART software (http://smart.embl-heidelberg.de/) [20]. The ATP binding site, Ser/Thr kinase active site and twin arginine translocation (Tat) signal domain were predicted by the ScanProsite software (http://prosite.expasy.org/scanprosite/).

Phylogenetic trees were constructed based on the full length amino acid sequences of TaRLK1, TaRLK2, and other LRR-RLKs. Full length of TaRLK1 and TaRLK2 were used as query sequences to identify their orthologs by the BLASTP in the Phytozome proteome database (http://www.phytozome.net/search.php?show=blast&method=Org_Cpapaya). Phylogenetic analysis was performed using MEGA 4 based on the Neighbor-Joining method and a bootstrap test of 1000 replicates [21].

Marker analysis
Genomic DNA was extracted from young leaves as previously described [22], and the detail sequence information of the primer pairs was listed in Qin et al. [23]. PCR was performed following the procedure of Ji et al. [18] and the PCR products were separated on 8 % non-denaturing polyacrylamide gels (Acr:Bis = 19:1 or 39:1) at room temperature with 1 × TBE buffer and visualized by silver staining [24].

Expression analysis of TaRLK1 and TaRLK2 by quantitative Real Time-PCR (qRT-PCR)
For Bgt treatments, the Prins and IGV1-465 were grown in a chamber for 16/8 h light/dark at 25/20 °C and then inoculated with Bgt spores onto the leaf surface at the second and fourth leaf stages. For chemical treatments, the seedlings of IGV1-465 at the first leaf stage were sprayed with 5 mM salicylic acid (SA), 100 mM methyl jasmonate (MeJ), 100 μM abscisic acid (ABA), or 7 mM hydrogen peroxide (H2O2) in 0.1 % ethanol solution, and with 0.1 % ethanol used as control. For the expression of transgenic lines, the Yangmai 158 and transgenic lines were grown in a chamber for 16/8 h light/dark at 25/20 °C. The leaves of each sample were frozen immediately in liquid nitrogen and stored at −80 °C. Total RNA was extracted using the TRIZOL reagent (Invitrogen, USA), and DNase I was used to remove the DNA before reverse transcription. The reverse transcript reaction was performed using AMV reverse transcriptase (TaKaRa, Japan) according to the manufacturer’s instructions.

qRT-PCR was performed to analyze the expression of TaRLK1/TaRLK2 using SYBR Green I Master Mix (TaKaRa, Japan) in a volume of 25 μL, and the 18S rRNA was used as a reference. The qRT-PCR reaction was performed using the ABI Prism 7000 system (Applied Biosystems, USA). The program used was as follows: 94 °C for 1 min; followed by 40 cycles of 94 °C for 5 s, 65 °C for 15 s and 72 °C for 30 s. The relative transcript level of TaRLK1/TaRLK2 was calculated using the 2−ΔΔCT method [25]. The sequence information of the primers (LRR-RLK-QPCR-F/R and 18S rRNA-F/R) used are listed in Additional file 1: Table S1.

Construction of pBI220:TaRLK and pWMB006:TaRLK vectors
The ORF of TaRLK1 and TaRLK2, amplified by primer pair LRR-RLK-ORF-F and LRR-RLK-ORF-R, were inserted into the expression vector pBI220 under the control of the CaMV35S promoter, respectively. The recombinant vectors pBI220:TaRLK1 and pBI220:TaRLK2 were used for single-cell transient over-expression assay and genetic transformation.

The binary vector pWMB006 (kindly provided by Dr. Xingguo Ye, Institute of Crop Science, Chinese Academy of Agricultural Sciences), which is under the control of the maize ubiquitin promoter Ubi, was used as an intermediate vector for RNAi vector construction [26]. The common 259 bp-fragment to both TaRLK1 and TaRLK2, amplified by primer pair LRR-RLK-RNAi-F and LRR-RLK-RNAi-R (Additional file 1: Table S1), was digested with the restrictive enzymes SpeI and SacI and then inserted into the SpeI-SacI site in sense orientation. The fragment amplified with the same above primer pair was digested by the restrictive enzymes BamHI and KpnI and then ligated into the BamHI-KpnI site in antisense orientation. Accordingly, the hairpin RNAi vector pWMB006:TaRLK was obtained and used for single-cell transient gene-silencing assay. The linker between the two reverse fragments is a 478 bp intron from rice.

Single-cell transient over-expression and gene-silencing assays
The single-cell transient over-expression and gene silencing assays were performed according to Shirasu et al. [27] and Douchkov et al. [28], respectively. In brief, primary leaf segments of seven-day-old wheat seedlings were transformed by tungsten particles coated with a mixture of pAHCC25 [29] containing the GUS gene and the recombinant vectors pBI220:TaRLK1 or pBI220-TaRLK2. The bombarded wheat leaves were transferred to 1 % agar plates supplemented with 85 μM benzinidazole.
and incubated at 18 °C for 6 h before high density inoculation with Bgt spores. When RNAi vectors containing pWMB006:TaRLK were transformed, the bombardment leaf segments were incubated for 72 h before the inoculation with Bgt mixed isolates. The leaves were stained with GUS to observe the epidermal cells and haustorium-containing transformed cells infected by Bgt spores at 48 hai. The haustorium index (number of haustoria in GUS-expressing cells relative to the total number of GUS-expressing cells) was presented as the mean of three or four independent replicated experiments. Each replicate included examination of 50–200 successful GUS-expressing cells upon inoculation with Bgt conidia.

**Genetic transformation**

The moderate susceptible wheat variety Yangmai 158 was used as the receptor to generate the transgenic wheat. The expression plasmid pBI220:TaRLK1 or pBI220:TaRLK2 was co-bombarded with the plasmid pAHC25 into the callus from the immature embryo of Yangmai 158. Gene gun bombardment of embryos, selection, and regeneration were carried out as described by Xing et al. [30].

The genomic DNA of the regenerated T₀ plants was isolated and used for PCR analysis using the primer pair 35S-F and LRR-RLK-R (Additional file 1: Table S1) to identify the positive transgenic plants. Semi qRT-PCR (sqRT-PCR) was performed with 30 cycles (95 °C for 1 min, 56 °C for 40 s, 72 °C for 35 s) to compare the expression of the target genes in the untransformed control and the transgenic wheat. The sequence information of the primers (LRR-RLK-RT-F and LRR-RLK-RT-R) is listed in Additional file 1: Table S1.

**Evaluation of powdery mildew resistance**

Powdery mildew resistance of the transgenic line and Yangmai 158 was evaluated by artificial Bgt inoculation as described below.

For seedling stage resistance, the detached leaf segments of T₀ transgenic plants and the control were maintained on the culture medium (0.5 % agar and 20 mg/l 6-BA) in a petridish, inoculated with Bgt isolates for 5–6 days under pathogen-free environment, and the infection types (ITs) were scored as grades 0 to 4, in which, IT 0–1 were resistant, and IT ≥ 2 were susceptible. The final results were the average of three independent experiments.

For adult stage resistance, at 6–7 days after Bgt inoculation of the T₀ transgenic plants and the control in the greenhouse, their ITs were scored as grades 0 to 8 [31], in which IT 0–3 was resistant, while IT > 3 was susceptible. All the plants were scored twice.

**Histochemical staining using 3, 3′-diaminobenzidine (DAB)**

*In vivo* H₂O₂ production in plants was detected by an endogenous peroxidase-dependent *in situ* histochemical staining procedure using DAB (Bio Basic Inc., Shanghai, China) [32]. The first detached leaves from 7-day-old seedlings after Bgt infection were stained with 1 mg/mL DAB dissolved in NaOH-acidified (pH 3.8) distilled water overnight, then discolored in boiling 95 % ethanol for 10 min, stored in 50 % glycerol. Representative phenotypes were captured with an Olympus microscope (MVX10, Olympus, Japan).

**Measurements of H₂O₂ production and antioxidant enzyme activities**

The H₂O₂ content was measured according to Bellincampi et al. [33]. Briefly, an aliquot of supernatant (500 μL) was added to 500 μL assay reagent (500 μM ferrous ammonium sulfate, 50 mM H₂SO₄, 200 μM xylene orange, 200 mM sorbitol). After 45 min of incubation, the peroxide-mediated oxidation of Fe²⁺ to Fe³⁺ was determined by measuring the absorbance at 560 nm of the Fe³⁺-xylene orange complex.

Super oxide dismutase (SOD) and catalase (CAT) enzyme activities were analyzed following the method described by Zhang et al. [34]. Total SOD activity was measured based on its ability to reduce nitroblue tetrazolium (NBT) by the superoxide anion generated by the riboflavin system under illumination. One unit of SOD (U) was defined as the amount of crude enzyme extract required to inhibit the reduction rate of NBT by 50 %. Determination of guaiacol peroxidases (POD) enzyme activity was carried out by measuring the oxidation of guaiacol extinction coefficient. CAT activity was spectrophotometrically measured by monitoring the consumption of H₂O₂ (extinction coefficient 39.4 mM⁻¹ cm⁻¹) at 240 nm for at least 3 min.

**Statistical analysis**

All data obtained were subjected to ANOVA, and the mean difference was compared by the LSD test at 95 % or 99 % levels of probability. In all figures, the spread of values is shown as error bars representing standard errors of the means.

**Results**

**Cloning and sequence analysis of TaRLK1 and TaRLK2**

Previous study suggested that a cluster of LRR-RLK genes located in the long arm of chromosome 2B of *T. aestivum* - *T. timopheevii* introgression line IGV1-465. In the synthetic regions of rice and *B. distachyon*, the cluster is conservely present and each has five and two LRR-RLK gene members, respectively [23]. Based on sequence of one of the two RLK gene members in *B.
**distachyon** (Bradi5g21860.1), two specific nest primer pairs (Additional file 1: Table S1) were designed and used for 5’ RACE (5’ RACE Inner and 5’ RACE Outer) and 3’ RACE (AUAP and AP) using cDNA of IGV1-465. The 5’- and 3’-end sequences of the LRR-RLK gene were obtained (Additional file 2: Table S2). According to the predicted open reading frame (ORF) of the obtained expressed sequence of a putative LRR-RLK, two primer pairs (LRR-RLK1-QC-F/LRR-RLK1-QC-R and LRR-RLK2-QC-F/LRR-RLK2-QC-R) were further designed and used to clone the full-length cDNA. Two members of the LRR-RLK gene family, namely TaRLK1 (Genbank acc. No.: KC700615) and TaRLK2 (Genbank acc. No.: KC700616) were obtained from IGV1-465, both containing an ORF of 3045 nucleotides encoding a 1014-amino-acid peptide.

Pair wise comparison showed that TaRLK1 and TaRLK2 share 99.31 % identity and are only different in seven amino acids, i.e. V7A, V21A, D23H, P56L, T82S, S433P and L672R (Fig. 1). The SMART software [20] predicted that both LRR-RLKs contain a putative signal peptide domain at the N-terminal region (residues 1-24), a putative extra-cellular domain (residues 103-376) with 11 tandem copies of a 24-amino acid LRR; a putative trans-membrane domain (residues 624-646) and a putative Ser/Thr protein kinase domain at the C-terminal region (residues 684-951) (Fig. 1). Each unit of the LRR domain has the LXXLXXLXXLXXLXXLXXLXXLXXNXXLGXXLPXX consensus, in which X represents any amino acid. Based on the structural properties predicted by the ScanProsite, both LRR-RLKs consist of an ATP binding site (LGGQGGSVYKGLTDGRFVAVK) and a Ser/Thr kinase active site (D) in their protein kinase catalytic domains. As expected, several highly conserved motifs, Val-Ala-Val-Lys (VAVK), His-Arg-Asp (HRD), or Asp-Phe-Gly (DFG) exist in the C-terminal Arg-Asp (RD) kinase domains for both LRR-RLKs [35]. The LRR domain of both LRR-RLKs contains 9 glycosylation sites (N-X-S/T). The N-terminal region of TaRLK2 contains a putative Tat (twin arginine translocation) signal domain, which is absent in the TaRLK1 (Fig. 1).

BLASTP was performed against the Phytozome proteome database for identifying the TaRLK1/2 orthologs based on the similarity of full-length amino acid sequence. TaRLK1/2 orthologs were identified from 31 plant species with available genome information. Phylogenetic analysis revealed that the two TaRLKs and their orthologs from different Gramineae species are in the same branches. Both TaRLK1 and TaRLK2 are highly homologous to Bradi5g21870.2, Os04g52600, Os04g52630.1, Os04g52606.1, Os04g52640.1, Os04g52614.1, GRMZM2G126858_T02, Si009240m, and Sb06g028570.1, indicating LRR-RLK orthologs are highly conserved (Additional file 3: Figure S1, Additional file 4: Table S2). The expression profiles of TaRLK1/TaRLK2 in the resistant line IGV1-465 and susceptible Prins were analyzed by qRT-PCR. In IGV1-465, the expression level of TaRLK1/TaRLK2 at the fourth leaf stage is 14.4 times higher than that at the second leaf stage. Whereas, in Prins, expression levels are similar for all tested time points, and remain at relatively low levels both at the second- and fourth-leaf stages (Fig. 3a). The gene

**Physical localization of TaRLK1/2**

In the scaffold 11030 from chromosome 2D of the released Aegilop tauschii (2n = 14, genome DD) genome sequence [36], a TaRLKs homolog (TaRLK-D) having highest sequence similarity, were identified. A primer pair (NAU-2 F/R) were designed and used for determine the chromosome location of TaRLKs from IGV1-465. NAU-2 F/R amplified three common amplicons (760 bp, 680 bp and 550 bp, respectively for chromosome 2D, 2A and 2B) in Prins and IGV1-465. A 450 bp amplicon was only present in T. timopheevii and four introgression lines IGV1-468, 458, 474, 466 (Fig. 2a). Six molecular markers previously mapped to the long arm of chromosome 2B [23] together with NAU-2 F/R were used for amplification in nine T. aestivum-T. timopheevii introgression lines. Based on the presence and absence of the specific amplicons for chromosome 2G, the TaRLK1/2 could be mapped to the same chromosome region as markers CINAU123 and CINAU124, while not as markers CINAU135 and CINAU141, in which the Pm6 is located (Fig. 2b).

Compared with the released sequence of Triticum urartu (Genome AA) [37], the TaRLK1 is from the 2A, and the TaRLK2 is from 2B. By PCR-based homologous cloning, 51, 19 and 16 full length sequences of TaRLKs were obtained from Bgt resistant IGV1-465 and T. timopheevii as well as the Bgt susceptible wheat variety Prins. Multiple sequence alignment with TaRLK1 and TaRLK2 identified 59 Single Nucleotide Polymorphisms (SNPs) (Table 1). Based on the distribution of the 59 SNPs, the TaRLKs can be classified into four different types, with the TaRLK1 and TiRLK were two original types. The TaRLK2 is presumed to be originated from a recombination between TaRLK1 and TiRLK at the region between 396-459 bp, and TaRLKPrins is presumed to be originated from a recombination between TaRLK1 and TiRLK, but at a different region (between 1054-1125 bp) (Additional file 5: Figure S2).

**The gene expression patterns for TaRLK1/TaRLK2 at two leaf stages and by Bgt infection**

*T. aestivum-T. timopheevii* introgression lines show high powdery mildew resistance only after the fourth leaf stage [38]. The IGV1-465 is susceptible at the first leaf stage but has a higher resistance level starting from the fourth leaf stage (Additional file 6: Figure S3) [23].

The expression profiles of TaRLK1/TaRLK2 in the resistant line IGV1-465 and susceptible Prins were analyzed by qRT-PCR. In IGV1-465, the expression level of TaRLK1/TaRLK2 at the fourth leaf stage is 14.4 times higher than that at the second leaf stage. Whereas, in Prins, expression levels are similar for all tested time points, and remain at relatively low levels both at the second- and fourth-leaf stages (Fig. 3a). The gene
expression patterns of TaRLK1/TaRLK2 in IGV1-465 are consistent with the development-dependent resistance phenotype. Further comparison of the expression of TaRLK1/TaRLK2 in IGV1-465 and Prins upon Bgt inoculation at the second and fourth leaf stages showed that, TaRLK1/TaRLK2 was slightly up-regulated in both genotypes (in Prins at 6 hai: 2.9 folds; in IGV1-465 at 6 hai: 3.0 folds) at the second leaf stage (Fig. 3b). However, their expression levels were significantly up-regulated in IGV1-465 (6.0 folds at 24 hai) than those in Prins (4.0 folds at 24 hai) at the fourth leaf stage when challenged with Bgt infection (Fig. 3c). Above results indicated that the expression of TaRLK1/TaRLK2 was development-dependent.

![Fig. 2](See figure on previous page.)

**Fig. 1** The deduced amino acid sequences of TaRLK1 and TaRLK2 proteins. Red characters represent seven amino acid differences. The open boxed region represents the N-terminal region of TaRLK2 which comprises a putative twin arginine translocation (Tat) signal domain. Characters in the green brackets represent conserved pairs of cysteines spaced by six or seven amino acids. Roman numerals mark the 11 tandem copies of a 24-amino acid LRR. Blue characters represent nine glycosylation sites (N-X-S/T). Green characters represent a putative protein kinase catalytic domain with ATP binding site. Black arrow heads indicate a Ser/Thr kinase active site (D). Characters underlined as red represent the conserved motifs (VAVK, HRD and DFG) in the RD kinases. SP: signal peptide domain; LRR: leucine-rich repeat domain; TM: transmembrane domain; PK: Ser/Thr protein kinase domain.
Table 1 SNPs in the TaRLK genes from powdery mildew resistant IGVI-465, T. timopheevii and powdery mildew susceptible Prins

| Gene Type | SNPs |
|-----------|------|
| TiRLK     | 20   |
| TaRLK1    | 62   |
| TaRLK2    | 67   |
| TaRLK <sub>inv</sub> | 183 |
| TaRLK     | 186  |
| TaRLK     | 213  |
| TaRLK     | 244  |
| TaRLK     | 343  |
| TaRLK     | 312  |
| TaRLK     | 318  |
| TaRLK     | 343  |
| TaRLK     | 396  |
| TaRLK     | 459  |
| TaRLK     | 468  |
| TaRLK     | 472  |
| TaRLK     | 483  |
| TaRLK     | 547  |
| TaRLK     | 612  |
| TaRLK     | 687  |
| TaRLK     | 692  |
| TaRLK     | 723  |
| TaRLK     | 750  |
| TaRLK     | 766  |
| TaRLK     | 779  |
| TaRLK     | 799  |
| TaRLK     | 853  |
| TaRLK     | 882  |
| TaRLK     | 909  |
| TaRLK     | 1054 |
| TaRLK     | 1125 |
| TaRLK     | 1128 |
| TaRLK     | 1137 |
| TaRLK     | 1221 |
| TaRLK     | 1225 |
| TaRLK     | 1248 |
| TaRLK     | 1306 |
| TaRLK     | 1319 |
| TaRLK     | 1362 |
| TaRLK     | 1404 |
| TaRLK     | 1455 |
| TaRLK     | 1593 |
| TaRLK     | 1668 |
| TaRLK     | 1847 |
| TaRLK     | 1887 |
| TaRLK     | 1894 |
| TaRLK     | 1911 |
| TaRLK     | 1917 |
| TaRLK     | 2088 |
| TaRLK     | 2091 |
| TaRLK     | 2142 |
| TaRLK     | 2146 |
| TaRLK     | 2172 |
| TaRLK     | 2220 |
| TaRLK     | 2223 |
| TaRLK     | 2276 |
| TaRLK     | 2487 |
| TaRLK     | 2490 |
| TaRLK     | 2514 |
| TaRLK     | 2517 |
| TaRLK     | 2526 |
| TaRLK     | 2972 |
| TaRLK     | 3022 |
| TaRLK     | 3076 |
| TaRLK     | 3487 |
| TaRLK     | 3490 |
| TaRLK     | 3514 |
| TaRLK     | 3517 |
| TaRLK     | 3526 |

Powdery mildew resistance evaluation of TaRLKs by transient over-expression or silencing assays

A single-cell transient over-expression assay, which has been successfully used to elucidate gene function in resistance against Bgt infection [9, 39], was used to elucidate the above assumption. The epidermal cells expressing reporter gene GUS and undergoing attacks by germinating Bgt spores were selected to observe the haustorium formation and calculate the Haustorium Index (HI). The interaction between the host and Bgt is considered compatible when haustorium and elongating secondary hyphae are formed (Fig. 4a, b). When Bgt fail to penetrate into the cells and no haustorium formed, the interaction is considered incompatible (Fig. 4c). Our analyses revealed that, compared with the 60.41 % HI in Prins transformed with pAHC25 only, transient over-expression of TaRLK1 or TaRLK2 in leaves of Prins by co-transformation of pBII220:TaRLK1 or pBII220:TaRLK2 with pAHC25 significantly decreased the HI to 53.72 % and 37.55 %, respectively.
The HI in the *Bgt* resistant line IGV1-465 transformed with *pAHC25* empty vector was 31.47 % (Fig. 4d). Whereas, when *TaRLKs* were transiently silenced in IGV1-465, the HI was significantly increased to 52.73 % (Fig. 4e). These results indicate that the *TaRLKs* positively regulate the powdery mildew resistance by suppressing *Bgt* haustorium formation.

**The function analysis of *TaRLK1* and *TaRLK2* in resistance to powdery mildew in transgenic lines**

Subsequently, the *pBI220:TaRLK1* and *pBI220:TaRLK2* were each co-transformed with *pAHC25* (having a *Bar* gene encoding phosphinothricin in acetyltransferase for selection) into the callus of Yangmai 158 by Genegun bombardment. After three rounds of herbicide bialaphos selection, a total of 158 and 187 regenerated plants were
obtained from 2,000 and 2,240 immature embryo callus transformed TaRLK1 and TaRLK2, respectively. PCR analysis using the combination primer pair CaMV35S-F and LRR-RLK-R identified 22 and 33 transgene-positive plants, respectively (Fig. 5a). The regeneration frequencies were 7.9 % (158/2,000) and 8.3 % (187/2,240), and the frequencies of transgene-positive plants were 1.1 % (22/2,000) and 1.5 % (33/2,240) for genes TaRLK1 and TaRLK2, respectively. qRT-PCR analysis of part of the selected positive plants verified that the expression levels of TaRLK1 or TaRLK2 were higher in the transgenic plants than those in the receptor Yangmai 158 and the negative regenerated plant TaRLK2-148 (Fig. 5b).

The powdery mildew resistance of transgenic plants at T0 generation against Bgt mixture isolates was evaluated at both seedling and adult stages. At seedling stage, for example, the infection types (ITs) of three transgenic plants over-expressing TaRLK2 (TaRLK2-17, TaRLK2-143, TaRLK2-144) were all grade 0. Whereas, the IT of non-transformed Yangmai 158 was grade 3 (Fig. 5c), indicating that the over-expression of TaRLK1 or TaRLK2 enhanced the powdery mildew resistance of Yangmai 158. Compared to that in Yangmai 158, three resistant transgenic plants of TaRLK2 had fewer germinated conidia, developed hyphae and conidiophores on their leaves (Fig. 5d). At adult stage, all the above six transgenic plants showed improved powdery mildew resistance (Grade 1), compared to Yangmai 158 (Grade 8) (Fig. 5e). These indicate that both TaRLK1 and TaRLK2 confer powdery mildew resistance in wheat, and TaRLK2 gene has superior effect at seedling stage.

**The involvement of SA and ROS homeostasis in the powdery mildew resistance conferred by TaRLK1 and TaRLK2**

Salicylic acid (SA), jasmonate (JA) and hydrogen peroxide (H₂O₂) are signaling molecules that regulate complex

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Fig. 4 The interaction of leaf epidermal cells challenged with Bgt and the statistics of Haustorium Index (HI) after single-cell transient over-expression assay. Representative of compatible (a and b) and incompatible interaction (c) wheat leaf epidermal cells challenged with Bgt, the haustorium index (HI) of Bgt after single-cell transient expression of TaRLK1 and TaRLK2 in the epidermal cells in Prins (d) and after transient induced gene silencing of TaRLK1/2 in IGV1-465 (e) co: conidia; ha: haustorium; hy: hyphae. Scale bars = 50 μm. Different letters indicate the significant difference at 0.05 levels. Data were from three independent replicated experiments.
Fig. 5 (See legend on next page.)
defense responses by inducing pathogenesis related (PR) genes [40]. The induction of ROS potentiates the programmed cell death (PCD). Effective defense against biotrophic pathogens is mainly due to PCD in the host, and to associated activation of defense responses regulated by the SA-dependent pathway [41].

To elucidate the mechanism of powdery mildew resistance conferred by TaRLK1/TaRLK2, the first or second leaves from 2-week-old plants of Prins, IGV1-465, Yangmai 158 and the TaRLK1/TaRLK2 transgenic plants at the T1 generation were DAB-stained to investigate H2O2 accumulation upon Bgt infection. At 0 hai, no oxidative production was observed in the leaves of any tested samples (Fig. 6a). At 24 hai, IGV1-465 exhibited higher ROS level than in Prins. The resistant transgenic lines TaRLK1-62 and TaRLK2-143 also showed more ROS than Yangmai 158 (Fig. 6a). At most of the Bgt interaction sites, no oxidative production was detected in Prins and Yangmai 158 (Fig. 6b). However, in TaRLK1-62, TaRLK2-143, and IGV1-465, massive H2O2 accumulation was observed at either the Bgt interaction sites (Fig. 6c) or in the whole cells (Fig. 6d). Time course tests of endogenous H2O2 levels showed that, at both seedling and adult stages, there was a fast endogenous H2O2 production in the two positive transgenic lines (24 hai), IGV1-465 (12 hai), IGV1-466 (12 hai) and T. timopheevii (12 hai and 6 hai at the second and fourth leaf stages, respectively) upon Bgt infection. No significant change was observed in the susceptible lines (Fig. 6e, f), such as Yangmai 158, Prins and another negative transgenic plant TaRLK2-8 which was an alternative of TaRLK2-148. These suggested that ROS pathway participated in the powdery mildew resistance conferred by TaRLKs.

The activities of enzymes responsible for H2O2 production and ROS scavenging were further tested. In response to Bgt inoculation, the H2O2 producing SOD enzyme activities significantly increased in the powdery mildew resistant materials, although different genotypes showed different patterns. At the second leaf stage, the SOD activities in the transgenic lines of both TaRLKs increased and reached the peak at 24 hai, while at the fourth leaf stage, significant increase advanced to 6 hai. In IGV1-465 and IGV1-466, the SOD activity increase was observed at 12 hai at the second leaf stage; however, no significant change was observed at the fourth leaf stage. In T. timopheevii, a significant increase of SOD activity was only observed at the fourth leaf stage. However, both Yangmai 158 and the negative transgenic line TaRLK2-8 showed significant decrease of SOD activities at 24 hai and 6 hai, respectively (Additional file 7: Figure S4).

In response to Bgt inoculation at two developmental stages, the activities of ROS scavenging enzymes CAT and POD increased significantly in all the resistant materials, but decreased in Yangmai 158 and Prins. In the transgenic lines of both TaRLKs, the increased POD activity occurred at 24 hai at the second leaf stage, and advanced to 12 hai at the fourth leaf stage; the increase of CAT activity occurred at 24 hai and 12 hai at the second leaf stages respectively in TaRLK1 and TaRLK2, but advanced to 6 hai at the fourth leaf stage. In T. timopheevii, the POD activity increase occurred earlier than CAT (Additional file 7: Figure S4). The distinct difference of ROS production and scavenging enzymes in the resistant and susceptible materials at different developmental stages further support the importance of ROS in powdery mildew resistance mediated by the two TaRLKs.

Gene expression patterns for TaRLK1/2 in Prins and IGV1-465 when treated with SA, MeJA, ABA and H2O2 were compared. In IGV1-465, TaRLK1/TaRLK2 was moderately up-regulated by MeJa (2.4 folds at 6 hai, Fig. 7a), ABA (3.1 folds at 0.5 hai, Fig. 7c) and H2O2 (3.2 folds at 2 hai, Fig. 7d), and was dramatically induced by SA (13.4 folds higher, Fig. 7b) at 24 hai. While in Prins, no increased expression was detected, except when treated with H2O2, a delayed induction at 24 hai was observed (Fig. 7d). We also found that the expression of TaPR1, TaPR2 and TaPR3, three marker genes of SA signaling pathway, was constitutively expressed at the seedling stage without Bgt inoculation in the transgenic lines over-expressing TaRLKs. The expression of CAT, scavenger of ROS, was up-expressed in TaRLK1/TaRLK2 transgenic lines without Bgt inoculation (Additional file 8: Figure S5). These indicate multiple signal pathways may influence the resistance conferred by TaRLK1/TaRLK2 in wheat.

Discussion

Plant LRR-RLKs, for example, Arabidopsis FLS2 and rice Xa21, were found to be involved in plant immune responses to the microbe attacks [4, 42]. Both TaRLK1 and TaRLK2 contain signal peptide, LRR, TM, S/TPK domain, and several N-glycosylation sites, which have also been identified in the LRR-containing ectodomain of cell-surface receptors that can recognize MAMPs to activate PTI.
Fig. 6 (See legend on next page.)
response in both animals and plants [43–45]. Arabidopsis defense-associated LRR-RLKs, such as brassinosteroid insensitive 1-associated receptor kinase [46], Flg22-induced receptor-like kinase 1 (FRK1) [47], and the PEPtide 1 and PEPtide 2 Receptors [48], are all RD kinases. Similar to Arabidopsis RKF1, which is responsible for triggering a cascade of intracellular events during defense responses [49], TaRLK1 and TaRLK2 containing the N-glycosylation sites and RD motif belong to the VIII-2 subfamily due to their high homology to Bradi5g21870.2, which is a member of the VIII-2 subfamily in Brachypodium (http://www.brachypodium.org/).

Both genes contributed to powdery mildew resistance, but showed distinct resistance level. The transgenic plants over-expressing TaRLK1 were moderately resistant at seedling stage, and highly resistant at adult stage, while transgenic plants for TaRLK2 exhibited high resistance at both stages, implying that both TaRLKs contributed to the defense response to Bgt infection, and TaRLK2 had larger effects than TaRLK1. The Tat domain of LRR-RLKs was reported to function in transporting folded proteins across energy-transducing membranes [50]. Therefore, we assume that differential resistance may be due to their sequence differentiation at the Tat signal domain and spacer region of same gene family, which driven by co-evolution with the pathogen. The two genes had different resistance spectrums, so their different resistance levels to Bgt mixture may also be due to the composition of the Bgt isolates.

Many disease resistance genes belong to multi-gene families, indicating that gene duplication and subsequent diversification are common for gene evolution in plant [51, 52]. Recombination at the disease resistance loci, such as the disease resistance gene Rp1 in maize and wheat stem rust resistance gene Sr33 has been proven to be associated with the creation of novel resistance phenotypes [53, 54] and contribute to the diversification of plant gene families. Our results suggest a model for the evolution of the TaRLK gene family. Duplication and subsequent divergence of a progenitor TaRLK gene led to the emergence of TaRLK multi-gene families. The TaRLK2 and TaRLKPrins were originated from the types of sequence recombination between TaRLK1 and TiRLK1. The association of the gene structure with their function diversification will be further elucidated.

Fig. 6 H2O2 accumulation in the leaves and endogenous H2O2 contents in different materials. Microscope observation of H2O2 accumulation in wheat leaves of Prins, Yangmai 158, IGV1-465 and the transgenic lines at 0 and 24 hai of Bgt (a) and endogenous H2O2 contents of wheat at second (e) and fourth (f) leaf stages after Bgt treatments. b was the representative image at Bgt interaction sites in susceptible genotypes. c and d were the representative images at Bgt interaction sites in resistant genotypes. H2O2 was detected at Bgt interaction sites in leave epidermal cells of resistant IGV1-465 and transgenic lines (c and d), while not obvious in those of susceptible Prins and Yangmai 158 (b). ** indicates significant differences across different time point within each genotype at 0.05 levels, using non-inoculated sample as control. h: hours after Bgt inoculation. Results are replicated in three independent experiments of similar result.

Fig. 7 Gene expression patterns for TaRLK1/2 in response to the treatments of exogenous phytohormones MeJA (a), SA (b), ABA (c) and signal molecule H2O2 (d) in Prins and IGV1-465. ** indicates significant differences across different time point within each genotype at 0.05 levels, using non-inoculated sample as control. h: hours after treatment. N/A: data missing.
Pm6 was introgressed from T. timopheevii into common wheat [15]. Pm6 mediated powdery mildew resistance showed a developmentally-dependent, and has been widely used in wheat breeding programs. IGV1-465 show high resistance to the local Bgt population, starting from the fourth leaf stage [23]. In this paper, we observed that the expression of TaRLK1/TaRLK2 in IGV1-465 at the fourth leaf stage was higher than that at the second leaf stage (Fig. 3a). Over-expression of TaRLK1 in Yangmai 158 via stable transformation resulted in moderately higher powdery mildew resistance at both seedling and adult stages, and over-expression of TaRLK2 in Yangmai 158 significantly enhanced resistance at both stages (Fig. 5c, d, and e). According to these findings, we infer that both TaRLKs were regulated at different stages. Pm6 was previously mapped on chromosome 2BL with the fraction length (FL) 0.50-1.00 [16–18], flanked by two STS markers, NAU/STSBCD135-1 and NAU/STSBCD135-2, with a genetic distance of 0.8 cM [18]. In the present study the TaRLK1/TaRLK2 was mapped to homologous group 2 chromosomes of T. aestivum (Fig. 2a). However, we found that the specific amplicon for TaRLK1/TaRLK2 from the GG genome was only present in IGV1-466 and not in IGV1-465 (Fig. 2a). IGV1-465 has the smallest introgression fragment of 2G, while IGV1-466 has the largest [17]. The absence of the 2G specific amplicon (450 bp in Fig. 2a) reveal that TaRLK1/TaRLK2 were not located in the 2G chromosome introgression fragment in IGV1-465. However, they have clear function in powdery mildew resistance in wheat, and we speculated that TaRLKs are new powdery mildew resistance genes neighboring with the Pm6.

We observed that when challenged with Bgt, plants over-expressing TaRLK1/2 showed enhanced H₂O₂ accumulation at as early as 12 hai (Fig. 6a). The SOD, POD and CAT activities increased significantly in resistant lines (IGV1-465 and IGV1-466), as well as TaRLK1/2 over-expressing plants, mostly at 24 hai upon Bgt inoculation (Additional file 7: Figure S4), suggesting that the increased H₂O₂ production could in turn trigger the activity of ROS-scavenging enzymes, to maintain the appropriate levels of endogenous ROS. ROS, superoxide, hydrogen peroxide and nitric oxide, are produced at all levels in resistance reactions in plants. In basal resistance, they are linked to papilla formation and the assembly of barriers. In the R gene mediated defense, they may be linked to PCD, and resulted in systemic acquired resistance (SAR). They interact with SA in signaling, which is the typical pathway against a biotroph pathogen in Arabidopsis [41]. At least two distinct enzymes, POD and CAT, contribute to the removal of ROS [55, 56]. The HR is accompanied by a localized increase in the accumulation of ROS and is further characterized by rapid PCD at sites of infection [57]. Our data affirmed the association of TaRLK1/2 mediated powdery mildew resistance with the ROS homeostasis, which has been proven to be responsible for triggering defense response in plants [58].

The expression of TaRLK1/TaRLK2 was up-regulated significantly by SA. Moreover, the marker genes of the SA signaling pathway, TaPR1 and TaPR2, were all constitutively up-regulated at the seedling stage without Bgt inoculation in the positive T₀ transgenic plants over-expressing TaRLK1 or TaRLK2 (Additional file 8: Figure S5), indicating that both genes were involved in the SA-mediated defense pathway against Bgt. This implies the predominant role of the SA pathway in the TaRLK1 or TaRLK2 mediated powdery mildew resistance. The marker gene of ETH pathway, PR3, was also significantly up-regulated in the transgenic plants, indicating the possible involvement of ETH signaling pathway in the TaRLK1 or TaRLK2 mediated powdery mildew resistance.

Conclusions
In conclusion, we cloned two members of TaRLK family, named TaRLK1 and TaRLK2, from T. aestivum c.v. Prins-T. timopheevii introgression line IGV1-465. The two genes are present as a gene cluster on the long arm of chromosome 2B, and both TaRLK1 and TaRLK2 confer powdery mildew resistance, which was proved by single-cell transient over-expression, gene-silencing assays and stable genetic transformation. SA and altered ROS homeostasis are involved in defense responses of the transgenic wheat to Bgt infection.

Availability of supporting data
The data sets supporting the results of this article are included within the article and its additional files.

Additional files

Additional file 1: Table S1. Sequence information of primer pairs used in this study. (DOC 48 kb)

Additional file 2: Table S2. Biochemical characteristics of TaRLK1 and TaRLK2. (DOC 27 kb)

Additional file 3: Figure S1. Phylogenetic analysis of TaRLK1 and TaRLK2 with other LRR-RLKs. (DOC 27 kb)

Additional file 4: Figure S2. Biochemical characteristics of TaRLK1 and TaRLK2 with other LRR-RLKs. (DOC 27 kb)

Additional file 5: Figure S3. Biochemical characteristics of TaRLK1 and TaRLK2 with other LRR-RLKs. (DOC 27 kb)

Additional file 6: Figure S4. Biochemical characteristics of TaRLK1 and TaRLK2 with other LRR-RLKs. (DOC 27 kb)

Additional file 7: Figure S4. Biochemical characteristics of TaRLK1 and TaRLK2 with other LRR-RLKs. (DOC 27 kb)

Additional file 8: Figure S5. Biochemical characteristics of TaRLK1 and TaRLK2 with other LRR-RLKs. (DOC 27 kb)
We are grateful to Prof. Xingguo Ye for providing RNAi vector for the data. SZZ, WC and RQZ conducted the field works. TTC, HYW, Jin Xiao, XEW, TTC and BQ designed the experiments. TTC, Jun Xu, WTW and CD.

Competing interests
The authors declare that they have no competing interests.

Authors’ contributions
XEW, TTC and BQ designed the experiments. TTC, Jun Xu, WTW and CD performed the experiments. TTC, Jin Xiao, A2C, LPX, XQG and WBS analyzed the data. SZZ, WC and RO2 conducted the field works. TTC, HYW, Jin Xiao and XEW wrote the paper. All authors have read, edited and approved the current version of the manuscript.

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Abbreviations
Bgr: Bluniera graveanaum f.sp. tritic; ROS: reactive oxygen species; SA: salicylic acid; H2O2: hydrogen peroxide; JA: jasmonate acid; ABA: abscisic acid; PCD: programmed cell death; FRK1: Flg22-induced receptor-like kinase 1 of Arabidopsis; PTI: pattern triggered immunity; FLS2: Flagellin 22; associated molecular patterns; PRRs: pattern recognition receptors; PAMPs: pathogen-associated molecular patterns; PTI: pattern triggered immunity; FL25 Flagellin Sensitive 2; EPR-Tu receptor; Lys: lysine; motif; LeRCL- receptor kinase; ASCSK1: chitin elicitor receptor kinase 1 of Arabidopsis; Cs: Chinese Spring; WGGC: Wheat Genetics and Genomics Resources Center; Ta-bn: barley nitrate translocation; TIt: infection types; DAB: diaminobenzidine; SOD: super oxide dismutase; CAT: catalase; NBT: nitroblue tetrazolium; POD: guaiacol peroxidase; SNPs: Single Nucleotide Polymorphisms; HI: Haustorium Index; PCR: programmed cell death; FRK1: Flg22-induced receptor-like kinase 1.

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