Cloning of a Conserved Receptor-Like Protein Kinase Gene and Its Use as a Functional Marker for Homoeologous Group-2 Chromosomes of the *Triticeae* Species

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Abstract

Receptor-like kinases (RLKs) play broad biological roles in plants. We report on a conserved receptor-like protein kinase (RPK) gene from wheat and other *Triticeae* species. The TaRPK1 was isolated from the *Triticum aestivum* cv. Prins - *Triticum timopheevii* introgression line IGVI-465 carrying the powdery mildew resistance gene Pm6. The TaRPK1 was mapped to homoeologous chromosomes 2A (TaRPK1-2A), 2D (TaRPK1-2D) and the Pm6-carrier chromosome 2G (TaRPK1-2G) of IGVI-465. Under the tested conditions, only the TaRPK1-2G allele was actively transcribed, producing two distinct transcripts via alternative splicing. The predicted 424-amino acid protein of TaRPK1-2G contained a signal peptide, a transmembrane domain and an intracellular serine/threonine kinase domain, but lacked a typical extracellular domain. The expression of TaRPK1-2G gene was up-regulated upon the infection by *Blumeria graminis* f.sp. *triticum* (*Bgt*) and treatment with methyl jasmonate (MeJA), but down-regulated in response to treatments of SA and ABA. Over-expression of TaRPK1-2G in the powdery mildew susceptible wheat variety Prins by a transient expression assay showed that it slightly reduced the haustorium index of the infected *Bgt*. These data indicated that TaRPK1-2G participated in the defense response to *Bgt* infection and in the JA signaling pathway. Phylogenetic analysis indicated that TaRPK1-2G was highly conserved among plant species, and the amino acid sequence similarity of TaRPK1-2G among grass species was more than 86%. Based on its conservation, the RPK gene-based STS primers were designed, and used to amplify the RPK orthologs from the homoeologous group-2 chromosomes of all the tested *Triticeae* species, such as chromosome 2G of *T. timopheevii*, 2R of *Secale cereale*, 2H of *Hordeum vulgare*, 2S of *Aegilops speltoides*, 2S* of *Ae. longissima*, 2M9 of *Ae. geniculata*, 2S* and 2U* of *Ae. peregrina*. The developed STS markers serve as conserved functional markers for the identification of homoeologous group-2 chromosomes of the *Triticeae* species.

Introduction

Receptor protein kinases (RPKs) play essential roles in the signal perception in animals in response to various growth factors and hormones [1]. These receptors generally have an extracellular domain, a single transmembrane domain, and an intracellular catalytic kinase domain. Ligands bound by the extracellular domain stimulates receptor autophosphorylation on tyrosine residues within the cytoplasmic protein kinase domain. Then the binding of the ligand to the extracellular domain causes receptor dimerization thereby activating the cytoplasmic kinase domain by intermolecular phosphorylation and transduction of the signal to the downstream effectors [2]. Based on the primary structure, plants also have a large gene family named as receptor-like kinases (RLKs) similar to the animal’s RPKs, however, the auto-phosphorylation in plant RLKs is mostly specific to the serine and/or threonine [3]. Plant RLKs include receptor kinases and receptor-like cytoplasmic kinases (RLCKs) with no typical signal sequence or transmembrane domain, which have been implicated in the perception and transduction of extracellular signals into the cell [4]. The RLKs are usually encoded by hundreds of genes in plant genomes, for example, *Arabidopsis* has more than 600 predicted RLKs representing nearly 2.5% of all the coding genes, and *rice* (*Oryza sativa*) has nearly twice as many RLKs members as...
Arabidopsis does [5]. Due to the large gene family, RLKs vary greatly for both their domain organization and the extracellular domains, and the RLK family can be divided into more than 40 sub-families based on their distinct extracellular domains [4]. The diversity in the ligand binding domain endows the RLKs a wide range of biological function, such as growth and development, responses to biotic and abiotic stresses, and nodulation and rhizobial symbiosis [5].

Plant RLKs have been implicated in their roles in diverse signaling pathways. For example, Pit provides resistance against Pseudomonas syringae in tomato [6], SLG of Brassica oleracea may be required for self-incompatibility in the recognition between pollen and stigma [7,8], and the rice Xa21 confers broad spectrum resistance to Xanthomonas oryzae pv. oryzae [9]. Several RLKs have also been identified in wheat. The WLRK (wheat leaf rust kinase) gene family, which is located at the Lr10 locus and conserved in wheat and related species, confers resistance to leaf rust disease [10–12]. Three receptor-like kinase genes (TaRLK-R1, 2 and 3) were found to participate in the wheat hypersensitive response to the stripe rust fungus [13]. A recent research in our lab showed that a putative serine and threonine protein kinase gene, Spk-V, which located to the Pm21 locus from Hagnalida villoosa, conferred broad-spectrum powdery mildew resistance in wheat [14]. However, compared to the large population of the RLK genes in wheat, information on their biological role is still very limited. Thus, the identification of more wheat RLK genes is critical for better understanding of their biological roles in wheat.

In our previous studies, a barley RFLP probe BCD135 was found to be closely linked with the powdery mildew resistance gene Pm6, which has been introduced from the tetraploid wheat T. timopheevii into the hexaploid common wheat. The sequence of BCD135 was highly conserved among several species including barley, wheat, rye, and rice [15]. At the genome region of BCD135 in barley and rice, there were two conserved genes, one was a putative receptor-like protein kinase gene (HrRPK), and the other had no putative function [16]. Based on the sequence of the second gene in barley, Ji et al. developed two STS markers closely linked to the gene Pm6 in common wheat [17]. In the present study, the conserved TaRPK1 gene in this region was explored in grass species based on the barley HrRPK, and a functional marker was developed for the identification of the homoeologous group-2 chromosomes of Triticeae species. The TaRPK1 genes belonging to this conserved family were further cloned and characterized from hexaploid wheat, and the putative biological function of TaRPK1-2G was investigated.

Materials and Methods

Plant materials

Different Triticeae species with various genome constitution including Secale cereale L. cv. ‘BLANCO’ (2n = 2x = 14, RR), Hordeum vulgare L. cv. ‘BETZES’ (2n = 2x = 14, HH), Aegilops speltoides Tausch (2n = 2x = 14, SS), Ae. longissima Schw. et Musch (2n = 2x = 14, SSS), Ae. geniculata Roth (2n = 2x = 14, M2M2), and Ae. peregrina (Hack.) Maire & Weiller (2n = 4x = 28, SSSS[U^6L^6]), and their genetic stocks, i.e. addition lines with the alien chromosomes added in the background of wheat variety ‘Chinese spring’ (CS) (Triticum aestivum L., 2n = 6x = 42, AABBBDD) were introduced from Wheat Genetics & Genomic Resources Center (WGGRC), Kansas State University, USA (Table 1). The Swedish wheat variety Pm6, which is susceptible to powdery mildew, one T. timopheevii (2n = 4x = 28, AAGG) accession with the powdery mildew resistance gene Pm6, two Pm6-carrying T. aestivum-T. timopheevii introgression lines (IGVI-465 [FAO 163b/7*Prins] and IGVI-466 [Kenya Lemphi 50-13596/7*Prins]) were kindly provided by Dr. J. MacKey, Swedish Agricultural University, Uppsala, Sweden, and were used for gene cloning. A set of CS nulli-tetrasomic lines of homoeologous group-2 were also provided by the WGGRC and were used for mapping of wheat RPK genes.

Development of STS markers based on the sequence of the barley RPK gene HvRPK

The sequence of the barley gene HvRPK from the barley BAC clone AF474072 was downloaded from the Genbank [16]. Two SSRs (simple sequence repeats) were identified within the HvRPK sequence using the SSRHUNTER [18], and several primers flanking the two SSR motifs were designed using Primer 3 Software (http://www.genome.wi.mit.edu/cgi-bin/primer/primer3.cgi). The detailed information on all the primers used in this study is given in Table 2.

PCR amplification

For polymorphic analysis of the STS markers and chromosomal location of TaRPK1, PCR was performed following the procedure of Ji et al. (2008) [17]. The PCR products were separated in the 8% non-denaturing polyacrylamide gels (AcC: Bis = 19: 1 or 39: 1) at room temperature with 1×TBE buffer and visualized by silver staining [19]. High fidelity Taq DNA polymerases (QIAGEN, HotStar HiFidelity Polymerase Kit) were used for PCR amplifications for cloning of the gene TaRPK1. Amplification was performed at 94°C for 5 min; 32 cycles of 94°C for 15 s, 65 to 68°C for 1 min, and 72°C for 2 min; followed by 10 min at 72°C. PCR products were analyzed in 1% agarose gels.

BAC library screening and RPK gene cloning in wheat

The BAC library of an elite common wheat variety Xiaoyan 54, kindly provided by Dr. Hongjing Ling, Institute of Genetics and Development Biology Chinese Academy of Sciences, China, was used to isolate the TaRPK1 gene. The positive BAC was identified by PCR using a STS marker STSRPK-F1/R4 specific to the RPK sequence following the procedure of Dong et al. [20]. HvRPK was located in a 4.3 kb fragment when the BAC clone AF147042 was digested with XbaI [16]. To sub-clone the TaRPK1 from the positive BAC clone of Xiaoian 54, the BAC was also digested with XbaI and the expected full-length DNA sequence of TaRPK1 gene was obtained. Based on the TaRPK1 sequence obtained from Xiaoian 54, PCR primers flanking the ORF of TaRPK1 were designed and used to clone the full-length genomic DNA and cDNA sequences of the TaRPK1 gene from IGVI-465 and T. timopheevii.

Splicing feature, protein architecture and phylogenetic analyses

To characterize the splicing feature of the gene TaRPK1-2G, the genomic DNA and cDNA sequences of TaRPK1-2G were used for alignment and similarity analysis by DNAMAN version 5.2.2. TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) [21] and SMART (http://smart.embl-heidelberg.de/) [22] were used to identify the architectures of the protein domain.

The kinase domain of TaRPK1-2G was used as query sequence for identifying the TaRPK1-2G orthologs by the BLASTP searches in the Phytozome proteome database (http://www.phytozome.net/search.php?show = blast&method = Org_Cnapaya), and the best hits in various plant species were used for further analysis when the amino acid identity was >50% identical (e-values<e^-40) for at least 100 amino acid of the query sequence. The
| Accession number | Materials | Genome or Chromosome constitution |
|------------------|-----------|-----------------------------------|
| TA9001           | *H. vulgare* L. cv. 'BETZES' | HH                                |
| TA3698           | CS- BETZES DA 2H               | 21*M*2H arm unknown               |
| TA3699           | CS- BETZES DA 3H               | 21*M*3H                           |
| TA3700           | CS- BETZES DA 4H               | 21*M*4H                           |
| TA3701           | CS- BETZES DA 5H               | 21*M*5H                           |
| TA3702           | CS- BETZES DA 6H               | 21*M*6H                           |
| TA7591           | CS-H. chilense DA 7H ch        | 21*M*7H ch                        |
| TA9020           | Secale L.cv.'BLANCO'           | RR                                |
| TA7501           | CS- BLANCO DA 1R               | 21*M*1R                           |
| TA7502           | CS- BLANCO DA 2R               | 21*M*2R                           |
| TA7503           | CS- BLANCO DA 3R               | 21*M*3R                           |
| TA7505           | CS- BLANCO DA 4R               | 21*M*4R                           |
| TA7506           | CS- BLANCO DA 5R               | 21*M*5R                           |
| TA7507           | CS- BLANCO DA 6R               | 21*M*6R                           |
| TA7508           | CS- BLANCO DA 7R               | 21*M*7R                           |
| TA1910           | *Ae. longissima*               | S5%                              |
| TA7543           | CS- *Ae. longissima* DA 1S l #3 | 21*M*1S [1S l #3]               |
| TA7544           | CS- *Ae. longissima* DA 2S l #3 | 21*M*2S [2S l #3]               |
| TA7545           | CS- *Ae. longissima* DA 3S l #3 | 21*M*3S [3S l #3]               |
| TA7546           | CS- *Ae. longissima* DA 4S l #3 | 21*M*4S [4S l #3]               |
| TA7547           | CS- *Ae. longissima* DA 5S l #3 | 21*M*5S [5S l #3]               |
| TA7548           | CS- *Ae. longissima* DA 6S l #3 | 21*M*6S [6S l #3]               |
| TA7550           | CS- *Ae. longissima* DA 7S l #3 | 21*M*7S [7S l #3]               |
| TA7579           | CS- *Ae. longissima* DA 7S l #3 | 21*M*8S [8S l #3]               |
| TA2899           | *Ae. geniculata*               | M#M#                            |
| TA7655           | CS- *Ae. geniculata* DA 1M g #1 | 21*M*1[1M g #1]                  |
| TA7656           | CS- *Ae. geniculata* DA 2M g #1 | 21*M*2[2M g #1]                  |
| TA7657           | CS- *Ae. geniculata* DA 3M g #1 | 21*M*3[3M g #1]                  |
| TA7658           | CS- *Ae. geniculata* DA 4M g #1 | 21*M*4[4M g #1]                  |
| TA7659           | CS- *Ae. geniculata* DA 5M g #1 | 21*M*5[5M g #1]                  |
| TA7660           | CS- *Ae. geniculata* DA 6M g #1 | 21*M*6[6M g #1]                  |
| TA7661           | CS- *Ae. geniculata* DA 7M g #1 | 21*M*7[7M g #1]                  |
| TA2775           | *Ae. peregrina*                | UPU5#S5#                         |
| TA7594           | CS- *Ae. peregrina* DA 1S v #1 | 21*M*1[1S v #1]                  |
| TA7595           | CS- *Ae. peregrina* DA 2S v #1 | 21*M*2[2S v #1]                  |
| TA7596           | CS- *Ae. peregrina* DA 3S v #1 | 21*M*3[3S v #1]                  |
| TA7597           | CS- *Ae. peregrina* DA 4S v #1 | 21*M*4[4S v #1]                  |
| TA7598           | CS- *Ae. peregrina* DA 5S v #1 | 21*M*5[5S v #1]                  |
| TA7600           | CS- *Ae. peregrina* DA 7S v #1 | 21*M*7[7S v #1]                  |
| TA7614           | CS- *Ae. peregrina* DA 1U v #1 | 21*M*1[1U v #1]                  |
| TA7615           | CS- *Ae. peregrina* DA 2U v #1 | 21*M*2[2U v #1]                  |
| TA7616           | CS- *Ae. peregrina* DA 3U v #1 | 21*M*3[3U v #1]                  |
| TA7617           | CS- *Ae. peregrina* DA 4U v #1 | 21*M*4[4U v #1]                  |
| TA7618           | CS- *Ae. peregrina* DA 5U v #1 | 21*M*5[5U v #1]                  |
| TA7619           | CS- *Ae. peregrina* DA 6U v #1 | 21*M*6[6U v #1]                  |
| TA7620           | CS- *Ae. peregrina* DA 7U v #1 | 21*M*7[7U v #1]                  |
| TA2780           | *Ae. speltoides*               | S5                              |
| TA7689           | CS- *Ae. speltoides* DA 1S #3  | 21*M*1[1S #3]                   |
| TA7690           | CS- *Ae. speltoides* DA 2S #3  | 21*M*2[2S #3]                   |

Table 1. Plant materials introduced from the WGGRC, KSU, USA.
amino acid sequences of identified orthologs in fasta format were retrieved from the Phytozome proteome database, and the kinase domains of these orthologs were identified by the SMART protein domain prediction server [22]. The kinase domain sequences of TaRPK1-2G and its orthologs were aligned using the CLUSTALX [23]. Phylogenetic analysis was conducted and viewed using MEGA version 4.1 [24] based on the Neighbor–Joining method with a Poisson correction model and a bootstrap test of 1000 replicates.

Expression analysis of TaRPK1-2G by quantitative RT-PCR

Seedlings of the ‘IGVI-465’ were grown in a growth chamber at 16/8 h light/dark and 25/20°C temperature regime until the second leaf stage, when inoculation of *Blumeria graminis* f.sp. *tritici* (*Bgt*) was conducted by dusting the conidia from a susceptible wheat variety Sumai 3 onto the surface of the leaves of ‘IGVI-465’. The source of *Bgt* inoculum was a local natural mixture collected in the wheat fields of Nanjing, Jiangsu province, China. For chemical treatments, the seedlings of ‘IGVI-465’ at the first leaf stage were sprayed with 5 mM salicylic acid (SA), 100 mM methyl jasmonate (MeJA), 100 mM abscisic acid (ABA), and 7 mM H2O2, respectively. Total RNA of each sample was extracted using the TRIZOL reagent (Invitrogen, USA), and 2 μg of RNA per sample was used for synthesizing the first-strand cDNA by the AMV reverse transcriptase (Takara) following the manufacturer’s instruction.

Responses of the *TaRPK1-2G* upon infection with *Bgt* and the application of chemical agents were analyzed by the real-time quantitative RT-PCR (Q-RT-PCR) with the specific primer pair of *TaRPK1-2G* (RPK-QPCR-F and RPK-QPCR-R), and the 18S rRNA (amplified with primers 18rRNA-F and 18S rRNA-R) was used as a control. The PCR reaction was performed in a 25 μl reaction mixture containing 1×SYB Premix Ex Taq (Takara), 0.2 mM of each primer, 1×Rox Reference Dye, and about 30 ng cDNA per sample using the ABI Prism 7000 system (Applied Biosystems, USA). The program used was as follows: 1 min at 94°C; followed by 40 cycles of 5 s at 94°C, 15 s at 68°C and 30 s at 72°C. After completion of the reactions, the cycle threshold (CT) value was calculated and subsequently, the ΔΔCT algorithm was used [25]. The final value of relative gene quantification was expressed as n-fold change in *TaRPK1-2G* transcript level in tested samples in comparison with the untreated controls. The results are presented as the mean ± standard deviation (SD) of three independent analyses.

Table 1. Information of primers used in this study.

| Primer name | Primer sequence (5′-3′) |
|-------------|-------------------------|
| RPK-F1/F2   | CGACTACGTGACGCTCAAGA  |
| RPK-F2     | CAAGAGCCTCGACAAGATCC  |
| RPK-R1     | GCGAAGAGGATCTTGTCGAG  |
| RPK-R2     | ACTTGTCGCGAGGAGATG   |
| RPK-R3     | ACACCAAGCATACCTCTTC  |
| RPK-R4     | AGGCCGTATCATCCAGTAGC |
| TaRPK-ORF-F | ATGGCAGGCCGAGACACATTCAA |
| TaRPK-ORF-R | TCAATGCGGCTAATCATCGTAG |
| 18S rRNA-F | AACACTTACCGGAGCATCA |
| 18S rRNA-R | CGTCCCTGGCTTGGTACAC |

Primers were used to amplify the full length of *TaRPK1* and the annealing temperature was 61°C.

Table 2. Information of primers used in this study.

| Accession number | Materials | Genome or Chromosome constitution |
|------------------|-----------|-----------------------------------|
| TA7691           | CS- *Ae. speltoides* DA 35#3 | 21+1[35#3] |
| TA7692           | CS- *Ae. speltoides* DA 45#3 | 21+1[45#3] |
| TA7693           | CS- *Ae. speltoides* DA 55#3 | 21+1[55#3] |
| TA7694           | CS- *Ae. speltoides* DA 65#3 | 21+1[65#3] |
| TA7695           | CS- *Ae. speltoides* DA 75#3 | 21+1[75#3] |

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Single-cell transient expression assay

The ORF of TaRPK1-2G, amplified using the primer pair TaRPK1-2G-F and TaRPK1-2G-R, was inserted into the expression vector pBI220 by placing the TaRPK1-2G under the control of the CaMV35S promoter. The recombinant vector pBI220-TaRPK1-2G was used for single-cell transient expression assay according to Shirasu et al. [26] and Cao et al. [14]. The reporter plasmid pAHC25 containing the β-glucuronidase (GUS) gene and the expression plasmid pBI220-TaRPK1-2G were mixed before coating of the particles (molar ratio of 1:1; 1 μg of total DNA). The bombarded leaves were transferred to 1% agar plates supplemented with 85 μM benzimidazole and incubated at 18°C for 8 h before high density inoculation with Bgt spores. Leaves were stained for GUS to identify the TaRPK1-2G transformed cells at 48 h after Bgt inoculation. The haustorium index (percentage of GUS-staining cells with haustoria in the total GUS-staining cells attacked by Bgt) was computed based on mean of three independent experiments, each based at least 50 independent interaction events.

Statistical analysis

For the results of Q-RT-PCR and single-cell transient expression assay, the obtained data were analyzed by using SPSS analytical software package (version 19.0, IBM Corporation, New York, USA), and presented as the mean ± standard deviation (SD) of three independent replicates (n = 3). Comparisons between the controls and the tested samples were made ANOVA test and the statistical differences between the groups were tested by the Duncan’s test. All statistically significant differences were tested at the level of P<0.05. Figures were drawn by Origin Data Analysis and Graphing Software, OriginPro® (Version8.5.1, OriginLab Corporation, Massachusetts, USA).

Results

Development of RPK gene-based STS markers and analysis of their potential use as functional marker in wheat and other Triticeae species

The barley RPK gene (HvRPK) and the RFLP marker BCD135, which closely linked with gene Pm6 in wheat, are both present in the barley BAC clone AF474072. To detect whether the orthologs of HvRPK was also linked with Pm6 in wheat, two forward (RPK-F1 and RPK-F2) and four reverse (RPK-R1, RPK-R2, RPK-R3, and RPK-R4) primers, which flanked the SSR motifs of the HvRPK gene, were designed. Eight primer pairs were combined and used to test the polymorphism between the Pm6-carrying lines and their susceptible recurrent parent ‘Prins’. It was found that four primer combinations, STSRPK-F1/R1, STSRPK-F1/R2, STSRPK-F2/R3 and STSRPK-F1/R4 could produce clear polymorphism (Figure 1A and B). They produced distinct specific bands for both the Pm6-carrying lines and ‘Prins’, indicating they could serve as co-dominant markers. Linkage analysis in a previous study has shown that the wheat RPK gene is closely linked to the Pm6 [27].

The amplification of STSRPK-F1/R4 in the Chinese Spring (CS) nulli-tetrasomic lines involving homoeologous group-2 chromosomes enabled the clear discrimination of the RPK genes on the chromosomes 2G and 2B, while the two RPK gene copies on chromosomes 2A and 2D could not be discriminated due to their similar size (Figure 1B). The presence of RPK in the 2A, 2B and 2D indicated their conservation in the three wheat genomes. To further demonstrate the conservation of the RPK gene in homoeologous group-2 chromosomes in the genomes of wheat relatives, the RPK genes were amplified using the above four STS markers in wheat relatives and their corresponding alien addition lines in the background of Chinese Spring. The results showed that all these STS markers could produce specific fragments in the

Figure 1. Polymorphic analysis of HvRPK-based STS primer pair amplicons and chromosome location of the RPK amplicons in common wheat. A. Polymorphisms between the Pm6-carrying powdery mildew resistant lines and susceptible lines (Arrows indicate the polymorphic bands). M: DNA fragment marker DL2000; 1 and 2: Triticum timopheevii (with Pm6); 3 and 4: Pm6-carrying T. aestivum-T. timopheevii introgression line IGVI-465; 5 and 6: susceptible wheat variety Prins. B. Chromosome location of the RPK gene using the homoeologous group-2 ‘Chinese spring’ (CS) nulli-tetrasomic (NT) lines and Pm6-carrying T. aestivum-T. timopheevii introgression lines. The left arrows show the specific bands from chromosomes 2A or 2D and 2B, and arrow on the right shows the specific band from the chromosome 2G of Pm6-carrying lines. 1: Nulli2A/Tetra2B; 2: Nulli2A/Tetra2D; 3: Nulli2B/Tetra2A; 4: Nulli2B/Tetra2D; 5: Nulli2D/Tetra2A; 6: Nulli2D/Tetra2B; 7: CS; 8: Prins; 9: Pm6-carrying T. aestivum-T. timopheevii introgression line IGVI-465; 10: Pm6-carrying T. aestivum-T. timopheevii introgression line IGVI-466; 11: T. timopheevii (with Pm6).

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A Conserved Receptor-Like Protein Kinase Gene

Isolation of TaRPK gene in the common wheat variety Xiaoyan 54

The fragment amplified by the STS_{RPK-F1/R1} only included a ~1900 bp specific band from chromosome 2G of *T. timopheevii* (Figure 1B), a ~400 bp specific band from chromosome 2H of barley (Figure 2A), a ~1000 bp specific band from 2R of rye (Figure 2B), three specific bands (~480 bp, ~500 bp, and ~1000 bp) from 2S of *Ae. speltoides* (Fig. 2C), two specific bands (~1200 bp and ~500 bp) from 2S* of *Ae. longissima* (Figure 2D), two specific bands (~1000 bp and ~600 bp) from 2M* of *Ae. geniculata* (Figure 2E), a ~500 bp and a ~1000 bp specific band from 2S* and 2U* of *Ae. peregrina*, respectively (Figure 2F). STS_{RPK-F1/R1} could clearly discriminate the chromosomes 2S* and 2U* in the tetraploid *Ae. peregrina*. All these indicated highly conservation of RPK genes among diverse species, and the STS markers derived from RPK gene can be served as conserved functional markers to identify specific homoeologous group-2 chromosomes or chromosome segments of wheat relatives.

Cloning of TaRPK homologs from different genomes, analysis of its splicing feature and putative protein domain architectures in the *Pm6*-carrying line IGV1-465

As TaRPK was tightly linked with *Pm6* [27], and mapped to the introgression segment of 2G. It is interesting to isolate the TaRPK homologous gene from the *Pm6*-carrying line IGV1-465 and to figure out its putative biological function. Based on the sequence of TaRPK from Xiaoyan 54, PCR primers (TaRPK-ORF-F and TaRPK-ORF-R) flanking the ORF (open reading frame) of TaRPK were used to clone the full-length genomic DNA and cDNA sequences of the TaRPK homologs from IGV1-465 and *T. timopheevii*. Sequence analysis indicated that three TaRPK homologs were present in IGV1-465 on chromosomes 2A (TaRPK1-2A, 1,691 bp, GenBank accession number JX065225), 2D (TaRPK1-2D, 1,692 bp, GenBank accession number JX065228), and the introgressed chromosome segment of 2G (TaRPK1-2G, 1,650 bp, GenBank accession number JX065231). Two TaRPK homologs were present in *T. timopheevii*, each one on chromosomes 2A* (TaRPK1-2A*, 1,692 bp, GenBank accession number JX065227) and 2G (TaRPK1-2G, 1,650 bp). This indicated that in wheat variety CS, there were also three copies of RPK, and the two copies from chromosomes 2A and 2D in both CS and IGV1-465 were hard to separate and visualized as a single band in the non-denaturing polyacrylamide gels (as shown in Figure 1B) due to
their similar sequence size (with only one base pair difference in size as shown in IGVI-465). The 100% identity of the two TaRPK1-2G from IGVI-465 and T. timopheevii further proved that TaRPK1-2G was located on the introgressed chromosomal fragment of 2G in IGVI-465. Multiple sequence alignment showed that the TaRPK from Xiaoyan 54 and TaRPK1-2A had the highest similarity (being 93.27% identity) indicating that TaRPK was originally from the chromosome 2A in Xiaoyan 54.

The comparison of the genomic DNA of the three TaRPK1 homologs with their corresponding cDNA sequence in IGVI-465 showed that TaRPK1-2G was the gene actively transcribed under the tested conditions. TaRPK1-2G produced two different transcripts, probably due to alternative splicing (Figure 3). One type (TaRPK1-2G -1, GenBank accession number JX065229) had the complete ORF and generated the putative functional protein, whereas the other (TaRPK1-2G-2, GenBank accession number JX065230) had 13 bp intronic sequence from the second intron, and resulted in a truncated protein due to premature termination (Figure 3, indicated by the star). Alignment of the TaRPK1-2G-1 and TaRPK1-2G indicated that TaRPK1-2G had 4 introns and 5 exons (Figure 3), and TaRPK1-2G -1 coded for a predicted protein of 424 amino acids. Based on structural properties indicated by the TMHMM [21] and SMART programs [22], the predicted protein was a receptor protein kinase (RPK). The predicted protein TaRPK1-2G has a transmembrane domain (TM, from the 7th to 29th amino acid), an N-terminal hydrophobic signal peptide (from the 1st to 26th amino acid), and a cytoplasmic serine/threonine protein kinase domain (from the 80th to 358th amino acid), but does not have the typical extracellular structure such as eLRRs. We found that, in the transmembrane domain and the signal peptide, there were 20 amino acids overlapping, which has not been found in the Arabidopsis RLPks.

Analysis of TaRPK1-2G orthologs in plant species

To reveal the relationship of TaRPK1-2G with other RPKs, BLASTP was performed against the Phytozome proteome database for identifying TaRPK1-2G orthologs based on the similarity in their kinase domains, which was considered to be the most conserved domain among the RPKs. Orthologs were identified from 30 plant species with available genomic information, and together with the barley HvRPK, a total of 31 orthologs were used for phylogenetic analysis. These orthologs share a conserved serine/threonine protein kinase domain and belong to serine/threonine protein kinase by KOG classification (Form S1). The results indicated that TaRPK1-2G was highly conserved among the 32 selected species, and the amino acid sequence identity in the kinase domain was more than 86% among the RPKs from various grass species, including wheat (TaRPK1-2G), barley (HvRPK), Oryza sativa (OsRPK), Brachypodium distachyon (BdRPK), Sorghum bicolor (SbRPK), Setaria italica (SiRPK), Z. mays (ZmRPK), and Panicum virgatum (PavRPK) (Figure 4, detail information on these orthologs were provided in Form S1). Comparative genome mapping of the TaRPK1-2G orthologs in grass species showed that they were well retained in the collinear regions of TaRPK1-2G which have been revealed by whole-genome comparison [28,29], such as TaRPK1-2G and Pn6 colocated on 2BL [fraction length (FL) 0.50–1.00 [27], HvRPK on chromosome 2H: 140.1 cM, OsRPK on Chr4: 33,594,296 bp–33,596,099 bp, BdRPK on Bd5: 26,167,244 bp–26,168,882 bp, and SbRPK on chromosome 6: 59,793,399 bp–59,795,140 bp (The locations of these RPKs in their corresponding genomes are shown in Form S1). Moreover, these orthologs from the grass species formed into a group, which was separate from the dicots.
Figure 5. Expression of TaRPK1-2G in the leaves of IGVI-465. (A) inoculated with Bgt. (B) treated with 100 μM MeJA, 5 mM SA and 100 μM ABA. (C) treated with 7 mM H₂O₂. Bars with different letters show significant differences at the P<0.05 level. doi:10.1371/journal.pone.0049718.g005
with a high bootstrap support value (99%). However, TaRPK1-2G was most similar (with 97% identity) to the putative HvRPK from the barley BAC clone AF474072 (AAM13439). In *Arabidopsis*, the putative LRR-RLK (AT1G36140) showed the highest identity (53.9%) with TaRPK1-2G. AT1G36140 has been classified as the LRR protein of the VIII-2 subfamily [4], suggesting that TaRPK1-2G, although without the LRR motifs at its extracellular structure, also belongs to the VIII-2 subfamily (Figure 4).

**Expression profiling of TaRPK1-2G under stress and hormone treatments**

Since a large number of RPKs were proved to participate in plant defense response. To investigate whether TaRPK1-2G also participates in the defense response in wheat, the expression patterns of TaRPK1-2G upon the attack of Bgt infection and different hormone applications were analyzed by Q-RT-PCR. It was found that expression of TaRPK1-2G was up-regulated by the Bgt infection in the *Pm6*-containing line IGV1-465 at 1 h after inoculation (hai), but then reduced its expression at 6 hai and maintained at a low level from 24 hai to 72 hai (Figure 5A), suggesting that TaRPK1-2G might take part in the active defense response to Bgt. Expression of TaRPK1-2G was significantly induced by MeJA, but down-regulated by SA and ABA (Figure 5B). Application of H2O2 also regulated the expression of TaRPK1-2G, although not dramatically (Figure 5C). The induction of TaRPK1-2G transcription by Bgt and MeJA treatments in IGV1-465 suggested that this gene played a role in defense response to the infection by Bgt or other biotic or abiotic stresses via the JA pathway.

**TaRPK1-2G-mediated resistance to Bgt revealed by single-cell transient expression assay**

The localization of TaRPK1-2G to the same chromosome region of *Pm6* on the chromosome 2G fragment and its up-regulation by Bgt infection hinted the possible correlation of TaRPK1-2G with powdery mildew resistance. This assumption was tested using a single-cell transient expression assay. Single-cell transient expression assay is highly efficient in assessing the function of defense-related genes in response to Bgt both in wheat and barley, especially for those genes with pre-haustorium resistance. Haustorium is a key structure for nutrient extraction during Bgt development. The haustorium index (HI) can be used as a criterion to estimate the compatibility of interaction between the host and Bgt. In the single-cell transient assay, the epidermal cells expressing GUS gene and challenged by the Bgt were selected as the targets. If Bgt failed to penetrate into the cell and no haustorium formed, the interaction was considered to be incompatible (Figure 6A-a); on the other hand, if the haustorium and elongating secondary hyphae were observed, the interaction was considered to be compatible (Figure 6A-b, c, d). In the susceptible variety Prins, the HI was 64.16% when transformed with the GUS gene alone, and 59.33% when co-transformed with GUS and TaRPK1-2G. In the resistant line IGV1-465, the HI was 37.47% when transformed with the GUS gene (Figure 6B). Statistical analysis indicated that there was no significant difference for the HI between the *GUS* alone transformation and the TaRPK1-2G+GUS co-transformation in the susceptible variety Prins, while significant difference was observed for the HI between the GUS alone transformation in Prins and IGV1-465. These indicated that transiently over-expression of TaRPK1-2G could prevent the formation of haustoria to a certain extent, but it could not improve the resistance level of the susceptible variety to Bgt. We deduced that the TaRPK1-2G play a role in the powdery mildew resistance pathway of IGV1-465, but it should not be the best candidate for the *Pm6* gene.

**Discussion**

In this study, a conserved wheat RPK gene associated with the introgressed *Pm6* from *T. timopheevii* was cloned and characterized. Based on the *HR-PK* gene, STS markers were developed and they could be used to identify the alien chromosomes belonging to the homoeologous group-2 in the wheat background. Molecular mapping and bioinformatics analysis revealed orthologous copies of *RPK* in the *Triticeae* and apparent orthologs were present in various plant species. The orthologs of *RPK* were conserved in the collinear regions of rice, *Brachypodium*, *S. bicolor*, and *Z. mays* (The locations of these *RPK* in their corresponding genomes are shown in Form S1), and the collinear regions of *TaRPK1-2G* orthologs in these grass species have been revealed by whole-genome comparison [28,29]. This implied that the conserved *RPK* gene may confer important biological roles in plant species.

The presence of a putative hydrophobic signal peptide, a hydrophobic membrane-spanning segment, and a highly conserved kinase domain supported that TaRPK1-2G is a receptor-like protein kinase. Although the kinase domain-based multiple sequence alignment and phylogenetic analysis supported the TaRPK1-2G could be classified into the LRR VIII-2 subfamily, the fact that it lacks of the LRR motifs at the extracellular structure implied that TaRPK1-2G may be a new member of the LRR VIII-2 subfamily. Among those genes closely related to TaRPK1-2G, none of their function has been elucidated. TaRPK1-2G was found to be up-regulated by both Bgt and MeJA treatments, suggesting that it might be involved in the defense response. However, the exact function of TaRPK1-2G and its orthologs in the grass species remains to be further elucidated.

Alternative splicing of pre-mRNA is a prominent post-transcriptional mechanism, which contributes to increased protein diversity and the regulation of gene function in eukaryotic cells [30,31]. It was estimated that about 40–60% genes in human, at least 42% in *A. thaliana* and 56% in maize undergo alternative splicing [32–34]. The impact of the alternative splicing has been frequently reported in human, which sometimes leads to many diseases [35–38]. In plants, alternative splicing was reported to partake in the regulation of flowering, disease resistance, stress tolerance, grain quality and so on [39]. For example, the photoperiod gene *Ppd-B1*, which plays a major role in controlling the heading time, yield, and adaptability in wheat, was alternatively spliced [40]. The disease resistance gene *RPS4* in *Arabidopsis* produces multiple transcripts via alternative splicing, and *RPS4*-mediated resistance requires the combined presence of transcripts encoding both the full-length and the truncated ORFs [41]. In wheat, the transcription factor *DREB2* in response to different abiotic stresses can produce three types of transcripts in varying amounts through alternative splicing. Alternative splicing of *RPS4* undergoes dynamic changes specifically during the resistance response [42], and alternative splicing of *DREB2* was regulated by the ABA-dependent (response to drought and salt stress) and ABA independent pathway (response to low temperature stress) [43]. These findings indicate the importance of alternative splicing in regulation of responses to various stresses. In this study, we found that TaRPK1-2G was also an alternative splicing gene producing two different transcripts, *TaRPK1-2G-1* and *TaRPK1-2G-2*, however, only the *TaRPK1-2G-1* encoded the full-length ORF, and it was mainly up-regulated by the MeJA. The relationship between the alternative splicing of *TaRPK1-2G* and JA signaling defense pathway is still unknown, and it will be
important to characterize the function of TaRPK1-2G and its regulation at the post-transcriptional level.

Supporting Information

Form S1 The information of TaRPK1-2G orthologs. The kinase domain of TaRPK1-2G was used as query sequence for identifying the TaRPK1-2G orthologs by BLASTP searches in the Phytozome proteome database. The best hits in each species were retrieved. This form provided the information about the species of the orthologs, accession number & locus name, location in corresponding genome, description of putative protein, and scores, e-values, identities, and positives of the BLAST results.

Author Contributions

Conceived and designed the experiments: BQ XW. Performed the experiments: BQ TC HW LX JX JJ XC CY. Analyzed the data: BQ AC DW PC DL XW. Contributed reagents/materials/analysis tools: HL. Wrote the paper: BQ AC XW.

References

1. Fantl WJ, Johnson DE, Williams LT (1993) Signalling by receptor tyrosine kinases. Annu Rev Biochem 62: 453–481.
2. Song YZ, Carraway KL, Eck MJ, Harrison SC, Feldman RA, et al. (1995) Catalytic specificity of protein-tyrosine kinases is critical for selective signalling. Nature 373: 536–539.
3. Walker JC (1994) Structure and function of the receptor-like protein kinases of higher plants. Plant Mol Biol 26: 1599–1609.
4. Shiu SH, Bleecker AB (2001) Receptor-like kinases from Arabidopsis form a monophyletic gene family related to animal receptor kinases. Proc Natl Acad Sci U S A 98: 10763–10768.

Figure 6. Functional analysis of the TaRPK1-2G by single cell transient expression assay in interaction with Bgt. A. The compatible and incompatible interactions between the positive transformed cells of the target gene with Bgt fungus. a: incompatible interaction in which the formation of haustorium failed. b, c, d: compatible interaction in which the formation of haustorium succeeded. co: conidia; pp: penetration peg; ha: haustorium; hy: hyphae. B. The haustorium index in the GUS transformed cells or in the GUS and TaRPK1-2G co-transformed cells. Bars with different letters show significant differences at the P<0.05 level; differences of the haustorium index were compared between the GUS transformed cells and the TaRPK1-2G+GUS co-transformed cells using Prins (susceptible) as the receptor, and between the GUS transformed cells using IGVI-465 (resistant) as the receptor.
doi:10.1371/journal.pone.0049718.g006
5. Shiu SH, Karlovski WM, Pan R, Tseng YH, Mayer KE, et al. (2004) Comparative analysis of the receptor-like kinase family in *Arabidopsis* and rice. Plant Cell 16: 1220–1234.

6. Martin GB, Brommmonschenkel SH, Chunwongse J, Frary A, Canol MW, et al. (1995) Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science 262: 1432–1436.

7. Walker JC (1993) Receptor-like protein kinase genes of *Arabidopsis thaliana*. Plant J 3: 451–456.

8. Torii KL: Revised gene 100: 564–568.

9. Song WY, Wang GL, Chen LL, Kim HS, Pi LY, et al. (1995) A receptor kinase-like protein encoded by the rice disease resistance locus, *Xa21*. Science 270: 1804–1806.

10. Feuillet C, Rezeau-C, Kjellbom P, Keller B (1997) Molecular characterization of a new type of receptor-like kinase (wlk) gene family in wheat. Plant Mol Biol 37: 943–953.

11. Feuillet C, Schachermayr G, Keller B (1997) Molecular cloning of a new receptor-like kinase gene encoded at the *Li10* disease resistance locus of wheat. Plant J 11: 45–52.

12. Feuillet C, Peng C, Gebler K, Matt A, Keller B (2001) Molecular evolution of receptor-like kinase genes in hexaploid wheat. Independent evolution of orthologs after polyploidization and mechanisms of local rearrangements at paralogous loci. Plant Physiol 125: 1300–1313.

13. Zhou H, Li S, Deng Z, Wang X, Chen T, et al. (2007) Molecular analysis of three new receptor-like kinase genes from hexaploid wheat and evidence for their participation in the wheat hypersensitive response to stripe rust fungus infection. Plant J 52: 420–434.

14. Cao A, Xing L, Wang X, Yang X, Wang W, et al. (2011) Serine/threonine kinase gene *Stpk-V*, a key member of powdery mildew resistance gene *Pm6b*, confers powdery mildew resistance in wheat. Proc Natl Acad Sci U S A 108: 7727–7732.

15. Tao W, Liu D, Liu J, Feng Y, Chen P (2000) Genetic mapping of the powdery mildew resistance gene *Pm6b* in wheat by RFLP analysis. TAG Theoretical and Applied Genetics 11: 45–52.

16. Feuillet C, Peng C, Gebler K, Matt A, Keller B (2001) Molecular evolution of receptor-like kinase genes in hexaploid wheat. Independent evolution of orthologs after polyploidization and mechanisms of local rearrangements at paralogous loci. Plant Physiol 125: 1300–1313.

17. Zhou H, Li S, Deng Z, Wang X, Chen T, et al. (2007) Molecular analysis of three new receptor-like kinase genes from hexaploid wheat and evidence for their participation in the wheat hypersensitive response to stripe rust fungus infection. Plant J 52: 420–434.

18. Cao A, Xing L, Wang X, Yang X, Wang W, et al. (2011) Serine/threonine kinase gene *Stpk-V*, a key member of powdery mildew resistance gene *Pm6b*, confers powdery mildew resistance in wheat. Proc Natl Acad Sci U S A 108: 7727–7732.

19. Tao W, Liu D, Liu J, Feng Y, Chen P (2000) Genetic mapping of the powdery mildew resistance gene *Pm6b* in wheat by RFLP analysis. TAG Theoretical and Applied Genetics 11: 45–52.

20. Park YJ, Dixit A, Yoo JW, Bennetzen J (2004) Further evidence of microcolinearity between barley and rice genomes at two orthologous regions. Mol Cells 17: 492–502.

21. Ji J, Qin B, Wang H, Cao A, Wang S, et al. (2008) STS markers for powdery mildew resistance gene *Pm6b* in wheat. Euphytica 163: 159–165.

22. Li Q, Wan JM (2005) SSRHunter: development of a local searching software for SSR sites. Yi Chuan 27: 808–810.

23. Basset RJ, Caetano-Anolles G, Gresshoff PM (1991) Fast and sensitive silver staining of DNA in polyacrylamide gels. Anal Biochem 196: 80–83.

24. Dong L, Zhang X, Liu D, Fan H, Sun J, et al. (2010) New insights into the organization, recombination, expression and functional mechanism of low molecular weight glutenin subunits genes in bread wheat. Planta 230: 15348.

25. Krogh A, Larsson B, von Heijne G, Sonhammer EL (2001) Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. J Mol Biol 305: 567–580.

26. Letunic I, Copley RR, Cattaneo FD, Doerks T, et al. (2004) SMART 4.0: towards genomic data integration. Nucleic Acids Res 32: D142–144.

27. Thompson JD, Gibson TJ, Higgins DG (2002) Multiple sequence alignment using ClustalW and ClustalX. Curr Protoc Bioinformatics Chapter 2: Unit 2.3.

28. Kumar S, Tamura K, Jakobsen IB, Nei M (2001) MEGA2: molecular evolutionary genetics analysis software. Bioinformatics 17: 1244–1245.

29. Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2^-Delta Delta Cq Method. Methods 25: 402–408.

30. Shi Y, Nielen K, Pillanelli P, Oliver R, Schulze-Lefert P (1999) Cell-autonomous complementation of fungal resistance using a biolistic transient expression system. The Plant Journal 17: 293–299.

31. Qin B, Cao A, Wang H, Chen T, You FM, et al. (2011) Collinearity-based marker mining for the fine mapping of *Pm6*, a powdery mildew resistance gene in wheat. Theor Appl Genet 120: 207–218.

32. Salse J, Boto S, Throude M, Jouffe V, Piegus B, et al. (2008) Identification and characterization of shared duplications between rice and wheat provide new insight into grass genome evolution. Plant Cell 20: 11–24.

33. The International Barley Genome Initiative (2010) Genome sequencing and analysis of the model grass *Hordeum distichum*. Nature 468: 763–768.

34. Stamm S, Ben-Ari S, Rafalska I, Tang Y, Zhang Z, et al. (2005) Function of alternative splicing. Gene 344: 1–20.

35. Larrea LF, Green RE, Bhattacharjee BS, Bennetse SF, et al. (2004) The evolving roles of alternative splicing. Curr Opin Struct Biol 14: 273–282.

36. Modrek B, Lee C (2002) A genomic view of alternative splicing. Nat Genet 30: 13–19.

37. Filichkin SA, Priest HD, Givan SA, Shen R, Bryant DW, et al. (2010) Genome-wide mapping of alternative splicing in *Arabidopsis thaliana*. Genome Res 20: 45–58.

38. Li P, Ponnala L, Gandotra N, Wang L, Si Y, et al. (2010) The developmental dynamics of the maize leaf transcriptome. Nat Genet 42: 1060–1067.

39. Soleymanlou N, Wu Y, Wang JX, Todros T, Ietta F, et al. (2005) A novel Mtd splice isoform is responsible for trophoblast cell death in pre-eclampsia. Cell Death Differ 12: 441–452.

40. Tan EK, Shen H, Tan JM, Lim KL, Fook-Chong S, et al. (2003) Differential expression of splice variant and wild-type parkin in sporadic Parkinson’s disease. Neurogenetics 6: 179–184.

41. Agravat S, Eng C (2006) Differential expression of novel naturally occurring splice variants of *PTEV* and their functional consequences in Cowden syndrome and sporadic breast cancer. Hum Mol Genet 15: 777–787.

42. Venables JP (2006) Unbalanced alternative splicing and its significance in cancer. Bioessays 28: 378–386.

43. Reddy AS (2007) Alternative splicing of pre-messenger RNAs in plants in the genomic era. Annu Rev Plant Biol 58: 267–294.

44. Guo Z, Zhao G, Ren Z, Jia J (2009) Alternative splicing of photoperiod response gene *Ppd-B1* in wheat. Acta Agronomica Sinica 35: 1764–1766.

45. Zhang XC, Gassmann W (2003) *RPS4*-mediated disease resistance requires the combined presence of *RPS4* transcripts with full-length and truncated open reading frames. Plant Cell 15: 2333–2342.

46. Zhang XC, Gassmann W (2007) Alternative splicing and mRNA levels of the disease resistance gene *RPS4* are induced during defense responses. Plant Physiol 145: 1577–1587.

47. Egawa C, Kobayashi F, Shishibashi M, Nakamura T, Nakamura C, et al. (2006) Differential regulation of transcript accumulation and alternative splicing of a *DRD2* homolog under abiotic stress conditions in common wheat. Genes Genet Syst 81: 77–91.