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The somatic embryogenesis receptor kinase TaSERK1 participates in the immune response to *Rhizoctonia cerealis* infection by interacting and phosphorylating the receptor-like cytoplasmic kinase TaRLCK1B in wheat

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ABSTRACT

The sharp eyespot, caused by necrotrophic pathogen *Rhizoctonia cerealis,* often causes serious yield loss in wheat (*Triticum aestivum*). However, the mechanisms underlying wheat resistant responses to the pathogen are still limited. In this study, we performed a genome-wide analysis of somatic embryogenesis receptor kinase (SERK) family in wheat. As a result, a total of 26 *TaSERK* candidate genes were identified from the wheat genome. Only 6 *TaSERK* genes on the chromosomes 2A, 2B, 2D, 3A, 3B, and 3D showed obvious heightening expression patterns in resistant wheat infected with *R. cerealis* compared than those un-infected wheat. Of them, the transcripts of 3 *TaSERK1* homoeologs on the chromosomes 2A, 2B, and 2D were significantly up-regulated in the highest level compared to other *TaSERKs*. Importantly, silencing of *TaSERK1* significantly impaired wheat resistance to sharp eyespot. Further bio-molecular assays showed that TaSERK1 could interact with the defence-associated receptor-like cytoplasmic kinase TaRLCK1B, and phosphorylated TaRLCK1B. Together, the results suggest that TaSERK1 mediated resistance responses to *R. cerealis* infection by interacting and phosphorylating TaRLCK1B in wheat. This study sheds light on the understanding of the wheat SERKs in the innate immunity against *R. cerealis*, and provided a theoretical fulcrum to identify candidate resistant genes for improving wheat resistance against sharp eyespot in wheat.

1. Introduction

As one of the major staple crops, wheat (*Triticum aestivum*) is widely cultivated in the world. The yield of wheat affects the global food security and economy [1]. The sharp eyespot, caused mainly by necrotrophic pathogens *Rhizoctonia cerealis,* often causes serious yield loss in wheat [2,3]. However, the sharp eyespot disease-resistant germplasms available in breeding are still rare. A few quantitative trait locus (QTLs) or genes were reported to participate in wheat resistant reactions to sharp eyespot in wheat [3–5]. Especially, the molecular mechanisms underlying host defence reactions against the pathogen have been largely elusive untill now.

In plants, the leucine-rich repeat (LRR) receptor-like kinases (RLKs) are a large group of proteins, which often have an extracellular LRR receptor domain to perceive external signals, a transmembrane region, and an intracellular kinase domain to transmit signals in immune reactions [6]. For example, the somatic embryogenesis receptor-like kinases (SERKs), which usually act as co-receptors for other RLKs, have been well studied in *Arabidopsis* [7]. The SERKs belong to the subgroup II of LRR RLK subfamily, and are distinguished by several characteristics. Firstly, in the genome sequences, SERKs are characterized by the presence of 11 exons with conserved splicing boundaries. Secondly, for protein sequences, each protein of SERKs usually contains a signal peptide, five LRR motifs, a proline-rich domain, a transmembrane domain, and a cytosolic Serine/Thrine protein kinase domain [8]. In *Arabidopsis*, a total of 5 *SERK* genes have been identified, and subdivided into two subgroups. One includes *AtSERK1* and *AtSERK2*, another is consisted of *AtSERK3, AtSERK4,* and *AtSERK5* [9]. Especially, the *AtSERK3* and *AtSERK4* are often associated with programmed cell death and defence responses [10]. When plants were infected by pathogens,

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the AtSERK3/BAK1 could form heterodimerization with certain receptor kinases, including flagellin sensing 2 (FLS2) receptor kinase, phytosulfokine peptide receptor 1 (PSKR1), and male discoverer 1-interacting receptor –like kinase 2 (MIK2), as well as damage-associated molecular pattern peptide 1 receptors AtPEPR1 and AtPEPR2 [11–14]. In rice, *OsSERK1* was involved in resistance to the blast fungus. Overexpression of OsSERK1 led to an increasing in host resistance to the blast fungus [15]. OsSERK2 also positively regulates immunity against *Xanthomonas oryzae pv. oryzae* (Xoo) by interacting with the intracellular domains of XA21, XA3, and FLS2 in a kinase activity-dependent manner [16]. The SERKs have been identified as one of the most important components among the plant immune system. However, the functions of SERKs involved in resistance to fungal pathogens in wheat were scarcely reported.

In this study, to investigate whether the *SERK* family genes play a crucial role in wheat resistant responses against *R. cerealis*, we firstly performed a genome-wide analysis of *SERKs* family genes in wheat, and in turn a total of 26 *TaSERK* candidate genes were identified from the wheat genome. By means of RNA-sequencing (RNA-Seq) of wheat against *R. cerealis* and barley stripe mosaic virus induced silencing (VIGS) assays, we found that 3 *TaSERK1* homoeologous genes on the chromosome 2A, 2B and 2D showed higher elevated expression than other 23 *SERK* genes, and silencing of *TaSERK1* significantly impaired wheat resistance to sharp eyespot. Multiple molecular biological assays showed that TaSERK1 can interact with and phosphorylate a previous reported defence-associated receptor-like cytoplasmic kinase TaRLCK1B [17] in wheat. This study identifies *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* involved in wheat resistant responses to *R. cerealis* in wheat, and validates that TaRLCK1B is a substrate of TaSERK1 in wheat immune responses to sharp eyespot.

2. Materials and methods

2.1. Plants and fungal materials

The wheat cultivar CI12633, showing middle resistance to sharp eyespot, was used for Barley yellow dwarf virus-mediated silencing (VIGS) assays. Two middle resistant cultivars (CI12633 and Shanhongmai) and two highly susceptible wheat cultivars (Wenmai 6 and Yangmai 9) were used to investigate *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* transcript profiles upon *R. cerealis* infection. All wheat seedlings were planted in greenhouse at 23 °C for 14 h in light, and 15 °C for 10 h in darkness. The pathogen *Rhizoctonia cerealis* strain Rc207 was isolated and provided by Prof. Jinfeng Yu and Dr. Li Zhang (Shandong Agricultural University, China) [18].

2.2. Identification of SERK gene family members in wheat

The whole *SERK* genes in wheat genome were idenficated according to the previous studys [19,20]. Firstly, genenome and protein sequences of all five *Arabidopsis AtSERK* genes and whole genome sequences of wheat were downloaded from Ensembl Plants database (http://plants.ensembl.org/index.html). Secondly, the protein sequences of five AtSERKs were used as queries to conduct local BLAST searches (e-value <1e-10) genome-widely in wheat by using the software Tbtools [21]. Thirdly, the candidate sequences were used to build HMM profile through hmm build program and gene structure analysis by the software Tbtools. Subsequently, candidate genes with 11 exons were screened out according to the gene structural characteristics of *SERKs.* And, all the remained candidate sequences were manually checked by the programs SMART (http://smart.embl-heidelberg.de/). Eventually, proteins with a signal peptide, five LRR motifs, a proline-rich domain, a transmembrane domain, and an cytosolic Ser/Thr kinase domain, were regarded as SERK family members in wheat. And the duplicated gene pairs of wheat *SERK* genes were calculated using MCScanX (e-value ≤1e-10) and visualized via Circos software [22].

2.3. Phylogenetic, gene structure and conserved motifs analysis

A phylogenetic tree of all the wheat SERK family proteins was built by the neighbor-joining method with 1000 bootstrap replicates using the program MEGA 7 (https://www.megasoftware.net/). The online MEME program 9 were used to identifiy the conserved motifs of TaSERK family members [23]. The gene structure as well as the conserved motifs of TaSERK family members were visualized using TBtools software [21]. The molecular weight (MW) and isoelectric point (pI) values of TaSERKs were predicted by online tool ExPASy7.

2.4. Plants and fungal materials

The wheat cultivar CI12633, a middle resistant germplasm to sharp eyespot, was used for Barley yellow dwarf virus-mediated silencing (VIGS) assays. Two middle resistant cultivars (CI12633, Shanhongmai) and two highly susceptible wheat cultivars (Wenmai 6, Yangmai 9), were used to investigate *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* transcript profiles upon *R. cerealis* infection. All wheat seedlings were planted in greenhouse at 23 °C for 14 h in light, and 15 °C for 10 h in darkness. The pathogen strain *R. cerealis* strain Rc207 was isolated by Prof. Jinfeng Yu and Dr. Li Zhang (ShandongAgricultural University, China).

2.5. RNA-seq data analysis

In this study, at 0, 4 and 10 dpi with *R. cerealis*, the purified RNA were extracted from the resistant recombinant inbred lines (RILs-R) derived from the cross 'Shanhongmai' × 'Wenmai 6', were used for RNA-Seq (RNA-sequencing). The resistant RILs were kindly probided by by Prof. Jizeng Jia (ICS, CAAS). The RILs parent cultivar 'Shanhongmai' is a resistant germplasm to *R. cerealis*, and another parent cultivar 'Wenmai 6' is a highly susceptible wheat cultivar to *R. cerealis*. The methods of RNA-Seq data analysis was same as previously described by Guo et al. [24].

2.6. RNA extraction and qRT-PCR

At 0, and 4 dpi with *R. cerealis*, the pirified RNA were extracted from CI12633, Shanhongmai and Wenmai 6, Yangmai 9 wheat plants using a Trizol reagent (Invitrogen, USA). Then, the RNA was purified and reverse-transcribed into cDNA for further real-time quantitative PCR (RT-qPCR) experiments using the FastQuant RT Kit (Tiangen, China).

In VIGS assays, at 15 days after virus infection, the pirified RNA were extracted from BSMV: GFP- (control) or BSMV:TaSERK1- infected CI12633 seedlings. Then, the transcript level of a BSMV *coat protein* (*CP*) gene was used to check whether the BSMV was successfully infected. The.

specific primers were used to measure the transcript level of the *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* to check whether the genes was successfully silenced or not. The transcript level of target genes was calculated using the $2^{-\Delta\Delta CT}$ method, and the wheat actin gene used as internal reference gene.

2.7. VIGS and assessment for wheat resistance to R. cerealis

A Barley yellow dwarf virus-mediated silencing (VIGS) assay were used to analysis the resistant function of *TaSERK1* to *R. cerealis*. A 210 bp fragment of *TaSERK1* was subcloned in the antisense orientation into the RNAγ of BSMV, to form a BSMV: TaSERK1 recombinant construct (Fig. S1). Then, the tripartite cDNA chains of BSMV: TaSERK1 or the control BSMV: GFP virus genomes were separately transcribed into RNAs, mixed, and used to infect CI12633 seedlings at thethree-leaf stage. At 15 days after virus infection, the fourth leaves of the inoculated seedlings were collected to monitor BSMV infection and to evaluate the silencing efficinecy of *TaSERK1*. Then, the *TaSERK1* silenced or

the BSMV: GFP-infected (control) CI12633 seedlings were further used in assessment for Wheat resistance to *R. cerealis* inoculation and assessment methods were same as previously described by Chen et al. [25].

2.8. Yeast two-hybrid and BiFC assays

For yeast two-hybrid assays, the coding sequence of *TaSERK1-2D* (TraesCS2D02G321400.1) was amplified and sub-cloned into bait plasmid pBT-STE, *TaRLCK1B* was amplified and subcloned into the prey vector pPR—3C. The appropriate plasmid pairs were co-transformed into NMY51 competent cells following the supplier's instructions (shanhaiweidi, YC1040). For the BiFC assays, the coding sequences of *TaSERK1-2D* or *TaRLCK1B* were amplified and subcloned into 35S: TaSERK1-cYFP, and 35S: TaRLCK1B-nYFP. Then, the appropriate pairs of plasmids were co-transformed into wheat mesophyll protoplasts through the PEG-mediated transfection methods [26]. After incubation at 25 °C for 16 h in darkness, the YFP fluorescence was detected by a laser scanning confocal microscope (ZeissLSM700).

2.9. Phosphorylation assay and phosphorylation sites prediction

To define whether TaRLCK1B could be phosphorylated by TaSERK1-2D, Pcold-His-TaSERK1-2D and Pcold-HA-TaRLCK1B plasmids were heterologously expressed using the *E. coli* DE3 and purified as directed by the manufacturer (Transgen). HA-TF-TaRLCK1B alone or with His-TF-TaSERK1-2D were incubated in kinase reaction buffer (20 mM HEPES, pH¼7.5, 10 mM$MgCl_2$, 1 mM DTT, 1 mM ATP) at 25 °C for 60 min, then a 50 μM phos-tag™ Acrylamide AAL-107 assay with anti-HA monoclonal antibody (Transgen, HT301) was used to test

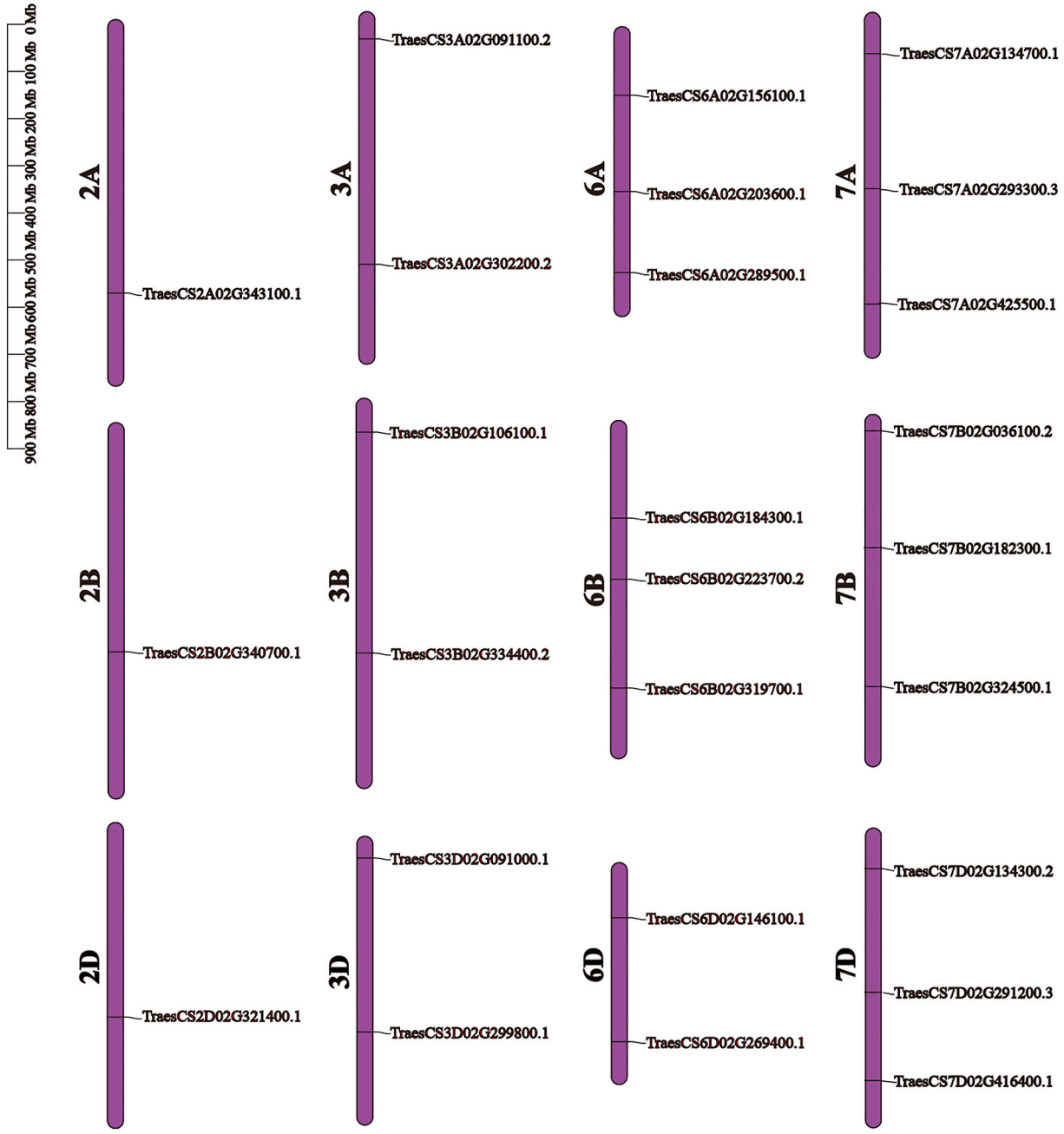

Fig. 1. Chromosomal distribution of the 26 *TaSERK* genes. The 26 *TaSERK* genes were unevenly distributed on 12 wheat chromosomes. The chromosome numbers are denoted left of each chromosome, and the bar locates on the left side shows the size of chromosome in megabases (MB).

phosphorylation proteins. The phosphorylation sites of TaSERK1 or TaRLCK1B were predicted using the online software NetPhos-3.1 (https://services.healthtech. dtu.dk/service.php NetPhos-3.1) [27].

3. Results

3.1. Identification of SERK family genes in wheat

To identify *TaSERK* genes in wheat genome, five *Arabidopsis* AtSERK proteins were used as queries for genome-wide identification in the genome database of wheat (http://plants.ensembl.org/index.html). Based on conserved domains and the gene structure of SERK family members, a total of 26 *TaSERK* candidate genes were identified in the wheat genome. As shown in Fig. 1, these candidate genes were located on the chromosome 2A, 2B, 2D, 3A, 3B, 3D, 6A, 6B, 6D, 7A, 7B, and 7D, respectively. The amino acid sequences ranged from 589 to 653 (amibo acid, aa) in length, with an average of 623 aa. The molecular weight (MW) values ranged from 64.55 kDa to 71.88 kDa, with an average of 68.29 KDa, and isoelectric point (pI) values ranged from 5.13 to 7.47, with an average of 6.32 (Supplementary Table 1).

To analyze the expansion mechanism within *TaSERK* gene family, a collinearity analysis of *TaSERK* genes of the wheat genome were used to evaluate duplication events of *TaSERK* genes [28,29]. As shown in Fig. 2, there were a total of 26 pairs of duplicated genes. Almost all *TaSERK* genes contained three duplicated genes, except the two homolog genes TraesCS6B02G223700.2 on the chromosome 6B and TraesCS6A02G203600.1 on the chromosome 6A, only have two duplicated copies. Moreover, all 26 *TaSERK* genes were derived from whole genome duplication, implied whole genome duplication is the main driving factor of *TaSERK* genes in the wheat genome.

3.2. Phylogenetic, gene structure analysis and conserved motif analysis of TaSERK members

To analyze evolutionary relationships of wheat TaSERK members, we conducted phylogenetic analysis of the TaSERK proteins. As shown in Fig. 3A, based on amino acid sequence similarities and genome sequence length, TaSERK proteins were divided into 5 groups (I-V). Meanwhile, the exon-intron structures were compared between the 26 *TaSERK* members (Fig. 3B). All of the 26 *TaSERK* members in wheat contain 11 exons and 10 introns, which were in line with the typical gene structure characteristics of the *Arabidopsis AtSERK* family. Subsequently, the conserved motifs of TaSERK members were analyzed by MEME (Fig. 3C). There were 10 conserved motifs identified from the TaSERK

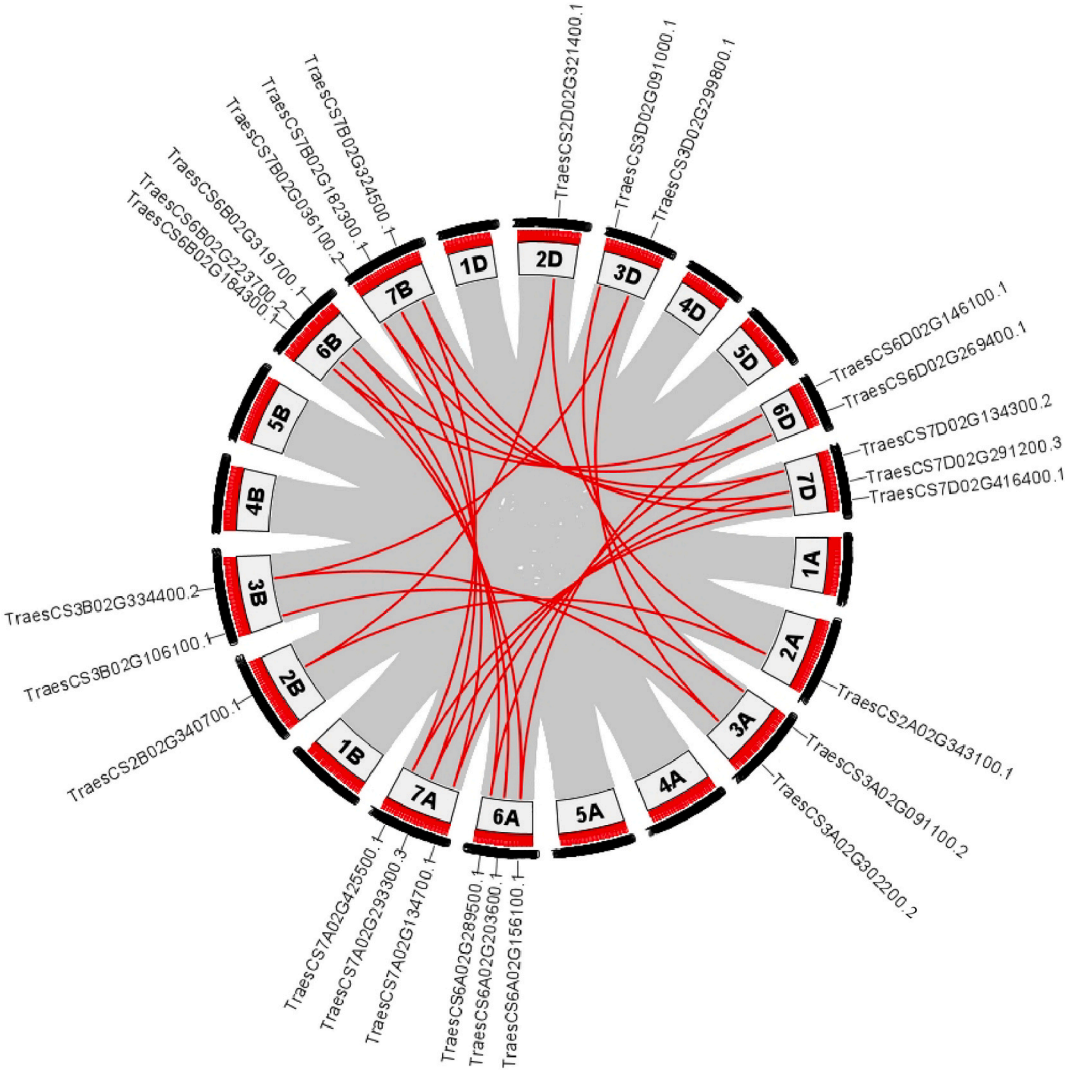

Fig. 2. Distribution and duplication events of *TaSERK* genes in wheat genome. All duplications of *TaSERK* genes were mapped to their respective locations using Circos. Gray regions indicate all synteny blocks and synteny blocks of *TaSERK* genes within the wheat genome were indicated by red lines. The chromosome numbers were in inside of the circle.

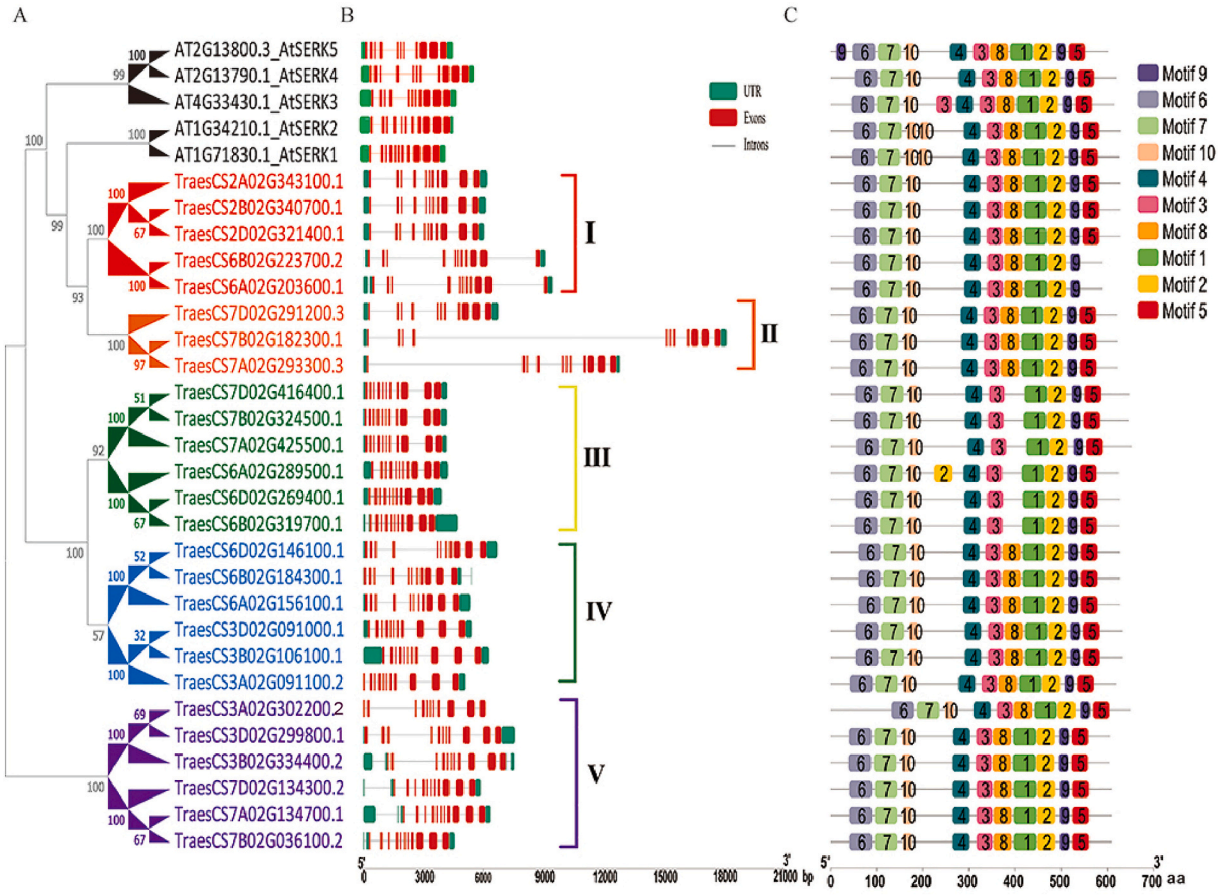

Fig. 3. Phylogenetic analysis of SERK proteins in wheat and *Arabidopsis*. (A) A total of 26 SERK proteins of wheat and 5 SERK proteins of *Arabidopsis* were used to construct the maximum-likelihood (ML) phylogenetic tree by MEGA X with 1000 bootstrap replicates. The wheat SERK proteins were divided into nine groups (I-V), which were marked with different colors. (B) The gene structure analysis of 26 *TaSERK* genes. All *TaSERK* genes contain 11 Exons and 10 introns. Red boxes represent exons, black lines represent introns, and UTRs were marked with green boxes. (C) Motif distribution of 26 TaSERK proteins. 10 motifs in TaSERK proteins were represented by different colors.

members. Among them, 9 motifs (1, 2, 3, 4, 5, 6, 7, 9, and 10) were present in all wheat TaSERK and *Arabidopsis* AtSERK proteins. The motif 8 was present in almost all wheat TaSERK and *Arabidopsis* AtSERK proteins, except in 6 homoeologous TaSERK proteins of group III. Overall, the motifs of different SERK members were conserved between wheat and *Arabidopsis*, impling that SERK family proteins were conserved in different plant species, and might have functional redundancy.

3.3. *TaSERK1 are involved in wheat response against R. cerealis*

SERK family proteins play a key role in the resistant responses against pathogens. However, none of specific *SERK* genes was reported to play an important role against *R. cerealis* in wheat. In this study, to identify the potential roles of *TaSERKs* in resistant responses against the fungal pathogen, we performed RNA-Seq (RNA-sequencing) analysis upon *R. cerealis* infection. The results showed that, after inoculation with *R. cerealis* Rc207, the transcript levels of 20 *TaSERK* genes were not significantly changed compared with mock-treatment. Only 6 *TaSERK* genes on the chromosome 2A, 2B, 2D, 3A, 3B, and 3D were significantly up-regulated upon *R. cerealis* infection (Fig. 4A).

Then, to further analyze the expression patterns of these six genes, we respectively calculated the transcript fold change of the 6 *TaSERK* genes in the sharp eyespot-resistant recombinant inbred lines (RILs-R) derived from the cross 'Shanhongmai' × 'Wenmai 6'. As shown in Fig. 4B, all the 6 *SERK* genes exhibited higher transcript fold change in the RIL-R after inoculation with *R. cerealis* Rc207 relative to mock-treatment, especially the transcript folds of *TaSERKs* on the chromosome 2A, 2B, 2D were higher than those on the chromosome 3A, 3B, 3D. At 10 dpi, the transcript levels of *TaSERK-2A, TaSERK-2B, and TaSERK-2D* were elevated to 3.92, 3.28 and 2.95 fold compared with mock-treatment in the RIL-R, while the *TaSERK-3A, TaSERK-3B,* and *TaSERK-3D* were only upregulated to 2.50, 1.81 and 2.46 fold in the RIL-R compared to mock-treatment. These data suggested that all the 6 homoeologous *TaSERK* genes, especially 3 homoeologous *TaSERK* genes (TraesCS2A02G343100.1, TraesCS2B02G340700.1, and TraesCS 2D02G321400.1) were involved in wheat resistant responses against *R. cerealis*. Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi) results indicated that these three *TaSERK* genes on chromosome 2A, 2B, 2D were homologous to *Arabidopsis thaliana AtSERK1*. Thus these three homologous *TaSERK* genes were named as *TaSERK1-2A, TaSERK1-2B,* and *TaSERK1-2D*. Sequence analysis showed that the TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D proteins were highly similar, with 99.47 % identity, especially their intracellular kinase domains are 100 % identical. As shown in Fig. S2, the TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D were consisted of 627 aa residues, with a signal peptide (no. 1–30 aa), a leucine zipper (no. 31–77 aa), five LRR motifs (no. 78–197 aa), a proline rich domain (no. 198–235 aa), a transmembrane region (no. 236–278 aa), a juxtamembrane domain (no. 279–302 aa), a cytosolic Ser/Thr protein kinase domain (no. 303–579 aa), and a c-terminal motif (no. 580–627 aa).

Furthermore, we deployed RT-qPCR to examine the transcript profiles of *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* in two middle resistant cultivars (CI12633, Shanhongmai) and two highly susceptible

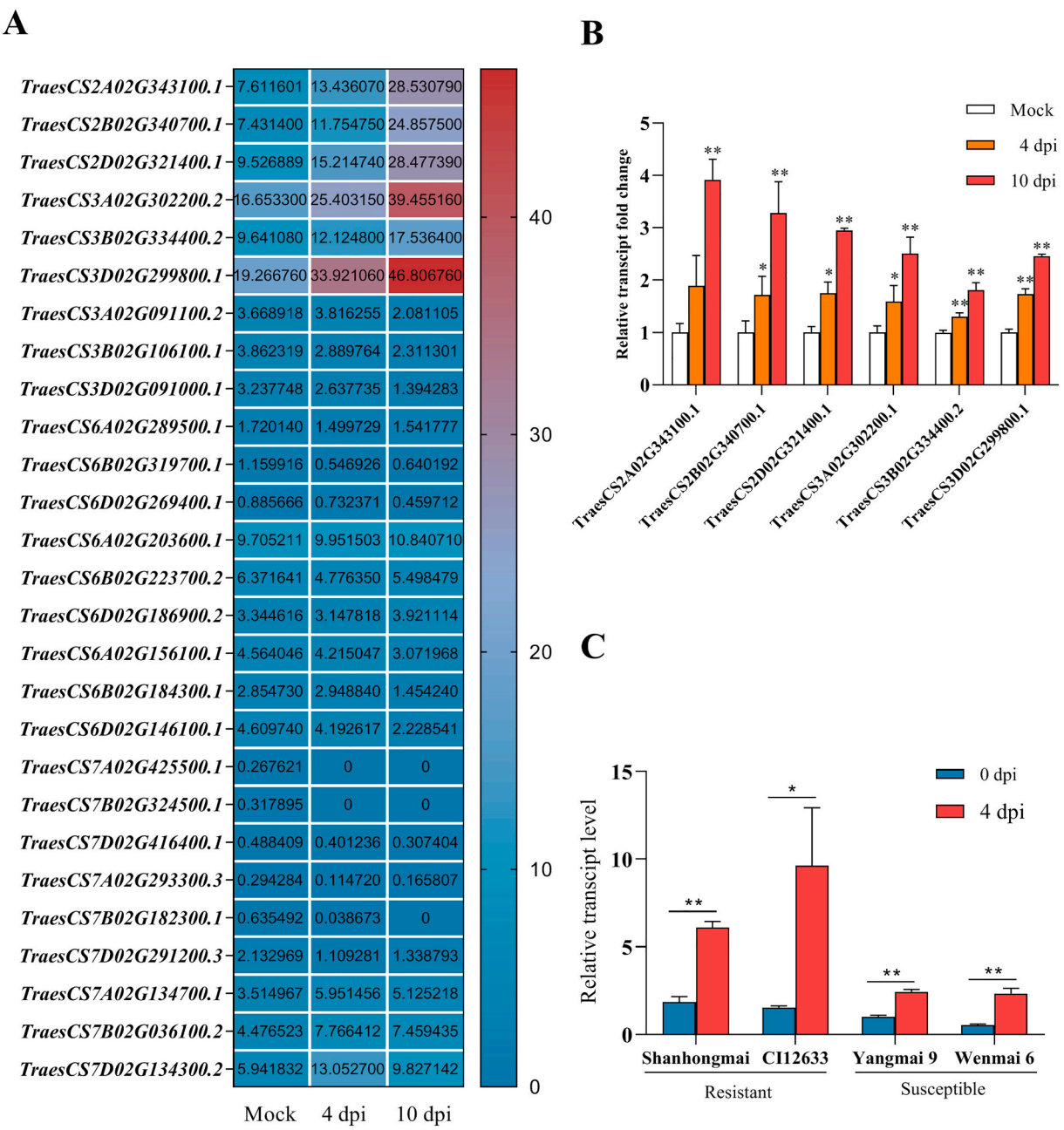

Fig. 4. *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* were involved in wheat response against *R. cerealis*. (A) The heatmap of *TaSERK* family genes in RNA-seq data in the resistant recombinant inbred lines (RILs) derived from the cross Shanhongmai × Wenmai 6 upon *R. cerealis* infection. (B) The transcript fold change of the 6 homoeolog *TaSERK* genes in the *R. cerealis* resistant (RIL-R). The transcript levels of mock treatment were set to 1. (C) Expression patterns of *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* in four wheat cultivars with different resistance degrees at 4 dpi with *R. cerealis*. The expression level of *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* at 0 dpi in Yangmai 9 was set to 1.

wheat cultivars (Wenmai 6, Yangmai 9) at 0 and 4dpi with *R. cerealis* strain Rc207. The results showed that after inoculation with *R. cerealis*, *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* transcript levels were significantly higher in resistant wheat germplasms CI12633 and Shanhongmai, than those in the susceptible wheat cultivars Wenmai 6 and Yangmai 9 (Fig. 4C). These results suggested that the transcript abundance of *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* was corresponding to the *R. cerealis* resistant degree of the four wheat cultivars.

Taken together, the *TaSERK1* genes on chromosome 2A, 2B, and 2D were in response to *R. cerealis* infection, and may play an important role in wheat resistant reactions against the fungal pathogen.

3.4. Silencing of TaSERK1 impairs wheat resistance to sharp eyespot

To further identidy the function of *TaSERK1* in wheat resistant responses against *R. cerealis*. A Barley stripe mosaic virus induced silencing (VIGS) experiment to investigate the defence role of *TaSERK1* against wheat sharp eyespot. As shown in Fig. 5A, two weeks after infection with BSMV, typical infection symptoms appeared in the newly emerged leaves and the transcript of BSMV *coat protein* (*CP*) gene was detected, indicating that the BSMV had infected wheat plants. At the same time, RT-qPCR assays were used to measure the silencing efficiency. The results showed that the transcript levels of *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* in BSMV: TaSERK1 plants were significantly lower than that in BSMV: GFP-infected CI12633 plants, indicating that *TaSERK1-*

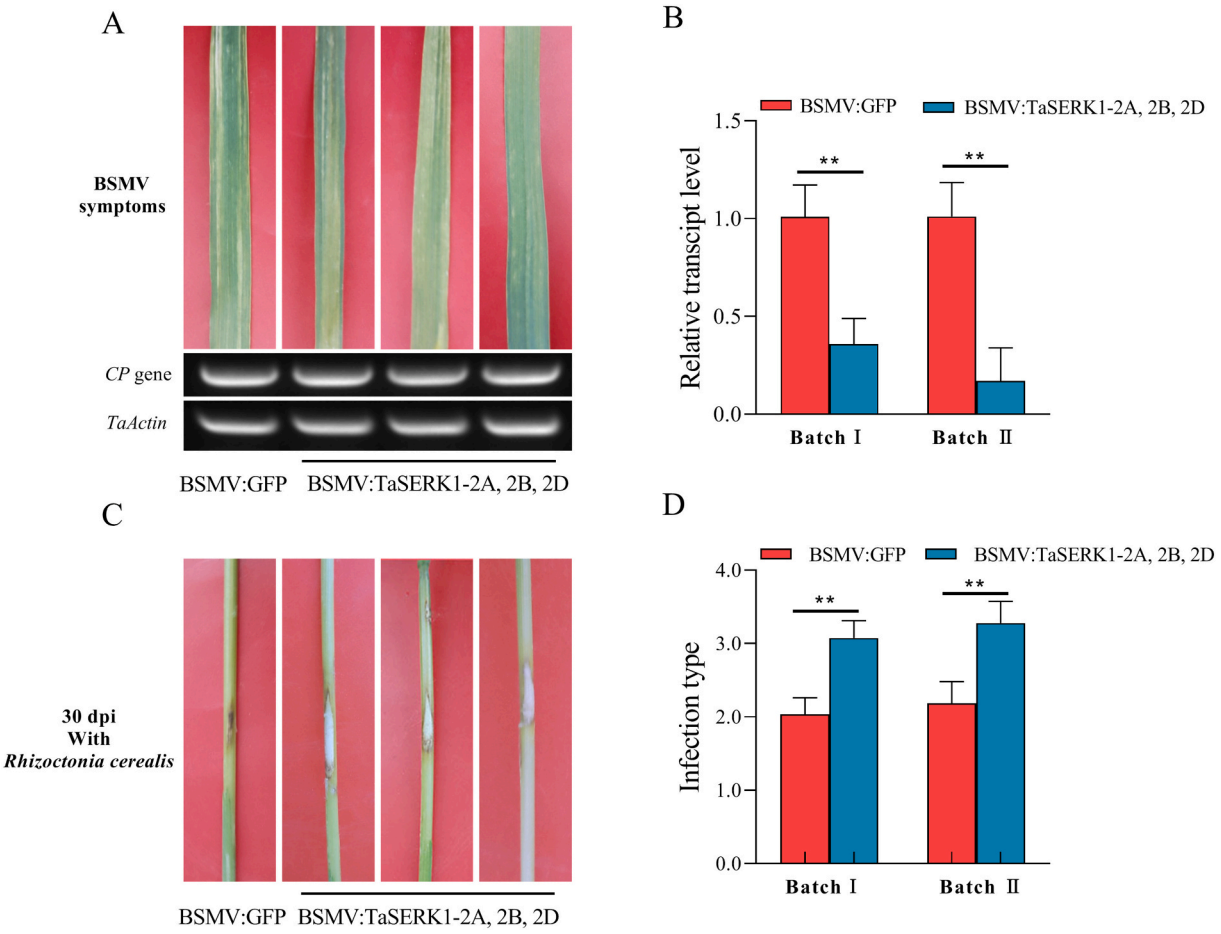

Fig. 5. Silencing of *TaSERK1* increases wheat susceptibility to *R. cerealis*. (A) The typical symptom of BSMV on wheat leaves after infected by BSMV: GFP or BSMV: TaSERK1 for two weeks. The transcript of the BSMV *coat protein (CP)* was used as a marker gene to detect the BSMV infection. (B) The transcript level of *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* in TaSERK1-silenced and BSMV: GFP-infected wheat CI12633 plants. The transcript level of *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D* in BSMV: GFP- infected wheat plants was set to 1. Significant differences were determined based on three technical repeats (*t*-test: $^{**}P < 0.01$, $^{*}P < 0.05$). (C) Sharp eyespot symptoms on *TaSERK1*-silenced and control wheat plants at 30 dpi with *R. cerealis*. (D) Mean Infection type (IT) of *TaSERK1*-silenced and control wheat plants at 30 dpi with *R. cerealis* in two independent batches (t-test: $^{**}P < 0.01$). Bars indicate SEs of the mean.

2A, TaSERK1-2B, and TaSERK1-2D was successfully silenced (Fig. 5B). Subsequently, these *TaSERK1* -silenced and control wheat plants were subjected to resistance assessment against sharp eyespot after inoculation with *R. cerealis* strain Rc207.

The results showed that, after inoculated with *R. cerealis* Rc207 for 30 days, *TaSERK1* silenced wheat plants exhibited more serious disease symptoms of sharp eyespot, including larger necrotic areas, and bigger infection types (ITs) (Fig. 5C). The disease scoring results showed that in two VIGS batches, the average ITs of *TaSERK1* silenced wheat plants were 3.07 and 3.28, and their disease indexes were 61.5 and 65.6; while the ITs of control BSMV: GFP -infected CI12633 plants were 2.03–2.18, their disease indexwere 40.7 and 43.7 (Fig. 5D, Supplementary Fig. S3). The results indicated that *TaSERK1* were required for wheat resistance to *R. cerealis*.

3.5. TaSERK1 interacts and phosphorylates TaRLCK1B

In *Arabidopsis*, AtSERK3/BAK1 interacts with and directly phosphorylates *Botrytis*-induced kinase 1 (BIK1) and positively regulates plant immunity [30]. In previous study, a receptor-like cytoplasmic kinase TaRLCK1B was shown to be involved in resistance response to *R. cerealis* in wheat [17]. To investigate whether TaSERK1 could interact with TaRLCK1B, we conducted a yeast two-hybrid and Bi-molecular fluorescence complementation (BiFC) assays. As TaSERK1-2A, TaSERK1-2B, TaSERK1-2D proteins were highly conserved, especially their intracellular kinase domains are identical (Fig. S2). Thus, one of the three homologues, TaSERK1-2D, was used in the following protein interaction and phosphorylation assays.

The results showed that, TaSERK1-2D can interact with TaRLCK1B in vitro and vivo (Fig. 6A, B). Subsequently, a phosphorylation assay was used to investigate whether TaSERK1-2D phosphorylates TaRLCK1B. In this assay, the purified HA-TF-TaRLCK1B alone, or HA-TF-TaRLCK1B with His-TF-TaSERK1-2D proteins were incubated in kinase reaction buffer, then 50 μM phos-tag™ Acrylamide AAL-107 assay with anti-HA monoclonal antibody was used to test phosphorylation proteins. As shown in Fig. 6C, TaRLCK1B has slightly autophosphorylation in vitro, and when TaSERK1-2D co-incubated with HA-TF-TaRLCK1B, the phosphorylation degree of TaRLCK1B was significantly enhanced. The phosphorylation assay indicated TaSERK1-2D could phosphorylate TaRLCK1B.

Furthermore, we predicted the potential phosphorylation sites on the TaSERK1 and TaRLCK1B. The results showed that, the predicted phosphorylation sites on TaSERK1 were identical with *Arabidopsis* AtSERK3/AtBAK1, including the vital phosphorylation sites required for interacting with and transphosphorylation of AtBIK1 in plant immune responses (287D，450 T, 455 T on AtBAK1, or 303D，466 T and 471 T on TaSERK1, Fig. 7A) [30,31]. There were some different phosphorylation sites were predicted between TaRLCK1B and AtBIK1, however the major phosphorylated sites were conserved between them (Fig. 7B). In *Arabidopsis*, AtBIK1 T237 is an essential phosphorylation site mediated by

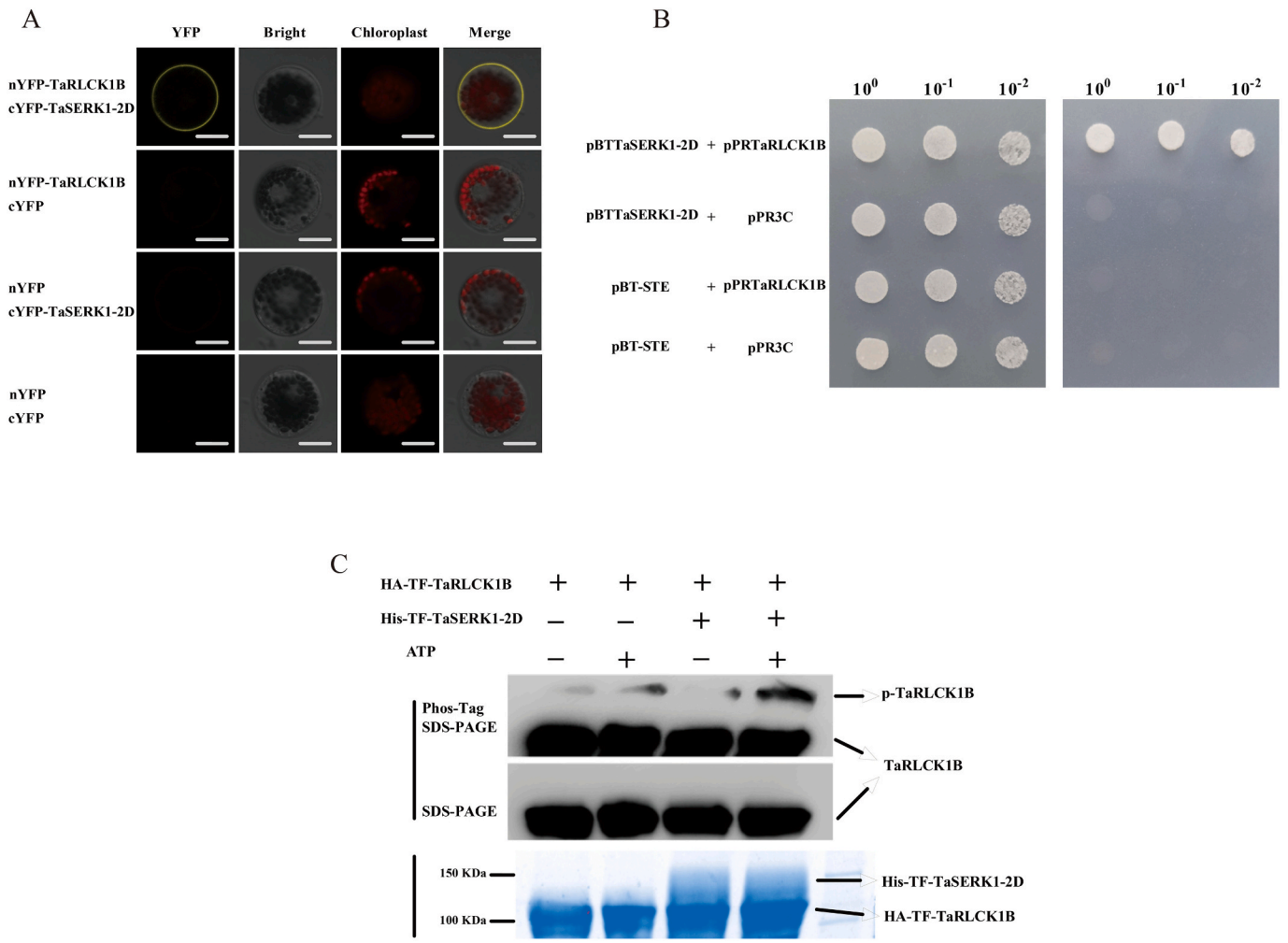

Fig. 6. TaSERK1-2D interact with TaRLCK1B and phosphorylate it. (a) The Bi-FC assay shows that TaSERK1-2D and TaRLCK1B interacted in wheat protoplasts. Bar = 50 μm. (b) The Y2H assay shows that TaSERK1-2D interacted with TaRLCK1B. The interaction is verified based on colony yeast cells grown on SD-LTHA plates. (c) The phosphorylation assay shows that TaRLCK1B was phosphorylated by TaSERK1-2D in vitro. The phosphorylated proteins were detected by Phos-tag SDS-PAGE.

AtSERK3/AtBAK1, and the corresponding major transphosphorylation site mediated by TaSERK1 in wheat was TaRLCK1B 244 T. Moreover, the 157Y, 250Y and 257Y on TaRLCK1B were conserved with 150Y, 243Y, 250Y on AtBIK1, which were required for plant innate immunity in *Arabidopsis* [30]. These results suggested that TaSERK1 and TaRLCK1B likely shared similar regulatory mechanisms with *Arabidopsis* AtBAK1 and AtBIK1 in plant innate immunity.

Additionally, we analyzed the phosphorylation sites required for AtBAK1/AtBIK1 interaction on all *Arabidopsis* and wheat SERKs. As shown in Fig. S4, although the key sites required for phosphorylation of BIK1 (387D，450 T and 455 T on BAK1) were present on the *Arabidopsis* AtSERK1–4 and 17 wheat TaSERKs, actually only several of them were required for plant immunity. On the contrary, unlike the conserved kinase domains, the extracellular domains of different SERK proteins showed more diversity, especially in the signal peptide, leucine zipper, LRR1, LRR5, and proline-rich domains. The results imply the defensive function of TaSERK1 was affected by the important phosphorylation sites, and their specific extracellular LRR regions.

4. Discussion

The wheat sharp eyespot is one of the most devasting diseases of wheat grain production [2]. When wheat plants infected by *R. cerealis*, the pathogen usually causes necrosis of the wheat stem basal tissues, and stunts the growth of wheat seedlings, leads to serious yield loss and wheat grain quality decreasing. To solve with these problems, it is vital to unravel the mechanisms and identify the effective disease-resistant genes during wheat resistant responses against the pathogen. In this study, we performed a genome-wide analysis of SERK family in wheat, and a total of 26 *TaSERK* candidate genes were identified from the wheat genome (*Triticum aestivum*). By analysising the wheat RNA-Seq data upon *R. cerealis*, we found that the transcript levels of 20 *TaSERK* genes were not significant changed, only 6 homoeologs of two *TaSERK* genes on the chromosome 2A, 2B, 2D and 3A, 3B, 3D were up-regulated upon *R. cerealis*. Especially, *TaSERK1*-2A, *TaSERK1*-2B, and *TaSERK1*-2D were up-regulated in higher levels compared with other *TaSERK* genes. Together with a VIGS assays, we found that silencing of *TaSERK1* significantly impaired wheat resistance to sharp eyespot. Furthermore, we found that TaSERK1 could directly interacted with a defence-associated receptor-like cytoplasmic kinase TaRLCK1B, and phosphorylated it in vitro, which provides insights into the role of the wheat TaSERK1/RLCK1B complex in innate immunity.

For the sessile plants, the *SERK* genes play a crucial role in plant immune responses [6,32]. In *Arabidopsis*, the AtSERK3/BAK1 acted as a important regulator in both pattern-triggered immunity (PTI) and effector-triggered immunity (ETI) [32]. On the one hand, multiple pattern recognition receptors (PRRs) should bind with AtSERK3/BAK1 to activate the PTI responses, on the other hand, mutant of *AtSERK3/BAK1* impaired the ETI responses when challenged with bacteria [33,34]. In rice, *OsSERK1* was significantly induced by the rice blast

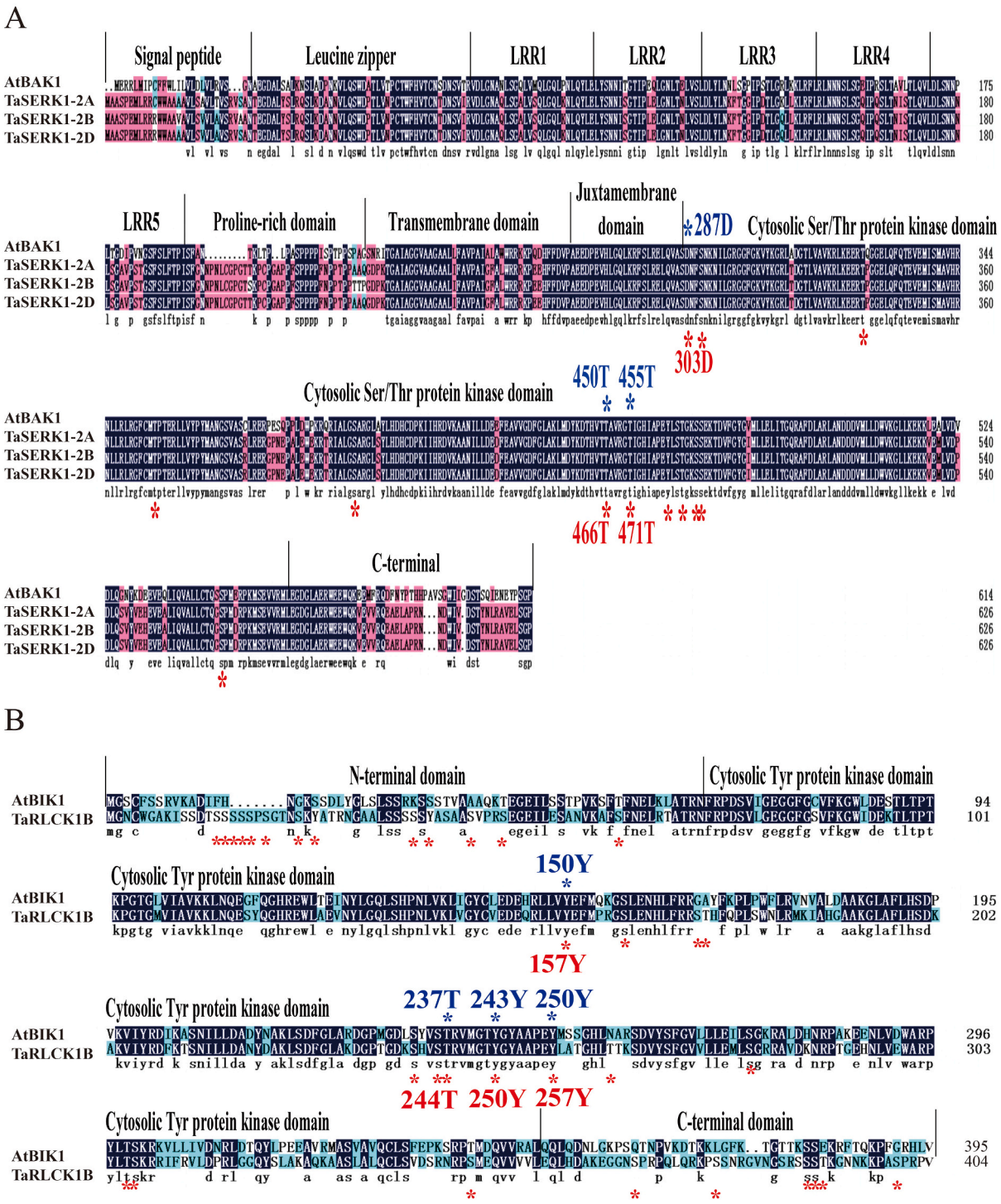

Fig. 7. The potential phosphorylation analyses of the TaSERK1 and TaRLCK1B. (a) The potential phosphorylation sites on the TaSERK1. * indicates the residues identified required for interacting with and transphosphorylation of AtBIK1 in *Arabidopsis* AtSERK3/AtBAK1. * indicates the potential phosphorylation sites on the TaSERK1 predicted by the online software NetPhos-3.1. (b) The potential phosphorylation sites on the TaRLCK1B. * indicates the residues identified involving in plant innate immunity or AtSERK3/AtBAK1-mediated transphosphorylation on AtBIK1. * indicates the potential phosphorylation sites on the TaRLCK1B predicted by the online software NetPhos-3.1.

fungus, overexpression of *OsSERK1* enhanced the rice host resistance to the blast fungus [15]. The OsSERK2 also proved positively regulates immunity against *Xanthomonas oryzae pv. oryzae (Xoo)*, by forming a constitutive complex with XA21 [16]. However, it remains unknown that how many *SERK* genes are available in wheat genome, and which *SERKs* were involved in wheat resistantance to sharp eyespot. Our study firstly pointed out the specific *TaSERK* genes, *TaSERK1-2A, TaSERK1-2B, and TaSERK1-2D*, were involved in wheat resistant responses against *R. cerealis*.

The phosphorylation of the receptor-like cytoplasmic kinases (RLCKs) by PRRs is an essential step to initiate immune signaling. In *Arabidopsis*, the receptor-like cytoplasmic kinase1 (BIK1) and PBS1-like 1 (PBL1), have been reported could interacted and phosphorylated by BAK1, FLS2, PEPR1,and PEPR2 [30,35,36]. However, little is known

about the specific TaRLCKs were directly interacted with PRRs, and involved in defence signaling in wheat. Here, we reported a defence associated receptor-like cytoplasmic kinase TaRLCK1B, could directly interacted with TaSERK1, and phosphorylated by TaSERK1 in vitro. The phosphorylation prediction results also showed that, the potential phosphorylation sites involved in wheat innate immunity and interacting between TaSERK1 and TaRLCK1B were same with AtBAK1/AtBIK1 complex in *Arabidopsis*, implying TaSERK1 and TaRLCK1B were likely sharing similar regulatory mechanisms with *Arabidopsis* AtBAK1/AtBIK1 complex in plant innate immunity.

In addition to the key phosphorylation sites in intracellular kinase domains, the specific extracellular domains were also playing an important role in perceiving different pathogen- or microbe-associated molecular patterns (PAMPs or MAMPs) or damage-associated molecular patterns (DAMPs) and initiating pattern-triggered plant innate immunity. In *Arabidopsis*, when the plants infected by bacterial, the conserved bacterial N-termianl epitope flg22 and Elongation factor-Tu EF-Tu were respectively perceived by the FLS2 and EFR, inducing heteromerization with AtSERK3/AtBAK1 in ectodomains and initiating plant immunity [37,38]. Recently, it was reported that Serine-rich endogenous peptides (SCOOPs) deriving from *Arabidopsis* plants or several pathogens, induce the AtMIK2/AtBAK1 complex formation, and relay the signaling through BIK1 to trigger plant immune responses [13,39]. To deepen understanding of the pathogenicity mechanisms of the *R. cerealis*, our lab has established a high-quality genome assembly of *R. cerealis* Rc207, identified 831 candidate secretory effectors and validated the functions of a series up-regulated candidate effectors during pathogen infection [18,40–43]. However, how the TaSERK1 involved in perception of effectors or secreted proteins of *R. cerealis* to initiate immune responses are still unknown. It is very interesting to investigate how the extracellular LRR regions of TaSERK1 involved in interaction with other immunity associated PRRs and perception of effectors or secreted proteins of *R. cerealis* in future.

Taken togather, TaSERK1 could interact with and phosphorylate TaRLCK1B to mediate immunity to *R. cerealis* in wheat. Our study sheds light on the understanding of the wheat SERKs in innate immunity against *R. cerealis*, and provided a theoretical bait to identify more candidate resistant genes for improving wheat resistance against sharp eyespot in wheat.

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CRediT authorship contribution statement

Z. Z. and W. S. designed the research, supervised the work, revised, and edited the manuscript. H. Q. performed the majority of the experiments, analyzed the data, and wrote the draft manuscript. J. Y. and X. Y. planted and assessed these wheat materials. All authors contributed to the article and approved the submitted version.

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Declaration of competing interest

The authors declare that they have no competing interests.

Data availability

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

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