Overexpression of TaSTT3b-2B improves resistance to sharp eyespot and increases grain weight in wheat

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Summary

STAUROSPORINE AND TEMPERATURE SENSITIVE3 (STT3) is a catalytic subunit of oligosaccharyltransferase, which is important for asparagine-linked glycosylation. Sharp eyespot, caused by the necrotrophic fungal pathogen Rhizoctonia cerealis, is a devastating disease of bread wheat. However, the molecular mechanisms underlying wheat defense against R. cerealis are still largely unclear. In this study, we identified TaSTT3a and TaSTT3b, two STT3 subunit genes from wheat and reported their functional roles in wheat defense against R. cerealis and increasing grain weight. The transcript abundance of TaSTT3b-2B was associated with the degree of wheat resistance to R. cerealis and induced by both R. cerealis and exogenous jasmonic acid (JA). Overexpression of TaSTT3b-2B significantly enhanced resistance to R. cerealis, grain weight, and JA content in transgenic wheat subjected to R. cerealis stress, while silencing of TaSTT3b-2B compromised resistance of wheat to R. cerealis. Transcriptomic analysis showed that TaSTT3b-2B affected the expression of a series of defense-related genes and JA biosynthesis–related genes, as well as genes coding starch synthase and sucrose synthase. Application of exogenous JA elevated expression levels of the abovementioned defense- and grain weight–related genes, and rescuing the resistance of TaSTT3b-2B–silenced wheat to R. cerealis, while pretreatment with sodium diethyldithiocarbamate, an inhibitor of JA synthesis, attenuated the TaSTT3b-2B–mediated resistance to R. cerealis, suggesting that TaSTT3b-2B played critical roles in regulating R. cerealis resistance and grain weight via JA biosynthesis. Altogether, this study reveals new functional roles of TaSTT3b-2B in regulating plant innate immunity and grain weight, and illustrates its potential application value for wheat molecular breeding.

Keywords: Grain weight, jasmonic acid, Rhizoctonia cerealis, STAUROSPORINE AND TEMPERATURE SENSITIVE3 (STT3), transgenic wheat, Triticum aestivum.

Introduction

The wheat sharp eyespot disease, primarily caused by Rhizoctonia cerealis, is a destructive disease of wheat in warm humid regions of the world, including China (Chen et al., 2008, 2013), the United Kingdom (Clarkson and Cook, 1983), New Zealand (Cromey et al., 2002), and Egypt (Hammouda, 2003). Dark-bordered lesions appear on the stem bases of young and mature wheat plants about seven days after infection by R. cerealis. Subsequently, peduncle rot occurs, leading to lodging at the internode and even premature spike senescence or ripening, which is also known as white heads (Ren et al., 2020). Although chemical control is available for wheat fungal pathogens, fungicide resistance has become increasingly prominent due to long-term use of a single chemical fungicide (Chen et al., 2007). Breeding resistant cultivars with resistance against R. cerealis is a more cost-effective and environmental friendly alternative. Therefore, it is urgent to identify genes that play important roles in plant defense response and unravel their underlying functional mechanisms. Although few quantitative trait loci (QTLs) have been reported to be associated with resistance to sharp eyespot disease in wheat (Chen et al., 2013; Wu et al., 2017), no QTL has been applied to molecular breeding. Thus, the molecular basis for the defense and signaling pathways that underlie the interaction between the host wheat with R. cerealis remains largely unknown.

Asparagine N-glycosylation is a major class of posttranslational modification, which is catalyzed by the oligosaccharyltransferase (OST) complex in the lumen of the endoplasmic reticulum (ER). In plants, N-glycosylation has been shown to play vital roles in diverse aspects of development and physiology, including salt tolerance and plant immunity (Häweker et al., 2010; Jia et al., 2020; Jiao et al., 2020; Koiva et al., 2003; Lerouge et al., 1998; Liebminger et al., 2009; Liu and Howell, 2010; Nagashima et al., 2018; Saijo et al., 2009). As one subunit of the OST complex, the STAUROSPORINE AND TEMPERATURE SENSITIVE3 (STT3) subunit is critical for the catalytic activity of OST that transfers a preassembled glycan chain (Glc3Man9GlcNAc2) to a special asparagine residue in the sequon N-X-Ser/Thr (X is except Pro) of acceptor proteins (Aebi, 2013; Lu et al., 2018; Ruiz-Canada et al., 2009). Mammalian cells contain two isoforms of STT3. STT3a is primarily required for co-translational glycosylation of the nascent polypeptide and STT3a can also mediate posttranslational glycosylation of the sites that have been missed by STT3a (Ruiz-Canada et al., 2009).
In recent years, a number of major QTLs and regulatory genes, which control grain size and grain weight of wheat, have been isolated. These cloned grain weight–related genes can be divided into several types, such as serine carboxypeptidase, ubiquitin E3 RING ligase, G protein, sucrose synthase, ubiquitin receptor, mitogen-activated protein kinase, purine permease, and B3-type RING ligase, G protein, sucrose synthase, ubiquitin receptor, serine carboxypeptidase, ubiquitin E3 (Häweker et al., 2010; Koiva et al., 2003). Interestingly, the STTa gene cloned from Spartina alterniflora completely complements the salt-sensitive phenotype of the Arabidopsis stt3a mutant (Jiang et al., 2015), suggesting that both STT3a genes are conserved in the two plant species. All these studies imply that STT3a is essential for N-glycosylation and stress tolerance of plants (Jeong et al., 2018; Koiva et al., 2003). In Arabidopsis, BRASSINOSTEROID-INSENSITIVE 1–associated receptor kinase 1 (BAK1), somatic embryogenesis receptor kinase4 (SERK4), and BAK1-INTERACTING RECEPTOR-LIKE KINASE 1 (BIR1) negatively regulate the process of cell death (He et al., 2007; Kemmerling et al., 2007; Gao et al., 2009), and STTa was identified as an important regulator of bak1/serk4 cell death (de Oliveira et al., 2016). In the stt3a mutant, the functions of the receptor kinase EF-TU RECEPTOR1 (Häweker et al., 2010) and the BAK1/SERK4 pathway (de Oliveira et al., 2016) involved in plant innate immunity pathways were impaired. Similarly, loss of function of STT3a also suppressed the autoimmune phenotype in bir1-1 (Zhang et al., 2015). The latest research indicated that a mutation in STT3a impaired N-glycosylation and showed greater susceptibility to Pst DC3000 infection (Jia et al., 2020). However, little is known about the involvement of STT3 in defense responses of wheat against various pathogens.

Jasmonic acid (JA) signaling pathways have been reported to induce resistance against necrotrophs, such as Alternaria brassicicola (Chen et al., 2021; Thomma et al., 1998), Botrytis cinerea (Chen et al., 2021; Liu et al., 2019), Bipolaris sorokiniana (Singh et al., 2019), and R. solani (Taheri and Tarighi, 2010). In rice (Oryza sativa), cpm2 and hebiba mutants, which are defective for allene oxide cyclase activity and JA production, show increased susceptibility to Magnaporthe oryzae. Application of exogenous JA restored resistance to M. oryzae in these mutants, suggesting that JA was required for this response (Riemann et al., 2013). Exogenous JA application to rice also induced resistance against R. solani (Taheri and Tarighi, 2010).

In recent years, a number of major QTLs and regulatory genes, which control grain size and grain weight of wheat, have been isolated. These cloned grain weight–related genes can be divided into several types, such as serine carboxypeptidase, ubiquitin E3 RING ligase, G protein, sucrose synthase, ubiquitin receptor, mitogen-activated protein kinase, purine permease, and B3-type transcription factor (Hou et al., 2014; Li et al., 2008, 2011, 2021; Su et al., 2011; Sun et al., 2018; Xia et al., 2013; Xu et al., 2018; Yin et al., 2020). Nevertheless, no study has reported the regulatory role of STT3 in grain weight. As an essential plant hormone, JA plays critical roles in plant defense and development (Lyons et al., 2013). However, studies about JA involved in regulation of grain weight are scarce.

In this study, we identified two STT3 subunit genes TaSTT3a and TaSTT3b in wheat and provided evidence that TaSTT3b-2B positively regulated defense against R. cerealis. We explored the mechanism underlying TaSTT3b-2B–mediated resistance to R. cerealis, in which TaSTT3b-2B enhanced the transcript levels of various defense-related genes. TaSTT3b-2B also affected the JA content and the expression of JA-synthesis-related genes, and application of exogenous JA increased resistance to R. cerealis, whereas pretreatment with sodium diethylthiocarbamate (DIECA), an inhibitor of JA synthesis, reduced the TaSTT3b-2B–mediated resistance to R. cerealis. Furthermore, we found that TaSTT3b-2B overexpression up-regulated the expression of the starch synthase and sucrose synthase genes, leading to larger grains produced in transgenic wheat infected with R. cerealis. Thus, our results indicate an essential role of TaSTT3b-2B in wheat defense response to R. cerealis and in controlling grain size, and overall, its potential application in wheat molecular breeding.
highly conserved with STT3b proteins in Arabidopsis and rice, suggesting that they may be orthologs of TaSTT3b proteins (Figure S2). The genomic sequences of TaSTT3b-2B were also obtained from the susceptible wheat cultivar Zhoumai18. Genomic sequence alignment showed that TaSTT3b-2B from CI12633 and Zhoumai18 had 98.79% identity, and many single-nucleotide polymorphisms (SNPs) existed (Figure S3). We summarized TaSTT3b and the reported QTLs controlling sharp eyespot resistance on the chromosome 2B (Jiang et al., 2016; Liu et al., 2021; Wu et al., 2017), with their genomic distributions annotated using the wheat genome database. TaSTT3b-2B located on the chromosome 2B was near the SSR marker Xwmc149 associated with the Qses.jaas-2B locus (Figure S4).

To investigate the evolutionary relationships of TaSTT3a and TaSTT3b, a neighbor-joining phylogenetic tree was constructed using ClustalW to align the protein sequences of TaSTT3a and TaSTT3b and their homologs from various plant species. The dendrogram showed that these proteins were clustered into two clades, STT3a and STT3b. Both TaSTT3b and TaSTT3a proteins

Figure 1  Identification of TaSTT3b in wheat. (a) Expression patterns of the three TaSTT3b homologs in the wheat line CI12633 at 0, 4, 7, 10, 14, and 21 dpi with *R. cerealis* infection. TaSTT3b transcription level in mock-treated plant (0 dpi) was set to 1. (b) Relative transcript levels of TaSTT3b-2B in five wheat cultivars at 4 dpi with *R. cerealis* infection. The expression level of TaSTT3b-2B in Wenmai6 was set to 1. Data were normalized to wheat TaActin. Statistically significant differences are derived from the results of three independent replications (t-test: *P* < 0.05, **P** < 0.01). Bars indicate standard errors of the means. (c) Genomic structure of the TaSTT3b-2B gene. Frames and dotted lines represent exons and introns, respectively. (d) Phylogenetic analysis of the TaSTT3a, TaSTT3b, and other STT3 proteins. The phylogenetic tree was constructed from a complete alignment of 26 STT3 protein sequences using the neighbor-joining method with 1000 bootstrap repetitions with the MEGA 6.0 program.
were most closely related to their homologs in *Hordeum vulgare* (Figure 1d).

**Subcellular localization of the TaSTT3b-2B protein**

Prediction of subcellular localization with a transmembrane domain hidden Markov model (TMHMM version 2.0; http://www.cbs.dtu.dk/services/TMHMM) suggested that the TaSTT3b-2B protein was a typical transmembrane protein with 11 transmembrane domains (Figure S5a). To experimentally confirm this prediction, the full-length coding sequence of TaSTT3b-2B was fused to the C-terminus of green fluorescent protein (GFP). The GFP-TaSTT3b-2B fusion sequence and an ER marker mCherry-HDEL (Nelson et al., 2007) were coexpressed in wheat mesophyll protoplasts. The results showed that the fluorescence signal of GFP-TaSTT3b-2B could be merged with that of mCherry-HDEL in the ER (Figure S5b), indicating that the TaSTT3b-2B protein localizes to the ER.

**Silencing of TaSTT3a and TaSTT3b genes reduced wheat resistance to *R. cerealis***

To determine the potential functions of TaSTT3a and TaSTT3b in wheat defense response to *R. cerealis* infection, we generated TaSTT3a- and TaSTT3b-silenced wheat plants through the barley stripe mosaic virus (BSMV)-based virus-induced gene silencing (VIGS) technique. Two specific fragments of TaSTT3a-1B and TaSTT3b-2B were amplified to construct two recombinant BSMV vectors, BSMV-TaSTT3a and BSMV-TaSTT3b, respectively (Figure S6). Figure S7a showed the BSMV-VIGS experiment flowchart. Off-target prediction of the TaSTT3a-1B VIGS fragment by Si-Fi software indicated efficient siRNA hits of TraesCS1B02G352700.1, TraesCS1D02G342400.1, and TraesCS1A02G340400.1 (Figure S7b), while off-target prediction of the TaSTT3b-2B VIGS fragment showed efficient siRNA hits of TraesCS2B02G587900.1, TraesCS2D02G558800.1, and TraesCS2A02G55600.1 (Figure S7c). The prediction analyses suggested efficient silencing of TaSTT3a and TaSTT3b, and no off-target was predicted for the VIGS constructs in wheat. At 10 dpi with BSMV, all CI26333 wheat seedlings inoculated with BSMV: GFP (control), BSMV:TaSTT3a, and BSMV:TaSTT3b showed mild chlorotic mosaic symptoms on the fourth leaves, and the expression of the BSMV coat protein (cp) gene was clearly detected (Figure 2a and Figure S8a), indicating that the BSMV-VIGS system functioned well. To confirm silencing efficiency, qRT-PCR was used to detect the transcript levels of TaSTT3a, as well as TaSTT3b-2A, TaSTT3b-2B, and TaSTT3b-2D in the sheaths of BSMV-infected wheat plants at 7 dpi with *R. cerealis*. As shown in Figure 2b, compared with the control (BSMV:GFP) plants, the transcriptional levels of TaSTT3b-2A, TaSTT3b-2B, and TaSTT3b-2D in the BSMV:TaSTT3b-infected plants were reduced by 74.23%, 90.02%, and 86.26%, respectively. Similarly, in BSMV:TaSTT3a-infected wheat seedlings, the transcriptional level of TaSTT3a was reduced by 77.32% (Figure S8b). These results suggest that TaSTT3a and TaSTT3b were successfully knocked down in BSMV:TaSTT3a- and BSMV:TaSTT3b-infected plants, respectively.

Subsequently, these BSMV-infected wheat plants were inoculated with the *R. cerealis* isolate WK207. At 14 dpi with *R. cerealis* inoculation, the sheaths of BSMV:TaSTT3b-infected CI26333 plants displayed more serious necrosis due to sharp eyespot than did BSMV:GFP-infected (control) plants (Figure 2c). The infection types (ITs) of control plants were 0 and 1, while those of 21.88% and 3.13% of the BSMV:TaSTT3b-infected plants were 2 and 4, respectively (Figure 2d). The average IT value of the BSMV:TaSTT3b-infected plants was 1.13 and was higher than that of BSMV:GFP-treated plants (0.60) at 14 dpi with *R. cerealis* inoculation (Figure 2e). The average area of lesions on TaSTT3b-infected plants was 1.50 cm², whereas the average area on the sheaths of the control plants was smaller at 0.95 cm² 14 days after *R. cerealis* inoculation (Figure 2f). At 28 dpi with *R. cerealis* inoculation, the lesions on the BSMV:TaSTT3b-infected plants were more obvious (Figure 2c). The IT value of 10.00% of BSMV:TaSTT3b-infected plants was 4, and that of 6.67% of BSMV:TaSTT3b-infected plants was 5. No BSMV:GFP-infected plants had an IT value of 4 or 5 (Figure 2d). The average IT value of BSMV: TaSTT3b-infected plants was higher than that of BSMV:GFP-infected plants at 28 dpi with *R. cerealis* inoculation (Figure 2e). The average area of lesions on stems of BSMV:TaSTT3b-infected plants was 1.73 cm², which was significantly higher than that of BSMV:GFP-infected plants (0.68 cm²) at 28 dpi with *R. cerealis* inoculation (Figure 2f). These results indicate that down-regulation of TaSTT3b significantly impaired resistance of the wheat cv. CI26333 to sharp eyespot, which suggests that TaSTT3b is required for host immune response to *R. cerealis* inoculation.

The BSMV:TaSTT3a-infected plants were also inoculated with the *R. cerealis* isolate WK207 (Figure S9a). The ITs of both control and BSMV:TaSTT3a-infected plants were 2, 3, 4, and 5 (Figure S9b). Based on the disease scoring at 28 dpi with *R. cerealis* inoculation, the average area of lesions on stems of BSMV: TaSTT3a-infected plants was 2.99 cm², which was significantly higher than that of BSMV:GFP-infected plants (2.36 cm²) (Figure S9c). However, there was no significant difference between the average IT values of BSMV:TaSTT3a- and BSMV:GFP-infected plants (Figure S9d). The results suggest that compared to TaSTT3b, TaSTT3a plays a minor role in wheat resistance response to the infection of *R. cerealis*.

**Overexpression of TaSTT3b-2B boosted wheat resistance to *R. cerealis***

To further investigate potential functions of TaSTT3b in wheat defense response to *R. cerealis*, we constructed an overexpression vector pWMB-TaSTT3b-2B-His (Figure S10) and transformed this construct into a hexaploid wheat cv. Zhoumai 18, generating TaSTT3b-2B transgenic wheat lines. In total, 55 wheat regeneration seedlings were obtained. Based on PCR detection, 14 T0 transgenic positive plants were verified (Figure S11), among which three lines (OX49, OX74, and OX89) carried two copies (Figure 3b, 3c), and two lines (OX49 and OX74) contained two copies of introduced *TaSTT3b-2B* transgene copy number in the transgenic wheat lines. Meanwhile, *Pmnb-D1b* (*Purinolindole-b D1b*) was used as the reference with two copies in hexaploid wheat (Collier et al., 2017). The ddPCR results showed that the transgenic lines OX49 and OX74 contained two copies of introduced *Ubi-TaSTT3b-2B-Tnos* chimeric, respectively, and OX89 carried three copies (Figure 3b, Figure S12). The expression levels of TaSTT3b-2B transgene individuals from the T1–T9 generations of the OX49, OX74, and OX89 lines were detected using qRT-PCR. As shown in Figure 3c, the transcriptional levels of TaSTT3b-2B in these three transgenic lines were significantly higher than those in the un-transformed (wild type, WT) Zhoumai18, indicating the introduced TaSTT3b-2B was overexpressed in these three lines. Western blotting
Figure 2  Barley stripe mosaic virus (BSMV)-induced TaSTT3b-silencing impairs resistance of wheat CI12633 to *Rhizoctonia cerealis*. (a) Mild chlorotic mosaic symptoms on leaves of CI12633 plants infected by BSMV:GFP or BSMV:TaSTT3b for 10 days. Detection of the BSMV coat protein (cp) gene was detected by RT-PCR. (b) Relative transcriptional levels of the three TaSTT3b homologs (TaSTT3b-2A, TaSTT3b-2B, and TaSTT3b-2D) in BSMV:GFP- and BSMV:TaSTT3b-infected wheat plants by qRT-PCR analysis. The relative transcriptional levels of TaSTT3b homologs in BSMV:TaSTT3b-infected CI12633 plants is relative to those in BSMV:GFP-infected plants (set to 1). (c) Sharp eyespot symptoms on CI12633 plants infected with BSMV:GFP and BSMV: TaSTT3b at 14 and 28 dpi with *R. cerealis* infection. (d) Percentages of BSMV:GFP- and BSMV:TaSTT3b-infected CI12633 plants with different sharp eyespot infection types (ITs) at 14 and 28 dpi with *R. cerealis* infection. (e) Average ITs of BSMV:GFP- and BSMV:TaSTT3b-infected CI12633 plants at 14 and 28 dpi with *R. cerealis* infection. (f) Average size of lesions in BSMV:GFP- and BSMV:TaSTT3b-infected CI12633 plants at 14 and 28 dpi with *R. cerealis* infection. Bars indicate standard errors of the means (n varies for each column and is shown in each case directly on the graphs). Single and double asterisks represent significant differences between BSMV:GFP- and BSMV:TaSTT3b-infected CI12633 plants determined by Student’s *t*-test at *P* < 0.05 and *P* < 0.01, respectively.
analysis revealed that the introduced TaSTT3b-2B-His fusion protein constitutively accumulated in these transgenic wheat lines (Figure 3d).

Because the resistance/susceptibility of wheat to *R. cerealis* is easily influenced by environmental conditions, we tested the resistance of transgenic and WT wheat plants to *R. cerealis* in Beijing and Nanjing in 2018, 2019, and 2020. The three TaSTT3b-2B overexpressing wheat lines, OX49, OX74, and OX89, displayed significantly enhanced resistance to sharp eyespot caused by *R. cerealis* (Figure 3e). Mean disease index values of the WT Zhoumai18, 76.96 (2018) and 39.46 (2019), were greater than mean disease index values of the three transgenic lines, 38.50–59.73 (2018) and 28.99–32.89 (2019), in Beijing (Figure 3f, g). Compared with the WT Zhoumai18, the three transgenic lines also displayed markedly elevated resistance to sharp eyespot in plants grown in Nanjing. For example, the disease index values of three overexpressing lines OX49, OX74, and OX89 were separately 21.16, 21.73, and 22.80 in 2019, and 37.07, 36.49, and 36.76 in 2020, respectively, and they were all lower than those of the WT Zhoumai18 (Figure 3h,i). These results indicate that TaSTT3b-2B overexpression improved resistance of the transgenic wheat to sharp eyespot, and TaSTT3b-2B positively participates in wheat resistance to *R. cerealis* infection.

**Figure 3** Molecular characterizations of TaSTT3b-2B overexpressing wheat plants and plant responses to *Rhizoctonia cerealis* infection. (a) PCR patterns of three TaSTT3b-2B transgenic lines (OX49, OX74, and OX89) and wild-type (WT) wheat Zhoumai18 using the primers specific to the TaSTT3b-2B-Tnos cassette. Marker, DL2, 000 DNA marker; P, the transformation vector pWMB122-TaSTT3b-2B as the positive control. (b) Transgene copy number measurements in TaSTT3b-2B transgenic and WT lines. Primers and probes were designed to detect the sequence of the maize Ubi promoter, which was used to control TaSTT3b-2B expression. (c) qRT-PCR analyses of the relative transcriptional levels of TaSTT3b-2B in TaSTT3b-2B transgenic lines in the T1–T3 generations. Mean values of TaSTT3b-2B in WT Zhoumai18 were defined as 1. Three biological replicates per line were averaged (t-test; **P < 0.01). Bars indicate standard errors of the means. (d) Western blot pattern of the three TaSTT3b-2B overexpressing lines and WT Zhoumai18 using an anti-6×His antibody. Similar results were obtained from three independent replicates. (e) Typical symptoms of sharp eyespot in the three TaSTT3b-2B overexpressing and WT Zhoumai18 lines at 60 dpi with *R. cerealis* infection. (f–i) Disease index of TaSTT3b-2B overexpressing and WT Zhoumai18 lines. The phenotypes were identified in wheat grown in Beijing in 2018 and 2019, as well as in Nanjing in 2019 and 2020. Bars indicate standard errors of the means (n varies for each column and is shown in each case directly on the graphs), and asterisks indicate significant differences between WT and transgenic lines using Student’s t-tests (*, P < 0.05; **, P < 0.01).
RNA-seq analysis identifies defense-related genes modulated by TaSTT3b-2B

To further dissect the molecular basis of TaSTT3b-2B–mediated wheat defense response to *R. cerealis*, we performed RNA-seq analysis on TaSTT3b-2B overexpressing and WT Zhoumai18 plants at 4 dpi with *R. cerealis* inoculation (Table S2). A total of 2264 differentially expressed genes (DEGs) were obtained based on the criteria of a fold change of 2 and false discovery rate (FDR) < 0.05. Among them, 1751 genes were highly expressed in TaSTT3b-2B overexpressing wheat lines in comparison to the WT, and 513 genes were expressed at a lower level (Figure S13, Table S3). Gene Ontology (GO) analysis of the DEGs indicated that TaSTT3b-2B overexpression affected multiple processes in the transgenic wheat plants. Notably, some of the DEGs were clustered into stress response–related terms such as ‘response to stimulus’, ‘signaling’, and ‘immune system process’ (Figure S14), suggesting that overexpression of TaSTT3b-2B affected the expression levels of genes involved in stress and immune responses. The genes for diverse processes were also enriched, which includes metabolic, cellular, and single organism processes, implying that the TaSTT3b-2B–mediated defense response to *R. cerealis* infection might be a consequence of multiple biological processes in wheat (Figure S14).

As shown in Figure 4a, 297 of the DEGs were categorized for their involvement in defense response to pathogens based on GO analysis, Pfam annotation, and SWISS PROT annotation (Table S4). These genes encoded cytochrome P450, GDSL-like lipase (GLIP), O-methyltransferase; ABC transporter; pathogenesis-related protein; protein kinase; oxygenase; 1,3-beta-glucosidase; lipoxygenase (LOX); chitinase; and some transcription factors (TFs), such as WRKY TFs, basic helix-loop-helix TFs, myeloblastosis-related (MYB) TFs, ethylene-responsive factors (ERFs), MADS-box TFs, basic leucine zipper (bZIP) TFs, and GATA TFs. It is worth mentioning that 27 GLIP genes were identified in the DEGs, 26 of which were significantly up-regulated in TaSTT3b-2B overexpressing lines (Figure S15). To verify the reliability of the RNA-seq data, eight defense-related genes, including TaBRRI (BRASSINOSTEROID INSENSITIVE 1, TaBriss1), TaCOTMT (caffeic acid O-methyltransferase, TraesCS3B02G612000), TaLTP (lipid transfer protein, TaesCS2B02G501000), TaGLu1 (glucosidase, TaesCS2B02G600200), TaMAH1 (midchain alkane hydroxylase, TraesCSU02G1014000), TaGLP1 (TraesCS4A02G397000), TaLOX (TraesCS5B02G382600), and TaAOS2 (allene oxide synthase 2, TraesCS4A02G601800), were chosen to be validated by qRT-PCR. As shown in Figure 4b, the relative transcript levels of these eight genes in TaSTT3b-2B overexpressing plants were significantly higher than those of the WT plants. Because of being generally consistent with the RNA-seq data, these results suggested that the RNA-seq data should be reliable. Expectedly, the transcriptional levels of these defense-related genes were significantly down-regulated in the TaSTT3b-silenced wheat plants in comparison with the BSMV:GFP-infected (control) wheat plants (Figure S16). These results indicated that these eight defense-related genes were regulated by TaSTT3b-2B and were possibly downstream of TaSTT3b-2B.

Overexpression of TaSTT3b-2B increased grain size in transgenic wheat with *R. cerealis* infection

To explore if overexpression of TaSTT3b-2B influences yield-affected factors, spike number per m², spike length, spikelet number per spike, grain number per spike, grain length, grain width, and thousand-grain weight (TGW) were examined in both TaSTT3b-2B transgenic and WT plants infected with *R. cerealis*. Notably, there was a significant difference in grain size (grain length and grain width) between TaSTT3b-2B overexpressing wheat lines and the WT Zhoumai18 line. The overexpressing wheat lines produced bigger kernels than the WT Zhoumai18 line (Figure 5a). The grain length (Figure 5b), grain width (Figure 5c), and TGW (Figure 5d) all were significantly higher in these three TaSTT3b-2B overexpressing wheat lines (OX49, OX74, and OX89) than those in the WT Zhoumai18 plants. In contrast, there were no significant differences in spike number per m², spike length, spikelet number per spike, and grain number per spike between TaSTT3b-2B overexpressing wheat lines and the WT Zhoumai18 line (Figure S17).

To investigate the relationship between TaSTT3b-2B expression and grain size, we analyzed the expression profile of TaSTT3b-2B in wheat varieties with different TGWs. As shown in Figure 5e and 5f, the expression level of TaSTT3b-2B was higher in wheat varieties with higher TGW (40–50 g) than in those with low TGW (<40 g), and these varieties with the highest TGW (>50 g) showed the highest expression level of TaSTT3b-2B. These data suggested that the expression level of TaSTT3b-2B was positively associated with the grain size in wheat and support the abovementioned results, that is, greater grain weight of the TaSTT3b-2B–overexpressing wheat.

The high molecular weight glutenin (HMW-GS), starch synthase, starch branching enzyme, and sucrose synthase have shown significant associations with grain size in wheat or rice (Daba et al., 2018; Jiang et al., 2011; Li et al., 2011). In order to explore the mechanisms underlying the increased grain weight of the TaSTT3b-2B overexpression, we examined relative transcript abundances of TaSsil (encoding starch synthase Ila), TaSBel1b (encoding starch branching enzyme Iib), the HMW-GS genes [TaGl1u-1B1 (TraesCS1B02G239992), TaGl1u-1B2 (TraesCS1B02G329711), TaGl1u-1D1 (TraesCS1D02G317201), TaGl1u-1D2 (TraesCS1D02G317211), and TaGl1u-4A (TraesCS4A02G485400)], the sucrose synthase genes, including TaSuSy1 (TraesCS7D02G519800), TaSuSy2A (TraesCS2A02G168200), and TaSuSy2D (TraesCS2D02G175600), and the grain size gene TaGSS (TraesCS6B02G261700, a wheat homologous gene of rice GSS) in TaSTT3b-2B overexpressing and WT wheat lines. The qRT-PCR analyses showed that the expression levels of these grain size–related genes were markedly up-regulated in TaSTT3b-2B overexpressing wheat lines compared to those in the WT Zhoumai18 line (Figure 5g). These data suggest that overexpression of TaSTT3b-2B increased grain weight by up-regulating the expression of these grain size–related genes. Interestingly, treatment with exogenous JA increased the transcript levels of these grain size–related genes except of those of TaGl1u1 (Figure 5h), implying that JA biosynthesis might contribute to grain size in wheat.

TaSTT3b-2B–mediated resistance to *R. cerealis* possibly through the JA signal pathway

JA is a crucial signal molecule in plant resistance against pathogens, especially in resistance to necrotrophic pathogens (Pieterse et al., 2009). Thus, we endeavored to investigate whether overexpression of TaSTT3b-2B alters the defense response of wheat to *R. cerealis* through the JA signal pathway.
The expression profile of TaSTT3b-2B in wheat response to exogenous JA was detected. As shown in Figure 6a, the expression of TaSTT3b-2B was significantly induced from 1 to 8 hours post treatment with JA.

The JA pathway, including JA biosynthesis and the subsequent JA signal transduction, has been characterized (Lyons et al., 2013). In the DEGs identified in this study, 30 JA biosynthesis-related genes, comprising 21 lipoxygenase (LOX) genes, five allene oxide synthase (AOS) genes, and four OPDA reductase (OPR) genes, were identified (Figure 6b, Table S5). With the exceptions of two OPR genes (TraesCS1D02G013600 and TraesCS1A02G015800), the transcript abundances of the other 28 JA-related genes were significantly higher in TaSTT3b-2B overexpressing wheat plants than those in the WT plants based on a log2 fold-change analysis (Figure 6b, Table S5). Accordingly, compared to the WT line, high accumulation of JA was observed in the TaSTT3b-2B transgenic lines (Figure 6c). These results suggested that TaSTT3b-2B positively regulated JA biosynthesis in wheat.

As shown in Figure 6c, R. cerealis infection induced JA biosynthesis, and the transgenic wheat lines still had a higher level of JA content than the WT plants. Seven JA biosynthesis-related genes, TaLOX-5A, TaLOX-5D, TaLOX-6D, TaAOS-4A, TaAOS-4D, TaOPR-1D, and TaOPR-4B, were chosen for further gene expression analysis in wheat after infection by R. cerealis. The expression levels of these seven genes were significantly induced by R. cerealis infection (Figure 6d). These data indicated that TaSTT3b-2B positively regulated the transcripts of these JA biosynthesis-related genes, which were also induced by R. cerealis infection.

To further explore the relationship between the TaSTT3b-2B-mediated resistance and the JA biosynthesis, we first examined the transcriptional levels of two JA-related genes, TaLOX and TaOPR, in TaSTT3b-silenced and BSMV:GFP-infected wheat plants. The data showed that silencing of TaSTT3b-2B down-regulated the transcriptional levels of TaLOX and TaOPR (Figure 7a). Next, TaSTT3b-silenced and BSMV: GFP-infected wheat plants were pretreated with MeJA and then inoculated...
with R. cerealis. Compared with the mock group given 0.1% Tween-20, MeJA pretreatment increased wheat resistance to R. cerealis, and there was no significant difference in the observed disease symptoms between TaSTT3b-silenced and BSMV:GFP-infected plants (Figure 7b). Accordingly, the expression level of R. cerealis actin mRNA (RcActin), used as a measure of fungal biomass in the pathogen-inoculated wheat, was consistent with the symptoms of these plants (Figure 7c). Furthermore, JA treatment elevated the transcriptional levels of some defense-related genes, including TaCOMT, TaBRI1, TaLTP, TaGlu, TaMAH1, TaGLIP1, TaLOX, and TaAOS2 (Figure S18). In contrast, pretreatment with the JA biosynthesis inhibitor DICEA attenuated the TaSTT3b-2B-mediated resistance to R. cerealis (Figure 7d), and the detection of the RcActin expression level supported the altered disease symptoms described above (Figure 7e). Overall, these results suggest that TaSTT3b-2B-mediated resistance to R. cerealis was partially dependent on JA biosynthesis.

Figure 5 TaSTT3b-2B regulates grain size in transgenic wheat infected by Rhizoctonia cerealis. (a) Grains of TaSTT3b-2B-overexpressing and WT Zhoumai18 lines. Scale bar = 1 cm. (b–d) Grain size–related phenotypes of TaSTT3b-2B-overexpressing and WT Zhoumai18 lines: grain length (b), grain width (c), and TGW (thousand-grain weight) (d). (e) TGW of different wheat varieties. Three replicates were used, each with approximately 20 g grains. (f) Expression levels of TaSTT3b-2B in different wheat varieties. (g) qRT-PCR analysis of TaSSIIa, TaSBEIIb, TaGlu1, TaGlu-4A TaSuSy1, TaSuS-2A, TaSuS-2B, TaSuS-2D, and TaGSS in three TaSTT3b-2B transgenic lines (OX49, OX74, and OX89) and WT Zhoumai18. (h) Expression patterns of these grain size–related genes in wheat response to exogenous JA. Wheat plants at the four-leaf stage were sprayed with 0.1 mM MeJA. qRT-PCR data were normalized to wheat TaActin. The data from three replicates are shown with ±SD. The single and double asterisks represent significant differences determined by Student’s t-test at P < 0.05 and P < 0.01, respectively.

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Discussion

The majority of the reports on STT3 subunits mainly focus on involvement of STT3a in plant resistance to abiotic (Jeong et al., 2018; Koiwa et al., 2003) and biotic (Häweker et al., 2010; Jia et al., 2020; de Oliveira et al., 2016; Zhang et al., 2015) stresses. For instance, cell wall biosynthesis was abnormal in Arabidopsis stt3a mutants, which led to compromised salt tolerance (Koiwa et al., 2003). STT3a has also been shown to play a key role in BAK1/SERK4-regulated cell death (de Oliveira et al., 2016). However, no study of STT3b involved in plant immunity to various pathogens has been reported. In this study, two OST subunit genes, TaSTT3a and TaSTT3b, were identified from wheat. The qRT-PCR analysis showed that TaSTT3b-2B expression, but not

Figure 6 TaSTT3b-2B-mediated resistance to Rhizoctonia cerealis is associated with the JA biosynthesis. (a) Expression patterns of TaSTT3b-2B in leaves of Zhoumai18 wheat in response to applications of exogenous JA. Wheat plants at the four-leaf stage were sprayed with 0.1 mM MeJA. (b) JA biosynthesis–related genes in differentially expressed genes (DEGs). These genes were identified by transcriptome analysis as DEGs identified between TaSTT3b-2B overexpressing and WT Zhoumai18 plants (based on log2 fold change). TaSTT3b-1, TaSTT3b-2, and TaSTT3b-3 indicate samples of three TaSTT3b-2B overexpressing lines. ZM18-1, ZM18-2, and M18-3 indicate three leaf sheath samples of the WT Zhoumai18 line. (c) JA contents of TaSTT3b-2B overexpressing and WT Zhoumai18 plants before and after R. cerealis infection for 4 days. (d) Expression patterns of JA-related genes in wheat response to R. cerealis infection for 4 and 7 days. Data were normalized to wheat TaActin. Statistically significant differences were derived from the results of three independent replications (Student’s t-test: *P < 0.05, **P < 0.01). Bars indicate standard errors of the means.

Figure 7 Jasmonic acid positively regulates TaSTT3b-2B–mediated resistance to Rhizoctonia cerealis. (a) Expression levels of TaLOX and TaOPR in TaSTT3b-2B–silenced wheat plants. The expression levels of target genes in BSMV/GFP-infected plants were set to 1. Data were normalized to wheat TaActin. (b) Sharp eyepoint symptoms of TaSTT3b-2B–silenced and BSMV/GFP-infected plants after treatment with exogenous JA. C12633 plants at 20 dpi with BSMV: GFP or BSMV:TaSTT3b were sprayed with 0.1 mM MeJA and 0.1% Tween-20 (mock). (c) qRT-PCR analysis of the R. cerealis actin (RcActin) gene in leaf sheaths of TaSTT3b-2B–silenced and control C12633 plants after JA or mock treatment. (d) Sharp eyepoint symptoms of TaSTT3b-2B overexpressing and WT plants after pretreatment with the JA biosynthesis inhibitor DIECA. Seedlings sprayed with 0.1% Tween-20 were used as mock. (e) qRT-PCR analysis of the RcActin gene in leaf sheaths of TaSTT3b-2B overexpressing and WT plants after DIECA or mock treatment. The expression level represents the biomass of R. cerealis. Statistically significant differences were derived from the results of three independent replications (Student’s t-test: *P < 0.05, **P < 0.01). Bars indicate standard errors of the means.
TaSTT3a, was markedly induced by *R. cerealis* infection, and TaSTT3b-2B produced significantly higher transcriptional levels in *R. cerealis*-resistant wheat genotypes than in the susceptible wheat genotypes. The functional dissection data indicate that TaSTT3b-2B, acting as a positive regulator, was required for wheat resistance response to *R. cerealis*. To our knowledge, this is probably the first investigation to uncover the positive regulation of STT3b in plant resistance responses to necrotrophic fungal pathogens. This study undoubtedly deepens understanding of biological functions of the STT3 subunit in plant species.

To explore the molecular mechanism underlying the defensive role of TaSTT3b-2B, RNA-seq-based transcriptomic analyses were deployed to identify a number of defense-associated genes, being differently expressed between TaSTT3b-2B overexpressing and WT wheat lines. In addition to MYB, ERF, GATA, COMT, chitinase, pathogenesis-related (PR) protein, and 1,3-beta-glucosidase that have been reported to be involved in the wheat defense response to *R. cerealis* infection (Liu *et al*., 2009, 2020; Wang *et al*., 2018; Wei *et al*., 2016; Zhang *et al*., 2007; Zhu *et al*., 2014, 2018), some other types of genes, such as those coding the cytochrome P450 enzyme, GLU, LOX, and AOS, were also identified. The qRT-PCR assay results on eight defense-related genes in TaSTT3b-2B-silenced and overexpressing plants, as well as the control wheat plants, supported that the RNA-seq data were reliable. These data suggest that TaSTT3b-2B expression is required for the expression of these defense-related genes during wheat resistance to *R. cerealis* infection. Thus, overexpressing TaSTT3b-2B increased the expression of these defense-related genes, resulting in enhanced resistance of the transgenic wheat to *R. cerealis*.

JA is an essential plant hormone that regulates certain types of disease resistance, especially resistance to necrotrophic pathogens (Bari and Jones, 2009; Peng *et al*., 2012). In plants, increasing evidence has shown that JA is able to induce the expression of certain defense-related genes (Liu *et al*., 2020; McGrath *et al*., 2005; Peng *et al*., 2012; Pieterse *et al*., 2009; Thomma *et al*., 1998). For example, an increase in endogenous JA and the expression of JA synthesis–related genes LOX, AOS2, and a subset of PR genes, such as PR3 and PR10, conferred enhanced resistance to sheath blight and blast fungi in *WRKY30* overexpression rice (Peng *et al*., 2012). Both WRKY4 and WRKY80 have been found to enhance rice resistance to sheath blight by inducing the up-regulated expression of JA- and ET-responsive PR genes (Peng *et al*., 2016). In this study, transcriptional abundance of TaSTT3b-2B was induced by *R. cerealis* and JA treatments, and higher accumulation of JA was observed in the resistant TaSTT3b-2B overexpressing wheat lines than that of the WT wheat line, suggesting that the TaSTT3b-2B–mediated resistance to *R. cerealis* infection was positively associated with JA biosynthesis/signaling. Consistently, TaSTT3b-2B positively promoted JA synthesis by positively regulating the expression of the JA synthesis–related genes TaLOX-5A, TaLOX-5D, TaLOX-6D, TaAOS-4A, and TaAOS-4D, whose transcripts were also responsive to *R. cerealis* infection. Importantly, exogenous JA application could counteract the reduced resistance of TaSTT3b-2B–silenced wheat plants to *R. cerealis*, and the JA also induced expression of the TaSTT3b-2B–regulated defense-related genes. Similarly, in rice, application of exogenous JA induced resistance to *R. solani* (Taheri and Tarighi, 2010) and *M. oryzae* (Riemann *et al*., 2013). Meanwhile, pretreatment with DIECA, an inhibitor of JA synthesis, reduced the TaSTT3b-2B–mediated resistance to *R. cerealis*. Taken together, these data suggest that JA biosynthesis might play an important role in TaSTT3b-2B–mediated immune response to *R. cerealis*. Our study not only showed the positive regulatory function of TaSTT3b-2B in the expression of JA biosynthesis–related genes but also demonstrated the importance of JA treatment in expression of TaSTT3b-2B–mediated defense-related genes and disease resistance in wheat.

Grain weight, grain number per spike, and spike number per plant are the most important traits in relation to achieving greater grain yield per plant. In this study, analyses of the agronomic traits and yield parameters showed that the TaSTT3b-2B overexpressing wheat lines possessed greater grain length and width and higher TGW than those possessed by WT after both groups were infected by *R. cerealis*. Starch and sucrose synthases have been shown to contribute to grain yield (Fan *et al*., 2019; Hou *et al*., 2014; Xie *et al*., 2018). In maize, overexpression of mutated ZmDAt1 or ZmDAt1 improved kernel yield by promoting starch synthesis (Xie *et al*., 2018). In rice, overexpression of a sucrose synthase gene, OsSUS3, significantly improved grain weight by dynamically regulating cell division and starch accumulation (Fan *et al*., 2019). In wheat, the sucrose synthase genes TaSuS1 and TaSuS2 showed significant association with grain yield (Hou *et al*., 2014; Daba *et al*., 2018). Here, the results showed that the transcriptional levels of two starchy synthase–related genes, TaSSila and TaSHEllb, and four sucrose synthase genes, TaSuS1y, TaSuS-2A, TaSuS-2D, and TaSuS-2D, were higher in TaSTT3b-2B overexpressing plants than in WT plants. These data indicate that TaSTT3b-2B promoted grain weight most possibly by affecting the expression of starch synthase and sucrose synthase genes. In a most recent article, in wheat, mutation of the JA synthetic gene, KAT-2B, produces smaller grains and accumulates less JA, while overexpression of KAT-2B increases grain weight and yield in plants grown in field trials (Chen *et al*., 2020). Rice mutants defective in JA biosynthesis (dfo2 and Opsor7) or signaling (Osmyc2) exhibited various degrees of abnormality in spikelet development, including abnormal number or morphology of lemma, palea, glume, lodicule, and floral organs, which can be partially rescued by application of exogenous JA (You *et al*., 2019). The TCP (Teosinte branched/Cycloidea/PCF) transcription factor mutant msdT had 50% less JA than WT plants and showed double the grain number per panicle. Meanwhile, application of exogenous JA can rescue the msdT phenotype in sorghum (Jiao *et al*., 2018). Arabidopsis mutants defective in JA biosynthesis and signaling pathway, such as opr3, myc2/3/4/5, and coi1, displayed a male sterility phenotype (Browse, 2009; Ishiguroet al., 2001; Qi *et al*., 2015; Stintzi and Browse, 2000). In our study, the exogenous JA treatment increased the expression levels of sucrose and starch synthase genes and the grain-related gene TaG55, suggesting the potential effect of JA on regulation of TaSTT3b-2B in wheat grain weight. These investigations suggested an essential role of JA in regulating grain yield in different plant species; however, the downstream regulatory mechanisms differ in distinct taxa. These field trials were conducted in the same location for two years. In order to validate the wider applicability of the TaSTT3b-2B gene in wheat breeding, repeating this study while using multiple sites with different soil types and environmental conditions is needed.

**Experimental procedures**

**Plant and fungal materials and treatments**

Five wheat lines/cultivars including CI12633, ShanHongmai, Yangmai158, Zhoumai18, and Wenmai 6, showing different
degrees of resistance to *R. cerealis* (Zhu et al., 2015) and about 50 other cultivars with different TGWs were used in this study. The pathogenic *R. cerealis* strain, WK207, which possesses high virulence and is prevalent in northern China, was provided by Prof. Jinfeng Yu of Shandong Agricultural University, China.

All wheat plants used in this research were grown in field plots or in a greenhouse under 14-h light (22 °C/10-h dark (12 °C) conditions (Zhu et al., 2017). Wheat plants at the tillering stages were inoculated with WK207 using the toothpick inoculation method (see below). Samples were collected from leaf sheaths of wheat plants at 0, 4, 7, 14, and 21 dpi with *R. cerealis* infection and stored at ~80 °C.

**Sequence identification and analysis of TaSTT3b**

Based on the sequence of TraesCS2B02G5879001.1 (Ensembl Plants, http://plants.ensembl.org/index.html), two pairs of primers (Table S6) were designed and used for nested PCR to amplify the full-length sequences of TaSTT3b from cDNA and genomic DNA of CI12633 stems. The conserved motifs were predicted by an online smart software package (http://smart.embl-heidelberg.de/). Multiple protein sequence alignments were performed using DNAMAN software. A phylogenetic tree was constructed by using MEGA 6.0 software.

**Subcellular localization of TaSTT3b**

The coding sequence of TaSTT3b was amplified by the primers GFP-TaSTT3b-F/R (Table S6) and cloned into the 3′ end of the GFP vector, TaAction, which was used to control the expression levels of the target genes.

**qRT-PCR analysis**

Primers of target genes for qRT-PCR were designed by Primer Premier 5 software and are listed in Table S6. The qRT-PCR was performed using SYBR Green SuperReal PreMix (TIANGEN, China). The cDNA was diluted to a 1:50 ratio with RNase-free water, and 5 µL of the diluted cDNA was used as the template. The TaAction gene was used as an internal control for qRT-PCR. The relative expression levels of the target genes were calculated using the comparative 2−ΔΔCT method (Livak and Schmittgen, 2001).

**BSMV-induced gene silencing of TaSTT3** in wheat CI12633

To generate the BSMV:TaSTT3a and BSMV:TaSTT3b recombinant constructs, a specific fragment (333 bp) of TaSTT3a and a specific fragment (192 bp) of the TaSTT3b gene with efficient siRNA generation were selected using SiFi software (Nowara et al., 2010) and amplified from cDNA of CI12633. The purified PCR product digested with Nhe I was ligated in an antisense orientation into the RNAi of BSMV. Clones containing the fragments in the γ- vector were confirmed by sequencing. Capped *in vitro* transcription of α-, β-, and γ-RNAs of the BSMV genome were prepared using the RibomAX® Large Scale RNA Production System-T7 kit (Promega, USA) and the Ribom m7G Cap Analog (Promega, USA) as described by the manufacturer’s instructions. The BSMV-VIGS experiment flowchart is shown in Figure S7a. Briefly, at the three-leaf stage, the third fully expanded leaves of CI12633 wheat plants were inoculated with *in vitro* synthesized BSMV RNAs by gently sliding pinched fingers from the leaf base to the leaf tip. BSMV: GFP served as the control. After incubation for 48 h in a humid environment (95% relative humidity), seedlings were transferred to a greenhouse under 14-h light (22 °C/10-h dark (12 °C) conditions. After 10 dpi with BSMV, samples were collected from the four leaves to monitor BSMV infection based on the transcript abundance of the BSMV cp gene. After 20 dpi with BSMV, wheat plants were inoculated with *R. cerealis* WK207 (the inoculation method is described below). And then, seven days after *R. cerealis* inoculation, the leaf sheaths were collected and used to evaluate the transcriptional levels of target genes. The experiment was repeated three times. For one repeat, at least 15 plants were infected by BSMV:GFP, BSMV:TaSTT3a, or BSMV: TaSTT3b. The primers used in this assay are listed in Table S6.

**Generation and identification of transgenic wheat**

To generate the TaSTT3b overexpression vector, the full ORF sequence of TaSTT3b-2B plus a 6× His epitope tag was amplified using the primers containing the BarnHI and SacI restriction sites and then inserted into the monocot transformation vector pWMB122 (Wang et al., 2017). In the pWMB122-TaSTT3b-2B vector (Figure S10), the TaSTT3b-2B-His fusion protein is driven by the maize ubiquitin (Ubi) promoter and terminated by the 3′-non-transcribed region of the *Agrobacterium tumefaciens* nopalin synthase (Tnos) gene. To generate TaSTT3b transgenic wheat plants, the pWMB122-TaSTT3b-2B vector was introduced into hexaploid wheat (*cv. Zhounail8*) via *Agrobacterium*-mediated transformation. Herbicide spraying and PCR were used to select the positive transgenic plants. Herbicide spraying was performed as described by Wang et al. (2017). For the PCR experiment, reactions containing about 200 ng genomic DNA, 10 µL 2× Taq MasterMix (TransGen Biotech, China), and 1 µL of each primer (10 µM) were prepared. The PCR products were resolved on a 1.5% agarose gel and visualized after ethidium bromide staining. The primers used in this assay are listed in Table S6.

**Transgene copy number measurement by ddPCR**

We developed a ddPCR assay using the *PINb-D1b* gene as a reference (Collier et al., 2017) to estimate the copy number of the transgene. Primers and probes were designed to detect the sequence of the maize *Ubi* promoter, which was used to control TaSTT3b-2B expression. The *Ubi* probe was 5′ FAM (6-fluorescein)-labeled and the *PINb-D1b* probe was 5′ ROX (6-Carboxyl-X-Rhodamine)-labeled (Table S6). ddPCR analysis was performed using a Pilot Gene Droplet Digital PCR System (Pilot Gene Technology Company, Hangzhou, China) following the manufacturer’s protocol. Briefly, the ddPCR master mix for each testing panel contained 1× ddPCR premix, 1 µM of each primer pair (for the reference and transgene), 250 nM of each probe, and 10 µL of template DNA (~1 ng/µL), and the final volume was adjusted to 15 µL with DNase-free water. The reaction mixture was gently mixed and added into a ready-to-use disposable plastic chip to generate droplets. Chips were then amplified in a thermal cycling protocol entailed incubation at 95 °C for 5 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. After PCR amplification, chips were loaded into a chip scanner for fluorescence signal reading and further data analysis. The synthesized DNA fragment was used as a positive control, DNase-free water served as a negative control.
Western blotting assay on the transgenic wheat

Total proteins of leaf samples (0.1 g each) from the transgenic and WT wheat lines were extracted using a plant protein extraction kit (CWBIO, China). Total proteins were separated on a 12% SDS-PAGE gel and then transferred to a PVDF membrane (Millipore, USA). The TaSTT3b-2B-His protein was incubated with 2000-fold diluted anti-His antibody followed by a secondary antibody conjugated to horseradish peroxidase (HRP). After incubating overnight at 4 °C, the TaSTT3b-2B-His protein was visualized using the Pro-light HRP Chemiluminescent Kit (TIANGEN, China).

*R. cerealis* inoculation and wheat sharp eyespot evaluation

The *R. cerealis* isolate WK207 was used to inoculate wheat plants by two methods, the toothpick inoculation and wheat kernel inoculation methods. An aliquot of the fungal stock, maintained on potato dextrose agar (PDA) at 4 °C, was activated on fresh PDA plates under artificial incubation at 25 °C in the darkness (Figure S19a). For the toothpick inoculation method, according to the method of Ren et al. (2020) with slight modification, ordinary wooden toothpicks were cut into approximately 0.5-cm-long pieces and then autoclaved at 121 °C for 20 min. About 50 sterilized toothpick segments were evenly placed onto each PDA plate under a strict asepsis procedure. A piece of active medium with WK207 mycelia was symmetrically added to each PDA plate with toothpick segments, and then the plates were placed in an incubator at 25 °C in the darkness. After 15 days, the colonized toothpick segments were used to inoculate each stem of wheat plants (Figure S19b). The inoculated region on the stem was wrapped with wet cotton and sprayed with water twice per day during the first week and then once a day until the final disease was recorded. For the wheat kernel inoculation method, the wheat kernels were first soaked in water overnight and then transferred into Erlenmeyer flasks for autoclaving at 121 °C for 20 min. After cooling, five pieces of activated medium with WK207 mycelia were placed into each Erlenmeyer flask and mixed thoroughly before being placed in the incubator to grow at 25 °C in the darkness. The flasks were fully shaken every two days to promote uniform colonization. After 15 days, the kernels full of white mycelia were used to inoculate wheat seedlings. At the tillering growth stage, the WT and transgenic wheat plants were inoculated at the base of each stem with 8 full of white mycelia were used to inoculate wheat seedlings. At 4 dpi with *R. cerealis* infection and tested using an HPLC-MS/MS system as previously reported (Xu et al., 2016). JA content of seedlings without *R. cerealis* infection was also measured as the control.

Transcriptome analysis of transgenic wheat after *R. cerealis* infection

The transcriptomes of three TaSTT3b-2B transgenic lines (OX49, OX74, and OX89) and WT Zhoumai18 were examined using RNA-seq analysis. At 4 dpi with *R. cerealis* infection, sheaths were collected from wheat seedlings and used for RNA-seq analysis. Three replicates were used. Total RNA was extracted using TRizol (Invitrogen, California, USA) following the manufacturer’s protocol. RNA integrity was evaluated using a 2100 Bioanalyzer (Agilent Technologies, California, USA). Samples with an RNA Integrity Number (RIN) > 7 were selected for subsequent analysis. The RNA-seq analysis was completed by Biomarker Biotechnology (Beijing, China). The filtered clean reads were mapped to the wheat reference genome and genes (http://plants.ensembl.org/Triticum_aestivum/Info/Index). Thresholds of FDR<0.05 and a log2 fold change >2 or <0.5 were used to confirm DEGs between transgenic lines and WT Zhoumai18. Gene Ontology analysis and Kyoto Encyclopedia of Genes and Genomes pathway analyses were carried out using BMKCloud (www.biocloud.net).

Analyses of TaSTT3b-2B expression and grain weight in various wheat varieties

Seeds of various wheat varieties were planted at the experimental farm in Beijing, one variety in three repeated rows, each with a length of 1.5 m and a spacing of 25 cm. At the three-leaf stage, leaves of at least ten plants per variety were collected and mixed for qRT-PCR analysis of TaSTT3b-2B. The method of qRT-PCR refers to the “qRT-PCR analysis” section. Mature seeds were harvested and seeds from at least 20 plants for each variety were mixed for phenotypic assessment. A Seed Counter-G system (WSeen, China) was used to measure grain weight, grain width, and grain length. Three replicates were used, each with approximately 20 g seeds.

Measurements of agronomic traits

Wild type Zhoumai18 and T3 generation plants of transgenic wheat lines (OX49, OX74, and OX89) were grown at the experimental farm at the Institute of Crop Sciences, Chinese Academy of Agricultural Sciences, Beijing, China (39°93′ N, 116°40′ E). The planting area of each line was 13.5 m² (Figure S20). Spike number per m², spike length, spikelet number per spike, and grain number per spike were measured as described previously by Ulukan and Kun (2007) prior to harvest. After harvest, at least 300 grains were randomly selected from each line and used to measure average grain length, grain width, and TGW.
using a Seed Counter-G system (WSeen, China). The measurements of agronomic traits described above were performed with three replicates, and each contained at least 20 plants.

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Conflict of interest
The authors declare no competing interests.

Author contributions
Z.Z. and X.Z. conceived the study, designed the experiments, and wrote the manuscript. X.Z. performed most of the experiments. X.Z. and W.R. identified the TaSTT3b gene. K.W. performed subcellular localization analysis of TaSTT3b-2B. W.G., M.Z., J.W., and X.W. participated in phenotyping the transgenic wheat. X.Y. provided technical assistance on wheat transformation.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1 Expression patterns of TaSTT3a from the wheat line CI12633 at 0, 4, 7, 10, 14, and 21 days post infection with R. cerealis.

Figure S2 Alignment of TaSTT3b homologs of AtSTT3b and OsSTT3b.

Figure S3 Alignment of TaSTT3b genomic sequences from CI12633 and Zhoumai18.

Figure S4 Identified QTLs controlling wheat resistance to sharp eyespot.

Figure S5 Subcellular localization of the TaSTT3b-2B protein.

Figure S6 Schematics of genomic RNAs of the BSMV construct and the construct of the recombinant virus expressing the wheat gene TaSTT3b, BSMV: TaSTT3b.

Figure S7 BSMV-VIGS experiment flowchart and off-target prediction of TaSTT3a and TaSTT3b VIGS fragments.

Figure S8 Silencing of TaSTT3a by barley stripe mosaic virus–induced gene silencing in the sharp eyespot-resistant wheat line CI12633.

Figure S9 Response to Rhizoctonia cerealis by TaSTT3a-silenced and control wheat lines.

Figure S10 Diagram of TaSTT3b-2B overexpressing transformation vector pWMB122-TaSTT3b-2B.

Figure S11 PCR detection of TaSTT3b-2B in the T0 generation of transgenic lines.

Figure S12 Droplets visualized in two dimensions for transgenic and wild type lines.

Figure S13 Volcano map of differentially expressed genes.

Figure S14 Classification of differentially expressed genes by Gene Ontology analysis.

Figure S15 GDSL-like lipase genes in differentially expressed genes.

Figure S16 Transcription analysis of eight defense-related genes in TaSTT3b-silenced and control wheat plants.

Figure S17 Yield-affected factors of WT and transgenic plants infected by Rhizoctonia cerealis.

Figure S18 Expression patterns of eight defense-related genes in wheat response to exogenous JA.

Figure S19 Schematic diagram of seedling inoculation identification for sharp eyespot.

Figure S20 Examples of field plots of wheat grown to obtain agronomic trait data.

Table S1 Identities between amino acids of TaSTT3b-2A, TaSTT3b-2B, and TaSTT3b.

Table S2 Genes expressed in all samples.

Table S3 List of differentially expressed genes.

Table S4 List of defense-related genes in differentially expressed genes.

Table S5 List of genes involved in JA biosynthesis in differentially expressed genes.

Table S6 List of primers.