A unique wheat disease resistance-like gene governs effector-triggered susceptibility to necrotrophic pathogens

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Plant disease resistance is often conferred by genes with nucleotide binding site (NBS) and leucine-rich repeat (LRR) or serine/threonine protein kinase (S/TPK) domains. Much less is known about mechanisms of susceptibility, particularly to necrotrophic fungal pathogens. The pathogens that cause the diseases tan spot and Stagonospora nodorum blotch on wheat produce effectors (host-selective toxins) that induce susceptibility in wheat lines harboring corresponding toxin sensitivity genes. The effector ToxA is produced by both pathogens, and sensitivity to ToxA is governed by the *Tsn1* gene on wheat chromosome arm 5BL. Here, we report the cloning of *Tsn1*, which was found to have disease resistance gene-like features, including S/TPK and NBS-LRR domains. Mutagenesis revealed that all three domains are required for ToxA sensitivity, and hence disease susceptibility. *Tsn1* is unique to ToxA-sensitive genotypes, and insensitive genotypes are null. Sequencing and phylogenetic analysis indicated that *Tsn1* arose in the B-genome diploid progenitor of polyploid wheat through a gene-fusion event that gave rise to its unique structure. Although *Tsn1* is necessary to mediate ToxA recognition, yeast two-hybrid experiments suggested that the Tsn1 protein does not interact directly with ToxA. *Tsn1* transcription is tightly regulated by the circadian clock and light, providing further evidence that *Tsn1*-ToxA interactions are associated with photosynthesis pathways. This work suggests that these necrotrophic pathogens may thrive by subverting the resistance mechanisms acquired by plants to combat other pathogens.

disease resistance | host–pathogen interaction | map-based cloning | necrotrophic fungus | *Triticum*

Plants have evolved sophisticated innate immune systems to protect themselves from invading pathogens and pests. The direct or indirect recognition of pathogen-produced effectors by host resistance (R) genes leads to a resistance response known as effector-triggered immunity (ETI), which includes localized programmed cell death (PCD), known as the hypersensitive response (HR), to restrict pathogen growth (1). R gene proteins serve to recognize pathogen effectors either through direct interaction or as guards for target molecules and are known to confer resistance to bacteria, viruses, nematodes, oomycetes, insects, and biotrophic fungi (2). The largest class of plant R genes consists of the N-terminal nucleotide-binding site (NBS)–C-terminal leucine-rich repeat (LRR) proteins characterized by an NBS and LRRs. Another, albeit smaller, class of R proteins are those composed of serine/threonine protein kinase (S/TPK) domains such as the barley *Rpg1* stem rust R gene (3). The barley *Rpg5* stem rust R gene represents a unique structure among R genes in that it contains an N-terminal NBS-LRR and a C-terminal S/TPK (4).

Stagonospora nodorum and *Pyrenophora tritici-repentis* are necrotrophic fungal pathogens of wheat that cause the diseases Stagonospora nodorum blotch (SNB) and tan spot, respectively, each of which can cause substantial economic losses worldwide (5, 6). Both pathogens produce numerous proteinaceous host-selective toxins (HSTs) that function as effectors to elicit severe necrosis in wheat lines harboring corresponding dominant sensitivity genes (7, 8). A compatible interaction requires both the effector (HST) and the host gene and results in susceptibility as opposed to ETI. The absence of either the effector or the host gene results in an incompatible interaction (insensitivity) and leads to resistance unless other compatible host-effector interactions are present.

Among the host–effector interactions identified in the wheat–*P. tritici-repentis* and wheat–*S. nodorum* systems, the *Tsn1*–ToxA interaction is the best characterized. The *ToxA* gene encodes a 13-kDa polypeptide (9) and was horizontally transferred from *S. nodorum* to *P. tritici-repentis*, an event that likely resulted in tan spot becoming an economically significant disease (10). The wheat *Tsn1* gene confers sensitivity to ToxA produced by both pathogens (11), and compatible *Tsn1*–ToxA interactions play major roles in conferring susceptibility in both systems (12, 13). Here, we isolated and characterized the *Tsn1* gene to gain understanding of the mechanisms associated with compatible interactions that lead to effector-triggered susceptibility (ETS) in these wheat-necrotrophic fungus pathosystems.

Results

Chromosome Walking and Identification of *Tsn1* Candidate Genes. We previously reported the development of BAC contigs flanking *Tsn1* on chromosome 5B and anchored to a high-resolution genetic linkage map (Fig. 1 *A*–*C*) developed in a population of 5,438 gametes based on the durum wheat cultivar Langdon (LDN) (14, 15). Beginning with marker *Xfcg26* located at the distal end of the larger contig (ctg548) (Fig. 1*C*), chromosome walking was conducted to assemble a physical map spanning the *Tsn1* locus. This delineated *Tsn1* to an ~350-kb region, and bioinformatic analysis of the DNA sequence of this interval pre-

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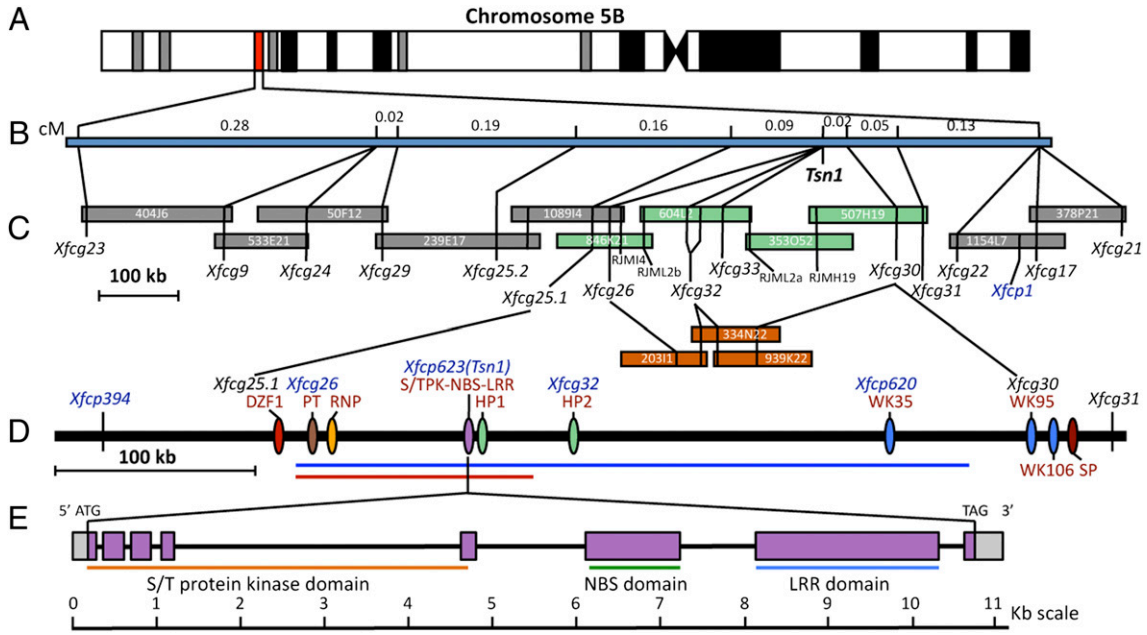

Fig. 1. Map-based cloning of the *Tsn1* gene. (*A*) Genomic region containing the *Tsn1* gene on the long arm of wheat chromosome 5B is shown in red. (*B*) Genetic linkage map of the *Tsn1* region. (*C*) BAC-based physical maps of the *Tsn1* region anchored to the genetic map. LDN BACs previously described (14) are shown in gray. The 5B and 5A BACs reported in the current work are shown in green and orange, respectively. (*D*) Predicted genes (ovals with names in dark red) and markers (names beginning with an "X"). Markers used for association mapping of the 386 *Triticum* accessions are indicated in blue. The blue and red lines indicate the candidate gene regions as defined by recombination in the mapping population and association mapping of the 386 *Triticum* accessions, respectively. (*E*) Exons and UTRs of *Tsn1* are shown in purple and gray, respectively.

dicted six genes that cosegregated with *Tsn1*. Further examination by association mapping using 386 *Triticum* accessions phenotyped for ToxA and genotyped with markers derived from these genes (Tables S1 and S2) disassociated two of them from *Tsn1* and left four candidate genes in a 120-kb interval; these included a hypothetical protein, a potassium transporter, a U2 small nuclear (sn) ribonucleoprotein (RNP) auxiliary factor, and, potentially, a single gene encoding S/TPK-NBS-LRR domains (Fig. 1*D*).

Validation and Structural Characterization of *Tsn1*. We treated seed of LDN and the hexaploid wheat variety Bobwhite with the chemical mutagen ethylmethane sulfonate and identified 13 independent ToxA-insensitive/disease-susceptible mutants (Fig. 2) from the screening of 2,115 M_2 families. Sequence analysis of the four candidate genes from mutants LDNems114, LDNems230, and LDNems937 indicated that the S/TPK-NBS-LRR–like gene of each mutant harbored a mutation (Table 1), whereas the sequences of the other three genes were identical to the wild type (WT). The S/TPK-NBS-LRR gene was then fully characterized by molecular analysis. The full-length cDNA was amplified from LDN using primers located at the 5′ end of the translation start site and the 3′ end of the stop codon to verify that the S/TPK and NBS-LRR domains are encoded by a single transcript (Fig. S1), and the cDNA sequence was aligned to the genomic sequence to determine splicing structure. The gene has eight exons and is 10,581 bp from the start to stop codons with a coding sequence of 4,473 bp and 5′ and 3′ UTRs of 161 and 391 bp, respectively (Fig. 1*E*). The predicted protein of 1,490 amino acids harbors three highly conserved domains. The first five exons encode the S/TPK domain, which harbors the expected conserved motifs, including the ATP-binding site, substrate-binding site, and activation loop (Fig. S2). The sixth exon encodes the NBS domain, which also contains all the expected conserved motifs. The seventh exon encodes 24 LRRs. The last exon has no obvious conserved domains and, unlike most monocot NBS-LRRs, the gene does not appear to contain a coiled-coil domain. In addition, the gene does not contain any apparent transmembrane domains, and is therefore likely located in the cytoplasm.

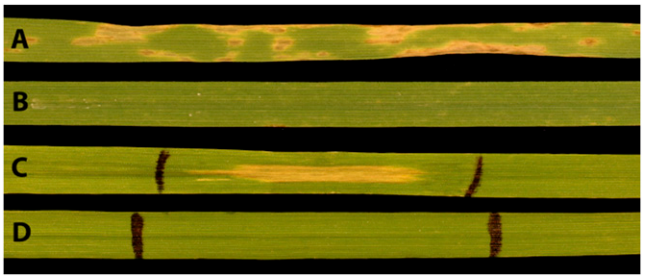

Fig. 2. Leaves of Kulm (*Tsn1*) (*A* and *C*) and Kems103 (*Tsn1* mutant) (*B* and *D*) inoculated with *S. nodorum* (*A* and *B*) and infiltrated with ToxA (*C* and *D*).

To confirm further that the S/TPK-NBS-LRR gene was *Tsn1*, we sequenced the gene from the remaining 10 mutants along with 3 mutants previously developed in the hexaploid variety Kulm (16), and analysis revealed that all 13 harbored mutations consisting of missense, nonsense, and splice site mutations (Table 1). The splice site mutations occurred in mutants LDNems230 and Kems103, and they were further validated by RT-PCR (Fig. S1) and sequence analysis. LDNems230 had a point mutation in the third intron at position 778, 5 bp from the splice site junction, and Kems103 had a point mutation at position 7,074, which was the first base of intron 6 (Table 1). The LDNems230 fragment was 226 bp smaller than the fragment amplified in LDN. Subsequent sequence analysis indicated this was attributable to the absence of exon 3 in the transcribed sequence. The fragment amplified in Kems103 was larger than that of Kulm. Sequence analysis revealed that the exon 6/intron 6 WT splice site was abolished; instead, splicing occurred at position 7,116, which was 43 bp downstream of the WT splice site.

Southern and PCR analysis of 24 wheat cultivars indicated that the S/TPK-NBS-LRR gene was specific to ToxA-sensitive genotypes and that a null allele occurred in insensitive lines (Fig. 3). In addition, genotyping of the 386 *Triticum* accessions with the

Table 1. Descriptions of induced and natural mutations identified within the *Tsn1* gene

	Mutation type	Position*	Exon	Domain	Codon change	Amino acid change
Induced mutant						
LDNems114	Missense	86	1	S/TPK	GGG→GAG	Gly→Glu
Kems37-5†	Missense/nonsense	602/9,767	3/7	S/TPK/LRR	TGT→TAT/TGG→TGA	Cys→Tyr/Trp→stop
BWems123	Nonsense	625	3	S/TPK	GGA→TGA	Gly→stop
LDNems230‡	Splice	778	—	—	—	—
Kems103†	Missense/splice	5,983/7,074	6/–	NBS/–	AGG→AAG/–	Arg→Lys/–
LDNems391	Nonsense	6,663	6	NBS	CAA→TAA	Gln→stop
LDNems346	Missense	6,705	6	NBS	GGT→AGT	Gly→Ser
BWems687	Missense	6,721	6	NBS	GCT→GTT	Ala→Val
Kems322	Missense	6,847	6	NBS	TCC→TTC	Ser→Phe
LDNems299	Nonsense	6,959	6	NBS	TGG→TGA	Trp→stop
LDNems138	Missense	7,008	6	NBS	CTC→TTC	Leu→Phe
LDNems355	Missense	9,184	7	LRR	ACT→ATT	Thr→Ile
LDNems403	Missense	9,552	7	LRR	CCT→TCT	Pro→Ser
LDNems937	Missense	9,792	7	LRR	CTC→TTC	Leu→Phe
BWems952	Missense	9,792	7	LRR	CTC→TTC	Leu→Phe
BWems258	Missense	9,817	7	LRR	TGC→TAC	Cys→Tyr
Natural mutant						
TA2601	Frameshift	4,610	5	S/TPK	—	—
Ching Feng	Frameshift	5,983	6	NBS	—	—
Siu Mak	Frameshift	8,144	7	LRR	—	—
Huo Mai	Nonsense	9,767	7	LRR	TGG→TGA	Trp→stop
Novo	Nonsense	9,767	7	LRR	TGG→TGA	Trp→stop
Puseas	Nonsense	9,767	7	LRR	TGG→TGA	Trp→stop

*Base pair position counting from the translation start site.
†Kems37-5 and Kems103 each contained two mutations. RT-PCR and sequence analysis indicated that the splice mutation in Kems103 results in a product 43 bp larger than the WT (Fig. S1).
‡RT-PCR and sequence analysis of LDNems230 showed that exon 3 is eliminated from the coding sequence (Fig. S1).

PCR marker *Xfcp623* indicated that, with six exceptions, only ToxA-sensitive lines harbored the S/TPK-NBS-LRR sequence (Table S1). Sequence analysis of these six lines indicated that Novo, Puseas, and Huo Mai all had a nonsense mutation at the same position within the LRR domain (Table 1). The lines Siu Mak, Ching Feng, and TA2601 all had frameshift mutations at different positions within the gene. Together, these results indicated that the S/TPK-NBS-LRR–like gene was *Tsn1* and that all three major domains are essential for *Tsn1* function.

Comparative Analysis. Comparative analysis of the *Tsn1* genomic region of wheat chromosome 5B with the homologous region of wheat chromosome 5A, rice, and *Brachypodium* indicated a conserved level of colinearity with wheat chromosome 5A, rice chromosome 9, and *Brachypodium* chromosome 4, but *Tsn1* homologs were not present in any of the colinear segments (Fig. 4 and Table S3). Southern analysis confirmed the absence of *Tsn1* homoalleles on wheat chromosome 5A as well as on wheat chromosome 5D (Fig. 3). Separate homologs of the S/TPK and NBS-LRR regions of *Tsn1* were present on rice and *Brachypodium* chromosomes 11 and 2, where they are separated by 8.5 kb and 2.1 Mb, respectively. Comparisons indicated that the rice and *Brachypodium* NBS-LRR homologs contain no additional domains or motifs compared with the NBS-LRR region of *Tsn1*, but the S/TPK homologs in *Brachypodium* and rice harbor C-terminal domains with similarity to hypothetical and WD40 repeat-containing proteins, respectively (Fig. 4).

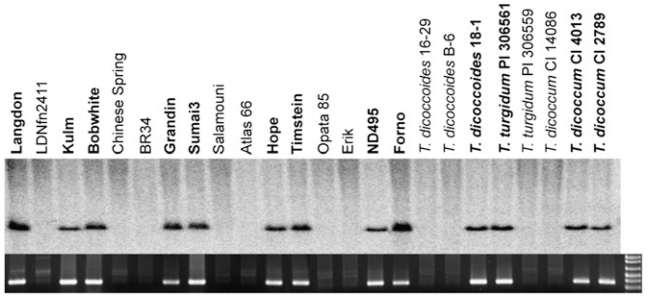

Fig. 3. Southern and PCR analysis of 24 selected wheat lines. (*Top panel*) Southern hybridization of DNA digested with restriction enzyme *XbaI* and probed with FCG34, which is derived from the NBS region of *Tsn1*. (*Bottom panel*) PCR amplification with primers for marker *Xfcp623(Tsn1)* derived from intron five of *Tsn1*. The wheat genotypes labeled in bold and not bold are sensitive and insensitive to ToxA, respectively.

Tsn1 has no significant similarity to any sequence in the National Center for Biotechnology Information (NCBI) nonredundant (nr) database at the nucleotide level. At the amino acid level, the S/TPK and NBS-LRR portions of *Tsn1* are most similar to rice homologs (Table S4). Phylogenetic analysis using the amino acid sequences of the S/TPK and NBS domains separately was conducted to determine relationships with other known plant genes. The S/TPK domain of *Tsn1* is closely related to those of the barley stem rust R gene *Rpg1* (3) and its homologs in monocots, whereas the NBS domain of *Tsn1* is closely related to the maize *Rp3* rust R gene (17) and its homologs (Fig. S3).

***Tsn1* Allelic Diversity.** Evaluation the 386 *Triticum* accessions indicated that *Tsn1* is present among B genome-containing hexaploid and tetraploid wheat species, including wild emmer wheat (*Triticum turgidum* ssp. *dicoccoides*) (Table S1), which is a primitive tetraploid ancestor of domesticated durum and common wheat. *Aegilops speltoides* (SS genomes) is a close relative of the diploid B-genome progenitor of polyploid wheat (18). Therefore, we screened 127 accessions of *A. speltoides* for reaction to ToxA to determine if any harbored functional *Tsn1* alleles (Table S5). Two accessions were sensitive to ToxA, indicating that they harbor functional copies of *Tsn1*.

We sequenced *Tsn1* from a total of 42 diverse *Tsn1*-containing lines. Phylogenetic analysis indicated that *Tsn1* alleles of the two

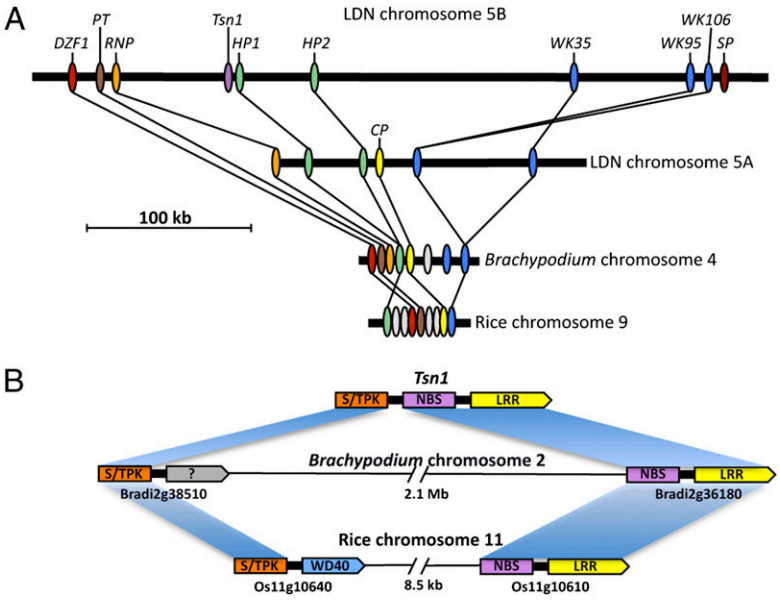

Fig. 4. Colinearity at the *Tsn1* locus. (*A*) Colinearity of genes (colored ovals) within the *Tsn1* region of wheat chromosome 5B, the homologous region of wheat chromosome 5A, *Brachypodium* chromosome 4, and rice chromosome 9. Gene descriptions are presented in Table S4. Note that *Tsn1* is present only in wheat 5B. (*B*) Comparisons of the *Tsn1* domains with homologous domains/genes on rice chromosome 11 and *Brachypodium* chromosome 2. The NBS-LRR region of *Tsn1* corresponds to the full coding regions of the predicted rice and *Brachypodium* NBS-LRR genes, but the genes containing homologous S/TPK domains in rice and *Brachypodium* harbor additional domains not found in *Tsn1*.

A. speltoides accessions were more divergent compared with the *Triticum* accessions. Among *Triticum* species, wild emmer *Tsn1* alleles were the most divergent with *T. dicoccoides* accession 18-10 showing 97% identity with the LDN allele, but nucleotide variation among the *Tsn1* alleles of durum and bread wheat varieties was nearly nonexistent (Fig. S3).

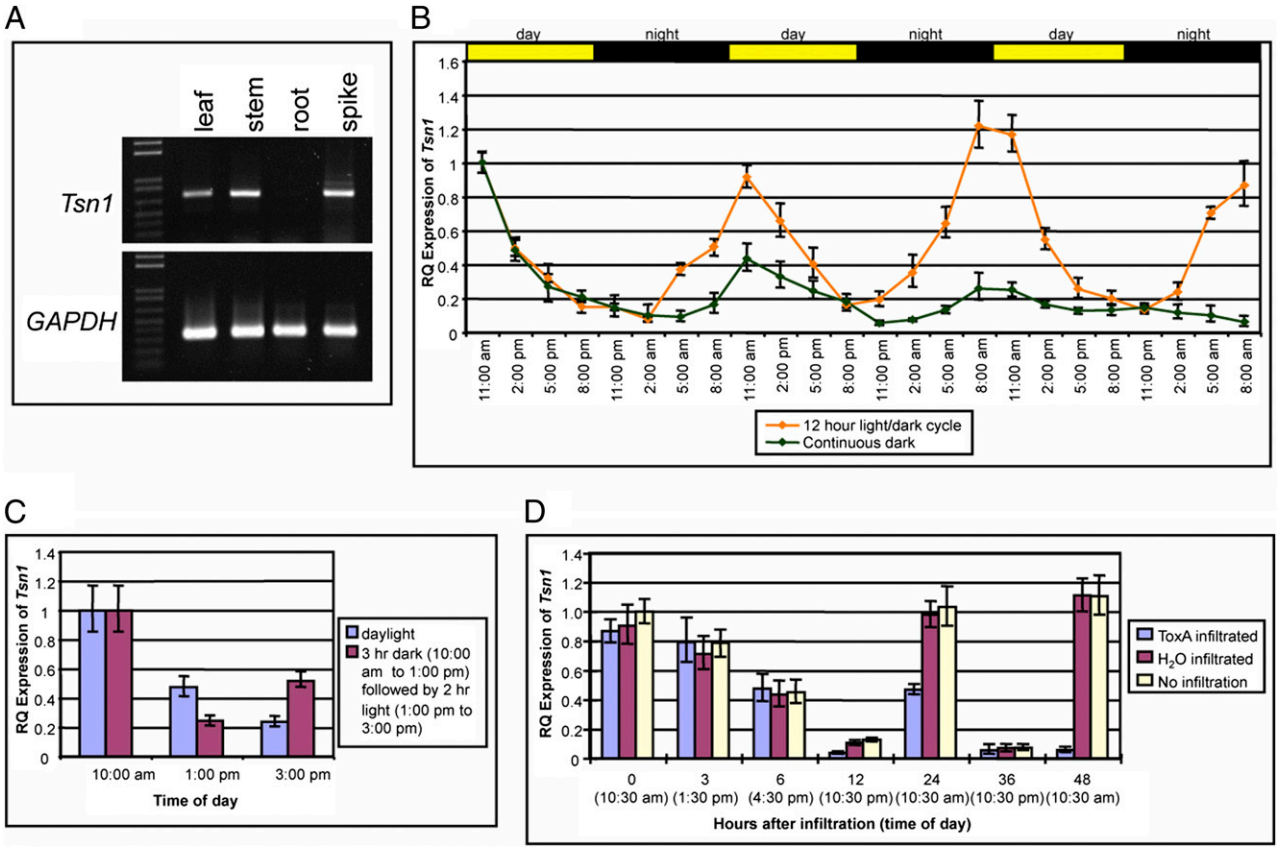

Fig. 5. Transcriptional expression of *Tsn1*. (*A*) *Tsn1* expression survey by RT-PCR with *GAPDH* as an endogenous control. (*B*) *Tsn1* expression levels in 2-wk-old plants entrained with a 12-h light/dark cycle evaluated every 3 h over a 72-h period (orange) and in plants subjected to continuous dark for the same time periods (dark green) using RQ-PCR. (*C*) RQ-PCR evaluation of *Tsn1* expression in 2-wk-old plants entrained with a 12-h light/dark cycle (control; blue bars) and plants subjected to 3 h of dark followed by 2 h of light (red bars). (*D*) RQ-PCR evaluation of *Tsn1* expression in ToxA-challenged plants (blue bars, ToxA infiltrated; red bars, H_2O infiltrated; yellow bars, no infiltration).

Transcriptional Regulation of *Tsn1*. Evaluation of *Tsn1* expression in different plant tissues of LDN by RT-PCR indicated that it is expressed in the leaf, stem, and immature spike but not in the roots (Fig. 5*A*). Initial attempts to quantify transcriptional expression levels of *Tsn1* in leaf tissue were inconsistent. Therefore, we collected samples of leaf tissue every 3 h for 3 d from 2-wk-old plants grown in the growth chamber with a 12-h light/dark cycle (9:00 AM/9:00 PM) and from plants placed under continuous darkness at the first time point. Relative quantitative (RQ)-PCR analysis of plants grown under the 12-h light/dark cycle indicated that *Tsn1* expression is regulated by the circadian clock (Fig. 5*B*). *Tsn1* expression levels in the plants placed under continuous darkness mimicked those of the light/dark cycle-grown plants for the first 15 h; however, at 24 h, expression levels increased to less than half of the level observed in the light/dark-grown plants. At 48 h, expression levels increased to only 20% of those observed for the light/dark cycle-grown plants, and no elevated expression levels were observed at 72 h. The modest increase in *Tsn1* expression at 24 and 48 h was probably attributable to rhythmic entrainment.

To investigate the effects of light on *Tsn1* expression further, we evaluated 2-wk-old plants entrained with a 12-h light/dark cycle and plants subjected to 3 h of dark followed by 2 h of light. The light/dark cycle-grown control plants showed a continuous decline in *Tsn1* expression levels (Fig. 5*C*), which agreed with the previous experiment (Fig. 5*B*). However, *Tsn1* expression in the plants exposed to 3 h of darkness was significantly less than that of the control at this time point. When the plants were then exposed to light for 2 h, *Tsn1* expression was significantly greater than in the control plants. Therefore, *Tsn1* transcription was significantly reduced under darkness and significantly increased by exposure to light, which further demonstrates that light is an important factor in *Tsn1* regulation.

To determine whether ToxA influences *Tsn1* transcription levels, we evaluated expression in 2-wk-old plants infiltrated with ToxA and included water-infiltrated and noninfiltrated plants as controls. Expression levels of *Tsn1* in the water-infiltrated and noninfiltrated controls agreed with those observed in the previous experiments (Fig. 5*D*). *Tsn1* expression levels in the ToxA-treated plants declined steadily for the first 12 h just as in the controls but increased to only half the level of the controls at 24 h. Expression levels then declined to the minimum level at 36 h, and no upregulation occurred at 48 h. Therefore, the pattern of *Tsn1* expression in ToxA-treated plants closely mimicked that observed for plants grown under continuous darkness.

The Tsn1 Protein Does Not Interact Directly with ToxA in Yeast Two-Hybrid Assays. Yeast two-hybrid analysis was conducted to determine if the Tsn1 protein interacts directly with ToxA. The full-length *Tsn1* gene and individual S/TPK, NBS, and LRR domains were tested for interaction with ToxA, but all showed negative results, suggesting that the Tsn1 protein does not interact directly with ToxA (Fig. S4).

Discussion

Here, we report the cloning and characterization of the gene conferring sensitivity to ToxA produced by the necrotrophic pathogens that cause tan spot and SNB of wheat. *Tsn1* contains features commonly associated with disease R genes, including S/TPK and NBS-LRR domains, but it is the only gene known to possess this structure (i.e., N-terminal S/TPK and C-terminal NBS-LRR). Two other plant genes that condition sensitivity to necrotrophic effectors have recently been isolated. The Arabidopsis *LOV1* gene, which governs sensitivity to the effector known as victorin produced by the oat pathogen *Cochliobolus victoriae*, was found to belong to the NBS-LRR class of genes (19). The *Pc* gene of sorghum, which confers sensitivity to the effector known as Pc-toxin produced by *Periconia circinata*, was also shown to be a member of the NBS-LRR class (20). Although *LOV1* and *Pc* mediate recognition of effectors that are small molecular weight secondary metabolites, *Tsn1* is unique in that it mediates recognition of a proteinaceous HST.

To date, only one other gene, the barley *Rpg5* stem rust R gene (4), has been shown to possess S/TPK, NBS, and LRR domains in a single transcript. However, the domains are arranged differently in *Rpg5* compared with *Tsn1*, with the S/TPK domain occurring at the N terminus in *Tsn1* and at the C terminus in *Rpg5*. Phylogenetic analysis indicated that the two proteins are not closely related. These results indicated that *Tsn1* and *Rpg5* probably do not share recent ancestry, and they probably arose through independent gene fusion events that gave rise to their unique structures.

Tetraploid wild emmer wheat (*T. turgidum* ssp. *dicoccoides*, 2n = 4× = 28, AABB genomes) arose through hybridization of two diploid species, *T. urartu* (2n = 2× = 14, AA genome) and what is thought to be a close relative of *A. speltoides* (2n = 2× = 14, SS genome). Wild emmer gave rise to domesticated emmer wheat (*T. turgidum* ssp. *dicoccum*, 2n = 4× = 28, AABB genomes), which was later fully domesticated in the form of durum (macaroni) wheat (*T. turgidum* ssp. *durum*, 2n = 4× = 28, AABB genomes). Hexaploid wheat (bread or common wheat, *T. aestivum*, 2n = 6× = 42, AABBDD genomes) arose under cultivation from a spontaneous hybridization between an AB-tetraploid and the diploid goatgrass *A. tauschii* (2n = 2× = 14, DD genomes). Here, we found functional *Tsn1* alleles to exist at all ploidy levels of the B-genome lineage. Phylogenetic analysis indicated that the wild progenitor alleles were more divergent compared with the alleles of domesticated wheats. This suggests that the gene fusion event that gave rise to *Tsn1* occurred in the diploid B-genome progenitor of polyploid wheat and was passed through the tetraploids and the bottleneck that gave rise to hexaploid wheat. Comparative analysis with wheat chromosome 5A, rice, and *Brachypodium* suggests that an ancestor of the diploid progenitors likely harbored separate S/TPK and NBS-LRR genes that were ultimately lost from the A and D genomes. This analysis also suggests that the primitive S/TPK gene involved in the gene-fusion event may have harbored additional domains that were lost on fusion with the complete NBS-LRR gene.

Previous work by Manning and Ciuffetti (21) demonstrated that ToxA is imported within the cell in *Tsn1* lines but not in lines lacking *Tsn1*. The Tsn1 protein contains no apparent transmembrane domains, and is therefore likely located within the cell. This and the fact that Tsn1 does not appear to interact directly with ToxA suggest that Tsn1 is not likely to be the ToxA receptor. Tsn1 may instead act to monitor the ToxA receptor or another ToxA-associated target. ToxA has been reported to interact directly with plastocyanin (22) and with another chloroplast localized protein known as ToxABP1 (23). Our preliminary data from yeast two-hybrid experiments indicate that the Tsn1 protein does not directly interact with either plastocyanin or ToxABP1, and therefore does not likely function as a guard of these two ToxA interactors. We are currently performing experiments to identify proteins that directly interact with Tsn1 and to identify possible ToxA targets guarded by Tsn1. It is possible that the sensing of ToxA-induced perturbations of a target guarded by Tsn1 could trigger events that lead to cell importation of ToxA. Under this scenario, Tsn1 may interact with ToxA indirectly and initiate signaling that leads to importation of ToxA. Although this hypothesis is yet to be tested, the current work demonstrates that *Tsn1* mediates effector recognition and that a functional *Tsn1* is necessary for sensitivity to ToxA.

In this work, we demonstrate that *Tsn1* expression is regulated by the circadian clock and light. The low expression of *Tsn1* in the dark provides a possible explanation for the light dependency of compatible *Tsn1*–ToxA interactions (21). There is considerable evidence indicating that the *Tsn1*–ToxA pathway is associated with photosynthetic pathways (24), including the observation that ToxA is located to the chloroplast (21) and directly interacts with plastocyanin (22), which is a vital component of electron transport in photosystem II, and ToxABP1 (23). Manning et al. (24) suggested that ToxA induces photosystem alterations, which would affect photosynthesis. This could lead to perturbed regulation of *Tsn1* transcription similar to that observed under continuous darkness, providing a possible explanation for the similarities observed in *Tsn1* expression patterns in ToxA-challenged plants and plants exposed to con-

tinuous darkness (Fig. 5). One might speculate that once ToxA is recognized by *Tsn1*, the necessary signaling events leading to PCD are soon in motion and ToxA no longer would require *Tsn1*. The reduction of *Tsn1* expression may be attributable to perturbation of photosynthesis pathways, which are exploited by the pathogen, rather than direct down-regulation by ToxA. Although these hypotheses need further exploration, others have demonstrated that the HR and defense response associated with other host–pathogen interactions are also influenced by the circadian clock and light (25). It would be interesting to evaluate the effects of light and circadian rhythms on the transcriptional regulation of classic disease R genes.

Among the 513 wheat accessions evaluated in this study, 44 were developed through breeding and artificial selection (*T. aestivum* spp. *aestivum* and *compactum* as well as *T. turgidum* ssp. *durum* accessions in Table S1). Among these, 22 (50%) had functional *Tsn1* alleles. In contrast, only 60 (13%) of the remaining 469 accessions had *Tsn1*. This group consists mostly of wild wheats exposed to natural selection. Oliver et al. (26) tested 53 wheat cultivars for reaction to ToxA and found that all but 8 (85%) possessed *Tsn1*. Therefore, it seems apparent that *Tsn1*, a disease susceptibility gene, has been retained to some degree in the cultivated wheats via artificial selection. One possible explanation is that *Tsn1* may have, or have had, an alternate function, such as to confer resistance to another pathogen, as is the case with the oat victorin sensitivity gene (19).

The results of this work, along with a growing amount of evidence indicating that common signaling pathways are associated with both biotroph resistance and necrotroph susceptibility (19, 27), suggest that host response mechanisms associated with ETS to necrotrophs and ETI to other pathogens are very similar. The differences in the outcomes may be attributed to the biology of the pathogen (i.e., necrotrophs are equipped to thrive in environments that would be detrimental to pathogens with biotrophic lifestyles).

Methods

Materials and methods are described in detail in *SI Text*. The following is a brief summary of methods used. Chromosome walking was conducted using the population and methods previously described (14). In total, 386 *Triticum* accessions (Table S1) were used for haplotype analysis to identify additional recombination events and to reduce the *Tsn1* candidate gene region further. Putative genes within and flanking the *Tsn1* candidate gene region were subjected to BLASTx searches of the NCBI database (Table S4) and tBLASTx searches of the rice and *Brachypodium* genomes in October, 2009, to identify putative orthologs and determine colinearity (Table S3). *Tsn1* candidates were validated by PCR-amplifying genomic sequences (Table S2) from the mutants and WTs, followed by comparative sequence analysis. *Tsn1* structure was determined by PCR-amplifying cDNA sequences from LDN and *A. speltoides* (Table S2), aligning the full-length cDNA sequence with the genomic DNA sequence, and by performing 5′ and 3′ RACE. Southern analysis using a probe derived from the NBS region of *Tsn1* (Table S2) was done to determine genome/genotype specificity. A total of 127 *A. speltoides* accessions were screened for reaction to ToxA to determine if any harbored a functional *Tsn1* allele (Table S5). To determine allelic diversity, *Tsn1* genomic sequences were obtained from 42 *Tsn1*-containing genotypes and a phylogenetic tree was constructed from CLUSTALW alignments of the complete coding region using the unweighted pair group method with arithmetic mean (UPGMA) method. The conserved NBS domains or S/TPK domains encoded in *Tsn1* and related plant genes were determined, and phylogenetic analysis was performed using the neighbor-joining method. RT-PCR was performed to evaluate *Tsn1* transcription in different tissues. RQ-PCR was performed on RNA collected from leaves of 2-wk-old plants to determine the effects of the circadian clock, light, and ToxA infiltrations on *Tsn1* expression levels using the *GAPDH* gene as an endogenous control. Yeast two-hybrid analysis was performed using the Matchmaker Library Construction and Screening Kit (Clontech) following the manufacturer's instructions.

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