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-like gene 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.

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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 Xfg26 located at the distal end of the larger contig (ctg548) (Fig. 1C), 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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Data deposition: BAC sequences have been deposited in Genbank under accession numbers GU256282 to GU256287, and Tsn1 sequences were deposited under GU593618 to GU593657, and GU593050 to GU593053.

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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 M₁ populations. 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. 1E). 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.

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

![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”) used for association mapping of the 386 Triticum accessions. (E) Exons and UTRs of Tsn1 are shown in purple and gray, respectively.](image)

![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).](image)
PCRand other 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 homoeoalleles 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).

Tsn1 has no significant similarity to any sequence in the National Center for Biotechnology Information (NCBI) non-redundant (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 Rps3 rust R gene (17) and its homologs (Fig. S3).

**Tsn1 Allelic Diversity.** Evaluation of 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

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

<table>
<thead>
<tr>
<th>Induced mutant</th>
<th>Mutation type</th>
<th>Position*</th>
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<th>Domain</th>
<th>Codon change</th>
<th>Amino acid change</th>
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<td>LDNems114</td>
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<td>GGA→TGA</td>
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<td>AGG→AAG/—</td>
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<td>Cys→Tyr</td>
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<table>
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<tr>
<th>Natural mutant</th>
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<th>Position*</th>
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<th>Domain</th>
<th>Codon change</th>
<th>Amino acid change</th>
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<tr>
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<td>Frameshift</td>
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<td>Novo</td>
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<td>LRR</td>
<td>TGG→TGA</td>
<td>Trp→stop</td>
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<tr>
<td>Puseas</td>
<td>Nonsense</td>
<td>9,767</td>
<td>7</td>
<td>LRR</td>
<td>TGG→TGA</td>
<td>Trp→stop</td>
</tr>
</tbody>
</table>

*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).

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**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 FG343, 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.
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. 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.

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, H2O infiltrated; yellow bars, no infiltration).
Transcriptional Regulation of 

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. 3A). 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 (RO)-PCR analysis of plants grown under the 12-h light/dark cycle indicated that Tsn1 expression is regulated by the circadian clock (Fig. 5B). 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 darkness by exposure to light. Light/dark-cycle-grown control plants showed a continuous decline in Tsn1 expression (Fig. 5C), which agreed with the previous expression experiments (Fig. 5D). 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 inoculated 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. 5D). 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 up-regulation 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 virginin produced by the oot pathogen Coelothruss 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 = 4x = 28, AABB genomes) arose through hybridization of two diploid species, T. urartu (2n = 2x = 14, AA genome) and what is thought to be a close relative of A. speltoides (2n = 2x = 14, SS genome). Wild emmer gave rise to domesticated emmer wheat (T. turgidum ssp. dicoccum, 2n = 4x = 28, AABB genomes), which was later fully domesticated in the form of durum (macaroni) wheat (T. turgidum ssp. durum, 2n = 4x = 28, AABB genomes). Hexaploid wheat (bread or common wheat, T. aestivum, 2n = 6x = 42, AABBD genomes) arose under cultivation from a spontaneous hybridization between an AB-tetraploid and the diploid goatchest A. tauschii (2n = 2x = 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. The high expression of Tsn1 at 24 h in the progenitors (Fig. 5A) may have occurred in the diploid B-genome progenitor of polyplloid wheat and was passed through the tetraploids and出现了"的小写和大写错误。
tuous 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 voinorin sensitivity gene (19).

The results of this work, along with a growing amount of evidence indicating that common signaling pathways are associated with both biotrophic resistance and necrotrophic 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. speltoideus (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. speltoideus accessions were screened for reactions to ToxA, and 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 neighboring method. RT-PCR was performed to evaluate Tsn1 transcription in different tissues. RQ-PCR was performed on RNA collected from leaves of 2-week-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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