A wheat COP9 subunit 5-like gene is negatively involved in host response to leaf rust

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SUMMARY

The COP9 (constitutive photomorphogenesis 9) signalosome (CSN) is a protein complex involved in the ubiquitin proteasome system and a common host target of diverse pathogens in Arabidopsis. The known deubiquitylation function of the COP9 complex is carried out by subunit 5 encoded by AtCSN5A or AtCSN5B in Arabidopsis. A single CSN5-like gene (designated as TaCSN5) with three homeologues was identified on the long arms of wheat (Triticum aestivum L.) group 2 chromosomes. In this study, we identified and characterized the function of TaCSN5 in response to infection by the leaf rust pathogen. Down-regulation of all three TaCSN5 homeologues or mutations in the homeologues on chromosomes 2A or 2D resulted in significantly enhanced resistance to leaf rust. Enhanced leaf rust resistance corresponded to a seven-fold increase in PR1 (pathogenesis-related gene 1) expression. Collectively, the data indicate that the wheat COP9 subunit 5-like gene acts as a negative regulator of wheat leaf rust resistance.

Keywords: in silico cloning, leaf rust, qRT-PCR, Triticum aestivum, VIGS.

INTRODUCTION

The Arabidopsis COP9 (constitutive photomorphogenesis 9) gene encodes a 22.5-kDa protein which is a component of a protein complex called the COP9 signalosome (CSN) that represses photomorphogenic development (Wei et al., 1994a). The COP9 signalosome comprises eight subunits designated CSN1–CSN8 (Wei and Deng, 1999). The CSN genes were first identified in morphological screens for constitutive photomorphogenesis phenotypes in Arabidopsis (Deng, 1994; Wei and Deng, 1999). The CSN genes are involved in diverse cellular processes, including hormone signalling and development (Dohmann et al., 2008; Gusmaroli et al., 2004, 2007; Wei and Deng, 1998), cell cycle progression (Dohmann et al., 2008) and stress response (Hind et al., 2011; Liu et al., 2002). The COP9 signalosome is conserved among divergent organisms, including fission yeast, Drosophila melanogaster, Arabidopsis, humans and probably cyanobacteria (Chamovitz et al., 1996; Freilich et al., 1999; Kaniol et al., 1999; Mundt et al., 1999; Seeger et al., 1998; Wei et al., 1998). The only known biochemical function of the COP9 signalosome is to remove ubiquitin-like protein RUB (Related to Ubiquitin) via the deubiquitylation of cullins (Gusmaroli et al., 2004). The catalytic centre of deubiquitylation resides in subunit 5 (CSN5), which is encoded by homologous genes CSN5A and CSN5B in Arabidopsis (Gusmaroli et al., 2004).

The involvement of the COP9 signalosome in the plant defence response was first documented in relation to the Tobacco mosaic virus (TMV) resistance gene N (Liu et al., 2002). The COP9 signalosome associates with NbRar1 and NbSGT1 in another protein complex involved in protein degradation in the ubiquitin proteasome pathway. Silencing subunits CSN4 and CSN8 of the COP9 signalosome compromise N-mediated TMV resistance, suggesting a positive role of the COP9 signalosome in N-mediated resistance to TMV (Liu et al., 2002). More recently, the CSN5A subunit of the Arabidopsis COP9 signalosome has been found to interact with 29 distinct effectors from Hyaloperonospora arabidopsidis (Hpa) and Pseudomonas syringae (Psy), two evolutionarily diverse pathogens. CSN5A mutations enhance resistance to Hpa and Psy (Mukhtar et al., 2011), indicating that evolutionarily diverse pathogens can target the same host genes.

In this study, our goal was to identify the bread wheat (Triticum aestivum L.) homologues of CSN5 (designated as TaCSN5) and to assess their roles in the host defence response to leaf rust. Three rust pathogens infect wheat, namely Puccinia triticina (Pt), P. graminis f. sp. tritici (Pgt) and P. striiformis f. sp. tritici (Pst), causing leaf rust, stem rust and stripe rust, respectively. The rust pathogens are biotrophic fungi that infect living cells and obtain nutrients via haustoria (Dodds et al., 2004). The leaf rust pathogen forms its first haustorium as early as 24 h post-inoculation (hpi), and establishes five to seven haustoria per infection site produced from a single urediospore by 3 days post-inoculation (dpi) (Talajoor et al., 2016).

To identify wheat CSN5-like genes, we searched wheat sequence databases using the AtCSN5A and AtCSN5B sequences and identified three homeologues of one CSN5-like gene located
on the long arms of wheat group 2 chromosomes. In this article, we demonstrate the role of these genes during the infection process.

RESULTS

Identification of AtCNS5 homologues in rice and wheat

Searches of the Oryza sativa (japonica cultivar group) RefSeq RNA databases at the National Center for Biotechnology Information (NCBI) and the wheat genomic DNA sequence database at the International Wheat Genomic Sequence Consortium (IWGSC) for homologues of Arabidopsis AtCSN5A (At1g22920.1) and AtCSN5B (At1g71230) genes revealed one rice gene and three contigs in the wheat genome sequence database (Table 1). The rice gene Os04g0654700, annotated as ‘COP9 signalosome complex subunit 5b’ in the Rice Genome Annotation Project, is the homologue of both AtCSN5A and AtCSN5B. Alignments of Os04g0654700 with AtCSN5A and AtCSN5B showed the same maxi score, total score and maxi identity (Table 1), but the rice gene Os04g0654700 was more similar to AtCSN5B (77%) than to AtCSN5A (71%). Therefore, the rice gene is more similar to AtCSN5B. Three wheat homeologues were identified in contig6404307 (6403 bp) from chromosome 2AL, contig8034625 (16206 bp) from 2BL and contig9910009 (4797 bp) from 2DL (Table 1). Gene prediction analysis using ‘SoftBerry’ suggested the presence of two genes in contig6404307_2AL, five genes in contig8034625_2BL and one gene in contig9910009_2DL (Fig. 1). Gene annotation using the ‘Rice Genome Annotation Project’ suggested that gene 1 in contig6404307_2AL, gene 4 in contig8034625_2BL and the gene in contig9910009_2DL were homologues of both AtCSN5A and AtCSN5B, but with higher maxi scores, longer coverage and higher maxi identities to AtCSN5B than to AtCSN5A (Table 1). Among the three wheat homeologues, gene 4 of contig8034625_2BL showed the highest similarity to both AtCSN5A and AtCSN5B, and was therefore used to blast the nucleotide collection database (nr/nt) at NCBI. A full-length cDNA (accession no AK331742.1) was identified. The sequence shared 99% similarity with the CSN5 homologue on

| Table 1 | Similarities of homologous rice gene and wheat contigs with AtCSN5A (At1g22920.1) and AtCSN5B (At1g71230). |
| Arabidopsis thaliana gene | Homologues | Maxi score* | Total score† | Coverage (%)‡ | E value§ | Maxi identity (%)¶ |
|--------------------------|------------|-------------|-------------|-------------|---------|-------------------|
| At1g22920.1 | Rice gene  | Os04g0654700 | 645  | 645  | 71  | 0.0  | 74 |
| Wheat contigs  | 2AL_6404307 | 250 | 750 | 61 | 4e-69 | 80 |
| 2BL_8034625 | 250 | 1017 | 65 | 1e-68 | 80 |
| 2DL_9910009 | 253 | 517 | 37 | 3e-70 | 80 |
| At1g71230 | Rice gene  | Os04g0654700 | 645  | 645  | 77  | 0.0  | 74 |
| Wheat contigs  | 2AL_6404307 | 257 | 686 | 63 | 3e-71 | 81 |
| 2BL_8034625 | 257 | 868 | 65 | 7e-71 | 81 |
| 2DL_9910009 | 262 | 551 | 41 | 5e-73 | 81 |

*Overall score of HSPs (high scoring pairs) between sequences; the higher the maxi score, the better the alignment between the hit and the query.
†The sum of scores from all HSPs from the same sequence.
‡The amount of query sequence that overlaps the subject sequence.
§The significance of each sequence alignment hit to the query. The lower the E value, the more significant the alignment.
¶The highest percentage identity for a set of aligned segments to the same subject sequence.

Fig. 1 In silico cloning of the wheat TaCSN5 gene. Gene prediction of the three homeologous wheat contigs and alignment of AK331742.1 to TaCSN5-2B. TaCSN5 homologues are indicated as CSN5.
Table 2. Sequences of primers used for virus-induced gene silencing, real-time polymerase chain reaction (PCR) and mutation screening.

| Primer name | Primer sequence (5′–3′) |
|-------------|-------------------------|
| COP9 VIGSF  | TCCTGGTTATGGATGCTGGCTGTCA |
| COP9 VIGSR  | CTATCTGTTGATGCTGGCTGTCA |
| COP9 RTF    | GGATATAAACCCTCTGCTATC |
| COP9 RTR    | GAAGTGTTGATGAAATGCTGGG |
| COP9 MR     | CAAGATGCTGGAGCAGAGT |
| COP9 MDF    | GGTAGGAGAAGAAAGCATAG |
| COP9 MDR    | TGATAGTGGAGGGTAAATCAG |
| ACTB F      | CCAACATGATGATGCAGCCATCC |
| ACTB R      | CCCAAGCTCCAAACGAGG |
| PR1 F       | CTTGAGCAGAAGCTGCAG |
| PR1 R       | CGAGTCTGGAGCCTTCAG |
| PDF1.2 F    | CCGTGTGTCCTCTATATT |
| PDF1.2 R    | CATCACACACACACACAG |

2B and was 100% identical to the CSN5 homeologue on 2D. The cDNA of each homeologue was confirmed by direct sequencing of polymerase chain reaction (PCR) products amplified from wheat cDNA using gene-specific primers. The comparison between the cDNA and gDNA of the three homeologues revealed six exons and five introns in each gene (Figs 1 and S1, see Supporting Information). The deduced protein sequences (Fig. S2, see Supporting Information) of the three homeologues contain MPN (Mpr1-Pad-N-terminal) and NES (nuclear export signal) domains, characteristics of CSN5 and CSN6. To conclude, only one CSN5-like gene (referred to as TaCSN5) was identified in the wheat genome, but with three homeologues located on 2AL, 2BL and 2DL, and hereafter referred to as TaCSN5-2A, TaCSN5-2B and TaCSN5-2D, respectively (Fig. S1).

Expression profile of the TaCSN5 gene

Expression of the TaCSN5 gene was studied via real-time quantitative PCR at six time points after leaf rust inoculation of the susceptible spring wheat cultivar Alpowa. The three TaCSN5 homeologues were measured collectively using primers COP9 RTF + COP9 RTR, which were designed based on regions conserved among the three homeologues [Tables 2 and S1 (see Supporting Information), Fig. S1]. Leaf samples were taken from Alpowa seedlings inoculated with race PBJJG at 0, 1, 2, 3, 5, 8 and 10 dpi. Alpowa leaves inoculated with the buffer Soltrol 170 Isoparaffin and sampled at the same time points were used as controls. TaCSN5 expression levels were almost unchanged in samples taken at the first four time points (0–3 dpi) between the infected and non-infected Alpowa (Fig. 2). A significant increase in TaCSN5 transcript abundance was detected at 5 dpi in the inoculated Alpowa compared with the control plants, and TaCSN5 levels returned to the original level at 8 dpi.

Silencing of TaCSN5

Under the experimental conditions, one haustorium per infection site was present at 1 dpi and increased to more than five haustoria per infection site after 3 dpi (Talajoor et al., 2016). We observed that the TaCSN5 expression level correlated with the increased number of haustoria, suggesting that the gene may contribute to host susceptibility during infection. To test this hypothesis, we reduced the TaCSN5 transcript levels via Barley stripe mosaic virus (BSMV)-induced gene silencing. Considering the potential functional redundancy of the homeologues as a result of triplication, we knocked down the three homeologues of TaCSN5 simultaneously using a silencing vector with a 214-bp TaCSN5 fragment that was conserved in all three homeologues. The sequences of the primers COP9 VIGSF and COP9 VIGSR used to amplify the fragment are given in Table 2 and the locations of the primers are illustrated in Fig. S1. Twenty Alpowa seedlings were rub inoculated with BSMV: CSN5 RNAs (labelled as γCSN5 in Fig. 3). Seedlings of Alpowa inoculated with the BSMV genome alone (labelled as γ00), or BSMV plus a 183-bp phytoene desaturase (PDS) fragment inserted into the silencing vector (labelled as γPDS), together with non-inoculated Alpowa (labelled as CK), were used as controls. Gene silencing triggered by BSMV started 9 days after viral inoculation as evidenced by a photobleaching phenotype on Alpowa–γPDS. Pt race PBJJG was inoculated 10 days after BSMV inoculation. Alpowa–CK showed a susceptible infection type at 8 dpi (Fig. 3). Silenced leaves from Alpowa–γCSN5 plants showed highly resistant reactions to Pt race PBJJG (Fig. 3). Real-time PCR using primers COP9 RTF + COP9 RTR (Tables 2 and S1) also confirmed that the expression level of TaCSN5 in three randomly selected silenced plants inoculated with γCSN5 was reduced to 31%–35% of the Alpowa–γ00 control (Table 3), indicating that the overall transcript abundances of TaCSN5 homeologues were knocked down. These
results suggest that knock down of TaCSN5 in wheat enhances host resistance to leaf rust.

Relative importance of each TaCSN5 homeologue

TaCSN5 in bread wheat has three homeologues in the A, B and D genomes. To determine the importance of each homeologue during leaf rust development, we used wheat cultivar Chinese Spring (CS) group 2 chromosome nullisomic–tetrasomic (NT) lines N2AT2B, N2AT2D, N2BT2A, N2BT2D, N2DT2A and N2DT2B to assess the host reaction to \textit{Pt} race PBJJG when one homeologue was removed. In each NT line, a pair of group 2 chromosomes is missing, but is compensated by an extra pair of group 2 homeologues. For example, in line N2AT2B, a pair of 2A chromosomes is missing and compensated by the additional pair of 2B chromosomes. As shown in Fig. 4, N2AT2B and N2AT2D exhibited different reactions to the pathogen. Both N2AT2B and N2AT2D lack 2A chromosomes and therefore the TaCSN5-2A homeologue. With compensation by the additional pair of 2B chromosomes in N2AT2B, the NT line showed less severe disease, suggesting that the function of TaCSN5-2A for leaf rust susceptibility was not fully compensated by TaCSN5-2B. However, when an additional pair of 2D chromosomes was present in the N2AT2D line, the NT line had the same level of susceptibility as normal CS, suggesting that TaCSN5-2D fully compensated for the function of TaCSN5-2A. The same enhanced resistance was observed for N2DT2B, but not N2DT2A and N2DT2B to assess the host reaction to \textit{Pt} race PBJJG when one homeologue was removed. In each NT line, a pair of group 2 chromosomes is missing, but is compensated by an extra pair of 2B chromosomes. As shown in Fig. 4, N2AT2B and N2AT2D exhibited different reactions to the pathogen. Both N2AT2B and N2AT2D lack 2A chromosomes and therefore the TaCSN5-2A homeologue. With compensation by the additional pair of 2B chromosomes in N2AT2B, the NT line showed less severe disease, suggesting that the function of TaCSN5-2A for leaf rust susceptibility was not fully compensated by TaCSN5-2B. However, when an additional pair of 2D chromosomes was present in the N2AT2D line, the NT line had the same level of susceptibility as normal CS, suggesting that TaCSN5-2D fully compensated for the function of TaCSN5-2A. The same enhanced resistance was observed for N2DT2B, but not N2DT2A and N2DT2B, again confirming that the function of TaCSN5-2D was compensated by TaCSN5-2A, but not by TaCSN5-2B. Little change in disease phenotype was observed when TaCSN5-2B was missing, as evidenced by the same level of susceptibility of both N2BT2A and N2BT2D, and suggesting that TaCSN5-2B is less important for the pathogen during colonization. Therefore, the pathogen may tolerate some TaCSN5 homeologues more than others during the infection process; the relative importance of each of the TaCSN5 homeologues to \textit{Pt} PBJJG is TaCSN5-2A $\geq$ TaCSN5-2D $>$ TaCSN5-2B.

Identification of TaCSN5 mutants and their response to the leaf rust pathogen

Transient knock down assays and tests of NT lines revealed the function and relative importance of TaCSN5 homeologues in the...
defence response of wheat to leaf rust. To search for mutants of one or other of the three TaCSN5 homeologues, two sets of primers, COP9 VIGSF + COP9 RTR and COP9 MDF + COP9 MDR (2D genome-specific primers) (Tables 2 and S1), were used to screen for mutations of TaCSN5 from an ethylmethane sulfonate (EMS)-mutagenized Alpowa population (Feiz et al., 2009). By screening a 433-bp genomic DNA segment of the TaCSN5 gene region among 376 individuals from the mutagenized population, six point mutations were identified, including three located in introns. Among the other three mutations, two (P306S and S327L) were identified with the 2D-specific primers COP9 MDF + COP9 MDR; therefore, the mutations were located in TaCSN5-2D. Mutation R192K was identified with primers COP9 VIGSF + COP9 RTR which amplify a conserved region in all three TaCSN5 homeologues, suggesting that the mutation could have occurred on any of the three homeologues. Additional primer COP9 MR was designed to be combined with COP9 VIGSF to determine the chromosome location of the mutation. Sequence analysis revealed that the mutation R192K was located in TaCSN5-2A, was a G to A change, and resulted in an amino acid change from arginine (Arg) to lysine (Lys) at position 192 (Table 4). The mutation P306S in TaCSN5-2D was a C to T change, resulting in an amino acid change at position 306 from proline (Pro) to serine (Ser). A similar C to T change was present in the mutation S327L on TaCSN5-2D, but with a different amino acid change from Ser to leucine (Leu) at position 327. The SIFT scores of mutations R192K, P306S and S327L were 0.23, 0.70 and 0.30, respectively, all >0.05, suggesting that amino acid substitutions at these positions are tolerated.

The three characterized mutants were tested with Pt race PBJJG. Mutants R192K and P306S showed less severe disease compared with wild-type Alpowa, and mutant S327L showed a similar infection type to the wild-type (Fig. 5). Consistent with the silencing and NT results, knock down [via virus-induced gene silencing (VIGS)] or knock out (via EMS mutagenesis) of TaCSN5-2A or TaCSN5-2D reduced susceptibility to leaf rust, suggesting a negative role of TaCSN5 in wheat defence to leaf rust.

To further confirm that the enhanced resistance phenotype resulted from the mutated TaCSN5, we used an F2 population derived from a cross between mutants R192K (the TaCSN5-2A mutant) and P306S (the TaCSN5-2D mutant), and mapped the resistance to the CSN5 locus with 120 individuals.

### Table 4 Summary of TaCSN5 mutations that were identified from the ethylmethane sulfonate (EMS)-induced Alpowa population.

| Mutation ID | Genome | Nucleotide change | Mutated amino acid position | Amino acid changes | SIFT score* |
|-------------|--------|-------------------|----------------------------|-------------------|-------------|
| R192K       | 2A     | G to A            | 192                        | R to K            | 0.23        |
| P306S       | 2D     | C to T            | 306                        | P to S            | 0.70        |
| S327L       | 2D     | C to T            | 327                        | S to L            | 0.30        |

*SIFT predicts whether an amino acid substitution affects protein function, and ranges from 0 to 1. The amino acid substitution is predicted to be damaging if the score is <0.05, and tolerated if the score is >0.05.

**Expression of PDF1.2 (plant defensin 1.2) and PR1 (pathogenesis-related gene 1) in TaCSN5-silenced Alpowa**

The COP9 signalosome is involved in the biotic stress response through the regulation of jasmonate synthesis and response (Hind et al., 2011; Feng et al., 2003; Schwechheimer et al. 2002). Consistent with this, the Arabidopsis Atcsn5a mutant shows a high accumulation of PR1 protein (Mukhtar et al., 2011). This suggests the possibility that the biotrophic leaf rust pathogen may hijack TaCSN5 as a strategy for its colonization. It is well known that plants possess two major defence response pathways, the salicylic acid (SA)-mediated and jasmonic acid (JA)-mediated signalling pathways, and the two pathways may be antagonistic to each other (Kunkel and Brooks, 2002). To investigate how TaCSN5 silencing leads to leaf rust resistance in Alpowa, the expression of PDF1.2 and PR1 in TaCSN5-silenced and non-silenced plants at 0, 1, 2 and 3 days after Pt PBJJG inoculation was monitored. As PDF1.2, also called Thi2-1, is JA dependent (Bohlmann et al., 1998; Penninckx et al., 1998; Turner et al., 2002), it was used as a marker gene for the JA-mediated pathway. PR1 is SA dependent (Shah et al., 1999), and was used as a marker gene for the SA-
mediated pathway. In the control Alpowa-g00 at 24 hpi, both PR1 (Fig. 6A) and PDF1.2 (Fig. 6B) were up-regulated compared with that at 0 dpi. The increase in PR1 was greater than that in PDF1.2. However, at the same time point in TaCSN5-silenced Alpowa plants, the PR1 level was about four-fold higher than that of the control (Fig. 6A). In contrast, PDF1.2 was significantly reduced in the silenced plants (Fig. 6B), indicating that, when TaCSN5 is reduced, the JA-mediated pathway is suppressed. In other words, enhanced TaCSN5 activates the JA signalling pathway and suppresses the SA-mediated pathway.

To confirm this finding, we measured the expression level of PR1 in leaf rust-inoculated mutants R192K and P306S at 2 dpi. As shown in Fig. 7, the two mutants exhibited a significantly higher PR1 expression than did wild-type Alpowa.

**DISCUSSION**

In silico cloning of the TaCSN5 gene was conducted via a comparative genomics approach. The coding region was confirmed by subsequent resequencing of wheat cDNA. The search for wheat and rice CSN5 homologues using the two Arabidopsis CSN5 genes (AtCSN5A and AtCSN5B) found only a single CSN5-like locus in the rice genome and three TaCSN5-like homeologous loci in the wheat genome with higher similarity and more overlap with AtCSN5B. It is unclear whether TaCSN5 or OsCSN5 has a pleiotropic role in photomorphogenesis regulation because we focused here only on the role of TaCSN5 in the wheat defence response to leaf rust. No abnormal photomorphogenic phenotypes associated with mutations in either TaCSN5-2A or TaCSN5-2D were observed.

TaCSN5 contributes to host susceptibility to leaf rust, as evidenced by gene expression analysis and knock down assays. TaCSN5 transcript abundance was higher in RNA extracted from the susceptible cultivar Alpowa after inoculation with Pt race PB134 than in non-inoculated controls. TaCSN5 expression reached a maximum at 5 dpi, which is consistent with the timing of haustorial formation; the number of haustoria in the host at 5 dpi was five to seven times higher than that at 1 dpi, suggesting that the up-regulation of TaCSN5 was pathogen induced. Down-regulation of TaCSN5 via VIGS significantly enhanced the resistance to Pt race PB134 (Fig. 3), supporting a negative role of TaCSN5 on leaf rust infection.

Mukhtar et al. (2011) found that AtCSN5A was a common target of Hpa and Psy in Arabidopsis. The double-mutant csn5A-cu13A line displayed enhanced resistance to these pathogens and
a higher level of PR1 protein accumulation. Without characterized pathogen effectors from *Puccinia* spp., it is unclear whether TaCSN5 is physically targeted by the rust effectors. However, our study revealed the same negative role of TaCSN5 genes in response to leaf rust, as AtCSN5 to other pathogens. In contrast, the COP9 signalosome was also shown to be positively involved in N gene-mediated resistance in tobacco (Liu et al., 2002). Down-regulation of CSN5 in tomato resulted in reduced resistance to the herbivorous *Manduca sexta* and necrotrophic fungal pathogen *Botrytis cinerea* (Hind et al., 2011), suggesting a positive role of CSN5 in JA-dependent resistance. COP9 signalosome-mediated proteasomal protein degradation was shown to be involved in the regulation of JA synthesis (Hind et al., 2011).

Enhanced JA-dependent PDF1.2 gene expression and decreased SA-dependent PR1 gene expression, observed after inoculation with *Pt* race PBJG, may implicate the same mechanism of manipulation of JA signalling to suppress SA signalling as observed with the bacterial pathogen *Psy* infecting *Arabidopsis* (Katsir et al., 2008; Kloek et al., 2001; Xie et al., 1998). The bacterium produces the phytotoxin coronatine to favour the JA defence response pathway and, ultimately, suppresses SA-mediated defence responses that are effective against *Psy* (Katsir et al., 2008). An *Arabidopsis* coronatine-insensitive mutant (coil) exhibited a robust resistance to *Psy* (Kloek et al., 2001).

We observed the highest TaCSN5 expression at 5 dpi (Fig. 2), later than the highest PR1 expression at 1 dpi after leaf rust inoculation of TaCSN5-silenced leaves (Fig. 6A), and 2 dpi in the mutants of R192K and P306S (Fig. 7). The difference in timing may imply that TaCSN5 is manipulated by a haustorially secreted effector. At 1 dpi, only one haustorium was established per infection site, such that expression levels of TaCSN5 were diluted when extracting total RNA from early infected cells. However, detectable enhancement in the PR1 level in inoculated leaves at 1 dpi suggests that the increased level was highly significant. This implies that a high level of PR1 occurred not only in the cells with haustoria, but also in neighbouring cells without haustoria, a paradigm called ‘systemic acquired resistance’ (Ryals et al., 1996; Ward et al., 1991). Although little change could be seen on the surfaces of infected leaves at 5 dpi, there were at least five times more haustoria inside the leaves than at 1 dpi, so that the amount of TaCSN5 extracted from the same amount of leaf tissue became more significant. The study also suggested that a rapid increase in PR1 level at early time points (e.g. at 1–2 dpi) was critical in determining whether or not *Pt* race PBJG could successfully colonize the host. Once past the early time points, the host was unable to maintain an adequate level of PR1 to prevent rust development.

One of the great challenges of studying gene function in polyploid species is functional redundancy. Bread wheat is an allohexaploid containing three related genomes, thus being triplicated for most gene loci. Consistent with this, there were three copies of the TaCSN5 homeologues, one for each genome. Leaf rust assays on the NT lines revealed functional compensation of the TaCSN5—2A and TaCSN5—2D homeologues (Fig. 4). Mutations at single loci affecting only one copy had a small impact on the infection process (Fig. 5). However, the use of BSMV-VIGS to reduce the transcript abundances of all three gene transcripts simultaneously resulted in significantly enhanced disease resistance (Fig. 3), suggesting that only the TaCSN5-2A and TaCSN5-2D double mutant could provide significant leaf rust resistance.

### EXPERIMENTAL PROCEDURES

#### Plant materials

Alpowa (PI 566596), a soft white spring wheat cultivar, was obtained from the USDA National Plant Germplasm System (NPGS). CS used in this study was provided by Dr Luther Talbert (Montana State University, Bozeman, MT, USA) and the CS NT lines were provided by Dr Bikram S. Gill (Kansas State University, Manhattan, KS, USA).

#### Pathogen

The *Pt* race PBJG used for leaf rust assays was kindly provided by Dr Robert Bowden (USDA-ARS, Manhattan, KS, USA).

#### Plant growth conditions and pathogen inoculation

**Plant growth conditions**

Before inoculation, all wheat seedlings were grown in a growth room at the Plant Growth Center at Montana State University under the following conditions: 22°C/14°C day/night temperatures and a 16-h photoperiod. Plants were watered and fertilized every day with Peters General Purpose Plant Food (Scotts-Miracle-Gro Company, Marysville, OH, USA) at a concentration of 150 ppm N-P-K.

**Leaf rust inoculation**

Leaf rust inoculations and disease assessments were performed as described in Campbell et al. (2012).

#### Gene expression analysis by quantitative real time-polymerase chain reaction (qRT-PCR)

**TaCSN5 gene expression during leaf rust development**

The time course study of TaCSN5 gene expression was assessed by qRT-PCR. Wheat cultivar Alpowa (susceptible to *Pt* race PBJG) was inoculated with *Pt* race PBJG suspended in the inoculation buffer Soltrol 170 Isoparaffin (Chempoint, Bellevue, WA, USA) or with Soltrol 170 Isoparaffin alone as a mock control. Three biological replicates were performed. Leaf tissues were collected at 0, 1, 2, 3, 5, 8 and 10 dpi, snap frozen in liquid nitrogen and stored at −80°C until RNA isolations were performed. Total RNA was isolated and treated with DNase I on a column using a Qiagen RNeasy Plant Mini Kit (Qiagen, Valencia, CA, USA) following the manufacturer’s instructions. The quality and concentration of total RNA were assessed via agarose gel electrophoresis and 260/280Abs measurements on a
Expression of PR1 and PDF1.2 in TaCSN5-silenced Alpowa
Relative expression profiles of genes PR1 and PDF1.2 in TaCSN5-silenced Alpowa (BSMV: CSNS) were measured during leaf rust development, and non-silenced Alpowa (BSMV: 00) was used as the control. Silenced and non-silenced Alpowa were challenged with Pt race PBJJG at 10 days after BSMV inoculation; leaf tissues were sampled at 0, 1, 2, and 3 dpi, snap frozen in liquid nitrogen and stored at −80°C until RNA isolations. Three biological replicates were performed. RNA isolations, qRT-PCR and data analysis were performed as described above. Expression levels of PR1 and PDF1.2 at 1, 2 and 3 dpi in silenced plants were compared with those in non-silenced plants.

Expression of PR1 in wild-type Alpowa and mutants challenged with leaf rust
Alpowa and its mutants were inoculated with Pt race PBJJG, and sampled at 2 dpi. Three biological replicates were performed. The relative expression of PR1 in the mutants was compared with that in wild-type Alpowa.

Primer sequences, annealing temperatures and sizes of qRT-PCR products for the ACTB, PR1 and PDF1.2 genes are listed in Tables 2 and S1.

Virus-induced gene silencing
The original BSMV vectors were obtained from Dr Andrew O. Jackson (UC Berkeley, CA, USA). The target fragment for the silencing assay was inserted into modified γ vector ready for direct PCR cloning as described by Campbell and Huang (2010). Infectious RNA transcripts were synthesized in vitro using T7 RNA polymerase (New England Biolabs, Ipswich, MA, USA) from linearized α, β and γ plasmids. The BSMV inoculum was prepared with 1 μL of each of the in vitro transcription reactions and 22.5 μL of inoculation buffer. The inoculum was then used to rub inoculate the first leaf of two-leaf-stage plants. Leaf tissue was sampled 9 days after virus inoculation to test the silencing efficiency. Leaf rust tests were conducted following the method described by Campbell et al. (2012). Disease assessments were made when disease on control plants (wild-type Alpowa or CS) was fully developed at 8 dpi.

Mutant screen
The mutagenized population was generated by EMS (Feiz et al. 2009). The population was selfed and advanced to the M5 generation by single seed descent, and then bulked to generate M6 seed pools used in mutation screening. The primers used in mutant identification are listed in Tables 2 and S1. Primer designs are illustrated in Fig. S1.

For D genome mutation screening, the designs of D genome-specific primers COP9 MDF + COP9 MDR were based on the location of an insertion/deletion variation among the TaCSN5 genes on the A, B and D genomes to ensure D genome specificity (Fig. S1). Verification of the specificity of the D genome-specific primer is shown in Fig. S3 (see Supporting Information). To detect the A genome mutation, the COP9 VIGSF + COP9 RTR primers were first used to screen the EMS-induced population, and then an additional primer, COP9 MR, was designed to extend the region and make the PCR products A genome specific.

PCR amplifications were conducted in total volumes of 20 μL containing 20 mM tris(hydroxymethyl)aminomethane (Tris)-HCl (pH 8.3), 100 mM KCl, 3.0 mM MgCl2, 0.4 mM deoxynucleoside triphosphate (dNTP), 50 ng of each primer, 50 ng genomic DNA and 1.5 U Taq DNA polymerase. Amplifications were performed at an initial denaturation of 94°C for 5 min, followed by 40 cycles of 94°C for 45 s, 56–60°C for 45 s (specific annealing temperatures for different primers as listed in Table S1) and 72°C for 45–60 s, with a final extension at 72°C for 10 min.

The PCR products were then purified using a Qiagen gel purification kit, sequenced and compared. First, the sequence from wild-type Alpowa was compared with the TaCSN5 gene sequences of CS from IWGSC, and then sequences from individual mutagenized lines were compared with the sequence of wild-type Alpowa for mutation identification.

Database and sequence analysis software
All BLAST searches were conducted using the NCBI (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and IWGSC (http://wheat-urgi.versailles.inra.fr/Seq-Repository/BLAST) databases; all sequences were also downloaded from these two websites. The Softberry database (http://linux1.softberry.com/berry.phtml?topic=genesh&group=programs&subgroup=gfind) was used for gene prediction. Gene annotations were conducted on the rice genome annotation project website (http://rice.plantbiology.msu.edu/index.shtml). SIFT scores were predicted at the J. Craig Venter website (http://sift.jcvi.org/). Sequence alignments were conducted using the BioEdit (IBIS Biosciences, Carlsbad, CA, USA) sequence alignment editor.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher’s website.

Fig. S1 Comparison of wheat genome sequences of homologous genes TaCSN5-2A, TaCSN5-2B and TaCSN5-2D, and cDNA (accession# AK331742.1) and primer locations. Dots denote the same nucleotide, dashes indicate deletions and introns are highlighted in grey.

Fig. S2 Alignment of the deduced protein sequences of the three TaCSN5 homologues. Conserved domains of CSN5 are labelled and highlighted.

Fig. S3 Polymerase chain reaction (PCR) products of D genome-specific primers COP9 (constitutive photomorphogene-
sis 9) MDF and COP9 MDR. M, molecular weight marker; 1–3, genomic DNA of Chinese Spring nullisomic–tetrasomic lines NW3T2B, N2BT2A and N2DT2B as template; 4, Chinese Spring.

Table S1 Primer combinations and their specific purposes, annealing temperatures and product sizes.