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

HONGTAO ZHANG[1], XIAOJING WANG[1,2], MICHAEL J. GIROUX[1] AND LI HUANG[1,*]

[1]*Department of Plant Sciences and Plant Pathology, Montana State University, Bozeman, MT 59717-3150, USA*
[2]*State Key Laboratory of Crop Stress Biology for Arid Areas and College of Life Sciences, Northwest A&F University, Yangling, Shanxi 712100, China*

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 derubylation 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 *et al.*, 1994b). CSN mutants exhibit short hypocotyls, and open and expanded cotyledons, when grown in either light or dark conditions. The COP9 signalosome is involved in the ubiquitin proteasome system through modulation of cullin-ring E3 ubiquitin ligase (Wei *et al.*, 2008). This system regulates additional 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; Karniol *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 derubylation of cullins (Gusmaroli *et al.*, 2004). The catalytic centre of derubylation 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

* Correspondence: Email: lhuang@montana.edu

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.

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 (16 206 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* homeologue on
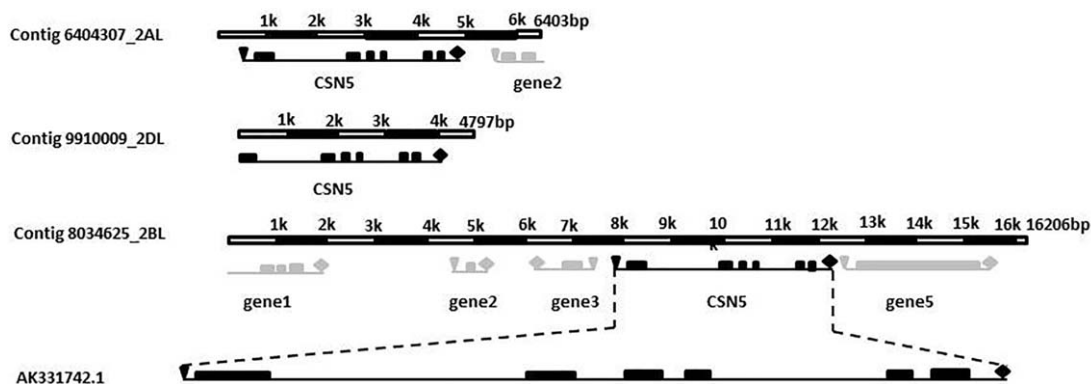
Fig. 1 *In silico* cloning of the wheat *TaCSN5* gene. Gene prediction of the three homeologous wheat contigs and alignment of AK331742.1 to *TaCSN5-2B*. *TaCSN5* homologues are indicated as CSN5.

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	CTATCTTGTTGAGTGGTATGGTCTG
COP9 RTF	GGATATAACCTATTTCAAGTCATCCC
COP9 RTR	GAAGTGGTGATGAAGATAATGTGTTG
COP9 MR	CAAGATAGTGCTGGACCAGT
COP9 MDF	GTTGAAGGAAGAAAGCATAG
COP9 MDR	TGTAGTAGTTAGGGGTAACATCAG
ACTB F	CCAGCAATGTATGTCGCAATCC
ACTB R	CCAGCAAGGTCCAAACGAAGG
PR1 F	CTGGAGCACGAAGCTGCAG
PR1 R	CGAGTGCTGGAGCTTGCAGT
PDF1.2 F	CGGTGTGTCCCTCTTATATT
PDF1.2 R	CATCACCACACACAAATCAG

Fig. 2 Relative expression of *TaCSN5* during leaf rust development. Relative expression is given as fold change, i.e. expression level of *TaCSN5* in leaf rust-infected Alpowa relative to that in the control (Soltrol 170 Isoparaffin-inoculated Alpowa) at corresponding time points. Error bars represent standard deviations of three biological replicates. **Statistical significance at $P \leq 0.01$.

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 *TaCSN5* 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.
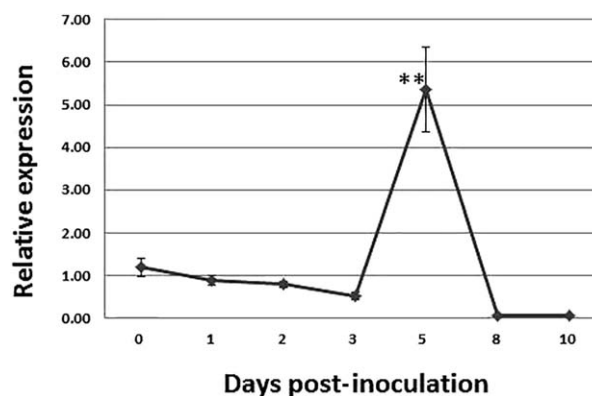
Silencing of *TaCSN5*

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
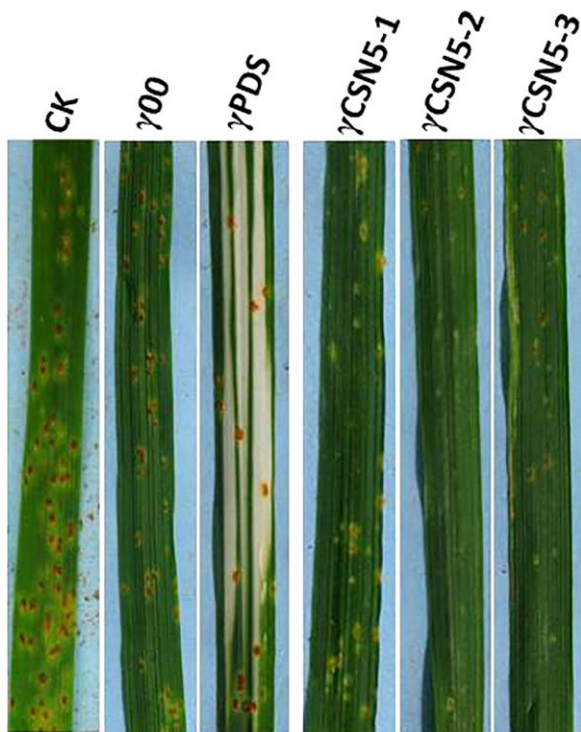
Fig. 3 Infection types of *TaCSN5*-silenced plants. The first leaf of each plant was rub inoculated with the indicated *Barley stripe mosaic virus* (BSMV) constructs at the two-leaf stage and then spray inoculated with *Puccinia triticina* (*Pt*) race PBJJG at 10 days after BSMV inoculation. The disease was assessed and photographed at 8 days post-inoculation (dpi). CK, Alpowa without any viral inoculation, used as a rust inoculation control; γ00, Alpowa inoculated with BSMV: 00; γPDS, Alpowa inoculated with BSMV: PDS; γCSN5, Alpowa inoculated with BSMV: CSN5.

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)

Table 3 Quantitative real time-polymerase chain reaction (qRT-PCR) analysis of suppression of *TaCSN5* gene expression by *Barley stripe mosaic virus*-induced gene silencing (BSMV-VIGS) for leaf rust-challenged Alpowa.

Gene	Relative expression*				
	Rep. 1	Rep. 2	Rep. 3	Average relative expression	SD
TaCSN5	0.35	0.31	0.31	0.32	0.02

*Relative expression was calculated by dividing the expression value for *TaCSN5* in silenced plants by the *TaCSN5* expression value measured in plants infected with BSMV: 00.
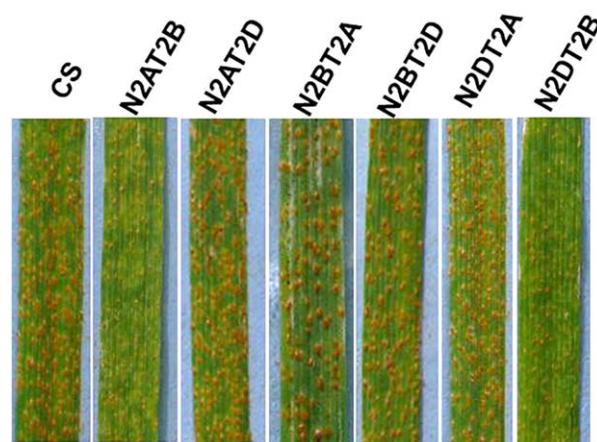
Fig. 4 Disease assay with *Puccinia triticina* (*Pt*) race PBJJG on Chinese Spring (CS) nullisomic–tetrasomic (NT) lines. Leaves of wheat cultivar CS, together with six CS group 2 NT lines, inoculated with *Pt* race PBJJG. The N2AT2B and N2DT2B lines showed less disease than CS and the other four NT lines. The photograph was taken 9 days after *Pt* PBJJG inoculation.

group 2 chromosome nullisomic–tetrasomic (NT) lines N2AT2B, N2AT2D, N2BT2A, N2BT2D, N2DT2A and N2DT2B to assess the host reaction to *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 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, 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 *Pt* PBJJG is *TaCSN5-2A* ≥ *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

Table 4 Summary of *TaCSN5* gene 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.

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.

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-
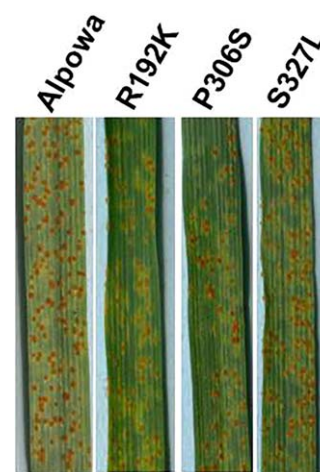

Fig. 5 Infection types on *TaCSN5* mutants. Three ethylmethane sulfonate (EMS)-derived *TaCSN5* mutants and wild-type (WT) Alpowa were challenged with *Puccinia triticina* (*Pt*) race PBJJG. Mutants R192K and P306S showed less severe disease; mutant S327L showed a similar infection type to Alpowa.
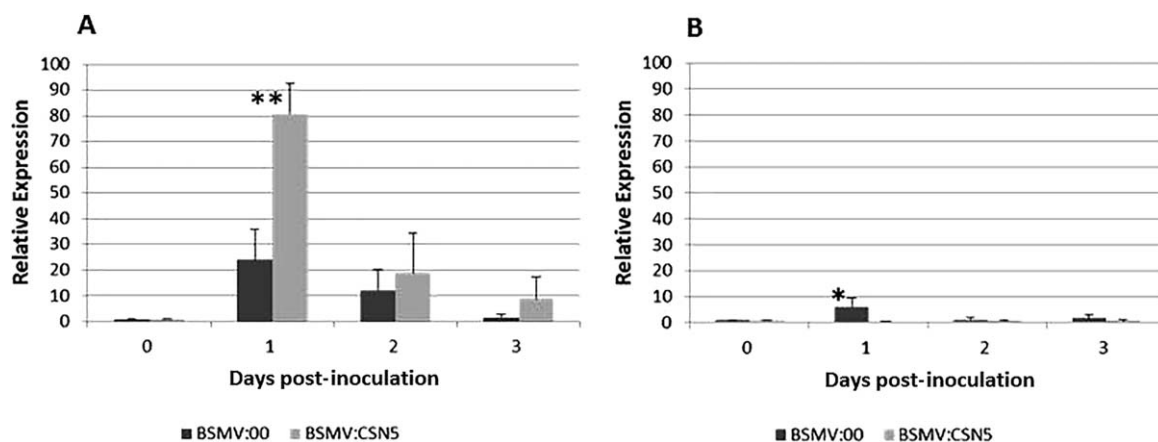
Fig. 6 Relative expression profiles of genes *PR1* (*pathogenesis-related gene 1*) (A) and *PDF1.2* (*plant defensin 1.2*) (B) during leaf rust development on BSMV: 00- and BSMV: CSN5-treated Alpowa. Relative expression is given as fold change, i.e. expression level of *PR1* and *PDF1.2* at 1, 2 and 3 days post-inoculation (dpi) relative to that at 0 dpi. Error bars represent standard deviations of three biological replicates. The *TaCSN5* gene was confirmed to be silenced in BSMV: CSN5-treated plants. *Statistical significance at $P \leq 0.05$. **Statistical significance at $P \leq 0.01$.

mediated pathway. In the control Alpowa-γ00 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
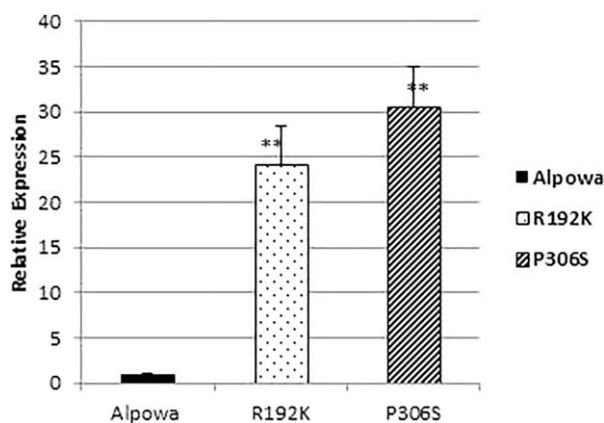
Fig. 7 Relative expression of *PR1* (*pathogenesis-related gene 1*) in wild-type Alpowa and *TaCSN5* mutants at 2 days after *Puccinia triticina* (*Pt*) inoculation. Relative expression is given as fold change, i.e. expression level of *PR1* in *TaCSN5* mutants relative to Alpowa. Error bars represent standard deviations of three biological replicates. *Statistical significance at $P \leq 0.05$. **Statistical significance at $P \leq 0.01$.

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 *TaCN5*-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 PBJJG 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 PBJJG (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-cul3A* 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 PBJJG, may imply 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 (*coi1*) 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 PBJJG 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 PBJJG 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 PBJJG) was inoculated with *Pt* race PBJJG 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 $260/280_{ABS}$ measurements on a

NanoDrop 1000 spectrophotometer (Thermo Fisher Scientific Inc., Wilmington, DE, USA). The time course of *TaCSN5* gene expression after inoculation was quantified by qRT-PCR with the same amount of total RNA employed as template in each reaction using the iScript One-Step RT-PCR Kit with SYBR Green (Bio-Rad, Hercules, CA, USA) following the manufacturer's recommended protocol. Transcript abundance was normalized to the reference gene *ACTB* (β-*actin*) (Kozera and Rapacz, 2013), and performed on a CFX96 real-time PCR detection system (Bio-Rad). Each reaction was conducted in triplicate on each of the three biological replicates. Data were used only if the Ct standard deviation between replicates was $\leq$0.2. Relative expression was calculated using the $\Delta\Delta$Ct method as described in the CFX96 manual (Bio-Rad), where fold change $= 2^{-\Delta\Delta Ct}$. The relative expression of the *TaCSN5* gene is presented as the expression level of the gene in *Pt*-inoculated plants relative to that in the control (Soltrol 170 Isoparaffin-inoculated plants). Standard deviations were calculated among different biological replicates. Mean relative expressions were calculated using the $\Delta\Delta$Ct method between biological replicates $\pm$ standard deviation. An unpaired two-tailed *t*-test was conducted to compare means of *Pt*-inoculated and buffer Soltrol 170 Isoparaffin-inoculated plants at each time point.

Expression of PR1 and PDF1.2 in TaCSN5-silenced Alpowa

Relative expression profiles of genes *PR1* and *PDF1.2* in *TaCSN5*-silenced Alpowa (BSMV: CSN5) 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 M$_5$ generation by single seed descent, and then bulked to generate M$_{5:6}$ 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 MgCl$_2$, 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=fgenesh&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.

REFERENCES

Bohlmann, H., Vignutelli, A., Hilpert, B., Miersch, O., Wasternack, C. and Apel, K. (1998) Wounding and chemicals induce expression of the *Arabidopsis thaliana* gene Thi2.1, encoding a fungal defense thionin, via the octadecanoid pathway. *FEBS Lett.* **43**, 281–286.

Campbell, J. and Huang, L. (2010) Silencing of multiple genes in wheat using Barley stripe mosaic virus. *J. Biotech. Res.* **2**, 12–20.

Campbell, J., Zhang, H., Giroux, M.J., Feiz, L., Jin, Y., Wang, M., Chen, X. and Huang, L. (2012) A mutagenesis-derived broad-spectrum disease resistance locus in wheat. *Theor. Appl. Genet.* **125**, 391–404.

Chamovitz, D.A., Wei, N., Osterlund, M.T., vonArnim, A.G., Staub, J.M., Matsui, M. and Deng, X.W. (1996) The COP9 complex, a novel multisubunit nuclear regulator involved in light control of a plant developmental switch. *Cell*, **86**, 115–121.

Deng, X.W. (1994) Fresh view of light signal-transduction in plants. *Cell*, **76**, 423–426.

Dodds, P.N., Lawrence, G.J., Catanzariti, A.M., Ayliffe, M.A. and Ellis, J.G. (2004) The *Melampsora lini* AvrL567 avirulence genes are expressed in haustoria and their products are recognized inside plant cells. *Plant Cell*, **16**, 755–768.

Dohmann, E.M.N., Levesque, M.P., De Veylder, L., Reichardt, I., Juergens, G., Schmid, M. and Schwechheimer C. (2008) The Arabidopsis COP9 signalosome is essential for G2 phase progression and genomic stability. *Development*, **135**, 2013–2022.

Feiz, L., Beecher, B.S., Martin, J.M. and Giroux, M.J. (2009) In planta mutagenesis determines the functional regions of the wheat puroindoline proteins. *Genetics*, **183**, 853–860.

Feng, S.H., Ma, L.G., Wang, X.P., Xie, D.X., Dinesh-Kumar, S.P., Wei, N. and Deng, X.W. (2003) The COP9 signalosome interacts physically with SCFCOI1 and modulates jasmonate responses. *Plant Cell*, **15**, 1083–1094.

Freilich, S., Oron, E., Kapp, Y., Nevo-Caspi, Y., Orgad, S., Segal, D. and Chamovitz, D.A. (1999) The COP9 signalosome is essential for development of *Drosophila melanogaster*. *Curr. Biol.* **9**, 1187–1190.

Gusmaroli, G., Feng, S.H. and Deng, X.W. (2004) The Arabidopsis CSN5A and CSN5B subunits are present in distinct COP9 signalosome complexes, and mutations in their JAMM domains exhibit differential dominant negative effects on development. *Plant Cell*, **16**, 2984–3001.

Gusmaroli, G., Figueroa, P., Serino, G. and Deng, X.W. (2007) Role of the MPN subunits in COP9 signalosome assembly and activity, and their regulatory interaction with Arabidopsis cullin3-based E3 ligases. *Plant Cell*, **19**, 564–581.

Hind, S.R., Pulliam, S.E., Veronese, P., Shantharaj, D., Nazir, A., Jacobs, N.S. and Stratmann, J.W. (2011) The COP9 signalosome controls jasmonic acid synthesis and plant responses to herbivory and pathogens. *Plant J.* **65**, 480–491.

Karniol, B., Malec, P. and Chamovitz, D.A. (1999) Arabidopsis FUSCA5 encodes a novel phosphoprotein that is a component of the COP9 complex. *Plant Cell*, **11**, 839–848.

Katsir, L., Schilmiller, A.L., Staswick, P.E., He, S.Y. and Howe, G.A. (2008) COI1 is a critical component of a receptor for jasmonate and the bacterial virulence factor coronatine. *Proc. Natl. Acad. Sci. USA*, **105**, 7100–7105.

Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F. and Kunkel, B.N. (2001) Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronatine-insensitive (coi1) mutation occurs through two distinct mechanisms. *Plant J.* **26**, 509–522.

Kozera, B. and Rapacz, M. (2013) Reference genes in real-time PCR. *J. Appl. Genet.* **54**, 391–406.

Kunkel, B.N. and Brooks, D.M. (2002) Cross talk between signaling pathways in pathogen defense. *Curr. Opin. Plant Biol.* **5**, 325–331.

Liu, Y., Schiff, M., Serino, G., Deng, X.W. and Dinesh-Kumar, S.P. (2002) Role of SCF ubiquitin-ligase and the COP9 signalosome in the N gene-mediated resistance response to Tobacco mosaic virus. *Plant Cell*, **14**, 1483–1496.

Mukhtar, M.S., Carvunis, A.R., Dreze, M., Epple, P., Steinbrenner, J., Moore, J., Tasan, M., Galli, M., Hao, T., Nishimura, M.T., Pevzner, S.J., Donovan, S.E., Ghamsari, L., Santhanam, B., Romero, V., Poulin, M.M., Gebreab, F., Gutierrez, B.J., Tam, S., Monachello, D., Boxem, M., Harbort, C.J., McDonald, N., Gai, L., Chen, H., He, Y., European Union Effectoromics Consortium, Vandenhaute, J., Roth, F.P., Hill, D.E., Ecker, J.R., Vidal, M., Beynon, J., Braun, P. and Dangl, J.L. (2011) Independently evolved virulence effectors converge onto hubs in a plant immune system network. *Science*, **333**, 596–601.

Mundt, K.E., Porte, J., Murray, J.M., Brikos, C., Christensen, P.U., Caspari, T., Hagan, I.M., Millar, J.B., Simanis, V., Hofmann, K. and Carr, A.M. (1999) The COP9/signalosome complex is conserved in fission yeast and has a role in S phase. *Curr. Biol.* **9**, 1427–1430.

Penninckx, I., Thomma, B., Buchala, A., Metraux, J.P. and Broekaert, W.F. (1998) Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in Arabidopsis. *Plant Cell*, **10**, 2103–2113.

Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y. and Hunt, M.D. (1996) Systemic acquired resistance. *Plant Cell*, **8**, 1809–1819.

Schwechheimer, C., Serino, G. and Deng, X.W. (2002) Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. *Plant Cell*, **14**, 2553–2563.

Seeger, M., Kraft, R., Ferrell, K., Bech-Otschir, D., Dumdey, R., Schade, R., Gordon, C., Naumann, M. and Dubiel, W. (1998) A novel protein complex involved in signal transduction possessing similarities to 26S proteasome subunits. *FASEB J.* **12**, 469–478.

Shah, J., Kachroo, P. and Klessig, D.F. (1999) The Arabidopsis ssi1 mutation restores pathogenesis-related gene expression in npr1 plants and renders defensin gene expression salicylic acid dependent. *Plant Cell*, **11**, 191–206.

Talajoor, M., Wang, X., Zhang, H., Zhang, P., Bhavani, S., Tabe, L., Lagudah, E., and Huang, L. (2016) Wheat mutant MNR220 delays haustoria formation during leaf rust pathogenesis at the seedling stage, *Can. J. Plant Pathol.*, doi: 10.1080/07060661.2016.1205663

Turner, J.G., Ellis, C. and Devoto, A. (2002) The jasmonate signal pathway. *Plant Cell*, **14**, S153–S164.

Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P. and Ryals, J.A. (1991) Coordinate gene activity in response to agents that induce systemic acquired-resistance. *Plant Cell*, **3**, 1085–1094.

Wei, N. and Deng, X.W. (1998) Characterization and purification of the mammalian COP9 complex, a conserved nuclear regulator initially identified as a repressor of photomorphogenesis in higher plants. *Photochem. Photobiol.* **68**, 237–241.

Wei, N. and Deng, X.W. (1999) Making sense of the COP9 signalosome – a regulatory protein complex conserved from Arabidopsis to human. *Trends Genet.* **15**, 98–103.

Wei, N., Chamovitz, D.A. and Deng, X.W. (1994a) Arabidopsis cop9 is a component of a novel signaling complex mediating light control of development. *Cell*, **78**, 117–124.

Wei, N., Kwok, S.F., Vonarnim, A.G., Lee, A., McNellis, T.W., Piekos, B., and Deng, X.W. (1994b) *Arabidopsis COP8, COP10, and COP11* genes are involved in repression of photomorphogenic development in darkness. *Plant Cell*, **6**, 629–643.

Wei, N., Tsuge, T., Serino, G., Dohmae, N., Takio, K., Matsui, M. and Deng, X.W. (1998) The COP9 complex is conserved between plants and mammals and is related to the 26S proteasome regulatory complex. *Curr. Biol.* **8**, 919–924.

Wei, N., Serino, G. and Deng, X.W. (2008) The COP9 signalosome: more than a protease. *Trends Biochem. Sci.* **33**, 592–600.

Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M. and Turner, J.G. (1998) COI1: an Arabidopsis gene required for jasmonate-regulated defense and fertility. *Science*, **280**, 1091–1094.

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 homeologous 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* homeologues. Conserved domains of CSN5 are labelled and highlighted.

Fig. S3 Polymerase chain reaction (PCR) products of D genome-specific primers COP9 (constitutive photomorphogenesis 9) MDF and COP9 MDR. M, molecular weight marker; 1–3, genomic DNA of Chinese Spring nullisomic–tetrasomic lines N2AT2B, N2BT2A and N2DT2B as template; 4, Chinese Spring.

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