

The COP9 signalosome controls jasmonic acid synthesis and plant responses to herbivory and pathogens

Sarah R. Hind¹, Sarah E. Pulliam¹, Paola Veronese², Deepak Shantharaj^{1,†}, Azka Nazir¹, Nekaiya S. Jacobs^{1,‡} and Johannes W. Stratmann^{1,*}

¹Department of Biological Sciences, University of South Carolina, Columbia, SC 29208, USA, and

²Department of Plant Pathology, North Carolina State University, Raleigh, NC 27695-7251, USA

Received 6 October 2010; revised 10 November 2010; accepted 18 November 2010; published online 4 January 2011.

*For correspondence (fax +1 803 777 4002; e-mail johstrat@biol.sc.edu).

†Present address: Department of Plant and Soil Sciences, University of Delaware, Newark, DE 1971, USA.

‡Present address: Medical University of South Carolina, Charleston, SC 29425, USA.

SUMMARY

The COP9 signalosome (CSN) is a multi-protein complex that regulates the activities of cullin-RING E3 ubiquitin ligases (CRLs). CRLs ubiquitinate proteins in order to target them for proteasomal degradation. The CSN is required for proper plant development. Here we show that the CSN also has a profound effect on plant defense responses. Silencing of genes for CSN subunits in tomato plants resulted in a mild morphological phenotype and reduced expression of wound-responsive genes in response to mechanical wounding, attack by *Manduca sexta* larvae, and *Prosystemin* over-expression. In contrast, expression of pathogenesis-related genes was increased in a stimulus-independent manner in these plants. The reduced wound response in CSN-silenced plants corresponded with reduced synthesis of jasmonic acid (JA), but levels of salicylic acid (SA) were unaltered. As a consequence, these plants exhibited reduced resistance against herbivorous *M. sexta* larvae and the necrotrophic fungal pathogen *Botrytis cinerea*. In contrast, susceptibility to tobacco mosaic virus (TMV) was not altered in CSN-silenced plants. These data demonstrate that the CSN orchestrates not only plant development but also JA-dependent plant defense responses.

Keywords: COP9 signalosome, jasmonic acid, plant–insect interactions, plant–pathogen interactions, systemin, virus-induced gene silencing.

INTRODUCTION

The COP9 signalosome (CSN) is a multi-protein complex that is conserved among all eukaryotes. It consists of eight subunits: CSN1–CSN8. A well-documented function of the CSN is its deneddylation activity (Wei and Deng, 2003). Multi-protein Cullin-RING-type E3 ubiquitin ligases (CRLs) comprise a cullin protein, which functions as a scaffold, a RING protein, an adapter protein and a substrate recognition protein. Once assembled, CRLs polyubiquitinate substrate proteins, thereby targeting them for proteasomal degradation. Many CRLs pass through a cycle of assembly and disassembly, and this cycling is regulated by the conjugation and removal of NEDD8 (deneddylation) (Wee *et al.*, 2005; Wei *et al.*, 2008). NEDD8/RUB1 is a ubiquitin-like protein that is covalently attached to cullins, and deneddylation is performed by the CSN subunit CSN5 via its metalloisopeptidase activity, which resides in a JAMM (JAB1/MPN domain metalloenzyme) motif located within the MPN (MPR1–PAD1–N-

terminal) domain (Lyapina *et al.*, 2001; Cope *et al.*, 2002; Wei and Deng, 2003; Gusmaroli *et al.*, 2007). None of the other CSN subunits are known to exhibit enzymatic activities, but they are required for assembly of the holocomplex and the deneddylation activity of CSN5. Null mutants of all Arabidopsis CSN subunits exhibit the same overall morphological features and seedling lethality (*cop/det/fus* mutant phenotype) (Kwok *et al.*, 1998; Dohmann *et al.*, 2005, 2008b; Gusmaroli *et al.*, 2007). The holocomplex is not assembled, and neddylated cullins accumulate. Furthermore, complete knockout of CSN5, 6, 7 and 8 resulted in destabilization of other CSN subunits, with the exception of CSN2 and 4 (Kwok *et al.*, 1998; Gusmaroli *et al.*, 2007). CSN reduction-of-function lines show a less dramatic morphological phenotype, intermediate cullin neddylation, and altered gene expression patterns (Mayer *et al.*, 1996; Schwechheimer *et al.*, 2001).

In *Arabidopsis*, *CSN5* and *CSN6* are both present as two copies. Unlike *CSN6A* and *CSN6B*, which seem to have the same function, *CSN5A* and *CSN5B* do not function in a redundant manner and are incorporated into distinct CSN complexes (Gusmaroli *et al.*, 2004). The null mutant *csn5a* shows severe morphological distortions, but is viable and can produce offspring, while the *csn5b* null mutant does not show a visual phenotype or a reduction in deneddylation activity. In contrast, the double null mutants *csn5a csn5b* show the typical *cop/det/fus* phenotype (Gusmaroli *et al.*, 2004, 2007; Dohmann *et al.*, 2005, 2008a). Additional functions of the CSN in *Arabidopsis* and other eukaryotes include prevention of auto-ubiquitylation-mediated proteasomal degradation of F-box proteins, the specific substrate recognition proteins within a CRL (Stuttman *et al.*, 2009), and regulation of the nuclear localization of COP1, an E3 ligase involved in light signaling (Chamovitz *et al.*, 1996; Wang *et al.*, 2009). In some eukaryotes, there is evidence for a CSN holocomplex-independent function of CSN5 (Wei *et al.*, 2008), but it appears that this is not the case in *Arabidopsis* (Dohmann *et al.*, 2008a).

CRLs that are known to interact with the CSN include SCF^{TIR1} (auxin signaling) (Schwechheimer *et al.*, 2001), SCF^{UFO} (flower development) (Wang *et al.*, 2003), SCF^{COI1} (jasmonate signaling) (Feng *et al.*, 2003) and SCF^{SLY1} (gibberellic acid signaling) (Dohmann *et al.*, 2010), in which TIR1, UFO, COI1, and SLY1 represent substrate-recognition F-box proteins. Indirect interactions of other CRLs with the CSN have also been demonstrated (Azevedo *et al.*, 2002; Stone *et al.*, 2003). As part of SCF^{COI1}, COI1 functions as a JA receptor by binding the JA conjugate JA-isoleucine. The SCF^{COI1}-JA-isoleucine complex then interacts with repressor proteins of the JAZ (jasmonate ZIM-domain) family. This interaction results in polyubiquitination of the JAZ proteins and their subsequent degradation by the proteasome. This de-repression liberates a MYC2 transcription factor that regulates expression of a subset of JA-responsive genes (Chini *et al.*, 2007; Thines *et al.*, 2007; Katsir *et al.*, 2008; Chung and Howe, 2009; Yan *et al.*, 2009). As a pleiotropic plant hormone, JA is involved in plant development and defenses. As a consequence, tomato (*Solanum lycopersicum*), *Arabidopsis* and *Nicotiana attenuata coi1* mutant plants show abnormal reproductive organ and trichome development, as well as reduced resistance to herbivory and necrotrophic fungal pathogens caused by compromised expression of JA-responsive defense genes (McConn *et al.*, 1997; Thomma *et al.*, 1998; Li *et al.*, 2004; Devoto *et al.*, 2005; Paschold *et al.*, 2007). In contrast, salicylic acid-dependent resistance against biotrophic pathogens is not reduced in *coi1* plants (Thomma *et al.*, 1998; Glazebrook, 2005). Other JA biosynthetic and signaling mutants generally show compromised resistance against a wide range of herbivores, including lepidopteran larvae, thrips, spider mites and isopod crustaceans, and

also to necrotrophic pathogens (Walling, 2000; Howe and Jander, 2008; Browse, 2009; Farmer and Dubugnon, 2009). Successful defense often depends on the concerted action of JA and ethylene (O'Donnell *et al.*, 1996; Penninckx *et al.*, 1998; Browse, 2009).

In solanaceous plants such as tomato and *N. attenuata*, the JA-dependent wound response to herbivory is well characterized. Extracellular primary signals, including fatty acid-amino acid conjugates present in the oral secretions of lepidopteran larvae (Halitschke *et al.*, 2001; Schmelz *et al.*, 2009), mechanical signals triggered by mechanical stimulation of insect mandibles (Mithofer *et al.*, 2005) and the wound signaling peptide systemin and functional systemin analogs (Ryan and Pearce, 2003), result in activation of an intracellular signaling network. Major nodes in this network include ion fluxes, reactive oxygen species, ethylene, mitogen-activated protein kinase (MAPK) cascades and jasmonic acid (Ryan, 2000; Kandathil *et al.*, 2007; Paschold *et al.*, 2007; Wu *et al.*, 2007).

JA-dependent genes encode an arsenal of plant defense proteins involved in resistance to insects. Wound response proteins comprise various groups of proteinase inhibitors (Ryan, 2000), amino acid-catabolizing enzymes such as arginase, threonine deaminase and leucine aminopeptidase (Chen *et al.*, 2005; Fowler *et al.*, 2009), and polyphenol oxidases (Constabel *et al.*, 1995). Most of these anti-digestive proteins function inside the insect digestive tract, resulting in reduced assimilation of essential amino acids. In lepidopteran larvae, the net effect is reduced growth and retarded larval development (Howe and Jander, 2008). As expression of these genes starts several hours after wounding, insect attack or application of wound signals such as systemin, they are classified as 'late' wound response genes. In contrast, genes encoding JA biosynthesis enzymes and prosystemin, the precursor protein for systemin, are expressed at basal levels in a JA-independent manner. However, JA perception leads to rapidly increased expression of these 'early' wound response genes (Li *et al.*, 2004). Wound response genes may also function in defenses against necrotrophic pathogens (Diaz *et al.*, 2002), while build-up of defenses against biotrophic pathogens includes SA-dependent pathogenesis-related (*PR*) genes. Furthermore, JA- and SA-induced signaling pathways negatively regulate each other (Glazebrook, 2005; Spoel *et al.*, 2007; Balbi and Devoto, 2008; Koornneef and Pieterse, 2008). However, only a few genes regulating the interplay between JA and SA signaling networks have been identified.

Here we show a function for the CSN in defenses against herbivory and necrotrophic pathogens. JA synthesis, expression of wound response genes and resistance against *M. sexta* larvae and *B. cinerea* fungi are attenuated in tomato plants with reduced CSN levels, and expression of *PR* genes is up-regulated without a concomitant increase in SA levels.

RESULTS

CSN5 genes in tomato

CSN5 is one of the two MPN domain-containing subunits of the CSN, and is encoded by two genes in Arabidopsis, *AtCSN5A* and *AtCSN5B*, which share 86% sequence identity (88% at the amino acid level) (Kwok *et al.*, 1998; Gusmaroli *et al.*, 2004). We identified two tomato (*Solanum lycopersicum*) *CSN5* genes, *CSN5-1* (GenBank accessions BT014209, AF175964, AK329790) and *CSN5-2* (GenBank accessions AK328186 and AK329574), that share 92% sequence identity (94% at amino acid level). The JAMM/MPN+ domain for deneddylation activity is highly conserved among the Arabidopsis and tomato CSN5 proteins. Although *CSN5-1* with *AtCSN5B* and *CSN5-2* shares a few amino acids with *AtCSN5A* at the C-terminus, this may not constitute true orthology because of the overall similarity between the two tomato *CSN5* genes (Figure S1).

Developmental effects of *CSN5/4/3* silencing

To test whether the CSN plays a role in tomato defense responses, we reduced *CSN5* expression by virus-induced gene silencing using a tobacco rattle virus-based system (TRV-VIGS) (Ratcliff *et al.*, 2001; Liu *et al.*, 2002a,b; Kandoth *et al.*, 2007). The vectors pTRV1 and pTRV2 carry the bipartite TRV genome inserted between left and right border sequences of *Agrobacterium tumefaciens* T-DNA. A 367 bp sequence from the *CSN5-1* cDNA was cloned into a multiple cloning site present in the viral genome on pTRV2 (*CSN5-VIGS*). Due to the high sequence homology between *CSN5-1* and *CSN5-2* (Figure S1), this construct targets both *CSN5* sequences for silencing. *A. tumefaciens* cultures containing pTRV1 and pTRV2-*CSN5* were co-infiltrated into tomato cotyledons of 11–14-day-old seedlings. As controls, plants were infiltrated with *A. tumefaciens* transformed with pTRV2 containing a partial green fluorescent protein cDNA (GFP) sequence (control VIGS plants), or with *A. tumefaciens* containing no pTRV vectors (mock treatment). Four weeks later, control VIGS plants appeared normal and did not show morphological differences compared to mock-treated plants (Figure 1a). This shows that the presence of the virus does not interfere with tomato development. Growth and development of control VIGS and mock-treated plants was slightly delayed (approximately 3 days) compared to completely untreated plants due to a short recovery period after the infiltration treatment at the cotyledon stage. In contrast to mock-infiltrated and control VIGS plants, *CSN5-VIGS* plants (Rio Grande, MicroTom and Castlemart varieties) showed stunted growth (height reduced by approximately 50% compared to controls), and slightly curled leaves, but otherwise normal development (Figures 1a and S2). This is a relatively mild phenotype compared to Arabidopsis *csn5a* or *csn5a csn5b* null mutants (Gusmaroli *et al.*, 2004, 2007; Dohmann *et al.*, 2005). CSN5

protein and transcript levels were strongly reduced in *CSN5-VIGS* plants compared to controls (Figures 1b and 2a). The mean reduction in CSN5 protein levels was 50–60%, and the maximal reduction was $\geq 90\%$. Occasionally, plants that were infiltrated with pTRV2-*CSN5* did not show an altered growth phenotype, and these plants also did not have reduced CSN5 protein levels (Figure S3a). The correlation between growth phenotype and reduction in CSN5 protein levels in *CSN5-VIGS* plants was very reliable.

In Arabidopsis, *CSN5A* and *CSN5B* appear to be functionally different (Gusmaroli *et al.*, 2007). We were unable to silence the two tomato *CSN5* genes individually using dissimilar sequences found in the untranslated regions. Therefore, we do not know the relative contribution of both genes to the observed effects in *CSN5*-silenced plants. As the anti-CSN5 antibody recognizes both Arabidopsis CSN5 proteins, it is highly likely that it also recognizes the two very similar tomato CSN5 proteins.

A morphological phenotype similar to *CSN5-VIGS* plants was observed when *CSN4* or *CSN3* were silenced by VIGS (Figure S2). We identified only one copy each of *CSN4* (accession number AK325540 on chromosome 4) and *CSN3* (accession number AK322699 on chromosome 2) in the tomato genome.

We tested whether silencing of *CSN5* and *CSN4* in tomato plants caused a change in the composition and abundance of the CSN complex. The Arabidopsis CSN holocomplex elutes from gel filtration columns with a peak at approximately 500 kDa. We found a similar elution pattern in control VIGS tomato plants (Figure 1c,d). In *CSN5-VIGS* plants, CSN5, but not CSN4, was reduced by 85% (Figure 1c, input). Accordingly, levels of the CSN5-containing CSN complex were also strongly reduced in these plants (Figure 1c, gel filtration fractions, upper panel). However, when testing for CSN4 in *CSN5-VIGS* plants, a similar amount of CSN complex as in control VIGS plants was detected (Figure 1c, gel filtration fractions, lower panel). This indicates that the CSN complex assembles in *CSN5-VIGS* plants but that the majority of complexes do not contain CSN5. In *CSN4-VIGS* plants, the levels of CSN4-containing CSN complex were strongly reduced (Figure 1d, gel filtration fractions, lower panel), correlating with a reduction of CSN4 protein by 88% (Figure 1d, input, lower panel). CSN5 protein levels were also reduced in *CSN4-VIGS* plants by 63% (Figure 1d, input, upper panel), and this correlated with a reduced amount of CSN complex compared to control VIGS plants (Figure 1d, gel filtration fractions, upper panel). Therefore, silencing of *CSN4* has a more striking effect on CSN complex abundance than silencing of *CSN5*. These data are consistent with gel filtration experiments in Arabidopsis *csn4* and *csn5* null and partial loss-of-function mutants (Kwok *et al.*, 1998; Serino *et al.*, 1999; Dohmann *et al.*, 2005, 2008a; Gusmaroli *et al.*, 2007; Stuttmann *et al.*, 2009).

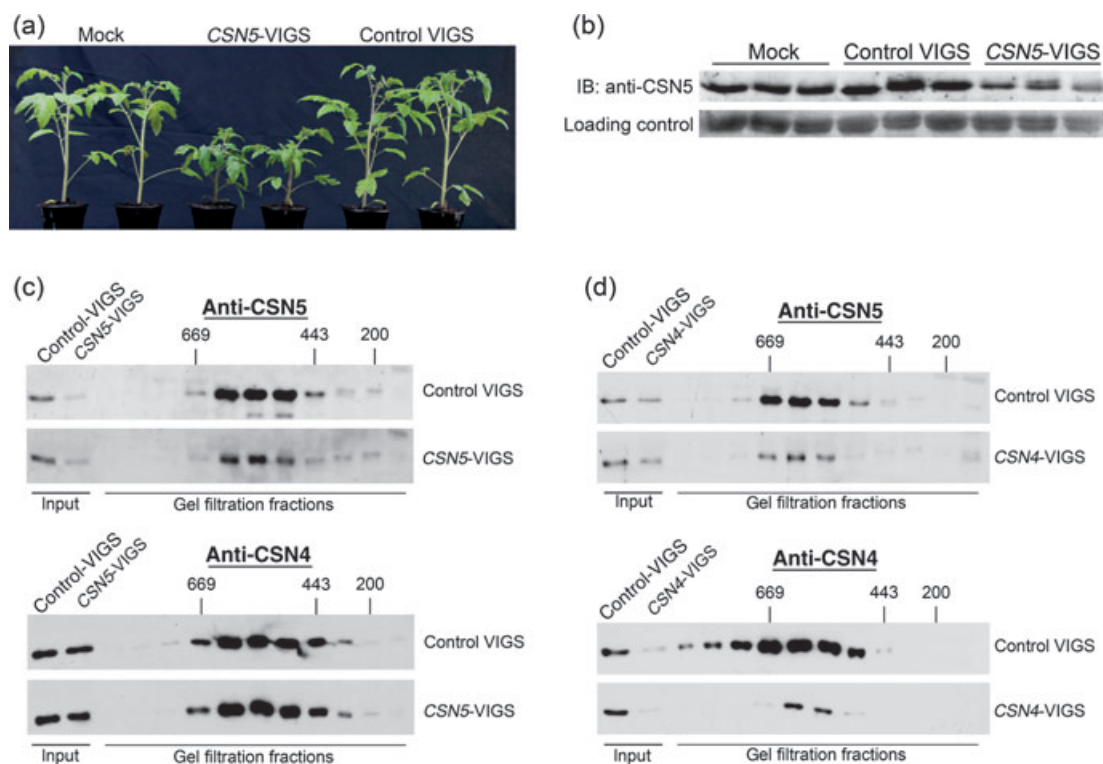


Figure 1. Virus-induced gene silencing of *CSN5* and *CSN4*.

(a) Rio Grande plants 4 weeks after infiltration with either *Agrobacterium* without TRV (mock), *Agrobacterium* containing pTRV2-*GFP* (control VIGS) or *Agrobacterium* containing pTRV2-*CSN5* (*CSN5*-VIGS).

(b) Immunoblot analysis of *CSN5* protein levels in leaves of *CSN5*-VIGS and control plants using anti-*CSN5* antibody. After immunodetection, proteins on membranes were stained to demonstrate equal protein loading (loading control).

(c, d) Gel filtration analysis of the CSN complex in *CSN5*-VIGS plants (c, d), *CSN5*-VIGS (c) or *CSN4*-VIGS (d) plants were separated by gel filtration, and fractions were analyzed by immunoblotting with anti-*CSN5* antibody (upper panel) and anti-*CSN4* antibody (lower panel). Unfractionated total protein inputs (30 μ g) are shown in left lanes (input: control VIGS and *CSN*-VIGS). The numbers refer to peak fractions of molecular weight markers. Low-molecular-weight fractions showing low levels of *CSN5* and *CSN4* monomer in control VIGS plants are not shown.

When testing additional plants, we confirmed that *CSN5* levels are slightly reduced in *CSN4*-VIGS plants compared to controls (Figure 1d, input, upper panel, and Figure S3b). We also found that *CSN4* levels were somewhat reduced when the silencing efficiency of *CSN5* was 98% (Figure S3b, lanes 5,6); however, in plants that had a lower *CSN5* silencing efficiency, *CSN4* levels remained unchanged (Figure 1c, input, lower panel, and Figure S3b, lane 4).

Taken together, VIGS of *CSN* subunits in tomato plants results in strong similarities to Arabidopsis *csn* reduction-of-function plants with regard to overall morphology and the composition and assembly of the CSN complex.

The CSN controls defense gene expression

We measured the expression of tomato defense genes in control VIGS and *CSN5*-VIGS plants. *CSN5* was silenced in plants of the MicroTom variety, including plants that over-express the *Prosystemin* gene (*35S:PS* plants) (Chen *et al.*, 2006). *CSN5* transcript levels were strongly reduced in *CSN5*-VIGS plants compared to control VIGS plants, but transcripts of three housekeeping genes (*Actin*, *Ubiquitin*

and *eIF4A*) were unaltered (Figures 2a and S4a). Expression of the 'late' strictly JA-dependent wound response genes *Proteinase Inhibitor-I (PI-I)*, *PI-II* and *Leucine Aminopeptidase (LAP)* was strongly reduced in *CSN5*-VIGS MicroTom plants compared to control plants, even in the absence of a wound stimulus (Figure 2a). Over-expression of *Prosystemin (PS)* is known to up-regulate late wound response genes, but this up-regulation was strongly reduced in *35S:PS* MicroTom plants silenced for *CSN5* (Figure S4a). Expression of the 'early' JA-independent wound response genes *Lipoxygenase D (LOXD)* and *Allene Oxide Cyclase (AOC)* was only reduced in *35S:PS* MicroTom *CSN5*-VIGS plants (Figures 2a and S4a).

Consistent with the reduced transcript levels of late wound response genes in *CSN5*-VIGS plants, we found that protein levels of PI-II in *35S:PS* MicroTom plants were reduced by $73 \pm 5\%$ when *CSN5* was silenced (Figure 2b). In non-transgenic *CSN5*-VIGS MicroTom plants, mechanical wounding- and methyl JA (MeJA)-induced levels of PI-II were reduced by 50 ± 9 and $39 \pm 6\%$, respectively, compared to control plants (Figure 2b).

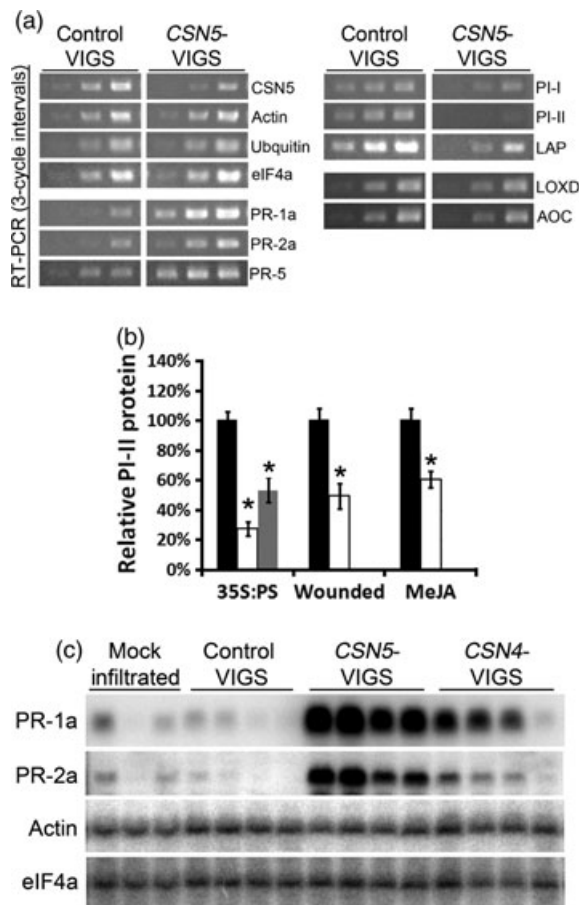


Figure 2. Reduced expression of wound response genes and increased expression of *PR* genes in *CSN*-VIGS plants.

MicroTom plants were infiltrated with *Agrobacterium* (mock infiltrated), pTRV2-*GFP* (control VIGS), pTRV2-*CSN5* (*CSN5*-VIGS) or pTRV2-*CSN4* (*CSN4*-VIGS), and analyzed 4 weeks later.

(a) Transcript levels in control VIGS and *CSN5*-VIGS plants were assessed by semi-quantitative RT-PCR in leaf tissue. Ethidium bromide-stained agarose gels containing PCR products are shown. For cycle numbers, see Table S2.

(b) PI-II protein levels in *CSN5*-VIGS plants (white bars) and *CSN4*-VIGS plants (gray bars) were quantified in leaf tissue of *35S:Prosystemin* MicroTom plants (*35S:PS*) ($n = 30$, four independent experiments for *CSN5*-VIGS; $n = 10$, one experiment for *CSN4*-VIGS), and in non-transgenic MicroTom plants 24 h after wounding (wounded) ($n = 20$; two independent experiments) or MeJA treatment ($n = 26$; three independent experiments). The levels in VIGS plants were expressed as percentages of the mean levels in control VIGS plants (black bars), which were defined as 100%, with bars representing the mean \pm SE. * $P < 0.05$ between controls and VIGS plants (Student's t test).

(c) *PR* gene expression in four replicate samples of mock-infiltrated, control VIGS, *CSN5*-VIGS and *CSN4*-VIGS plants, assessed by RNA blotting.

Expression of the pathogenesis-related genes *PR-1a* (accession number AJ011520), *PR-2a* (accession number M80604; β -1,3-glucanase) and *PR-5* (accession number AJ277064) was much higher in *CSN5*-VIGS plants compared to controls (Figure 2a,c). In *35S:PS* MicroTom plants, silencing of *CSN5* resulted in up-regulation of *PR-1a* and *PR-2a*, while *PR-5* was down-regulated, indicating that these *PR* genes are differentially regulated by over-expression of *PS* (Figure S4a). When *CSN5*, *CSN4* and *CSN3* were silenced in

the Rio Grande variety, the *CSN*-silenced plants accumulated relatively high levels of *PR-1a* and *PR-2a* transcripts, but *PR* gene transcripts in control plants were barely detectable (Figures 2c and S4b).

The *CSN* controls synthesis of jasmonic acid, but not salicylic acid

Late wound response genes in tomato are strictly JA-dependent, and their expression is reduced in JA biosynthetic or signaling mutants (Li *et al.*, 2003, 2004). In contrast, expression of *PR-1a*, *PR-2a* and *PR-5* is induced in response to pathogens and SA (Van Loon and Van Strien, 1999; Schaller *et al.*, 2000). To determine whether the *CSN* modulates gene expression by regulating JA and SA synthesis, we measured the levels of the two plant hormones in tomato Rio Grande plants. JA levels in untreated tomato leaves are below the detection threshold, but strongly and rapidly increase after wounding (Stenzel *et al.*, 2003; Li *et al.*, 2005; Kandoth *et al.*, 2007). In *CSN5*-VIGS plants, synthesis of JA after wounding was strongly reduced compared to control VIGS plants (Figure 3a). This corresponded with the reduced expression of JA-dependent wound response genes in *CSN5*-VIGS plants. SA levels in control and *CSN5*-VIGS plants were not significantly different (Figure 3b). This indicates that constitutive up-regulation of *PR* genes in *CSN5*-VIGS plants does not depend on increased accumulation of SA. We also tested SA levels 1 h after wounding, and found no change compared to unwounded plants. Similar results were obtained for the MicroTom variety and transgenic *35S:PS* MicroTom plants (Figure S5). Taken together, these data show that the *CSN* positively regulates expression of JA-dependent wound response genes but negatively regulates expression of SA-responsive *PR* genes.

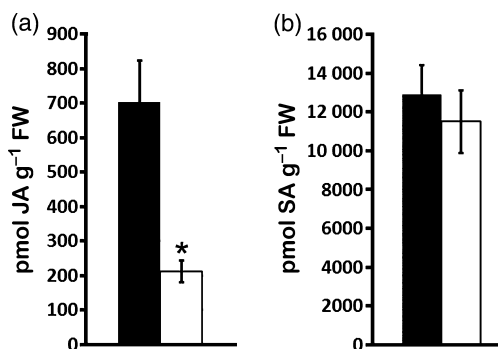


Figure 3. JA and SA levels in *CSN5*-VIGS Rio Grande plants.

Plants were infiltrated with pTRV2-*GFP* (black bars) or pTRV2-*CSN5* (white bars).

(a) Leaves were wounded 4 weeks after infiltration, and JA levels were measured 1 h after wounding. Bars represent the mean of ≥ 4 plants \pm SE. * $P < 0.05$ between controls and *CSN5*-VIGS plants (Student's t test).

(b) SA levels were measured in unwounded plants 4 weeks after infiltration. Bars represent the mean of 15 plants \pm SE from two independent experiments.

CSN function is required for defenses against herbivorous insects

Late wound response genes encode an arsenal of anti-digestive proteins that protect tomato plants against insects. We reasoned that *CSN5*-VIGS plants with reduced expression of wound response genes would be more susceptible to insect attack than control plants. After 14 days of constant unrestricted feeding of lepidopteran *Manduca sexta* larvae on *CSN5*-VIGS and control VIGS Rio Grande plants, *CSN5*-VIGS plants were almost completely defoliated but control plants retained much of their foliage (Figure 4a). The defoliation of the *CSN5*-VIGS plants can partially be explained by their smaller stature. However, *M. sexta* larvae that consumed these plants showed an overall larger size (Figure 4b) and a significantly increased growth rate compared to larvae from control plants (Figure 4c). Also, larval mortality on control VIGS plants was five times higher than on *CSN5*-VIGS plants. When protein levels of PI-II were measured in the remaining tissue of insect-exposed plants 14 days after onset of feeding, *CSN5*-VIGS plants were found to contain very low levels of PI-II, while control VIGS plants had high PI-II levels (Figure 4d). Similar results were obtained using plants of the Castlemart and Micro-Tom varieties (Figure S6).

The CSN plays a variable role in defenses against pathogens

A trend in Arabidopsis research suggests that resistance to necrotrophic pathogens is mainly mediated by JA and ethylene, while resistance to biotrophic pathogens is dependent on SA and often characterized by a hypersensitive response. We therefore tested whether *CSN5*-VIGS plants with reduced JA synthesis exhibit altered resistance against the necrotrophic fungal pathogen *Botrytis cinerea*. Excised tomato leaves were drop-inoculated with a *B. cinerea* conidial suspension. *CSN5*-VIGS plants showed increased susceptibility to the pathogen 48 and 72 h later, characterized by larger lesion sizes and necrosis spreading to major veins (Figure 5a,b). Five days after infiltration, cell death occurred outside the infiltration zones and spread over the entire leaf, while expansions of cell death zones in control plants were more confined (Figure 5b, lower panel).

Infection of tomato plants with tobacco mosaic virus (TMV) and other viruses results in mosaic patterns of chlorosis and up-regulation of some *PR* genes without concomitant resistance to the virus (Brederode *et al.*, 1991; Xu *et al.*, 2003). We tested whether susceptibility to TMV was altered in *CSN5*-VIGS plants that express *PR* genes constitutively (Figures 2 and S3). In uninoculated *CSN5*-VIGS plants, *PR-1a* expression was higher than in control plants (Figure S7, lanes 1 and 4). After TMV inoculation, *PR-1a* transcript levels in control plants increased strongly, reaching levels similar to uninoculated *CSN5*-VIGS plants, but *PR-1a* expression in infected *CSN5*-VIGS plants did not

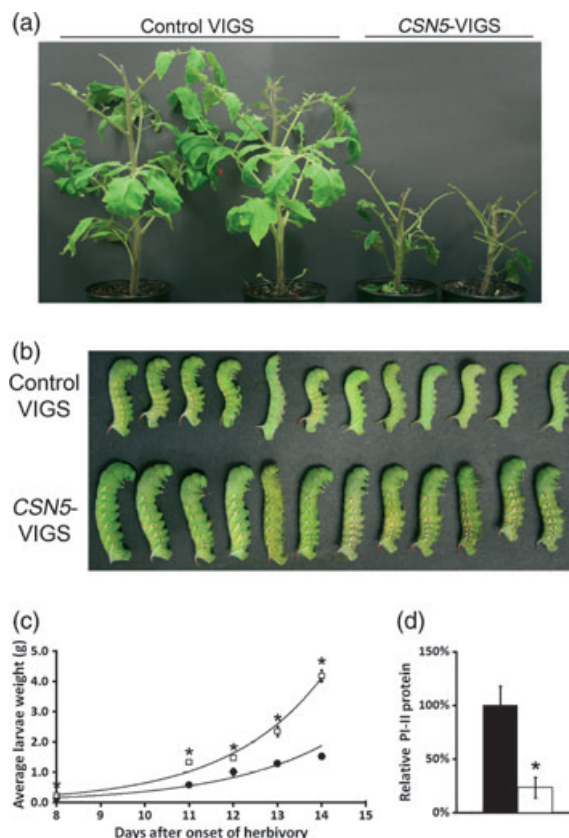


Figure 4. *CSN5*-VIGS plants exhibit increased susceptibility to herbivory. Rio Grande plants were infiltrated with either pTRV2-*GFP* (control VIGS) or pTRV2-*CSN5* (*CSN5*-VIGS). Four weeks after infiltration, newly hatched *M. sexta* larvae were placed on upper leaves of plants (one larva per plant) and allowed to feed *ad libitum* for 14 days. (a) Representative plants are shown after 14 days of herbivory. (b) *M. sexta* larvae after 14 days of feeding. Similar results were obtained in three independent experiments. (c) Larval growth rate ($n \geq 18$; two independent experiments). (d) PI-II protein levels after 14 days of herbivory. The levels in VIGS plants (mean \pm SE) (white bars) are expressed as percentages of the mean levels in control plants (black bars), which were defined as 100% ($n = 18$; two independent experiments). For (c) and (d), $*P < 0.05$ between controls and *CSN5*-VIGS plants (Student's *t* test).

increase above the levels of uninoculated *CSN5*-VIGS plants (Figures 5c and S7). However, in all infected plants, transcripts for the TMV coat protein increased in a similar manner (Figures 5c and S7), and this increase correlated with the development of disease symptoms. Therefore, constitutive expression of *PR* genes in *CSN5*-VIGS plants did not alter propagation of TMV.

In both the *B. cinerea* and TMV experiments, mock-infiltrated plants (no TRV) were compared to control VIGS plants and no differences were observed. This excludes the possibility of confounding effects of the TRV on defense responses to the two pathogens.

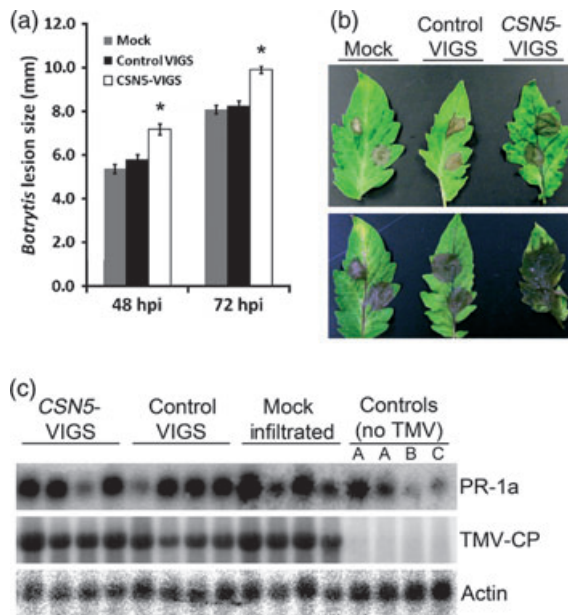


Figure 5. *CSN5*-VIGS plants show increased susceptibility to *B. cinerea* but not to TMV.

Rio Grande plants were infiltrated with *Agrobacterium* (mock), *Agrobacterium* containing pTRV2-GFP (control VIGS) or *Agrobacterium* containing pTRV2-*CSN5* (*CSN5*-VIGS).

(a) Four to five weeks after infiltration, leaflets were excised from plants and inoculated with *B. cinerea*. The diameter of disease lesions was measured at 48 h ($n \geq 37$; one experiment) or 72 h ($n \geq 56$; one experiment) after inoculation. * $P < 0.05$ between controls and *CSN5*-VIGS plants (Student's *t* test).

(b) Excised inoculated leaflets 3 days (upper panel) and 5 days (lower panel) after *B. cinerea* inoculation.

(c) Four to five weeks after infiltration, terminal leaflets were inoculated with TMV. Ten days after inoculation, transcript levels of *PR-1a*, *TMV coat protein* (*TMV-CP*) and *Actin* were assessed in systemic leaflets by RNA blotting. Four replicate samples each of *CSN5*-VIGS, control VIGS and mock-infiltrated plants are shown. Uninoculated controls (no TMV) are shown for *CSN5*-VIGS (lane A), control VIGS (lane B) and mock-treated (lane C) plants. Similar results were obtained in two independent experiments.

CSN5 transcript and protein levels are not changed during defense responses

Plant responses to herbivory or pathogens have pronounced effects on primary metabolism and plant development. Involvement of the CSN in developmental processes and responses to stress suggests that CSN activity is regulated by environmental and developmental cues. CSN-associated kinases and phosphorylation of CSN subunits have been described previously (Harari-Steinberg and Chamovitz, 2004; Malec and Chamovitz, 2006), but it is not known how these factors affect CSN activity. As a first attempt to assess possible regulation of CSN activity, we tested whether the *CSN5* subunit is regulated at the transcriptional level or post-translationally through changes in protein abundance in response to application of the microbe-associated molecular pattern flg22, the wound signaling peptide systemin, MeJA or wounding by *M. sexta* larvae over a period of 6 h. Both

CSN5 transcript and *CSN5* protein levels remained unchanged (Figure S8).

DISCUSSION

By reducing levels of CSN subunits and the CSN holocomplex, we have demonstrated that the CSN controls wound-inducible JA synthesis and defense responses against an insect herbivore and a necrotrophic pathogen in tomato. VIGS of CSN subunits resulted in a reduction of CSN function that affected plant development marginally but had a pronounced effect on JA synthesis and plant defense.

The relatively moderate morphological phenotype in *CSN5*-VIGS plants combined with strongly reduced *CSN5* protein levels and the absence of virus-induced disease symptoms or developmental defects in control VIGS plants show that VIGS is an appropriate method to test the effect of *CSN5* silencing on plant defense responses. In order to assess the efficiency of a reduction-of-function approach such as VIGS, it is important to correlate reductions in transcript and protein levels. The use of CSN-specific antibodies enabled us to demonstrate that VIGS of *CSN5* and *CSN4* resulted in significant reductions in *CSN5* and *CSN4* protein levels. However, the remaining CSN protein appears to be sufficient for a relatively normal growth pattern compared to *csn* null mutants, which are lethal at the seedling stage, at least in *Arabidopsis*. It is conceivable that the presence of the virus (TRV) induces defense responses in VIGS plants. However, when mock-treated plants (no TRV present in the plant) were compared to control VIGS plants (TRV present in the plant) with regard to growth and defense parameters, no differences were observed. Also, responses to another virus, TMV, were not altered in control VIGS plants compared to mock-treated plants. Moreover, we identified a similar pattern of gene expression in *Arabidopsis csn* reduction-of-function mutants (see below). Taken together, these results largely exclude confounding effects of TRV on the data presented, and validate VIGS as a rapid, convenient and efficient gene silencing tool in tomato.

The CSN is required for JA signaling through its control of SCF^{COI1} activity. However, it is not known whether JA synthesis is affected in *Arabidopsis csn* mutants. We envisage two mechanisms for CSN effects on JA synthesis. First, as the CSN regulates SCF^{COI1} activity, JA signaling is likely to be impaired in *CSN*-silenced plants. Reduced JA perception and signaling may in turn have a negative feedback effect on JA synthesis via reduced gene expression of JA-biosynthetic genes. Consistent with this, we found that expression of the JA-biosynthetic genes *AOC* and *LOXD* is reduced in *CSN5*-VIGS plants that over-express *Prosystemin* (Figure S4a). Moreover, the *Arabidopsis coi1* mutant shows reduced wound-inducible JA synthesis (Glauser *et al.*, 2008). This suggests that SCF^{COI1} is at least one target of the CSN that affects defense responses. Alternatively, the CSN may regulate other CRLs that control JA biosynthesis via

ubiquitination of JA-biosynthetic enzymes or proteins that regulate those enzymes. If SCF^{COI1} were the sole CSN target for JA-mediated defenses, *coi1* mutants would not only show a reduced expression of wound response genes, but also exhibit constitutive *PR* gene expression. Reduced JA levels in *coi1* or *CSN5*-VIGS plants may mitigate the negative effects of JA on *PR* gene expression. However, we did not find evidence for increased *PR* gene expression in the tomato *coi1* (*jai-1*) or Arabidopsis *coi1* mutant to support this (Kloek *et al.*, 2001; Feng *et al.*, 2003; Zhao *et al.*, 2003; Li *et al.*, 2004; Mur *et al.*, 2006; Hruz *et al.*, 2008). In Arabidopsis, several SCF-type CRLs have been implicated in defenses against pathogens (Azevedo *et al.*, 2002; Kim and Delaney, 2002; van den Burg *et al.*, 2008; Craig *et al.*, 2009; Gou *et al.*, 2009). Therefore, it is possible that our results may be the consequence of CSN interactions with E3 ligases other than SCF^{COI1}. This is a likely scenario with regard to the characteristic defense gene expression pattern in *CSN*-VIGS plants, which is not found in *coi1* mutants. Alternatively, recent developments suggest a more direct role of the CSN in regulation of transcription (Wei *et al.*, 2008; Chamovitz, 2009). The pleiotropy of the CSN is an important aspect of its well-established role in development, based on known CSN interactions with SCF^{TIR1} and SCF^{UFO}, which regulate auxin signaling and flower development (Schwechheimer *et al.*, 2002; Wang *et al.*, 2003; Wei *et al.*, 2008).

As resistance to herbivorous insects and necrotrophic pathogens is known to be compromised in *coi1* mutants of Arabidopsis, tomato and *Nicotiana attenuata* (Thomma *et al.*, 1998; Li *et al.*, 2004; Paschold *et al.*, 2007; AbuQamar *et al.*, 2008), it is likely that the reduced synthesis of JA in *CSN5*-VIGS plants is the cause of their impaired defenses against *M. sexta* and *B. cinerea*. This suggests that interaction between the CSN and SCF^{COI1} is not only critical for proper plant development, but is also necessary for plant defenses.

We also tested whether *CSN*-VIGS plants would be less susceptible to TMV because of their constitutively elevated *PR* gene transcripts. However, *CSN5*-VIGS plants were as susceptible to TMV as control plants. In the only other study examining the role of the CSN in defenses to pathogens, *CSN3/8*-VIGS tobacco plants carrying the *N* resistance gene did show reduced resistance against TMV (Liu *et al.*, 2002c), implicating the CSN in *R* gene-mediated resistance against pathogens. Interestingly, the F-box protein ACRE189/ACIF1 is part of a SCF-type E3 ligase, and tobacco plants with reduced levels of ACIF1 showed compromised N-mediated resistance to TMV, Pto-mediated resistance to *Pseudomonas syringae* pv. *tabaci*, and Cf-9-mediated resistance to *Cladosporium fulvum* (van den Burg *et al.*, 2008). Taken together, our findings suggest a relatively specific role for the CSN in defense responses to various pathogens.

An altered gene expression pattern of defense genes was observed for Arabidopsis *csn8* (*cop9*) mutants, which

constitutively express three SA-responsive *PR* genes (Mayer *et al.*, 1996). It has also been shown that expression of most JA-responsive genes in Arabidopsis requires a functional CSN (Feng *et al.*, 2003). Furthermore, microarray experiments in public databases reveal a similar gene expression pattern for JA- and SA-responsive genes in Arabidopsis *csn* mutants as in *CSN5*-VIGS tomato plants: for example, expression of SA-responsive genes such as *PR-1* and *NPR1* was strongly increased, while expression of JA-responsive genes such as *VSP1* was strongly decreased in the Arabidopsis *csn4-1* (Schwager *et al.*, 2007) (Gene Expression Omnibus accession numbers GSM88049–GSM88051), *csn5a-2*, *csn5b-1* and *csn3-1* mutants (Gene Expression Omnibus accession number GSE9728) (Dohmann *et al.*, 2008a). As these studies were concerned with general or developmental aspects of the CSN, neither herbivory or pathogenesis studies nor direct measurements of JA and SA were performed in these *csn* mutants. The similar gene expression pattern in *CSN*-VIGS tomato plants (Solanaceae) and Arabidopsis (Brassicaceae) *csn* mutants indicates a conserved role for the CSN in controlling plant defenses.

In *Drosophila*, the CSN is known to play a role in immunity (Harari-Steinberg *et al.*, 2007), but most previous analyses of CSN function in plants focused on development and hormone signaling. Reduced sensitivity to JA in Arabidopsis *csn* mutants and increased *PR* gene expression have been noted previously (Mayer *et al.*, 1996; Schwechheimer *et al.*, 2002); however, no integrated view of the role of the CSN in plant development and defense has been provided. Here, we bring the various aspects of CSN signaling together, and show that the CSN, by controlling JA synthesis and inversely affecting wound response and *PR* gene expression, exerts a profound effect on plant defense responses. These findings reveal a function of the CSN as a central signaling node that connects environmental and developmental signaling networks.

EXPERIMENTAL PROCEDURES

Plant materials and growth conditions

Tomato plants (*Solanum lycopersicum*, formerly *Lycopersicon esculentum*) of the MicroTom, Rio Grande or Castlemart varieties were grown in AR66L growth chambers (Percival Scientific, <http://www.percival-scientific.com>) under 17 h light ($120 \pm 20 \mu\text{E m}^{-2} \text{sec}^{-1}$) and 7 h dark periods. For optimal VIGS conditions, plants were grown at 20°C (day) and 18°C (night). One week before sampling, the day and night temperatures were increased to 27 and 22°C, respectively, in order to increase PI protein synthesis. The transgene in *35S:Prosystemin* MicroTom plants had been backcrossed five times from Castlemart (Chen *et al.*, 2006).

Identification of *CSN5* genes in tomato

GenBank accessions BT014209, AF175964 and AK329790 are referred to as *SICSN5-1* and accessions AK328186 and AK329574 are referred to as *SICSN5-2*. The tomato genome is currently being assembled, and a tentative genome shotgun sequence has been

released by the International Tomato Genome Sequencing Consortium via the Solanaceae Genome Network (<http://solgenomics.net>). A BAC clone containing *CSN5-1* is currently being sequenced by the 'chromosome 4 sequencing project', but it is not clear whether it maps to chromosome 4. A BAC clone containing the *CSN5-2* sequence is being sequenced by the chromosome 6 sequencing project. ESTs corresponding to both *CSN5-1* and *CSN5-2* were found by the Solanaceae Genome Network to be present in various tissues, including leaves (*CSN5-1* unigene ID: SGN-U585559; *CSN5-2* unigene ID: SGN-U585560).

VIGS

The TRV-based VIGS vectors pTRV-*RNA1* (pTRV1) and pTRV-*RNA2* (pTRV2) were obtained from S.P. Dinesh-Kumar (Department of Molecular, Cellular and Developmental Biology, Yale University, New Haven, CT, USA). The pTRV2-*GFP* construct has been described previously (Kandath *et al.*, 2007). *CSN* gene fragments were amplified from a cDNA library generated from MicroTom plants. Primers used for amplification of *CSN* gene fragments are shown in Table S1. PCR products were inserted into the multiple cloning site of pTRV2. Cotyledons of 11–14-day-old seedlings were infiltrated with *Agrobacterium tumefaciens* GV3101 containing both pTRV1 and pTRV2 cultures using a 1 ml syringe as described previously (Ekengren *et al.*, 2003; Kandath *et al.*, 2007).

Wounding and MeJA treatments

Four weeks after infiltration, VIGS plants were wounded or treated with MeJA as previously described (Kandath *et al.*, 2007). Plants were wounded using a hemostat and incubated for 24 h before sampling, or were treated with MeJA for 12 h and then sampled 12 h after the end of treatment.

Insect feeding assays

Tobacco hornworm (*Manduca sexta*) eggs were hatched on an artificial diet at room temperature. Immediately after hatching, larvae were placed on the upper leaves of plants (one larva per plant) and allowed to feed *ad libitum*.

Botrytis and TMV assays

Growth and culturing of *Botrytis cinerea* strain B05 were performed as described previously (Mengiste *et al.*, 2003). Four weeks after VIGS infiltration, leaflets were excised from plants, placed on wet filter paper and inoculated with 10 µl droplets of 2×10^5 conidia per ml suspension. Leaf material was kept under 100% humidity, and lesion diameter was scored at 48 and 72 h after inoculation. Three independent inoculation experiments were performed, each including at least 10 of mock, control VIGS and *CSN5*-VIGS plants. For each experiment, at least 20 detached leaves per treatment were drop-inoculated.

TMV was propagated on 30-day-old *Nicotiana clevelandii* plants. Infected leaves were excised, air-dried, and stored at 4°C. TMV inoculum was prepared by homogenizing 1 g of TMV-containing dried *N. clevelandii* leaves in 0.05 M phosphate buffer (pH 7.0). Carborundum was applied to the terminal leaflet of a tomato leaf, and inoculum was carefully rubbed onto the leaf surface using a cotton swab. At the times indicated after inoculation, 20 leaf discs were obtained using a hole puncher from random leaves of the plant (not including the inoculated leaf).

Tissue sampling

Leaf material was collected as described previously (Kandath *et al.*, 2007). For RNA transcript analysis, 20–25 leaf disks (approximately

100 mg fresh weight) were punched with a hole puncher and frozen, and two or three leaflets were harvested for immunoblotting and radial immunodiffusion analysis.

Protein extraction, gel electrophoresis and immunoblotting analysis

Frozen leaf material was homogenized in extraction buffer containing 50 mM Hepes/KOH (pH 7.6), 2 mM DTT, 1 mM EDTA, 1 mM EGTA, 20 mM β-glycerophosphate, 20% v/v glycerol, 10 µM leupeptin, 1 mM Na₃VO₄, 1 mM NaF, 0.5% w/v polyvinylpyrrolidone and 1 mM phenylmethanesulfonylfluoride. Homogenates were centrifuged twice for 10 min at 18 000 *g* at 4°C. Protein concentrations were determined using Bio-Rad protein assay solution (<http://www.bio-rad.com/>) using BSA as the standard.

Protein aliquots (30 µg) were separated using 7.5 or 10% polyacrylamide gels, and transferred to Immobilon-P PVDF membranes (Millipore, <http://www.millipore.com/>) using a mini trans-blot electrophoretic transfer cell (Bio-Rad) according to the manufacturer's recommendations. After transfer, membranes were blocked in 5% w/v BSA (fraction V, Fisher Scientific, <http://www.fishersci.com>) in TBST (10 mM Tris/HCl pH 7.5, 150 mM NaCl, 0.1% v/v Tween-20) for 1 h at room temperature. Blots were washed twice in TBST before incubating with the primary antibody. All primary antibodies were incubated overnight at 4°C. After five washes with TBST, blots were incubated with alkaline phosphatase-conjugated secondary antibody for 1 h at room temperature. Blots were washed five times with TBST, and then incubated for 5 min with a LumiPhos chemiluminescence detection system (Thermo Fisher/Pierce, <http://www.piercenet.com>) and visualized using HyBlot CL autoradiography film (Denville Scientific Inc., <http://www.denvillescientific.com>). Primary antibodies used were anti-*CSN5* (both 1:2000 in 1% w/v BSA; Enzo Life Sciences, <http://www.enzolifesciences.com>), and proteins for loading controls were determined using a MemCode reversible protein stain kit for PVDF membranes (Thermo Fisher/Pierce). The secondary antibody used was monoclonal mouse anti-rabbit alkaline phosphatase-conjugated antibody (1:20 000; Sigma-Aldrich, <http://www.sigmaaldrich.com/>).

Semi-quantitative RT-PCR

Total RNA was isolated from approximately 100 mg of leaf tissue using TRIzol reagent according to the manufacturer's recommendations (Invitrogen, <http://www.invitrogen.com/>). RNA was treated with a TURBO DNA-free kit (Applied Biosystems/Ambion, <http://www.appliedbiosystems.com/>) to remove contaminating DNA. First-strand cDNA synthesis was performed with 6 µg of RNA using a SuperScript II first-strand synthesis kit (Invitrogen). The cDNA (0.3 µl) was used for subsequent PCR amplifications using 0.3 µl of PCR Extender System enzyme (5 PRIME Inc., <http://www.5prime.com>) in a 30 µl reaction volume. Thermal cycling conditions were as follows: one cycle of 3 min at 95°C, up to 35 cycles of denaturing for 30 sec at 95°C, annealing for 30 sec at 56°C and extension for 45 sec at 72°C, and then one cycle of 7 min at 72°C. During the PCR, 5 µl samples were removed 30 sec into the extension step at three-cycle intervals (see Table S2). Samples were separated on 1.5% w/v agarose-TAE gels by electrophoresis, and stained with ethidium bromide for visualization and documentation.

RNA gel-blot analysis

Total RNA was isolated from approximately 100 mg of leaf tissue using TRIzol reagent according to the manufacturer's recommendations (Invitrogen). The total RNA (10 µg) was separated on a 1.2% formaldehyde agarose gel and transferred by overnight capillary

transfer to Hybond NX nylon membrane (Amersham Pharmacia Biotech, <http://www5.amershambiosciences.com/>). Radiolabeled DNA probes were generated using the random primer DECAprime II kit (Applied Biosystems/Ambion). Unincorporated radionucleotides were removed using Micro Bio-Spin 30 columns (Bio-Rad). The membrane was blocked for 3 h in ULTRAhyb hybridization buffer (Applied Biosystems/Ambion) before addition of the radiolabeled probe, and incubated overnight at 42°C (38°C for eIF4A). Blots were washed twice with 2 × SSC/0.1% SDS heated to 65°C for 15 min each, and then washed once in 0.1 × SSC/0.1% SDS heated to 65°C for 10 min (no last wash for eIF4A). Radiolabeled RNA was visualized using a Storm phosphoimaging system (Amersham).

PI-II analysis

PI-II protein levels in leaf juice were measured using a radial immunodiffusion assay as described previously (Kandoth *et al.*, 2007).

JA and SA analysis

Samples were collected from VIGS plants 4 weeks after infiltration, and jasmonic acid analysis was performed as described previously (Kandoth *et al.*, 2007). Salicylic acid analysis was performed as described by Schmelz *et al.* (2004). Tissue from the upper three or four leaves of 5-week-old plants was collected for hormone analysis. Briefly, one half of each leaflet was excised to act as unwounded controls, and thereafter, the other half of the leaflet was wounded using a hemostat. One hour later, the wounded half of the leaflet was excised from the midvein. Each sample was weighed before freezing the tissue in liquid nitrogen, and samples were stored at -70°C. Approximately 300 mg of leaf tissue was collected for each sample. The internal standards (100 ng) were added during grinding of the plant tissue in liquid nitrogen before continuing with the hormone extraction as described by Schmelz *et al.* (2004). JA analysis using dihydro-JA (dhJA) as an internal standard (Kandoth *et al.*, 2007) and SA analysis using 2-hydroxybenzoic acid-d₆ (C/D/N Isotopes Inc., <https://www.cdnisotopes.com/>) as an internal standard were performed as described previously (Schmelz *et al.*, 2004; Kandoth *et al.*, 2007). The retention time for JA (224.1412) was 23 min 39 sec, and that for dihydro-JA (226.1569) was 23 min 49 sec. The retention time for SA (152.0473) was 10 min 49 sec, and that for 2-hydroxybenzoic acid-d₆ (156.0725) was 10 min 46 sec.

Gel filtration chromatography

Protein extracts were prepared as described previously (Gusmaroli *et al.*, 2004). Frozen leaf material (three plants per sample) was homogenized in extraction buffer, centrifuged twice for 10 min each at 13 000 g at 4°C, and then filtered through 0.2 μm filters. Total proteins (1500–2000 μg) were loaded onto a Superose 6 10/300 GL column (GE Healthcare Bio-Sciences, <http://www.gehealthcare.com>) that had been equilibrated using equilibration buffer (50 mM Tris/HCl pH 7.5, 150 mM NaCl, 10 mM MgCl₂ and 2.5 mM EDTA). A total of 22 fractions of 0.5 ml were collected at a flow rate of 0.5 ml min⁻¹. The column was calibrated using gel filtration molecular weight standards (Sigma-Aldrich). Fractions corresponding to the column void volume (7 ml) were not subsequently analyzed. Other fractions were concentrated using 5 μl StrataClean resin (Agilent Technologies, <http://www.agilent.com>), and eluted in 5 μl of 3× SDS sample buffer. The entire sample volume and resin was subjected to immunoblotting.

ACKNOWLEDGEMENTS

We thank G. Martin (Boyce Thompson Institute for Plant Research, Ithaca, NY and Department of Plant Pathology, Cornell University,

Ithaca, NY) for Rio Grande seeds, G. Pruss for TMV inoculum and advice on TMV disease assays, P. Thompson, J. Jones, Q. Wang, D. Menasco and G. Horvath for providing equipment and advice on gel filtration, C. Bequette for searching the tomato genome for *CSN3* and *CSN4* genes, and W. Franklin III and R. Vogt for supplying *M. sexta* larvae. This research was supported by National Science Foundation grants IOB-0321453 and IOS-0745545 (to J.W.S.), by a grant from the University of South Carolina Magellan Scholar Program (to S.P.), and by a scholarship from the South Carolina Alliance for Minority Participation (SCAMP) (to N.S.J.).

SUPPORTING INFORMATION

Additional Supporting information may be found in the online version of this article:

Figure S1. Multiple sequence alignment of Arabidopsis and tomato CSN5 proteins.

Figure S2. Morphology of *CSN5*- and *CSN4*-VIGS plants.

Figure S3. *CSN4/5* protein levels in *CSN4/5*-VIGS plants.

Figure S4. Reduced expression of wound response genes and increased expression of *PR* genes in *CSN*-VIGS plants.

Figure S5. JA and SA levels in *CSN5*-VIGS MicroTom and *35S:PS* MicroTom plants.

Figure S6. *CSN5*-VIGS plants (Castlemart and MicroTom varieties) show increased susceptibility to herbivory.

Figure S7. Susceptibility to TMV is not altered in *CSN5*-VIGS plants.

Figure S8. *CSN5* transcript and protein levels do not change in response to herbivory-related stimuli.

Table S1. Sequences of primers used to clone *CSN5/4/3* VIGS constructs.

Table S2. Sequences of primers used for gene expression analysis.

Please note: As a service to our authors and readers, this journal provides supporting information supplied by the authors. Such materials are peer-reviewed and may be re-organized for online delivery, but are not copy-edited or typeset. Technical support issues arising from supporting information (other than missing files) should be addressed to the authors.

REFERENCES

- AbuQamar, S., Chai, M.-F., Luo, H., Song, F. and Mengiste, T. (2008) Tomato protein kinase 1b mediates signaling of plant responses to necrotrophic fungi and insect herbivory. *Plant Cell*, **20**, 1964–1983.
- Azevedo, C., Sadanandom, A., Kitagawa, K., Freialdenhoven, A., Shirasu, K. and Schulze-Lefert, P. (2002) The RAR1 interactor SGT1, an essential component of R gene-triggered disease resistance. *Science*, **295**, 2073–2076.
- Balbi, V. and Devoto, A. (2008) Jasmonate signalling network in *Arabidopsis thaliana*: crucial regulatory nodes and new physiological scenarios. *New Phytol.* **177**, 301–318.
- Brederode, F.T., Linthorst, H.J.M. and Bol, J.F. (1991) Differential induction of acquired resistance and PR gene expression in tobacco by virus infection, ethephon treatment, UV light and wounding. *Plant Mol. Biol.* **17**, 1117–1125.
- Browse, J. (2009) Jasmonate passes muster: a receptor and targets for the defense hormone. *Annu. Rev. Plant Biol.* **60**, 183–205.
- van den Burg, H.A., Tsitsigiannis, D.I., Rowland, O., Lo, J., Rallapalli, G., MacLean, D., Takken, F.L.W. and Jones, J.D.G. (2008) The F-box protein ACRE189/ACIF1 regulates cell death and defense responses activated during pathogen recognition in tobacco and tomato. *Plant Cell*, **20**, 697–719.
- Chamovitz, D.A. (2009) Revisiting the COP9 signalosome as a transcriptional regulator. *EMBO Rep.* **10**, 352–358.
- Chamovitz, D.A., Wei, N., Osterlund, M.T., von Arnim, 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.
- Chen, H., Wilkerson, C.G., Kuchar, J.A., Phinney, B.S. and Howe, G.A. (2005) Jasmonate-inducible plant enzymes degrade essential amino

- acids in the herbivore midgut. *Proc. Natl Acad. Sci. USA*, **102**, 19237–19242.
- Chen, H., Jones, A.D. and Howe, G.A. (2006) Constitutive activation of the jasmonate signaling pathway enhances the production of secondary metabolites in tomato. *FEBS Lett.* **580**, 2540–2546.
- Chini, A., Fonseca, S., Fernandez, G. et al. (2007) The JAZ family of repressors is the missing link in jasmonate signalling. *Nature*, **448**, 666–671.
- Chung, H.S. and Howe, G.A. (2009) A critical role for the TIFY motif in repression of jasmonate signaling by a stabilized splice variant of the JASMONATE ZIM-domain protein JAZ10 in Arabidopsis. *Plant Cell*, **21**, 131–145.
- Constabel, C.P., Bergery, D.R. and Ryan, C.A. (1995) Systemin activates synthesis of wound-inducible tomato leaf polyphenol oxidase via the octadecanoid defense signaling pathway. *Proc. Natl Acad. Sci. USA*, **92**, 407–411.
- Cope, G.A., Suh, G.S.B., Aravind, L., Schwarz, S.E., Zipursky, S.L., Koonin, E.V. and Deshaies, R.J. (2002) Role of predicted metalloprotease motif of Jab1/Csn5 in cleavage of Nedd8 from Cul1. *Science*, **298**, 608–611.
- Craig, A., Ewan, R., Mesmar, J., Gudipati, V. and Sadanandom, A. (2009) E3 ubiquitin ligases and plant innate immunity. *J. Exp. Bot.* **60**, 1123–1132.
- Devoto, A., Ellis, C., Magusin, A., Chang, H.S., Chilcott, C., Zhu, T. and Turner, J.G. (2005) Expression profiling reveals COI1 to be a key regulator of genes involved in wound- and methyl jasmonate-induced secondary metabolism, defence, and hormone interactions. *Plant Mol. Biol.* **58**, 497–513.
- Diaz, J., ten Have, A. and van Kan, J.A. (2002) The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. *Plant Physiol.* **129**, 1341–1351.
- Dohmann, E.M.N., Kuhnle, C. and Schwechheimer, C. (2005) Loss of the CONSTITUTIVE PHOTOMORPHOGENIC9 signalosome subunit 5 is sufficient to cause the *cop/det/fus* mutant phenotype in Arabidopsis. *Plant Cell*, **17**, 1967–1978.
- Dohmann, E.M., Levesque, M.P., Isono, E., Schmid, M. and Schwechheimer, C. (2008a) Auxin responses in mutants of the Arabidopsis CONSTITUTIVE PHOTOMORPHOGENIC9 signalosome. *Plant Physiol.* **147**, 1369–1379.
- Dohmann, E.M.N., Levesque, M.P., De Veylder, L., Reichardt, I., Jurgens, G., Schmid, M. and Schwechheimer, C. (2008b) The Arabidopsis COP9 signalosome is essential for G₂ phase progression and genomic stability. *Development*, **135**, 2013–2022.
- Dohmann, E.M., Nill, C. and Schwechheimer, C. (2010) DELLA proteins restrain germination and elongation growth in *Arabidopsis thaliana* COP9 signalosome mutants. *Eur. J. Cell Biol.* **89**, 163–168.
- Ekengren, S.K., Liu, Y., Schiff, M., Dinesh-Kumar, S.P. and Martin, G.B. (2003) Two MAPK cascades, NPR1, and TGA transcription factors play a role in Pto-mediated disease resistance in tomato. *Plant J.* **36**, 905–917.
- Farmer, E.E. and Dubugnon, L. (2009) Detritivorous crustaceans become herbivores on jasmonate-deficient plants. *Proc. Natl Acad. Sci. USA*, **106**, 935–940.
- Feng, S., Ma, L., Wang, X., Xie, D., Dinesh-Kumar, S.P., Wei, N. and Deng, X.W. (2003) The COP9 signalosome interacts physically with SCF^{COI1} and modulates jasmonate responses. *Plant Cell*, **15**, 1083–1094.
- Fowler, J.H., Narvaez-Vasquez, J., Aromdee, D.N., Pautot, V., Holzer, F.M. and Walling, L.L. (2009) Leucine aminopeptidase regulates defense and wound signaling in tomato downstream of jasmonic acid. *Plant Cell*, **21**, 1239–1251.
- Glauser, G., Grata, E., Dubugnon, L., Rudaz, S., Farmer, E.E. and Wolfender, J.-L. (2008) Spatial and temporal dynamics of jasmonate synthesis and accumulation in Arabidopsis in response to wounding. *J. Biol. Chem.* **283**, 16400–16407.
- Glazebrook, J. (2005) Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. *Annu. Rev. Phytopathol.* **43**, 205–227.
- Gou, M., Su, N., Zheng, J., Huai, J., Wu, G., Zhao, J., He, J., Tang, D., Yang, S. and Wang, G. (2009) An F-box gene, CPR30, functions as a negative regulator of the defense response in Arabidopsis. *Plant J.* **60**, 757–770.
- Gusmaroli, G., Feng, S. 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.
- Halitschke, R., Schittko, U., Pohnert, G., Boland, W. and Baldwin, I.T. (2001) Molecular interactions between the specialist herbivore *Manduca sexta* (Lepidoptera, Sphingidae) and its natural host *Nicotiana attenuata*. III. Fatty acid-amino acid conjugates in herbivore oral secretions are necessary and sufficient for herbivore-specific plant responses. *Plant Physiol.* **125**, 711–717.
- Harari-Steinberg, O. and Chamovitz, D.A. (2004) The COP9 signalosome: mediating between kinase signaling and protein degradation. *Curr. Protein Pept. Sci.* **5**, 185–189.
- Harari-Steinberg, O., Cantera, R., Denti, S., Bianchi, E., Oron, E., Segal, D. and Chamovitz, D.A. (2007) COP9 signalosome subunit 5 (CSN5/Jab1) regulates the development of the *Drosophila* immune system: effects on Cactus, Dorsal and hematopoiesis. *Genes Cells*, **12**, 183–195.
- Howe, G.A. and Jander, G. (2008) Plant immunity to insect herbivores. *Annu. Rev. Plant Biol.* **59**, 41–66.
- Hruz, T., Laule, O., Szabo, G., Wessendorp, F., Bleuler, S., Oertle, L., Widmayer, P., Gruissem, W. and Zimmermann, P. (2008) Genevestigator v3: a reference expression database for the meta-analysis of transcriptomes. *Adv. Bioinformatics*, **2008**, 420747.
- Kandath, P.K., Ranf, S., Pancholi, S.S., Jayanty, S., Walla, M.D., Miller, W., Howe, G.A., Lincoln, D.E. and Strattmann, J.W. (2007) Tomato MAPKs LeMPK1, LeMPK2, and LeMPK3 function in the systemin-mediated defense response against herbivorous insects. *Proc. Natl Acad. Sci. USA*, **104**, 12205–12210.
- Katsir, L., Schillmiller, 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.
- Kim, H.S. and Delaney, T.P. (2002) Arabidopsis SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. *Plant Cell*, **14**, 1469–1482.
- Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klässig, 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.
- Koornneef, A. and Pieterse, C.M. (2008) Cross talk in defense signaling. *Plant Physiol.* **146**, 839–844.
- Kwok, S.F., Solano, R., Tsuge, T., Chamovitz, D.A., Ecker, J.R., Matsui, M. and Deng, X.-W. (1998) Arabidopsis homologs of a c-Jun coactivator are present both in monomeric form and in the COP9 complex, and their abundance is differentially affected by the pleiotropic *cop/det/fus* mutations. *Plant Cell*, **10**, 1779–1790.
- Li, C., Liu, G., Xu, C., Lee, G.I., Bauer, P., Ling, H.-Q., Ganai, M.W. and Howe, G.A. (2003) The tomato *Suppressor of pro-systemin-mediated responses2* gene encodes a fatty acid desaturase required for the biosynthesis of jasmonic acid and the production of a systemic wound signal for defense gene expression. *Plant Cell*, **15**, 1646–1661.
- Li, L., Zhao, Y., McCaig, B.C., Wingerd, B.A., Wang, J., Whalon, M.E., Pichersky, E. and Howe, G.A. (2004) The tomato homolog of CORONATINE-INSENSITIVE1 is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. *Plant Cell*, **16**, 126–143.
- Li, C., Schillmiller, A.L., Liu, G. et al. (2005) Role of β -oxidation in jasmonate biosynthesis and systemic wound signaling in tomato. *Plant Cell*, **17**, 971–986.
- Liu, Y., Schiff, M., Marathe, R. and Dinesh-Kumar, S.P. (2002a) Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for N-mediated resistance to tobacco mosaic virus. *Plant J.* **30**, 415–429.
- Liu, Y., Schiff, M. and Dinesh-Kumar, S.P. (2002b) Virus-induced gene silencing in tomato. *Plant J.* **31**, 777–786.
- Liu, Y., Schiff, M., Serino, G., Deng, X.-W. and Dinesh-Kumar, S.P. (2002c) 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.
- Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsuge, T., Zhou, C., Wolf, D.A., Wei, N., Shevchenko, A. and Deshaies, R.J. (2001) Promotion of NEDD8-CUL1 conjugate cleavage by COP9 signalosome. *Science*, **292**, 1382–1385.
- Malec, P. and Chamovitz, D.A. (2006) Characterization and purification of kinase activities against Arabidopsis COP9 signalosome subunit 7. *Isr. J. Chem.* **46**, 239–246.

- Mayer, R., Raventos, D. and Chua, N.H. (1996) *det1*, *cop1*, and *cop9* mutations cause inappropriate expression of several gene sets. *Plant Cell*, **8**, 1951–1959.
- McConn, M., Creelman, R.A., Bell, E., Mullet, J.E. and Browse, J. (1997) Jasmonate is essential for insect defense in Arabidopsis. *Proc. Natl Acad. Sci. USA*, **94**, 5473–5477.
- Mengiste, T., Chen, X., Salmerson, J. and Dietrich, R. (2003) The *BOTRYTIS SUSCEPTIBLE1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in Arabidopsis. *Plant Cell*, **15**, 2551–2565.
- Mithofer, A., Wanner, G. and Boland, W. (2005) Effects of feeding *Spodoptera littoralis* on lima bean leaves. II. Continuous mechanical wounding resembling insect feeding is sufficient to elicit herbivory-related volatile emission. *Plant Physiol.* **137**, 1160–1168.
- Mur, L.A.J., Kenton, P., Atzorn, R., Miersch, O. and Wasternack, C. (2006) The outcomes of concentration-specific interactions between salicylate and jasmonate signaling include synergy, antagonism, and oxidative stress leading to cell death. *Plant Physiol.* **140**, 249–262.
- O'Donnell, P.J., Calvert, C., Atzorn, R., Wasternack, C., Leyser, H.M.O. and Bowles, D.J. (1996) Ethylene as a signal mediating the wound response of tomato plants. *Science*, **274**, 1914–1917.
- Paschold, A., Halitschke, R. and Baldwin, I.T. (2007) Co(i)-ordinating defenses: NaCOI1 mediates herbivore-induced resistance in *Nicotiana attenuata* and reveals the role of herbivore movement in avoiding defenses. *Plant J.* **51**, 79–91.
- Penninckx, I.A.M.A., Thomma, B.P.H.J., 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.
- Ratcliff, F., Martin-Hernandez, A.M. and Baulcombe, D.C. (2001) Tobacco rattle virus as a vector for analysis of gene function by silencing. *Plant J.* **25**, 237–245.
- Ryan, C.A. (2000) The systemin signaling pathway: differential activation of plant defensive genes. *Biochim. Biophys. Acta*, **1477**, 112–121.
- Ryan, C.A. and Pearce, G. (2003) Systemins: a functionally defined family of peptide signals that regulate defensive genes in Solanaceae species. *Proc. Natl Acad. Sci. USA*, **100**, 14577–14580.
- Schaller, A., Philippe, R. and Amrhein, N. (2000) Salicylic acid-independent induction of pathogenesis-related gene expression by fusicoccin. *Planta*, **210**, 599–606.
- Schmelz, E.A., Engelberth, J., Tumlinson, J.H., Block, A. and Alborn, H.T. (2004) The use of vapor phase extraction in metabolic profiling of phytohormones and other metabolites. *Plant J.* **39**, 790–808.
- Schmelz, E.A., Engelberth, J., Alborn, H.T., Tumlinson, J.H. and Teal, P.E.A. (2009) Phytohormone-based activity mapping of insect herbivore-produced elicitors. *Proc. Natl Acad. Sci. USA*, **106**, 653–657.
- Schwager, K.M., Calderon-Villalobos, L.I.A., Dohmann, E.M.N., Willige, B.C., Knierer, S., Nill, C. and Schwachheimer, C. (2007) Characterization of the *VIER F-BOX PROTEINE* genes from Arabidopsis reveals their importance for plant growth and development. *Plant Cell*, **19**, 1163–1178.
- Schwachheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M. and Deng, X.W. (2001) Interactions of the COP9 signalosome with the E3 ubiquitin ligase SCF^{TIR1} in mediating auxin response. *Science*, **292**, 1379–1382.
- Schwachheimer, C., Serino, G. and Deng, X.-W. (2002) Multiple ubiquitin ligase-mediated processes require COP9 signalosome and AXR1 function. *Plant Cell*, **14**, 2553–2563.
- Serino, G., Tsuge, T., Kwok, S., Matsui, M., Wei, N. and Deng, X.-W. (1999) Arabidopsis *cop8* and *fus4* mutations define the same gene that encodes subunit 4 of the COP9 signalosome. *Plant Cell*, **11**, 1967–1980.
- Spoel, S.H., Johnson, J.S. and Dong, X. (2007) Regulation of tradeoffs between plant defenses against pathogens with different lifestyles. *Proc. Natl Acad. Sci. USA*, **104**, 18842–18847.
- Stenzel, I., Hause, B., Maucher, H., Pitzschke, A., Miersch, O., Ziegler, J., Ryan, C.A. and Wasternack, C. (2003) Allene oxide cyclase dependence of the wound response and vascular bundle-specific generation of jasmonates in tomato – amplification in wound signalling. *Plant J.* **33**, 577–589.
- Stone, S.L., Anderson, E.M., Mullen, R.T. and Goring, D.R. (2003) ARC1 is an E3 ubiquitin ligase and promotes the ubiquitination of proteins during the rejection of self-incompatible Brassica pollen. *Plant Cell*, **15**, 885–898.
- Stuttmann, J., Lechner, E., Guerois, R., Parker, J.E., Nussaume, L., Genschik, P. and Noel, L.D. (2009) COP9 signalosome- and 26S proteasome-dependent regulation of SCF^{TIR1} accumulation in Arabidopsis. *J. Biol. Chem.* **284**, 7920–7930.
- Thines, B., Katsir, L., Melotto, M., Niu, Y., Mandaokar, A., Liu, G., Nomura, K., He, S.Y., Howe, G.A. and Browse, J. (2007) JAZ repressor proteins are targets of the SCF^{COI1} complex during jasmonate signalling. *Nature*, **448**, 661–665.
- Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A. and Broekaert, W.F. (1998) Separate jasmonate-dependent and salicylate-dependent defense-response pathways in Arabidopsis are essential for resistance to distinct microbial pathogens. *Proc. Natl Acad. Sci. USA*, **95**, 15107–15111.
- Van Loon, L.C. and Van Strien, E.A. (1999) The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. *Physiol. Mol. Plant Pathol.* **55**, 85–97.
- Walling, L.L. (2000) The myriad plant responses to herbivores. *J. Plant Growth Regul.* **19**, 195–216.
- Wang, X., Feng, S., Nakayama, N., Crosby, W.L., Irish, V., Deng, X.W. and Wei, N. (2003) The COP9 signalosome interacts with SCF^{UFO} and participates in Arabidopsis flower development. *Plant Cell*, **15**, 1071–1082.
- Wang, X., Li, W., Piqueras, R., Cao, K., Deng, X.W. and Wei, N. (2009) Regulation of COP1 nuclear localization by the COP9 signalosome via direct interaction with CSN1. *Plant J.* **58**, 655–667.
- Wee, S., Geyer, R.K., Toda, T. and Wolf, D.A. (2005) CSN facilitates Cullin-RING ubiquitin ligase function by counteracting autocatalytic adapter instability. *Nat. Cell Biol.* **7**, 387–391.
- Wei, N. and Deng, X.W. (2003) The COP9 signalosome. *Annu. Rev. Cell Dev. Biol.* **19**, 261–286.
- Wei, N., Serino, G. and Deng, X.W. (2008) The COP9 signalosome: more than a protease. *Trends Biochem. Sci.* **33**, 592–600.
- Wu, J., Hettenhausen, C., Meldau, S. and Baldwin, I.T. (2007) Herbivory rapidly activates MAPK signaling in attacked and unattacked leaf regions but not between leaves of *Nicotiana attenuata*. *Plant Cell*, **19**, 1096–1122.
- Xu, P., Blancaflor, E.B. and Roossinck, M.J. (2003) In spite of induced multiple defense responses, tomato plants infected with Cucumber mosaic virus and D satellite RNA succumb to systemic necrosis. *Mol. Plant Microbe Interact.* **16**, 467–476.
- Yan, J., Zhang, C., Gu, M. et al. (2009) The Arabidopsis CORONATINE INSENSITIVE1 protein is a jasmonate receptor. *Plant Cell*, **21**, 2220–2236.
- Zhao, Y., Thilmony, R., Bender, C.L., Schaller, A., He, S.Y. and Howe, G.A. (2003) Virulence systems of *Pseudomonas syringae* pv. *tomato* promote bacterial speck disease in tomato by targeting the jasmonate signaling pathway. *Plant J.* **36**, 485–499.