

Role of SCF Ubiquitin-Ligase and the COP9 Signalosome in the *N* Gene–Mediated Resistance Response to *Tobacco mosaic virus*

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The tobacco *N* gene confers resistance to *Tobacco mosaic virus* (TMV) and encodes a toll–interleukin-1 receptor/nucleotide binding/Leu-rich repeat class protein. Recent evidence indicates that the *Nicotiana benthamiana Rar1* gene (*NbRar1*), which encodes a protein with a zinc finger motif called CHORD (Cys- and His-rich domain), is required for the function of *N*. To investigate the role of *NbRar1* in plant defense, we identified its interaction partners. We show that the *NbRar1* protein interacts with *NbSGT1*, a highly conserved component of the SCF (Skp1/Cullin/F-box protein)-type E3 ubiquitin ligase complex involved in protein degradation. In addition, we show that *NbSGT1* interacts with *NbSKP1*. Suppression of *NbSGT1* and *NbSKP1* shows that these genes play an important role in the *N*-mediated resistance response to TMV. Both *NbRar1* and *NbSGT1* associate with the COP9 signalosome, another multiprotein complex involved in protein degradation via the ubiquitin-proteasome pathway. Silencing of the *NbCOP9* signalosome also compromises *N*-mediated resistance to TMV. Our results reveal new roles for SCF and the COP9 signalosome in plant defense signaling.

INTRODUCTION

Plants mount various defense responses to survive the challenge of pathogen attack. One such response involves recognition of pathogen-encoded ligands by plant disease resistance (*R*) gene products (Gabriel and Rolfe, 1990). This recognition event initiates signaling pathways that lead to hypersensitive response (localized cell death) at the site of pathogen ingress, rapid oxidative burst, cell wall strengthening, protein phosphorylation, and activation of various defense response genes (McDowell and Dangl, 2000). These events are followed by a nonspecific general defense response throughout the plant called systemic acquired resistance (Ryals et al., 1996). During systemic acquired resistance, salicylic acid levels increase throughout the plant, defense genes such as pathogenesis-related genes are expressed, and the plant becomes increasingly resistant to further pathogen attack. Evidence suggests that in addition to salicylic acid, jasmonic acid and ethylene play roles in plant defense (Thomma et al., 2001).

In the last decade, the cloning of a number of plant *R* genes that confer resistance to various pathogens has brought significant progress in our understanding of host–

pathogen interactions (Dangl and Jones, 2001). Different classes of cloned *R* proteins contain similar structural features. Members of the most prominent class of *R* genes, the NB-LRRs, contain a nucleotide binding domain (NB) and C-terminal Leu-rich repeats (LRRs) of various lengths. The NB-LRR class of *R* genes can be further classified into toll–interleukin-1 receptor (TIR)–NB-LRR or coiled-coil (CC)–NB-LRR based on their N-terminal sequences. Members of the TIR–NB-LRR class of proteins contain an N-terminal domain that is similar to the cytoplasmic domains of Toll, interleukin-1 receptor (TIR domain), and Myd88 (Rock et al., 1998). The CC–NB-LRR proteins contain a CC domain at the N terminus. Other classes of *R* genes contain a kinase and/or an LRR domain.

The conserved domains of *R* proteins from different plant species suggest that activation of common signaling pathways occurs upon pathogen perception (Baker et al., 1997). For example, the functional TIR–NB-LRR class of *R* genes requires *EDS1*, and a subset of the CC–NB-LRR class of *R* genes requires *NDR1*, for resistance to pathogens (Aarts et al., 1998). Another example of a converging point in disease resistance is the requirement for the *Rar1* gene in resistance to powdery mildew by several unlinked CC–NB-LRR genes (Schulze-Lefert and Vogel, 2000). Recent evidence suggests that *Nicotiana benthamiana Rar1* (*NbRar1*) also is an essential component of the *N* gene–mediated resistance response to *Tobacco mosaic virus* (TMV) (Liu et al., 2002). This is

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interesting because the *N* gene belongs to the TIR-NBS-LRR class of *R* genes (Whitham et al., 1994). Thus, *Rar1* may represent an example of a signaling component involved in pathways shared by both the CC-NB-LRR and the TIR-NB-LRR resistance gene classes.

Genetic studies in barley suggest that *Rar1* functions downstream of pathogen perception and upstream of H₂O₂ accumulation and host cell death (Shirasu et al., 1999). *Rar1* encodes a protein with a novel zinc finger motif called CHORD (Cys- and His-rich domain) that is present in all eukaryotes except yeast. *Rar1* lacks a CS domain that is found in metazoan CHORD proteins. The CS domain shares homology with yeast SGT1 and human SIP, both of which are novel subunits of a multiprotein ubiquitin ligase (E3) complex (the SCF [Skp/Cullin/F-box] complex) (Kitagawa et al., 1999; Matsuzawa and Reed, 2001). This complex mediates the degradation of multiple proteins involved in diverse signaling pathways through a ubiquitin-proteasome pathway (Deshaies, 1999). Different components of the SCF also have been shown to interact with the COP9 signalosome (Lyapina et al., 2001), a multiprotein complex involved in protein degradation through the 26S proteasome (Wei and Deng, 1999).

Shirasu et al. (1999) hypothesized that SGT1-like proteins in plants that contain a CS domain might provide a link between *Rar1* and the SCF complex and to the protein degradation pathway. To understand the role and regulation of *NbRar1* in protein degradation and plant defense mechanisms, we used a yeast two-hybrid screen to identify its interactors. We report here that *NbRar1* interacts directly with *NbSGT1* of the SCF ubiquitin ligase complex. In turn, *NbSGT1* interacts with *NbSKP1*. Both *NbRar1* and *NbSGT1* associate with the COP9 signalosome. The *N* gene plants that are knocked out for the expression of *NbSGT1*, *NbSKP1*, and the COP9 signalosome are compromised in their resistance response to TMV. These findings are consistent with the most recent work published by Azevedo et al. (2002) and Austin et al. (2002).

RESULTS

Identification of *NbRar1*-Interacting Proteins

Previous studies in our laboratory suggested that *NbRar1* is an essential component of *N*-mediated signaling (Liu et al., 2002). To identify proteins that interact with *NbRar1*, we conducted a yeast two-hybrid screen (Kolonin et al., 2000). The *NbRar1* sequence was fused to a nuclear localization signal sequence (NLS) and a LexA DNA binding domain (NLS-LexA-BD) under the control of a Gal-inducible promoter (GAL10). This construct was used as the bait to screen a tomato cDNA library that was fused to the B42 activation domain (AD) under the control of a Gal-inducible promoter (GAL1).

We identified 24 *Leu* prototrophs that showed activation of the *LacZ* reporter gene. The cDNA inserts of these 24 positive clones were sequenced. A database search for homology using the BLAST program suggested three classes of *NbRar1* interactors: SGT1-like (7 clones), *hsp90* (13 clones), and OTU-like Cys protease (4 clones).

We focused specifically on the *NbRar1* interactors that showed significant homology with the SGT1 proteins found in yeast, human, Arabidopsis, and barley. SGT1 in yeast is a novel subunit of the SCF complex that is involved in kinetochore assembly (Kitagawa et al., 1999). In human, an SGT1 homolog called SIP (Siah-interacting protein) complements defects in yeast strains that contain SGT1 mutant alleles (Kitagawa et al., 1999; Matsuzawa and Reed, 2001). SIP interacts with the SCF complex and controls β -catenin levels, which affect the activity of β -catenin-dependent Tcf/LEF transcription factors (Matsuzawa and Reed, 2001). The longest SGT1 cDNA clone we obtained from the yeast two-hybrid screen contained only 237 amino acids corresponding to the C-terminal region.

We cloned the full-length cDNA of *SGT1* from *N. benthamiana* using reverse transcriptase-mediated (RT) PCR. The predicted *N. benthamiana* SGT1 protein (*NbSGT1*) encodes an open reading frame of 370 amino acids and shows substantial homology with SGT1 proteins from different organisms (Figure 1). The *NbSGT1* protein shares 90% identity and 95% similarity with tomato SGT1. In addition, it shares homology with barley SGT1 (61% identity and 75% similarity) (Azevedo et al., 2002) and Arabidopsis SGT1a and SGT1b (60% identity and 70% similarity) (Austin et al., 2002). It also shares homology with human SGT1 (44% identity and 63% similarity), human SIP (20% identity and 31% similarity), and yeast SGT1 (27% identity and 44% similarity) (Kitagawa et al., 1999; Matsuzawa and Reed, 2001).

NbSGT1 Interacts Directly with *NbRar1*

Specific interaction between full-length *NbRar1* and *NbSGT1* proteins was confirmed using yeast two-hybrid, in vitro pull-down, and in planta interaction assays. Yeast colonies containing both LexA-BD-*NbRar1* and AD-*NbSGT1* grew on *Leu*⁻ plates containing Gal (Figure 2A, top) and turned dark blue on 5-bromo-4-chloro-3-indolyl β -D-galactoside plates (Figure 2A, third from top). Yeast colonies containing both LexA-BD-*NbRar1* and AD-*NbSGT1* did not grow on *Leu*⁻ plates containing Glc (Figure 2A, second from top) and were white on *Leu*⁺ plates containing Glc and 5-bromo-4-chloro-3-indolyl β -D-galactoside (Figure 2A, bottom). Furthermore, no interaction was observed between LexA-BD-*NbRar1* and control bait pLexA-p53 (Figure 2A). These results suggest that the expression of both reporter genes (*Leu* and *LacZ*) is dependent on the interaction and the Gal-inducible expression of *NbRar1* and *NbSGT1*.

NbRar1 interaction with *NbSGT1* was further confirmed by in vitro binding assays. *NbRar1* and *NbSGT1* were ex-

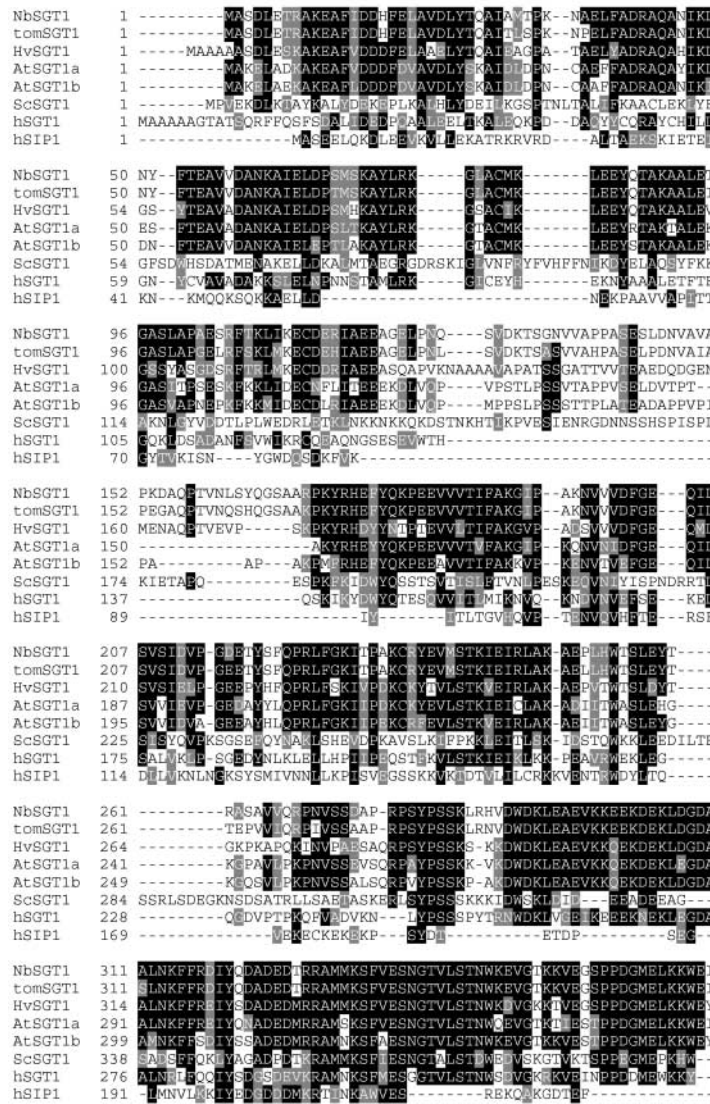


Figure 1. Comparison of the *N. benthamiana* SGT1 Amino Acid Sequence with Those of Its Homologs from Other Organisms.

Alignment of the predicted *N. benthamiana* SGT1 (NbSGT1) protein with its homologs from tomato (tomSGT1), barley (HvSGT1), Arabidopsis (AtSGT1a and AtSGT1b), yeast (ScSGT1), and human (hSGT1 and hSIP1). Numbers at left indicate the positions of the amino acid residues. Identical residues are shaded in black, and similar residues are shaded in gray.

pressed in *Escherichia coli* as glutathione S-transferase (GST) fusion proteins and purified using glutathione-Sepharose beads. The identity of these fusion proteins was confirmed using a GST antibody (data not shown). Purified GST-NbSGT1 and GST-NbRar1 fusion proteins were mixed with 35S-radiolabeled NbRar1 or NbSGT1 translated in vitro. Analysis of the bound fraction by SDS-PAGE and autoradiography showed that GST-NbSGT1 bound to NbRar1 (Figure 2B) and GST-NbRar1 bound to NbSGT1 (Figure 2C).

Neither NbRar1 nor NbSGT1 protein translated in vitro interacted with the GST control alone (Figures 2B and 2C).

We performed in planta interaction assays using a TMV-based viral expression system to further confirm the interaction between NbRar1 and NbSGT1. Hemagglutinin (HA)-tagged NbSGT1 and FLAG-tagged NbRar1 were expressed alone or together by transfecting *N. benthamiana* leaves with TMV-based viral vectors. Immunoblot analysis of total protein extracts from the leaves transfected with HA-tagged

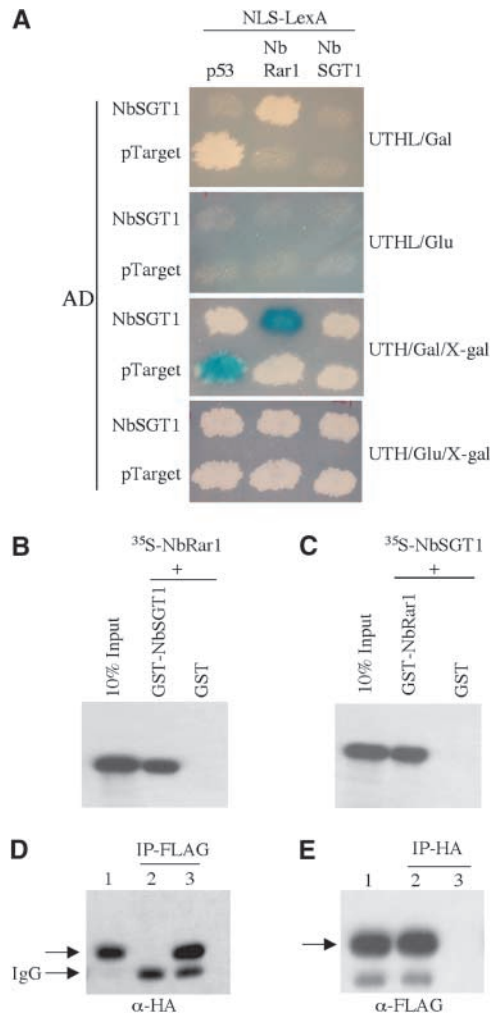


Figure 2. Analysis of NbRar1 Interaction with NbSGT1.

(A) Growth of yeast strains containing NLS-LexA-p53 (control) or NLS-LexA-NbRar1 or NLS-LexA-NbSGT1 baits transformed with AD-NbSGT1 or AD-pTarget (control) on Leu-deficient medium containing Gal or Glc after 3 days at 30°C (top and second from top). Induction of *LacZ* expression was monitored on X-Gal-containing medium with Gal or Glc after 3 days at 30°C (third from top and bottom).

(B) One microgram of GST-NbSGT1 or GST was incubated with ³⁵S-labeled NbRar1. Beads were washed and analyzed by SDS-PAGE and fluorography. As a control, 10% of input ³⁵S-labeled NbRar1 was loaded directly on the same gel.

(C) One microgram of GST-NbRar1 or GST was incubated with ³⁵S-labeled NbSGT1 and analyzed as in **(B)**. As a control, 10% of input ³⁵S-labeled NbSGT1 was loaded directly on the same gel.

(D) *N. benthamiana* plants were transfected with TMV expression vectors, producing FLAG-tagged NbRar1 alone (lane 2) or together with HA-tagged NbSGT1 (lane 3). Total cell extracts were normalized for protein quantity, and immunoprecipitation performed using anti-FLAG antibody-conjugated Sepharose beads. After washing, immune complexes were analyzed by SDS-PAGE followed by immunoblot analysis using an anti-HA antibody. As a control, input total

NbSGT1 or FLAG-tagged NbRar1 detected single protein bands of the expected size for NbSGT1 (Figure 2D, lane 1) and NbRar1 (Figure 2E, lane 1), respectively.

Immunoprecipitation of protein extracts derived from cotransfected leaves using FLAG monoclonal antibody followed by immunoblot analysis with anti-HA antibody detected NbSGT1 (Figure 2D, lane 3). This finding demonstrates that NbSGT1 and NbRar1 proteins interact in plant cells. In a reciprocal experiment, the HA antibody immunoprecipitated NbRar1 (Figure 2E, lane 2). This interaction is specific because control immunoprecipitation experiments performed using tissue transfected with TMV vector carrying FLAG-tagged NbRar1 alone (Figure 2D, lane 2) and TMV vector carrying HA-tagged NbSGT1 alone (Figure 2E, lane 3) failed to detect any protein bands.

NbSGT1 Interacts Directly with the SCF Component NbSKP1

In yeast and human, SGT1 interacts with SKP1, a component of the SCF-type E3 ubiquitin ligase complex (Kitagawa et al., 1999; Matsuzawa and Reed, 2001). Therefore, we performed yeast two-hybrid library screening to search for NbSGT1-interacting partners. Our two-hybrid screen yielded 20 NbSGT1-interacting partners: 14 were homologs of Rar1, and 6 clones encoded an unknown protein. Because our screen failed to identify SKP1, we tested direct interaction between SKP1 and NbSGT1.

We cloned *NbSKP1*, which encodes a protein of 153 amino acids and shares substantial homology with Arabidopsis SKP1/ASK1 (74% similarity and 83% identity) and ASK2 (67% identity and 79% similarity) (Figure 3). Homology with other Arabidopsis SKP1-like proteins ranges from 35 to 57% identity and 52 to 71% similarity. *NbSKP1* bears homology with human SKP1 (58% identity and 71% similarity) and yeast SKP1 (46% identity and 67% similarity).

NbSKP1 interacted specifically with NbSGT1 in a yeast two-hybrid assay (Figure 4A). This interaction was confirmed by in vitro binding assays using ³⁵S-labeled NbSKP1

tal cell extract containing HA-tagged NbSGT1 was loaded directly on the same gel (lane 1). IgG corresponds to the light IgG chain of the anti-FLAG antibody.

(E) *N. benthamiana* plants were transfected with TMV expression vectors producing HA-tagged NbSGT1 alone (lane 3) or together with FLAG-tagged NbRar1 (lane 2). The immunoprecipitation of total cell extracts using anti-HA antibody-conjugated Sepharose beads followed by immunoblot analysis using an anti-FLAG antibody was performed as described for **(D)**. As a control, input total cell extract containing FLAG-tagged NbRar1 was loaded directly on the same gel (lane 1).



Figure 3. Comparison of the *N. benthamiana* SKP1 Amino Acid Sequence with Those of Its Homologs from Other Organisms.

Alignment of the predicted *N. benthamiana* SKP1 (NbSKP1) protein with its homologs from Arabidopsis (AtSKP1 and AtSKP2), yeast (ScSKP1), and human (hSKP1). Numbers at left indicate the positions of the amino acid residues. Identical residues are shaded in black, and similar residues are shaded in gray.

and purified GST-NbSGT1 fusion protein (Figure 4B). These results show that NbSGT1 interacts directly with the SCF protein complex component NbSKP1, as its counterparts do in yeast and human.

We performed in planta interaction assays using a TMV-based viral expression system to further confirm the interaction between NbSKP1 and NbSGT1 or NbRar1. Myc-tagged NbSKP1 and HA-tagged NbSGT1 or FLAG-tagged NbRar1 were coexpressed by transfecting *N. benthamiana* leaves. Immunoprecipitation of protein extracts derived from cotransfected leaves using anti-Myc antibody followed by immunoblot analysis with anti-HA or anti-FLAG antibodies detected NbSGT1 (Figure 4C, top, lane 2) and NbRar1 (Figure 4C, bottom, lane 3), respectively. Control immunoprecipitation experiments performed using tissue transfected with TMV vector carrying Myc tag alone failed to detect any protein bands (Figure 4C, top and bottom, lane 1). These results suggest that NbSKP1 exists in a complex with NbSGT1 and NbRar1 in planta.

NbRar1 and NbSGT1 Interact with the Components of SCF in Vivo

SGT1 associates with the SCF-type E3 ubiquitin ligase complex proteins SKP1 and Cdc53p (a homolog of Cullin1) in yeast (Kitagawa et al., 1999). This prompted us to investigate whether NbRar1 and NbSGT1 associate with components of the SCF ubiquitin ligase complex in planta. We explored the hypothesis that Cullin1 (CUL1) and NbSKP1 coimmunoprecipitate with NbRar1 and NbSGT1. For this purpose, we used Arabidopsis Cullin1 (AtCUL1) and AtSKP1

antibodies because they immunoreact with *N. benthamiana* Cullin1 (NbCUL1) (Figure 5A, lane 2) and NbSKP1 (Figure 5B, lane 2), respectively.

First, we transfected FLAG-tagged Rar1 and HA-tagged SGT1 into *N. benthamiana* leaves using a TMV-based viral expression vector. Total protein extracts derived from these transfected leaves were immunoprecipitated using anti-FLAG or anti-HA antibodies linked covalently to agarose beads and probed with anti-AtCUL1 or anti-AtSKP1 antibodies. Our results suggest that NbRar1 and NbSGT1 exist in a complex with NbCUL1 (Figure 5A, lanes 4 and 5) and NbSKP1 (Figure 5B, lanes 4 and 5). Control immunoprecipitations with TMV vector carrying FLAG tag alone did not detect NbCUL1 (Figure 5A, lane 3) or NbSKP1 (Figure 5B, lane 3).

NbRar1 and NbSGT1 Interact with the COP9 Signalosome in Vivo

Recently, the Cullin1 subunit of the SCF complex has been shown to interact with the COP9 signalosome (CSN) (Lyapina et al., 2001). The COP9 signalosome was discovered originally in Arabidopsis as a suppressor of photomorphogenesis (Wei and Deng, 1999). The eukaryotic COP9 signalosome is a multiprotein complex with eight distinct subunits (CSN1 to CSN8) that is involved in protein degradation via the ubiquitin proteasome pathway (Wei and Deng, 1999). We tested NbRar1 and NbSGT1 interaction with all eight subunits of the COP9 signalosome using the yeast two-hybrid system. None of the individual subunits interacted with NbRar1 or NbSGT1 (data not shown).

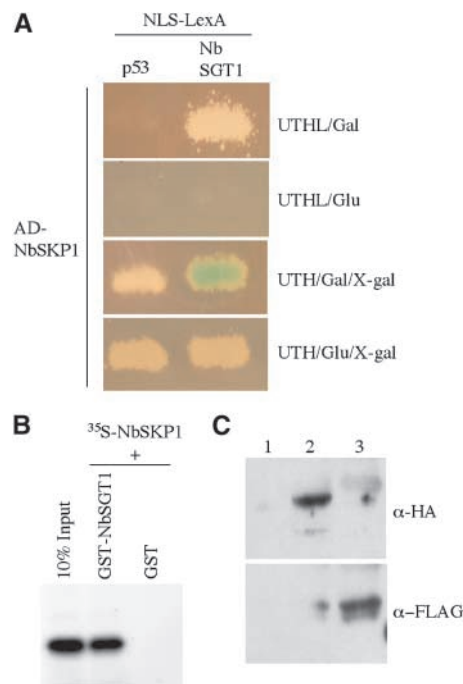


Figure 4. Analysis of NbSGT1 Interaction with NbSKP1.

(A) Yeast strains containing NLS-LexA-NbRar1 or NLS-LexA-p53 (control) bait were transformed with AD-NbSKP1. Growth on Leu-deficient medium containing Gal or Glc was examined for 3 days at room temperature (top and second from top). Induction of *LacZ* expression was monitored on X-Gal-containing medium with Gal or Glc for 3 days at room temperature (third from top and bottom).

(B) One microgram of GST-NbSGT1 or GST was incubated with ³⁵S-labeled NbSKP1. Beads were washed and analyzed by SDS-PAGE and fluorography. As a control, 10% of input ³⁵S-labeled NbSKP1 was loaded directly on the same gel.

(C) *N. benthamiana* plants were transfected with TMV expression vector carrying Myc tag alone (lane 1), TMV expression vector producing Myc-tagged NbSKP1 and HA-tagged NbSGT1 (lane 2), or TMV expression vector producing Myc-tagged NbSKP1 and FLAG-tagged NbRar1 (lane 3). The immunoprecipitation of total cell extracts using anti-Myc antibody-conjugated Sepharose beads followed by immunoblot analysis using anti-HA (top) and anti-FLAG (bottom) antibodies was performed as described for Figure 2D.

We performed immunoprecipitation assays to investigate whether NbRar1 and NbSGT1 form a complex with the COP9 signalosome in vivo. Immunoblot analysis of total protein extract derived from *N. benthamiana* leaves detected NbCSN4 using AtCSN4 antibodies (Figure 5C, lanes 1 and 2). We probed the immunocomplex of NbRar1 and NbSGT1 with anti-AtCSN4 antibodies and observed the association of NbCSN4 with NbRar1 and NbSGT1 (Figure 5C, lanes 4 and 5). Control immunoprecipitation with TMV

vector carrying the FLAG tag alone did not detect NbCSN4 (Figure 5C, lane 3). These results suggest that NbRar1 and NbSGT1 associate with the COP9 signalosome because the CSN4 subunit is always present in the COP9 multiprotein complex.

Suppression of *NbSGT1* and *NbSKP1* Leads to the Loss of *N*-Mediated Resistance to TMV

We used a *Tobacco rattle virus* (TRV)-based virus-induced gene silencing (VIGS) technique as a gene knockout system to examine the biological role of *NbSGT1* and *NbSKP1* in *N*-mediated resistance to TMV (Ratcliff et al., 2001; Liu et al., 2002). VIGS is initiated when a plant is infected with recombinant TRV carrying an insert homologous with a host gene. Endogenous gene transcripts homologous with the VIGS vector insert are degraded by a post-transcriptional gene silencing mechanism or by cosuppression (Baulcombe, 1999). VIGS is a rapid, transient assay with the phenotype of the silenced plant mimicking a loss-of-function phenotype.

We performed VIGS to silence *NbSGT1* and *NbSKP1* genes in transgenic *N. benthamiana* lines containing the tobacco *N* gene (referred to as *NN*). Transgenic *NN* plants confer resistance to TMV, as well as to recombinant TMV carrying the green fluorescent protein (TMV-GFP), by inducing the hypersensitive response at the site of infection and containing virus at the infection site (Liu et al., 2002). To suppress *NbSGT1* and *NbSKP1* using TRV-VIGS, we cloned fragments of these genes into pTRV2 (RNA2 cDNA of TRV) as described in Methods. Mixtures of *Agrobacterium tumefaciens* cultures containing pTRV1 (RNA1 cDNA of TRV) with pTRV2, pTRV2-*N*, pTRV2-*NbSGT1*, or pTRV2-*NbSKP1* T-DNAs were infiltrated onto *NN* plants at the four-leaf stage.

Ten days after infiltration, the upper leaves of these plants were infected with TMV-GFP. These plants were visualized under UV light for at least 20 days to observe whether TMV-GFP spread from the infection site. In wild-type *N. benthamiana* plants (referred to as *nn*) infected with TRV alone, TMV-GFP spread to the upper leaves (Figure 6A). In *NN* plants, TMV-GFP was restricted to the infection site (Figure 6B). Interestingly, in *NbSGT1*- and *NbSKP1*-suppressed *NN* plants, TMV-GFP spread into the upper leaves (Figures 6D and 6E). Loss of resistance to TMV-GFP in these plants was similar to that observed in *NN* plants suppressed for the *N* transgene (Figure 6C). Therefore, the phenotypic data of the VIGS assays suggest that the *NbSGT1* and *NbSKP1* genes are required for *N* gene-mediated TMV resistance.

We confirmed the presence of TMV-GFP RNA in the upper uninfected leaves of *N*-, *NbSGT1*-, and *NbSKP1*-silenced plants by RT-PCR analysis using primers that anneal to the TMV movement protein gene (Figure 7A). No TMV-RNA was detected in *NN* control plants infected with TRV alone (Figure 7A).

We performed semiquantitative RT-PCR to examine the effects of VIGS-induced suppression on the endogenous

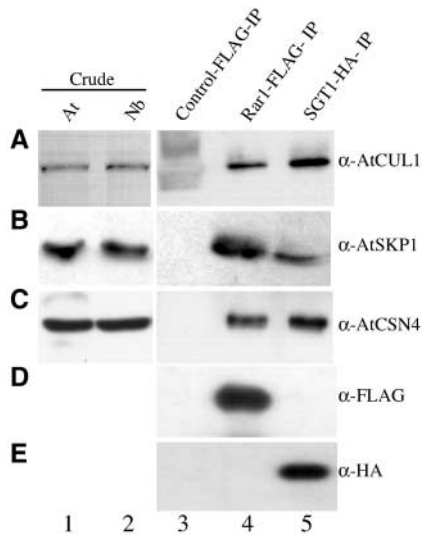


Figure 5. NbRar1 and NbSGT1 Association with SCF and the COP9 Signalosome.

N. benthamiana plants were transfected with empty TMV expression vector carrying FLAG tag alone (lane 3), TMV expression vector producing FLAG-tagged NbRar1 (lane 4), or TMV expression vector producing HA-tagged NbSGT1 (lane 5). Total cell extracts were normalized for protein quantity, and immunoprecipitation was performed using anti-FLAG or anti-HA antibody-conjugated Sepharose beads. After washing, immune complexes were eluted using FLAG or HA peptides. The resulting complex was analyzed by SDS-PAGE followed by immunoblot analysis using anti-AtCUL1 (A), anti-AtSKP1 (B), anti-AtCSN4 (C), anti-FLAG (D), and anti-HA (E) antibodies. As a control, whole cell extracts from Arabidopsis (At; lane 1) and *N. benthamiana* (Nb; lane 2) plants were analyzed by immunoblotting.

amounts of mRNA for the *NbSGT1* and *NbSKP1* genes. In *NbSGT1*- and *NbSKP1*-silenced plants, the *SGT1* and *SKP1* messages were reduced by >82 and 71%, respectively, compared with the TRV-infected control (Figures 7C and 7D). However, EF1 α RNA levels were similar (Figure 7B, TRV-*NbSGT1*) and served as an internal control for RNA quality and RT-PCR amplification.

Suppression of the COP9 Signalosome Leads to the Loss of *N*-Mediated Resistance to TMV

We used the VIGS assay described above to examine the biological role of the COP9 signalosome in *N*-mediated resistance to TMV. Suppression of the *NbCSN3* and *NbCSN8* genes in *NN* plants resulted in the spread of TMV-GFP to the upper leaves (Figures 8A and 8B). We confirmed the presence of TMV-GFP RNA in these upper leaves (Figure 8C). RT-PCR analysis found 79% reduction of *NbCSN3* and 72% reduction of *NbCSN8* endogenous mRNA levels (Figure 8D).

Studies from COP9 signalosome mutants suggest that the

loss of one subunit of the CSN results in the loss of the entire protein complex (Schwechheimer et al., 2001). We tested the level of the COP9 signalosome subunits CSN8 and CSN4 in *NbCSN8*-silenced plants. Total protein was extracted from *NbCSN8*-silenced and nonsilenced *NN* plants, and immunoblot analyses using anti-AtCSN4 and anti-AtCSN8 antibodies were performed. We observed ~75 and 70% reduction of NbCSN8 and NbCSN4 subunit levels, respectively, in *NbCSN8*-silenced plants compared with nonsilenced *NN* plants (Figure 8E). Together, our phenotypic and molecular data suggest that suppression of the COP9 signalosome compromises the *N*-mediated resistance response to TMV.

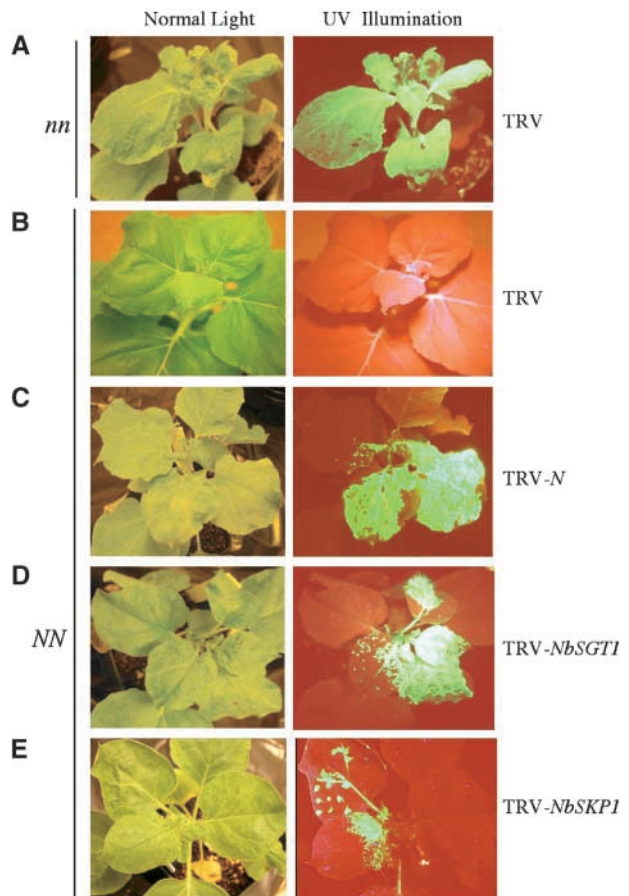


Figure 6. Analysis of the Effect of *NbSGT1* and *NbSKP1* Suppression on *N* Gene-Mediated Resistance to TMV.

Wild-type *nn* (A) and transgenic *NN* ([B] to [E]) *N. benthamiana* plants were first silenced for *N* (C), *NbSGT1* (D), and *NbSKP1* (E) at the four-leaf stage. (A) and (B) show nonsilenced controls. The upper leaves of these plants then were infected with TMV-GFP virus to monitor resistance or susceptibility responses. The spread of TMV-GFP from the inoculated leaf into the upper uninoculated leaves indicates loss of resistance to TMV.

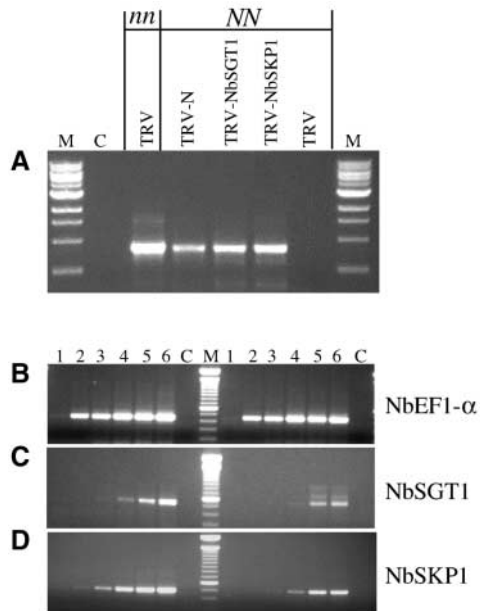


Figure 7. Molecular Analysis of *NbSGT1* and *NbSKP1* Suppression.

(A) RT-PCR results for the presence of TMV-GFP RNA in *NbSGT1*- and *NbSKP1*-suppressed plants. First-strand cDNA was synthesized using total RNA from *N*-, *NbSGT1*-, and *NbSKP1*-silenced *NN* plants and nonsilenced (*nn* and *NN*) plants. This cDNA was used in PCR to amplify the TMV movement protein (M) gene. For the control reaction (C), the RT mix without reverse transcriptase was used as a template.

(B) to (D) RT-PCR to confirm the silencing of *NbSGT1* and *NbSKP1* expression. First-strand cDNA was synthesized using total RNA isolated from *NbSGT1*- and *NbSKP1*-silenced *NN* plants. This cDNA was used in PCR with gene-specific primers.

(B) Typical PCR products for EF1 α derived from *NbSGT1*-silenced (right) and nonsilenced (left) plants.

(C) PCR products of *NbSGT1* derived from *NbSGT1*-silenced (right) and nonsilenced (left) plants.

(D) PCR products of *NbSKP1* derived from *NbSKP1*-silenced (right) and nonsilenced (left) plants.

Lanes 1 to 6 correspond to products from PCR cycles 15, 18, 21, 24, 27, and 30. Lane C represents the control reaction, in which the RT mix without reverse transcriptase was used as a template.

Arabidopsis *SGT1b* Rescues the *NbSGT1* Suppression Phenotype

SGT1 is a highly conserved component of the SCF-type E3 ubiquitin ligase complex in eukaryotes (Kitagawa et al., 1999; Lyapina et al., 2001; Matsuzawa and Reed, 2001). In fact, human SGT1 and SIP complement yeast *sgt1* mutant defects (Kitagawa et al., 1999; Matsuzawa and Reed, 2001). Because *NbSGT1* has similarity to two Arabidopsis SGT1-like genes (*AtSGT1a* and *AtSGT1b*), we tested whether these Arabidopsis genes can complement the *NbSGT1* suppression pheno-

type in *NN* plants. We overexpressed *AtSGT1a* and *AtSGT1b* using a *Potato virus X* (PVX) expression system. *NN* plants were coinfiltrated with a mixture of Arabidopsis containing pTRV-*NbSGT1* (silencing construct) and pPVX-*AtSGT1a*-2xHA or pPVX-*AtSGT1b*-2xHA (overexpression constructs).

Ten days after infiltration, these plants were challenged with TMV-GFP. *AtSGT1b* fully rescued the loss-of-resistance phenotype induced by the suppression of *NbSGT1* (cf. Figures 9A and 6D). However, *AtSGT1a* failed to complement the suppression effect of *NbSGT1*, because we observed TMV-GFP on the upper, uninfected leaves of the plants containing pPVX-*AtSGT1a*-2xHA (Figure 9B). Infection of pPVX-*AtSGT1a*-2xHA and pPVX-*AtSGT1b*-2xHA alone in *NN* and wild-type *nn* plants had no effect on TMV-GFP resistance or susceptibility (data not shown).

To exclude the possibility that rescue of the suppression phenotype by *AtSGT1b* is not attributable simply to the lack of suppression of endogenous *NbSGT1*, we performed semiquantitative RT-PCR. We found that *NbSGT1* is suppressed in these plants (data not shown). In addition, we reasoned that the region targeted for *NbSGT1* silencing shows only 71% homology with *AtSGT1a* and *AtSGT1b* at the nucleotide level; therefore, TRV-*NbSGT1* does not suppress the PVX constructs. We confirmed the expression of *AtSGT1a* and *AtSGT1b* proteins using HA antibodies (Figure 9C, top). In addition, our immunoprecipitation experiments suggest that *AtSGT1a* and *AtSGT1b* associate with *NbRar1* (Figure 9C, bottom). Together, these results suggest that the function of *SGT1b* is highly conserved in plants and plays a role in plant defense. In addition, *AtSGT1a* may function in different defense pathways or have some other function.

DISCUSSION

SCF Function in TMV Resistance

We report here that a CHORD-containing *NbRar1* protein that is required for the *N*-mediated resistance to TMV interacts with *NbSGT1* in yeast two-hybrid and in vitro coimmunoprecipitation assays. These results are consistent with the most recent findings by Azevedo et al. (2002) in that *HvRar1* has been shown to interact directly with *HvSGT1*. SGT1 is a novel, highly conserved component of the SCF-type E3 ubiquitin ligase complex (Kitagawa et al., 1999; Lyapina et al., 2001; Matsuzawa and Reed, 2001). SGT1, which plays an important role in kinetochore function in yeast, forms complexes with the other SCF components SKP1 and Cdc53p, a Cullin1 homolog (Kitagawa et al., 1999). In human, an SGT1 homolog, SIP, which regulates β -catenin levels in the *Wnt* signaling pathway, also forms a complex with SKP1 (Matsuzawa and Reed, 2001).

We show that *NbSGT1* associates physically with *NbSKP1* in vitro and in vivo. Suppression of *NbSGT1* and

NbSKP1 in *NN* transgenic plants by VIGS resulted in the loss of resistance to TMV. This finding indicates that *NbSGT1* and *NbSKP1* are required for *N* function. VIGS- or RNA interference-like gene knockout systems are known to silence highly homologous genes. This makes the interpretation of our results difficult. To overcome this shortcoming, we overexpressed SGT1 homologs from heterologous *Arabidopsis* in *NbSGT1*-silenced *NN* plants.

Arabidopsis has two *SGT1*-like genes, *AtSGT1a* and *AtSGT1b* (Austin et al., 2002; Azevedo et al., 2002). Our *N. benthamiana* genomic DNA gel blot analysis suggested that *NbSGT1* is a single-copy gene (data not shown). Consistent with this observation, we recovered only a single sequence from our RT-PCR cloning effort of *NbSGT1*. In addition, all seven clones derived from our tomato two-hybrid library also belong to a single sequence. Of the two *Arabidopsis* *SGT1*-like genes, only *AtSGT1b* was able to rescue the *NbSGT1* suppression phenotype.

It is not clear why *AtSGT1a* failed to rescue the suppression phenotype even though *NbSGT1* shares 60% identity with *AtSGT1b* and *AtSGT1a* at the amino acid level. Interestingly, Austin et al. (2002) recently reported that *AtSGT1b* is required for the function of another TIR-NB-LRR-containing *RPP5* resistance gene in *Arabidopsis*. Even though *AtSGT1a* and *AtSGT1b* are highly similar, the presence of a functional copy of *AtSGT1a* in a *AtSGT1b* mutant background failed to provide *RPP5*-mediated resistance to *Peronospora parasitica*.

Together, our results from protein-protein interactions, the suppression phenotype, and its rescue indicate that *NbSGT1* is required for *N* gene function. We did not attempt to rescue the *NbSKP1* suppression phenotype because there are 19 *SKP1*-like genes in *Arabidopsis*. In our VIGS of *NbSKP1*, we cannot exclude the possibility of the silencing of other *SKP1*-like genes. Therefore, the data presented here indicate that the *NbSKP1*-like gene is required for the function of the *N* gene. Thus, SCF seems to play an important role in plant defense, in addition to the auxin response in plants.

COP9 Signalosome Function in TMV Resistance

The role of the COP9 signalosome in plant photomorphogenesis is well established (Wei and Deng, 1999). Because mutations in the CSN subunits of the COP9 signalosome result in lethality, it is difficult to investigate the role of the COP9 signalosome in other signal transduction processes. However, recently, Schwachheimer et al. (2001) made a nonlethal phenotype of CSN5 in an antisense transgenic plant, showing that the COP9 signalosome plays an important role in the degradation of the SCF^{TIR} substrate PSIAA6 in the auxin response. VIGS, used as a reverse genetics approach as described here, provides a new way to study the function of a gene whose knockout leads to a lethal phenotype.

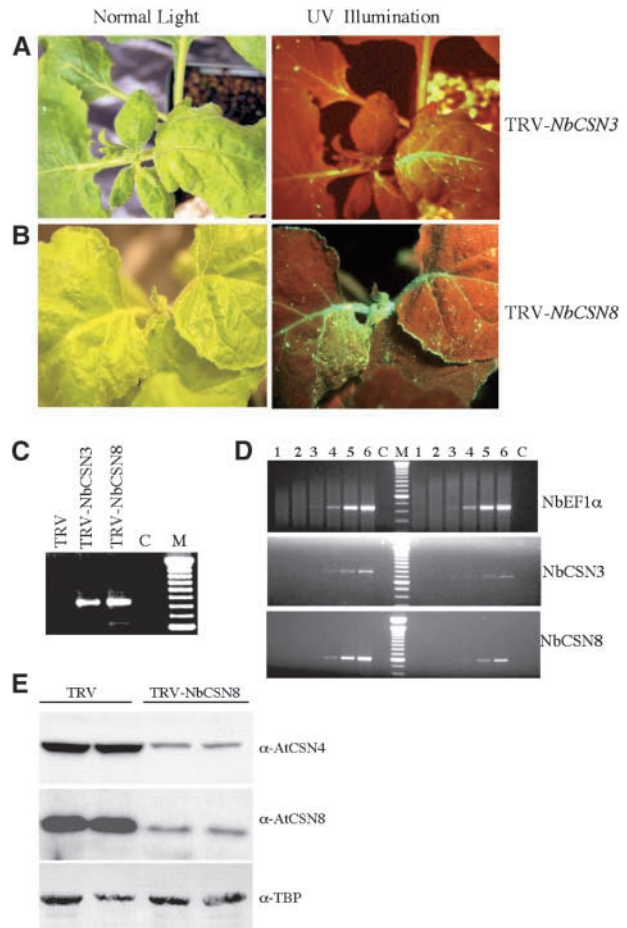


Figure 8. Analysis of the Effect of COP9 Signalosome Suppression on the *N*-Mediated Resistance to TMV.

(A) and (B) Transgenic *NN* plants were first silenced for *NbCSN3* (A) or *NbCSN8* (B) at the four-leaf stage. The upper leaves of these plants were infected with TMV-GFP virus to monitor resistance or susceptibility responses as described for Figure 6.

(C) RT-PCR to detect TMV-GFP RNA in *NbCSN3*- and *NbCSN8*-suppressed plants, as described for Figure 7A.

(D) RT-PCR to confirm the silencing of *NbCSN3* and *NbCSN8* expression, as described for Figures 7B to 7D. The top shows typical PCR products for EF1 α derived from *NbCSN3*-silenced (right) and nonsilenced (left) plants. The middle shows PCR products of *NbCSN3* derived from *NbCSN3*-silenced (right) and nonsilenced (left) plants. The bottom shows typical PCR products of *NbCSN8* derived from *NbCSN8*-silenced (right) and nonsilenced (left) plants. Lanes 1 to 6 correspond to products from PCR cycles 15, 18, 21, 24, 27, and 30. Lane C represents the control reaction, in which the RT mix without reverse transcriptase was used as a template.

(E) *NbCSN4* and *NbCSN8* silencing results at the protein level. Total protein extracted from nonsilenced and *NbCSN8*-silenced plants was normalized for protein quantity and analyzed by SDS-PAGE followed by immunoblot analysis using anti-*AtCSN4* and anti-*AtCSN8* antibodies. As a loading control, an anti-TATA box binding protein (TBP) antibody was used.

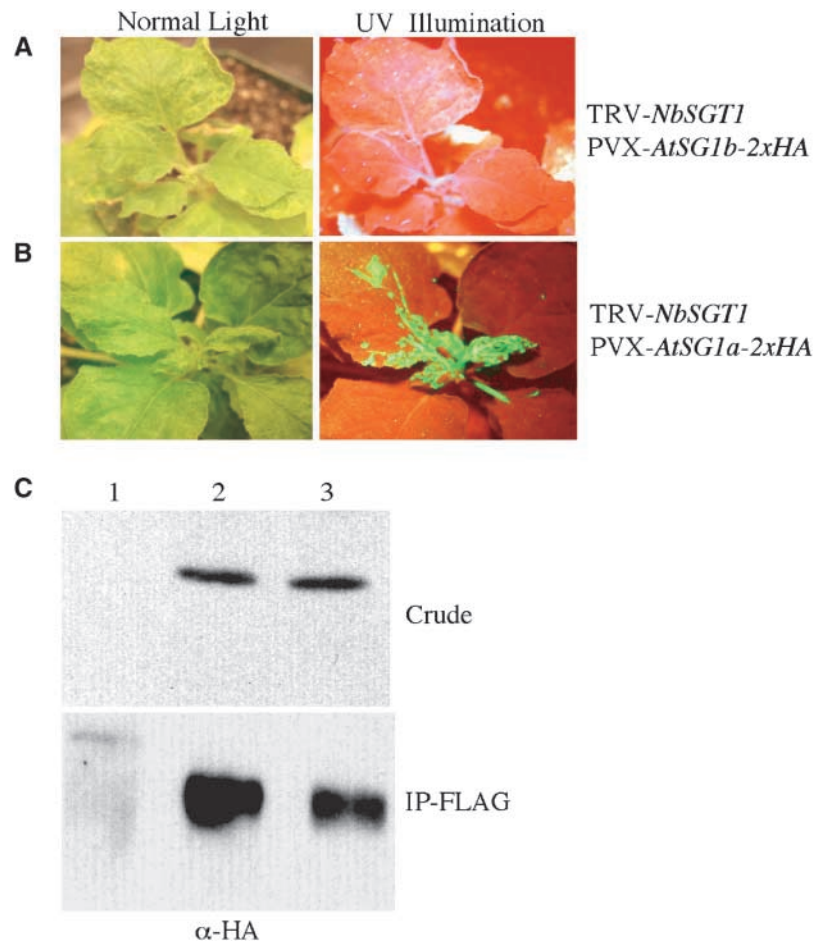


Figure 9. Conserved SGT1 Function in Plant Defense.

(A) and **(B)** Transgenic *NN* plants were first silenced for *NbSGT1*. In these plants, *AtSGT1b* **(A)** and *AtSGT1a* **(B)** were overexpressed using a PVX-based expression vector. The upper leaves of these plants were infected with TMV-GFP virus to monitor resistance or susceptibility responses, as described for Figure 6.

(C) *N. benthamiana* plants were transfected with the TMV expression vector carrying FLAG tag alone (lane 1) or the TMV expression vector producing HA-tagged *AtSGT1a* (lane 2) and HA-tagged *AtSGT1b* (lane 3). Whole cell extracts from transfected plants were analyzed by immunoblotting using HA antibodies (top). Total cell extracts were normalized for protein quantity and immunoprecipitation using anti-FLAG antibody-conjugated Sepharose beads. After washing, immune complexes were eluted using FLAG peptide. The resulting complex was analyzed by SDS-PAGE followed by immunoblot analysis with anti-HA antibodies (bottom).

Recent evidence indicates that the subunits of the COP9 signalosome interact directly with components of the SCF-type E3 ubiquitin ligase complex (Lyapina et al., 2001). Because *NbSKP1* is a component of the SCF complex and interacts with *NbSGT1*, we tested whether *NbSGT1* interacts with subunits of the COP9 signalosome using a yeast two-hybrid assay. We found no interaction between *NbSGT1* and any of the eight subunits of the COP9 signalosome. In human, Cullin1 (also a subunit of the SCF) has been shown to interact strongly with the CSN2 subunit and weakly with the CSN6 subunit in yeast two-hybrid assays, but the SKP1

component of SCF fails to interact with any subunit of the COP9 signalosome.

However, in vivo, the SCF components Cullin1, SKP1, HRT1/Rbx1, and SKP2 (F-box protein) associate with the COP9 signalosome (Lyapina et al., 2001). In addition, SKP2 also associates with SGT1 and hsp90 β . These results prompted us to test the association of *NbSGT1* with the COP9 signalosome in vivo. Our studies using immune complexes from plant cells suggest that *NbSGT1* associates with the COP9 signalosome. Interestingly, a recent report from Azevedo et al. (2002) indicates that *HvSGT1* also asso-

ciates with the COP9 signalosome. In addition, suppression of the CSN3 and CSN8 subunits of the COP9 signalosome resulted in loss of resistance to TMV. Our results provide evidence for the new role of the COP9 signalosome as an integral part of plant defense.

What Is the Role of SCF Ubiquitin Ligase and the COP9 Signalosome In Plant Defense?

The SCF-type E3 ubiquitin protein ligases act with E2 enzymes to ubiquitinate short-lived cytoplasmic and nuclear proteins and target them for degradation (Hochstrasser, 2000). Recent evidence indicates that the COP9 signalosome plays an important role in mediating SCF-type E3 ubiquitin ligase function. In the SCF model, Cullin and Rbx1/Hrt/Roc (RING finger proteins) first dock SKP1 with an E2 enzyme, then SKP1 binds to an F-box protein, which then binds the substrate that is targeted for degradation. Therefore, in SCF, a variable F-box protein component serves as a substrate receptor for ubiquitination and subsequent degradation (Deshaies, 1999).

There are >300 F-box proteins in plants (Arabidopsis Genome Initiative, 2000). Only a few plant F-box proteins, such as TIR1 (Gray et al., 1999), COI1 (Xie et al., 1998), UFO (Samach et al., 1999), ZTL (Somers et al., 2000), and FKF1 (Nelson et al., 2000), have been studied. COI1 is involved in defense, and others are involved in developmental processes. Moreover, only TIR1 has been shown to assemble into SCF complexes *in vivo*.

Based on the model discussed above, R proteins may relay signals through Rar1 and SGT1 into a plant SCF complex for ubiquitin-mediated protein degradation. Our results suggest that Rar1 interacts directly with SGT1 and exists in a functional SCF complex. However, it is not clear whether Rar1 is an additional component of the SCF that participates in SCF ubiquitination activity or is simply an interaction partner of SGT1. SGT1 is found in the SKP1 (F-box) complex from human (Lyapina et al., 2001). However, it was not detected in the Grr1 (F-box) complex in yeast and does not participate in SCF ubiquitination activity *in vitro* (Kitagawa et al., 1999). Therefore, it is unclear at present whether SGT1 is a core component of the SCF ligase complex or participates independently. As shown here and in other systems, SGT1 seems to interact with potentially functional forms of SCF complexes.

Unlike yeast SGT1, plant and human SGT1 proteins contain three TPR repeats at the N terminus. TPR domains play an important role in protein-protein interactions that mediate binding to other proteins (Lamb et al., 1995). Interestingly, in our yeast two-hybrid assays, NbRar1 interacts with the C-terminal domain (amino acid residues 134 to 370) of NbSGT1 but not with the N-terminal domain (residues 1 to 133) that contains TPR (Y. Liu and S.P. Dinesh-Kumar, unpublished observation). It is possible that the TPR domain of NbSGT1 interacts with proteins that target Rar1 and SGT1

to the functional SCF complex. In fact, in the anaphase-promoting E3 ligase complex, many TPR-containing proteins (Apc3/Cdc27, Apc6/Cdc16, and Apc8/Cdc23) form an independent complex and are predicted to serve as a scaffold for the anaphase-promoting E3 ligase complex (Jackson et al., 2000).

What are the targets of protein degradation in plant defense? In the SCF-mediated protein degradation pathways, SKP1 binds to F-box proteins, which in turn bind to the specific substrate that is targeted for degradation (Hochstrasser, 2000). Recent evidence suggests that Skp1 or Skp1 homologs also can bind to non-F-box-containing proteins such as Rav1 (Seol et al., 2001) and adenovirus E4orf6 (Querido et al., 2001). Further studies will be required to determine whether F-box proteins or other candidate proteins involved in the Rar1-SGT1-SKP1 complex function in plant defense.

It is possible that this complex may recruit target substrates, presumably negative regulators of defense response, and subject them to degradation through the COP9 signalosome via the 26S proteasome. These negative regulators may be inhibitors of cell death, transcription factors that are required for the induction of defense response genes, or other regulatory proteins.

In the future, it will be interesting to determine to what extent the role of protein degradation in plant defense is general. It is necessary to determine the roles of SGT1, SCF, and the COP9 signalosome in other *R* gene-mediated resistance signaling pathways. Identification of the SCF targets for plant defense using interaction cloning, affinity purification of protein complexes followed by mass spectrometry, and VIGS knockout approaches will increase our understanding of the role of the SCF-type E3 ligase and the COP9 signalosome in plant disease resistance signaling.

METHODS

Plasmid Construction

Yeast Two-Hybrid Vectors

The LexA DNA binding domain containing bait vectors was prepared by cloning full-length *NbRar1* and *NbSGT1* PCR products into pTBS1, in which the nuclear localization signal sequence LexA DNA binding domain is under the control of a GAL10 promoter (T. Burch-Smith and S.P. Dinesh-Kumar, unpublished data). The B42 DNA activation domain (AD) containing prey vectors was prepared by cloning full-length *NbSKP1* and *NbSGT1* into pJG4-5 (Kolonin et al., 2000). Plasmids pLexA-p53 and pAD-Target were purchased from Origene Technologies (Rockville, MD).

Bacterial Expression Vectors

NbSGT1 and NbRar1 were amplified by PCR and recombined into pDEST15 using the GATEWAY system (Invitrogen, Carlsbad, CA) to

express glutathione S-transferase (GST)-tagged fusion proteins in *Escherichia coli*.

T7 Promoter-Based Vectors for *In Vitro* Translation

NbSGT1, NbRar1, and NbSKP1 were amplified by PCR and recombined into pDEST14 using the GATEWAY system.

Tobacco mosaic virus-Based Expression Vectors

pYL257, a *Tobacco mosaic virus* (TMV)-based expression vector, was created by cloning the attR1-Cmr-ccdB-attR2 PCR product from GATEWAY conversion system cassette B into TMV binary vector pSPDK661 (Liu et al., 2002). pTMV-NbSGT1-2xHA, pTMV-NbRar1-2xFLAG, and pTMV-NbSKP1-2xMyc were created by recombining full-length PCR products of NbSGT1 with 2 × hemagglutinin (HA) tag, NbRar1 with 2 × FLAG tag, and NbSKP1 with 2 × Myc tag, respectively, into pYL257 using the GATEWAY system.

Potato virus X-Based Expression Vectors

pSPDK658, a *Potato virus X* (PVX) T-DNA vector containing the full-length PVX cDNA from pCambia2300 (from David Baulcombe, Sainsbury Lab, Norwich, UK), was cloned into pYL41, a pBin19 derivative carrying the duplicated 35S promoter of *Cauliflower mosaic virus* and nopaline synthase terminator to give pSPDK658. attR1-Cmr-ccdB-attR2 was amplified by PCR from GATEWAY conversion system cassette B and cloned into pSPDK658 to give pYL254, a PVX-based expression vector. pPVX-AtSGT1a-2xHA and pPVX-AtSGT1b-2xHA were generated by recombining full-length *Arabidopsis thaliana* SGT1a and SGT1b, respectively, with 2 × HA tag into pYL254 using the GATEWAY system.

Tobacco rattle virus 2 Derivatives

cDNA fragments of putative *Nicotiana benthamiana* SGT1 (nucleotides 1 to 479), SKP1 (nucleotides 1 to 411), CSN3 (nucleotides 43 to 582), and CSN8 (nucleotides 1 to 298) were amplified from cDNA clones and cloned into pTRV2 (Liu et al., 2002). pTRV2-*N* has been described (Liu et al., 2002). The identities of the vectors described above were confirmed by sequencing. Primer sequences used for this purpose will be available upon request.

Yeast Two-Hybrid Screen and Interaction Assays

The yeast two-hybrid prey library was constructed in pJG4-5. A cDNA library construction kit (Stratagene) was used to generate cDNAs using poly(A)⁺ RNA isolated from tissues taken from TMV-resistant (VF36:*NN*) (Whitham et al., 1996) and TMV-susceptible (VF36) plants 1, 4, and 8 h after TMV infection. The cDNA library was fused to the B42 AD under the control of a Gal-inducible promoter (GAL1). The yeast two-hybrid screen and interaction assays were performed as described (Kolonin et al., 2000).

Isolation of cDNAs from *N. benthamiana*

Total RNA was extracted from *N. benthamiana* leaves using RNAwiz solution (Ambion, Austin, TX), and single-stranded cDNA was pre-

pared from 2 μg of total RNA with Superscript II reverse transcriptase (Invitrogen) and oligo(dT) primer. Full-length cDNAs of putative NbCullin1, NbCSN3, NbCSN4, and NbCSN8 were amplified by PCR with primer combinations based on putative tomato Cullin1 (TC84853), putative tomato CSN3 (TC94454), putative tomato CSN4 (BG134323 and TC91324), and CSN8 (AW030374 and AW624151). The 5' and 3' ends of *NbSGT1* were cloned using the SMRT rapid amplification of cDNA ends kit (Clontech, Palo Alto, CA). PCR products were cloned into pCR-TOPO vector (Invitrogen), and their identities were verified by DNA sequencing. Primer sequences used for this purpose will be available upon request.

GST Pulldown Assays

GST-NbSGT1 and GST-NbRar1 fusion proteins were produced in BL21 codon plus cells (Stratagene) and affinity purified using glutathione-Sepharose beads. Approximately 1 μg of purified GST fusion proteins were incubated for 3 h with ³⁵S-Met-labeled in vitro translated proteins (TNT; Promega) at 4°C in 0.2 mL of buffer A (100 mM NaCl, 50 mM Tris-Cl, pH 7.5, 0.1 mM EDTA, 0.1 mM EGTA, 50 mM NaF, 0.2% Triton X-100, 0.1% β-mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor). The beads were washed four times with ice-cold buffer B (100 mM NaCl, 50 mM Hepes, pH 7.5, 0.1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, and complete protease inhibitor) at 4°C. The washed beads were boiled in SDS sample buffer, and proteins were separated by SDS-PAGE. Gels were fixed, fluorographed using Amplify solution (Amersham Pharmacia), dried, and exposed to x-ray film.

Immunoprecipitation

Total protein from nontransfected and transfected leaves was extracted using the protocol described in Serino et al. (1999). Immunoprecipitations were performed using EZview Red anti-FLAG M2 agarose (Sigma) and anti-HA antibody affinity matrix (CRP, Berkeley, CA) at 4°C overnight. After washing four times with PBS solution, proteins were eluted using 3 × FLAG or HA peptide (Sigma). Immune complexes were analyzed by SDS-PAGE and immunoblotting using various antibodies and were detected using Westpico SuperSignal enhanced chemiluminescence (Pierce).

Virus-Induced Gene Silencing Assay, Green Fluorescent Protein Imaging, and Detection of TMV by Reverse Transcriptase-Mediated PCR

Wild-type *nn* and transgenic *NN* plants were grown in pots at 25°C in a growth chamber under a 16-h-light/8-h-dark cycle. For the virus-induced gene silencing assay, pTRV1 or pTRV2 and its derivatives were introduced into *Agrobacterium tumefaciens* strain GV2260. *Agrobacterium* cultures (OD₆₀₀ = 0.8) containing pTRV1 or pTRV2 derivative plasmids were mixed at a 1:1 ratio and infiltrated into the lower leaves of four-leaf-stage plants using a 1-mL needleless syringe. Ten days after TRV infiltration, the upper leaves of the plants were infected with TMV-green fluorescent protein virus. In the overexpression experiments, pPVX derivatives and pTRV derivatives were coinfiltrated. Each silencing experiment was repeated at least five times, and each experiment included at least four independent plants. Green fluorescent protein imaging was performed using UV il-

lumination, and photographs were taken using an Olympus Camedia E10 digital camera (Tokyo, Japan).

To detect the presence of TMV, total RNA extracted from upper uninoculated leaves was used to generate the first-strand cDNA using a primer that anneals to the 3' end of the movement protein gene of TMV. This first strand was used in PCR reactions to amplify the movement protein gene, and the resulting PCR products were analyzed by agarose gel electrophoresis.

Analysis of Transcript Levels by Reverse Transcriptase-Mediated PCR

Total RNA was extracted from *N. benthamiana* leaves and treated using the Message Clean Kit (Genhunter, Houston, TX) to remove DNA contamination. This RNA (1 μ g) was used to synthesize the first-strand cDNA. Samples from each reaction (1.5 μ L) were used in a 30- μ L PCR mixture containing the cDNA template and Taq DNA polymerase. Primers that anneal outside the region of the virus-induced gene silencing target sequence were used. The sequences of primers used in PCR will be available upon request. PCR was performed for 15, 18, 21, 24, 27, and 30 cycles. Control PCR (using RNA without reverse transcription) was performed for 30 cycles. The intensities of the PCR bands were analyzed and quantified using Gel Doc 2000 and Quantity One Version 4.2.1 (Bio-Rad).

Accession Numbers

The accession numbers for Arabidopsis SGT1a and SGT1b are A439975 and AF43976, respectively.

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REFERENCES

- Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E. (1998). Different requirements for *EDS1* and *NDR1* by disease resistance genes define at least two *R* gene-mediated signaling pathways in *Arabidopsis*. *Proc. Natl. Acad. Sci. USA* **95**, 10306–10311.
- Arabidopsis Genome Initiative. (2000). Analysis of the genome sequence of the flowering plant *Arabidopsis thaliana*. *Nature* **408**, 796–815.
- Austin, M.J., Muskett, P., Kahn, K., Feys, B.J., Jones, J.D.G., and Parker, J.E. (2002). Regulatory role of *SGT1* in early *R* gene-mediated plant defense. *Science* **295**, 2077–2080.
- 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.
- Baker, B., Zambryski, P., Staskawicz, B., and Dinesh-Kumar, S.P. (1997). Signaling in plant-microbe interactions. *Science* **276**, 726–733.
- Baulcombe, D.C. (1999). Fast forward genetics based on virus-induced gene silencing. *Curr. Opin. Plant Biol.* **2**, 109–113.
- Dangl, J.L., and Jones, J.D.G. (2001). Plant pathogens and integrated defense responses to infection. *Nature* **411**, 826–833.
- Deshaies, R.J. (1999). SCF and Cullin/Ring H2-based ubiquitin ligases. *Annu. Rev. Cell Dev. Biol.* **15**, 435–467.
- Gabriel, D., and Rolfe, B. (1990). Working models of specific recognition in plant-microbe interactions. *Annu. Rev. Phytopathol.* **28**, 365–391.
- Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H., and Estelle, M. (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. *Genes Dev.* **13**, 1678–1691.
- Hochstrasser, M. (2000). Evolution and function of ubiquitin-like protein-conjugation systems. *Nat. Cell Biol.* **2**, E153–E157.
- Jackson, P.K., Eldridge, A.G., Freed, E., Furstenthal, L., Hsu, J.Y., Kaiser, B.K., and Reimann, J.D.R. (2000). The role of the RINGs: Substrate recognition and catalysis by ubiquitin ligases. *Trends Cell Biol.* **10**, 429–439.
- Kitagawa, K., Skowyra, D., Elledge, S.J., Harper, J.W., and Hieter, P. (1999). SGT1 encodes an essential component of the yeast kinetochore assembly pathway and a novel subunit of the SCF ubiquitin ligase complex. *Mol. Cell* **4**, 21–33.
- Kolonin, M.G., Zhong, J., and Finley, R.L. (2000). Interaction mapping methods in two-hybrid systems. *Methods Enzymol.* **328**, 26–46.
- Lamb, J.R., Tugendreich, S., and Hieter, P. (1995). Tetratricopeptide repeat interactions: To TPR or not to TPR? *Trends Biochem. Sci.* **20**, 257–259.
- Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S.P. (2002). Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for *N*-mediated resistance to tobacco mosaic virus. *Plant J.* **30**, 415–429.
- Lyapina, S., Cope, G., Shevchenko, A., Serino, G., Tsude, 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.
- Matsuzawa, S.-I., and Reed, J.C. (2001). Siah-1, SIP, and Ebi collaborate in a novel pathway for β -catenin degradation linked to p53 responses. *Mol. Cell* **7**, 915–926.
- McDowell, J.M., and Dangl, J.L. (2000). Signal transduction in the plant immune response. *Trends Biochem. Sci.* **25**, 79–82.
- Nelson, D.C., Lasswell, J., Rogg, L.E., Cohen, M.A., and Bartel, B. (2000). *FKF1*, a clock-controlled gene that regulates the transition to flowering in *Arabidopsis*. *Cell* **101**, 331–340.
- Querido, E., Blanchette, P., Yan, Q., Kamura, T., Morrison, M., Boivin, D., Kaelin, W.G., Conaway, R.C., Conaway, J.W., and Branton, P.E. (2001). Degradation of p53 by adenovirus E4orf6

- and E1B55K proteins occurs via a novel mechanism involving a Cullin-containing complex. *Genes Dev.* **15**, 3104–3117.
- 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.
- Rock, F.L., Hardiman, G., Timans, J.C., Kastelein, R.A., and Bazan, J.F.** (1998). A family of human receptors structurally related to *Drosophila Toll*. *Proc. Natl. Acad. Sci. USA* **95**, 588–593.
- 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.
- Samach, A., Klenz, J.E., Kohalmi, S.E., Risseuw, E., Haughn, G.W., and Crosby, W.L.** (1999). The *UNUSUAL FLORAL ORGANS* gene of *Arabidopsis thaliana* is an F-box protein required for normal patterning and growth in the floral meristem. *Plant J.* **20**, 433–445.
- Schulze-Lefert, P., and Vogel, J.** (2000). Closing the ranks to attack by powdery mildew. *Trends Plant Sci.* **5**, 343–348.
- Schwechheimer, C., Serino, G., Callis, J., Crosby, W.L., Lyapina, S., Deshaies, R.J., Gray, W.M., Estelle, M., and Deng, X.-W.** (2001). Interaction of the COP9 signalosome with the E3 ubiquitin ligase SCF^{TIR1} in mediating auxin response. *Science* **292**, 1379–1382.
- Seol, J.H., Shevchenko, A., Shevchenko, A., and Deshaies, R.J.** (2001). Skp1 forms multiple protein complexes, including RAVE, a regulator of V-ATPase assembly. *Nat. Cell Biol.* **3**, 384–391.
- 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.
- Shirasu, K., Lahaye, T., Tan, M.-W., and Schulze-Lefert, P.** (1999). A novel class of eukaryotic zinc-binding proteins is required for disease resistance signaling in barley and development in *C. elegans*. *Cell* **99**, 355–366.
- Somers, D.E., Schultz, T.F., Milnamow, M., and Kay, S.** (2000). *ZEITLUPE* encodes a novel clock-associated PAS protein from *Arabidopsis*. *Cell* **101**, 319–329.
- Thomma, B.P.H.J., Penninckx, I.A.M.A., Broekaert, W.F., and Cammue, B.P.A.** (2001). The complexity of disease signaling in *Arabidopsis*. *Curr. Opin. Immunol.* **13**, 63–68.
- Wei, N., and Deng, X.-W.** (1999). Making sense of the COP9 signalosome, a conserved regulatory protein complex from *Arabidopsis* to human. *Trends Genet.* **15**, 98–103.
- Whitham, S., Dinesh-Kumar, S.P., Choi, D., Hehl, R., Corr, C., and Baker, B.** (1994). The product of the tobacco mosaic virus resistance gene *N*: Similarity to toll and the interleukin-1 receptor. *Cell* **78**, 1101–1115.
- Whitham, S., McCormick, S., and Baker, B.** (1996). The *N* gene of tobacco confers resistance to tobacco mosaic virus in transgenic tomato. *Proc. Natl. Acad. Sci. USA* **93**, 8776–8781.
- Xie, D., 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.