

The U-Box Protein CMPG1 Is Required for Efficient Activation of Defense Mechanisms Triggered by Multiple Resistance Genes in Tobacco and Tomato ^W

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We previously identified three *Avr9/Cf-9 Rapidly Elicited (ACRE)* genes essential for *Cf-9*- and *Cf-4*-dependent hypersensitive response (HR) production in *Nicotiana benthamiana*. Two of them encode putative E3 ubiquitin ligase components. This led us to investigate other *ACRE* genes associated with the ubiquitination pathway. *ACRE74* encodes a U-box E3 ligase homolog, highly related to parsley (*Petroselinum crispum*) CMPG1 and *Arabidopsis thaliana* PLANT U-BOX20 (PUB20) and PUB21 proteins, and was called Nt CMPG1. Transcript levels of Nt CMPG1 and the homologous tomato (*Solanum lycopersicum*) *Cmpg1* are induced in Cf9 tobacco (*Nicotiana tabacum*) and Cf9 tomato after Avr9 elicitation. Tobacco CMPG1 possesses *in vitro* E3 ligase activity. *N. benthamiana* plants silenced for Nt CMPG1 show reduced HR after Cf-9/Avr9 elicitation, while overexpression of Nt CMPG1 induces a stronger HR in Cf9 tobacco plants after Avr9 infiltration. In tomato, silencing of *Cmpg1* decreased resistance to *Cladosporium fulvum*. Overexpression of epitope-tagged tobacco CMPG1 mutated in the U-box domain confers a dominant-negative phenotype. We also show that Nt CMPG1 is involved in the Pto/AvrPto and Inf1 responses. In summary, we show that the E3 ligase Nt CMPG1 is essential for plant defense and disease resistance.

INTRODUCTION

Plants are continuously exposed to pathogen attack, but successful infection is rare. Many plant–microbe interactions can be described by the gene-for-gene model, in which a plant, via a resistance (*R*) gene, has the capacity to recognize a pathogen carrying the corresponding avirulence (*Avr*) gene (Dangl and Jones, 2001). This interaction induces a hypersensitive response (HR), comprising localized cell death around the infection site. Many *R* genes have been cloned from plants (Meyers et al., 2005), but the mechanisms involved in signal transduction after pathogen recognition are poorly understood.

Cladosporium fulvum is a biotrophic fungus that causes leaf mold in tomato (*Solanum lycopersicum*). Several resistance genes to *C. fulvum* have been cloned, of which *Cf-9* is one of the best characterized (Rivas and Thomas, 2005). *Cf* genes encode receptor-like proteins, type I transmembrane protein with extracellular leucine-rich repeats, and a short cytoplasmic region (Thomas et al., 1998; Fritz-Laylin et al., 2005).

Cf-9 confers resistance to *C. fulvum* strains carrying the *Avr9* gene, and infiltration of Avr9 peptide induces HR in tobacco (*Nicotiana tabacum*) and tomato plants carrying *Cf-9* (Hammond-

Kosack and Jones, 1997). Since the cloning of *Cf-9*, we have investigated how the defense response is produced after Cf-9/Avr9 recognition. Several early responses have been characterized that are activated in a Cf-9/Avr9-dependent manner, including ion fluxes (Blatt et al., 1999), production of reactive oxygen species (Piedras et al., 1998), and activation of mitogen-activated and calcium-dependent protein kinases (Romeis et al., 1999, 2000, 2001). An essential role for calcium-dependent kinases (Romeis et al., 2001) has been shown, as well as for SUPPRESSOR OF G-2 ALLELE OF SKP1 (SGT1), a protein putatively required for the function of SCF (for Skp1/Cullin/F-box) ubiquitin ligases (Peart et al., 2002). More recently, Cf-9–interacting thioredoxin was identified, which negatively regulates the Cf-9–dependent responses (Rivas et al., 2004).

To get further insight into the molecular mechanism regulating *Cf-9* function, many *Avr9/Cf-9 Rapidly Elicited (ACRE)* genes were identified that change their expression rapidly after Avr9 elicitation in Cf9 tobacco cells (Durrant et al., 2000). Most of those genes encode components of signaling pathways, including transcription factors, protein kinases, and ubiquitination pathway-related proteins. Virus-induced gene silencing (VIGS) of 42 *ACRE* genes identified three genes essential for Cf-9/Avr9 and Cf-4/Avr4 HR: a protein kinase–encoding gene (*Avr9/Cf-9-INDUCED KINASE [ACIK1]*), an F-box–encoding gene (*ACRE189*), and a U-box–encoding gene (*ACRE276*) (Rowland et al., 2005). The F-box and U-box proteins are ubiquitin ligase components (Deshaies, 1999; Hatakeyama et al., 2001), indicating a major role for this regulatory mechanism in the control of Cf-9–dependent responses.

Until recently, ubiquitination was primarily associated with proteasome-mediated protein degradation, but it is now clear

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that ubiquitination also regulates protein function in a proteasome-independent way. Ubiquitination alters protein localization, activity, and interactions (Schnell and Hicke, 2003). Ubiquitin is a 76-amino acid polypeptide that is attached to a protein target through an enzymatic cascade comprising a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3) (Glickman and Ciechanover, 2001). The E3 ligase confers the specificity of the reaction (Hershko et al., 1983; Finley et al., 2004) and can be a single protein or a protein complex. Single protein E3 ligases include HECT, RING finger, and U-box domain proteins (Moon et al., 2004). U-box domains resemble the RING finger domain (Aravind and Koonin, 2000). The relevance of the U-box domain for the ubiquitin activity of U-box proteins has been shown in different ways. The U-box domain interacts with E2 proteins (Pringa et al., 2001), and deletion of the U-box domain leads to a lack of ubiquitination activity (Ohi et al., 2003; Stone et al., 2003; Zeng et al., 2004). *Arabidopsis thaliana* encodes many U-box proteins compared with other organisms, and plant U-box proteins have been classified in different groups based on the presence of other domains (Azevedo et al., 2001; Mudgil et al., 2004). Few physiological roles for plant U-box proteins have been established. Plant U-box proteins are involved in hormone regulation (Amador et al., 2001), biotic and abiotic response (Yan et al., 2003; Zeng et al., 2004), self-incompatibility (Stone et al., 2003), and plant development (Kim et al., 2003).

In plants, ubiquitination modulates environmental and endogenous signals, including responses to pathogen attack (Hare et al., 2003). Despite much effort showing regulation of defense by the ubiquitination pathway, the identity of the involved E3 ligases remains almost unknown (Devoto et al., 2003). The only E3 ligases involved in plant pathogen response identified to date are the rice (*Oryza sativa*) U-box SPOTTED LEAF11 (SPL11; Zeng et al., 2004), the *Arabidopsis* RING finger proteins RPM1-INTERACTING PROTEIN2 (RIN2) and RIN3 (Kawasaki et al., 2005), and two *ACRE* genes identified in our laboratory (Rowland et al., 2005). In animals, ubiquitination regulates innate immune responses through Toll-Like Receptor (TLR) in different ways. Tumor necrosis factor Receptor-Associated Factor6 (TRAF6) is a RING domain-containing protein that is essential in Nuclear Factor- κ B activation downstream of TLRs (Akira and Takeda, 2004). Another example is the RING finger protein Triad3A that ubiquitinates TLR4 and TLR9, controlling signaling through those receptors and avoiding a harmful effect after activation (Chuang and Ulevitch, 2004). Recent work also shows that the *Shigella flexneri* effector Outer *Shigella* protein G (OspG) controls human innate immunity through binding of ubiquitin-conjugating enzymes (Kim et al., 2005).

The discovery that two out of the three *ACRE* genes essential for *Cf-9*- and *Cf-4*-dependent HR encode E3 ligases led us to consider other *ACRE* genes encoding putative ubiquitin ligases for analysis in *Cf*-mediated defense mechanisms. In this work, we describe the characterization of tobacco CMPG1, a U-box protein essential for *Cf-9*-dependent HR production and for *Cf-9*-mediated resistance to *C. fulvum*. Tobacco CMPG1 can ubiquitinate in vitro, and mutation in the U-box domain abolishes this activity and confers a dominant-negative function on Nt CMPG1, indicating that this domain is essential for the physiological role of the protein. We also describe a role of tobacco

CMPG1 in the Pto/AvrPto interaction and in the response to Inf1 elicitor.

RESULTS

ACRE74 Is a U-Box Protein with High Similarity to Parsley CMPG1 and *Arabidopsis* PLANT U-BOX20 and 21

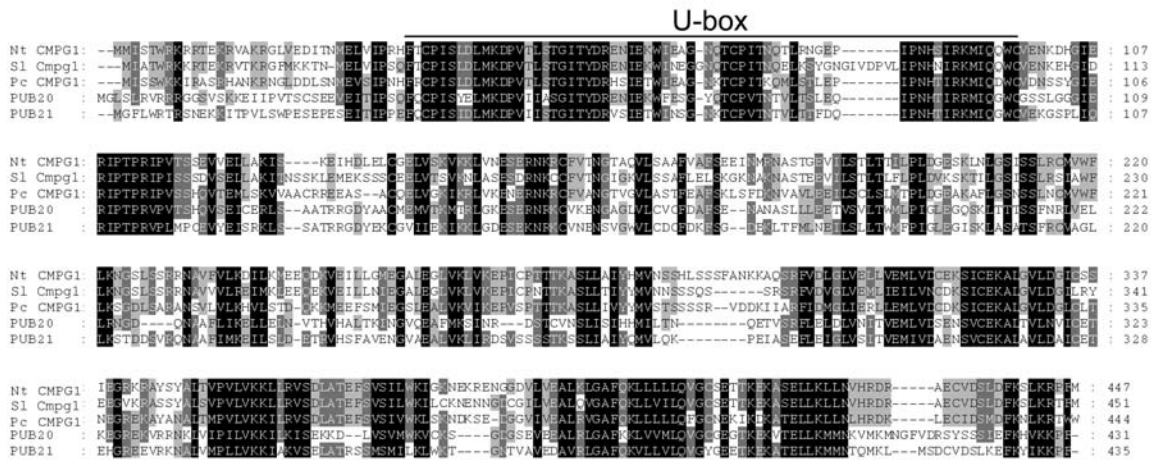
We earlier reported the homologies of 178 cDNA-amplified fragment length polymorphism (AFLP) fragments, corresponding to ~110 *ACRE* genes (Rowland et al., 2005). A BLAST search using one of those fragments, *ACRE74*, identified parsley (*Petroselinum crispum*) *CMPG1*, a rapidly induced gene after elicitation with Pep13 (Kirsch et al., 2001). We therefore refer to *ACRE74* as Nt *CMPG1*. Tobacco *CMPG1* also showed high similarity with *Arabidopsis* *PLANT U-BOX20* (*PUB20*) and *PUB21*, both encoding U-box proteins (Azevedo et al., 2001). These sequence similarities suggest a role for Nt *CMPG1* in early signaling in defense and in protein modification by ubiquitination, making tobacco *CMPG1* an interesting candidate for further investigation in plant defense.

An Nt *CMPG1* cDNA-AFLP fragment was used as a probe to screen a cDNA library from Avr9-elicited tobacco cells (Durrant et al., 2000). Several independent cDNA clones were sequenced, and a clone containing the exact sequence of the Nt *CMPG1* cDNA-AFLP fragment was selected for further studies.

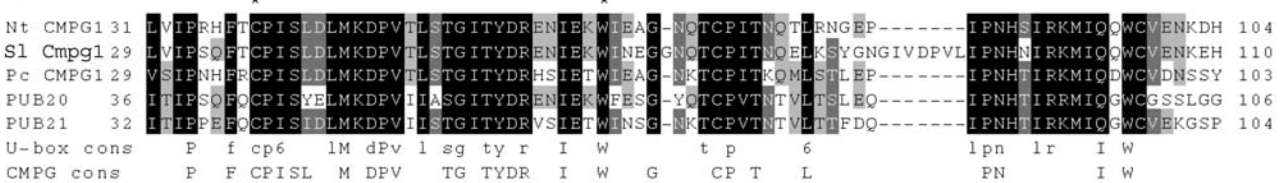
We identified in The Institute for Genomic Research (TIGR) tomato database an EST sequence with 73.6% identity to Nt *CMPG1* (sequence identification TC159549) that we designated Sl *Cmpg1*. Primers were designed for 3'-rapid amplification of cDNA ends (RACE) of tomato *Cmpg1* to obtain the full-length cDNA sequence. Three RACE fragments were identified that overlap with TC159549. Primers were designed based on the 5' and 3' cDNA sequences to isolate the full length of tomato *Cmpg1* cDNA. Tobacco *CMPG1* was predicted to encode a protein of 447 amino acids with a molecular mass of 50 kD. A SMART (Schultz et al., 1998; Letunic et al., 2004) search reveals an N-terminal U-box domain (Figure 1A) in the Nt *CMPG1* protein. For tomato *Cmpg1*, the predicted protein had 451 amino acids with a molecular mass of 50 kD. These protein sequences share 73.8% identity.

A BLAST search revealed parsley *CMPG1* as the closest homolog of tobacco *CMPG1* in the database. Two highly similar proteins are also found in *Arabidopsis*, *PUB20* and *PUB21*. Figure 1A shows the protein alignment of Nt *CMPG1* with putative orthologs in tomato (Sl *Cmpg1*), parsley (Pc *CMPG1*), and *Arabidopsis* (At *PUB20* and At *PUB21*). Parsley *CMPG1* shares 62.8% identity with tobacco *CMPG1* protein. Of the two *Arabidopsis* proteins, Nt *CMPG1* shows 43.1% identity to *PUB20* and 36.2% identity to *PUB21*. The U-box domain is highly similar in all these proteins (Figures 1A and 1B). A comparison of U-box and *CMPG1* consensus domains (Figure 1B) showed that most of the residues are conserved between them. An ARMADILLO (ARM) repeat domain has been identified in Pc *CMPG1*, At *PUB20*, and At *PUB21*. Parsley *CMPG1* contains three ARM repeats from residue 255 to 375 (Zeng et al., 2004). *Arabidopsis* *PUB20* and *PUB21* were originally described as class III plant U-box proteins, which were characterized by a C-terminal leucine-rich region (Azevedo et al., 2001). However, using a sophisticated

A



B



C

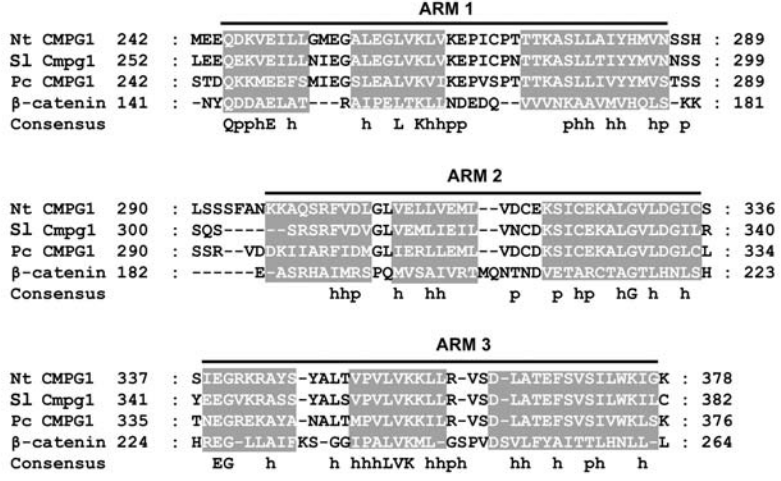


Figure 1. Sequence of Nt CMPG1 Protein and Alignment with Homologs.

(A) Protein sequence comparison between tobacco Nt CMPG1, tomato Sl Cmpg1, parsley Pc CMPG1, and *Arabidopsis* PUB20 and PUB21 was performed using ClustalW software. Position of the U-box domain is indicated. Shading represents the conservation of the residues among the sequences of the alignment: black, the residue is conserved in all the sequences; dark gray, the residue is conserved in four proteins of the alignment; light gray, the residue is conserved in three proteins of the alignment.

(B) Protein sequence alignment of the U-box domain of Nt CMPG1 homologs and comparison with U-box and CMPG consensus sequences. Asterisks show the amino acid residues mutated in this work to generate the dominant-negative versions of Nt CMPG1.

(C) Protein sequence comparison between the ARM repeats of Nt CMPG1, Sl Cmpg1, Pc CMPG1, and β-catenin (from human). The gray boxes indicate the protein region where an α-helix secondary structure was predicted. The consensus sequence is indicated, where p indicates polar residue and h indicates hydrophobic residue.

approach, Mudgil et al. (2004) identified ARM repeats in proteins of this class, including PUB20 and PUB21 where four and five repeats were found, respectively. Despite the low similarity of Sl Cmpg1 and Nt CMPG1 protein sequences with other ARM repeat-containing proteins, analysis of protein secondary struc-

ture using PSIPRED (Jones, 1999; McGuffin et al., 2000) and JPRED servers (Cuff et al., 1998) revealed a series of short α-helices consistent with the presence of three ARM repeats in both tobacco and tomato CMPG1 at the same position as for parsley CMPG1 (Figure 1C). Figure 1C shows the predicted

secondary structure of the putative ARM repeat domain of tomato, tobacco, and parsley CMPG1. The same pattern of α -helices as for the β -catenin ARM repeat domain was found in the C-terminal region of the three proteins, indicating a conserved ARM repeat domain among tomato, tobacco, and parsley CMPG1.

Nt CMPG1 and SI Cmpg1 mRNAs Are Induced after Avr9 Elicitation in Cf9 Tobacco and Tomato

To confirm the *Cf-9/Avr9*-dependent induction of tobacco *CMPG1* gene expression, we analyzed Nt *CMPG1* transcript levels after Avr9 elicitation. Cf9 tobacco cell cultures were elicited with Avr9 peptide (+Avr9) or mock inoculated (control), and tobacco *CMPG1* transcript levels were analyzed at different times after elicitation. RT-PCR was conducted with tobacco *CMPG1*-specific primers. *Actin* primers were used as a control for a constitutively expressed gene (Figure 2A). Consistent with the cDNA-AFLP data, Nt *CMPG1* mRNA levels were upregulated 30 min after Avr9 elicitation, reaching a maximum level by 4 h. No transcript induction was detected in the negative control. We also observed an induction of Nt *CMPG1* in Cf9 tobacco leaves after infiltration with a solution of intercellular fluid (IF) from Avr9-transgenic *N. tabacum* plants (+Avr9) or IF from tobacco wild-type plants (–Avr9). Elevated transcript levels were detected 15 min after infiltration with a maximum level at 30 min in both treatments (Figure 2B). However, the addition of IF(+Avr9) produced a stronger induction of tobacco *CMPG1* gene expression at the 30-min time point, and the expression was maintained over 2 h of the time course. Transient upregulation of gene expression by the mechanical stress caused by infiltration has been observed for other *ACRE* genes (Durrant et al., 2000; Rowland et al., 2005).

To investigate the role of tomato *Cmpg1* as a putative ortholog of tobacco *CMPG1*, we analyzed the expression pattern of SI *Cmpg1* after Avr9 elicitation. Cf9 tomato plants were infiltrated with IF with or without Avr9, and tomato *Cmpg1* transcript levels were analyzed by RT-PCR (Figure 2C). As observed for Nt *CMPG1*, SI *Cmpg1* transcript levels were induced after infiltration with both IF(+Avr9) and IF(–Avr9), but again, the induction was stronger and lasted longer after infiltration with IF(+Avr9). These results suggest a role for tomato *Cmpg1* in the *Cf-9/Avr9*-dependent response and the possibility that SI *Cmpg1* is the Nt *CMPG1* ortholog.

Tobacco CMPG1 Has in Vitro Ubiquitination Activity

We wanted to determine if tobacco *CMPG1* has in vitro ubiquitination activity. A full-length clone of Nt *CMPG1* was expressed in *Escherichia coli* as a C-terminal fusion with glutathione S-transferase (GST) and purified by affinity chromatography. Ubiquitination assays were performed using yeast E1 and three different E2 enzymes (UbcH5a, UbcH5b, and UbcH5c). As a positive control, the *Arabidopsis* U-box protein PUB26 (Azevedo et al., 2001) fused to GST was assayed. The reactions were separated on an SDS gel and transferred to nitrocellulose membranes. Ubiquitinated proteins were detected using anti-ubiquitin antibody, and *CMPG1* was detected using anti-GST antibody (Figures 3A and 3B). Tobacco *CMPG1*-GST displayed E3 ligase activity in the presence of UbcH5b and UbcH5c but not with UbcH5a (Figure 3A, lines 6 to 8). E3 ligase activity was not observed in the absence of any of the essential components of the reaction (E1, E2, E3, ubiquitin, or ATP; Figure 3, lines 1 to 5, respectively) or using GST alone as ubiquitin ligase (Figure 3, line 11). Thus, tobacco *CMPG1* has in vitro ubiquitination activity.

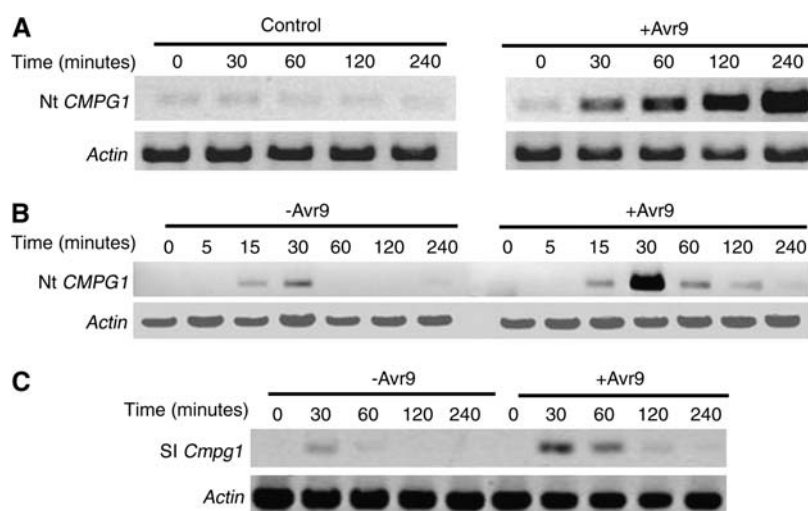


Figure 2. Expression Patterns of Nt *CMPG1* and SI *Cmpg1* after Avr9 Elicitation.

Cf9 tobacco cell cultures (A) were treated with Avr9 peptide (+Avr9) or untreated (control), and samples were harvested at the time points indicated. Cf9 tobacco plants (B) or Cf9 tomato plants (C) were infiltrated with IF that contains Avr9 (+Avr9) or that does not contain Avr9 (–Avr9), and plant leaf discs were harvested at the time points indicated. Total RNA was isolated and used for RT-PCR with specific primers for Nt *CMPG1*, SI *Cmpg1*, and *Actin*.

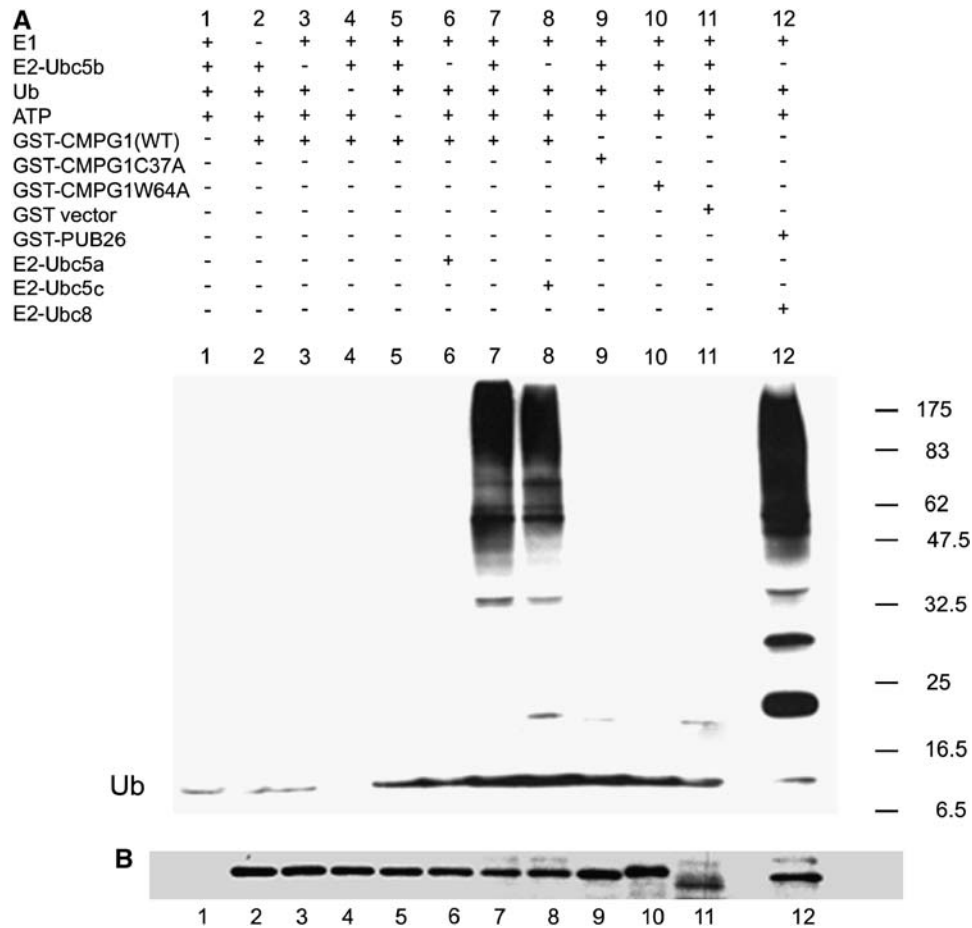


Figure 3. Nt CMPG1 Is an E3 Ligase, and Mutations in Its U-Box Domain Abolish Its Activity.

GST-Nt CMPG1, GST-Nt CMPG1C37A, and GST-Nt CMPG1W64A proteins were expressed and purified from *E. coli* and tested for E3 ligase activity using yeast E1 and three different E2s. GST-PUB26 with the E2 Ubc8 was used as a positive control (line 12). Lines 1 to 5 and 11 are negative controls. The reactions were analyzed by protein gel blots using anti-ubiquitin antibody (**A**) or anti-GST antibody (**B**).

We then investigated if, as for other U-box proteins described up to date, the U-box domain is essential for the E3 ligase activity of CMPG1. The U-box domain of E3 ligases is predicted to be involved in the interaction with the E2 ubiquitin-conjugating enzyme (Pringa et al., 2001), and several of its amino acids have been described to be essential for this interaction (Pringa et al., 2001; Ohi et al., 2003). The putatively E2-interacting amino acid Trp-64 and the highly conserved Cys-37 were mutated to Ala in the tobacco CMPG1 protein (Figure 1B). Both mutated proteins were expressed as GST fusions, and the E3 ligase activity was assayed as for the wild type. Figure 3 shows that neither of the CMPG1 mutants displayed ubiquitination activity (lines 9 and 10). Therefore, an intact U-box domain is essential for Nt CMPG1 E3 ligase activity.

Tobacco CMPG1 Is Required for Cf-9/Avr9-Dependent HR in *Nicotiana benthamiana* Plants

To investigate if CMPG1 protein is essential for Cf-9/Avr9-dependent HR, we performed VIGS in *N. benthamiana* plants

using tobacco rattle virus (TRV; Ratcliff et al., 2001) as a viral vector. A fragment of 374 bp from the 3' end of tobacco *CMPG1* was cloned in a TRV vector (TRV:Nt CMPG1). TRV empty vector (TRV:00) was used as a negative control. No differences in morphology were observed between *CMPG1*-silenced plants and those silenced with the empty vector. *SGT1* has been previously shown to be essential in Cf-9/Avr9-dependent HR production (Peart et al., 2002); therefore, the TRV:Nb SGT1 construct (Peart et al., 2002) was used as a positive control. *N. benthamiana* seedlings were inoculated via *Agrobacterium tumefaciens* with TRV:00, TRV:Nb SGT1, or TRV:Nt CMPG1. Three weeks later, agrobacteria expressing Cf-9 and Avr9 were delivered into the silenced plants, and HR was assessed after 5 d. No HR was observed in plants silenced for *SGT1*, consistent with previous results (Figure 4A, Table 1). We observed strong HR in 36 out of 43 TRV:00-silenced plants. The number of *CMPG1*-silenced plants that developed HR was much lower than in the TRV:00-silenced plants (Figure 4A, Table 1). Figure 4B shows that *CMPG1* transcript accumulation is reduced in two different plants carrying the TRV:Nt CMPG1 construct compared with

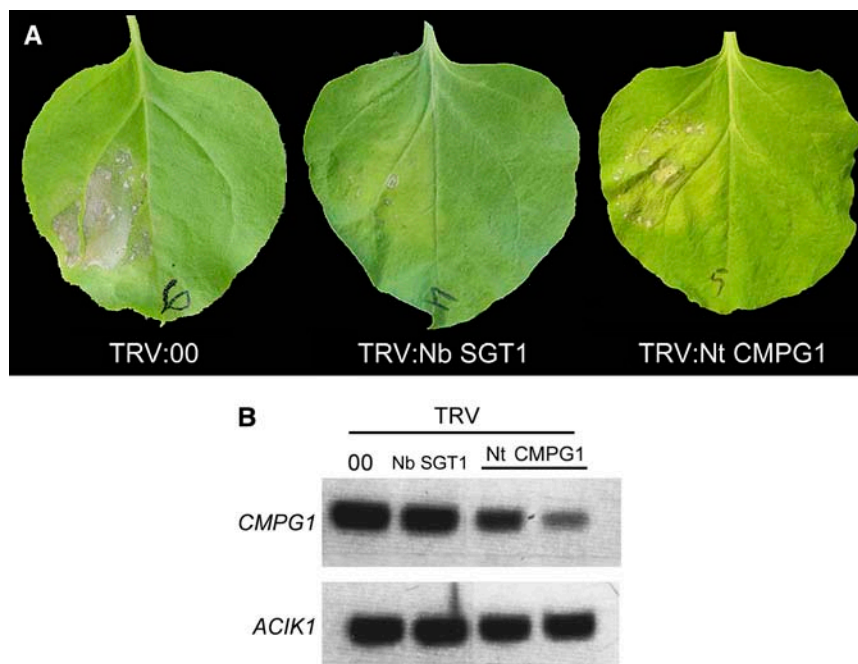


Figure 4. Nt *CMPG1* Is Required for Efficient HR Mediated by Cf-9 in *N. benthamiana*.

(A) VIGS in *N. benthamiana* using TRV. Seedlings were inoculated with TRV:Nt *CMPG1*, TRV:Nb SGT1, or empty vector as a control (TRV:00). Three weeks after inoculation, leaves were infiltrated with agrobacteria delivering Cf-9 and Avr9. Pictures were taken 4 d after agrobacteria infiltration.

(B) RT-PCR from silenced plants. *N. benthamiana* leaves silenced with the indicated TRV construct were infiltrated with water to induce Nb *CMPG1* expression. Samples were collected 30 min after infiltration, and RNA was isolated and used for RT-PCR. Nb *ACIK1* was used as a control for induction by flooding (bottom panel).

control. This experiment was repeated three times, and in each repetition, lower levels of tobacco *CMPG1* were observed in at least three plants carrying TRV:Nt *CMPG1* and showing low HR symptoms. These data indicate that *CMPG1* is essential for efficient production of Cf-9/Avr9-mediated HR in *N. benthamiana* plants.

Overexpression of Nt *CMPG1* Increases the HR in Cf9 Tobacco Plants after Avr9 Elicitation

To confirm the role of tobacco *CMPG1* in HR, we overexpressed Nt *CMPG1* in tobacco plants. We engineered a construct to produce tandem affinity purification (TAP)-tagged (Rigaut et al., 1999) *CMPG1* protein under the control of the 35S promoter, and stable transformants in Cf9 tobacco were generated. Ten independent transgenic lines were analyzed for HR and for *CMPG1*:TAP protein accumulation. Tobacco leaves from transgenic and

nontransgenic (control) plants were infiltrated with 1/16 dilution of IF(+Avr9) and IF(-Avr9) (Figure 5A). A weak HR was produced at this low concentration of Avr9 in control plants (Figure 5A, left panel), while 3 out of the 10 transgenic tobacco lines developed strong HR symptoms (Figure 5A, lines B, C, and J). *CMPG1*:TAP protein levels were analyzed by immunoblots in the same tobacco leaves that were infiltrated for HR production (Figure 5B). We observed that the three tobacco lines that responded with a strong HR accumulate high levels of *CMPG1* protein. A higher molecular mass (~75 kD) second band can be detected in some of the tobacco transgenic lines. This band was found in different lines in different experiments, and it does not correlate with the strong HR phenotype. We speculate that this higher molecular form of tobacco *CMPG1* might be due to a posttranslational modification that has not been further investigated. We chose the line with the strongest HR phenotype (line J) to analyze the response to Avr9 using a different approach. Ten-day-old seedlings from tobacco *CMPG1*:TAP line J and control were transferred to Murashige and Skoog (MS) medium containing different concentrations of IF(+Avr9) or IF(-Avr9) (Figures 5C and 5D). The presence of Avr9 in the medium elicited a response in Cf9 seedlings that is visualized by a growth inhibition effect in an Avr9 concentration-dependent manner (Figure 5C, left panel). Plants overexpressing *CMPG1* are more sensitive to Cf-9/Avr9-dependent growth inhibition at two different Avr9 concentrations (Figures 5C and 5D). In conclusion, by two different approaches, we have

Table 1. Number of TRV-Silenced Leaves of *N. benthamiana* Showing HR after Cf-9/Avr9 Elicitation

Silencing Construct	Leaves Producing HR	Total Leaves Infiltrated	Leaves Producing HR (%)
TRV:00	36	43	83.7
TRV:Nb SGT1	2	43	4.6
TRV:Nt <i>CMPG1</i>	13	40	32.5

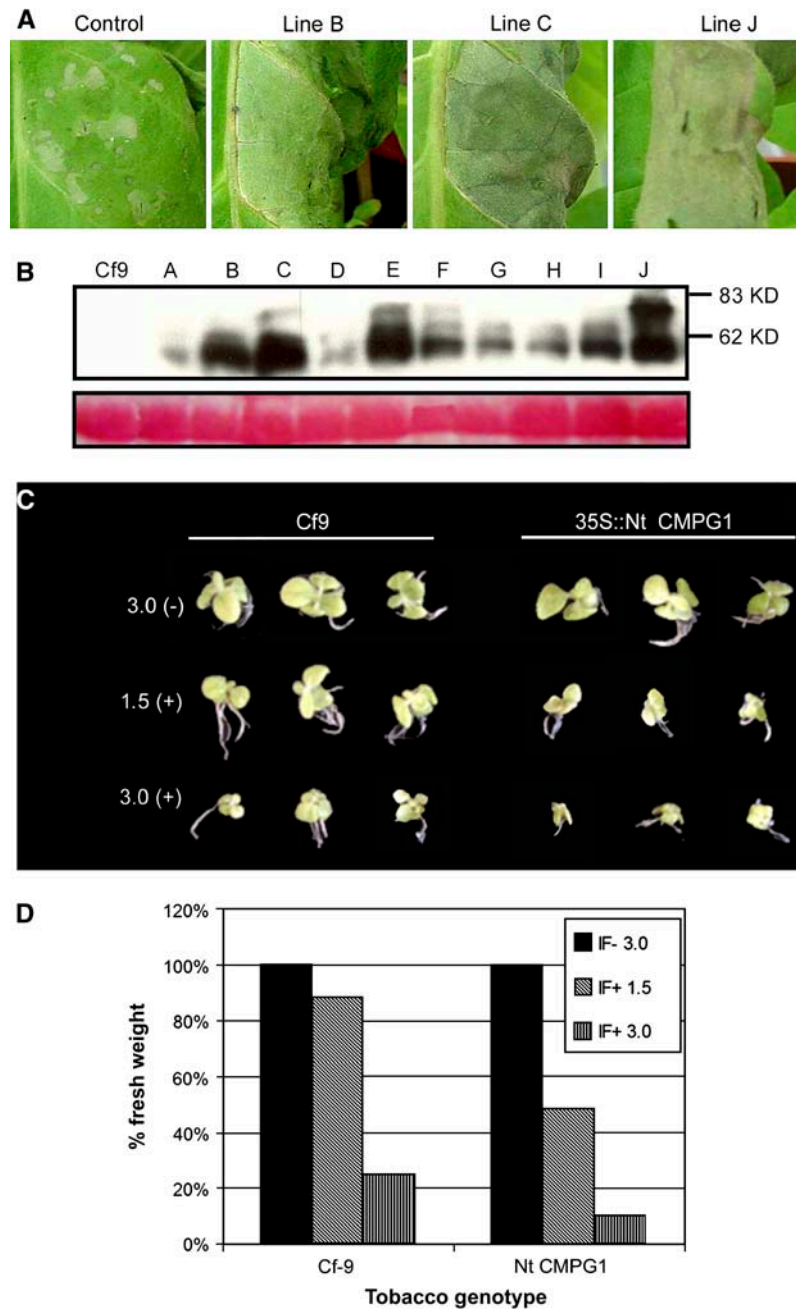


Figure 5. Overexpression of Nt CMPG1 Induces a Stronger HR Response to Avr9 in Cf9 Tobacco Plants.

(A) HR in *N. tabacum*. Cf9 tobacco plants were transformed with 35S:Nt CMPG1:TAP and analyzed for Avr9-dependent HR production. Tobacco leaves from three independent transgenic lines and a control were infiltrated with a 1/16 dilution of IF(+Avr9). Pictures were taken 3 d after infiltration.

(B) Nt CMPG1 protein levels were analyzed by protein gel blots. Leaf discs were harvested from different Cf9 tobacco lines expressing 35S:Nt CMPG1:TAP or control leaves. Total proteins were separated in SDS gels and analyzed by immunoblotting using PAP antibody for Nt CMPG1:TAP detection. Bottom panel shows Ponceau S Red staining of ribulose-1,5-bisphosphate carboxylase/oxygenase for confirmation of equal loading.

(C) Growth inhibition assay. Cf9 tobacco seedlings expressing Nt CMPG1:TAP (line J) and control were transferred to MS medium containing the indicated amount of IF(+Avr9) (+) or IF(-Avr9) (-) (μL per mL of MS medium). Pictures were taken 10 d after transfer to MS medium.

(D) Growth inhibition assay. Cf9 tobacco seedlings were transferred to MS medium containing IF(-Avr9) or IF(+Avr9) as described in **(C)**. Ten days after the transfer, the same number of seedlings was weighted for each treatment. The percentage was calculated based on the weight of seedling growth in IF(-Avr9) containing MS medium.

observed a stronger Avr9-dependent HR in tobacco plants overexpressing tobacco *CMPG1*. These results suggest that Nt *CMPG1* is a rate-limiting protein in HR production triggered by *Cf-9*.

Mutations in Essential Amino Acids of Nt *CMPG1* U-Box Domain Confer a Dominant-Negative Effect in *Cf9* Tobacco Plants

We wanted to investigate if the role of tobacco *CMPG1* in defense is dependent on its E3 ligase activity. For this purpose, we compared *Cf-9*-dependent HR production in tobacco plants overexpressing tobacco *CMPG1* wild type and the two mutants (*CMPG1W64A* and *CMPG1C37A*) that did not show activity in the ubiquitination experiments (Figure 3).

We generated transgenic plants carrying 35S promoter fusions for overexpression in *Cf9* tobacco of TAP-tagged *CMPG1W64A* (11 plants) and *CMPG1C37A* (eight plants). Untransformed (control), wild-type Nt *CMPG1*-transformed, and mutated Nt *CMPG1*-transformed tobacco leaves were infiltrated with IF(+Avr9), and HR was observed 3 d after infiltration. We observed a reduced HR compared with controls after Avr9 elicitation in 2/8 primary transformants carrying *CMPG1C37A* (Figure 6A), suggesting a dominant-negative effect of this mutation. By contrast, none of the *CMPG1W64A* T1 transgenic lines showed a clear HR reduction compared with untransformed controls (Figure 6A). Immunoblot analysis indicated the presence of the protein in the leaves tested for HR. The differences observed in protein level accumulation are correlated with the locus number of the transgene (based on segregation analysis) (Figure 6B; see Supplemental Table 1 online). Segregating T2 tobacco plants carrying either tobacco *CMPG1* mutant were analyzed for HR and protein levels (Figures 6C and 6D). We observed no HR in the tobacco plants that accumulated high levels of *CMPG1C37A* protein. Similar results were found in the analysis of *CMPG1W64A* lines B and D, where six segregating T2 tobacco plants were analyzed for protein accumulation and HR production. High levels of *CMPG1W64A* protein were correlated with a reduced HR (see Supplemental Table 1 and Figure 1 online) in both lines. Supplemental Figure 1 online shows the HR production and *CMPG1W64A* protein accumulation of the line D. Similar results were found for line B. Only two of the lines that carried more than one T-DNA locus (based on segregation results; data not shown) showed a dominant-negative phenotype in the T2 for the Nt *CMPG1W64A* mutant (see Supplemental Table 1 online).

Tobacco *CMPG1* Is Involved in Pto/AvrPto and Inf1-Mediated Cell Death

To investigate if tobacco *CMPG1* function is specific to *Cf-9*-dependent responses or if it is involved in different defense mechanisms, we analyzed the Pto/AvrPto-dependent (gene-for-gene system) and Inf1-dependent (general elicitor) HR in tobacco plants overexpressing *CMPG1* and *CMPG1C37A*. Pto is a tomato R protein that confers resistance against *Pseudomonas syringae* carrying *AvrPto* (Pedley and Martin, 2003). By contrast, Inf1 is a secreted protein from *Phytophthora infestans* that induces HR in *Nicotiana* spp (Kamoun et al., 1998).

Tobacco leaves were infiltrated with different densities of agrobacteria that confer transient expression of *Pto* and *AvrPto* (Figure 6E) or Inf1 (see Supplemental Figure 2A online) and HR assessed. Pictures were taken 4 d after infiltration. Tobacco *CMPG1* proteins levels are homogeneous among the individual plants in each line. Interestingly, Figure 6E showed that after *Pto/AvrPto* infiltration, HR was stronger in tobacco leaves overexpressing *CMPG1* compared with nontransgenic control, while no HR was observed in tobacco plants expressing *CMPG1C37A*. A weaker but consistent effect on HR production was observed in similar experiments with Inf1 elicitor (see Supplemental Figure 2A online). To confirm the involvement of tobacco *CMPG1* in Inf1-dependent HR production, stable tobacco lines carrying a hairpin tobacco *CMPG1* fragment were engineered. Ten independent tobacco *Cf9* transformants were generated, and two lines showing low *Cf-9*-dependent HR production were selected (see Supplemental Figure 2B online). Inf1 elicitor was infiltrated via agrobacteria in the same tobacco lines carrying *CMPG1* hairpin and untransformed controls. Supplemental Figure 2C online shows that the HR production is clearly reduced in the two lines silenced for tobacco *CMPG1*. RT-PCR experiments showed that the accumulation of *CMPG1* transcript was clearly reduced in both lines (see Supplemental Figure 2D online). These results suggest that tobacco *CMPG1* is involved in plant defense mechanisms triggered by various elicitors.

Cmpg1 Is Involved in Resistance to *C. fulvum* in Tomato *Cf9* Plants

To investigate if *CMPG1* is involved in resistance of tomato to *C. fulvum*, in addition to HR production, we silenced Sl *Cmpg1* in tomato plants using two different approaches, VIGS and double-stranded RNA hairpin-induced silencing. Similar to *CMPG1*-silenced tobacco plants, no differences in morphology were observed when tomato *Cmpg1* was silenced with any of the approaches. For the first approach, the TRV-based vector described by Liu et al. (2002a) was used. A 200-bp fragment of the 5' coding sequence of tomato *Cmpg1* was cloned in the TRV vector (TRV:*Cmpg1*). As controls, TRV empty vector (TRV:00) and TRV carrying a *Cf-9* fragment previously described (TRV:*Cf9*; Rowland et al., 2005) were used. TRV constructs were inoculated via agrobacteria in cotyledons of either *Cf0* tomato seedlings or tomato lines transgenic for the *Cf-9* gene. Three weeks after inoculation, tomato plants were analyzed for HR, *C. fulvum* infection, and tomato *Cmpg1* transcript accumulation. Three independent tomato plants were silenced with each construct in each experiment. Tomato-silenced plants were infiltrated with IF(+Avr9) or IF(-Avr9) (Figure 7A, right and left halves of the leaf, respectively) to confirm the HR phenotype observed in *N. benthamiana* plants silenced for tobacco *CMPG1* (Figure 4A). Figure 7A shows HR production in *Cf9* tomato plants silenced with the different TRV constructs. As expected, HR was highly reduced in tomato plants silenced for *Cmpg1* and *Cf-9*, but strong HR was observed in tomato plants silenced with the empty vector (Table 2). These data confirm those obtained using VIGS in *N. benthamiana*.

To study the role of *Cmpg1* in resistance to *C. fulvum*, tomato plants were infected with *C. fulvum* race 4 β -glucuronidase

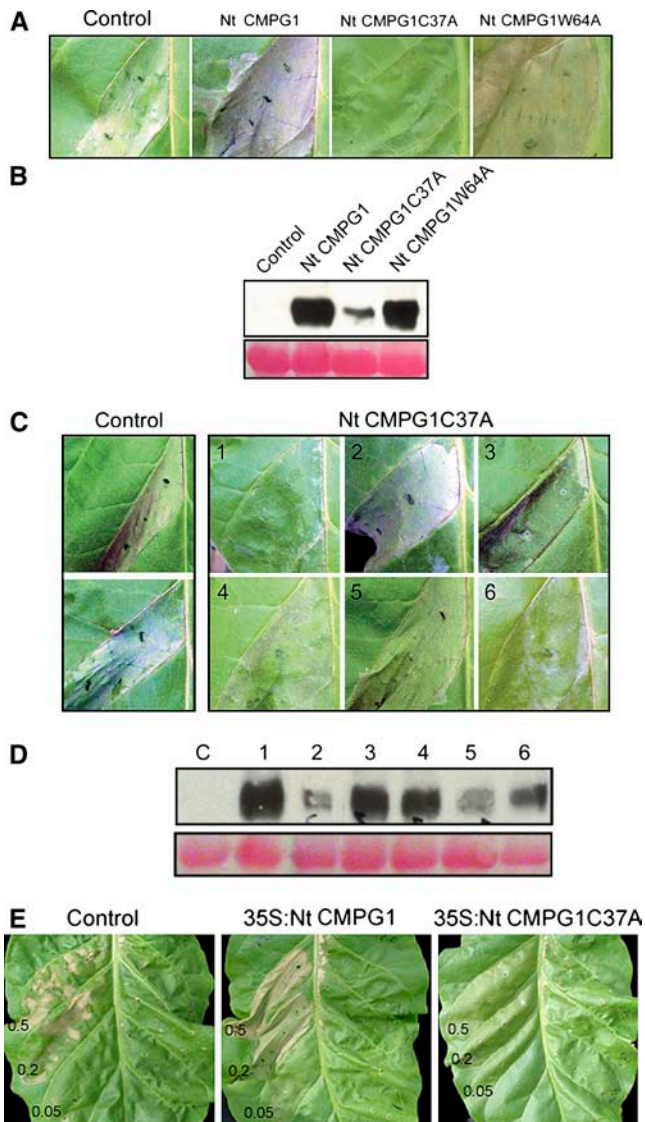


Figure 6. Overexpression of Nt CMPG1 Dominant-Negative Mutants.

(A) Leaf panels from Cf9 tobacco control plants (control) and Cf9 tobacco plants transgenic for Nt CMPG1 wild type and mutants (C37A line I and W64A line B) were infiltrated with IF(+Avr9) for HR induction. Pictures were taken 3 d after infiltration.

(B) Tobacco leaf discs were harvested from the same leaves shown in **(A)**, and Nt CMPG1:TAP protein levels were determined as described in Figure 5B.

(C) The presence of high levels of Nt CMPG1C37A correlates with a reduced HR production. Two leaves of Cf9 tobacco control plants (left panels) and of six different plants of the segregating population of transgenic Nt CMPG1C37A tobacco (right panels) were infiltrated with IF(+Avr9) for HR induction. Pictures were taken 3 d after infiltration.

(D) Nt CMPG1C37A protein levels were analyzed by protein gel blots. Tobacco leaf discs were harvested from the same leaves shown in **(C)**, and Nt CMPG1C37A:TAP protein levels were determined as described in Figure 5B. Numbers on top of the lanes correspond to the numbers of the leaves shown in **(C)**. C, control leaf.

(E) Leaf panels of Cf9 tobacco control and Cf9 tobacco overexpressing Nt CMPG1 wild type or Nt CMPG1C37A were infiltrated with agro-

(GUS) 3 weeks after silencing. The expression of the GUS gene in the fungal race allows the detection of the fungus after staining with 5-bromo-4-chloro-3-indolyl- β -glucuronide (X-gluc). Three weeks after inoculation, at least 12 leaves from three different plants were stained with X-gluc for each TRV construct (Figure 7B, top panel). We observed blue staining in the leaves of Cf0 tomato plants, indicating fungal infection. We examined the blue spots under the microscope, where we observed the conidiophores emerging from the stomata, indicating that the fungus completed its cycle (Figure 7B, bottom panel). Interestingly, we also observed X-gluc staining in the Cf9 tomato leaves from plants silenced for *Cf-9* or tomato *Cmpg1*. The same reproductive structures as in Cf0 plants were observed under the microscope. This result indicates that *Cmpg1* is essential for full tomato resistance to *C. fulvum*. The number of infection patches was much lower in Cf9 plants silenced for *Cf-9* or *Cmpg1* in comparison with Cf0 plants. However, this phenotype was consistent, and for tomato *Cmpg1*-silenced plants, 18 out of 50 leaves showed at least one blue spot (Table 2), while in *Cf-9*-silenced plants, 16 out of 42 leaves presented at least one blue spot. No blue staining was detected in Cf9 tomato leaves silenced with the empty vector, indicating that the phenotype observed is not due to the virus. Finally, we confirmed that *Cmpg1* transcript levels were reduced in the silenced plants. mRNA levels were determined by RT-PCR using tomato *Cmpg1*-specific primers and *Actin* primers as a control, in both empty vector and *Cmpg1*-silenced plants (Figure 7C). We could observe highly reduced levels of transcript accumulation in comparison with the empty vector in three independent silenced plants showing low HR accumulation.

To confirm VIGS results, RNA interference (RNAi)-induced silencing was performed. Cf9 tomato plants carrying *Cf-9* and -4 linked paralogs (Parniske et al., 1997) were transformed with the pHellsgate8 vector (Wesley et al., 2001; Helliwell and Waterhouse, 2003) carrying a tomato *Cmpg1* hairpin fragment. Eight independent transformant plants were obtained. Six leaves from each line were infiltrated with IF(+Avr9), and HR was observed. Six out of eight transformants showed clear reduction in HR in the six infiltrated leaves, while the other two lines showed similar HR to the nontransformed Cf9 tomato plants. Three lines were further analyzed, and we could correlate the levels of tomato *Cmpg1* mRNA with *PATHOGENESIS-RELATED5* (*PR5*) mRNA levels, HR development, and *C. fulvum* infection symptoms (see Supplemental Figure 3 online). *C. fulvum* infection was only observed in the line showing the lowest *Cmpg1* and *PR5* mRNA accumulation. The result obtained with the best line is shown in Figure 7. RT-PCR was conducted on RNA from untransformed (control) and transgenic (*Cmpg1* RNAi) Cf9 plants after elicitation with flooding (Figure 7F, top panel). Tomato *Acik1* (Rowland et al., 2005) and *Actin* primers were used as a control for an inducible and a constitutively expressed gene, respectively. Tomato *Cmpg1* transcript levels were highly reduced in Cf9 plants carrying the hairpin construct. Untransformed (control) and transgenic (*Cmpg1* RNAi)

bacteria delivering Pto and AvrPto at the indicated cell densities. Pictures were taken 4 d after infiltration.

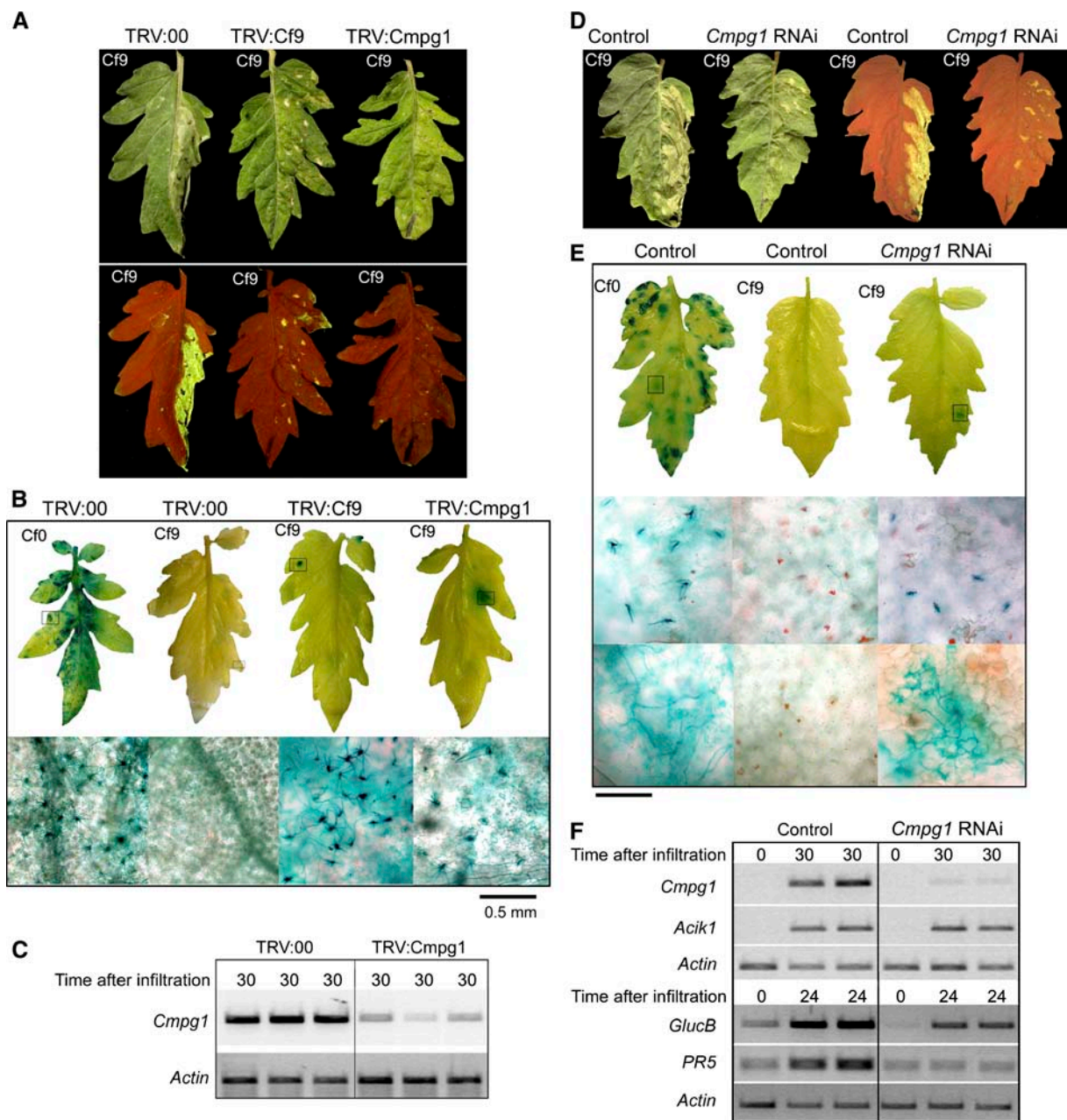


Figure 7. Tomato *Cmpg1* Is Required for Full *Cf-9*-Mediated Resistance to *C. fulvum* Expressing Avr9.

(A) VIGS in tomato. Transgenic *Cf9* tomato cotyledons were inoculated with agrobacteria carrying the indicated TRV construct. Three weeks after infiltration, the right half of the tomato leaf was infiltrated with IF(+Avr9), and pictures were taken 3 d after infiltration under daylight (top panel) or UV light (bottom panel).

(B) Transgenic *Cf9* or *Cf0* tomato seedlings were silenced with the indicated TRV construct as described in **(A)**. Three weeks later, plants were infected with *C. fulvum* race 4 GUS. Leaves were stained with X-gluc 3 weeks after *C. fulvum* inoculation, when pictures were taken (top panel). The fungal cycle was complete, as indicated by the conidiophores extruding from the stomata observed under the microscope (bottom panel).

(C) Tomato *Cmpg1* transcript levels were analyzed by RT-PCR. Leaf discs from the indicated silenced tomato plants were harvested 30 min after infiltration with water. Total RNA was extracted and used for RT-PCR analysis with SI *Cmpg1*-specific primers. Equal amounts of cDNA were used, as shown by the amplification with the constitutively expressed *Actin* gene.

(D) RNAi in tomato. *Cf9* tomato plants were transformed with pHellsgate containing a tomato *Cmpg1* hairpin. One leaf half from the transgenic (*Cmpg1* RNAi) and untransformed (control) *Cf9* tomato plants was infiltrated with IF(+Avr9) for HR induction. Pictures were taken 3 d after infiltration under daylight (left leaves) and UV light (right leaves).

(E) *Cf0* and *Cf9* untransformed (control) and *Cf9* transgenic (*Cmpg1* RNAi) tomato plants were infected with *C. fulvum* race 4 GUS. Leaves were

Table 2. Proportion of VIGS-Silenced Tomato Leaves Developing HR or Fungal Infection

Silencing Construct	Tomato Genotype	HR	<i>C. fulvum</i> Growth
TRV:00	Cf0	NT ^a	35/35
TRV:00	Cf-9	25/32	0/33
TRV:Cf-9	Cf-9	11/30	16/42
TRV:Cmpg1	Cf-9	4/29	18/50

^a NT, not tested.

Cf9 tomato plants were also infiltrated with IF(+Avr9) or IF(-Avr9) (Figure 7D, right and left half of the leaf, respectively). Three days later, HR was examined under daylight (left leaves) and UV light (right leaves). Sixteen out of 18 control leaves showed a strong HR as in the picture, while only 6 out of 18 *Cmpg1* RNAi leaves showed HR (Figure 7D, Table 3). Cf9 control, Cf9 *Cmpg1* RNAi, and Cf0 control plants were infected with *C. fulvum* race 4 GUS as before. Three weeks after infection, leaves were stained with X-gluc to score for fungal growth. Again, we could observe strong X-gluc staining in Cf0 tomato plants, where the presence of conidiophores was confirmed under the microscope (Figure 7E). We also detected patches of fungal growth in *Cmpg1* RNAi Cf9 tomato. As before, the infection in *Cmpg1*-silenced Cf9 plants was much less severe than in the Cf0 controls. We could observe fungal hyphae growing inside the leaves in all the blue patches of the transgenic plants (Figure 7E, bottom panel, Table 3), but we only detected reproductive structures in 10 out of the 19 leaves with blue patches (Figure 7E, middle panel, Table 3).

To further characterize the Cf-9-dependent defense response in tomato *Cmpg1*-silenced plants, the induction of defense-related genes was analyzed by RT-PCR. *Cmpg1* RNAi and control tomato plants were infiltrated with IF(+Avr9), and samples were taken at 0 and 24 h after infiltration. Transcript levels of two defense-related genes, *PR5* (Cornelissen et al., 1986; Hejgaard et al., 1991) and *basic β-1, 3-glucanase (GlucB)*; Niki et al., 1998), were analyzed (Figure 7F, bottom panel). *Cmpg1* RNAi plants showed a reduced induction of both genes at 24 h after infiltration in two independent samples.

DISCUSSION

Tobacco *CMPG1* Encodes a U-Box E3 Ligase and Is Rapidly Upregulated after Stress Stimuli

We sought to identify genes upregulated during the Cf-9/Avr9 defense response to gain further insight into the mechanisms involved. This generated a list of upregulated genes that was

scrutinized for potential signaling components and assessed for whether they are required for the Cf-9-mediated HR. Previously, we reported that two of the three *ACRE* genes identified as essential for Cf-9-mediated HR are predicted to encode components of the ubiquitylation machinery (Rowland et al., 2005). Further analysis of the previously published *ACRE74* cDNA fragment revealed homology to parsley *CMPG1*, a rapidly elicited gene after elicitor treatment (Kirsch et al., 2001). A full-length *ACRE74* cDNA was named Nt *CMPG1*. It shows strong similarity to *Arabidopsis* PUB20 and PUB21 and encodes a protein with an N-terminal U-box domain. Tobacco *CMPG1* displays E3 ligase activity that is dependent on an intact U-box domain. Three highly similar E2 proteins (UbcH5a, UbcH5b, and UbcH5c; Kraft et al., 2005) were used in our experiments. We showed that Nt *CMPG1* ubiquitinates with two E2 proteins (UbcH5b and UbcH5c), while no activity was observed with UbcH5a. These data indicate that the E2 specificity is a determining factor in the activity of tobacco *CMPG1*.

Tobacco and tomato *CMPG1* gene expression are induced after elicitor and wounding treatments (Figure 2), a typical *ACRE* gene expression pattern (Durrant et al., 2000; Rowland et al., 2005). Parsley *CMPG1* gene expression is induced 5 min after elicitation in parsley cell cultures (Kirsch et al., 2001), reaching maximum levels 1 h after elicitor treatment. Expression of the *Arabidopsis* *CMPG1* homologs is also elevated after pathogen treatment (Heise et al., 2002; Navarro et al., 2004). In a search for *Arabidopsis* orthologs of parsley *CMPG1*, Heise et al. (2002) identified and classified several At PUB proteins as At *CMPG*-like. In this classification, At PUB20 corresponds to At *CMPG1*, and At PUB21 to At *CMPG5*. *Arabidopsis* PUB20 expression is induced after treatment with the pathogen elicitors *Pmg* and *flg22* and after infection with *P. syringae* pv *tomato* strain DC3000, reaching maximum expression between 30 and 60 min after elicitation (Heise et al., 2002; Navarro et al., 2004). These data suggest a similar role for tobacco, tomato, *Arabidopsis*, and parsley *CMPG1* in plant defense signaling.

An alignment of Nt *CMPG1* with Sl *Cmpg1*, Pc *CMPG1*, At PUB20, and At PUB21 revealed high similarity in the U-box domain and also in the C-terminal region of the proteins (Figure 1A). The presence of ARM repeats has been described for Pc *CMPG1*, PUB20, and PUB21. ARM repeats are highly divergent in class III U-box proteins to which tomato and tobacco *CMPG1* belong (Mudgil et al., 2004). Based purely on sequence homology, ARM repeats were not found in Nt *CMPG1* and Sl *Cmpg1* proteins but were revealed by analysis of secondary structure. The putative ARM repeats are conserved among tobacco, tomato, and parsley *CMPG1* proteins (Figures 1A and 1C; Zeng et al., 2004), suggesting that this domain is essential for the function of *CMPG1*-like proteins.

Figure 7. (continued).

stained with X-gluc 3 weeks after infection (top panel). Fungal cycle was complete, as indicated by the conidiophores extruding from the stomata observed under the microscope (middle panel). Hyphal growth inside the leaves was confirmed under the microscope (bottom panel).

(F) Tomato *Cmpg1* mRNA levels were analyzed by RT-PCR. Leaf discs from the indicated tomato lines were harvested 30 min after IF(+Avr9) infiltration and processed as described in (C). Additionally, equal induction treatment is shown by the amplification of Sl *Acik1*. From the same leaves, samples were harvested after 24 h, and transcript levels for the defense-related genes *GlucB* and *PR5* were determined using gene-specific primers.

Table 3. Proportion of Hairpin-Silenced Tomato Leaves Developing HR or Fungal Infection

Transgene	Tomato Genotype	HR	<i>C. fulvum</i> Infection/Growth
–	Cf0	NT ^a	43/43
–	Cf9	16/18	0/55
<i>Cmpg1</i> RNAi	Cf9	6/18	19/60

^a NT, not tested.

Tobacco CMPG1 Is a Positive Regulator of Plant Defense Mechanisms

Using VIGS, we showed that tobacco and tomato *CMPG1* are essential for efficient HR activated by Cf-9 (Figures 4 and 7). Also, overexpression of tobacco *CMPG1* enhances the Cf-9-mediated HR in tobacco, consistent with the idea that CMPG1 positively contributes to the HR (Figure 5). Because a loss of HR is not always correlated with a loss of resistance (Bendahmane et al., 1999; Sharma et al., 2003), we also analyzed the response of SI *Cmpg1*-silenced Cf-9-containing tomato plants to *C. fulvum*. Using two different silencing methods, we showed that Cf9 tomato plants silenced for *Cmpg1* allowed *C. fulvum* growth (Figures 7B and 7E). *C. fulvum* infection symptoms were weaker in tomato *Cmpg1*-silenced plants using hairpin-induced silencing in comparison with VIGS. This difference might be due to the degree of silencing obtained with each method or because VIGS was performed on plants into which Cf-9 was introduced by transformation, and the hairpin silencing was performed in Cf9 plants that carried other Cf homologs, including Cf-9B (Panter et al., 2002).

Although with both silencing techniques the fungal colonization was weaker in Cf9 plants silenced for *Cmpg1* than in Cf0 control plants, in our VIGS experiments the results from silencing tomato *Cmpg1* and Cf-9 were comparable. Similar experiments showed that silencing of *R* genes does not lead to complete elimination of mRNA or resistance (Liu et al., 2002b; Brigneti et al., 2004). This suggests that tomato *Cmpg1* has a rate-limiting role in Cf-9-mediated resistance to *C. fulvum*. Finally, we showed that tomato *Cmpg1* regulates the expression of two different PR genes, *GlucB* and *PR5* (Figure 7F), identifying one of the defense pathways through which *Cmpg1* activates Cf-9-mediated resistance.

VIGS and hairpin-induced silencing of tomato *Cmpg1* were performed using a 200- and 400-bp fragment, respectively. Searches in the tomato TIGR database reveal no sequences with matches of 25 nucleotides or more with the fragments of *Cmpg1* used for silencing, indicating that the phenotype we observe is likely due to the specific silencing of *Cmpg1* and not to cross-silencing of other genes. Sequence analysis of the closest *Arabidopsis* homologs (PUB20 and PUB21) showed only one stretch with 25 identical nucleotides, which is not enough to permit PUB20 to silence PUB21 or vice versa. However, since the tomato and tobacco EST databases are not complete, we cannot exclude the possibility that other closely related genes might be targeted in our silencing experiments.

While rapid induction after elicitation has been previously shown for tobacco CMPG1 homologs in parsley and *Arabidopsis* (Kirsch et al., 2001; Heise et al., 2002; Navarro et al., 2004), our

data provide a functional assessment of this class of protein for defense, both for Cf-9- and Pto-mediated resistance and Inf1-triggered elicitation. The importance of the ubiquitylation pathway in plant defense mechanisms has been previously indicated (Devoto et al., 2003). The first evidence was obtained for SGT1 (Azevedo et al., 2002; Peart et al., 2002). The exact role of this protein is not clear yet, but yeast SGT1 interacts with the SCF complex, a ubiquitin E3 ligase (Kitagawa et al., 1999). Silencing of SGT1 compromises gene-for-gene and general defense mechanisms (Peart et al., 2002). Other reports suggest that the SCF complex is involved in regulation of plant defense mechanisms (Liu et al., 2002b; Xu et al., 2002). SPL11 is a U-box and ARM repeat domain-containing protein. Rice *sp11* mutants display spontaneous necrosis and enhanced resistance to fungal and bacterial pathogens, which surprisingly and paradoxically, is the opposite effect to the one observed for loss of CMPG1 function (Zeng et al., 2004). SPL11 could be involved in the activation of a negative regulator of defense, and its disruption would induce defense activation. This would reflect how the multiple regulators of plant defense need to be tightly coordinated in time and space and how this coordination has to be performed before (like SPL11, activating negative regulators) and after (like CMPG1, activating positive regulators; see below) pathogen elicitation.

Finally, a role in HR production has been shown for the two RING finger E3 ligases RIN2 and RIN3. The double mutant *rin2 rin3* develops a weaker RPM1- and RPS2-dependent HR, but it does not affect the growth of the pathogen in the plant (Kawasaki et al., 2005). As with CMPG1, these two proteins are positive regulators of HR, but distinct from CMPG1, they are not involved in restricting pathogen proliferation. This indicates the presence of common and independent signaling pathways between HR production and resistance, both controlled by E3 ligases.

It is interesting to note that all the E3 ligases identified in defense seem to play different roles in the signaling pathway. This variety of function is consistent with the presence of a high number of E3 ligases in plants (Downes and Vierstra, 2005), since the E3 ligase confers the specificity of the ubiquitination reaction (Hershko et al., 1983; Finley et al., 2004). The mode of action of the previously described E3 ligases is not known in any case. Only the targets of RIN2 and RIN3 have been identified, but the mechanism by which they positively regulate HR production is not understood (Kawasaki et al., 2005).

Mutations in the Tobacco CMPG1 U-Box Domain Confer a Dominant-Negative Phenotype

Tobacco CMPG1 displayed E3 in vitro ligase activity (Figure 3). To investigate if this activity is essential in the role of CMPG1 in plant defense mechanisms, we analyzed the defense response in Cf9 tobacco plants expressing the Nt CMPG1:TAP protein mutated in the U-box domain. We predicted that if CMPG1 with a mutated U-box binds to its target, it might be unable to complete the next step, resulting in a dominant-negative allele. We mutated the Trp in position 64 and the Cys in position 37 (of tobacco CMPG1). Several reports suggest that Trp is involved in the interaction with the E2 ubiquitin-conjugating enzyme in RING finger and U-box proteins (Joazeiro et al., 1999; Zheng et al., 2000; Andersen et al., 2004), and mutations in the Cys confer a

dominant-negative phenotype to the RING finger E3 ligase c-Cbl (Waterman et al., 1999). Mutation in either of these amino acids abolished the E3 ligase activity of tobacco CMPG1 (Figure 3). We also observed less *Cf-9/Avr9*-dependent HR when we overexpressed Nt CMPG1W64A and Nt CMPG1C37A (Figure 6; see Supplemental Table 1 online). In U-box domains, the Cys-37 residue is highly conserved and is adjacent to the E2-interacting amino acid Ile. In *Arabidopsis* PUB14, the Cys-37 corresponding residue is involved in the first hydrogen bond network of the U-box domain, and this network participates in the correct orientation of E2 binding residues (Andersen et al., 2004). Mutation of this Cys will prevent E2 interaction, which could explain the dominant-negative phenotype. The contrasting effect of overexpression of wild-type and mutant CMPG1 in tobacco suggests that the U-box domain is essential for CMPG1 function.

Model of the Role of CMPG1 in Plant Resistance

How might *CMPG1* contribute to *Cf-9*-triggered defense mechanisms? It could activate a positive regulator. There are several examples in the literature of activation of transcription factors through ubiquitination (Muratani and Tansey, 2003). Therefore, the positive regulator could be a transcription factor that activates different defense responses. Ubiquitination also regulates transcription by modifying histones and, therefore, chromatin (Bray et al., 2005). In animals, transcriptional activation of developmentally controlled genes is associated with the ubiquitination of histones (Bray et al., 2005). Protein kinases can also be activated through ubiquitination. The regulation of kinases by mono-ubiquitination has been recently shown in mammals (Aebersold et al., 2004), and it opens new possibilities of signaling control through the ubiquitination pathway. Another possible scenario would be that *CMPG1* activates defense by directing the degradation of negative regulators.

The fact that three E3 ligases (and two of them U-boxes) have been identified as positive regulators of *Cf-9*-dependent plant defense (Rowland et al., 2005) suggests that ubiquitination is a major regulator of this mechanism. This is consistent with the existence of a high number of U-box proteins in plants in comparison with other organisms (Azevedo et al., 2001), indicating that these proteins play multiple roles in plants. The U-box ACRE276 (Yang et al., 2006) regulates both HR production and pathogen arrest, while in the case of the F-box protein ACRE189, only a role in HR production has been found, and its involvement in inhibiting the pathogen growth has still to be shown (Rowland et al., 2005). How can three different ligases regulate the same defense pathway? The potential targets of ubiquitination in plant defense mechanisms are multiple; therefore, each E3 ligase could specifically modify one or several signaling components after *Cf-9* response activation (either activation of positive regulators or degradation of negative regulators). Also, any of the ligases could modulate activity of the receptor after the response has been elicited.

The data presented in this article do not discriminate between these possibilities. The identification of the target protein(s) of *CMPG1* and the two other E3 ligases is essential to understand the specific role of these proteins in defense mechanisms. The use of tobacco overexpressing a dominant-negative form of Nt

CMPG1 could be useful for this purpose. If the target protein is ubiquitinated for degradation, the overexpression of the dominant-negative *CMPG1* could result in its stabilization, facilitating its purification and identification. The dominant-negative form could facilitate the isolation of tobacco *CMPG1* protein targets, which will help us to understand how *CMPG1* controls *Cf-9*-mediated resistance in tomato.

METHODS

Plant Materials and Growth Conditions

Nicotiana benthamiana, *Solanum lycopersicum*, and *Nicotiana tabacum* plants were grown as previously described (Rowland et al., 2005). For tomato VIGS experiments, *S. lycopersicum* cv Moneymaker, carrying no known genes for resistance against *C. fulvum* (*Cf-0*), and the *Cf-9* transgenic tomato cv Moneymaker (Hammond-Kosack et al., 1998) were used. For pHellsgate transformation, *S. lycopersicum* cv Moneymaker containing an introgression segment that carries the *Cf-9* locus was used. For tobacco *CMPG1* TAP-tagged overexpression studies, the *Cf-9* transgenic *N. tabacum* cv Petite Havana line 8808J was used (Hammond-Kosack et al., 1998). Suspension cultures of *Cf9* tobacco cells derived from line 34.1B were subcultured as previously described (Piedras et al., 1998).

Isolation of Full-Length Tobacco *CMPG1* cDNA by Library Screening

The cDNA-AFLP fragment for ACRE74 was used as a probe to screen a cDNA library established from elicited tobacco cells (Durrant et al., 2000). Several clones were sequenced, and the clone containing the exact sequence of the ACRE74 cDNA-AFLP fragment was selected for further study.

Tomato *Cmpg1* 3'-RACE and Full-Length cDNA Cloning

The EST sequence TC159549 from the tomato TIGR gene index shared the highest identity with tobacco *CMPG1* along the 950 bp of the 5' end of Nt *CMPG1* cDNA and was named Sl *Cmpg1*. For tomato *Cmpg1* 3' cDNA cloning, total RNA was extracted from *Cf9* tomato plants after 30 min of infiltration with IF(+*Avr9*). cDNA was synthesized from 2 µg of total RNA using Superscript II reverse transcriptase (Invitrogen). Tomato *Cmpg1* 3' sequence was amplified with the gene-specific primer Le*CMPG1*F1 (5'-GAGGAAGTGATCTTGTCACACTCT-3') and oligo(dT) primer (5'-TTTTTTTTTTTTTTTTC/G-3'). Nested PCR was performed with the specific primer Le*CMPG1*F2 (5'-AAGTAGGAGGAATGCAGTTGTTGTC-3') and the oligo(dT) primer. PCR products were cloned into the pGem-T Easy plasmid (Promega), and independent clones were sequenced. A cDNA fragment that matched the sequence of the 5' end of the tomato EST TC159549 was selected. Primers Le*CMPGF4* (5'-CTTCGTTAGCTGATATTTGTTAG-3') and Le*CMPG1*R1 (5'-AATTACTAGACTAG-ATACTAAACTC-3') were used for PCR amplification of tomato *Cmpg1* full-length cDNA. The PCR product was cloned into the pGem-T Easy plasmid, and several independent clones were sequenced. A clone that matched the sequence of the original 3' and 5' Sl *Cmpg1* fragments was selected.

Sequence Analysis

Protein alignment was done using ClustalW (Thompson et al., 1994) and edited using the GeneDoc program (K.B. Nicolas and H.B. Nicolas Jr., GeneDoc: a tool for editing and annotating multiple sequence alignment; <http://www.psc.edu/biomed/genedoc>). The protein domains were identified using the PRODOM (Servant et al., 2002) and SMART (Schultz et al., 1998; Letunic et al., 2004) programs. Protein secondary structure

predictions were done using the programs JPRED (Cuff et al., 1998) and PSIPRED (Jones, 1999; McGuffin et al., 2000).

RT-PCR

Total RNA from leaves or from cell cultures was extracted using the Tri Reagent method according to the manufacturer's recommendations (Sigma-Aldrich). First-strand cDNA was synthesized from 2 μ g of total RNA using Superscript II reverse transcriptase (Invitrogen). RT-PCR was performed as described before (Rowland et al., 2005) using the primers 74-1 (5'-GAGAAATGCTTCAACAGGGGGAAG-3') and 74-2 (5'-CAC-CTAATGCCTTTTCACATATGC-3') for amplification from tobacco; Le74F (5'-ATGATTGCAACATGGAGAAAAAGAG-3') and Le74R2 (5'-ACAAG-CCCTTTTCACCCC-3') primers were used for amplification of the tomato *Cmpg1*. For *Actin* control amplification, primers NtActin1 (5'-ATGGCAGCGGTGAGGATATTCA-3') and NtActin2 (5'-GCCTTTGCAATCCACATCTGTTG-3') were used. LeACIK-ffwd (5'-AGTATAACAGCTATGGCTACTGCGGA-3') and LeACIK-RTPCRrev (5'-CCAAATCAAGCAATT-TAACAG-3') primers were used for *LeACIK1* amplification; finally, RT-LePR5F (5'-ATGGGGTAAACCACCAACA-3') and RT-LePR5R (5'-AAG-TGAACCAGGGCATTAC-3') primers were used for amplification of *PR5*. For amplification of the basic glucanase gene, primers were used as described before (Rivas et al., 2004).

Recombinant Protein Purification and E3 Ubiquitin Ligase Activity Assay

The creation of the GST-Nt *CMPG1* fusion constructs (wild type, Nt *CMPG1C37A*, and Nt *CMPG1W64A*) was conducted as follows. The modified primers *CMPG-GST-BamHI-F* (5'-ATGCTGGATCCATGATGATTTCAACATGGAGAAAAG-3') and *CMPG-GST-XhoI-R* (5'-GTC-AGACTCGAGTCAGAATGGCCTTTTGAGACTCTT-3'), constructed with *BamHI* and *XhoI* sites, respectively, were used to amplify the tobacco *CMPG1* cDNA fragments (wild-type and dominant-negative mutants) using the proofreading DNA polymerase Pfu (Stratagene). PCR products were gel purified, digested with *BamHI* and *XhoI*, and ligated into the *BamHI-XhoI*-digested pGEX 4T1 (Pharmacia Biotech). The generated GST:Nt *CMPG1* plasmids were transformed into *Escherichia coli* BL21(DE3) (Invitrogen) bacteria cells for protein expression. Overnight cultures of the bacteria were diluted 100 times in a total volume of 50 mL Luria-Bertani medium and further incubated at 28°C to reach an OD of 0.8. Induction of the GST:Nt *CMPG1* proteins was performed with 0.1 mM isopropylthio- β -galactoside for 3 h at 28°C. After the induction, cells were harvested by centrifugation at 13,000 rpm for 15 min, washed with 1 \times PBS (140 mM NaCl, 2.7 mM KCl, 10 mM NaH₂PO₄·7H₂O, and 1.8 mM KH₂PO₄), and further resuspended in 2.5 mL of 1 \times PBS amended with protease inhibitors (Roche). Cells were sonicated, and the supernatants were incubated for an additional 30 min at 4°C with 1% Triton X-100 to aid in solubilization of the proteins. The protein extracts were centrifuged, the supernatants were incubated with Glutathione Sepharose beads (Amersham Pharmacia), and the proteins were purified according to the manufacturer's recommendations. The fused GST:Nt *CMPG1* wild-type and mutated proteins were eluted with 0.5 mM glutathione elution buffer and further used for the ubiquitination assays.

For the in vitro ubiquitination assays, each reaction (30 μ L final volume) contained 40 mM Tris-HCl, pH 7.4, 5 mM MgCl₂, 50 mM KCl, 2 mM ATP, 1 mM DTT, 10% glycerol, 10 mM phosphocreatine, 3.5 units of phosphocreatine kinase (Sigma-Aldrich), 0.6 units of inorganic pyrophosphatase (Sigma-Aldrich), 0.1 μ g of yeast E1 (Affiniti Research Products), 0.5 μ g of E2 UbcH5a, UbcH5b, or UbcH5c (Affiniti Research Products), and ~0.5 μ g of E3s (eluted GST-Nt *CMPG1* wild type or mutated proteins or GST-PUB26). The reactions were incubated at 30°C for 3 h and stopped by adding 2 \times SDS-PAGE sample buffer (0.5 M Tris-HCl, pH 6.8, 20% [v/v] glycerol, 10% [w/v] SDS, 100 mM DTT, and 1% bromophenol

blue) at 65°C for 5 min and analyzed by SDS-PAGE electrophoresis followed by protein gel blotting using anti-ubiquitin antibody raised in mouse (Santa Cruz Biotechnology) and anti-GST antibody raised in goat (Amersham Pharmacia).

TRV-Based VIGS in *N. benthamiana*

A 380-bp fragment of tobacco *CMPG1* was obtained by *HindIII* digestion and cloned into the *HindIII* site of pTV00 (Ratcliff et al., 2001) to create TRV:Nt *CMPG1*. The empty pTV00 vector (TRV:00) and the pTV00 vector containing the Nbsgt1.2 fragment (TRV:sgt1; Peart et al., 2002), were used as controls. All the plasmids were transformed in *Agrobacterium tumefaciens* strain GV3101.

Infection of plants by agroinfiltration was performed as previously described (Rowland et al., 2005). Three weeks after infiltration, plants were analyzed for RNA accumulation and HR production. Fourth and fifth leaves were infiltrated with agrobacteria carrying Cf-9/Avr9 (Thomas et al., 2000) for HR production.

Stable Transformation of Tobacco with Nt *CMPG1*:TAP Wild-Type and Mutant Overexpression Constructs

Mutations in tobacco *CMPG1* were introduced by overlapping PCR. Two independent fragments were generated for each mutation. The 5' cDNA fragments were amplified using the primers Nt*CMPG1F1* (5'-GTAATCGATATGATGATTTCAACATGGAGG-3') and Nt*CMPG1R1* (5'-ATTCC-CAGCCTCAATCGCTTTTCTCAATATTCTC-3') for the Trp-64 mutation and Nt*CMPG1F1* and Nt*CMPG1R4* (5'-GTCTAAGGAAATTGGAGCTGTGAAATGTCTAGG-3') for the Cys-37 mutation. For the 3' fragment amplification, the primers Nt*CMPG1F2* (5'-GAGAATATTGAGAAAGCG-ATTGAGGCTGGGAAT-3') and Nt*CMPG1R2* (5'-GTAGGATCCGAATGGCCTTTTGAGACTCTT-3') were used for the Trp-64 mutation and Nt*CMPG1F4* (5'-CCTAGACATTTACAGCTTGTGAAATGTCTAGG-3') and Nt*CMPG1R2* for the Cys-37 mutation. The 5' and 3' PCR fragments were gel purified and mixed in a 1:1 ratio for overlapping PCR with the primers Nt*CMPG1F1* and Nt*CMPG1R2* for each construct. For the Nt*CMPG1* wild type, cloning PCR was performed from the original cDNA clone using the primers Nt*CMPG1F1* and Nt*CMPG1R2*. The restriction sites *Clal* and *BamHI* were introduced with the primers Nt*CMPG1F1* and Nt*CMPG1R2*, respectively. The amplification fragments were cloned into pGem-T Easy plasmid and digested with *Sall* and *Clal*. The digestion products were cloned into the epiGreenB4 binary vector containing a 35S:*GUS*:TAP cassette at the same sites to generate C-terminal TAP tag-fused tobacco *CMPG1* driven by the 35S promoter. To generate epiGreenB4 plasmid, pBIN19 h was digested with *EcoRI* and *HindIII* to obtain the expression cassette 35S:*GUS*:TAP:*NOS*, which was further cloned into a pGreen-derived vector (Hellens et al., 2000) at the same restriction sites. Each construct was cotransformed with the pSoup plasmid in *A. tumefaciens* strain Ag1, and tobacco stable transformants were generated as described (Hammond-Kosack et al., 1998).

Preparation of Protein Extract

Leaves from transgenic tobacco overexpressing Nt *CMPG1*:TAP and controls were homogenized in liquid nitrogen, thawed in 2 volumes of extraction buffer [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 2 μ g/mL antipain, 2 μ g/mL leupeptin, and 2 μ g/mL aprotinin], filtered through two layers of Miracloth, and centrifuged at 1000g for 10 min at 4°C. The supernatant was collected for SDS-PAGE.

SDS-PAGE and Immunoblotting

SDS-PAGE and immunoblotting experiments were performed as previously described (Rivas et al., 2002), except that 8% SDS gels were used for separation.

Growth Inhibition Assay

Homozygous *N. tabacum* seeds were germinated on plates without any selection. At day 10, tobacco seedlings were transferred to MS liquid medium containing IF(-Avr9) or IF(+Avr9) previously filter-sterilized. Ten days later, the fresh weight was determined and pictures were taken.

TRV-Based VIGS in *S. lycopersicum*

A 150-bp fragment from tomato *Cmpg1* cDNA was PCR amplified using the primers SulphurCMPGF (5'-AACATTGAGAACTCGGTGGGAATC-AAACA-3') and Le74seqR1 (5'-TGAAATCGGGATTCTTGGAG-3'). The fragment was cloned in the *Sma*I site of pTRV-RNA2 vector (Liu et al., 2002a). As a control, the pTRV-RNA2 empty vector and the pTRV-RNA2 vector containing a 150-bp Cf-9 fragment (Rowland et al., 2005) were used. Silencing of tomato plants and *C. fulvum* race 4 GUS infections were performed as before (Rowland et al., 2005). Fungal growth was scored 3 weeks later by GUS staining and light microscopy (Axiophot; Zeiss).

Stable Transformation of Tobacco and Tomato with a Hairpin-Driven RNAi Construct

For hairpin-induced RNAi in tomato and tobacco, the pHellsgate8 vector was used (Helliwell et al., 2002). A fragment of 400 bp was amplified using the primers Le74RNAi5 (5'-ACCGTCGACAGTGACTTTATCAACAGG-GATT-3') and Le74RNAi3 (5'-ACCCTCGAGGCATTTTTCGCGTTTTTCCC-3') for tomato and NtCMPGF8 (5'-ACCGTCGACAGTGACCTTGTC-AACAGGGATC-3') and NtCMPGR8 (5'-ACCCTCGAGCGATTCTCATATTATTTCC-3') for tobacco CMPG1. The amplification products were digested with *Sal*I and cloned into the gateway-compatible vector pENTR4 (Invitrogen) previously digested with *Sal*I and *Eco*RV to give pEntry4LeCMPG1 and pEntry4NtCMPG1, respectively. An *in vitro* BP clonase recombination reaction was performed with pEntry4LeCMPG1, pEntry4NtCMPG1, and pHellsgate8 according to the manufacturer's instructions (Invitrogen). The recombination reaction product was transformed into DH5 α *E. coli*. The positive clones were digested to check the intron orientation and sequenced. The selected clones were transformed into agrobacteria for transformation of tomato plants as described (Hammond-Kosack et al., 1998).

Agrobacteria-Mediated Transient Expression in Tobacco

For analysis of Pto- and Inf1-dependent HR production, agrobacteria carrying the binary construct containing Pto/AvrPto and Inf1 (Peart et al., 2002) were infiltrated at different optical densities in 7-week-old tobacco leaves. HR development was observed 3 and 4 d after infiltration.

Accession Numbers

The accession numbers of the sequences included in the alignment are as follows: parsley CMPG1 (AAK69402), tobacco CMPG1 (AAP03884), *Arabidopsis* PUB20 (AAG51307), and *Arabidopsis* PUB21 (NP_198565). Sequence data for tomato *Cmpg1* have been deposited in the GenBank data library under accession number DQ118759.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table 1. Protein levels and Phenotypes of Tobacco Lines Carrying 35S:Nt *CMPG1* Construct.

Supplemental Figure 1. The Presence of High Levels of Tobacco CMPG1W64A Correlates with Reduced HR Production.

Supplemental Figure 2. Tobacco CMPG1 Is Involved in Inf1-Mediated HR Production.

Supplemental Figure 3. Correlation between Tomato *Cmpg1* mRNA Levels and Defense Responses in Tomato Hairpin Lines.

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