

The E3 Ubiquitin Ligase Activity of *Arabidopsis* PLANT U-BOX17 and Its Functional Tobacco Homolog ACRE276 Are Required for Cell Death and Defense

Cheng-Wei Yang,^a Rocío González-Lamothe,^b Richard A. Ewan,^a Owen Rowland,^{b,1} Hirofumi Yoshioka,^{b,2} Matt Shenton,^a Heng Ye,^a Elizabeth O'Donnell,^a Jonathan D.G. Jones,^b and Ari Sadanandom^{a,3}

^aPlant Science Group, Division of Biochemistry and Molecular Biology, Institute of Biomedical and Life Sciences, University of Glasgow, Glasgow G12 8QQ, United Kingdom

^bSainsbury Laboratory, John Innes Centre, Norwich NR4 7UH, United Kingdom

Previous analysis of transcriptional changes after elicitation of Cf-9 transgenic tobacco (*Nicotiana tabacum*) by Avr9 peptide revealed a rapidly upregulated gene, ACRE276. We show that ACRE276 is transiently induced in wounded leaves within 15 min, but upon Avr9 elicitor treatment, this upregulation is enhanced and maintained until cell death onset in Cf-9 tobacco. ACRE276 RNA interference (RNAi) silencing in tobacco results in loss of hypersensitive response (HR) specified by Cf resistance genes. ACRE276 RNAi plants are also compromised for HR mediated by the tobacco mosaic virus defense elicitor p50. Silencing tomato (*Lycopersicon esculentum*) ACRE276 leads to breakdown of Cf-9–specified resistance against *Cladosporium fulvum* leaf mold. We confirmed that tobacco ACRE276 is an E3 ubiquitin ligase requiring an intact U-box domain. Bioinformatic analyses revealed *Arabidopsis thaliana* PLANT U-BOX17 (PUB17) and *Brassica napus* ARC1 as the closest homologs of tobacco ACRE276. Transiently expressing PUB17 in Cf-9 tobacco silenced for ACRE276 restores HR, while mutant PUB17 lacking E3 ligase activity fails to do so, demonstrating that PUB17 ligase activity is crucial for defense signaling. *Arabidopsis* PUB17 knockout plants are compromised in RPM1- and RPS4-mediated resistance against *Pseudomonas syringae* pv *tomato* containing avirulence genes AvrB and AvrRPS4, respectively. We identify a conserved class of U-box ARMADILLO repeat E3 ligases that are positive regulators of cell death and defense across the Solanaceae and Brassicaceae.

INTRODUCTION

The capacity of plants to protect themselves against pathogens depends on detection mechanisms that recognize pathogen-derived molecules and then activate host defense responses. For example, bacterial flagellin triggers host defenses (Ausubel, 2005). Pathogen effector molecules, such as bacterial AvrPto, can suppress all or some of these defense responses (Lee et al., 2005). Plant resistance (*R*) genes encode proteins that can recognize these effector molecules, in which case they are termed avirulence (*Avr*) proteins (Dangl and Jones, 2001). In the *R/Avr* gene system, R protein-mediated recognition could either be direct or indirect through the effects of Avr proteins on their host targets (Dangl and Jones, 2001).

The Cf-9 gene from tomato (*Lycopersicon esculentum*) confers resistance to races of leaf mold fungus *Cladosporium fulvum* expressing the corresponding Avr9 gene (Rivas and Thomas, 2005). The Cf-9 gene encodes an extracytoplasmic membrane-anchored glycoprotein. The Avr9 gene product is made by the fungus as a preprotein that is processed to a 28–amino acid secreted peptide (Joosten et al., 1994). Purified Avr9 peptide induces a battery of defense responses in tomato cultivars carrying the Cf-9 gene (Rivas and Thomas, 2005), and cells near the infection site die rapidly upon Avr9 perception. This localized programmed cell death is referred to as the hypersensitive response (HR). The HR has been proposed to release signals that condition the whole plant to become more responsive to *C. fulvum* elicitors and hence activate systemic acquired resistance (Dorey et al., 1999).

Tobacco (*Nicotiana tabacum*) plants and cell cultures carrying a Cf-9 transgene respond to Avr9 peptide with rapid induction of HR and associated responses in a strict gene-for-gene manner, indicating that all components required for efficient execution of the HR are present in this heterologous model system (Hammond-Kosack and Jones, 1997; Piedras et al., 1998). Cf-9 tobacco cell cultures provide an amenable experimental system in which to study rapid Avr9 responses (Piedras et al., 1998). We used cDNA amplified fragment length polymorphisms (AFLP) to identify Avr9/Cf-9 Rapidly Elicited (ACRE) genes (Durrant et al., 2000). Genetic analysis using virus-induced gene silencing (VIGS)

¹ Current address: Department of Biology, Carleton University, Ottawa, Ontario K1S 5B6, Canada.

² Current address: Plant Pathology Laboratory, Graduate School of Bioagricultural Sciences, Nagoya University, Chikusa 464-8601, Japan.

³ To whom correspondence should be addressed. E-mail ari.sadanandom@bio.gla.ac.uk; fax 44-0141-3306341.

The author responsible for distribution of materials integral to the findings presented in this article in accordance with the policy described in the Instructions for Authors (www.plantcell.org) is: Ari Sadanandom (ari.sadanandom@bio.gla.ac.uk).

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and RNA interference (RNAi) methods provided conclusive evidence that at least one of these *ACRE* genes, *ACIK1*, is required for full Cf-mediated defense. Two other *ACRE* genes, encoding an F-box protein (*ACRE189*) and a U-box protein (*ACRE276*), were also suggested to be involved in Cf-mediated HR, implicating the ubiquitin-proteasome system in defenses against pathogens (Rowland et al., 2005).

The ubiquitin conjugation pathway involves the activity of three enzymes or protein complexes called the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin-protein ligase (E3). The E1 enzyme catalyzes the formation of a thiol-ester bond between the COOH terminus of ubiquitin and a conserved Cys within the E1 enzymes (Hershko et al., 1983). The ubiquitin moiety is then transferred to an E2 enzyme (Hershko et al., 1983), again forming a thiol-ester bond with a conserved Cys. Next, the E3 enzyme facilitates the formation of an isopeptide linkage between ubiquitin and the target protein. A polyubiquitin chain is subsequently formed by the addition of multiple ubiquitin monomers via an internal Lys residue within the ubiquitin protein (Scherer et al., 1995). E3 ligases recruit specific target proteins and catalyze their ubiquitination. This polyubiquitin chain is then recognized by the 26S proteasome where the target is degraded and the ubiquitin monomers are recycled (Ciechanover, 1998). Some ubiquitinated proteins can undergo endocytosis or cellular relocation rather than proteasomal degradation (Prag et al., 2003). Plants contain a small number of similar E1 isoforms with no apparent functional specificity (Vierstra, 2003). The E2 family is much larger, and several E2 enzymes have specialized cellular functions (Villalobo et al., 2002). E3 ligases play an important role in substrate specificity and form the largest group of proteins within the ubiquitin-enzyme cascade. Single protein E3 ubiquitin ligases can be subdivided into various groups based on the presence of Hect, F-box, RING, or U-box domains.

In plants, mutations in specific E3 ligases can block embryogenesis, circadian rhythms, photomorphogenesis, floral homeosis, hormone responses, and senescence (reviewed in Callis and Vierstra, 2000). U-box domain proteins were originally identified as modified RING domains (Aravind and Koonin, 2000), and some of the U-box proteins were subsequently shown to have in vitro E3 ubiquitin ligase activity (Hatakeyama et al., 2001). Most U-box proteins have an additional domain that is speculated to be the substrate binding domain (Azevedo et al., 2001). There is a large number of plant U-box domain proteins compared with other organisms (Azevedo et al., 2001). Plant U-box proteins are implicated in self-incompatibility (Stone et al., 2003), abiotic stress (Yan et al., 2003), and hormone regulation (Amador et al., 2001).

The parsley (*Petroselinum crispum*) *CMPG1* gene is rapidly elicited by PEP13 elicitor (Kirsch et al., 2001), and its *Arabidopsis thaliana* homologs PLANT U-BOX20 (PUB20) and PUB21 are rapidly induced by flagellin (Navarro et al., 2004). *CMPG1* homologs are required in both tomato and tobacco for Cf gene function (González-Lamothe et al., 2006). Apart from this class and tobacco *ACRE276*, only one other U-box protein, rice (*Oryza sativa*) SPL11 (Zeng et al., 2004), is implicated in biotic stress. However, there has been no direct evidence linking the E3 ubiquitin ligase activity of any U-box protein to its physiological role in plants.

In this study, we show that tobacco *ACRE276* encodes a U-box protein with ARMADILLO (ARM) repeat domains and is a bona fide E3 ubiquitin ligase. Silencing *ACRE276* greatly attenuated Cf- and N-mediated cell death in tobacco. Tomato *ACRE276* is required for resistance in Cf-9 tomato against *C. fulvum*. We also identified *Arabidopsis PUB17* as the closest homolog of *ACRE276*. Transient expression of *PUB17* in Cf-9 tobacco plants that are stably silenced for *ACRE276* restored full HR after Avr9 treatment, indicating that *PUB17* is a functional homolog of *ACRE276*. Furthermore, we were able to show that the E3 ligase activity of *Arabidopsis PUB17* is required for HR initiated by Cf-9/Avr9 interaction in tobacco. *Arabidopsis* T-DNA insertion mutants of *PUB17* attenuate *RPM1*- and *RPS4*-mediated resistance. These data implicate tobacco *ACRE276* and its functional homolog *Arabidopsis PUB17* as crucial positive regulators of plant disease resistance.

RESULTS

ACRE276 Encodes a U-Box ARM Repeat Protein and Is Rapidly Upregulated upon Avr9 Treatment

A full-length tobacco *ACRE276* cDNA of 2444 bp was previously isolated (Rowland et al., 2005). The tobacco *ACRE276* protein is predicted to contain 726 amino acids and has two notable domains. A single ~70-amino acid U-box domain is contained within the central region of the protein (shaded boxes in Figure 1A). This domain was identified in a number of proteins as the catalytic component responsible for covalently attaching ubiquitin molecules to protein substrates targeted for multiubiquitination-mediated proteasomal degradation (Hatakeyama et al., 2001). The C-terminal region shows ~30% identity to the ARM repeats in the mouse pendulin protein and the β -catenin/ARM family of proteins (Gu et al., 1998). From previously defined ARM repeats in the ARM protein, at least five potential ARM repeats were identified in tobacco *ACRE276*. The closest homologs of tobacco *ACRE276* in plants are *Arabidopsis PUB17* (70% identity over the entire protein sequence), the *Brassica oleracea* ARC1 protein (Stone et al., 2003) (50% identity), and rice SPL11 (Zeng et al., 2004) (30% identity; Figure 1B). Phylogenetic analysis indicated that *Arabidopsis PUB17* clusters with tobacco *ACRE276* and *B. napus* ARC1 rather than with other closely related *Arabidopsis* ARM repeat proteins (Figure 1B).

We tested whether *ACRE276* mRNA is induced in Cf-9 tobacco leaf tissue as in Cf-9 tobacco cell cultures (Durrant et al., 2000) by infiltrating Avr9 and Avr4 peptide into Cf-9 tobacco leaves and collecting tissue samples at various time intervals. RT-PCR analysis confirmed that the *ACRE276* transcript is induced by infiltration fluid (IF) (Avr9) within 15 min of infiltration, and the upregulation of the mRNA is maintained till the leaf patch becomes necrotic (Figure 1C). However, in leaf tissue infiltrated with Avr4 peptide, there was a small transient induction of *ACRE276* mRNA that subsided after 60 min to basal levels. This initial upregulation could be due to a wounding or flooding effect (Durrant et al., 2000). These data indicate that *ACRE276* transcripts are similarly upregulated by Cf-9/Avr9 in cell cultures and at the whole-plant level.

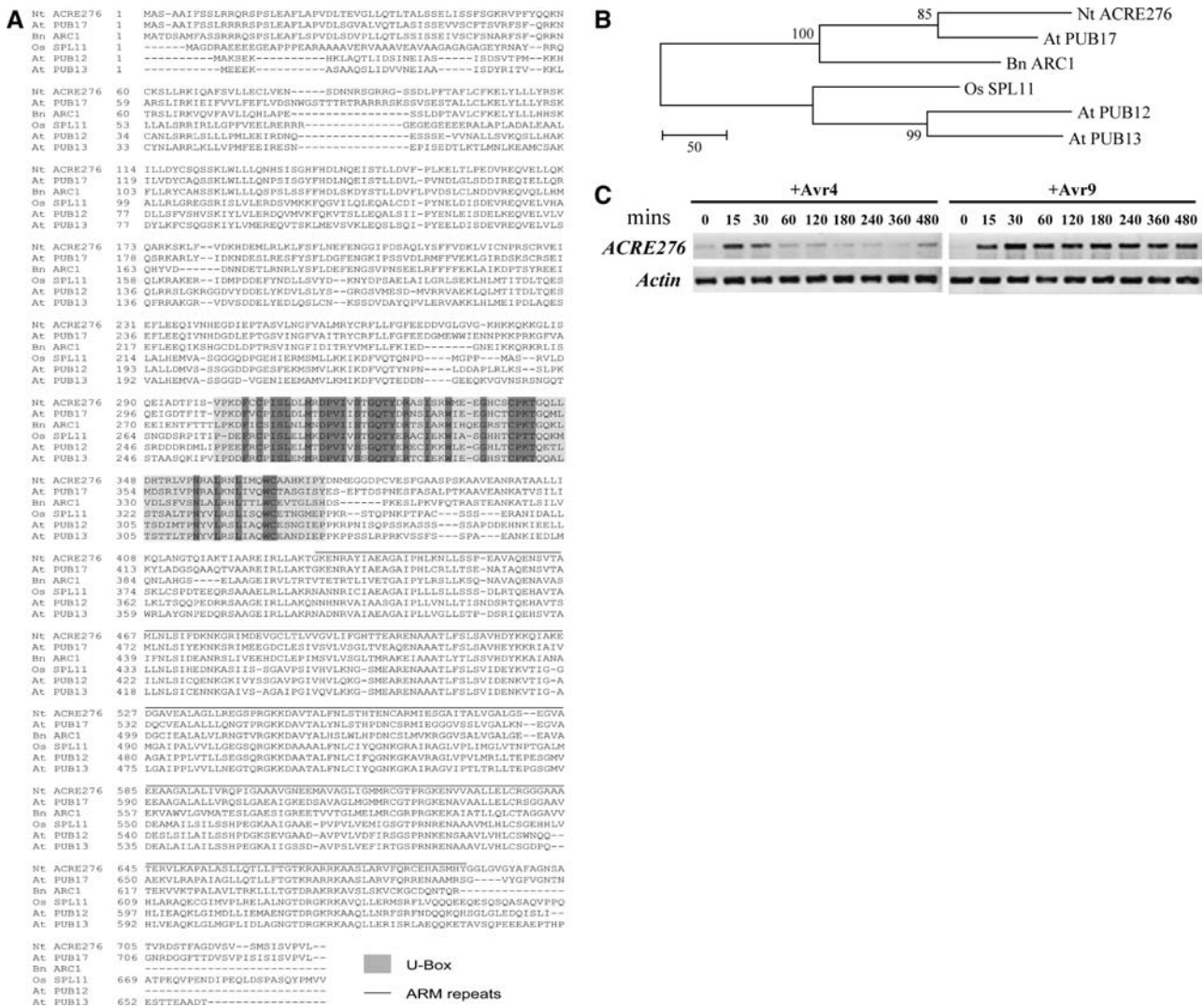


Figure 1. Protein Sequence Alignment of Tobacco ACRE276 with Related U-Box ARM Repeat Proteins and Expression Patterns of ACRE276 Transcripts after Elicitation.

(A) Amino acid sequence alignment of tobacco (Nt) ACRE276 with *Arabidopsis* (At) PUB17, PUB12, and PUB13, *Brassica napus* (Bn) ARC1, and rice (Os) SPL11. Gaps introduced to maximize the alignment are represented as dashes. Conserved U-box and ARM repeat regions are marked by gray highlighting and black lines, respectively.

(B) Phylogenetic relationships among tobacco ACRE276, *Arabidopsis* PUB17, and related ARM repeat proteins. Phylogeny generated by neighbor joining using rice SPL11 and *Arabidopsis* PUB12 and PUB13 as an outgroup using ClustalW. The phylogenetic tree was generated from the unmodified alignment using MEGA3.1 software (Kumar et al., 2004). Amino acid scoring was based on the JTT substitution model (Jones et al., 1992), and validity testing was based on 1000 bootstrap replicates.

(C) RT-PCR analysis of tobacco ACRE276 mRNA after treatment of Cf-9 tobacco leaves with Avr4 and Avr9 peptides. Tissues were infiltrated with IF containing Avr9 and Avr4, and leaf tissue was collected for RT-PCR analysis. The tobacco Actin transcript was used as a control for cDNA levels.

Cf-9:ACRE276 RNAi Lines Show Reduced Levels of ACRE276 mRNA and Loss of Cf- and N-Mediated Cell Death

Initial experiments in *Nicotiana benthamiana* using VIGS suggested that ACRE276 is required for full Cf-9-dependent HR (Rowland et al., 2005). To verify this, we silenced ACRE276 mRNA by constitutive expression of an intron-containing self-complementary ACRE276 hairpin DNA construct in Cf-9 tobacco

plants. Expression of such hairpin constructs results in the production of double-stranded RNA (dsRNA), triggering post-transcriptional gene silencing (Wesley et al., 2001).

To generate ACRE276-specific dsRNA, we cloned 500 bp from the 3' region of the ACRE276 cDNA, covering the ARM repeat domains in sense and antisense directions interrupted by the Pdk intron under the 35S promoter, using the pKannibal vector (Wesley et al., 2001). This construct should lead to the

generation of *ACRE276*-specific dsRNA. We transformed this construct into Cf-9 tobacco plants using *Agrobacterium tumefaciens*. We generated eight primary transformants and analyzed homozygotes in the T3 generation. Seedlings from the eight independent lines were tested for reduced levels of *ACRE276* mRNA by RT-PCR. Three of the eight transformants showed efficient silencing of *ACRE276* mRNA, and none of the three *ACRE276* suppressed plants showed any obvious abnormal developmental phenotypes. These *ACRE276*-silenced lines were then evaluated for their Cf-9/Avr9- and Cf-4/Avr4-mediated HR and further characterized.

Silencing *ACRE276* caused a marked reduction though not complete elimination of the Cf-9/Avr9- and Cf-4/Avr4-induced HR (Figures 2A and 2B). This reduced HR was consistently seen in all the independent lines that showed effective silencing of *ACRE276*, implicating this gene in Cf-mediated HR. The Cf-9 tobacco lines used in this study also contained the *N* transgene. It has been shown before that nonviral expression of a 50-kD tobacco mosaic virus (TMV) helicase fragment (*p50*) is sufficient to induce the *N*-mediated HR in tobacco plants carrying the *N* transgene (Erickson et al., 1999). We tested if *ACRE276* is involved in mediating *p50*-dependent HR by transiently expressing the *p50* transgene in Cf-9:*ACRE276* RNAi plants. Silencing *ACRE276* caused a marked reduction but not a complete elimination of the *p50*-induced HR (Figure 2C). These data indicate that tobacco *ACRE276* is required for the full spectrum of defense responses mediated by both the *Cf* and *N* resistance genes.

Since infiltrating Avr9 peptide causes a rapid upregulation of tobacco *ACRE276* in Cf-9 tobacco plants, we wanted to see if this upregulation was affected in *ACRE276* RNAi plants. RT-PCR analysis with *ACRE276*-specific primers showed that Avr9-mediated induction of *ACRE276* gene expression was abolished in Cf-9:*ACRE276* RNAi tobacco plants (Figure 2D). Thus, we can conclude that the reduction of Cf-mediated HR is due to the downregulation of *ACRE276* gene expression.

***N. tabacum* ACRE276 and Arabidopsis PUB17 Are Functional E3 Ligases**

Since it has been previously shown that U-box-containing ARM repeat proteins can function as E3 ligases, we tested whether tobacco *ACRE276* and PUB17 also possess E3 ligase activity. We expressed and affinity purified *ACRE276* and PUB17 proteins as glutathione S-transferase (GST) fusions from *Escherichia coli*. In the presence of yeast E1 and human E2 Hubs5b, ubiquitination activity was observed for the purified GST-*ACRE276* and -PUB17 fusion proteins (lane 5 in Figures 3A and 3B). No clear protein ubiquitination was detected in the absence of E1, E2, or E3 (Figures 3A and 3B). These results indicated that *ACRE276* and PUB17 are bona fide E3 ligases.

Since it is known that the U-box domain within ARM repeat proteins is essential for E3 ligase activity (Zeng et al., 2004), we wanted to determine if an intact U-box domain is critical for *ACRE276* E3 ligase activity. We made two mutant forms of *ACRE276* protein. The Δ U-box version of *ACRE276* had the entire U-box domain deleted (residues 299 to 365). The second version had a point mutation that results in the Val-316 to Ile

(V316I) amino acid substitution. The Val residue within different U-box domains is highly conserved, and mutating it to Ile causes impaired E3 activity in the yeast U-box protein Prp19 (Ohi et al., 2005) and the rice SPL11 protein (Zeng et al., 2004). In vitro ubiquitination assays indicated that the E3 activity was completely abolished in both mutant forms of *ACRE276* (Figure 3A). Furthermore, the corresponding V322I mutation in PUB17 yielded a similar loss of E3 ligase activity (Figure 3B).

Tomato ACRE276 Is Required for Tomato Cf-9-Mediated Resistance to *C. fulvum*

We tested whether tomato *ACRE276* is required not only for HR but also for resistance in tomato against *C. fulvum*. A specific 200-bp sequence at the end of the tomato *ACRE276* coding region was cloned into a tobacco rattle virus (TRV) vector described by Liu et al. (2002b). A 150-bp sequence encoding the Cf-9 C-terminal region was used as a positive control (Rowland et al., 2005) and the empty vector as negative control for the tomato silencing experiments. We silenced a transgenic tomato line that carries *Cf-9* but not the other four linked Cf-9 homologs that are present in Cf-9 stocks produced by breeding.

For each construct, we used at least five leaves of three different plants. The HR specified by Cf-9/Avr9 in tomato was checked in plants 3 weeks after the start of silencing from the different constructs. Consistent with the observation in *N. tabacum*, the HR induced by infiltration of IF (+Avr9) in tomato was weaker and delayed in tomato *ACRE276* and Cf-9-silenced plants when compared with control plants (Figure 4A). Tomato phytoalexins fluoresce in UV light and serve as visible markers for HR (Rowland et al., 2005). These phytoalexins were detected after Avr9 elicitation in the empty vector control plants, whereas these symptoms were much reduced in the tomato *ACRE276*- and Cf-9-silenced plants (Figure 4A). No clear necrosis or phytoalexin fluorescence was seen in Cf0 plants after infiltration with control IF without Avr9.

We also tested the effect of silencing tomato *ACRE276* on Cf-9-mediated resistance. Tomato plants were inoculated with *C. fulvum* spore cultures 3 weeks after initiating silencing (Figure 4B). The *C. fulvum* strain used expresses β -glucuronidase (GUS), which allows for easy visualization of fungal growth. After a further 3 weeks of fungal growth, leaves were stained with 5-bromo-4-chloro-3-indolyl- β -glucuronide (X-gluc). No X-gluc staining was detected in untreated or Cf-9 plants silenced with the empty vector, indicating no fungal growth (Figure 4B). By contrast, Cf0 tomato plants untreated or silenced with the empty vector (data not shown) showed clear X-gluc staining (Figure 4B), indicating extensive fungal growth. Leaves from Cf-9 plants silenced for either Cf-9 or tomato *ACRE276* showed increased fungal growth and X-gluc staining. The blue staining was a result of hyphae growing intracellularly and emerging through the stomata. These data indicated that tomato *ACRE276* silencing caused a breakdown of Cf-9-mediated resistance against *C. fulvum*. Fungal hyphae could be easily detected in leaves of Cf-9 tomato silenced for tomato *ACRE276* or Cf-9, but there were fewer hyphae compared with Cf0 plants infected with *C. fulvum* (Figure 4B). Nevertheless, *C. fulvum* was able to complete its full life cycle in tomato *ACRE276*-silenced tomato plants, indicating

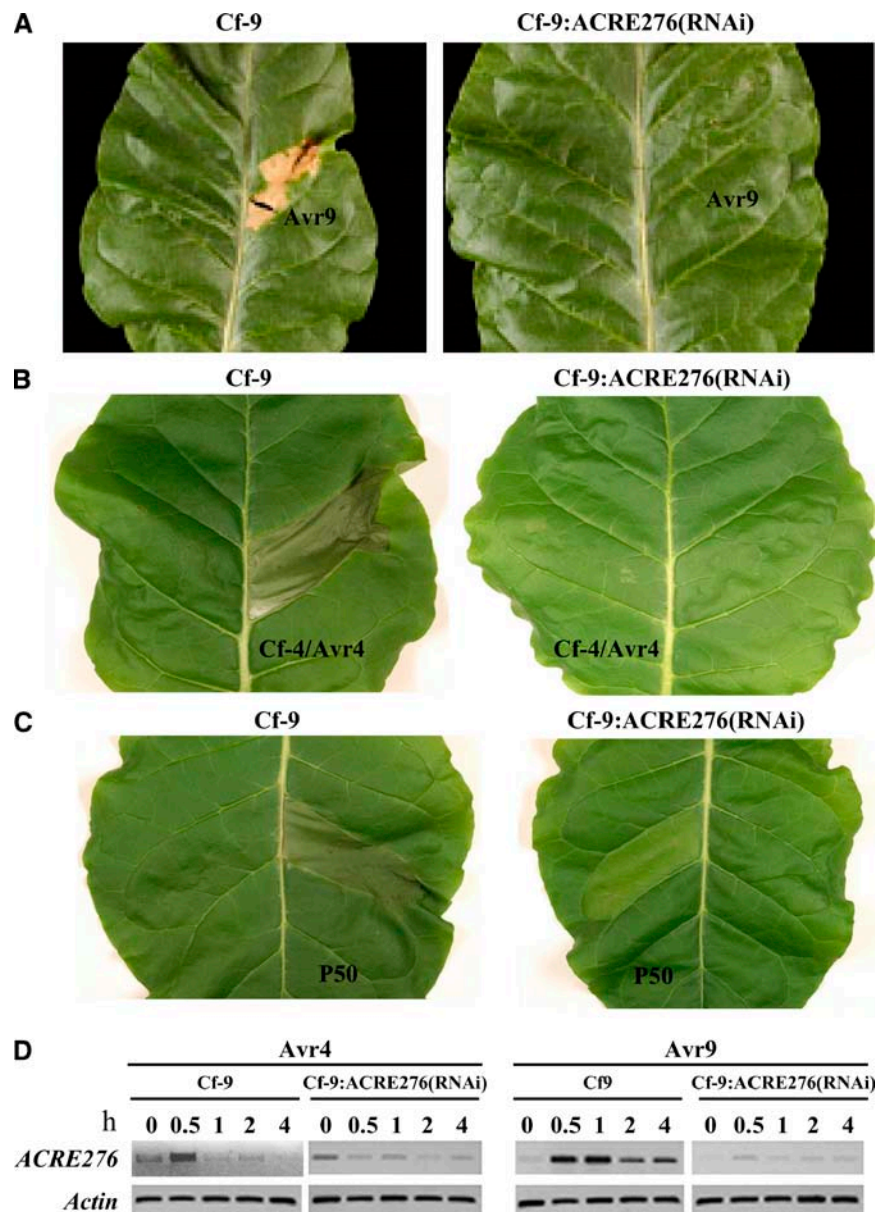


Figure 2. Cf-9:ACRE276 RNAi Tobacco Plants Show Reduced HR after Elicitor Treatment.

(A) Infiltration of Cf-9 tobacco and the Cf-9:ACRE276 RNAi lines with IF containing Avr9 peptide. IF containing Avr9 peptide was infiltrated into Cf-9 tobacco leaves (left panel) and Cf-9:ACRE276 RNAi plants (right panel). Pictures were taken 3 d after IF inoculation.

(B) Infiltration of Cf-9 tobacco and the Cf-9:ACRE276 RNAi lines with *Agrobacterium* containing Avr4 and Cf4 transgenes. An equal mixture of *Agrobacterium* cultures containing both Avr4 and Cf4 were infiltrated into Cf-9 plants (left panel) and Cf-9:ACRE276 RNAi plants (right panel) for transient expression and HR development. Pictures were taken 4 d after infiltration.

(C) Infiltration of Cf-9 tobacco and the Cf-9:ACRE276 RNAi lines treated with the TMV defense elicitor protein P50. *Agrobacterium* cultures containing 35S promoter-driven *p50* transgene encoding for the TMV defense elicitor protein P50 were infiltrated into Cf-9 (left panel) and Cf-9:ACRE276 RNAi plants (right panel) for transient expression and HR development. Pictures were taken 3 d after inoculation.

(D) RT-PCR analysis of *ACRE276* transcripts. Cf-9 and Cf-9:ACRE276 RNAi leaf tissues were infiltrated with IF containing Avr4 (nonelicitor) and Avr9 to induce *ACRE276* gene expression, and leaf tissue was collected for RT-PCR analysis. The tobacco *Actin* transcript was used as a control for cDNA levels.

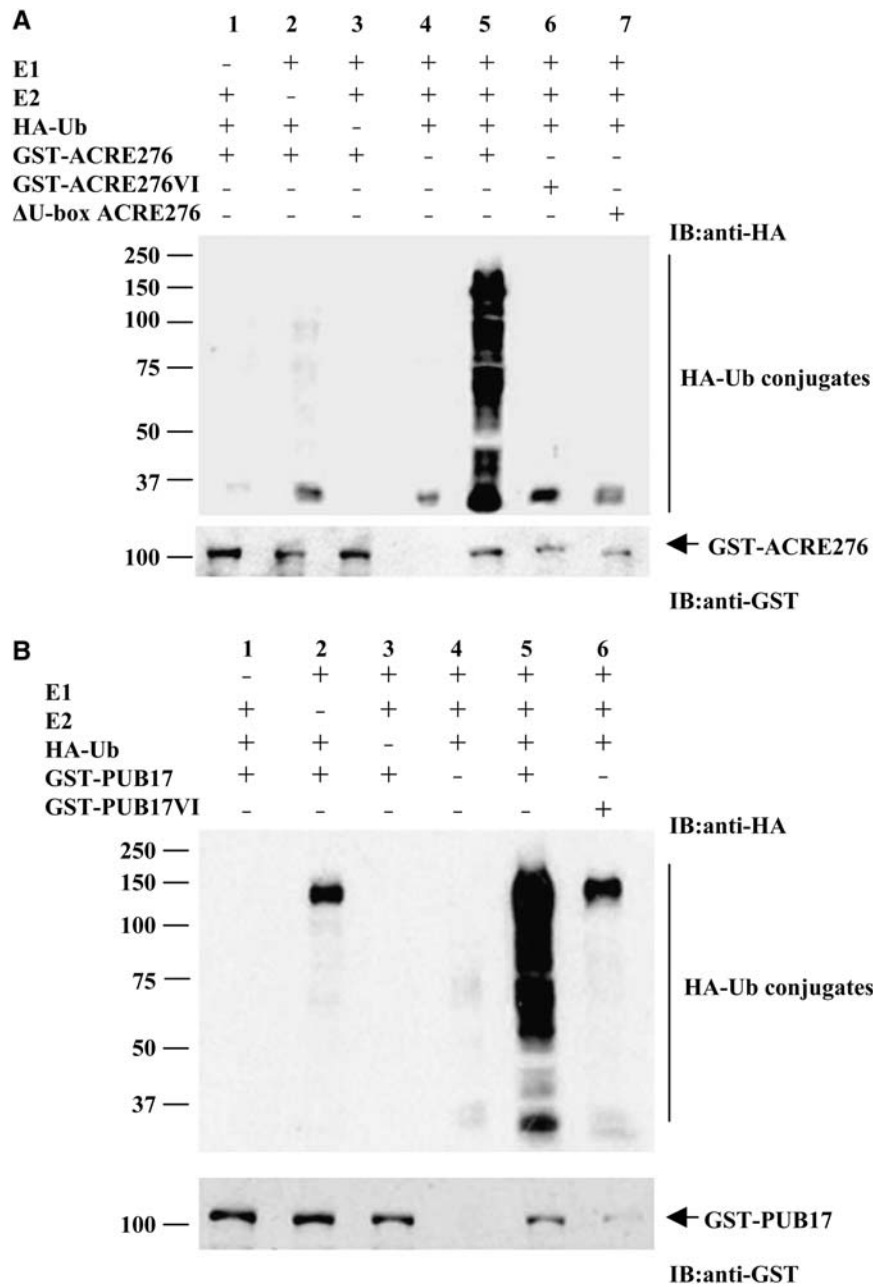


Figure 3. E3 Ubiquitin Ligase Activities of ACRE276 and PUB17.

(A) Ubiquitination assays with GST-NtACRE276 and mutant variants. GST-NtACRE276 was expressed and purified from *E. coli* and tested for ubiquitination activity in the presence of yeast E1, human E2 (*Hubc5b*), and *E. coli* extracts containing HA-tagged ubiquitin (HA-Ub). The immunoblots were probed with anti-HA antibodies (top panel) to detect ubiquitinated *E. coli* proteins. Anti-GST antibodies (bottom panels) were used to detect GST-ACRE276. As controls for the E3 activity, we used mutated versions of ACRE276 (Δ U-box mutant and a conserved U-box domain Val-316 to Ile substitution mutant, GST-ACRE276V316I).

(B) Ubiquitination assays with GST-PUB17 and mutant variants. GST-PUB17 was expressed and purified from *E. coli* and tested for ubiquitination activity in the presence of yeast E1, human E2 (*Hubc5b*), and *E. coli* extracts containing HA-tagged ubiquitin. The immunoblots were probed with anti-HA antibodies (top panel) to detect ubiquitinated *E. coli* proteins. Anti-GST antibodies (bottom panels) were used to detect GST-PUB17. As controls for the E3 activity, we used mutated versions of PUB17 (U-box domain conserved Val-322 to Ile substitution mutant, GST-PUB17V322I). Note the presence of two bands of identical sizes (~130 kD) in lanes 2 and 6; these bands are postulated to be unspecific ubiquitination of PUB17 and PUB17VI by yeast E1 protein.

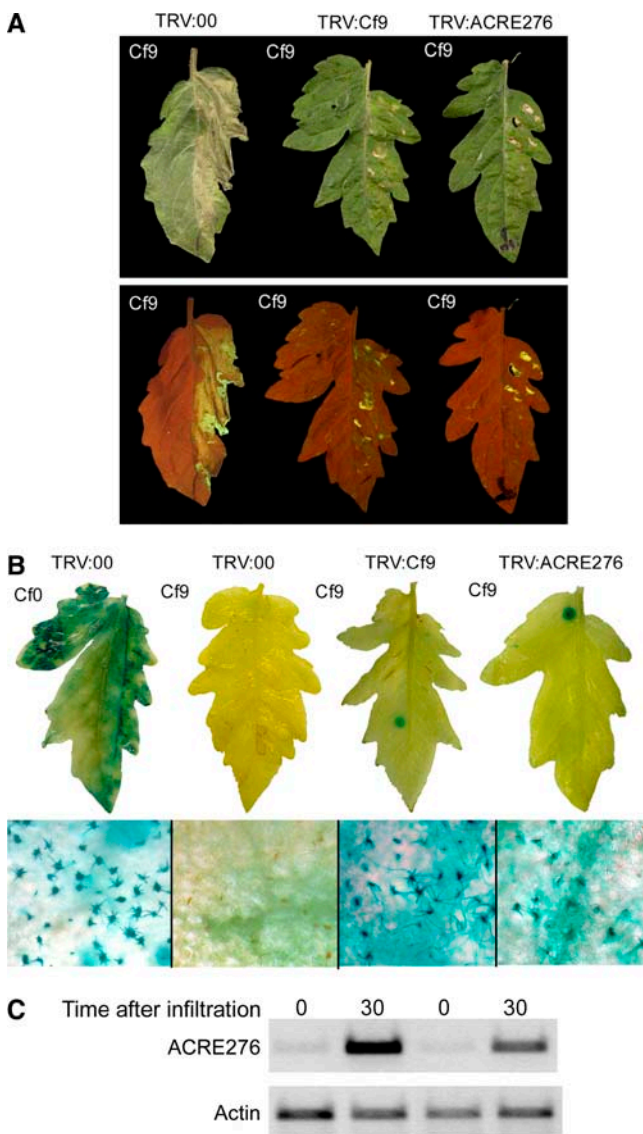


Figure 4. Tomato *ACRE276* Is Required for Cf-9-Mediated HR and Resistance to *C. fulvum* Expressing Avr9 in Tomato.

(A) VIGS in tomato. Cotyledons from Cf-9 tomato plants were inoculated with *Agrobacterium* expressing the indicated TRV construct. Three weeks after infiltration, tomato leaves were half-infiltrated with IF containing Avr9, and pictures were taken 3 d after infiltration under daylight (top panel) or UV light (bottom panel). TRV:00 indicates empty vector. TRV:Cf-9 and TRV:ACRE276 are the constructs for silencing Cf-9 and *ACRE276*.

(B) Cf-9 or Cf0 tomato plants were silenced with the indicated TRV construct as 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 *ACRE276* transcript levels were analyzed by RT-PCR. Leaf discs from the indicated tomato-silenced plants were harvested at 0 and 30 min after flooding with water. Total RNA was extracted and used for RT-PCR with *ACRE276* and tomato *Actin* primers. Suppression of *ACRE276* is significant when the amounts of constitutively expressed tomato *Actin* transcript levels are taken into account.

that reduction of tomato *ACRE276* in tomato (Figure 4C) can compromise resistance against *C. fulvum*. These results were obtained in three independent experiments using multiple leaves from two to three silenced tomato plants (Table 1).

To measure the levels of tomato *ACRE276* mRNA in the silenced plants, leaf discs from Cf-9 TRV:ACRE276-silenced tomato plants were harvested at 0 and 30 min after flooding with water. Total RNA was extracted and used for RT-PCR with tomato *ACRE276* and *Actin* primers. The results from these experiments confirmed that the induction of tomato *ACRE276* mRNA was reduced in the Cf-9 TRV:ACRE276-silenced tomato plants (Figure 4C), though the amount of reduction was considerably less than that seen in the *N. tabacum* dsRNAi plants.

PUB17 Can Restore Cf-9/Avr9-Specified Cell Death in ACRE276 RNAi Plants, and Its E3 Ligase Activity Is Required for Elicitation of the HR

There is greater homology between *PUB17* and *ACRE276* at the amino acid sequence than at the nucleotide level. We could not identify any consecutive stretch of identical nucleotide sequence longer than 17 bases between *ACRE276* and *PUB17*. At least a 22-base identity between the dsRNA and its target gene is required for efficient gene silencing to occur (Miki et al., 2005). This knowledge led us to hypothesize that the nucleotide sequence divergence between *PUB17* and *ACRE276* could allow us to perform functional complementation assays using the *PUB17* gene in Cf-9:ACRE276 RNAi lines.

To test whether *PUB17* is a true functional homolog of *ACRE276*, we transiently expressed *PUB17* in Cf-9:ACRE276 RNAi plants. If *PUB17* is a functional homolog of *ACRE276*, we should see the restoration of cell death specified by Cf-9/Avr9 interaction. Figure 5A clearly shows that Cf-9:ACRE276 RNAi tobacco leaf cells expressing *PUB17* can mount an effective HR that was previously lost due to silencing of *ACRE276*. Thus, *PUB17* can functionally substitute for *ACRE276* in signaling for cell death initiated by Cf-9/Avr9 interaction. There was no HR-associated cell death initiated by *PUB17* in the presence of the nonelicitor Avr4. Green fluorescent protein (GFP) gene expression driven by the 35S promoter was used as a control for transient assays, and GFP levels were comparable after transient expression in Cf-9:ACRE276-silenced and control plants (data not shown).

Since expressing *PUB17* in *ACRE276* RNAi plants led to the restoration of cell death specified by the Cf-9/Avr9 interaction, this bioassay enables structure/function analysis of *PUB17*. We therefore tested whether the E3 ligase activity of *PUB17* is required for initiating cell death after Cf-9/Avr9 elicitation.

Table 1. Proportion of VIGS-Silenced Tomato Leaves Developing HR or Fungal Infection

Silencing Construct	Tomato Genotype	HR	<i>C. fulvum</i> Growth
TRV:00	Cf0	0/28	28/28
TRV:00	Cf-9	18/23	0/26
TRV:Cf-9	Cf-9	9/25	15/34
TRV:ACRE276	Cf-9	10/24	13/33

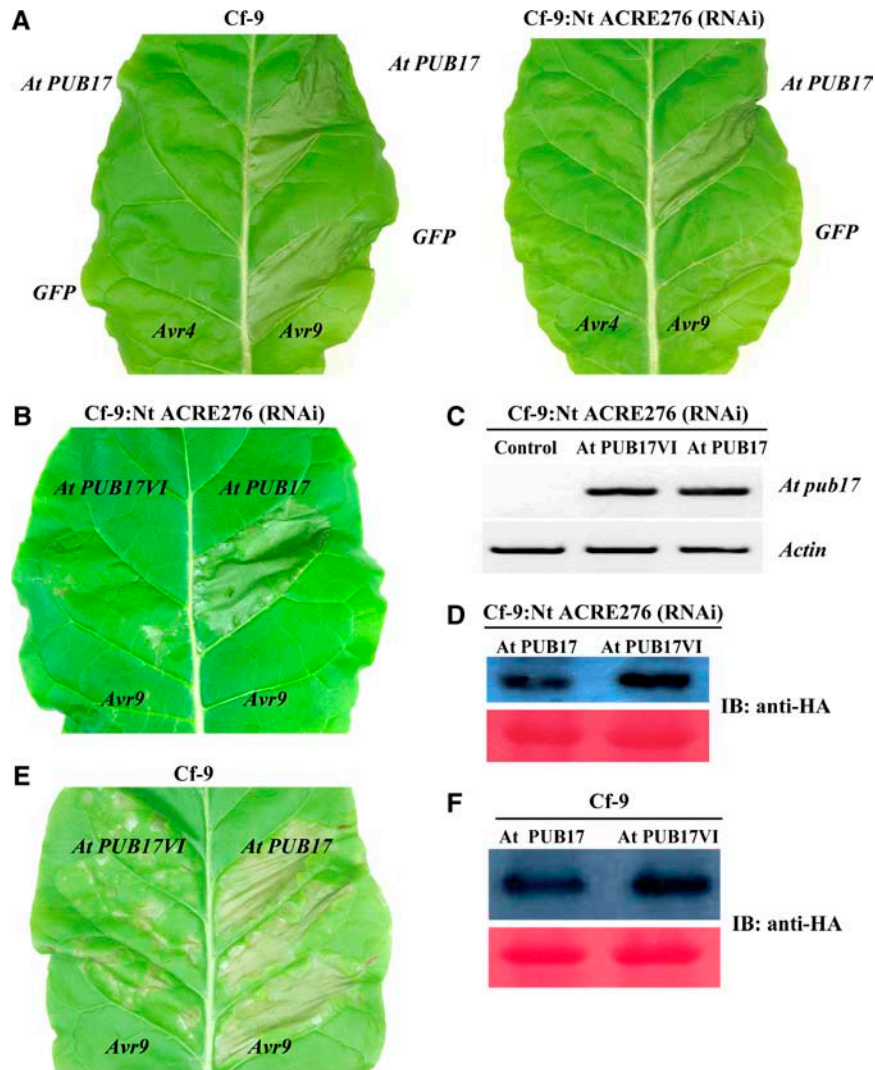


Figure 5. PUB17 Compensates for the Reduction of ACRE276 in Cf-9:ACRE276 RNAi Plants.

(A) Transient expression of HA-tagged PUB17. *Agrobacterium* cultures containing HA-tagged *PUB17* under its own promoter were infiltrated into Cf-9 (left panel) and Cf-9:ACRE276 RNAi (right panel) tobacco plants. Leaf patches infiltrated with *Agrobacterium* cultures containing 35S promoter-driven GFP were used as a transgene control. Three days after *Agrobacterium* inoculation, the same leaf patches were infiltrated with IF containing Avr4 or Avr9 to induce HR. Pictures were taken 2 d after IF treatment.

(B) Transient expression of HA-tagged *PUB17* and *PUB17VI* in Cf-9:ACRE276 RNAi tobacco. *Agrobacterium* cultures containing 35S promoter-driven HA-tagged *PUB17VI* (left side of leaf) and *PUB17* (right side of leaf) under its own promoter were infiltrated into Cf-9:ACRE276 RNAi tobacco plants. Three days after *Agrobacterium* inoculation, the same leaf patches were infiltrated with IF containing Avr9 to induce HR. Pictures were taken 2 d after IF treatment.

(C) RT-PCR analysis of *PUB17* and *PUB17VI* transcripts. *Agrobacterium* cultures containing 35S promoter-driven HA-tagged AtPUB17VI and *PUB17* under its own promoter were infiltrated into Cf-9:ACRE276 RNAi tobacco plants. Three days after *Agrobacterium* inoculation, the same leaf patches were infiltrated with IF containing Avr9 to induce HR. One hour after IF treatment, leaf samples were taken for cDNA preparation and RT-PCR. As negative controls for the RT-PCR, *Agrobacterium* cultures containing 35S promoter-driven GFP were used. Equal amounts of cDNA were used as shown by the amplification with the constitutively expressed tobacco *Actin*.

(D) Protein gel blot analysis of *PUB17* and *PUB17VI* in Cf-9:ACRE276 RNAi tobacco. Total protein was extracted from Cf-9:ACRE276 RNAi leaf patches expressing *PUB17* and *PUB17VI* 4 h after IF treatment. The immunoblots (IB) were probed with anti-HA antibodies (top panel) to detect HA-tagged *PUB17* and *PUB17VI* proteins. Ponceau S staining of ribulose-1-5-biphosphate carboxylase/oxygenase was performed (bottom panel) for confirmation of equal loading.

(E) Transient expression of HA-tagged *PUB17* and *PUB17VI* in Cf-9 tobacco. *Agrobacterium* cultures containing 35S promoter-driven HA-tagged *PUB17VI* (left side of leaf) and *PUB17* (right side of leaf) under its own promoter were infiltrated into Cf-9 tobacco plants. Three days after *Agrobacterium* inoculation, the same leaf patches were infiltrated with IF containing Avr9 to induce HR. Pictures were taken 2 d after IF treatment.

(F) Protein gel blot analysis of *PUB17* and *PUB17VI* in Cf-9 tobacco. Total protein was extracted from Cf-9 leaf patches expressing *PUB17* and *PUB17VI* 4 h after IF treatment. The immunoblots were probed with anti-HA antibodies (top panel) to detect HA-tagged *PUB17* and *PUB17VI* proteins. Ponceau S staining of ribulose-1-5-biphosphate carboxylase/oxygenase was performed (bottom panel) for confirmation of equal loading.

Figure 5B shows leaf tissue expressing both the wild type and a mutant form of PUB17 with the U-box domain V322I mutation. We have previously shown (Figure 3B) that this mutant variant of PUB17 has no discernible E3 ligase activity. In the transient bioassays, PUB17 V322I mutants were not able to restore HR after Cf-9/Avr9 elicitation in Cf-9:*ACRE276* RNAi plants. This result clearly indicates that the E3 ligase activity of PUB17 is required for its role in the Cf-9/Avr9-mediated HR and provides direct evidence for the requirement of E3 ubiquitin ligase activity by U-box domain proteins during plant disease resistance.

U-box domains within E3 ubiquitin ligases are suggested to interact with E2 ubiquitin-conjugating enzymes (Pringa et al., 2001), whereas the ARM repeat domain is hypothesized to interact with potential substrates (Azevedo et al., 2001). Disruption of E2 interaction by mutating one of the U-box core amino acids is predicted to result in impaired E3 ubiquitin ligase activity of PUB17 but still maintain interaction with possible substrates via the ARM repeat domain. Overproduction of such a mutant variant of PUB17, PUB17V322I, might act in a dominant-negative manner by sequestering and preventing subsequent ubiquitination of signaling substrates and interfering with the generation of the HR.

We tested whether transient overexpression of PUB17V322I in Cf-9:*ACRE276* wild-type leaf tissue can block HR after Avr9 treatment to determine if it can function as a dominant-negative allele of PUB17. Figure 5E shows Cf-9 tobacco leaf patches where we expressed both PUB17V322I (right side of leaf) and PUB17 (left side of leaf). After 3 d, we infiltrated the same patches with IF containing Avr9. Only patches expressing PUB17 showed extensive HR, while leaves expressing PUB17V322I had reduced HR. This indicates that PUB17V322I can inhibit the function of wild-type *ACRE276* in a dominant-negative fashion. RT-PCR and protein gel blotting experiments clearly indicated the expression of HA-tagged PUB17 and PUB17V322I mRNAs and proteins in Cf-9 and Cf-9:*ACRE276* RNAi tobacco leaf tissue (Figures 5C, 5D, and 5F).

PUB17 Is Required for Full Resistance Specified by *Arabidopsis* RPM1 and RPS4 Resistance Proteins against *Pseudomonas syringae* pv *tomato*

Since *PUB17* can functionally replace the role of *ACRE276* in signaling for Cf-9/Avr9 HR activation, we wanted to test if *PUB17* is required for disease resistance in *Arabidopsis*. We obtained *PUB17* knockouts (Figure 6A) in the Columbia-0 (Col-0) ecotype from the Syngenta Arabidopsis Insertion Library (SAIL) knockout facility and tested these for susceptibility to virulent and avirulent (carrying plasmids *AvrB* and *AvrRPS4*) strains of *Pseudomonas syringae* pv *tomato* (*Pst*). There were no detectable levels of *PUB17* mRNA in the SAIL *PUB17* insertion lines (Figure 6A). The *PUB17* knockout plants showed no obvious morphological phenotype. We could not see any effect on the growth of virulent *Pst* DC3000 in the *PUB17* knockout plants, suggesting that basal defense was not affected (Figure 6B). However, we saw a 5- to 10-fold increase in the growth of avirulent *Pst* (*AvrB* and *AvrRPS4*) 3 d after inoculation and increased chlorosis by day 4 (Figure 6C). These data indicate that *PUB17* knockout plants

are compromised for RPM1- and RPS4-mediated resistance against avirulent *Pst*.

DISCUSSION

Stress-Induced *ACRE276* Regulates Defense Gene Expression and Encodes a U-Box Protein Closely Related to PUB17 and *Brassica napus* ARC1

To identify signaling components involved in Cf-9/Avr9-mediated defense responses, cDNA-AFLP transcript profiling after elicitation was performed. This showed that *ACRE276* is induced by elicitation, and VIGS of this gene in *N. benthamiana* suggested that *ACRE276* may be involved in Cf-9- and Cf-4-mediated HR (Rowland et al., 2005). However, the genetic and biochemical roles of *ACRE276* in mediating the HR and disease resistance remained unclear. We defined full-length clones and verified by RT-PCR using gene-specific primers that *ACRE276* is induced transiently by wounding and in a sustained manner by Avr9 elicitor treatment until the onset of HR (Figure 1). This dual induction with wounding and pathogen stress has been previously reported for other *ACRE* genes (Durrant et al., 2000), suggesting that the cDNA-AFLP screen on cell culture-elicited material does not discriminate against genes involved in wounding stress.

To test the requirement for *ACRE276* during Cf-9/Avr9-mediated HR, we silenced *ACRE276* in Cf-9 tobacco plants using hairpin constructs based on a specific region of *ACRE276*. At least three independent transgenic *ACRE276* RNAi plants showed efficient silencing of the *ACRE276* transcript, and in each case, leaf tissue infiltrated with elicitors of Cf- or N-mediated defense responses showed reduced HR (Figure 2). The increased attenuation of HR in *ACRE276* dsRNAi plants is more robust and repeatable than the phenotypes we observed using VIGS in *N. benthamiana*. This patchy silencing effect probably reflects the uneven spread of the viral vector across the leaf tissue in *N. benthamiana* (Ruiz et al., 1998).

Sequence similarity searches using the *ACRE276* RNAi fragment against tobacco sequence databases revealed no significant nucleotide sequence matches with any other genes. This suggests that the loss of HR phenotype we observed is likely due to *ACRE276*-specific gene silencing. These data suggest that *ACRE276* gene expression is rate limiting for the development of HR during Cf- and N-mediated defense signaling.

Alignment of *ACRE276* with protein sequences of PUB17, PUB12, PUB13, *B. napus* ARC1, and rice SPL11 revealed it to be most closely related to PUB17 and *B. napus* ARC1 (Figure 1). PUB17 is more closely related to *ACRE276* and ARC1 than to its homologs in *Arabidopsis*. This indicates that *ACRE276*, PUB17, and ARC1 might perform a similar cellular function. However, secondary structure analysis indicated that *ACRE276* has at least five ARM repeats similar to PUB17, but ARC1 is predicted to have seven ARM repeats (Stone et al., 2003)

The rice *SPL11* gene is the only other known U-box ARM repeat-encoding gene that has been shown to be involved in HR and disease resistance. Interestingly, rice plants with a single base substitution causing a premature termination of SPL11

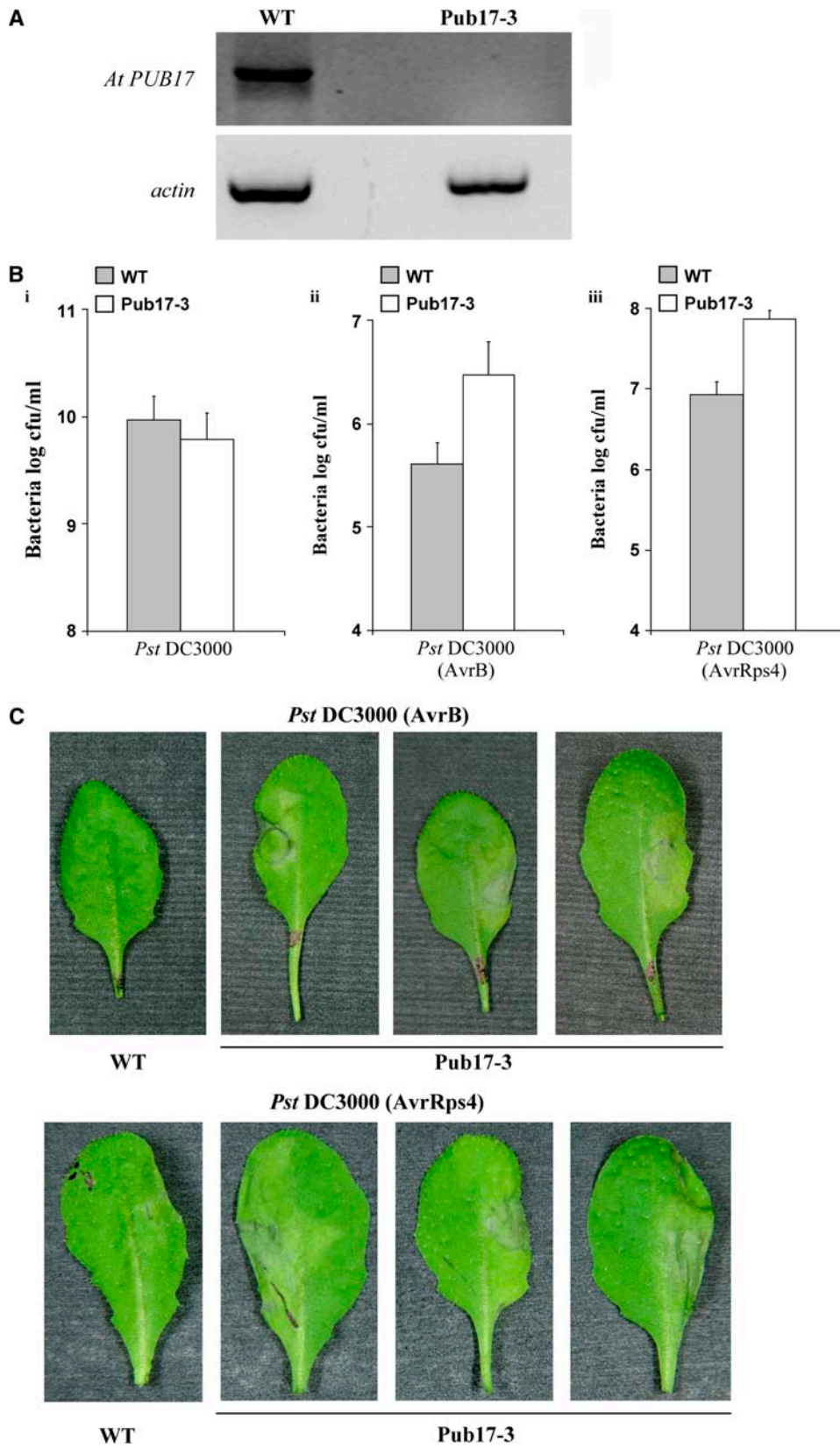


Figure 6. *PUB17* Is Required for Full Resistance Specified by RPM1 and RPS4 against *Pst*.

protein manifest a lesion mimic phenotype (Zeng et al., 2004), suggesting a negative regulatory role for SPL11 in defense.

ACRE276 and PUB17 Are Functional Homologs Whose E3 Ligase Activity Is Required for Plant Cell Death

In ubiquitin-mediated protein modification, E3 enzymes provide substrate specificity, and different types of E3s share little sequence or structural similarity. E3s can be a single polypeptide or multisubunit complexes (Vierstra, 2003). Nevertheless, to date, all identified E3s can be divided into five different groups: the N-end rule E3 (E3a), anaphase-promoting complexes, protein complexes containing SCF (Skp1/Cullin/F-box), Hect domain E3s, and RING finger domain E3s (Lorick et al., 1999). The U-box domain is a derived version of the RING finger domain that lacks characteristic metal chelating residues (Aravind and Koonin, 2000). Proteins containing U-box domains function similarly to the RING finger E3s in mediating ubiquitin conjugation to protein substrates (Jiang et al., 2001). However, modified RING domains have also been found in proteins whose function is to act as SUMO E3 ligases (Hochstrasser, 2001). In yeast, mutations within the Nse2 RING domain abolish its sumoylation activity (Andrews et al., 2005).

Although ACRE276 and PUB17 both contain all canonical amino acids (Figure 1) present in other U-box proteins, it was imperative to establish whether ACRE276 and PUB17 are actual ubiquitin E3 ligases. To achieve this, we overexpressed and purified GST-tagged ACRE276 and PUB17 from *E. coli* and performed in vitro ubiquitination assays. Both ACRE276 and PUB17 are able to conjugate ubiquitin moieties to *E. coli* proteins, clearly indicating that they are indeed ubiquitin E3 ligases (Figure 3). Furthermore, deleting the U-box domain or mutating the conserved Val (Val-316 in ACRE276 and Val-322 in PUB17) within the U-box domain completely abolishes ubiquitin E3 ligase activity without destabilizing the respective proteins (Figure 3). This effect is seen in a number of other U-box E3 ligases (Jiang et al., 2001; Murata et al., 2001; Ohi et al., 2005), illustrating that the in vivo function of ACRE276 and PUB17 is likely to be as an E3 ubiquitin ligase.

Even though ACRE276 and PUB17 proteins share extensive sequence similarity, there is considerable DNA sequence divergence between ACRE276 and PUB17 genes (only 63% identity). Thus, it is unlikely that PUB17 mRNA can be affected by ACRE276-triggered posttranscriptional gene silencing. To de-

termine whether PUB17 can functionally replace ACRE276, we expressed PUB17 under its native promoter in leaf patches of Cf-9 tobacco ACRE276 RNAi plants (Figure 5). Three days later, we infiltrated the same leaf patches expressing PUB17 with IF (Avr9). This treatment resulted in the restoration of full HR previously attenuated due to ACRE276 silencing, showing that PUB17 is a functional homolog of ACRE276.

This *Arabidopsis-Nicotiana* complementation assay has provided useful insights into the functions of several other similarly conserved genes involved in the ubiquitin-proteasome system and defense responses such as Sgt1 and Skp1 (Liu et al., 2002a). We used this bioassay to determine if the E3 activity of PUB17 is required for the development of the HR after Cf-9/Avr9 interaction in ACRE276 RNAi plants. We were unable to complement the loss of HR in ACRE276 RNAi plants by transiently expressing the catalytically inactive AtPUB17V322I mutant (Figure 5B). Taken together with the in vitro ubiquitination assays, this is direct evidence linking ubiquitin ligase activity of any E3 ligase to the plant defense response.

Mutating the U-box central core domain Val can abolish the E3 ligase activity of U-box proteins by disrupting the U-box tertiary structure (Ohi et al., 2003). Nevertheless, the AtPUB17V322I mutant may still interact with substrate but fail to complete its subsequent ubiquitination role. We conjectured that such an allele of PUB17 could act in a dominant-negative manner in the presence of wild-type ACRE276. Overexpressing PUB17V322I in Cf-9 tobacco ACRE276 wild-type leaves blocks the development of full HR after Avr9 treatment (Figure 5E). This provides further evidence for the importance of the ubiquitin E3 ligase activity of PUB17 in defense.

Tomato ACRE276 and PUB17 Are Required for Disease Resistance in Plants

Loss of HR is not always correlated with a loss of resistance (Sharma et al., 2003), so it was important to test whether VIGS of tomato ACRE276 in Cf-9 tomato can lead to a loss of resistance against *C. fulvum*. In accordance with our observation in *N. benthamiana* plants (Rowland et al., 2005) and tobacco ACRE276 RNAi plants, Cf-9 tomato plants silenced for tomato ACRE276 show reduced HR after Avr9 infiltration (Figure 4A). Furthermore, resistance of tomato ACRE276-silenced tomato plants against *C. fulvum* was compromised. *C. fulvum* strains carrying Avr9 were able to grow and complete their full life cycle,

Figure 6. (continued).

(A) RT-PCR analysis in the PUB17 T-DNA insertion line. Leaf tissue from 5-week-old *Arabidopsis* wild-type Col-0 and Pub17-3 plants was used for cDNA preparation and RT-PCR. cDNA derived from these plants was subjected to at least two rounds of 30 cycles, totaling 60 rounds of PCR amplification. The first 30 cycles of PCR amplification were performed with the original cDNA, and then a second round of 30 cycles of PCR was performed on amplified template derived from the previous 30 rounds of PCR. Both DNA amplifications were performed with Hotstart PCR for increased specificity. *Arabidopsis actin* was used as control for cDNA levels.

(B) Growth analysis of virulent and avirulent *Pst* in Col-0 and PUB17 insertion lines. *Pst* growth measurements 3 d after inoculations with (i) DC3000 (vector only), (ii) DC3000 (*AvrB*), and (iii) DC3000 (*AvrRPS4*). The 5×10^3 colony-forming units were infiltrated into individual leaves of 5-week-old *Arabidopsis* Col-0 (WT) and the PUB17 T-DNA insertion line (Pub17-3). Leaf samples were taken after 3 d of bacterial growth. Colony counts reflect the average of seven individual leaves. Error bars represent 95% confidence limits.

(C) Disease phenotype of Col-0 (WT) and PUB17 insertion mutant (Pub17-3) leaves infiltrated with *Pst* containing *AvrB* (top panel) and *AvrRPS4* (bottom panel). Photographs were taken 4 d after inoculation.

indicating that tomato *ACRE276*-silenced plants are reduced in *Cf-9* function. However, the fungal colonization was not as widespread in tomato *ACRE276*-silenced plants as in *Cf0* plants but was comparable to *Cf-9*-silenced plants (Figure 4B). This is probably due to residual levels of tomato *ACRE276* transcript in tomato *ACRE276*-silenced plants. It is known that *R* gene-silenced plants are rarely as susceptible as plants lacking *R* genes (Liu et al., 2002b). These data indicate that tomato *ACRE276* plays a crucial role in *Cf-9*-mediated resistance against *C. fulvum* in tomato.

In accordance with our observation in tomato, *Arabidopsis* *PUB17* knockout plants in Col-0 background were compromised in *RPM1*- and *RPS4*-mediated resistance against avirulent strains of *Pst* (Figure 6). However, we were not able to see any clear enhanced disease susceptibility to virulent strains of *Pst*, indicating that *PUB17* may primarily function downstream of *R* protein signaling pathways. Both tomato *ACRE276* silencing and *PUB17* T-DNA insertion mutant data identify *ACRE276* and *PUB17* as a new class of positive regulators of plant defense.

Mode of Action of ACRE276 and PUB17 during Plant Defense

From our results, it is clear that the principal mode of action of *ACRE276* and *PUB17* in plants is via the E3 ubiquitin ligase activity. Since U-box/RING domain proteins can directly interact with their substrates, *ACRE276* or *PUB17* may bind directly to their specific target proteins (Okui et al., 2005; Zhang et al., 2005). This interaction is likely to lead to the ubiquitination of the target proteins and subsequent proteosomal degradation. If the degraded protein is a negative regulator of defense, then removing this molecule has the effect of activating defense signaling.

Alternatively, *PUB17/ACRE276* could activate a positive regulator by ubiquitination. Similar ubiquitination-mediated activation of signaling components such as kinases and transcription factors has been reported in mammals and yeast (Muratani and Tansey, 2003; Aebersold et al., 2004).

An intriguing aspect of this work draws from the close sequence similarity of *ACRE276/PUB17* to *B. napus* ARC1 and the fact that interactors of *S*-receptor kinase (SRK) include ARC1 and members of the thioredoxin gene family (Bower et al., 1996). A negative regulator of *Cf-9*-mediated defense signaling is a thioredoxin (Rivas et al., 2004). It is possible that *ACRE276/PUB17* may form a signaling complex with a SRK1-like kinase analogous to the SRK/ARC1/thioredoxin complex in *B. napus* during rejection of self-incompatible pollen in *Brassica* (Gu et al., 1998; Cabrillac et al., 2001). Since programmed cell death is triggered in self-incompatibility interactions between pollen and stigma in *Papaver* (Thomas and Franklin-Tong, 2004), it is tempting to speculate that *ACRE276/PUB17/ARC1* may form part of a common signaling mechanism required for programmed cell death in self-incompatibility and in plant defense.

It is plausible that the *in vivo* substrates of *ACRE276/PUB17* are key signaling molecules that need to be degraded to generate the HR and establish disease resistance in plants. Such substrates will have two important features: covalent attachment of ubiquitin and an extremely short half-life upon avirulent pathogen infection. One can hypothesize that any bona fide target

would be rapidly tagged by ubiquitin and degraded in an *Avr9*-dependent manner but stabilized in *ACRE276*-suppressed plants. The *Arabidopsis-Nicotiana* transgenomic complementation assay, in conjunction with the dominant-negative allele of *PUB17*, provides a versatile tool for isolating and identifying this target and testing its efficacy as a negative regulator of plant cell death and defense.

METHODS

Plant Materials and Growth Conditions

Tobacco (*Nicotiana tabacum*) plants were grown in environmentally controlled cabinets at 24°C with 16-h-light/8-h-dark cycles. The *Cf-9* transgenic *N. tabacum* cv *Petite Havana* line 34.1B was previously described (Hammond-Kosack et al., 1998). Suspensions cultures of *Cf-9* tobacco cells derived from line 34.1B were subcultured as previously described (Piedras et al., 1998). *Lycopersicon esculentum* cv Money-maker was grown at ~20 to 25°C with extra lighting to provide a minimum of 16-h daylength. *Arabidopsis thaliana* plants were grown as described before (Muskett and Parker, 2003). *Arabidopsis* *PUB17* T-DNA insertion lines in Col-0 ecotype were obtained from the SAIL facility (www.tmri.org). The T-DNA insertion sites were confirmed by PCR using the T-DNA left border primer 5'-TGGTTCACGTAGTGGGCCATCG-3' and *PUB17*-specific primer 5'-AAGAAATTCGACTCTTTTGG-3'.

Bacterial Pathogen Strains and Pathology Tests

Pseudomonas syringae pv *tomato* (*Pst*) strains were cultured in King's Medium B (Uknes et al., 1992) at 28°C. *Arabidopsis* ecotype Col leaves were infiltrated with bacterial suspensions of virulent and avirulent *Pst* expressing *AvrB* and *AvrRPS4* in 10 mM MgCl₂. Inoculations were made into the abaxial leaf surface using a 1-mL syringe. Individual leaves were inoculated with 5 × 10³ colony-forming units of each respective *Pst* strain (Grant and Loake, 2000).

Bacterial populations in leaves were sampled by maceration in 1 mL of 10 mM MgCl₂ and serial dilutions plated on fresh King's agar containing appropriate selection antibiotics. Population sizes were examined on the days indicated; seven replicates were taken for each sampling.

Generation of Cf-9 Tobacco ACRE276 RNAi Plants and Transient Assays in N. tabacum

The 500-bp fragments of the *ACRE276* 3' ARM repeat domain were amplified by PCR in sense and antisense directions and ligated into the pKannibal RNAi vector (Wesley et al., 2001), resulting in plasmid SLJ20242. This plasmid was transformed into *Agrobacterium tumefaciens* strain LBA4404 and transformed into *Cf-9* tobacco plants as described previously (Horsch et al., 1985). The transgenic plants were selected on Murashige and Skoog plates containing Basta (20 µg/mL) for the tobacco *ACRE276* construct and kanamycin (300 µg/mL) for the *Cf-9* transgene. Positive transformants were moved onto soil and the T2 seeds collected for further characterization. IF containing *Avr4* and *Avr9* peptides was prepared from tobacco plants as described before (Hammond-Kosack et al., 1998). Transient assays were performed by cloning *PUB17*, *PUB17V322I* mutant, and GFP into the binary vector pBIN19. The resulting constructs were transformed into *Agrobacterium* strain GV3101. Growth and infiltration of *Agrobacterium* cultures into tobacco leaf tissue were performed essentially as described by Hammond-Kosack et al. (1998). All further analysis on the infiltrated leaf tissue was performed 3 d after inoculation.

Protein Gel Blot Analysis

Protein gel blot analysis was performed as previously described (Peart et al., 2002) with rat monoclonal anti-HA antibodies (Roche) and mouse anti-GST antibodies (Novagen). The primary antibodies were used at 1:1000 dilution, and the secondary horseradish peroxidase-conjugated secondary antibodies were used at a 1:10000 dilution. Amersham ECL-plus protein gel blotting chemiluminescence detection kits were used to detect levels of horseradish peroxidase and to develop the blots on light-sensitive autoradiograph films.

TRV-Based VIGS in *L. esculentum* and *Cladosporium fulvum* Pathotests

A 200-bp PCR fragment corresponding to the last three ARM repeats of the tomato *ACRE276* was amplified using primers TomNtACRE2761 (5'-GAAGAAGCTGCTGGTGCATT-3') and TomNtACRE2762 (5'-ATACTCTAGCAAGCGATGCT-3'). The PCR products were amplified from tomato cDNA and cloned into *Sma*I-digested *pTRV-RNA2* (Liu et al., 2002a). Silencing of tomato plant cultivar MoneyMaker transgenic for *Cf-9* was done essentially as described by Liu et al. (2002) with the exception that 10-d-old seedlings were infiltrated with *Agrobacterium*.

Three to four weeks after the onset of gene silencing, the plants were treated with paclobutrazol and dip-inoculated with 5×10^6 spores of *C. fulvum* race 9 as previously described (Thomas et al., 1997). Fungal growth was scored 3 weeks later by GUS staining and analyzed by light microscopy. In parallel, leaves of silenced tomato plants were infiltrated with Avr9 peptide and analyzed for the development of HR.

Protein Sequence Alignment and Phylogenetic Analysis

Plant U-box ARM repeat proteins used for analysis were as follows: *ACRE276* from *N. tabacum* (GI 30013679), *PUB17* from *Arabidopsis* (GI 15218915), *ARC1* from *Brassica napus* (GI 2558938), *SPL11* from *Oryza sativa* (GI 51038703), *PUB12* from *Arabidopsis* (GI 18401867), and *PUB13* from *Arabidopsis* (GI 15231445). Protein sequences were aligned using ClustalX (Thompson et al., 1997) with default gap penalty settings and BLOSUM series substitution matrices. Protein domains were identified with PSMART (Schultz et al., 1998; Letunic et al., 2004). The phylogenetic tree was generated from the unmodified alignment using MEGA3.1 software (Kumar et al., 2004). Phylogeny was inferred by maximum parsimony (Nei and Kumar, 2000) with default CNI values for tree searching. Amino acid scoring was based on the JTT substitution model (Jones et al., 1992), and validity testing was based on 1000 bootstrap replicates.

RT-PCR

Total RNA from two 2-cm-diameter leaf discs was isolated using the Tri Reagent (Sigma-Aldrich) method following the manufacturer's instructions. First-strand cDNAs were synthesized from 2 μ g of total RNA using Superscript II RNase H reverse transcriptase (Invitrogen). cDNAs for tobacco *ACRE276* and defense-related genes were amplified by PCR using the following primers: NtACRE276-F (5'-GTGGGGATCCATGTGTTGAAA-3') and NtACRE276-R (5'-AGACATCACCAGCAAAAGTTG-3'). *Actin* was used as an internal positive control for equal cDNA amounts using primers NtACT2a (5'-CTATTCTCCGCTTTGGACTTGGCA-3') and NtACT2b (5'-ACCTGCTGGAAGGTGCTGAGGGAA-3').

PUB17 gene expression in *Arabidopsis* Col-0 and the *PUB17* T-DNA insertion lines was verified using the *PUB17*-specific primers 5'-AGTCTTGTCGACATGGCCCTCAGCGGCGATATTCT-3' and 5'-AGTCTTCTAGACAACACTGGTACGGAGATTGAGA-3'.

Recombinant Protein Purification and E3 Ubiquitin Ligase Activity Assay

Wild-type and mutated *ACRE276* and *PUB17* cDNAs were introduced, via the Gateway system (Invitrogen), into the *pDEST15* vector to produce an in-frame fusion with the GST tag. All recombinant fusion proteins were retained mostly in the insoluble fraction of *Escherichia coli* strain BL21 (DE3)*pLysS*, the insoluble fraction was solubilized and dialyzed according to the protein refolding kit (Novagen), and the soluble protein was used for in vitro ubiquitination assay.

In vitro ubiquitination assays were performed as described previously (Hardtke et al., 2002). Each reaction (30- μ L final volume) contained 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, 1 mM ATP, 0.2 mM DTT, 10 mM phosphocreatine, 0.1 unit of creatine kinase (Sigma-Aldrich), 2 μ g purified 3xHA-ubiquitin, 50 ng of yeast E1 (Affiniti Research), 150 ng E2 UbcH5b (Affiniti Research), and 1 μ g of eluted/bead-bound GST-NtACRE276 and GST-NtACRE276VI (VI) protein, U-box deleted protein, Δ U-box NtACRE276, GST-AtPUB17, and GST-AtPUB17VI. The reactions were incubated at 30°C for 2 h and stopped by adding 4 \times SDS-PAGE sample buffer (125 mM Tris-HCl, pH 6.8, 20% [v/v] glycerol, 4% [w/v] SDS, and 10% [v/v] β -mercaptoethanol) at 100°C for 5 min and analyzed by SDS-PAGE electrophoresis followed by protein gel blotting using anti-HA antibodies (Roche) and anti-GST antibody (Novagen).

Accession Numbers

Sequence data for *ACRE276* and *PUB17* can be found in the GenBank/EMBL data libraries under the nucleotide accession numbers AY220483 and At1g29340, respectively.

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