Ubiquitin Extension Protein UEP1 Modulates Cell Death and Resistance to Various Pathogens in Tobacco

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ABSTRACT

Ubiquitin (Ub) extension proteins (UEPs) are fusion proteins of a Ub at the N terminus to a ribosomal protein. They are the main source of Ub and the only source of extension ribosomal protein. Although important roles of the Ub-26S proteasome system in various biological processes have been well established, direct evidence for the role of *UEP* genes in plant defense is rarely reported. In this study, we cloned a Ub-S27a-type *UEP* gene (*NbUEP1*) from *Nicotiana benthamiana* and demonstrated its function in cell death and disease resistance. Virus-induced gene silencing of *NbUEP1* led to intensive cell death, culminating in whole-seedling withering. Transient RNA interference (RNAi) of *NbUEP1* caused strong cell death in infiltrated areas, while stable *NbUEP1*-RNAi tobacco plants constitutively formed necrotic lesions in leaves. *NbUEP1*-RNAi plants exhibited increased resistance to the oomycete

Pythium aphanidermatum and viruses *Tobacco mosaic virus* and *Cucumber mosaic virus* while displaying decreased resistance to the nematode *Meloidogyne incognita* compared with non-RNAi control plants. Transcription profiling analysis indicated that jasmonate and ethylene pathways, lipid metabolism, copper amine oxidase-mediated active species generation, glycine-rich proteins, vacuolar processing enzyme- and RD21-mediated cell death and defense regulation, and autophagy might be associated with *NbUEP1*-mediated cell death and resistance. Our results provided evidence for the important roles of plant UEPs in modulating plant cell death and disease resistance.

Keywords: cell death, disease resistance, genetics and resistance, tobacco, ubiquitin, ubiquitin extension protein

Ubiquitin (Ub), comprising 76 amino acids (aa), is a highly conserved peptide in eukaryotes. Ubiquitylation, commonly mediated by a Ub-activating enzyme (E1), a Ub-conjugating enzyme (E2), and a Ub ligase enzyme (E3), is a well established system of post-translational modification (Vierstra 2012). The ubiquitylated proteins are generally degraded by 26S proteasome. The Ub-26S proteasome system is widely involved in regulation of various biological processes by targeting key regulatory proteins of these processes. Over the past years, the important roles of this pathway in plant–pathogen interactions have also been increasingly unveiled. The Ub-26S proteasome system contributes to a variety of defense mechanisms in plants against various pathogens, including the tiered plant defense system comprising pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) and effector-triggered immunity (ETI), while pathogens inhibit or usurp this system to favor their infection and disease development (Marino et al. 2012; Zhou and Zeng 2017).

Ub exists as Ub polymer or N-terminal Ub fusion to ribosomal proteins, the later being recognized as Ub extension proteins (UEPs) (Callis et al. 1995). Based on the fused ribosomal protein, the

currently known UEPs are divided into two types: ribosomal L40-type and ribosomal S27A-type. The two classes of ribosomal proteins differ in length and gene structure. The ribosomal L40 fused to Ub is approximately 52 aa in length and this type of *UEP* gene contain introns in the coding region, whereas the ribosomal S27A fused to Ub is approximately 81 aa in length and the corresponding *UEP* genes do not contain intron in the coding region (Callis et al. 1990). Functions of *UEP* genes in plants are still not fully understood. As in other higher eukaryotes, the initial translation products of *Arabidopsis UEP* genes are processed to separate the extension protein from Ub (Callis et al. 1990) by cleaving by Ub-specific proteases (Yan et al. 2000). The UEPs are not only the unique source for the extension ribosomal proteins but also the most important source for Ub. The extension ribosomal protein moiety of *Arabidopsis UEP* proteins is constituent of ribosomes, while the Ub moiety may act as a chaperone and assist in the formation of the holoribosome, as observed for other higher eukaryotes (Finley et al. 1989; Redman and Rechsteiner 1989; Spence et al. 2000). A study for a Ub-S27a-type UEP from *Nicotiana benthamiana* demonstrated that the Ub moiety did not affect the cellular localization of S27a but did alter its protein level, because expression of S27a without the Ub moiety caused a severe reduction in S27a protein level (Hanania et al. 2009). Additionally, the mature form of Ub released from UEPs after proteolysis may function as typical Ub in protein modification and, thus, is involved in various biological processes. Little is known about the function of extension ribosomal protein moiety. Given that they are assembled into ribosomes, they may be involved in protein biosynthesis. In addition, the ribosomal protein S27a of a UEP from *N. benthamiana* plays a critical role in the meristematic cell differentiation and proliferation. Overexpression of this S27a was correlated with increased proliferation of undifferentiated cells and arrest of morphologically “normal” shoot and leaf development (Hanania et al. 2009).

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Although the important roles of the Ub-26S proteasome system in plant development and plant–pathogen interactions have been well established, the direct evidence for the role of *UEP* genes in plant–pathogen interactions is rarely reported. The potato cyst nematode *Globodera rostochiensis* effector GrUBCEP12 is a UEP that consists of a signal peptide for secretion, a monoubiquitin domain, and a 12-aa carboxyl extension protein (CEP12) domain that does not have similarity to any known protein. This secreted noncanonical UEP effector is involved in its plant parasitism. When delivered into host plant cells, this UEP is processed into free Ub and a CEP12 peptide (GrCEP12) in planta, and GrCEP12 suppresses resistance-gene-mediated cell death and basal resistance against the nematode *G. rostochiensis*, an unrelated bacterial pathogen (*Streptomyces scabies*) (Chronis et al. 2013), and flg22-mediated PTI (Chen et al. 2013). Moreover, this UEP but not GrCEP12 potentially affects the host 26S proteasome to promote feeding cell formation (Chronis et al. 2013). Nevertheless, to date, the direct evidence for the function of plant UEP in disease resistance is still lacking.

In this study, we demonstrated the function of a tobacco Ub-S27a-type *UEP* gene in cell death and disease resistance, and analyzed the possible mechanisms underlying these functions. Our results provided evidence for the important roles of plant UEP in modulating cell death and disease resistance.

MATERIALS AND METHODS

Cloning and sequence analyses of *NbUEP1*. *NbUEP1* was cloned from *N. benthamiana* by real-time polymerase chain reaction (RT-PCR). Total RNA was extracted with TRIzol (Invitrogen), and reverse transcription from total RNA was conducted using the PrimeScript RT-PCR kit (TaKaRa Biotechnology). PCR was then performed from cDNAs using gene-specific primer pair NbUbi-F/NbRlp-R, designed according to the full-length cDNA sequence of the UEP (AY912494) (Supplementary Table S1). PCR products were ligated into T-vector and sequenced. The cloned sequence was compared with the other tobacco *UEP* sequences, including the *N. tabacum* *UEP* gene (AJ223329) and *N. benthamiana* *UEP* gene (AY912494) using GeneDoc program (Nicholas et al. 1997).

Virus-induced gene silencing assay. Virus-induced gene silencing (VIGS) analyses and their efficiency evaluation were conducted as described previously (Li et al. 2012). A 383-bp sequence of *NbUEP1*, including both Ub and ribosomal protein moieties, was amplified with primers NbUEP-F3 and NbUEP-R3 and inserted into *Tobacco rattle virus* (TRV)-based VIGS vector pYL156 with the sites *EcoRI* and *BamHI*. The recombinant pYL156-*NbUEP1* was transformed into the *Agrobacterium* strain GV3101, which was mixed with a pTRV1-transformed strain and then delivered into leaves of *N. benthamiana* plants by agro-infiltration with a needle-free syringe to initiate gene silencing. The VIGS analyses were repeated twice. For each experiment, 10 plants were analyzed.

Transient RNA interference assay. The full-length open reading frame (ORF) of *NbUEP1*, amplified using the primer pair NbUbi-F/NbRlp-R, was inserted in reverse and forward directions into the two sides of a bean nitrate reductase gene intron with the sites *XhoI/KpnI* and *XbaI/BamHI*, respectively, in the pCambia1301-based RNA interference (RNAi) vector (pCRNAi-*NbUEP1*) so that an inverted repeat (IR) sequence of *NbUEP1* could be formed to initiate efficient RNAi. The recombinant plasmid pCRNAi-*NbUEP1* was transformed into the *Agrobacterium* strain GV3101. The bacterial suspension, with an optical density at 600 nm (OD₆₀₀) value of 2.0 was infiltrated into leaves of *N. benthamiana* and *N. tabacum* plants at the six-leaf stage and flowering stage, respectively. The transient RNAi assays were repeated twice. For each experiment, eight plants were analyzed.

Transient overexpression assay. The full-length ORF of *NbUEP1* amplified using the primer pair NbUbi-F/NbRlp-R was

inserted into the overexpression vector pCHF3 after the CaMV 35 S promoter by *BamHI/SalI*. The recombinant plasmid pCHF3-*NbUEP1* was transformed into the *Agrobacterium* strain GV3101. The viral gene-silencing suppressor P19 was coexpressed to avoid possible silencing of *NbUEP1* in the transient overexpression assay. The mixed *Agrobacterium* suspension with a final OD₆₀₀ value of 2.0 for both *NbUEP1*- and *P19*-expressing bacteria was infiltrated into leaves of *N. benthamiana* plants at the six-leaf stage. The transient overexpression assays were repeated twice. For each experiment, six to eight plants were analyzed.

Construction of stable *UEP1*-RNAi plants. Stable *UEP1*-RNAi tobacco plants were constructed by *Agrobacterium*-mediated transformation of pCRNAi-*NbUEP1* into leaf discs of *N. tabacum* cultivar K326 following previously described procedures (Cai et al. 2007). The T₂ homozygous lines with single-copy insertion were selected for further functional analyses. The RT-quantitative PCR (RT-qPCR) assay was performed using primers NbUEP-F and NbUEP-R to detect the expression level of the *UEP1* gene in each line, as described in more detail below.

Gene expression analyses. Reverse-transcription PCR and RT-qPCR analyses and consequent statistical data analyses were conducted as described (Cao et al. 2016; Zhao et al. 2013). RT-qPCR data were statistically analyzed using SPSS software (version 19.0; IBM). Significant difference between the mean values of three independent experiments was determined with Duncan's multiple range test ($P < 0.05$) or *t* test ($P < 0.01$). The data represent means $\pm$ standard error (SE).

Trypan blue staining assay. Leaf discs were depigmentized in an ethanol/chloroform mixture (3:1 [vol/vol]) for 4 h, and then stained in staining solution (10 ml of lactic acid, 10 ml of glycerol, and 10 ml of trypan blue solution at 1 mg/ml) for 4 h, followed by destaining in saturated chloral hydrate for 2 h. The destained leaf discs were mounted on slides in 70% glycerol for microscopy observation. The trypan blue staining assays were conducted for *UEP1*-RNAi tobacco lines 2, 4, and 5 and repeated twice. For each experiment, six plants were analyzed.

Plant inoculation and resistance evaluation. Resistance of *UEP1*-RNAi tobacco plants to oomycete *Pythium aphanidermatum*, viruses *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV), and nematode *Meloidogyne incognita* was examined through inoculation analyses. For *P. aphanidermatum*, leaves were inoculated with mycelial plugs and disease lesion diameter was measured as described (Zhao et al. 2013). For TMV and CMV, virus was extracted from diseased *N. tabacum* K326 and *N. glutinosa* leaves, respectively, displaying strong viral symptoms (10 ml of phosphate-buffered saline [PBS] per 1 g of leaves). Two leaves per plant were dusted with carborundum and inoculated by gently rubbing with the viral crude extracts (200 μ l/leaf, PBS as control). Five minutes later, the inoculated leaves were rinsed with water to remove the dusts. The inoculated plants were grown at 25°C with a photoperiod of 16 h of light and 8 h of darkness. The cDNA sequences derived from coat protein and movement protein genes were detected by RT-qPCR using gene-specific primers. For *M. incognita*, each plant was inoculated with 5 ml of second-stage juvenile (J2) nematode inocula containing approximately 2,000 nematodes. The inocula were poured into a hole that was punched 8 to 10 cm into soils at approximately 2 cm from plant stem. The inoculated plants were grown at 25°C with a photoperiod of 16 h of light and 8 h of darkness. Root-knot formation of inoculated plants was investigated following a described 0-to-5 galling index system (Barker 1985). Disease index was calculated as $100 \times \Sigma(N \times R)/(8 \times 5)$, where *N* = number of plants in each rate, *R* = rate value, 8 = number of total plants, and 5 = the highest rate value.

The inoculation analyses were conducted three times in plants of *UEP1*-RNAi tobacco lines 2, 4, and 5 with similar results. Eight plants from each line were used for each inoculation experiment. The inoculation data were statistically analyzed using SPSS software (version 19.0; IBM). A significant difference between

the mean values of three independent experiments was determined with the *t* test ($P < 0.01$). The data represent means $\pm$ SE.

Microarray analysis for transcript profiling. The necrotic tissues of the lower leaves of *UEP1*-RNAi line 4 tobacco plants and the healthy tissues of the corresponding leaves of non-RNAi control plants were sampled. Total RNA was extracted using Trizol and purified using a NucleoSpin RNA clean-up kit, which was then subjected to labeling using the JingXin cRNA amplification label kit. Briefly, the first-strand cDNA was synthesized from purified total RNA using CbcScript enzyme and T7 Oligo (dT) primer. RNA in the generated product was cleaved by RNase H, and the second-strand cDNA was synthesized from the released short RNA fragments using DNA polymerase. The purified double-stranded cDNA was transcribed into cRNA by T7 Enzyme Mix. The purified cRNA was reverse transcribed into DNA by CbcScript II enzyme and Random primer. The DNA was labeled with fluorescent dNTP during synthesis of the second-strand DNA by Klenow Fragment enzyme and Random primer. The labeled DNA was hybridized with Agilent Tobacco mRNA 4-by-44k microarray chip. The hybridization signal was obtained by scanning the chip with an Agilent G2565CA Microarray Scanner, and analyzed by Feature Extraction software. The original data were further analyzed by GeneSpring GX software and normalized using a percentile shift approach. The fold change of gene expression in *UEP1*-RNAi plants relative to non-RNAi control plants was calculated. To solve the problem of lacking annotation of tobacco genes, agriGO software tools (gene ontology [GO] analysis toolkit and database for agricultural community, <http://bioinfo.cau.edu.cn/agriGO/>) were used to functionally annotate the genes under the setup of false discovery rate < 0.05 (Du et al. 2010). The upregulated and downregulated genes associated with each statistically significant ($P < 0.05$, *t* test) GO term were counted and analyzed respectively.

RESULTS

Cloning and sequence analyses of a *Nicotiana benthamiana* UEP gene *NbUEP1*. Searching against the *N. benthamiana* expressed sequence tag database retrieved a set of Ub-related sequences, among which was a full-length cDNA sequence encoding for a UEP protein (AY912494). Using the primers designed in accordance with this *UEP* gene, a *UEP* cDNA sequence was amplified and cloned from *N. benthamiana*. Sequencing results demonstrated that it is a full-length *UEP* gene of 471 bp in size, encoding for a fusion protein of a Ub (76 aa) at its N terminus to a s27a-type ribosomal protein (80 aa) at its C terminus and, thus, was provisionally named *NbUEP1* (National Center for Biotechnology Information accession number MG333694) (Fig. 1A). BLASTn searching analysis indicated that the *NbUEP1* nucleotide sequence was most similar to an *N. tabacum* *UEP* gene (AJ223329), with an identity of 96%, followed by to the *N. benthamiana* *UEP* gene (AY912494), with an identity of 91% (Supplementary Fig. S1).

VIGS of *NbUEP1* caused strong necrosis. Taking advantage of *N. benthamiana* as a model VIGS plant species, we preformed VIGS analysis to understand the function of *NbUEP1*. To ensure that effects from both Ub and ribosomal protein moieties could be observed, a 383-bp sequence of *NbUEP1*, including both Ub and ribosomal protein moieties, were cloned into TRV-based VIGS vector for silencing analyses. Remarkably, necrosis developed in the agroinfiltrated areas at 3 to 4 days postinfiltration (dpi) and spread beyond the infiltrated areas to cause the whole infiltrated leaves to wilt at 5 dpi. Later, the necrosis expanded beyond the infiltrated leaves to the stem and up uninfiltrated leaves and, ultimately, the whole plant withered at 7 to 8 dpi (Fig. 1B). Transcript accumulation analysis by RT-qPCR showed that the *NbUEP1* gene had been efficiently silenced (Fig. 1B). This result indicated that silencing of *NbUEP1* led to intensive cell death in plants.

Transient RNAi but not overexpression of *NbUEP1* resulted in strong cell death. To confirm the VIGS result and

avoid the effect from TRV virus, another gene-silencing strategy RNAi was employed to analyze the function of *NbUEP1* in cell death. The full-length ORF of *NbUEP1* was inserted in reverse and forward directions into the two sides of an intron, respectively, in the pCambia1301-based RNAi vector (pCRNAi-*NbUEP1*) so that an IR sequence of *NbUEP1* could be formed to initiate efficient RNAi (Fig. 2A). *Agrobacterium* strain GV3101 transformed with pCRNAi-*NbUEP1* was infiltrated into leaves of *N. benthamiana* plants. Cell death occurred strongly in the infiltrated areas and the time required for complete necrosis development depended on the stage of plant for infiltration. It took 4 days for leaves of six-leaf stage plants whereas it needed 6 days for those of flowering stage plants (Fig. 2B and C). In addition, infiltration of the same *Agrobacterium* in *N. tabacum* also caused necrosis as observed in *N. benthamiana* (Fig. 2D). Transcript detection by RT-qPCR showed that the *UEP1* gene in both plant species had been efficiently silenced (Fig. 2E). It is not unexpected considering that *NbUEP1* is 96% identical to an *N. tabacum* *UEP* gene (AJ223329).

Fig. 1. Virus-induced gene silencing (VIGS) of *NbUEP1* resulted in intensive necrosis. **A**, Structure of protein sequence predicted from *NbUEP1* cloned in this study. **B**, Symptoms of *Tobacco rattle virus*-induced gene silencing of *NbUEP1* in *Nicotiana benthamiana* at 7 days postagroinfiltration (up) and efficiency of *NbUEP1* gene silencing detected by quantitative real-time polymerase chain reaction (down). 18S ribosomal DNA serves as an inner control. Data represent mean $\pm$ standard error of three independent experiments. Double asterisks (**) indicate a significant difference of *NbUEP1* transcript accumulation between *UEP1*-silenced plants and empty vector (EV) control plants ($P < 0.01$, *t* test).

Additionally, the RNAi enhanced the expression of *HIN1*, a cell death-related gene (Fig. 2F), confirming in the molecular level that RNAi of *NbUEP1* resulted in cell death.

To further understand the role of *NbUEP1* in cell death, a transient overexpression assay was carried out for this gene. Agroinfiltrated areas already showed enhanced expression of *NbUEP1* at 3 dpi, with *Agrobacterium* expressing *NbUEP1* under CaMV 35S promoter, but did not exhibit visible necrosis even at 6 dpi (Supplementary Fig. S2).

Collectively, these results reveal that RNAi but not overexpression of *NbUEP1* induces strong cell death in both *N. benthamiana* and *N. tabacum*.

Stable *NbUEP1*-RNAi plants constitutively developed cell death and defense responses. To confirm the transient RNAi assay result and further understand the role of *NbUEP1* in disease resistance of common tobacco, stable *NbUEP1*-RNAi plants were constructed by transformation of pCRNAi-*NbUEP1* into *N. tabacum*. Six independent lines were generated. Genetic segregation analyses for T1 seed showed that lines 2, 4, and 5 contained a single-copy insertion of *NbUEP1*; thus, their T₂ homozygous lines were selected for further functional analyses. The RT-qPCR assay demonstrated that transcript of *UEP1* in lines 2, 4, and 5 was only 25.5, 17.5, and 24.3%, respectively, of that in control plants (Fig. 3A), demonstrating that the *UEP1* gene had been efficiently knocked down in these RNAi lines.

UEP1-RNAi plants showed some specific phenotypes compared with non-RNAi control plants. In *UEP1*-RNAi plants, the lower leaves narrowed and crinkled and formed water-soaked necrotic spots, which cohered at a later stage, while some leaves were mosaic (Fig. 3B and C). Nevertheless, severe phenotypes only exhibited in the lower leaves, and these plants could grow up and fruit normally.

Fig. 2. Transient RNA interference (RNAi) of *NbUEP1* caused strong cell death. **A**, Diagram for structure of *NbUEP1*-IR sequence inserted in RNAi vector. Symptoms of transient *NbUEP1*-RNAi (RNAi) in **B**, *Nicotiana benthamiana* at the six-leaf stage and **C**, flowering stage. EV = infiltration with *Agrobacterium* transformed with empty vector. **D**, Symptoms of transient *NbUEP1*-RNAi in *N. tabacum* at the six-leaf stage. Efficiency of **E**, *NbUEP1*-RNAi and **F**, *HIN1* expression in transient *NbUEP1*-RNAi leaves of *N. benthamiana* plants at 3 days postinfiltration (dpi). Efficiency of *NbUEP1*-RNAi was analyzed by quantitative real-time polymerase chain reaction (RT-qPCR). *HIN1* expression was detected by RT-PCR. Polyacrylamide gel electrophoresis profiles of RT-PCR products were shown. 18S ribosomal DNA served as an inner control for the loading check.

To further understand the cell death that appeared in *UEP1*-RNAi plants, a trypan blue staining assay was performed and expression of cell death-related gene *HIN1* and pathogenesis-related (*PR*) genes were monitored. A trypan blue staining assay showed that the necrotic tissues of *UEP1*-RNAi plants were clearly stained into blue, whereas no-symptom and mosaic tissues of *UEP1*-RNAi plants were not, as observed for those of non-RNAi control plants (Fig. 3D). This result confirmed that cell death, indeed, occurred in the necrotic tissues of *UEP1*-RNAi plants. Moreover, the RT-qPCR assay revealed that, compared with the non-RNAi control plants, expression of two *PR* genes (*PR1* and *PR5*), and the cell death-related gene *HIN1* was strongly upregulated by 19.1-fold, 26.2-fold, and 2.7-fold, respectively, in the necrotic tissues of *UEP1*-RNAi plants, whereas expression of these genes in the other two types of tissues of *UEP1*-RNAi plants was only slightly increased (Fig. 3E).

Taken together, our results obtained using *UEP1*-RNAi plants reveal that RNAi of *UEP1* constitutively activates strong cell death and defense responses in tobacco plants.

***UEP1* widely influences the resistance against various pathogens.** To probe the role of *UEP1* in disease resistance of common tobacco, inoculation analyses were conducted in stable *UEP1*-RNAi tobacco plants against a variety of pathogens, including oomycete *P. aphanidermatum*, viruses TMV and CMV, and nematode *M. incognita*.

RNAi of *UEP1* enhanced resistance to the oomycete pathogen *P. aphanidermatum*. The fully expanded symptomless leaves of *UEP1*-RNAi plants were inoculated with the pathogen mycelial plugs. Inoculation experiments demonstrated that, after inoculation, disease necrosis developed much slower in *UEP1*-RNAi plants than non-RNAi control plants. At 27 h postinfiltration, the lesion was only 2.0 cm in diameter in *UEP1*-RNAi plants whereas that had expanded to 2.8 cm in diameter in non-RNAi control plants (Fig. 4). This result suggests that *UEP1*-RNAi plants display increased resistance to *P. aphanidermatum*.

RNAi of *UEP1* augmented resistance to the viral pathogens TMV and CMV. The symptomless leaves of *UEP1*-RNAi plants were rubbing inoculated with the viral extracts. TMV inoculation experiments showed that, after inoculation, viral disease developed similarly in *UEP1*-RNAi plants and non-RNAi control plants. Growth and flowering were suppressed in both types of plants (Fig. 5A). However, the RT-qPCR assay showed that, in *UEP1*-RNAi plants, the cDNA sequences derived from TMV coat protein (CP) and movement protein (MP) genes only accumulated to less than half of those in non-RNAi control plants (Fig. 5B), indicating that *UEP1*-RNAi plants induced resistance to TMV. Additionally, CMV inoculation analysis showed that growth and development of inoculated non-RNAi control plants were severely repressed. These plants could not flower. However, growth and development of inoculated *UEP1*-RNAi plants were much less suppressed. They could grow up and flower (Fig. 5C). Furthermore, the RT-qPCR assay demonstrated that, in *UEP1*-RNAi plants, the cDNA sequences derived from CMV CP and MP genes only accumulated to approximately 30% of those in non-RNAi control plants (Fig. 5D), indicating that *UEP1*-RNAi plants enhanced resistance to CMV.

RNAi of *UEP1* reduced resistance to the nematode pathogen *M. incognita*. Roots of *UEP1*-RNAi plants were drench inoculated with the J2 nematodes. At 30 days after nematode inoculation, aboveground parts of *UEP1*-RNAi plants and non-RNAi control plants seemed to be similar. However, the roots of *UEP1*-RNAi plants formed many more root-knots than those of non-RNAi control plants. Approximately 80% of the roots of *UEP1*-RNAi plants formed root-knots whereas this percentage was only approximately 25% for non-RNAi control plants. Meanwhile, the number of root-knots per root was also much higher in *UEP1*-RNAi plants than in non-RNAi control plants (Fig. 6A). Moreover, a disease index, calculated based on a 0-to-5

galling index system, was significantly higher for *UEP1*-RNAi plants (89.2) than non-RNAi control plants (37.5) (Fig. 6B). This result indicates that *UEP1*-RNAi plants exhibit decreased resistance to *M. incognita*.

Collectively, our data provided evidence that *UEP1* is broadly involved in tobacco disease resistance against a variety of pathogens. This gene negatively affects resistance to oomycete pathogen *P. aphanidermatum* and viral pathogens TMV and CMV,

Fig. 3. Stable *NbUEP1*-RNA interference (RNAi) plants constitutively developed cell death and defense responses. **A**, Expression level of *UEP1* in stable RNAi tobacco lines relative to empty-vector (EV) control plants. Quantitative real-time polymerase chain reaction (RT-qPCR) results are shown. Data represent mean $\pm$ standard error of three independent experiments. Small letters indicate significant difference ($P < 0.05$, Duncan's multiple range test). **B**, Typical plants of RNAi tobacco. **C**, Magnified view for various symptoms developed in lower leaves of *UEP1*-RNAi plants. Both single and cohered necrotic spots were shown. NS = no symptoms. **D**, Light microscopy observations for trypan blue-stained tissues showing typical symptoms in line 4. Bar = 100 μ M. **E**, Expression of hypersensitive response marker and pathogenesis-related (PR) genes in *UEP1*-RNAi plants of line 4. Data were analyzed and shown as in A.

whereas it positively modulates resistance to nematode pathogen *M. incognita*.

Transcript profiling for *UEP1*-regulated resistance.

Given that Ub not only functions by protein degradation but also plays important roles in transcriptional regulation (Conaway et al. 2002), to explore the molecular mechanisms underlying *UEP1*-modulated tobacco disease resistance, comparative transcript profiling between necrotic tissues of *UEP1*-RNAi plants and healthy tissues of non-RNAi control plants were analyzed using the Agilent Tobacco mRNA 4-by-44k microarray. Genes differentially expressed (DE) between the two types of plants were selected according to the following criteria: (i) absolute fold change ≥ 2.0 , (ii) flag signal for both samples is detectable, and (iii) processed signal value for both samples ≥ 800 . In total, 915 DE genes were identified based on the above criteria, among which 400 were upregulated while 515 were downregulated in *UEP1*-RNAi plants compared with non-RNAi control plants (Supplementary Table S2). To solve the problem of lacking annotation of tobacco genes, agriGO software tools were used to functionally annotate the DE genes (Du et al. 2010). Consequently, 439 (48%) DE genes were successfully annotated. Among the statistically significant ($P < 0.05$, t test) GO terms, 13 of Biological Process, 10 of Molecular Function, and 9 of Cellular Component terms were enriched (comprising at least five genes) (Fig. 7). For the Biological Process class, DE genes associated with eight terms (electron transport chain, oxidation-reduction, nucleosome assembly, thiamine biosynthetic process, thiazole biosynthetic process, proteolysis, intracellular signal transduction, and starch biosynthetic process) were dominantly downregulated (at least five more than upregulated genes and described as “enriched” hereafter), which was the opposite of those associated with the terms calcium-mediated signaling and cellular glucan metabolic process (Fig. 7A). Regarding the Molecular Function class, downregulated DE genes were enriched for seven terms (metal ion binding, electron carrier activity, two iron–two sulfur cluster binding, copper ion binding, protein heterodimerization activity, oxidoreductase activity, and phosphatidylinositol phospholipase C activity), whereas upregulated DE genes were enriched for only one term (xyloglucan-xyloglucosyl transferase activity) (Fig. 7B). For the Cellular Component class, downregulated DE genes were enriched for intracellular localization-associated terms (chloroplast, chloroplast thylakoid membrane, chloroplast stroma, nucleosome, amyloplast, and intracellular part), while upregulated DE genes were enriched

for extracellular localization-associated terms (cell wall and extracellular region) (Fig. 7C). To verify the expression patterns of the DE genes, expression of eight DE genes, which are potentially associated with cell death or defense, was monitored in *UEP1*-RNAi plants and non-RNAi control plants, respectively, by RT-qPCR analysis. Results obtained from this RT-qPCR analysis were similar to those from the microarray transcript profiling assay (Fig. 8). Together, these results indicate that intracellular activities such as photosynthesis are strongly repressed while extracellular activities, including cell wall modification, are significantly promoted in the necrotic tissues of *UEP1*-RNAi plants compared with the healthy tissues of non-RNAi control plants.

To further find out the genes probably involved in *UEP1*-mediated resistance, the DE genes with an absolute fold change ≥ 4 and those encoding a known resistance-related protein were selected (Table 1). Among the most upregulated DE genes were those encoding for a set of glycine-rich proteins (GRPs) (approximately +4.9- to 21.6-fold), Cf-9/Avr9 rapidly elicited protein 44 (ACRE44)/linoleate 13S-lipoxygenase 3-1 (+12.6-fold), several jasmonate-ZIM-domain (JAZ) proteins (approximately +5.0- to 11.4-fold), a GRAS2 transcription factor (+8.6-fold), and a leucine-rich repeat (LRR) family protein (+8.4-fold) (Table 1), indicating that the JAZ-dependent jasmonate (JA) pathway, ACRE44-dependent lipid pathway, GRAS2, LRR family protein, and GRPs might be associated with *UEP1*-mediated resistance. In addition, DE genes encoding for Ca^{2+} -signaling components, including CaMs, CPKs, and CIPKs; a set of WRKY transcription factors, including WRKY6, -31, -33, and -40; and redox regulators, including peroxidase protein PER12 and thioredoxin H-type protein TH9, were also significantly upregulated (Table 1), suggesting that Ca^{2+} signaling, WRKY transcription factors, and redox modulation might also be related to *UEP1*-mediated resistance.

Additionally, in contrast to the above DE genes, the following were strongly downregulated in *UEP1*-RNAi plants: a copper amine oxidase (CuAO, -11.3-fold), three vacuolar processing enzymes (VPEs) (approximately -6.7- to -7.5-fold), RD21 (-4.3-fold), phospholipase A2 (-4.5-fold), glycerate dehydrogenases (-4.2-fold), a phosphoinositide binding protein (-4.2-fold), a set of EFEs/ACOs (approximately -2.0- to -3.3-fold), and two ATG8s (approximately -2.0- to -2.5-fold) (Table 1). These data demonstrate that the CuAO-mediated nitrous oxide (NO) and reactive oxygen species (ROS) production; VPE- and RD21-mediated cell

Fig. 4. RNA interference (RNAi) of *UEP1* enhanced resistance to the oomycete pathogen *Pythium aphanidermatum* in tobacco. **A**, Symptoms of upper (symptomless) leaves of *UEP1*-RNAi plants of line 4 after inoculation with *P. aphanidermatum* mycelial plugs. **B**, Statistical analysis of lesion diameter of inoculated plants; hpi = hours postinfiltration. Data represent mean $\pm$ standard error of three independent experiments. Double asterisks (**) indicate significant difference of lesion diameter between *UEP1*-RNAi plants and empty-vector (EV) control plants ($P < 0.01$, t test).

death and disease resistance; phospholipase A2, glycerate dehydrogenases, and the phosphoinositide binding protein-mediated lipid signaling; EFE/ACO-dependent ethylene (ET) biosynthesis; and ATG8-mediated autophagy, cell death, and resistance might be linked to *UEP1*-mediated resistance.

Interestingly, a set of Ub genes such as UBQ3 and UBQ10 were significantly downregulated by approximately 2.1- to 2.7-fold (Table 1), implying that Ub genes might be cosuppressed with the *UEP1* gene in *UEP1*-RNAi plants due to their high homology in sequence. Considering the molecular relationship between UEP and Ub, this provided another layer of evidence for the efficiency of *UEP1* gene silencing in *UEP1*-RNAi plants, and implied that

Ub-26S proteasome system-mediated protein modification is involved in regulating *UEP1*-mediated resistance.

DISCUSSION

The pivotal role of Ub as protein modifier in various biological processes has been well recognized in all eukaryotes. Nevertheless, the function of Ub fusion protein UEP in plants is rarely studied. In particular, direct evidence for the role of plant UEPs in plant defense against pathogens is still unavailable. In this study, we demonstrated that a tobacco Ub-S27a-type *UEP* gene, *NbUEP1*, regulates plant cell death and influences disease resistance against a variety of

Fig. 5. RNA interference (RNAi) of *UEP1* augmented resistance to the viral pathogens *Tobacco mosaic virus* (TMV) and *Cucumber mosaic virus* (CMV) in tobacco. **A**, Symptoms of *UEP1*-RNAi plants of line 4 after rubbing inoculation of upper (symptomless) leaves with TMV. EV = empty vector control. **B**, Expression of TMV movement protein (MP) and coat protein (CP) genes in inoculated plants. Quantitative real-time polymerase chain reaction (RT-qPCR) results are shown. Data represent mean $\pm$ standard error of three independent experiments. Double asterisks (**) indicate significant difference of expression level between *UEP1*-RNAi plants and EV control plants ($P < 0.01$, t test). **C**, Symptoms of *UEP1*-RNAi plants of line 4 after rubbing inoculation of upper (symptomless) leaves with CMV. **D**, Expression of CMV MP and CP genes in inoculated plants. RT-qPCR results are shown. Data were analyzed and shown as in B.

pathogens, potentially through modulating complex pathways, including JA and ET pathways; autophagy; and CuAO-, VPE-, and RD21-mediated pathways. Our results provided evidence for the contribution of plant UEP to modulating cell death and disease resistance.

Tobacco *UEP1* strongly regulates cell death. Three layers of evidence obtained in this study reveal the important role of tobacco *UEP1* in regulating cell death. First, silencing of *NbUEP1* through VIGS analysis in *N. benthamiana* led to rapid and strong systemic cell death and whole-seedling wilt within a week (Fig. 1). Second, to rule out the interference of viruses in VIGS assays, we conducted RNAi analyses. Transient RNAi of *NbUEP1* induces strong cell death in the agroinfiltrated areas of both *N. benthamiana* and *N. tabacum* plants (Fig. 2). Third, stable *UEP1*-RNAi plants were constructed and these plants constitutively developed cell death. To characterize the cell death, in addition to macroscopic observation, we performed trypan blue staining assays and cell-death-related and defense-related gene expression analysis. Results showed that the necrotic areas were deeply stained by trypan blue, and cell-death-related and defense-related genes were strongly expressed in the necrotic tissues (Fig. 3). Nevertheless, whether the cell death formed in the *UEP1*-RNAi plants is a hypersensitive response (HR) requires further verification by other analyses such as the TUNEL assay. It has been reported that tobacco plants transformed with a wild-type Ub gene showed less Ub accumulation due to cosuppression of the endogenous Ub gene, and these plants displayed strong necrosis (Becker et al. 1993). A similar phenomenon was also observed in a transient in planta expression assay for HR-eliciting gene screening (Karrer et al. 1998). In addition, tobacco plants transformed with a variant Ub gene (Ub R48) in which lys 48 is replaced by arg, acting as a specific inhibitor of Ub-dependent proteolysis, contained normal levels of free Ub but were deficient in Ub-dependent proteolysis. These plants not only exhibited necrotic lesions but also induced expression of *PR-1* gene and enhanced resistance to TMV (Becker et al. 1993). In addition, the Ub R48-transgenic plants showed leaf curling symptoms

(Backmair et al. 1990). Therefore, the phenotypes of the Ub R48-transgenic plants highly resemble those of the *UEP1* transgenic tobacco obtained in this study (Figs. 1, 2, and 3), indicating that, in the *UEP1* transgenic tobacco plants, the Ub system should have been perturbed.

Tobacco *UEP1* modulates disease resistance. Our results demonstrate that *UEP1* is widely involved in tobacco disease resistance against a variety of pathogens, including the oomycete *P. aphanidermatum*, viruses TMV and CMV, and nematode *M. incognita*. This is not unexpected considering that *UEP1* as a precursor of Ub may profoundly affect the accumulation level of Ub and, thus, alter the level of substrate protein ubiquitylation for 26S proteasome-dependent protein degradation, which has been reported to be substantially involved in plant resistance against various types of pathogens (Alcaide-Loridan and Jupin 2012; Dielen et al. 2010; Marino et al. 2012; Ustun et al. 2016). Notably, *UEP1* differentially regulates resistance to different pathogens: negatively to *P. aphanidermatum*, TMV, and CMV and positively to *M. incognita*. This may reflect the fact that contrast (negative versus positive) regulators of resistance are subjected to degradation by the Ub-26S proteasome system in these different pathosystems. The negative regulation of *UEP1* in tobacco resistance against TMV is reminiscent of the previous reports that Ub-transgenic tobacco plants showing cosuppression of endogenous Ub and Ub R48-transgenic tobacco plants manifesting perturbation of Ub-26S proteasome system exhibit increased resistance against this virus (Backmair et al. 1990; Becker et al. 1993). The effect of altered expression levels of plant endogenous *UEP* on resistance to nematodes has not been previously reported. However, GrUB-CEP12, the noncanonical UEP comprising a canonical Ub and CEP12, has been found to be an effector of the potato cyst nematode *G. rostochiensis*, playing important roles in plant parasitism (Chen et al. 2013; Chronis et al. 2013). The Ub moiety seems to affect the host Ub-26S proteasome system, because overexpression of GrUBCEP12 but not GrCEP12 strongly downregulated expression of the host 26S proteasome subunit RPN2a (Chronis et al. 2013). However, it is unclear whether and how the total Ub level in planta in GrUBCEP12 overexpression plants changes and, thus, it is not known whether the total Ub level in GrUBCEP12 overexpression plants significantly reduced due to the cosuppression of plant endogenous Ub genes as observed in Ub-transgenic and transiently expressed plants (Becker et al. 1993; Karrer et al. 1998). Determination of Ub level in planta in the GrUBCEP12 overexpression plants will clarify the functional mechanism of the Ub moiety underlying the resistance in these plants. In addition, as far as we know, this is the first report on the role of the *UEP* gene in plant resistance against an oomycete pathogen.

In addition to the resistance against the pathogens investigated in this study, *UEP1* may also modulate other layers of plant defenses such as PTI and ETI. PTI is initiated by recognition of PAMPs by PRRs, while ETI is initiated by recognition of effectors typically by nucleotide-binding site LRR receptors (NLRs). A list of PRRs and NLRs as well as their signaling components have been reported to be targeted for ubiquitination (Mithoe and Menke 2018; Zhou and Zeng 2017). In this context, given that *UEP1* is the main source of Ub and, thus, might affect ubiquitination, it is conceivable that *UEP1* might influence PTI and ETI. Identification of PTI and ETI components serving as substrates of *UEP1* is interesting research that remains to be conducted.

It needs to be mentioned that *UEP1* might also be indirectly involved in tobacco resistance. That is, RNAi of *UEP1* causes strong cell death in the lower leaves of plants, which might somehow activate resistance in the upper, asymptomatic leaves. In this scenario, resistance to multiple pathogens in *UEP1*-RNAi transgenic plants resembles systemic acquired resistance and the phenotype of some “lesion mimic” mutants (LMMs) (Bruggeman et al. 2015; Lorrain et al. 2003). The lesions of LMMs resulting

Fig. 6. RNA interference (RNAi) of *UEP1* reduced resistance to the nematode pathogen *Meloidogyne incognita* in tobacco. **A**, Symptoms of *UEP1*-RNAi plants of line 4 after drenching inoculation with *M. incognita* at second-stage juveniles at 30 days postinfiltration. Arrows indicate root knots. EV = empty vector. **B**, Disease index analysis for the inoculated plants. Calculation of disease index is based on the 0-to-5 galling index system described in the Materials and Methods. Data represent mean $\pm$ standard error of three independent experiments. Double asterisks (**) indicate significant difference of disease index between *UEP1*-RNAi plants and EV control plants ($P < 0.01$, t test).

from the altered regulation of cell death processes in the absence of pathogen attack resemble an HR-mediated cell death which, in some instances, leads to enhanced disease resistance to multiple pathogens due to constitutive accumulation of defense-related hormones and expression of the PR genes (Bruggeman et al. 2015; Lorrain et al. 2003). Meanwhile, mutants that show enhanced resistance to some pathogens while exhibiting increased susceptibility to others have been reported. For example, barley seedlings carrying the *mlo* mutation showed enhanced resistance to *Blumeria graminis* f. sp. *hordei* and *Phytophthora palmivora* and increased susceptibility to *Magnaporthe grisea* (Jarosch et al. 1999; Kusch and Panstruga 2017).

Mechanisms underlying tobacco *UEP1*-mediated modulation of cell death and disease resistance. To reveal the molecular mechanisms underlying *UEP1*-mediated modulation of cell death and disease resistance, the transcript profile of necrotic tissues of *UEP1*-RNAi plants was compared with that of non-RNAi control plants through tobacco mRNA microarray analyses, considering that Ub not only functions through protein degradation but also plays important roles in transcriptional regulation (Conaway et al. 2002). Notably, genes encoding several JAZ proteins, including JAZ1 and JAZ3, the repressors of JA signaling, were upregulated by approximately 5.0- to 11.4-fold, whereas genes coding for a set of EFEs/ACOs, the key enzymes to catalyze the biosynthesis of ET, were downregulated by approximately 2.0- to

Fig. 8. Expression analyses of eight differentially expressed genes by real-time quantitative polymerase chain reaction. 18S ribosomal DNA serves as an inner control. RNAi = RNA interference and EV = empty-vector control. Data represent mean $\pm$ standard error of three independent experiments.

Fig. 7. Gene ontology (GO) terms for genes differentially expressed between necrotic tissues of *UEP1*-RNA interference (RNAi) line 4 and healthy tissues of empty-vector (EV) control plants. Only statistically significant ($P < 0.05$, t test) GO terms associated with **A**, biological process; **B**, molecular function; and **C**, cellular component were presented. Numbers of upregulated and downregulated genes for each GO term are indicated.

3.3-fold in *UEP1*-RNAi plants, indicating that both JA and ET pathways were strongly suppressed in *UEP1*-RNAi plants (Table 1). Intriguingly, JAZ proteins are targets of the Ub-26S proteasome system and are rapidly degraded in the presence of JA, and JAZ genes are transcriptionally upregulated by JA (Chini et al. 2007; Thines et al. 2007). The dramatic upregulation of JAZ genes likely leads to accumulation of JAZ proteins, which likely can be attributed to the suppression of JAZ protein proteolysis by Ub-26S proteasome system caused by cosuppression of Ub genes such as UBQ3 and UBQ10 in *UEP1*-RNAi plants due to their high sequence homology with the *UEP1* gene (Table 1). Interestingly, JA has been reported to play vital roles in basal resistance to root-knot nematodes (Zhou et al. 2015). Thus, the suppression of the JA pathway in *UEP1*-RNAi plants might be the cause of the reduced resistance to *Meloidogyne incognita* (Fig. 6). In addition, the suppression of JA and ET pathways is indirectly confirmed by the promotion of the salicylic acid (SA) pathway, as manifested by the strong upregulation of gene *PRI*, the molecular marker of the SA pathway (Table 1), regarding a mutually antagonistic interaction between SA and JA or ET signaling pathways (An and Mou 2011). It is well known that SA plays crucial roles in resistance against various pathogens, including viruses and oomycetes (An and Mou 2011; Vlot et al. 2009). Therefore, promotion of the SA pathway may contribute to the enhanced resistance to *Pythium aphanidermatum*, TMV, and CMV in the *UEP1*-RNAi plants (Figs. 4 and 5).

Expressional alteration of genes encoding VPE, RD21, and ATG8 may be correlated with cell death and disease resistance in

UEP1-RNAi plants. VPEs are involved in stimuli-elicited cell death (Kuroyanagi et al. 2005; Rojo et al. 2004), developmentally regulated programmed cell death (Teper-Bamnolker et al. 2017), and disease resistance against various pathogens, including *Pseudomonas syringae*, *Botrytis cinerea*, and *Turnip mosaic virus* (Rojo et al. 2004). In particular, VPE γ exhibits caspase-like activity and, thus, contributes to HR and resistance (Rojo et al. 2004). RD21, like VPEs, is also a vacuolar protease. RD21 and its homologs play important roles in pathogen-induced cell death, disease resistance against necrotroph pathogens *B. cinerea* and *Sclerotinia sclerotiorum* (Lampl et al. 2013; Shindo et al. 2012), and plant development (Rustgi et al. 2017). ATG8 is a core component of autophagy, and autophagy has been increasingly and widely linked to cell death, HR, and disease resistance (Zhou et al. 2014). The roles of autophagy in cell death, HR, and disease resistance are complex, depending on the stimuli and pathosystems. For example, autophagy can play a positive role in plant antiviral defense by targeting viruses and viral RNAi suppressors for degradation, whereas it can also be hijacked by viral RNAi suppressors for the degradation of critical components of the host cell RNAi machinery (Han et al. 2015; Haxim et al. 2017; Zhou et al. 2014). Interestingly, there are complex crosstalk and interplay between the Ub-26S proteasome system and autophagy (Ji and Kwon 2017). In this study, we found that *UEP1* plays distinct roles in resistance against different pathogens: negatively against oomycete *Pythium aphanidermatum* and viruses TMV and CMV, and positively to nematode *M. incognita*. In *UEP1*-RNAi plants, VPE, RD21, and ATG8 all are significantly downregulated (Table 1), which might be partially

TABLE 1. Selected differentially expressed genes in comparison between *UEP1*-RNA interference (RNAi) and empty-vector (EV) control plants

Probe ID	Corresponding expressed sequence tag sequence	Description	Fold change (<i>UEP1</i> -RNAi/EV)
A_95_P103367	NtA.2985	Glycine-rich protein precursor	21.6
A_95_P104267	NtA.2985	Glycine-rich protein precursor	16.9
A_95_P125502	NtA.2985	Glycine-rich protein precursor	10.6
A_95_P103757	NtA.2985	Glycine-rich protein precursor	9.5
A_95_P000611	NtA.26956	Glycine-rich protein precursor	7.2
A_95_P000056	NtA.2714	Glycine-rich protein precursor	6.8
A_95_P158972	NtA.2714	Glycine-rich protein precursor	6.6
A_95_P164682	NtA.2985	Glycine-rich protein precursor	6.1
A_95_P164872	NtA.2985	Glycine-rich protein precursor	6
A_95_P104387	NtA.2714	Glycine-rich protein precursor	5.3
A_95_P109447	NtA.2714	Glycine-rich protein precursor	4.9
A_95_P297578	NtA.12079	Cf-9/Avr9 rapidly elicited protein 44 (ACRE44)	12.6
A_95_P234579	NtA.2780	Jasmonate ZIM-domain protein 3	11.4
A_95_P022406	NtA.2780	Jasmonate ZIM-domain protein 3	10.5
A_95_P104317	NtA.2780	Jasmonate ZIM-domain protein 3	9.9
A_95_P141952	NtA.22914	Jasmonate ZIM-domain protein 3	9.4
A_95_p293653	NtA.16863	Jasmonate-ZIM-domain protein 1	8
A_95_p159272	NtA.22914	Jasmonate-ZIM-domain protein 1	5
A_95_P163382	NtA.6418	SIGRAS2	8.6
A_95_P063575	NtA.21822	Leucine-rich repeat family protein	8.4
A_95_P293048	NtA.9459	Xyloglucosyl transferase	6.3
A_95_P121337	NtA.9459	Xyloglucosyl transferase	6.1
A_95_P258081	NtA.9459	Xyloglucosyl transferase	5.7
A_95_P112902	NtA.9459	Xyloglucosyl transferase	4.8
A_95_P158797	NtA.6400	Carboxylesterase	5.8
A_95_P183507	NtA.6400	Carboxylesterase	5.7
A_95_P164752	NtA.9510	NtERD10C	5.7
A_95_P028846	NtA.9510	NtERD10C	4.7
A_95_p195052	NtA.8125	AtCPK28	3.7
A_95_p021516	NtA.2727	NtCAM7	3.2
A_95_p258231	NtA.7696	AtCAM3	2.7
A_95_p291478	...	AtCDPK6	2.4
A_95_p034853	NtA.11596	AtCDPK32	2.2
A_95_p129262	NtA.972	AtCIPK3	2.1
A_95_P287768	NtA.15675	AtWRKY31	6.3
A_95_p161782	NtA.9092	AtWRKY31	2.3
A_95_p162582	NtA.9092	AtWRKY6	3.8

(Continued on next page)

responsible for the constitutive cell death and disease resistance in these plants against some pathogens checked in this study. The detailed mechanisms for how these genes were regulated by *UEP1* and how they function in modulating cell death and disease resistance in *UEP1*-RNAi plants await further dissection.

A CuAO gene was downregulated by 11.3-fold and is the most downregulated gene of the DE genes in *UEP1*-RNAi plants. CuAOs play important roles in production of active species such as H₂O₂ and NO and, therefore, are involved in regulation of development and defense (Angelini et al. 2010; Cona et al. 2006; Groß et al. 2017; Wimalasekera et al. 2011).

The most upregulated genes in *UEP1*-RNAi plants encoded a set of GRPs, which were detected by 11 probes, including the one whose expression is upregulated by 21.6-fold. Numerous GRPs are components of the plant cell wall. They are accumulated in the vascular tissues. Their expression is regulated developmentally and by external stimuli ranging from pathogen infection to environmental stresses (Mangeon et al. 2010; Mousavi and Hotta 2005). Thus, it is possible that the strong upregulation of GRPs in *UEP1*-RNAi plants somehow affects leaf development and plays a positive role in defense through reinforcing the cell wall by enhancing cell wall components, lignification, and callose deposition. In addition, some RNA-binding GRPs have been reported to regulate cell death, defense, and development (Mangeon et al. 2010). For instance, *Arabidopsis* glycine-rich RNA-binding protein7 (AtGRP7) is involved in

defense against a wide range of pathogens. Interestingly, AtGRP7 enhances resistance to TMV (Lee et al. 2012). This fits our observation that *UEP1*-RNAi plants show high levels of GRP gene expression and increased resistance to TMV. A pepper glycine-rich RNA-binding protein (CaGRP1) was found to interact with receptor-like cytoplasmic protein kinase 1 (CaPIK1) and negatively regulates CaPIK1-triggered cell death and defense responses by suppressing ROS accumulation (Kim et al. 2015). Intriguingly, a *Lilium* GRP LsGRP1 was ubiquitinated and induced by SA treatment and pathogen infection (Lin and Chen 2014). This indicates that the increased accumulation of GRPs in *UEP1*-RNAi plants might be directly caused by the reduction of Ub-26S proteasome system-mediated GRP protein degradation due to less accumulation of Ub resulting from the silencing of *UEP1* and consequent drop of GRP protein ubiquitylation. It would be worthwhile to identify whether the DE GRPs in this study are, indeed, targets of the Ub-26S proteasome system.

Conclusions. *NbUEP1* is a Ub-S27a-type *UEP* gene from *N. benthamiana*. Both VIGS and stable RNAi plant assays demonstrated that silencing of *NbUEP1* caused constitutive cell death. RNAi of *NbUEP1* enhanced resistance to oomycete *P. aphanidermatum* and viruses TMV and CMV, whereas it alleviated resistance to nematode *M. incognita*. Transcription profiling analysis revealed some potential pathways and regulators of *NbUEP1*-mediated cell death and resistance. These included JA

TABLE 1. (Continued from previous page)

Probe ID	Corresponding expressed sequence tag sequence	Description	Fold change (<i>UEP</i> -RNAi/EV)
A_95_p032421	NtA.4132	AtWRKY33	2.8
A_95_p010511	...	AtWRKY40	2.3
A_95_p034843	NtA.17662	Peroxidase 12 (PER12)	4.3
A_95_p021026	...	AtTH9 (thioredoxin H-type 9)	4.1
A_95_p250327	NtA.7214	AtTH9 (thioredoxin H-type 9)	2.7
A_95_P291458	NtA.16419	Copper amine oxidase family protein	-11.3
A_95_P269472	NtA.7223	AtGSR1	-8.1
A_95_P250807	NtA.7321	Vacuolar processing enzyme	-7.5
A_95_P163492	NtA.4071	Vacuolar processing enzyme	-6.9
A_95_P007441	NtA.4071	Vacuolar processing enzyme	-6.7
A_95_P239420	NtA.4048	Proline oxidase	-7.5
A_95_P218312	NtA.11690	Serine carboxypeptidase (<i>Arabidopsis thaliana</i>)	-6.2
A_95_P255594	NtA.8817	Serine carboxypeptidase (<i>A. thaliana</i>)	-5.6
A_95_P286283	NtA.15368	Bundle sheath-defective protein 2 family	-5
A_95_P029006	NtA.15368	Bundle sheath-defective protein 2 family	-4.7
A_95_P121002	NtA.6396	AtGLDP1 (glycine decarboxylase P 1)	-4.9
A_95_P247197	NtA.6396	AtGLDP1 (glycine decarboxylase P 1)	-4.4
A_95_P103707	NtA.7100	NtPLA2 (phospholipase A2)	-4.5
A_95_P292133	NtA.2590	AtRD21 (responsive to dehydration 21)	-4.3
A_95_P227359	NtA.954	Glycerate dehydrogenase	-4.2
A_95_P129652	NtA.954	Glycerate dehydrogenase	-4
A_95_P021996	NtA.8612	Phosphoinositide binding	-4.2
A_95_P279283	NtA.13943	AtFRO6 (ferric reduction oxidase 6)	-4.1
A_95_p150732	NtA.8560	Peptidase S41 family protein	-4.5
A_95_p045706	NtA.8560	Peptidase S41 family protein	-3.5
A_95_p010991	NtA.7063	AtEFE (ethylene-forming enzyme)	-3.3
A_95_p008226	NtA.7063	AtEFE (ethylene-forming enzyme)	-2.8
A_95_p179962	...	AtEFE (ethylene-forming enzyme)	-2.6
A_95_p130452	NtA.10728	AtEFE (ethylene-forming enzyme)	-2.4
A_95_p156612	NtA.7064	AtEFE (ethylene-forming enzyme)	-2.3
A_95_p020477	NtA.7063	AtEFE (ethylene-forming enzyme)	-2.2
A_95_p156027	NtA.7063	AtEFE (ethylene-forming enzyme)	-2.2
A_95_p179957	NtA.10728	AtEFE (ethylene-forming enzyme)	-2
A_95_p227174	NtA.911	C3HC4-type zinc-finger family protein	-2.9
A_95_p014336	NtA.9486	AtUBQ3	-2.7
A_95_p112762	NtA.9486	AtUBQ3	-2.4
A_95_p010476	NtA.9486	AtUBQ3	-2.2
A_95_p012331	NtA.22166	AtUBQ10	-2.1
A_95_p131717	NtA.881	ATG8c (autophagy 8c)	-2.5
A_95_p024721	NtA.2797	ATG8c (autophagy 8c)	-2

and ET pathways, lipid metabolism, CuAO-mediated active species generation, GRPs, VPE, RD21, and autophagy.

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