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Identification and Characterization of Plant Cell Death–Inducing Secreted Proteins From *Ustilaginoidea virens*

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Ustilaginoidea virens **(Cooke) Takah (telemorph *Villosiclava virens*) is an ascomycetous fungus that causes rice false smut, one of the most important rice diseases. Fungal effectors often play essential roles in host-pathogen coevolutionary interactions. However, little is known about the functions of *U. virens* effectors. Here, we performed functional studies on putative effectors in *U. virens* and demonstrated that 13 of 119 putative effectors caused necrosis or necrosis-like phenotypes in *Nicotiana benthamiana*. Among them, 11 proteins were confirmed to be secreted, using a yeast secretion system, and the corresponding genes are all highly induced during infection, except *UV_44* and *UV_4753*. Eight secreted proteins were proven to trigger cell death or defenses in rice protoplasts and the secretion signal of these proteins is essential for their cell death–inducing activity. The ability of UV_44 and UV_1423 to trigger cell death is dependent on the predicted serine peptidase and ribonuclease catalytic active sites, respectively. We demonstrated that UV_1423 and UV_6205 are *N*-glycosylated proteins, which glycosylation has different impacts on their abilities to induce cell death. Collectively, the study identified multiple secreted proteins in *U. virens* with specific structural motifs that induce cell death or defense machinery in nonhost and host plants.**

Defense and counter-defense are major themes in host-pathogen coevolutionary interactions (Jones and Dangl 2006). Biotrophic plant pathogens rely on viable host cells and tissues for colonization and multiplication, so these pathogens generally avoid disturbing plant growth and development for their survival. However, pattern recognition receptors in plants can recognize microbe-associated molecular patterns (MAMPs) such as fungal chitin and, thus, initiate pattern-triggered immunity (PTI) (Boller and He 2009; Macho and Zipfel 2014). In order to counteract plant defenses, phytopathogens secrete a great number of effectors into host cells that suppress immune responses by targeting essential components in plant defense signaling (Dou and Zhou 2012; Howden and Huitema 2012). Plants have evolved resistance (R) proteins that specifically recognize certain pathogen effectors, which often induce the hypersensitive response (HR) of host cells, called effector-triggered immunity (ETI) (Chisholm et al. 2006; Jones and Dangl 2006; Stergiopoulos and de Wit 2009).

Unlike bacterial type III effectors, which are all injected into the host cells, the effectors produced by filamentous plant pathogens belong to two major categories, apoplastic and cytoplasmic effectors (Giraldo and Valent 2013). Apoplastic effectors are recognized as avirulence proteins or function as virulence factors in the apoplast of host plants (van Esse et al. 2007), while cytoplasmic effectors have been demonstrated to be delivered into the cytoplasm and act inside the cell (Mentlak et al. 2012). Fungal pathogens are able to secrete a large array of effectors, but only a small subset of effectors has been characterized so far. For example, the apoplastic effector Avr4 in *Cladosporium fulvum* is required for full virulence and protects the fungus against chitinases when binding to chitin through the chitin-binding domain (van Esse et al. 2007). Another type of chitin-binding effectors, e.g., Slp1 in *Magnaporthe oryzae* and Ecp6 in *C. fulvum*, competes with chitin receptors to sequester chitin oligosaccharides through the LysM domains and, thus, perturb chitin-triggered immunity in hosts (de Jonge et al. 2010; Mentlak et al. 2012). The secreted lipase FGL1, which releases free fatty acids to inhibit the activity of callose synthase, is required for virulence of *Fusarium graminearum* (Blümke et al. 2014; Voigt et al. 2005). Pit2 in *Ustilago maydis* inhibits the activity of a maize cysteine protease 2 and is essential for full virulence to maize (Mueller et al. 2013). Pep1, a conserved core effector across smut pathogens of dicots and monocots, is required for *Ustilago maydis* penetration and for inhibition of plant apoplastic peroxidases (Hemetsberger et al. 2012, 2015). In addition, Cmu1 functions as a chorismate mutase, reduces salicylic acid level in maize, and suppresses plant defenses (Djamei et al. 2011). Heterologous expression of *M. oryzae* Avr-Piz-t in rice suppresses oxidative burst and defense-related gene expression induced by MAMPs (Park et al. 2012). Multiple SIX (secreted in xylem) effectors contribute to *F. oxysporum* f. sp. *lycopersici* virulence and suppress different types of immune responses in tomato (Ma et al. 2010). Barley powdery mildew candidate effectors CSEP0105 and CSEP0162 are required for haustorial formation and contribute to successful infection (Ahmed et al. 2015). Collectively, fungal effectors are among the most important virulence factors and suppress host immunity using various molecular mechanisms (Supplementary Table S1).

Besides functioning as virulence factors to cripple host defenses, fungal effectors are often recognized to trigger host immunity. Four apoplastic effectors Avr2, Avr4, Avr4E, and Avr9 in *C. fulvum* have been well-characterized to be recognized by receptor-like *C. fulvum* resistance proteins Cf-2, Cf-4, Cf-4E, and Cf-9, respectively, in tomato (Luderer et al. 2002; Van der Hoorn

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et al. 2000). Deletion of *six1* also disrupted I-3-mediated resistance against *F. oxysporum* f. sp. *lycopersici* in tomato (Rep et al. 2004). Multiple gene-for-gene interactions have been also revealed in the rice–*M. oryzae* model pathosystem. For example, Avr-Piz-t was shown to be delivered into the infected rice cells and recognized by Piz-t, which induced ETI in rice (Park et al. 2012). Eight other effectors in *M. oryzae*, PWL1, PWL2, Avr-Pita, Avr-Pia, Avr-Pii, Avr-Pik/km/kp, Avr1-CO39, and ACE1 are also recognized by the corresponding R proteins (Chauhan et al. 2002; Kanzaki et al. 2012; Okuyama et al. 2011; Takagi et al. 2013). Multiple effectors in *Phytophthora sojae* trigger defense responses in *Nicotiana benthamiana* (Wang et al. 2011). Recently, the apoplastic effector XEG1, a glycoside hydrolase family 12 (GH12) protein in *P. sojae* was identified to act as a MAMP to induce cell death in dicot plants (Ma et al. 2015). During attempted colonization by the nonadapted pathogens, nonhost recognition of effectors is proposed to play an important role in nonhost resistance (Schulze-Lefert and Panstruga 2011; Stam et al. 2014). Four secreted effectors in *M. oryzae* have been demonstrated to induce nonhost cell death when transiently expressed in *N. benthamiana* (Chen et al. 2013). Therefore, fungal effectors are often recognized to initiate resistance or defense signaling pathways in plants.

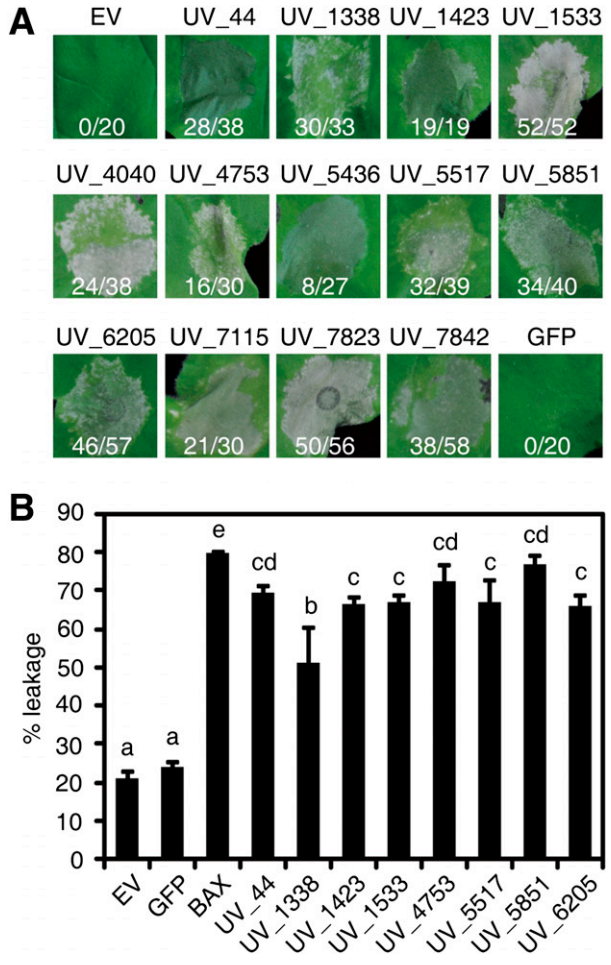

Fig. 1. The putative effectors in *Ustilaginoidea virens* induce necrotic cell-death phenotypes in *Nicotiana benthamiana*. **A,** Transient expression assays of 119 putative *U. virens* effectors revealed that 13 of them caused cell death or mottling symptoms in *N. benthamiana* leaves. Numbers, e.g., 28/38, indicate that 28 of 38 infiltrated leaves exhibited cell death or mottling phenotypes. EV = empty vector; GFP = green fluorescent protein. Representative photos were taken at 5 days after infiltration. **B,** Quantification of cell death in the infiltrated *N. benthamiana* leaves by measuring ion leakage. Ion leakage from the leaf discs infiltrated with different gene constructs was measured at 4 days after infiltration. The EV, *GFP*, and *BAX* constructs were infiltrated as negative controls and a positive control, respectively. Data are means ± standard error (SE) from three independent experiments.

Ustilaginoidea virens is the causal agent of rice false smut (RFS), which has recently expanded rapidly and become one of the most important rice diseases worldwide (Rush et al. 2000; Singh and Pophaly 2010; Tanaka et al. 2008; Tang et al. 2013). As a flower-infecting fungus, *U. virens* initially colonizes on rice florets during the booting stage and, consequently, chlamydospore-covered sporeballs are formed on the infected spikelets (Hu et al. 2014; Tang et al. 2013). The ovaries of infected spikelets remain alive at the late stage of infection. Therefore, *U. virens* is considered to be a biotrophic pathogen (Tang et al. 2013; Zhang et al. 2014). Different types of mycotoxins, including ustiloxins and ustilaginoidins, are produced in the sporeballs and are toxic to human and animals by inhibiting cell division (Shan et al. 2012). RFS not only causes significant yield loss in the staple food crop but, also, brings severe threat to food safety.

Based on genome sequence, *U. virens* encodes at least 193 small secreted cysteine-rich proteins, which are considered as effector candidates (Zhang et al. 2014). Transcriptome analyses during infection and HR inhibitory assays in *N. benthamiana* suggest that a subset of putative effectors is an essential component for successful infection of *U. virens* (Zhang et al. 2014). However, it is still a mystery whether the effectors in *U. virens* can be recognized and, thus, trigger cell death or defense responses in the host and nonhost plants. In this study, we investigated the cell death–inducing ability of 119 putative effectors in *U. virens* through transient expression assays. Eleven and eight putative effectors were found to induce cell death in *N. benthamiana* and rice protoplasts, respectively. The predicted active sites of a secreted serine peptidase and a putative ribonuclease-like effector were shown to be required for their cell death–inducing activity. Our results also indicate that *N*-glycosylation is a typical modification for these fungal cell death–inducing factors, which glycosylation might have important roles in their functions and stability.

RESULTS

A subset of putative effectors in *U. virens* induce cell death in *N. benthamiana*.

To experimentally identify potential *U. virens* effectors that induce cell death in plants, 119 predicted effector genes were amplified and were subcloned into the glucocorticoid-inducible pTA7001 binary vector (Aoyama and Chua 1997). The ability of these putative effectors to induce nonhost cell death was investigated through *Agrobacterium*-mediated transient expression in *N. benthamiana*. Among the 119 gene constructs, 13 were demonstrated to trigger cell death or mottling in *N. benthamiana* leaves, but infiltration of the empty vector or green fluorescent protein (*GFP*) construct did not induce necrosis (Fig. 1). Necrosis severity in the infiltrated leaf areas caused by these effector candidates varied. UV_1423 and UV_1533 induced severe cell death rapidly at 36 to 48 h after dexamethasone (DEX) spraying, which induces expression of the infiltrated gene constructs. Necrosis phenotypes induced by UV_1338, UV_4040, UV_4753, UV_5436, UV_5517, UV_5851, UV_6205, UV_7115, UV_7823, and UV_7842 appeared slightly later, at 3 days after DEX treatment. In addition, UV_44 induced a delayed but severe cell death at 5 to 6 days after agroinfiltration. Since ion leakage is positively correlated with cell death (Mittler et al. 1999), the molecular feature was quantified to further confirm cell death triggered by these putative effectors. The results showed that ion leakage from *N. benthamiana* leaves transiently expressing eight individual effector candidates was all significantly elevated compared with GFP-expressing or empty vector–infiltrated leaves (Fig. 1B). As a positive control,

BAX-expressing leaves exhibited the highest ion leakage (Fig. 1B). Together, the results suggest that the 13 putative effectors are recognized by the defense machinery of *N. benthamiana* or that they function as toxins to induce necrosis.

Functional validation of predicted signal peptides (SPs) of putative effectors.

An elegant yeast secretion system has been developed to track the secretion of proteins (Jacobs et al. 1997; Oh et al. 2009; Tian et al. 2011). Here, the system was exploited to experimentally corroborate SP prediction for the 13 nonhost cell death–inducing factors in *U. virens*. The predicted SP nucleotide sequence of each gene was fused in frame with the truncated *SUC2* gene that encodes invertase lacking its own SP. The fusion constructs were transformed into the invertase secretion–deficient yeast strain YTK12. In the assay, the invertase with bona fide SP, which guides the enzyme to secrete into the medium, can degrade raffinose into simple sugars so that YTK12 can grow on the medium with raffinose as sole carbon source. As a positive control, the secretion signal of *P. sojae* Avr1b led the secretion of invertase and, therefore, YTK12 grew on the raffinose-containing YPRAA (1% yeast extract, 2% peptone, 2% raffinose, and 2 µg of antimycin A per liter) medium (Fig. 2). Likewise, the predicted signal peptides of 11 putative effectors restored the ability of invertase-deficient yeast to grow on YPRAA medium. By contrast, the yeast strains transformed with the SP sequences of *UV_4040* and *UV_7823* as well as a negative control, the N-terminus of Mg87 in *M. oryzae* (Gu et al. 2011), did not grow on YPRAA plates, suggesting that the N-terminal peptides of the two proteins are not able to guide secretion of the truncated invertase (Fig. 2). These results indicate that the predicted SPs of the 11 putative *U. virens* effectors are functional to direct these proteins to the secretory pathway and that these proteins are bona fide secreted proteins.

Expression analysis of 11 putative effector genes during *U. virens* infection of young panicles.

The effector genes in filamentous plant pathogens are often transcriptionally induced during infection (Stergiopoulos and de Wit 2009). In order to reveal regulation of effector gene expression during *U. virens* infection, the highly virulent isolate P1 was artificially inoculated into young panicles of the rice cultivar IR28, which shows high field resistance to P1, and the cultivar LYP9, which is highly susceptible to P1 (Han et al. 2015). The expression of the 11 secreted protein-encoding genes at 0, 24, 48, and 96 h postinoculation (hpi) was detected by quantitative real time reverse transcription-polymerase chain reaction (RT-PCR) (Fig. 3). These genes are generally categorized into three groups based on their expression profiles during infection. The first group includes *UV_44* and *UV_4753*, in which expression was suppressed during infection (Fig. 3). The other two groups have nine genes, of which expression was all up-regulated to different levels from approximately twofold to approsimately 18-fold. The second group of effector genes, i.e., *UV_1533*, *UV_5436*, *UV_5517*, and *UV_7842*, were transcriptionally up-regulated and gradually increased during infection, while gene expression of the third group, i.e., *UV_1338* and *UV_5851*, was initially induced at the early infection stage and decreased at 96 hpi. The results demonstrated that all 11 secreted protein–encoding genes were differentially regulated during *U. virens* infection, indicating that these secreted proteins are likely effectors and might play important roles in the interaction of rice and the fungal pathogen.

Cell death in rice protoplasts induced by the 11 secreted proteins.

To determine whether the 11 secreted proteins trigger host cell death, transient expression in rice protoplasts was performed via a polyethylene glycol (PEG)-mediated transformation system (Chen et al. 2013). In this assay, the constructed plasmids

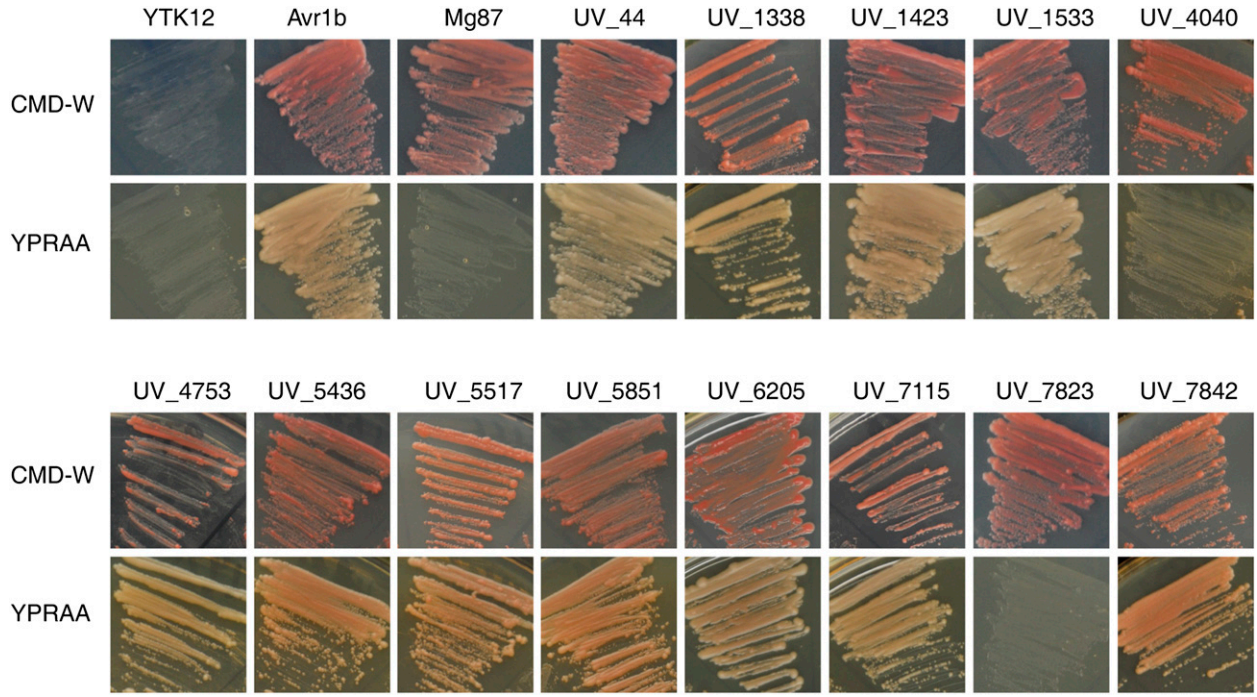

Fig. 2. Functional validation of signal peptides of putative *Ustilaginoidea virens* effectors using a yeast invertase secretion assay. The predicted SPs of 11 of 13 *U. virens* putative effectors, excepting UV_4040 and UV_7823, can guide the secretion of the truncated invertase lacking signal sequence, and therefore, these transformed yeast YTK12 strains were able to grow on YPRAA media with raffinose as sole carbon source (1% yeast extract, 2% peptone, 2% raffinose, and 2 µg of antimycin A per liter). The N-terminal sequences of *Phytophthora sojae* Avr1b and *Magnaporthe oryzae* Mg87 were used as positive and negative controls, respectively. The untransformed YTK12 did not grow on either CMD-W (0.67% yeast N base without amino acids, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar) or YPRAA media. Yeast growth on CMD-W media showed an equal viability of the transformed strains.

carrying the complete coding sequence of individual secreted protein–encoding genes were cotransformed into rice protoplasts with pJD301-*35S-LUC*, by which luciferase (LUC) is expressed under the control of the 35S promoter (Luehrsen et al. 1992). Induced cell death in rice protoplasts was evaluated by measurement of LUC activity in the protoplasts isolated from rice cv. Nipponbare (Okuyama et al. 2011; Yoshida et al. 2009). As compared with the luminescence intensity detected in the protoplasts cotransformed with the *GFP* construct, LUC activity in rice protoplasts was significantly reduced when eight individual secreted protein–encoding genes were coexpressed with LUC, respectively. In particular, transient expression of UV_1423 and UV_6205 caused cotransfected protoplasts to completely lose LUC activity (Fig. 4A). By contrast, expression of UV_5436, UV_7115, and UV_7842 in rice protoplasts did not have any inhibitory effect on LUC activity. Transient expression of these secreted proteins, except UV_5517, in rice protoplasts was clearly detected via immunoblotting (Fig. 4B). To confirm that

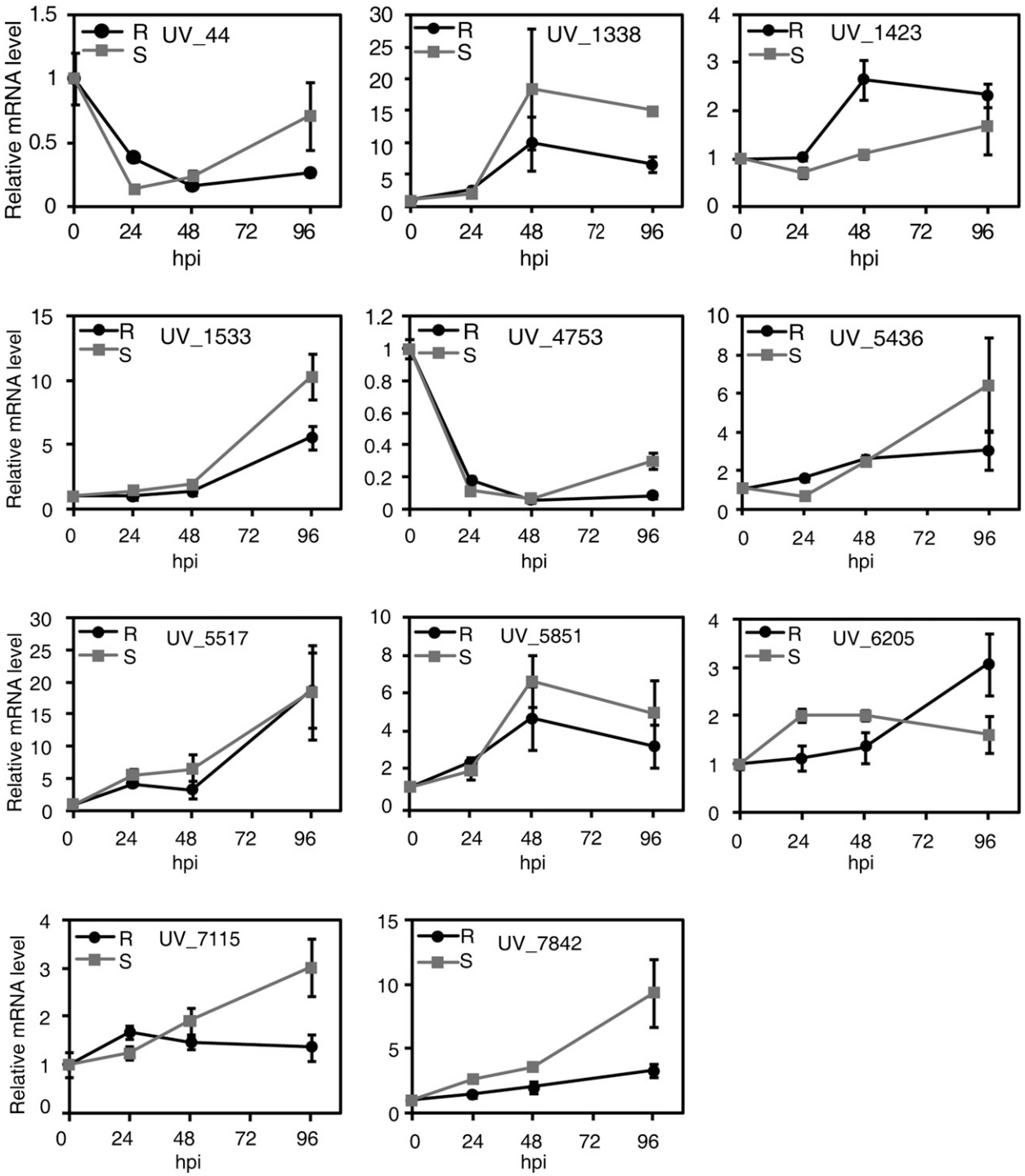

Fig. 3. Expression profiles of 11 putative effector genes during *Ustilaginoidea virens* infection of the false smut–resistant and -susceptible rice cultivars. The *U. virens*–inoculated panicles of the false smut–resistant cultivar IR28 (R) and susceptible cultivar LYP9 (S) were collected at 0, 24, 48, and 96 h post-inoculation (hpi) for gene expression analyses using quantitative real time reverse transcription-polymerase chain reaction assay. Data are means ± standard error. Gene expression patterns shown are representatives from three independent repeats with similar results.

the reduced LUC activity was associated with cell death, the viability of *UV_6205*-transfected protoplasts was determined after fluorescein diacetate (FDA) staining. The result showed that *UV_6205*-transfected protoplasts have a significantly lower surviving rate compared with *GFP*-transfected protoplasts (Fig. 4C). Furthermore, we investigated if the eight secreted proteins are able to induce cell death in the highly susceptible cultivar LYP9 (Han et al. 2015). Transient expression in rice protoplasts showed that these putative effectors, except UV_1338, inhibited LUC activity, which was similar to the result from Nipponbare (Supplementary Fig. S1). All these experimental data suggest that certain putative *U. virens* effectors are able to trigger cell death in rice.

SPs of putative *U. virens* effectors are required for their ability to trigger plant cell death.

The SP of multiple *M. oryzae* effectors has been demonstrated to be required for their ability to induce cell death in plants (Chen et al. 2013). Here, we determined whether these *U. virens* cell death–inducing factors without SPs remain to have the cell death–inducing ability. Transient expression assays in *N. benthamiana* leaves were first performed to determine the cell death–inducing ability of these putative *U. virens* effectors with or without SPs. The tested secreted proteins with SPs repeatably induced cell death, but all of the truncated proteins lacking SPs no longer caused any cell-death phenotypes (Fig. 5A). Protein gel blots showed that, in most cases, both full-length and truncated proteins were expressed in the infiltrated leaves (Fig. 5A). The requirement of SP for putative *U. virens* effectors to cause cell death was also evaluated in rice protoplasts. As compared with the full-length proteins, SP truncation of all tested putative effectors caused the proteins to completely or partially lose the ability to reduce the LUC activity in cotransfected protoplasts (Fig. 5B). The truncated proteins were all well-expressed in rice protoplasts, although some of them had a lower molecular weight than full-length proteins (Supplementary Fig. S2). In addition, FDA vital staining showed that viable cells in *GFP*- and *UV_6205NSP*-transfected protoplasts were much more than *UV_6205*-transfected protoplasts (Fig. 4C). Taken together, the results indicate that SPs of the tested secreted proteins in *U. virens* are all required for their ability to trigger cell death in *N. benthamiana* and in rice.

The predicted catalytic triad of serine protease in UV_44 is essential for its ability to induce cell death.

UV_44 was predicted to be a modular protein that contains a SP, an N-terminal inhibitor_I9 domain, and a conserved peptidase_S8 domain, through Pfam search. The protein belongs to a family of subtilisin-like serine proteases, which appear to have convergently evolved an Asp/His/Ser catalytic triad (Ekici et al. 2008). To analyze the importance of the predicted protease activity of UV_44 in inducing cell death, the residues Asp143, His175, and Ser330, which form the catalytic triad of UV_44, were replaced with alanine (Fig. 6A). The cell death–inducing ability of UV_44 and the mutant proteins UV_44^{D143A}, UV_44^{H175A}, and UV_44^{S330A} was first evaluated in *N. benthamiana*. While UV_44 expression caused an evident cell death, the mutant proteins UV_44^{D143A}, UV_44^{H175A}, and UV_44^{S330A} did not induce necrosis symptoms in the infiltrated areas (Fig. 6B). The wild type and three variants of *UV_44* have similar mRNA and protein expression levels detected by RT-PCR and Western blotting (Fig. 6C and D). Consistently, UV_44 but not the mutant proteins caused a significant reduction in LUC activity when coexpressing with LUC in rice protoplasts, suggesting that the mutant UV_44 proteins lose the ability to induce cell death in rice (Fig. 6E; Supplementary Fig. S3A). These results indicate that the predicted catalytic triad of serine protease is required for the ability of UV_44 to induce cell death in nonhost and host plants.

The predicted RNase active site of UV_1423 is essential for its cell death-inducing ability.

UV_1423 was predicted to contain a conserved fungus-specific RNase domain and a C-terminal transmembrane domain. To investigate whether the potential RNase activity of UV_1423 is required for its ability to induce cell death, point mutations were introduced into the predicted active site of the conserved RNase domain. The key residues His60, Glu79, or His113 of UV_1423 necessary for RNase activity were replaced with alanine (Fig. 7A).

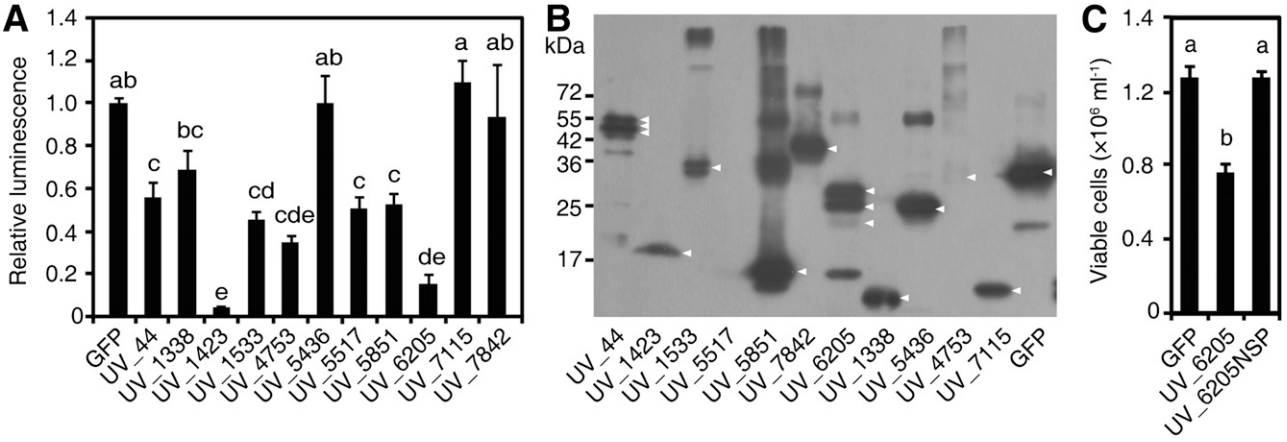

Fig. 4. Transient expression of the putative *Ustilaginoidea virens* effector genes induces cell death in the protoplasts of rice cv. Nipponbare. **A,** The activity of luciferase that was transiently expressed in rice protoplasts was significantly inhibited by coexpression of eight individual putative effector genes. Three secreted proteins, including UV_5436, UV_7115, and UV_7842, that induced cell death in *Nicotiana benthamiana* did not affect the luciferase activity when coexpressed in rice protoplasts. Data are means ± standard error (SE). Different letters (a through e) indicate significant difference in the luciferase activity at $P < 0.05$, according to Duncan's multiple-range test. **B,** Transient expression of different secreted proteins in rice protoplasts detected by immunoblotting with an anti-FLAG antibody. The correct bands for expressed proteins are indicated by white triangles. GFP = green fluorescent protein. **C,** Viability of rice protoplasts transfected with *GFP*, *UV_6205*, and *UV_6205NSP* gene constructs revealed by fluorescein diacetate (FDA) staining. The protoplasts that can be stained with FDA were counted as viable cells. The *GFP*-transfected protoplasts were stained as a control. NSP = truncated proteins lacking SPs. Data are means ± SE. Different letters (a or b) indicate significant difference in the number of viable cells at $P < 0.05$, according to Duncan's multiple-range test.

Cell-death symptoms were observed after different *UV_1423* variant constructs were infiltrated into *N. benthamiana* leaves. Consistently, the full-length UV_1423 caused rapid cell death in *N. benthamiana*. However, expression of UV_1423^{H60A} and UV_1423^{H113A} in *N. benthamiana* did not induce cell death, while UV_1423^{E79A} caused a less severe necrosis phenotype than the wild-type protein (Fig. 7B). Although mRNA expression of *UV_1423* and its variants was at the similar level, Western blot analysis demonstrated that accumulation of UV_1423 and UV_1423^{E79A} was surprisingly low in *N. benthamiana* leaves. By contrast, UV_1423^{H60A} and UV_1423^{H113A} were more abundant in the infiltrated leaves (Fig. 7C and D). Furthermore, detection of LUC activity in cotransfected rice protoplasts showed that the three mutant proteins completely or partially lost the ability to induce cell death in rice (Fig. 7E). These results indicate that the putative RNase active site of UV_1423 is essential for its ability to induce plant cell death.

N-linked glycosylation of putative effector proteins in *U. virens*.

Many effector proteins in filamentous fungal pathogens are predicted to be *N*-glycosylated. *N*-glycosylation of the *M. oryzae* effector Slp1 has been recently demonstrated to be required for the rice blast fungus to evade host innate immunity (Chen et al. 2014). To initially investigate the effect of *N*-glycosylation on the function of putative effector proteins, the online NetNGlyc 1.0 Server was used to predict *N*-linked glycosylation for the identified cell death–inducing factors in *U. virens*. The *N*-glycosylation sites were predicted in six secreted proteins including UV_44, UV_1423, UV_1533,

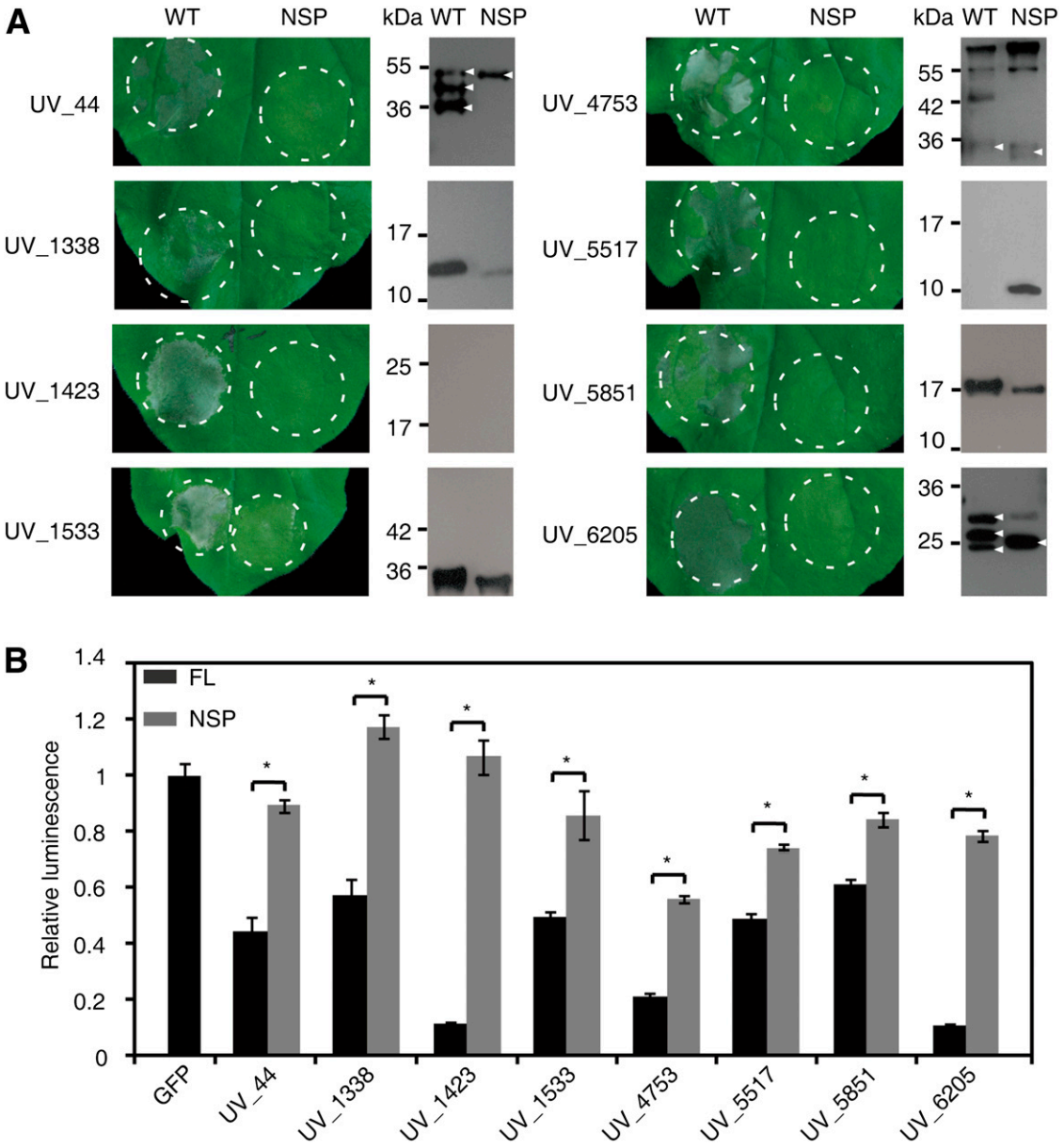

Fig. 5. The truncated *Ustilaginoidea virens* secreted proteins lacking signal peptides (SPs) are not able to induce plant cell death. **A,** The truncated *U. virens* secreted proteins without SPs did not cause cell death in *Nicotiana benthamiana* leaves. Left panels, cell-death symptoms caused by transient expression of full-length and truncated secreted proteins. Right panels, expression level of full-length and truncated proteins in *N. benthamiana* detected by Western blotting. The correct bands for expressed proteins were indicated by white triangles. **B,** The truncated *U. virens* secreted proteins without SPs completely or partially lost the inhibitory effect on the luciferase activity in rice protoplasts. Data are means ± standard error. Asterisks (*) indicate significant difference in the luciferase activity of full-length and truncated effector gene–transfected protoplasts ($P < 0.05$, Duncan's multiple-range test). WT = wild-type; NSP = truncated effectors lacking SPs; FL = full-length proteins.

UV_4753, UV_5436, and UV_6205. UV_1423 and UV_6205 were chosen to be studied further. To verify *N*-glycosylation of the two putative effectors, transiently expressed proteins in *N. benthamiana* were subject to immunoblotting after treatment with Endoglycosidase H (Endo H), which cleaves between the *N*-acetylglucosamine residues of the chitobiose core of high mannose and some hybrid structures from *N*-linked glycoproteins. The results showed that UV_1423^{H60A} and UV_6205 became smaller after the treatment with Endo H, indicating that both proteins are glycosylated (Supplementary Fig. S4). It is notable that UV_1423^{H60A} was used for deglycosylation experiments, because the expression of wild-type UV_1423 was barely detectable in *N. benthamiana* (Fig. 5). To further characterize the *N*-glycosylation sites of UV_1423 and UV_6205, mutant proteins with point mutations in their predicted *N*-glycosylation sites were transiently expressed in rice protoplasts. The apparent molecular weight of UV_6205^{N39G}, UV_6205^{N53G}, and UV_6205$^{N39G/N53G}$ was clearly lower than UV_6205, suggesting that both Asn39 and Asn53 are linked with *N*-glycans (Fig. 8A). Likewise, UV_1423^{H60A} expressed in rice protoplasts was cleaved by Endo H, while UV_1423$^{H60A/N49G}$ cannot be cleaved further. The molecular weight of UV_1423$^{H60A/N49G}$ is evidently smaller than UV_1423^{H60A} (Fig. 8B). The results suggest that Asn49 is the only *N*-glycosylation site in UV_1423. Collectively, the data indicate that UV_1423 and UV_6205 are both *N*-glycosylated in plant cells.

In order to determine whether *N*-glycosylation affects the ability of the putative effectors to trigger cell death, LUC activity was tested in rice protoplasts cotransfected with LUC and effector gene constructs. LUC activity in UV_1423^{N49G}-transfected protoplasts was significantly higher than that in UV_1423-transfected protoplasts, suggesting that the cell death–inducing ability of UV_1423^{N49G} is reduced (Fig. 8C). Although luminescence intensity in the protoplasts transfected with UV_6205 and its mutants with single point mutations was similar, LUC activity in UV_6205$^{N39G/N53G}$-transfected protoplasts was significantly lower, indicating that its ability to induce cell death is enhanced (Fig. 8C). Therefore, these results indicate that *N*-glycosylation at least partly affects the function of putative effector proteins in *U. virens*.

DISCUSSION

The effector proteins secreted by filamentous fungal pathogens play essential roles in the host-pathogen interactions (Giraldo and Valent 2013; Stergiopoulos and de Wit 2009). The *U. virens*

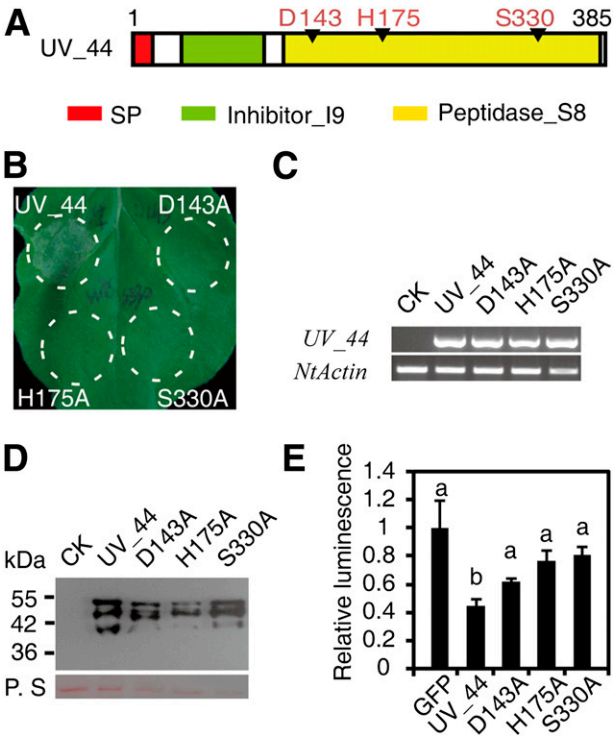

Fig. 6. The predicted catalytic triad of serine protease in UV_44 is essential for its cell death–inducing ability. **A,** The predicted domain structure of UV_44, a putative serine protease. SP = signal peptide; Inhibitor_I9 is the N-terminal peptidase inhibitor I9 domain and Peptidase_S8, a conserved protease domain, in which the residues Asp143, His175, and Ser330 form the catalytic triad. **B,** The mutant proteins UV_44^{D143A}, UV_44^{H175A}, and UV_44^{S330A} with point mutations in the predicted catalytic triad significantly lost the ability to induce cell death in *Nicotiana benthamiana*. **C,** The mRNA expression level of *UV_44* and its mutant variants in the infiltrated leaves detected by reverse transcription-polymerase chain reaction. *NtActin* was used as an internal reference gene. **D,** The protein level of the wild-type UV_44 and mutant proteins in the infiltrated leaves detected by Western blotting. Ponceau S staining (P. S) of same blot to detect total protein. CK = uninfiltrated leaves. **E,** The luciferase activity in rice protoplasts cotransfected with the luciferase and the green fluorescent protein gene (*GFP*), *UV_44*, or its mutant gene constructs. Data are means ± standard error. Different letters (a or b) indicate significant difference in the luciferase activity ($P < 0.05$, Duncan's multiple-range test).

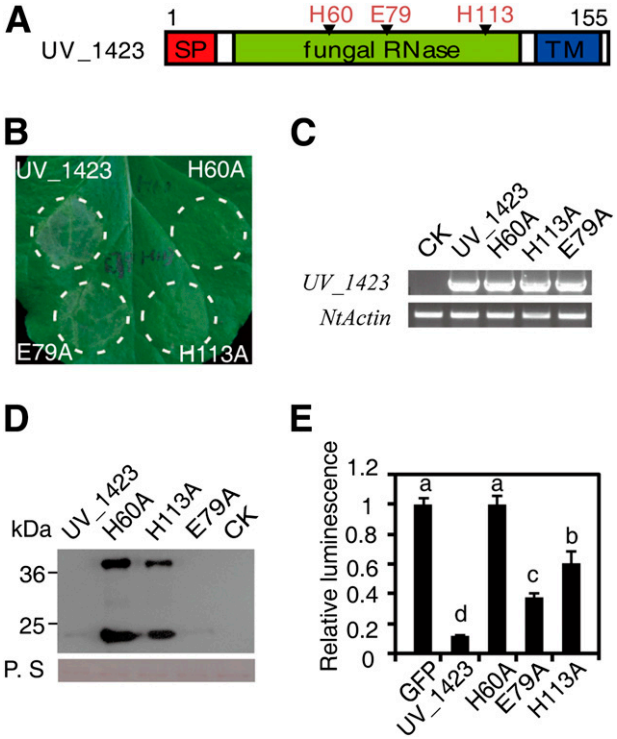

Fig. 7. The predicted RNase active site of UV_1423 is required for its cell death–triggering ability. **A,** The predicted domain structure of UV_1423, a putative fungus-specific RNase. SP = signal peptide; fungal RNase = a conserved fungus-specific RNase domain including His60, Glu79, and His113, the predicted key residues necessary for the RNase activity; TM = transmembrane domain. **B,** The mutant proteins UV_1423^{H60A} and UV_1423^{H113A} lost the ability to induce cell death, while UV_1423^{E79A} induced weak cell death in *Nicotiana benthamiana* leaves. **C,** The mRNA expression level of *UV_1423* and its mutant variants in the infiltrated leaves detected by reverse transcription-polymerase chain reaction. *NtActin* was used as an internal control gene. **D,** The protein expression level of the wild-type UV_1423 and mutant proteins in the infiltrated leaves detected by Western blotting. Ponceau S staining (P. S) of the same blot to detect total protein. CK = uninfiltrated leaves. **E,** The luciferase activity in rice protoplasts cotransfected with the luciferase and green fluorescent protein gene (*GFP*), *UV_1423*, or its mutant gene constructs. Data are means ± standard error. Different letters (a through d) indicate significant difference in the luciferase activity ($P < 0.05$, Duncan's multiple-range test).

genome encodes more than 600 secreted proteins, many of which are considered as candidates of effector proteins (Zhang et al. 2014). Expression profiling analyses demonstrated that many putative effector genes in *U. virens* were transcriptionally induced during rice infection, indicating that they play important roles in the interaction of rice and *U. virens* (Zhang et al. 2014). In this study, our experimental data demonstrated that 11 putative effectors caused nonhost cell death in *N. benthamiana* and that eight of them induced cell death or defense responses in rice. The National Center for Biotechnology Information gene ID, locus tag, geninfo identifier, and protein ID of these cell death-inducing factors in *U. virens* are summarized in Supplementary Table S2.

Transient expression assay in *N. benthamiana* by agroinfiltration has been used for identifying the *M. oryzae* effectors that induce nonhost cell death (Chen et al. 2013). Through this assay, 13 of 119 putative effectors in *U. virens* were demonstrated to trigger different levels of cell death phenotypes in *N. benthamiana*. Further experimental data indicate that 11 predicted secreted proteins among them are most likely effectors. First, the predicted SPs of the 11 putative effectors were functional to guide the secretion of the truncated invertase and, therefore, the invertase-deficient yeast was able to grow on YPRAA medium in the yeast secretion assay (Fig. 2). Second, the 11 putative effectors were all differentially regulated during *U. virens* infection of rice panicles (Fig. 3), which is a feature common to characterized effector proteins in filamentous fungal pathogens (Chen et al. 2013). A highly efficient transient protoplast system has been successfully utilized to detect hypersensitive cell death during gene-for-gene interactions in the rice–*M. oryzae* pathosystem (Okuyama et al. 2011; Yoshida et al. 2009). Using this assay, eight of the 11 putative effectors were demonstrated to have the ability to induce cell death in host rice (Fig. 4). These cell death–inducing factors might be recognized by *R* genes and, thus, trigger ETI. The hypothesis seems contradictory to the finding that most of these peptides induced cell death in the cultivar LYP9, which is highly susceptible to RFS (Han et al. 2015). Possibly, the ETI during infection is suppressed by other *U. virens* effectors, causing compatible interaction of rice and the pathogen. Our previous study identified multiple candidate effectors that can suppress HR triggered by *Burkholderia glumae* in *N. benthamiana* (Zhang et al. 2014). Similar observations have been reported in other adapted plant pathogens, such as *M. oryzae* and *P. sojae* (Chen et al. 2013; Wang et al. 2011). Alternatively, these proteins are recognized by the plant's MAMP recognition machinery and, thus, induce PTI. Recently, it has been demonstrated that the *P. sojae* effector XEG1, when transiently expressed, induces host and nonhost cell death and acts as a novel MAMP (Ma et al. 2015). Interestingly, our and other studies revealed that many putative filamentous pathogen effectors trigger cell death in nonhost *N. benthamiana* and in host plants as well, indicating that defense machinery in plants is highly conserved or that plants have convergently evolved to recognize the same defense elicitors.

The effector genes in filamentous plant pathogens are frequently located in regions that are either lineage-specific, gene-poor, or both, that are enriched for retrotransponson and other repetitive sequence elements (Ma et al. 2010; Xue et al. 2012). These effectors, when being recognized, are subject to high-speed positive selection, allowing the pathogen to diversify its effector repertoire rapidly and to evade recognition by plant *R* genes (Raffaele and Kamoun 2012; Zhang et al. 2014). As a consequence, fungal effectors generally share a relatively low amino acid sequence similarity among closely related fungal species (Zhang et al. 2014). Through Pfam and BLAST searches, no homolog or any identified functional motif or domain was identified for most of the 11 effectors except UV_44, UV_1423, and UV_5851. UV_5851 was predicted to have a novel MD-2-related lipid recognition (ML) domain implicated in lipid recognition. The ML domain–containing

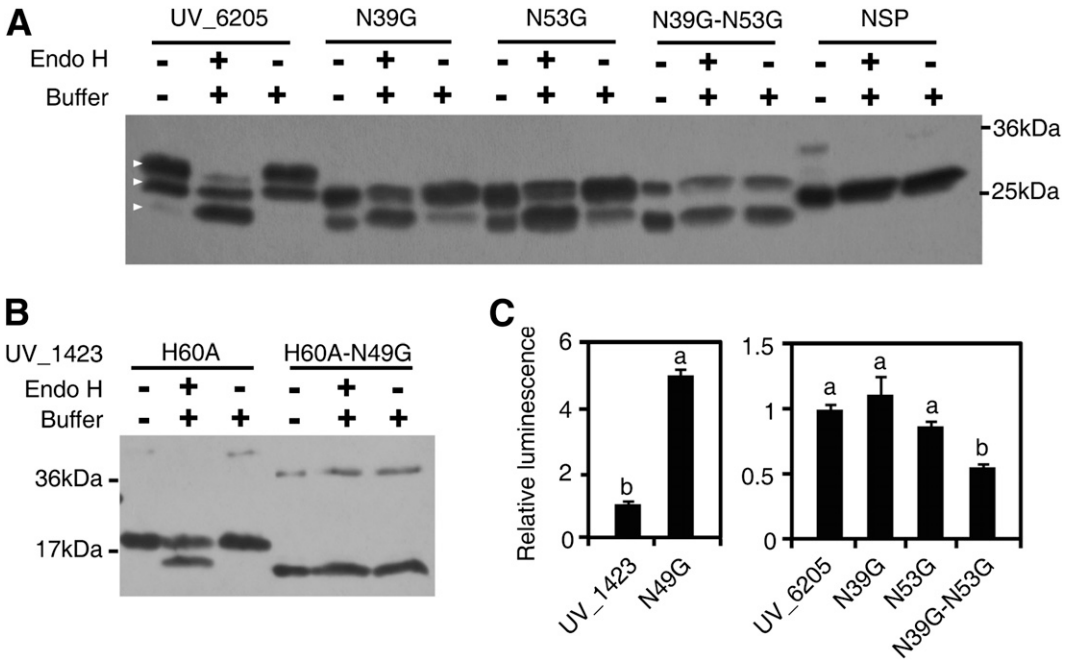

Fig. 8. The secreted proteins UV_6205 and UV_1423 are *N*-glycosylated proteins in plants. **A,** Deglycosylation analysis for the secreted protein UV_6205 and its mutant proteins with point mutations in putative *N*-glycosylaion sites Asn39 and Asn53 transiently expressed in rice protoplasts. **B,** Deglycosylation analysis for the mutant secreted proteins UV_1423^{H60A} and UV_1423$^{H60A/N49G}$ with a point mutation at the putative glycosylation site Asn49 transiently expressed in rice protoplasts. Total protein extracts were treated with Endo H or buffer only before immunoblotting. Different bands indicated by white triangles are caused by posttranslational *N*-glycosylation. **C,** The luciferase activity in rice protoplasts cotransfected with the luciferase and *UV_1423*, *UV_6205*, or its mutant gene constructs. Data are means ± standard error. Different letters (a or b) indicate significant difference in the luciferase activity ($P < 0.05$, Duncan's multiple-range test).

proteins have been reported to be related to innate immunity in mammals and in plants (Fridborg et al. 2013). However, no fungal effector with the ML domain has been reported so far and its functions in inducing cell death need to be further explored. UV_44 contains conserved peptidase inhibitor_I9 and peptidase_S8 domains. The UV_44 homolog in *Rhizoctonia solani* has been previously shown to induce HR in rice and maize (Zheng et al. 2013). We further revealed that the catalytic triad in the predicted peptidase_S8 domain was indispensable for its cell death–eliciting ability, indicating that the activity of serine peptidase is required for its function (Fig. 6). UV_1423 was predicted to be a fungus-specific RNase-like protein that triggered a rapid and severe cell death in *N. benthamiana* and in rice (Figs. 1 and 4). In contrast, BEC1011, a secreted RNase-like effector in *Blumeria graminis* f. sp. *hordei*, was shown to interfere with pathogen-induced host cell death specifically. Both BEC1011 and another RNase-like effector, BEC1054, contributed to *B. graminis* infection (Pliego et al. 2013). Different from the putative RNase active sites of BEC1011 and BEC1054, which are not conserved, key amino acid residues for RNase activity in UV_1423 were highly conserved. We showed that these conserved key residues were essential for its cell death–inducing activity, suggesting that UV_1423 is a functional RNase in plants (Fig. 7). By contrast, the contribution of BEC1011 and BEC1054 in *B. graminis* infection might not relate to RNase activity but, rather, host RNA binding or RNase scaffold (Pliego et al. 2013). These findings suggest that these similar effectors in different pathogenic fungi might function in the host-pathogen interaction with different mechanisms.

The SPs of the tested secreted proteins were all required for their ability to induce cell death in *N. benthamiana* and in rice protoplasts (Fig. 5). A similar phenomenon was observed for *M. oryzae* effector proteins. The full-length effectors MoCDIP1 to MoCDIP5 are able to trigger cell death in rice protoplasts, but the truncated versions of these proteins without SPs are not (Chen et al. 2013). The SP of XEG1, an apoplastic effector, is also required for its cell death–inducing ability (Ma et al. 2015). Interestingly, many oomycete RxLR effectors, such as $AVR3a^{KI}$, $ATR1^{NdWsB}$, and ATR13, when directly expressed in planta, did not require a SP to trigger HR and are, therefore, recognized inside the plant cytoplasm (Morgan and Kamoun 2007). The requirement of SP for secreted proteins to induce cell death in plants indicates that these proteins might function in the extracellular space. These proteins without SPs cannot be secreted into the extracellular matrix, in which proteins are recognized by plasma membrane–bound pattern recognition receptors. Alternatively, these secreted proteins might be translocated into the cell after their secretion and trigger cell death after being recognized by cytoplasmic receptors. However, functionality and recognition of these secreted proteins in the cytosol may require proper processing, correct folding, and specific modifications, such as posttranslational glycosylation during their passage through the secretory pathway in the endoplasmic reticulum (ER) and the Golgi apparatus. Therefore, the exact localization of these proteins in plant cells needs to be further elucidated.

As a common posttranslational modification in eukaryotic cells, *N*-linked glycosylation of secreted proteins has been demonstrated to be directly associated with protein folding, stability, quality control, sorting, and secretion, and even sometimes to be recognized as a "tag" indirectly (Helenius and Aebi 2004). Most effector glycoproteins require their *N*-linked glycans for more efficient secretion in the ER and the Golgi complex (Helenius and Aebi 2004). However, biological significance of *N*-glycosylation of effector proteins in plant-pathogenic fungi has not been accurately elucidated so far. Recently, *N*-glycosylation of effector proteins was shown to be important for *M. oryzae* to suppress host innate immunity (Chen et al. 2014). In this study, six of 11 putative effector proteins were predicted by the NetNGlyc 1.0 Server to be *N*-glycosylated. Through de-glycosylation analyses and site-directed mutagenesis of the predicted *N*-glycosylation sites, UV_1423 and UV_6205 were proven to be *N*-glycosylated (Fig. 8). The data suggest that *N*-glycosylation is a common feature for fungal effectors, particularly for apoplastic effectors. Notably, the fully deglycosylated UV_6205 protein showed double bands in Western blots, suggesting that the protein might be subject to one or more other posttranslational modifications (Fig. 8). More interestingly, experimental data demonstrated that the mutations on the *N*-glycosylation sites of UV_1423 and UV_6205 affected the ability of inducing cell death (Fig. 8C). UV_1423^{N49G} partially lost the cell death–inducing activity in rice protoplast, suggesting that *N*-glycosylation has a positive effect on the ability of UV_1423 to induce cell death. However, $UV\_6205^{N39G/N53G}$, with double point mutations, has a stronger effect on inhibition of LUC activity in rice protoplasts, indicating that *N*-glycosylation possibly masks cell death–eliciting determinants of the protein. A similar observation has been reported for flagellin of the rice pathogen *Acidovorax avenae*. *Acidovorax avenae* K1 flagellin induced much stronger immune responses after deglycosylation (Hirai et al. 2011). Therefore, *N*-glycosylation influences biological functions of secreted proteins in the plant-pathogen interaction through different mechanisms.

In the study, we identified and characterized several types of secreted proteins, probably effectors, in *U. virens* that induce plant cell death or defense machinery. However, precise molecular mechanisms describing how these secreted proteins are involved in the rice–*U. virens* interaction remain to be further elucidated.

MATERIALS AND METHODS

Bacterial strains, plant materials, and growth conditions.

The virulent *U. virens* isolates UV-8b and P1 used in this study were cultured in PSA medium (boiled extracts of 200 g of fresh potato, 20 g of sucrose, and 14 g of agar per liter). *N. benthamiana* plants were grown in growth chambers under a 25°C, 14-h day and 23°C, 10-h night cycle. *Agrobacterium tumefaciens* EHA105 was cultured in Luria Bertani medium (0.5% yeast extract, 1% tryptone, 1% NaCl) and was used for transient expression. The yeast strain YTK12 was cultured in YPDA medium (1% yeast extract, 2% peptone, 2% glucose, 0.003% adenine hemisulfate, 2% agar). Antibiotics were used at the following concentrations (μg ml^{-1}): ampicillin, 100; kanamycin, 50; rifampin, 25. All experiments were repeated at least three times with similar results, unless noted.

RNA isolation and plasmid construction of *U. virens* putative effector genes.

Total RNA was extracted from *U. virens*, *U. virens*–inoculated rice panicles or agrobacterium-infiltrated *N. benthamiana* leaves, using an Ultrapure RNA isolation kit according to the manufacturer's instructions (CWBIO). The quantity and quality of isolated RNA were determined by NanoDrop 2000 (Thermo Scientific). Complementary DNA was synthesized using reverse transcriptase M-MLV (Invitrogen). Secreted proteins and putative effectors in *U. virens* were predicted in our previous study (Zhang et al. 2014). The full-length and truncated secreted protein-encoding genes were amplified with fast *Pfu* polymerase (TransGen), using cDNAs as template with the respective primer sets (Supplementary Tables S3 and S4). PCR products were digested with the corresponding restriction enzymes and were subcloned into pUC19-35S-FLAG-RBS (Li et al. 2005). All constructs were confirmed by sequencing. The inserted DNA sequence, together with the FLAG sequence in pUC19-35S-FLAG-RBS, was

released by *Xho*I and *Spe*I and was religated into pTA7001 (Aoyama and Chua 1997).

Site-directed mutagenesis.

Site-directed mutagenesis was performed via circular PCR, using the pUC19-35S-FLAG-RBS gene constructs as templates. PCR was performed using *Pfu* Ultra DNA polymerase (Stratagene) for 18 thermal cycles (30 s at 95°C, 1 min at 55°C, and 5 min at 68°C). The resultant products were transformed into *Escherichia coli* DH5α competent cells after *Dpn*I digestion of the template plasmid DNA at 37°C for 1 h. The constructed plasmids with point mutations were all confirmed by sequencing (Supplementary Table S5). The mutated genes with the FLAG-tag sequence were cleaved and religated into pTA7001 (Aoyama and Chua 1997).

***Agrobacterium tumefaciens*–mediated transient gene expression.**

The pTA7001-based gene constructs were transformed into *A. tumefaciens* EHA105, using the freeze-thaw method (An et al. 1989). The overnight cultured *A. tumefaciens* strains with different gene constructs were harvested, were washed three times with sterile double-distilled H_2O, and were then resuspended in infiltration buffer (10 mM MES, pH 5.7, 10 mM $MgCl_2$, and 150 μM acetosyringone) to cell density at an optical density at 600 nm of 0.5. After standing at room temperature for 3 h, *A. tumefaciens* cultures were pressure-infiltrated into the leaves of 4-week-old *N. benthamiana* plants using needleless syringes. *A. tumefaciens* strains carrying the pTA7001 empty vector or pTA7001-*GFP* were infiltrated as controls. The infiltrated leaves were sprayed with a 30-μM DEX solution to induce gene expression at 16 h after infiltration. Cell-death symptoms were observed at 2 to 4 days after DEX spraying and were photographed at 5 days after infiltration.

Rice protoplast transformation and luminescence measurement.

Rice protoplast isolation and transformation were performed as described previously (Chen et al. 2006; Zhang et al. 2011). The seedlings of rice cultivars Nipponbare and LYP9 were grown on $^1/_2\times$ Murashige Skoog medium with 2% sucrose and 0.3% phytagel at 28°C in the dark for 10 to 12 days. The etiolated seedlings were cut into approximately 0.5-mm strips, using a surgical blade. Sliced stripes were incubated in 20 ml of enzyme solution (0.6 M mannitol, 1% cellulase RS, 0.5% macerozyme R10, 0.1% bovine serum albumin, 1 mM $CaCl_2$, 5 mM β-mercaptoethanol, 10 mM MES, pH 5.7) in a sterile Erlenmeyer flask at 28°C for 6 h in the dark with gentle shaking (approximately 70 rpm). The protoplasts were pelleted by centrifugation at 1,500 rpm (Centrifuge 5810R, Eppendorf) for 4 min after the enzyme solution was collected through filtration. The remaining debris was washed twice with W5 medium (154 mM NaCl, 125 mM $CaCl_2$, 5 mM KCl, 2 mM MES, pH 5.7) to release more protoplasts. For transfection, the protoplasts were resuspended with an appropriate volume of MMG buffer (0.8 M mannitol, 1 mM $MgCl_2$, 4 mM MES, pH 5.7) to the density of 1.5×10^6 to 2.5×10^6 protoplasts per milliliter. Individual pUC19-35S-FLAG-RBS construct and pJD301-35S-LUC plasmid DNA (10 μg each) were mixed with 200 μl of suspended protoplasts and 220 μl of PEG solution (40% PEG 4000, 1 mM $CaCl_2$, 0.6 M mannitol) by gently shaking and were incubated at 28°C for 10 min. W5 medium (1.0 ml) was then added into the protoplasts to dilute PEG. The protoplasts were pelleted, washed, and resuspended with W5 medium, and were then kept at 28°C in the dark. After 16 to 36 h of incubation, the protoplasts were collected and were incubated with 100 μl of extraction buffer (50 mM Tris-Cl, 25 mM EDTA, 250 mM NaCl, 10% glycerol, 0.1% Triton X-100, 20 mM dithiothreitol [DTT]) for protein extraction. The LUC activity was measured using a microplate reader after the mixture of extracted proteins (20 μl) with the substrate luciferin (1 mM, 20 μl) and 100 μl of Tricine buffer (20 mM Tricine, 27 mM $MgSO_4$+·$7H_2O$, 0.1 mM EDTA, 2 mM DTT, 5 μM ATP, pH 7.8). Viability of transfected protoplasts was determined by staining with 50 μg of fluorescein diacetate (FDA) per milliliter at 36 h after transfection. The FDA-stained vital cells were counted by thrombocytometer.

Measurement of ion leakage from leaf discs of *Nicotiana benthamiana*.

Ion leakage was measured to evaluate cell death in the inoculated leaves as described previously (Mittler et al. 1999; Yu et al. 2012). Briefly, five leaf discs (9 mm diameter) were collected and incubated on distilled water (5 ml) for 3 h at room temperature. The conductivity of the bathing solution was measured with a conductivity meter (FE30; Mettler Toledo). After leaf discs were returned to the bathing solution and boiled in sealed tubes, the conductivity of the solution was measured again. The conductivity ratio was calculated as ion leakage.

Validation of predicted SPs.

Functional validation of the predicted SP of putative effectors in *U. virens* was conducted using a yeast secretion assay (Jacobs et al. 1997). The yeast signal sequence trap vector pSUC2T7M13ORI (pSUC2), which carries a truncated invertase gene lacking the start codon and SP coding sequence, was used in this assay. The predicted SP coding sequences of *U. virens* candidate effector genes were amplified and cloned as *Eco*RI-*Xho*I fragments into pSUC2 (Oh et al. 2009; Tian et al. 2011) (Supplementary Table S6). The pSUC2-derived plasmid (0.5 μg) was transformed into the invertase-deficient yeast strain YTK12 (*SUC2*–) using the Frozen-EZ yeast transformation II kit (Zymo Research). Transformants were selected on yeast minimal tryptophan dropout medium (CMD-W medium, 0.67% yeast N base without amino acids, 0.075% tryptophan dropout supplement, 2% sucrose, 0.1% glucose, and 2% agar). Yeast colonies were replica-plated onto YPRAA plates (1% yeast extract, 2% peptone, 2% raffinose, and antimycin A at 2 μg l^{-1}) for invertase secretion assays.

***U. virens* inoculation and quantitative real time RT-PCR.**

Injection inoculation of the *U. virens* isolate P1 into rice panicles of the RFS resistant *Oryza sativa* L. subsp. *indica* cultivar IR28 and highly susceptible LYP9 was performed as described previously (Han et al. 2015). The inoculated panicles were harvested at 0, 24, 48, and 96 hpi, were immediately frozen in liquid nitrogen, and were then kept at −70°C for RNA isolation.

Quantitative real time (q)RT-PCR was performed with SYBR premix ex Taq (TaKaRa), using the ABI PRISM 7000 sequence detection system (Applied Biosystems) according to the manufacturer's instructions. The expression level of candidate effector genes was calculated relative to α-tubulin of *U. virens*, which was used as internal reference. The primer sets used for qRT-PCR and RT-PCR are listed in Supplementary Table S7.

Protein extraction and immunoblotting.

N. benthamiana leaves infiltrated with *A. tumefaciens* were harvested at 24 h after DEX spraying and were ground in liquid nitrogen. The powder was incubated with 1× sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis sample buffer and were then boiled for 10 min. Protein extracts from *N. benthamiana* leaves or transfected protoplasts were separated on a 12% SDS-polyacrylamide gel by electrophoresis. Separated proteins were

electrophoretically blotted onto nitrocellulose membranes (Millipore) and were stained with 0.1% Ponceau S to visualize sample loading. The membranes were blocked with 5% skimmed milk in TBS-T buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.05% Tween 20) for 1 h at room temperature, were incubated in an anti-FLAG antibody solution (1:5,000 dilution) for 1 h at room temperature, and were washed thoroughly with TBS-T buffer. The blots were then incubated in horseradish peroxidase–conjugated antimouse secondary antibody (1:5,000 dilution in TBS-T) for 1 h at room temperature. After rinsing thoroughly, the immunoblots were incubated with the eECL Western substrate (CWBIO) and were then exposed with X-films.

***N*-glycosylation analysis.**

Endo H (New England Biolabs) was used for the deglycosylation analysis of UV_1423 and UV_6205, according to the manufacturer's instructions. UV_1423 and UV_6205 with point mutations in the predicted *N*-glycosylation sites were transiently expressed in *N. benthamiana* and in rice protoplasts via the pTA7001 and pUC19-35S-FLAG-RBS constructs, respectively. Total proteins extracted from *N. benthamiana* leaves and rice protoplasts were treated with Endo H at 37°C for 1 h before immunoblotting.

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