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Verticillium dahliae manipulates plant immunity by glycoside hydrolase 12 proteins in conjunction with carbohydrate-binding module 1

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Summary

Glycoside hydrolase 12 (GH12) proteins act as virulence factors and pathogen-associated molecular patterns (PAMPs) in oomycetes. However, the pathogenic mechanisms of fungal GH12 proteins have not been characterized. In this study, we demonstrated that two of the six GH12 proteins produced by the fungus *Verticillium dahliae* Vd991, VdEG1 and VdEG3 acted as PAMPs to trigger cell death and PAMP-triggered immunity (PTI) independent of their enzymatic activity in *Nicotiana benthamiana*. A 63-amino-acid peptide of VdEG3 was sufficient for cell death-inducing activity, but this was not the case for the corresponding peptide of VdEG1. Further study indicated that VdEG1 and VdEG3 trigger PTI in different ways: BAK1 is required for VdEG1- and VdEG3-triggered immunity, while SOBIR1 is specifically required for VdEG1-triggered immunity in *N. benthamiana*. Unlike oomycetes, which employ RXLR effectors to suppress host immunity, a carbohydrate-binding module family 1 (CBM1) protein domain suppressed GH12 protein-induced cell death. Furthermore, during infection of *N. benthamiana* and cotton, VdEG1 and VdEG3 acted as PAMPs and virulence factors, respectively indicative of host-dependent molecular functions. These results suggest that VdEG1 and VdEG3 associate differently with BAK1 and SOBIR1 receptor-like kinases to trigger immunity in *N. benthamiana*, and together with CBM1-containing proteins manipulate plant immunity.

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Introduction

In the classic model of plant-pathogen interactions, plants employ at least two defense systems in response to pathogen attacks (Jones and Dangl, 2006; Zipfel, 2008). The first system is a basal defense that begins when plant cells recognize the presence of conserved pathogen-associated molecular patterns (PAMPs) via pattern recognition receptors (PRRs). This is termed PAMP-triggered immunity (PTI) and involves the rapid activation of downstream responses, including cell death, calcium ion (Ca^{2+}) level elevation, reactive oxygen species (ROS) bursts, callose deposition and induction of defense-related genes (Boller and Felix, 2009; Zipfel, 2009). Many PAMPs are widely conserved across genera, such as bacterial flagellin flg22 (Felix *et al.*, 1999), elongation factor Tu (Kunze *et al.*, 2004) and ethylene-inducing xylanase (EIX) (Enkerli *et al.*, 1999).

During the coevolution of host-microbe interactions, pathogens acquired the ability to deliver effector proteins to interfere with PTI, thus enabling pathogens to infect the host plant and cause disease (Chisholm *et al.*, 2006; Jones and Dangl, 2006; de Jonge *et al.*, 2011). Successful pathogens deliver effectors to interfere with PTI, resulting in effector-triggered susceptibility (ETS). As a countermeasure, plants have developed a second immunity system known as effector-triggered immunity (ETI), which involves additional resistance proteins that recognizes specific pathogen effectors, resulting in rapid activation of the defense response (Jones and Dangl, 2006; Zipfel, 2008). Several plant pathogen effectors suppress PTI and play key roles in establishing parasitic relationships (Kim *et al.*, 2004; Thomma *et al.*, 2011; Stassen and Van den Ackerveken, 2011). For example, the Ecp6 protein of *Cladosporium fulvum* prevents activation of host immunity by sequestering chitin fragments (de Jonge *et al.*, 2010). Recent studies have suggested that plant immunity is a

continuum between PTI and ETI, and that some effectors may also act as PAMPs (Thomma *et al.*, 2011).

The plant cell wall provides the first barrier to prevent pathogen infection, and pathogens secrete numerous cell wall-degrading enzymes (CWDEs) to depolymerize the polysaccharide components of the plant cell wall (Kubicek *et al.*, 2014). While CWDEs are virulence factors (Brito *et al.*, 2006; Tzima *et al.*, 2011; Kubicek *et al.*, 2014), some CWDEs such as xyloglucan-specific endoglucanase (XEG1) (Ma *et al.*, 2015b), fungal endopolygalacturonases (Zhang *et al.*, 2014) and EIX (Enkerli *et al.*, 1999) also function as PAMPs, and trigger PTI independent of their enzymatic activity. Specifically, CWDEs contain carbohydrate-binding modules (CBM), non-catalytic protein domains that are generally associated with carbohydrate hydrolases in fungi, which are known to act as elicitors of the PTI response in oomycetes (Gaulin *et al.*, 2006; Larroque *et al.*, 2012).

A recent study (Ma *et al.*, 2015b) showed that glycoside hydrolase family 12 (GH12) proteins are involved in cellulose degradation across microbial taxa and also act as PAMPs. One member of the GH12 family, *Phytophthora sojae* XEG1, acts as a PAMP to trigger cell death in several plant species, which in turn can be suppressed by effectors with an Arg-x-Leu-Arg motif (RXLR effectors) (Ma *et al.*, 2015b). However, oomycetes are evolutionarily distinct from fungi and although several fungal GH12 proteins can induce cell death in plants (Ma *et al.*, 2015b), the mechanism underlying the involvement of GH12 proteins in plant-fungus interactions remains unknown. It is especially unclear what types of effectors suppress GH12-triggered immunity and manipulate host immunity, given that fungi lack the typical RXLR effectors found in oomycetes.

Verticillium dahliae is a soilborne fungal plant pathogen that causes vascular wilt disease. The *V. dahliae* genome is enriched for more plant cell wall degrading enzymes than other fungi studied (Klosterman *et al.*, 2011; Chen *et al.*, 2016), and the CBM1-containing protein family is significantly expanded in *V. dahliae* (Klosterman *et al.*, 2011). Previous studies have shown that cellulases of phytopathogenic fungi are involved in pathogenicity (Sexton *et al.*, 2000; Eshel *et al.*, 2002). The *V. dahliae* VdLs.17 genome encodes a family of six cellulases with GH12 domains, one of which contains a CBM1 domain that is not present in the other five proteins. To understand the role of fungal GH12 proteins and the potential involvement of the CBM1 domain in particular, we identified and characterized *V. dahliae* GH12 proteins involved in virulence or the elicitation of plant immunity responses during infection.

The main objectives of the current study were to: 1) investigate which cell wall degrading enzymes (GH12 domain-containing proteins) from *V. dahliae* strain Vd991 trigger the PTI response in *N. bethamiana*; 2) identify the specific epitope(s) in GH12 domain-containing proteins that elicit PTI in *N. bethamiana*; 3) elucidate any additional proteins associated with PRR proteins that involved in triggering PTI; 4) study the role of CBM1 domains in suppressing the host cell death response in *N. bethamiana*; and 5) investigate the function of GH12 and CBM1 proteins in cotton pathogenesis, virulence and PTI.

Results

Verticillium dahliae *cellulases VdEG1 and VdEG3 trigger the PTI response in* N. Benthamiana *independent of their enzymatic activity*

Six GH12 domain-containing proteins (*VdEG1* to *VdEG6*) were identified in the *V. dahliae* (VdLs.17) genome, and cloned from the highly virulent isolate Vd991 from cotton (Supporting Information Figs S1 and S2; Supporting Information Table S1). Three of these six proteins (*VdEG1-VdEG3*) were predicted to be secreted proteins (Supporting Information Table S2). Transient expression of the *VdEG*s demonstrated that only *VdEG1* and *VdEG3* triggered cell death in *N. benthamiana* leaves 6 days post-inoculation (dpi) (Fig. 1A), although immunoblotting analysis confirmed the effective translation of all *VdEG*s in *N. benthamiana* (Fig. 1B). Amino acid sequence alignment indicated that VdEG1 and VdEG3 were typical GH12 family proteins, and that the highly conserved glutamic acid (E) catalytic residues were also conserved in VdEG1 (E137 and E223) and VdEG3 (E146 and E232) (Supporting Information Fig. S3A) (Sandgren *et al.*, 2001). Site-directed mutagenesis of the two conserved catalytic residues in VdEG1 and VdEG3, $EG1^{SM}$ and $EG3^{SM}$, respectively, resulted in almost complete loss of cellulase activity (Supporting Information Fig. S3B and C), further confirming that both were typical GH12 proteins. Interestingly, the non-enzymatically active mutant proteins ($EG1^{SM}$ and $EG3^{SM}$) still strongly triggered cell death 3 days after agro-infiltration or 24 h after infiltration with recombinant protein (Fig. 1C), indicating that the cellulase activities of VdEG1 and VdEG3 were not required for their cell death-inducing activities in *N. benthamiana*.

With the yeast signal trap assay system, fusion of the signal peptide of VdEG1 or VdEG3 into the invertase gene resulted in the secretion of invertase in yeast (Fig. 1D), indicating that VdEG1 and VdEG3 are most likely secreted into the extracellular space during host plant infection. Transient expression of the two genes lacking the signal peptide, $VdEG1^{-SP}$ and $VdEG3^{-SP}$, did not induce cell death 6 dpi in *N. benthamiana* (Fig. 1E), suggesting that the extracellular secretion of VdEG1 and VdEG3 is required to induce cell death in *N. benthamiana*. The above results suggested that VdEG1 and VdEG3 were targeted to the extracellular space (apoplast) in *N. benthamiana* tissues, providing evidence that VdEG1 and VdEG3 act as PAMPs to induce cell death.

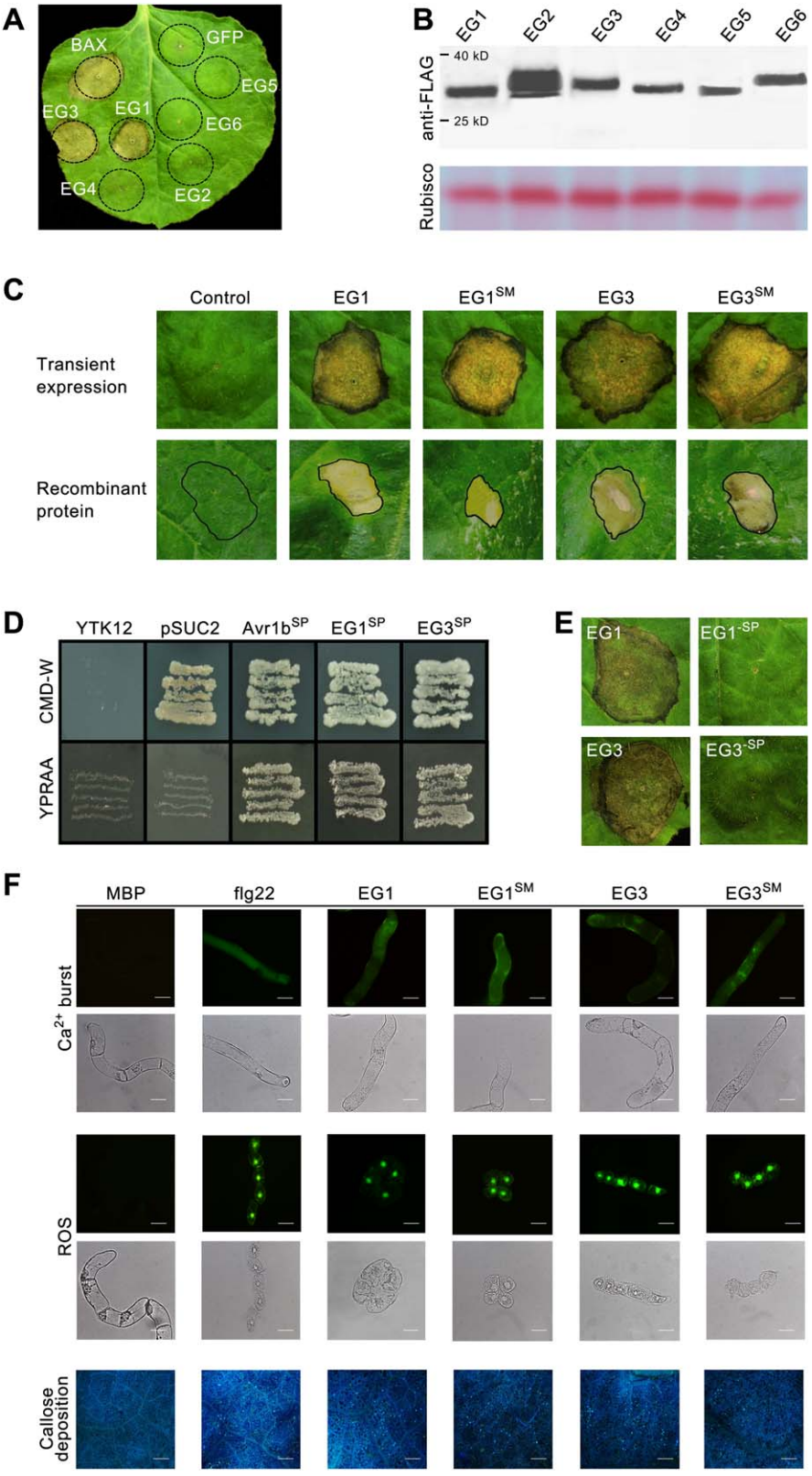

Fig. 1. Identification of cell death-inducing and PAMP activities of glycoside hydrolase 12 proteins in *Verticillium dahliae*.
A. Cell death induction was assessed for six *V. dahliae* genes in *N. benthamiana* leaves from 4-week-old plants 6 days after infiltration with *Agrobacterium* carrying the indicated genes. BAX and GFP were used as positive and negative controls respectively.
B. Immunoblotting analysis of transiently expressed *VdEGs* fused to the FLAG-tag in *Nicotiana benthamiana* leaves 60 h after infiltration. Ponceau S-stained Rubisco protein is shown as a total protein loading control.
C. Detection of the cell death-inducing activities of wild-type and site-directed mutagenized VdEG1 and VdEG3. Transient expression was assessed in *N. benthamiana* leaves from 4-week-old plants 6 days after agroinfiltration, and GFP was used as a control. Cell death induced by recombinant proteins was assessed in *N. benthamiana* leaves 2 days after infiltration with recombinant protein; 3.0 μM MBP tag was used as a control.
D. Validation of the function of the signal peptides of *VdEG1* and *VdEG3* by yeast signal trap assay. The yeast strain, YTK12, could not grow on CMD-W medium without tryptophan. The strain containing the pSUC2 vector can grew based on the function of the *Trp* operon. Fusion of the functional signal peptide of VdEG1 or VdEG3 in-frame with mature yeast invertase enabled secretion of invertase, resulting in growth on YPRAA medium. The functional signal peptide of Avr1b was used as a positive control.
E. A functional signal peptide is required for the cell death-inducing activities of VdEG1 and VdEG3 in *N. benthamiana*. Deletion of the signal peptide in VdEG1 and VdEG3 ($EG1^{-SP}$ and $EG3^{-SP}$) resulted in no induction of cell death in 4-week-old plants 6 days after infiltration.
F. Detection of PTI responses triggered by recombinant protein of VdEG1 and VdEG3 in *N. benthamiana*. MBP and flg22 were used as positive and negative controls respectively. Cytosolic Ca^{2+} accumulation was visualized under a fluorescence microscope with fluorescence (top) and phase contrast (bottom) in BY2 cells. ROS bursts in BY-2 cells were detected using the fluorescent probe DCFH-DA. ROS production was visualized under a fluorescence microscope with fluorescence (top) and phase contrast (bottom). Callose deposition in *N. benthamiana* leaves from 4-week-old plants were detected 2 days after infiltration of recombinant protein; leaves were stained with aniline blue. Bars = 50 μm.

To examine whether VdEG1 and VdEG3 could induce typical PTI responses, *N. benthamiana* leaves and suspension cells were treated with purified recombinant VdEG1/VdEG3 and $EG1^{SM}$/$EG3^{SM}$ proteins. The results showed each of these proteins triggered typical PTI responses, including the Ca^{2+} burst, ROS accumulation and callose deposition (Fig. 1F, Supporting Information Fig. S4A). Furthermore, agroinfiltration of VdEG1 and VdEG3 into *N. benthamiana* tissues also caused electrolyte leakage (Supporting Information Fig. S4B). Moreover, several genes associated with PTI and defense response were significantly activated in *N. benthamiana* leaves 12 h after treatment with the recombinant proteins (Supporting Information Fig. S4C). Taken together, these results strongly suggest that VdEG1 and VdEG3 act as PAMPs to trigger the PTI response, but independent of their enzymatic activities in *N. benthamiana*.

A small peptide from the GH12 domain of VdEG3 is sufficient for elicitor function in N. Benthamiana

Pathogen recognition receptors often recognize specific small protein epitopes of PAMPs. To delineate the elicitor-active peptides of VdEG1 and VdEG3, we assayed N-terminal and C-terminal deletion mutants for the ability to trigger cell death by agroinfiltration in *N. benthamiana*. In VdEG3, truncated proteins lacking the CBM1 domain (EG-N1), and deletion of one of the indispensable catalytic residues E232 (EG3-N2), still induced cell death in *N. benthamiana*. Further truncation from the C-terminal end (EG3-N3) prevented induction of cell death in *N. benthamiana* (Fig. 2A). Deletion of the region between the GH12 domain and the signal peptide (EG3-C1, EG3-C2 and EG3-C3) resulted in cell death-inducing activity, but the deletion of peptides 21 – 200 (EG3-C4), resulted in the loss of cell death-inducing activity in *N. benthamiana* (Fig. 2A). Finally, the small peptide consisting of the 63 residues from amino acid position 166 to 229 (EG3-MF), which did not include the indispensable catalytic residues E146 and E232, was identified as the functional fragment of VdEG3 responsible for inducing cell death in *N. benthamiana* (Fig. 2A). These findings suggest that the small peptide (EG3-MF) from the GH12 domain in VdEG3 is sufficient to induce cell death in *N. benthamiana*.

Sequence alignment demonstrated that the small peptide EG3-MF is conserved between VdEG3 and residues 157–220 of VdEG1 (EG1-MF) (Fig. 2B). However, EG1-MF did not induce cell death in *N. benthamiana*, although immunoblotting analysis confirmed effective protein translation of EG1-MF (Fig. 2C). Progressive deletion from the C- and N-termini showed that even very small truncations of VdEG1 from the two ends resulted in loss of cell death-inducing activity in *N. benthamiana* (Fig. 2C), indicating that the full length of VdEG1 is required for the elicitor function in *N. benthamiana*. The reactive oxygen species (ROS) detection further confirmed that infiltration of the conserved peptide (EG3-MF) from VdEG3 induced significant ROS accumulation, but the conserved peptide (EG1-MF) from VdEG1 did not (Fig. 2D). These results suggest that GH12 proteins have undergone sequence and functional divergence in *V. dahliae*, and therefore VdEG1 and VdEG3 may trigger cell death in different ways.

VdEG1 and VdEG3 associate with BAK1 and SOBIR1 receptor-like kinases differentially to trigger immunity in N. Benthamiana

Plants employ surface-localized PRRs, either as receptor-like kinases (RLKs) or receptor-like proteins (RLPs), to perceive PAMPs and then induce the PTI response (including

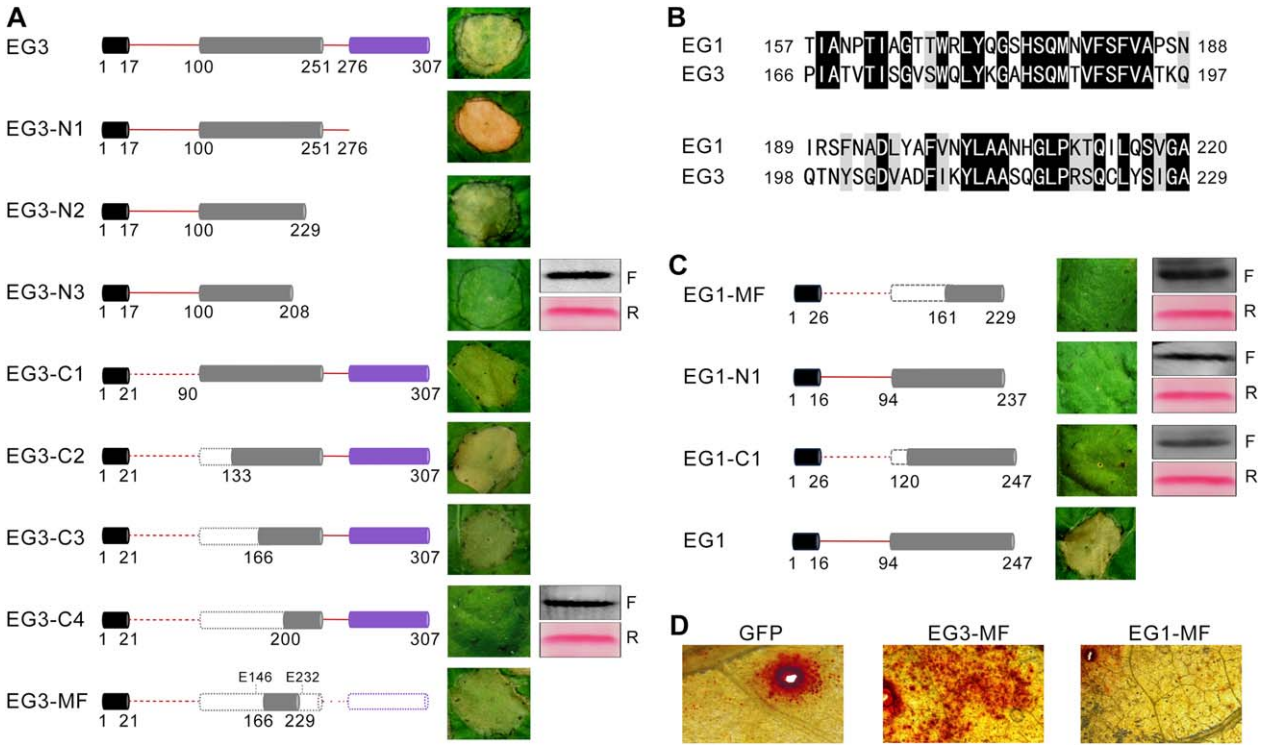

Fig. 2. A small peptide from the GH12 domain in VdEG3 is sufficient for elicitor function in *Nicotiana benthamiana*.
A. Identification of the elicitor active peptide of VdEG3 for inducing cell death. Various truncated *VdEG3* genes were constructed (boxes in black, gray, and purple symbolize the signal peptide, GH12 domain and CBM1 domain, respectively) and transiently expressed by agroinfiltration in *N. benthamiana* leaves from 4-week-old plants. Induction of cell death by the truncated genes was investigated 6 days after infiltration. Transient expression of *VdEG3* mutants that did not induce cell death was confirmed by immunoblotting analysis with anti-FLAG antibody (black bands, marked in 'F'), Ponceau S-stained Rubisco protein (pink bands, marked in 'R') is shown as a total protein loading control.
B. Amino acid sequence alignment of the elicitor-active peptides of VdEG3 to VdEG1. Conserved residues are shaded black.
C. Assays of the cell death-inducing activities of the various truncated VdEG1 mutants by agroinfiltration in *N. benthamiana* leaves from 4-week-old plants. Transient expression of *VdEG1* mutants that did not induce cell death was confirmed by immunoblotting analysis with anti-FLAG antibody (black bands), Ponceau S-stained Rubisco protein (pink bands) is shown as a total protein loading control.
D. ROS detection after agroinfiltration of the elicitor-active peptide of VdEG3 and the corresponding conserved peptide of VdEG1 in *N. benthamiana* leaves from 4-week-old plants.

cell death). BAK1 and SOBIR1 are proteins that play important roles in the regulation of PRRs by interacting with different ligand-binding receptors in a stimulus-dependent manner (Monaghan and Zipfel, 2012; Liebrand *et al.*, 2013; Zhang *et al.*, 2013). To determine whether BAK1 participates in the induction of cell death by VdEG1 and VdEG3, we generated virus-induced gene silencing (VIGS) constructs based on recombinant tobacco rattle virus (TRV) to target *NbBAK1* expression in tobacco. As expected, the BAX positive control retained cell death-inducing activity in the *NbBAK1*-silenced plants, while VdEG1 and VdEG3 failed to trigger cell death (Fig. 3A). Immunoblotting analysis confirmed that VdEG1 and VdEG3 were expressed in the areas of infiltration in the *NbBAK1*-silenced plants (Fig. 3B). The transcript level of *NbBAK1* was significantly reduced to only 19% of the control level (Fig. 3C). These results suggest that BAK1 acted with PRRs to mediate the PTI response, and was required for the induction of cell death by VdEG1 and VdEG3 in *N. benthamiana*.

The cell death-inducing activity of the truncation deletion mutants suggested that VdEG1 and VdEG3 may trigger cell death in different ways (Fig. 2). To determine whether PRRs perceive VdEG1 and VdEG3 in different manners, we further generated *NbSOBIR1*-silenced plants to study the cell death-inducing activities of VdEG1 and VdEG3. The *NbSOBIR1*-silenced plants were agroinfiltrated with VdEG1 and VdEG3 expression constructs, and intriguingly, the cell death-inducing activity was completely different between VdEG1 and VdEG3; VdEG1 did not trigger cell death in *NbSOBIR1*-silenced plants, while VdEG3 was still capable of inducing cell death (Fig. 3D). These results showed that tobacco SOBIR1 is specifically required for a cell death response triggered by VdEG1 in *N. benthamiana*. Immunoblotting analysis confirmed that VdEG1 and VdEG3 were successfully expressed in the *NbSOBIR1*-

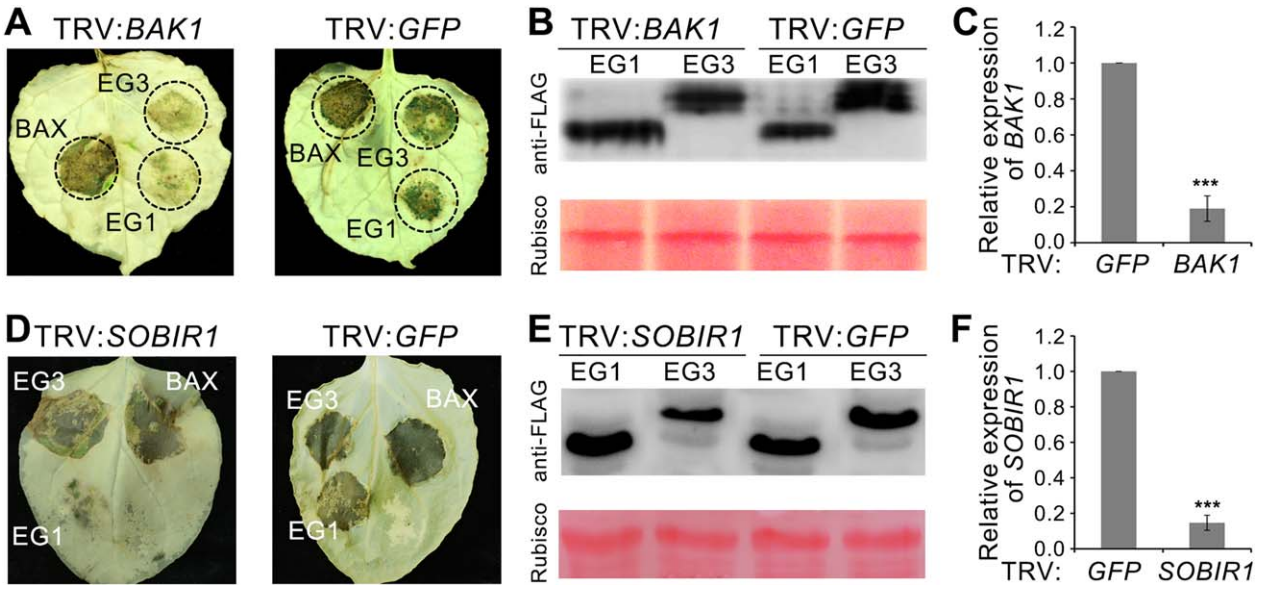

Fig. 3. Analysis of the associations of BAK1 and SOBIR1 receptor-like kinases with VdEG1 and VdEG3.
A. BAK1 is required for VdEG1- or VdEG3-triggered cell death in *N. benthamiana*. *VdEG1* and *VdEG3* were transiently expressed in *BAK1* gene-silenced plants that were subjected to VIGS by inoculation with TRV constructs for three weeks; VdEG1, VdEG3 and GFP were transiently expressed in the gene-silenced leaves, and the phenotypes of induced cell death were photographed 6 days later.
B. Immunoblotting analysis of VdEG1 or VdEG3 protein fused to a FLAG-tag transiently expressed in *BAK1*-silenced *N. benthamiana* leaves 6 days after agroinfiltration. Ponceau S-stained Rubisco protein is shown as a total protein loading control.
C. The silencing efficiency of *BAK1* was determined by qRT-PCR analysis, *EF-1α* was used as an endogenous control. Means and standard errors from three biological replicates are shown. Asterisks *** indicate significant differences ($P < 0.001$).
D. SOBIR1 is required for VdEG1-triggered cell death in *N. benthamiana*, but not for that of VdEG3. The cell death-inducing activities of VdEG1 and VdEG3 were detected in *SOBIR1*-silenced plants.
E. Immunoblotting analysis of VdEG1 or VdEG3 protein transiently expressed in *SOBIR1*-silenced *N. benthamiana* leaves 6 days after agroinfiltration. Ponceau S-stained Rubisco protein is shown as a total protein loading control.
F. The silencing efficiency of *SOBIR* was examined by qRT-PCR analysis. Means and standard errors from three biological replicates are shown. Asterisks *** indicate significant differences ($P < 0.001$).

silenced plants (Fig. 3E). qRT-PCR analysis confirmed that *NbSOBIR1* expression was markedly reduced upon inoculation with TRV:*NbSOBIR1*, with an expression level of only 15% compared with inoculation with TRV:*GFP* (Fig. 3F). Therefore, we inferred that VdEG1 and VdEG3 triggered cell death in different ways: the leucine-rich repeat receptor-like protein kinase/BAK1 (LRR-RLKs/BAK1) complex was required for VdEG3 to trigger immunity in *N. benthamiana*, whereas the leucine-rich repeat receptor-like protein (LRR-RLP/SOBIR1/BAK1) complex was required for VdEG1-triggered immunity.

Suppression of cell death in N. Benthamiana *via coupling of the CBM1 domains with a GH12 protein*

Of the six VdEGs in *V. dahliae* VdLs.17 and Vd991, only VdEG3 contains a CBM1 domain (Supporting Information Table S1). To determine the function of the CBM1 domain in VdEG3, truncation proteins lacking the GH12 or CBM1 domain ($EG3^{GH12}$ and $EG3^{CBM1}$, respectively) were engineered for cell death-inducing activity analysis. Transient expression of $EG3^{CBM1}$ failed to induce cell death in *N. benthamiana*, in contrast to the significant cell death-inducing activity of $EG3^{GH12}$ and full-length VdEG3 (Supporting Information Fig. S5A). Unexpectedly, infiltration of $EG3^{GH12}$ showed stronger cell death-inducing activity in *N. benthamiana* than VdEG3 at 3 dpi (Fig. 4A and B), although immunoblotting analysis confirmed that the levels of EG3 and $EG3^{GH12}$ expression were nearly equivalent (Fig. 4C). The above results suggested that the CBM1 domain of VdEG3 ($EG3^{CBM1}$) could probably suppress the cell death-inducing activities of $EG3^{GH12}$ and VdEG3. For further detection of the ability of $EG3^{CBM1}$ to suppress cell death, co-expression of $EG3^{CBM1}$ with $EG3^{GH12}$ in a time-course showed that $EG3^{CBM1}$ could suppress the cell death-inducing activity of $EG3^{GH12}$ to a certain degree before 92 hpi, although $EG3^{GH12}$ still caused cell death 108 h after co-infiltration with $EG3^{CBM1}$ (Supporting Information Fig. S5B). Furthermore, pre-infiltration with $EG3^{CBM1}$ also confirmed that the CBM1 domain could significantly suppress the cell death-inducing activity of $EG3^{GH12}$ (Supporting Information Fig. S5C).

To confirm the suppression activity of the CBM1 domain coupled with the GH12 domain, the cell death-inducing

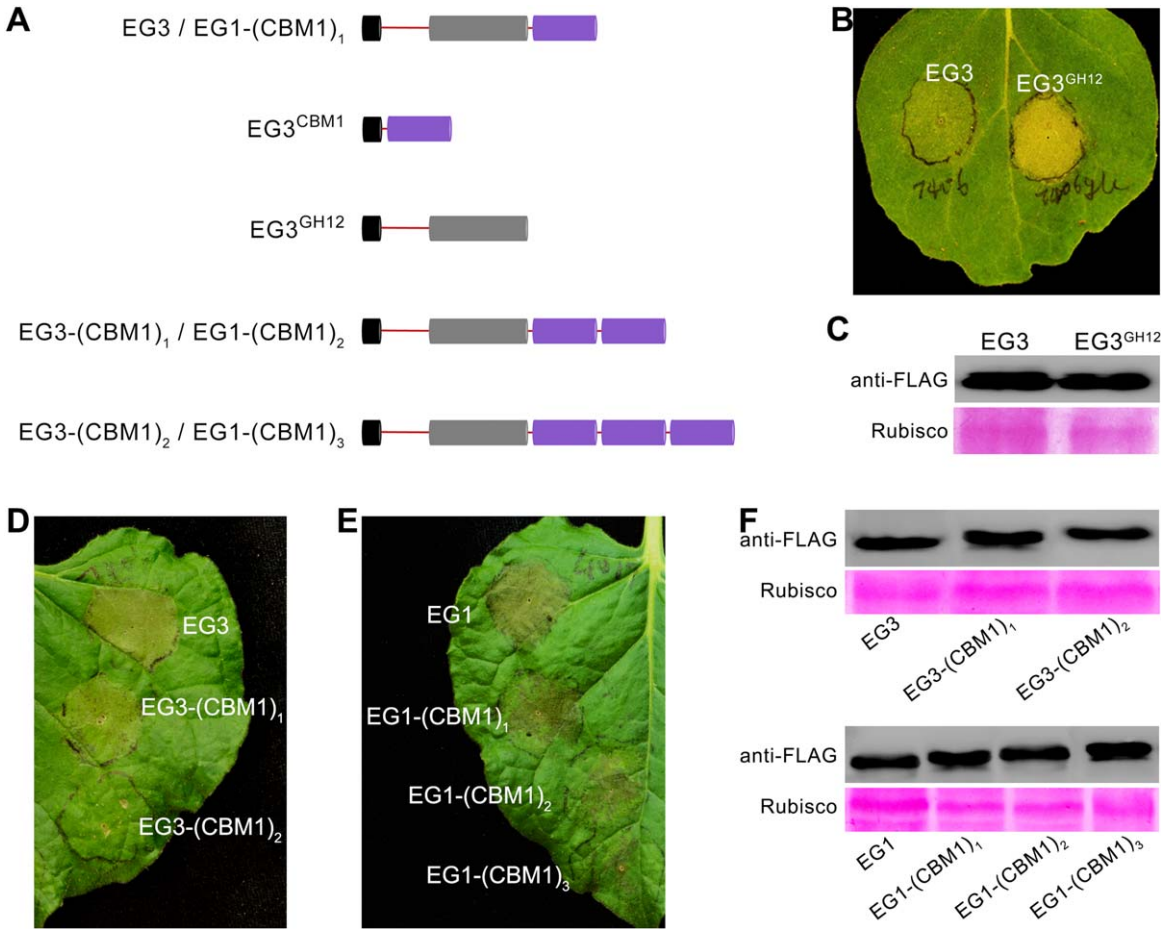

Fig. 4. Evidence that a GH12 protein coupled with CBM1 domains suppressed cell death-inducing activity in *N. benthamiana*.
A. Gene structures of VdEG1 and VdEG3 chimeric proteins. Blocks in black, gray, and purple represent the signal peptides, GH12 domains and CBM1 domains respectively.
B. Comparison of the cell death-inducing intensity of the full-length *VdEG3* and GH12 domain $EG3^{GH12}$ in *N. benthamiana* leaves from 4-week-old plants 4 days after agroinfiltration.
C. Immunoblotting analysis of proteins in *N. benthamiana* leaves transiently expressing VdEG3 and $EG3^{GH12}$. Ponceau S-stained Rubisco protein is shown as a total protein loading control.
D. Cell death-inducing activity was assayed on *N. benthamiana* leaves from 6-week-old plants 6 days after agroinfiltration of constructs of VdEG3 coupled with the CBM1 domain.
E. Detection of VdEG1 coupled with the CBM1 domain.
F. Immunoblotting analysis of proteins in *N. benthamiana* leaves transiently expressing VdEG1 and VdEG3 chimeric proteins, Ponceau S-stained Rubisco protein is shown as a total protein loading control.

activities of VdEG1 or VdEG3 coupled with multiple CBM1 domains were assayed in *N. benthamiana*. Interestingly, EG3-$(CBM1)_1$, VdEG3 coupled with an additional CBM1 domain, showed significant suppression of cell death-inducing activity compared with the wild-type protein in *N. benthamiana* 6 dpi. VdEG3 coupled with two additional CBM1 domains, EG3-$(CBM1)_2$, showed complete suppression of cell death-inducing activity (Fig. 4A and D). Similarly, the cell death-inducing activity of VdEG1 became gradually weaker when coupled with increasing numbers of CBM1 domains, and an almost complete loss of cell death-inducing activity was observed after fusion of VdEG1 with three CBM1 domains (Fig. 4A and E). Immunoblotting analysis confirmed that all of the chimeric VdEG1 and VdEG3 proteins coupled with CBM1 domains were successfully expressed in *N. benthamiana* (Fig. 4F). Taken together, these results suggested that CBM1 domains suppressed the elicitation of PTI of *N. benthamiana* by GH12 proteins during *V. dahliae* infection.

Multiple CBM1 domains can suppress GH12 protein-triggered immunity in N. Benthamiana

To test the hypothesis that the CBM1 domain/protein served as a suppressor of the PTI, we examined the cell death and defense responses triggered by VdEG1 and

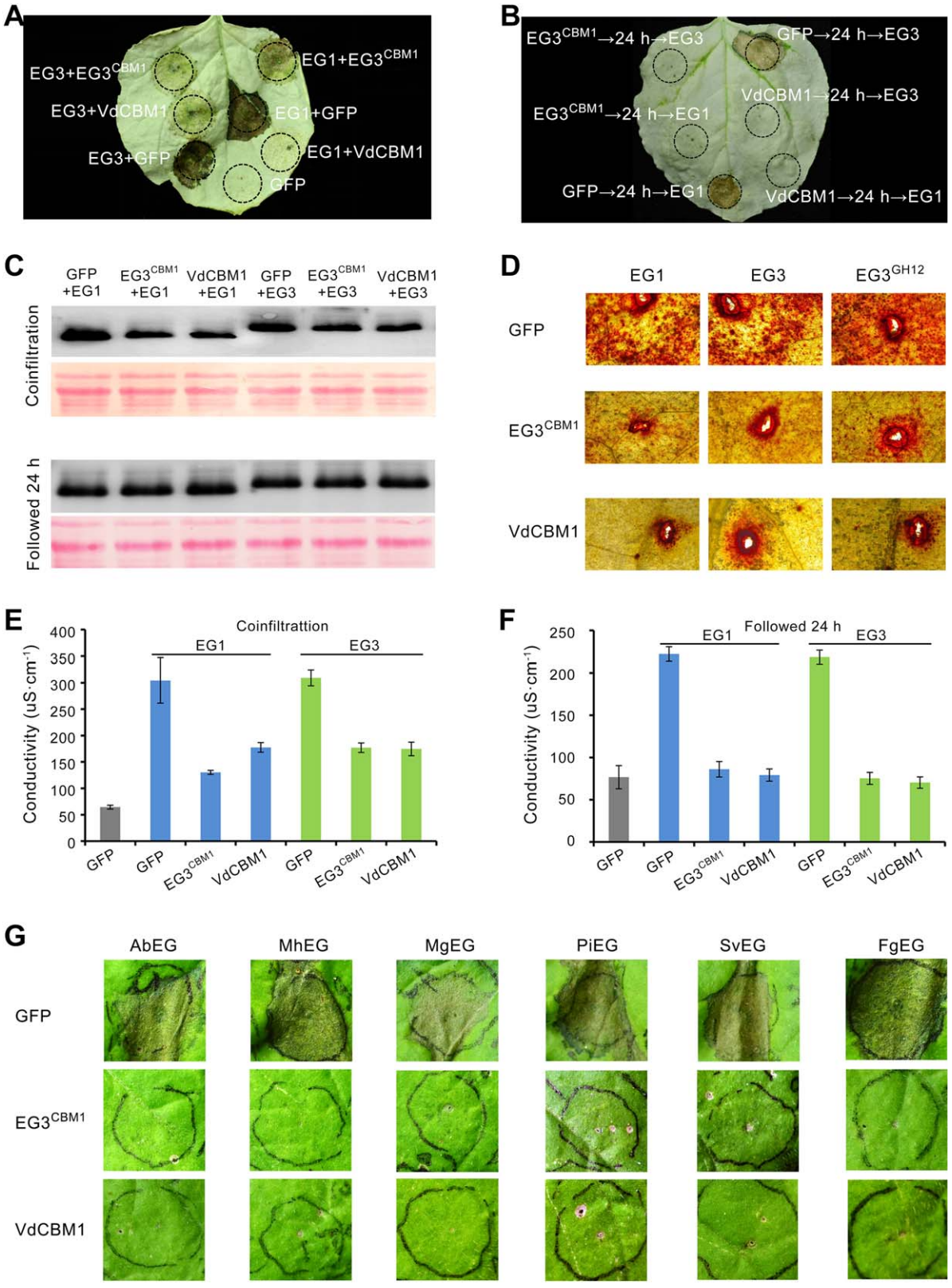

Fig. 5. The CBM1 domain/protein suppresses VdEG1- and VdEG3-tirggered immunity in *N. benthamiana*.
A. Assay for suppression of the cell death-inducing activity of $EG3^{CBM1}$ or VdCBM1 by co-agroinfiltration with *VdEG1* and *VdEG3* in *N. benthamiana* leaves from 4-week-old plants 6 days after agroinfiltration. GFP was used as a negative control.
B. Suppression of VdEG1- or VdEG3-triggered cell death-inducing activity by pre-infiltration for 24 h with $EG3^{CBM1}$ or VdCBM1. Suppression was assessed in *N. benthamiana* leaves from 4-week-old plants 6 days after infiltration with *VdEG1* and *VdEG3*, GFP was used as a negative control.
C. Immunoblotting analysis of proteins in *N. benthamiana* leaves transiently expressing *VdEG1* and *VdEG3* in the suppression experiment using $EG3^{CBM1}$ or VdCBM1, Ponceau S-stained Rubisco protein is shown as a total protein loading control.
D. ROS accumulation was assessed in *N. benthamiana* leaves from 4-week-old plants expressing VdEG1, VdEG3, or $EG3^{GH12}$ 24 h after pre-infiltration with $EG3^{CBM1}$ or VdCBM1.
E. Electrolyte leakage was assessed in *N. benthamiana* leaves from 4-week-old plants 48 h after co-infiltration of VdEG1 or VdEG3 with $EG3^{CBM1}$ or VdCBM1.
F. Electrolyte leakage was assessed in *N. benthamiana* from 4-week-old plants expressing VdEG1 or VdEG3 24 h after pre-infiltration with $EG3^{CBM1}$ or VdCBM1.
G. Detection of the suppression activity of $EG3^{CBM1}$ or VdCBM1 against other fungal cell death-inducing GH12 proteins in *N. benthamiana* leaves from 4-week-old plants. *N. benthamiana* leaves were infiltrated with *A. tumefaciens* cells carrying the fungal GH12 genes 24 h after pre-infiltration with $EG3^{CBM1}$ or VdCBM1, and cell death induction was assessed 6 days after infiltration, GFP was used as a negative control.

VdEG3 after treatment with the CBM1 domain ($EG3^{CBM1}$) or an additional *V. dahliae* protein containing a single CBM1 domain (VDAG_00170, designated as VdCBM1). In co-expression experiments, $EG3^{CBM1}$ and VdCBM1 had the ability to suppress VdEG1- and VdEG3-triggered cell death in *N. benthamiana* (Fig. 5A). Twenty four hours after the expression of $EG3^{CBM1}$ or VdCBM1, the cell death response induced by VdEG1 or VdEG3 was completely suppressed (Fig. 5B). Immunoblotting analysis confirmed effective protein translation of VdEG1 or VdEG3 (Fig. 5C). In addition, co-infiltration or pre-infiltration of VdCBM1 also suppressed the cell death-inducing activity of $EG3^{GH12}$ (Supporting Information Fig. S5B and C). Correspondingly, the PTI responses of ROS accumulation and electrolyte leakage triggered by VdEG1 and VdEG3 were also significantly suppressed by $EG3^{CBM1}$ or VdCBM1 (Fig. 5D–F). These results confirmed that both $EG3^{CBM1}$ and VdCBM1 efficiently suppressed VdEG1- and VdEG3-triggered immunity in *N. benthamiana*.

To determine whether the CBM1 domain/protein could suppress the immune response triggered by GH12 proteins from different fungi, we selected a set of cell death-inducing GH12 proteins for further investigation. Among these, six GH12 proteins from other fungi showed elicitor activity to induce cell death in *N. benthamiana* 24 h after expression of control GFP (Fig. 5G; Supporting Information Supporting Information Table S3). However, the cell death triggered by all six GH12 proteins was effectively suppressed by $EG3^{CBM1}$ or VdCBM1 (Fig. 5G). These findings confirmed that CBM1-containing proteins could suppress the fungal GH12 protein-triggered cell death and PTI responses during host-fungal interactions.

Pathogenic function diversification of VdEG1 and VdEG3 during plant infection

To assess the pathogenic functions of *VdEG1* and *VdEG3*, we generated gene deletion strains by homologous recombination (Supporting Information Fig. S6A and B), and also reintroduced the wild-type and catalytic site-directed gene mutants into the corresponding target gene deletion strains for analysis. Positive transformants of target gene deletion and complementation were determined by Southern and Western blotting analysis respectively (Supporting Information Fig. S6C–E). Pathogenicity assays showed that deletion of either *VdEG1* or *VdEG3* resulted in enhanced virulence and fungal biomass development in *N. benthamiana*. Reintroduction of the wild-type or site-directed mutant genes into the corresponding deletion strains resulted in reduced virulence and significantly less development of fungal biomass (Fig. 6A and B). These results indicated that the virulence of *V. dahliae* in *N. benthamiana* is affected by the defense response triggered by VdEG1 or VdEG3. The virulence phenotypes appeared to be consistent with the reduction in intensity of PTI response for gene deletion or PTI response restoration for *VdEG1* and *VdEG3* complementation, indicating that VdEG1 and VdEG3 acted as PAMPs to trigger the PTI response, which then inhibited *V. dahliae* infection in *N. benthamiana*. The transcript levels of VdEG1 and VdEG3 during tobacco infection, were significantly up-regulated at 2–3 days after inoculation (Supporting Information Fig. S6F), suggesting that the PTI response was strongly activated during this period as a plant response to prevent spread of pathogen. VdEG1 and VdEG3 triggered the PTI response independent of enzymatic activity in *N. benthamiana*; therefore, complementary transformants carrying the wild-type genes (EC^{EG1} and EC^{EG3}) should display similar virulence to complementary transformants carrying the site-directed mutagenized gene (EC^{EG1SM} and EC^{EG3SM}). Unexpectedly, the fungal biomass of complementary transformants carrying the site-directed mutagenized gene was significantly less than that of transformants carrying the wild-type gene (Fig. 6B), even though the relative expression levels of the reintroduced genes were similar during the infection of *N. benthamiana*

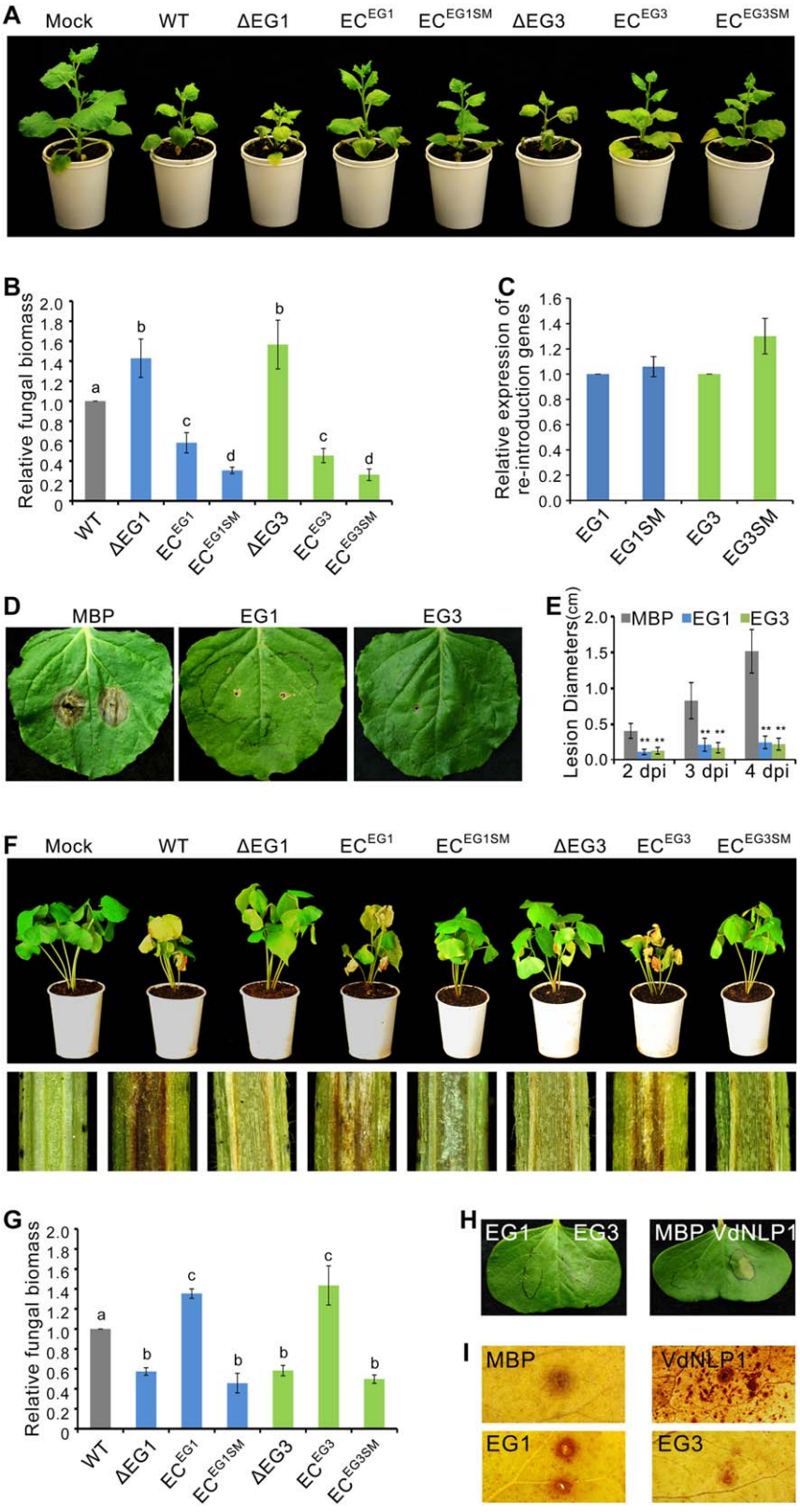

Fig. 6. Detection of the pathogenesis functional diversification of VdEG1 and VdEG3 during *N. benthamiana* and cotton infection.
A. Phenotypes of *N. benthamiana* plants inoculated with *VdEG1* and *VdEG3* gene deletion strains and corresponding complementary transformants by root-dip method. Five-week-old seedlings of *N. benthamiana* were inoculated with sterile water (Mock), wild-type *Verticillium dahliae* (WT), or *VdEG1* and *VdEG3* gene deletion strains. The virulence phenotypes were photographed 12 days postinoculation.
B. The fungal biomass of the gene deletion strain and complementary transformants on *N. benthamiana* were determined by qRT-PCR. Error bars represent standard errors. Columns with different letters represent statistical significance $P < 0.05$, according to unpaired Student's *t*-test.
C. The relative expression levels of reintroduced genes in the complementary transformants were determined by qRT-PCR. The infection samples of *N. benthamiana* shoots were collected 12 days postinoculation. Error bars represent standard errors. After RNA isolation and cDNA synthesis, qRT-PCR was performed to determine the relative expression levels of the wide-type (EG1 and EG3) and site-directed mutant genes (EG1SM and EG3SM) using *V. dahliae EF-1*α genes as endogenous controls.
D. Defense response induced by pretreatment with recombinant VdEG1 and VdEG3 proteins. *N. benthamiana* leaves from a 5-week-old plant were pre-treated with 100 nM of the indicated recombinant protein, and inoculated 12 h later with 5.0 μL of 2×10^6 conidia/ml *Botrytis cinerea*. Lesions symptoms were observed at 2 days postinoculation.
E. Lesion development of *B. cinerea* on *N. benthamiana* leaves was evaluated from 2 days postinoculation by determining the average lesion diameter on six leaves from six plants each. Error bars represent standard errors. ** significant differences ($P < 0.01$), according to unpaired Student's *t*-test.
F. Phenotypes of cotton seedlings inoculated with *VdEG1* and *VdEG3* gene deletion strains and complementary transformants. Two-week-old seedlings of susceptible cotton (cv. Junmian 1) were inoculated with sterile water (Mock), wild-type *V. dahliae* (WT), *VdEG1* and *VdEG3* gene deletion strains, and complementary transformants. The disease symptoms 3 weeks after inoculation are shown at the top, and the discoloration of the inoculation shoot longitudinal sections is shown at the bottom.
G. The fungal biomasses of the gene deletion strains and corresponding ectopic transformants on cotton were determined by qRT-PCR. Error bars represent standard errors. Columns with different letters indicate statistical significance ($P < 0.05$), according to unpaired Student's *t*-test.
H. Protein infiltration assays of the cell death-inducing activities of recombinant VdEG1 and VdEG3 proteins on cotton cv. Junmian 1 cotyledon leaves from 2-week-old plants 6 days after infiltration with recombinant protein. VdNLP1, which induces cell death in cotton leaves, was used as a positive control.
I. The ROS-inducing activities of the purified VdEG1 and VdEG3 proteins were determined in cotton cotyledons from 2-week-old seedlings. Purified VdNLP1 protein and the MBP tag were used as positive and negative controls respectively.

plants (Fig. 6C). These results suggest that the wild-type gene encoding VdEG1 and VdEG3 confers greater virulence than the site-directed mutagenized gene due to difference in enzymatic activity. The fact that the virulence of the complementary transformants was significantly less than that of the wide-type strain indicates host recognition of the PAMPs of VdEG1 and VdEG3 is important in minimizing the destructive enzymatic activities of these proteins in *N. benthamiana* (Fig. 6B). In addition, exogenous application of low concentrations of VdEG1 and VdEG3 onto the leaves of *N. benthamiana* resulted in significantly reduced disease severity and lesion diameter 48 h after inoculation with *B. cinerea* in VdEG1- or VdEG3-pretreaed leaves (Fig. 6D and E). These results suggested that VdEG1 and VdEG3 mainly act as PAMPs to elicit plant defense responses during *N. benthamiana* infection.

In cotton, the growth of *VdEG1* and *VdEG3* deletion mutants was significantly repressed in the vascular system (Supporting Information Fig. S7), resulting in reduced Verticillium wilt symptoms and fungal biomass development compared with the wild-type strain. Furthermore, highly virulent phenotypes were restored after reintroduction of the wild-type gene into the corresponding gene deletion mutant (Fig. 6F and G). However, reintroduction of the catalytic site-directed mutagenized gene ($EG1^{SM}$ and $EG3^{SM}$) could not restore the virulence on cotton, and also the fungal biomass was comparable to that observed in the VdEG1 or VdEG3 deletion strains (Fig. 6F and G). These results indicated that the enzymatic activities of VdEG1 and VdEG3 were required for full virulence in cotton. Further detection of the PAMP activity of recombinant VdEG1 and VdEG3 showed that neither caused necrosis on cotton cotyledons (Fig. 6H). In contrast to necrosis- and ethylene-inducing-like protein 1 (VdNLP1), ROS accumulation was not observed after treatment with VdEG1 or VdEG3 (Fig. 6I). Therefore, although the transcript levels of *VdEG1* and *VdEG3* showed significant accumulation during cotton infection (Supporting Information Fig. S6G), they did not activate the PTI response in cotton to prevent infection by *V. dahliae*. In contrast to *N. benthamiana*, *VdEG1* and *VdEG3* showed only a function in virulence by facilitating the infection of cotton (Fig. 6F and Supporting Information Fig. S7). These results suggested that VdEG1 and VdEG3 contributed to virulence on cotton due to their enzymatic activities, but did not act as PAMPs to induce the PTI response in cotton.

Discussion

Recent genomic and system-level studies have revealed a diversity of cell wall-degrading enzymes (CWDEs) in phytopathogenic fungi, suggesting that CWDEs play a critical role in pathogenicity (Kubicek *et al.*, 2014). Several CWDEs function as virulence factors in plant pathogens (Van Vu *et al.*, 2012; Zhang *et al.*, 2013; Nafisi *et al.*, 2014; Ma *et al.*, 2015b) in addition to acting as PAMPs during plant-pathogen interactions, if they are recognized by plant PRRs to trigger the PTI response (Enkerli *et al.*, 1999; Poinssot *et al.*, 2003; Zhang *et al.*, 2014; Ma *et al.*, 2015b). In this study, we identified two GH12 proteins in *V. dahliae*, VdEG1 and VdEG3, which induce cell death and PTI

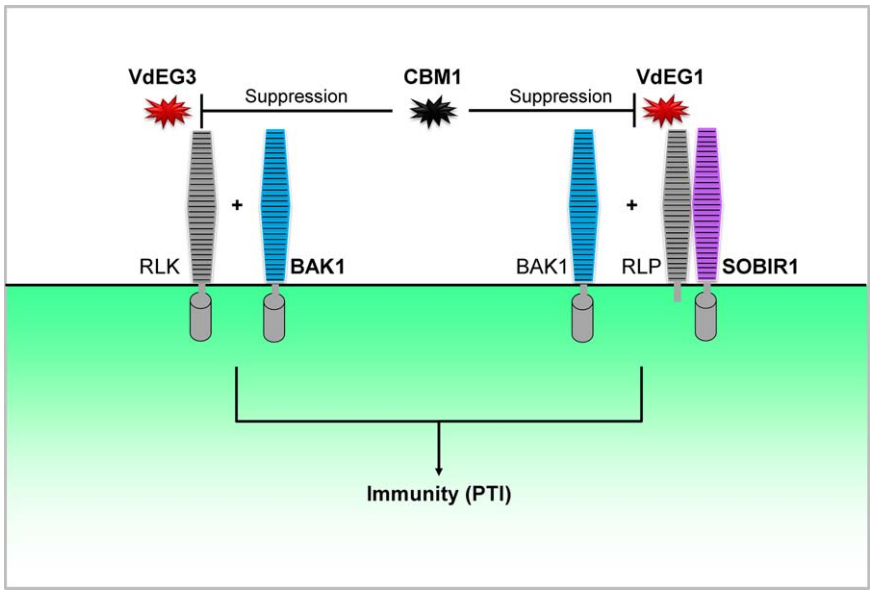

Fig. 7. Schematic overview of *Verticillium dahliae* manipulates plant immunity by VdEG1/VdEG3 in cooperation with CBM1 protein.
LRR-RLPs/SOBIR1/BAK1 and LRR-RLKs/BAK1 complex is required for VdEG1- and VdEG3-triggered immunity respectively. VdCBM1 can suppress the VdEG1- and VdEG3-induced cell death and PAMP-triggered immunity in *Nicotiana benthamiana*. VdEG1/VdEG3, the GH12 proteins in *V. dahliae*; CBM1, carbohydrate-binding module family 1; RLK: receptor-like kinase; RLP: receptor-like protein; BAK1: LRR-RLK BRI1-associated kinase-1; SOBIR1: LRR-receptor-like kinase suppressor of BIR1-1; PTI, PAMP-triggered immunity.

response independent of their enzymatic activity in *N. benthamiana*. Agroinfiltration of various VdEG3 deletion mutants showed that a small peptide from VdEG3 is sufficient for elicitor activity, but this was not the case for the corresponding peptide from VdEG1. VIGS assays showed that tobacco *BAK1* is required for VdEG1- and VdEG3-triggered cell death, but tobacco *SOBIR1* is specifically required for VdEG1-triggered cell death in *N. benthamiana*. These results suggested that VdEG1 and VdEG3 are perceived in two different ways to trigger immunity in *N. benthaniana*: through the LRR-RLPs/SOBIR1/BAK1 complex or through the LRR-RLKs/BAK1 complex (Fig. 7). To overcome plant defense responses, *V. dahliae* employed the CBM1 domain-containing protein to suppress cell death and PTI induced by GH12 proteins in *N. benthamiana* (Fig. 7). As expected, similar to the GH12 protein XEG1 in oomycetes (Ma *et al.*, 2015b), VdEG1 and VdEG3 in *V. dahliae* triggered immunity independent of their enzymatic activity. However, VdEG1 and VdEG3 triggered immunity through two different mechanisms and cooperated with novel effectors (CBM1-containing proteins) to manipulate immunity during *N. benthamiana* infection (Fig. 7).

Agroinfiltration is a versatile, rapid and simple technique that is widely used for the study of plant resistance and fungal avirulence (effector) genes in many plant species (Wroblewski *et al.*, 2005; Kanneganti *et al.*, 2007; Ma *et al.*, 2012), especially for the identification of fungal effectors that have cytotoxic activity or the ability to trigger the hypersensitive response. Previous studies of pathogen movement in plants showed that *Verticillium* spp. attack plants through the roots and can spread to the leaves through xylem vessels (Larsen *et al.*, 2007; Vallad and Subbarao, 2008). Compounds secreted by *V. dahliae*, a hemibiotrophic pathogen, causes vascular discoloration and foliar wilt symptoms (Fradin and Thomma, 2006). These studies suggest that agroinfiltration of plant leaves can be used to identify effectors in *V. dahliae*, even though roots are the natural point of entry for this pathogen. Several effectors secreted by *V. dahliae* have been identified through the employment of foliar agroinfiltration in tobacco, including Ave1 (de Jonge *et al.*, 2012), VdNLP1 and VdNLP2 (Zhou *et al.*, 2012; Santhanam *et al.*, 2013). In the current study, two GH12 proteins in *V. dahliae*, VdEG1 and VdEG3, displayed cell death-inducing activities independent of their enzymatic activity following agroinfiltration of *N. benthamiana* (Fig. 1A and C), suggesting that VdEG1 and VdEG3 trigger plant defense responses during *N. benthamiana* infection.

Plants activate immunity upon recognition of PAMPs to protect themselves from infection (Nurnberger *et al.*, 1994; Ausubel, 2005; Chinchilla *et al.*, 2007). During plant-pathogen interactions, some CWDEs, acting as PAMPs, are recognized by PRRs to trigger immunity (Enkerli *et al.*, 1999; Poinssot *et al.*, 2003; Zhang *et al.*, 2014; Ma *et al.*, 2015b). As classic PAMPs, VdEG1 and VdEG3 activate the PTI response independent of their enzymatic activities in *N. benthamiana* (Fig. 1C and F). Generally, most plant PRRs are plasma membrane-localized receptors with modular functional domains that recognize PAMPs outside of the plant plasma membrane (Monaghan and Zipfel, 2012). VdEG1 and VdEG3 were identified as apoplastic elicitors, as the signal peptide was required for cell death-inducing activity (Fig. 1E) and induced cell death was mediated by the plasma membrane-localized receptor BAK1 (Fig. 3A–C). In contrast, we also found that the recombinant VdEG1 and VdEG3 proteins, induced strong systemic resistance in *N. benthamiana*, which offered

protection against *B. cinerea* attack (Fig. 6D and E). Interestingly, further research showed that the gene deletion mutants exhibited increased virulence, and that constitutive expression of the gene in genetic complementary strains showed reduced virulence compared with the wild-type strains in *N. benthamiana* (Fig. 6A and B). Similar results have been obtained for other pathogen effectors (i.e., deletion enhances the virulence of the pathogen by preventing plant defense responses) (Li *et al.*, 2005; Lin and Martin, 2005; de Jonge *et al.*, 2012). For instance, deletion of the avirulence gene *Ave1* resulted in enhanced *V. dahliae* virulence on *Ve1* tomato (de Jonge *et al.*, 2012). Therefore, not only exogenous application of the purified elicitor, but also genetic evidence, indicated that VdEG1 and VdEG3 act as PAMPs to elicit strong resistance responses in *N. benthamiana*.

Proteinaceous PAMPs are often perceived by PRRs via specific epitopes. Thus, the elicitor activities of most PAMPs may be attributable to short amino acid sequences that are sufficient to stimulate immune responses (Nurnberger *et al.*, 1994; Felix *et al.*, 1999; Kunze *et al.*, 2004; Frias *et al.*, 2014; Oome *et al.*, 2014). Characterization of the elicitor sequences could lead to the development of special elicitors and facilitate their beneficial use in crop protection (Chen *et al.*, 2008; Che *et al.*, 2011). In this study, progressive truncation of the C- and N-termini of VdEG3 confirmed that a region of 63 consecutive amino acids was sufficient to trigger cell death and PTI in *N. benthamiana* (Fig. 2A and D), confirming that VdEG3 contains an epitope that is a typical PAMP, similar to flg22 (Felix *et al.*, 1999). However, the corresponding peptide of VdEG1 did not induce cell death in *N. benthamiana*, even with small bidirectional deletions (Fig. 2B and C).

Previous sequence analyses revealed several conserved residues in the GH12 protein family, including two conserved catalytic glutamic acid residues (Sandgren *et al.*, 2001), though GH12 proteins are also under diversifying selection pressure (Ma *et al.*, 2015b). Of the six GH12 proteins in *V. dahliae*, sequence analysis showed significant divergence in only a few conserved residues among VdEGs (Supporting Information Fig. S2). In comparison, the corresponding elicitor-active peptides of VdEG3 showed strong conservation among the functional GH12 proteins identified in this study. However, the most closely related peptide from MoEG (a *Magnaporthe oryzae* protein) could not induced cell death in *N. benthamiana* (Supporting Information Fig. S8A), similar to the second most conserved peptide from VdEG1 (Fig. 2B and C). It appears that the elicitor-active peptides of fungal GH12 proteins are under diversifying selective pressure, and that their cell death-inducing activity is dependent on variation in (rather than the conservation of) specific residues.

Plants rely on two types of PRRs (RLKs and RLPs) to detect PAMPs, and *BAK1* plays a general regulatory role in plasma membrane-associated receptor complexes comprising RLKs and/or RLPs (Liebrand *et al.*, 2014). VIGS assays confirmed that tobacco *BAK1* is required for VdEG1- and VdEG3-triggered cell death in *N. benthamiana* (Fig. 3A–C), indicating that *BAK1* mediates the detection of VdEG1 or VdEG3 by PRRs. However, progressive truncation experiments showed that a 63-amino acid peptide of VdEG3 was sufficient to trigger immunity, though the full-length VdEG1 was required for the elicitor function in *N. benthamiana* (Fig. 2). These results suggest that VdEG1 and VdEG3 may trigger cell death via different PRRs. Generally, PAMPs trigger immunity mediated by the plasma membrane-associated receptor complexes consisting of LRR-RLPs and/or LRR-RLKs, and SOBIR1 appears to be specifically required for the function of receptor complexes containing RLPs due to the lack of an intracellular signalling domain (Liebrand *et al.*, 2014). Interestingly, VdEG1 lost the ability to trigger cell death in *NbSOBIR1*-silenced plants (Fig. 3D–F). This suggests that *NbSOBIR1* is required for VdEG1-triggered immunity in *N. benthamiana*, similar to the case of Avr4, which is recognized by Cf-4 (Liebrand *et al.*, 2013). However, VdEG3-triggered cell death only required *BAK1*, suggesting that this is similar to the case of flg22 detected by FLAGELLIN-SENSING-2 (Boller and Felix, 2009). Therefore, we inferred that VdEG1 and VdEG3 act as PAMPs to trigger immunity in different ways: VdEG3-triggered immunity is mainly mediated by the LRR-RLKs/BAK1 complex, but the LRR-RLPs/SOBIR1/BAK1 complex is specifically required for VdEG1-triggered immunity in *N. benthaminana* (Fig. 7).

Successful pathogens deliver effector proteins to interfere with the host PTI response and establish infection (Jones and Dangl, 2006; Stergiopoulos and de Wit, 2009; de Jonge *et al.*, 2011). Several effectors have been identified as suppressors of the PTI response in different pathogens (Li *et al.*, 2005; Gimenez-Ibanez *et al.*, 2009; de Jonge *et al.*, 2010; Wang *et al.*, 2011). Oomycete pathogens are known to secrete several RXLR effectors to overcome XEG1-triggered immunity (Ma *et al.*, 2015b). However, typical RXLR motif proteins have not been found in fungal phytopathogens, although a few secreted proteins contain functional RXLR variants that could mediate their transduction into plant cells in the absence of the pathogen (Kale *et al.*, 2010; Rafiqi *et al.*, 2010; Plett *et al.*, 2011). VdEG3 contains a CBM1 domain in addition to the G12 domain, and loss of the CBM1 domain enhanced the cell death-inducing activity of VdEG3 in *N. benthamiana* (Fig. 4A and B). These results suggested that fungi probably utilize the CBM1 domain to suppress the PTI response during *N. benthamiana* infection. Several previous studies showed that the common functions of CBM1 domains involve promoting cellulase activity in fungi, or acting as elicitors in oomycetes (Gaulin *et al.*, 2006; Harris *et al.*, 2010; Beeson *et al.*, 2015). Fungal CBM1 proteins acting

as suppressors to manipulate plant immunity have seldom been reported. Our results suggest that the CBM1 domain ($EG3^{CBM1}$) and protein (VdCBM1) have the ability to suppress the cell death and PTI induced by VdEG1, VdEG3 and other GH12 proteins from different fungi (Fig. 5A,B,D and G). However, it is unclear whether this is due to a unique feature of the CBM1 domain in VdEG3 or VdCBM1, since the CBM1 domain is variable in other VdLs.17 genes except for a limited number of highly conserved residues (including four cysteine residues) (Supporting Information Fig. S8B). Interestingly, the cell death-inducing activities of VdEG1 and VdEG3 decreased when VdEG1 or VdEG3 were coupled with an increasing number of CBM1 domains (Fig. 4). This suggests that the effect of CBM1 on PAMP activity is dose-dependent. This hypothesis is partially supported by the outcome of pre- and co-infiltration of the CBM1 domain from VdEG3 or VdCBM1. The abundant accumulation of CBM1 following pre-infiltration had a significant effect on PAMP activity (Supporting Information Fig. S5B and C). Similar results have been obtained for the *C. fulvum* effector Ecp6, which sequesters chitin oligosaccharides in a dose-dependent manner to avoid eliciting host immunity (de Jonge *et al*., 2010). Therefore, we suggest that CBM1-containing proteins have the ability to suppress GH12 protein-triggered host immunity in a dose-dependent manner, facilitating the establishment of fungal infections in *N. benthamiana*.

This study indicated that VdEG1 and VdEG3 possess dual host-dependent biological activities, similar to EG1 and XEG1 (Zhang *et al*., 2014; Ma *et al*., 2015b). VdEG1 and VdEG3 do not trigger the PTI response in cotton, however (Fig. 6H and I). Targeted gene deletion of *VdEG1* and *VdEG3* significantly reduced the virulence of *V. dahliae* on cotton, and reintroduction of wild-type genes restored virulence in the gene deletion strains, but not for catalytic site-directed mutagenized gene mutants (Fig. 6F and G). This confirmed that VdEG1 and VdEG3 play key roles in pathogenicity and their enzymatic activity is required for *V. dahliae* virulence on cotton. Conversely, VdEG1 and VdEG3 act as PAMPs during *N. benthamiana* infection, as reduced virulence occurred after reintroducing the wild-type and site-directed mutagenized genes into the corresponding target gene deletion strains (Fig. 6A and B). Evidence of dual biological activities was also seen in the expression profiles of VdEG1 and VdEG3 in cotton and *N. benthamiana* plants. At key intervals, the transcript levels of VdEG1 and VdEG3 were significantly upregulated during cotton infection (Supporting Information Fig. S6G), resulting in enhanced *V. dahliae* virulence, with both genes acting as virulence factors. In *N. benthaminana*, however, the significant accumulation of VdEG1 and VdEG3 transcripts (Supporting Information Fig. S6F) corresponded to the elicitation of PTI, resulting in reduced infectivity of *V. dahliae*. Although VdEG1 and VdEG3 still functioned as enzymatic virulence factors (the complementary strains carrying the wild-type genes were more virulent than the catalytic residue mutagenized gene complementary strains (Fig. 6A and B), the virulence was attenuated due to VdEG1 and VdEG3 triggering defense response. These results suggested that VdEG1- and VdEG3-triggered immunity is the dominant effect during *N. benthamiana* infection, but *Gossypium hirsutum* cv. Junmian 1 lacked the receptors necessary for pathogen recognition and therefore displayed much more severe disease symptoms. We hypothesize that *V. dahliae* Vd991, a highly aggressive strain originally isolated from cotton, has evolved cotton-specific virulence factors and that the commercial cultivars susceptible to this strain lack recognition mechanisms that limit parasitic colonization. This was evident in the recombinant protein infiltration assays using cotton leaves, which showed no elicitation of cell-death in response to the GH12 domain containing proteins that triggered defense responses in *N. benthamiana*. Overall, these observations indicated that VdEG1 and VdEG3 showed host-dependent pathogenic function as either PAMPs or enzymatic activity virulence factors.

In conclusion, our results indicated that the *V. dahliae* GH12 proteins, VdEG1 and VdEG3, have two biological activities: cellulase activity required for virulence and elicitor activity that induces immunity during plant infection, depending on the host. The *V. dahliae* GH12 proteins, VdEG1 and VdEG3, appear to be perceived by different immune receptors in *N. benthamiana*. Meanwhile, *V. dahliae* secretes the CBM1 domain/protein to suppress GH12 protein-triggered immunity in plants. These results suggest that the vascular pathogen, *V. dahliae*, utilizes GH12 and CBM1 proteins cooperatively to manipulate the immunity of *N. benthamiana*.

Experimental procedures

Fungal culture, inoculation and pathogenicity assays

The *V. dahliae* strain Vd991 was cultured on potato dextrose agar medium or in liquid Czapek Dox medium for 7 d at 25°C. Cotton (*Gossypium hirsutum* cv. Junmian 1) and *N. benthamiana* were grown at 23°C and 27°C, respectively, in a greenhouse (14 h:10 h, day:night photoperiod). For inoculation with *V. dahliae*, 2-week-old cotton seedlings or 5-week-old *N. benthamiana* plants were inoculated with 5×10^6 conidia/ml or 1×10^6 conidia/ml, respectively, by the root-dip method (Zhou *et al*., 2013). Disease symptoms of inoculated plants were observed at 21 days postinoculation on cotton or 12 days postinoculation on *N. benthamiana*. Vascular discoloration of cotton was observed in longitudinal sections of the shoots 3 weeks after inoculation. Fungal biomass in cotton and *N. benthamiana* was determined as previously described (Santhanam *et al*., 2013). Real-time quantitative PCR (qPCR) was performed using a qPCR SYBR premix Ex Taq II kit (TaKaRa, Kyoto, Japan) with the primers listed in Supporting Information Table S4. *V. dahliae* elongation factor 1-α (*EF-1α*)

was used to quantify fungal colonization. The cotton *18S* gene and *N. benthamiana EF-1α* were used as endogenous plant controls. The growth of all test strains during cotton infection was investigated by transmission electron microscopy (TEM) and scanning electron microscopy (SEM), as described previously (Li *et al*., 2006; Jin *et al*., 2011).

PVX plasmid construction and agroinfiltration assay

The tested genes were amplified from *V. dahliae* cDNA using the indicated primers (Supporting Information Table S4), including *VdEGs*, *VdCBM1*, truncated *VdEG1* and *VdEG3* genes, and the genes without their signal peptides ($EG1^{-SP}$ and $EG3^{-SP}$ respectively). Site-directed mutagenesis of catalytic residues of *VdEG1* ($EG1^{SM}$, E137A/E223A) and *VdEG3* ($EG3^{SM}$, E146A/E232A) was performed using a Fast Mutagenesis System Kit (TransGen, Beijing, China). Chimeric VdEG1 and VdEG3 coupled with CBM1 were obtained by synthesis. All sequences were cloned separately into the PVX vector pGR107 and transformed into the *Agrobacterium tumefaciens* strain GV3101. Agroinfiltration assays were performed on *N. benthamiana* plants using the Bcl-2-associated X protein (BAX) and green fluorescent protein (GFP) as positive and negative controls respectively. To examine the suppression of cell death induction, *A. tumefaciens* cells carrying *VdEG1* and *VdEG3* were co-infiltrated or infiltrated 24 h after infiltration of pGR107:$EG3^{CBM1}$ or pGR107:VdCBM1. Symptom development was monitored at 3 d in a time-course experiment until 6 days post-infiltration (dpi). Each assay was performed on six leaves from three individual plants, and repeated at least three times. Total proteins were extracted using a P-PER Plant Protein Extraction Kit and Protease Inhibitor Cocktail Kit (Thermo Scientific, Waltham, MA, USA) from agroinfiltrated *N. benthamiana* leaves 60 h after infiltration. Transient protein expression in *N. benthamiana* was assessed using anti-FLAG antibodies (Sigma-Aldrich, St. Louis, MO, USA).

Yeast signal sequence trap system

Functional validation of the predicted signal peptide was performed as described previously (Jacobs *et al*., 1997). The predicted signal peptide sequences of *VdEG1* and *VdEG3* were fused in-frame to the secretion-defective invertase gene in the vector pSUC2. The resulting plasmids, pSUC2:$EG1^{SP}$ and pSUC2:$EG3^{SP}$, were transformed into the yeast strain YTK12 and screened on CMD-W (lacking tryptophan) medium. Positive clones were confirmed by PCR using vector-specific primers (Supporting Information Table S4). The positive transformants were incubated on YPRAA medium (2% raffinose). YTK12 transformed with pSUC2:$Avr1b^{SP}$ or an empty pSUC2 vector were used as positive and negative controls respectively.

Recombinant protein purification and enzyme activity assays

The wild-type genes (*VdEG1* and *VdEG3*) and site-directed mutagenized genes ($EG1^{SM}$ and $EG3^{SM}$) were cloned into the pMAL-2 vector with a maltose-binding protein (MBP) tag fused in-frame to the N-terminus. After transformation into *Escherichia coli* BL21(DE3), recombinant protein expression was induced by adding IPTG to a final concentration of 0.3 mM. The cells were lysed by freezing and thawing, followed by sonication, and the recombinant protein was purified with elution buffer (column buffer supplemented with 10 mM maltose) on a 5-ml amylose column MBPTrap™ HP (GE Healthcare Life Sciences, Issaquah, WA, USA). Enzyme activities were measured as described previously using CMC (C4888; Sigma, St. Louis, MO, USA) as the substrate (Ma *et al*., 2015a).

Virus-induced gene silencing (VIGS) in N. Benthamiana

Virus-induced gene silencing (VIGS) was assayed based on recombinant tobacco rattle virus (TRV) as described previously (Liu *et al*., 2002). The plasmid constructs pTRV1, pTRV2:BAK1, or pTRV2:SOBIR1 were introduced into *A. tumefaciens*. The plasmid pTRV2:*GFP* was used as the control. The silencing efficiency of *NbBAK1* or *NbSOBIR1* was validated by quantitative reverse transcription PCR (qRT-PCR). The experiment was performed three times, using five plants for each TRV construct.

Elicitor activity assay

The elicitor activity was detected by infiltration of recombinant wild-type and mutant proteins in *N. benthamiana*; the VdNLP1 and MBP were used as positive and negative controls respectively. Cytosolic Ca^{2+} was measured using the Ca^{2+}-sensitive fluorescent dye Fluo-4 acetoxymethyl ester (Fluo 4-AM) as described previously (Ma *et al*., 2015a). *Nicotiana tabacum* L. cv. Bright Yellow 2 (BY-2) cells were treated with 100 nM recombinant proteins at 25°C for 1 h. Fluorescence was detected using a fluorescence microscope (DM2500; Leica, Wetzlar, Germany), with an excitation filter of 340 – 380 nm and a barrier filter of 510 nm. The ROS generation in plant leaves was detected using 3'3-diaminobenzidine (DAB) solution as described previously (Bindschedler *et al*., 2006). Intracellular generation of ROS was performed as described previously (Ma *et al*., 2015a). Briefly, 1-ml aliquots of BY-2 cells were treated with 100 nM recombinant proteins and incubated for 3 h at 25°C and 150 rpm. The fluorescent probe 2',7'-dichlorofluorescein diacetate (DCFH-DA) (Beyotime, Haimen, China) was then added to the cells at a final concentration of 0.1 mM and incubated for 30 min at 37°C. The fluorescent signal was detected using a fluorescence microscope with an excitation filter of 450 – 490 nm and a barrier filter of 525 nm. Callose deposition was determined and counted under a fluorescence microscope using a UV filter. The *N. benthamiana* leaves were harvested 2 dpi with 100 nM recombinant proteins. Infiltrated leaf discs were destained with 75% ethanol at 37°C and then incubated in 150 mM phosphate buffer (pH 9.5) containing aniline blue (approximately 1% w/v; Sigma) for 2 h in the dark. Electrolyte leakage assays were performed as described previously (Oh *et al*., 2010). Ion conductivity was then measured using a conductivity meter with Probe LE703 (Mettler-Toledo, Shanghai, China).

Assays for suppression of Botrytis cinerea *infection by protein infiltration*

Whole leaves of 5-week-old *N. benthamiana* were infiltrated with 100 nM VdEG1 or VdEG3 recombinant protein. Aliquots of 5 μL of 2×10^6 conidia/ml *Botrytis cinerea* were placed on the infiltrated area 12 h after infiltration. The inoculated plants were placed in a light incubator at 25°C and 80% relative humidity. The diameter of the lesions was measured 48 h after inoculation. Infection of the plants was performed by inoculating three leaves per plant, which was repeated three times.

RNA extraction and qRT-PCR

The infection samples of cotton were prepared by the root-dip method (Liu *et al*., 2013). The treatment samples of *N. benthamiana* were collected 12 h after infiltration of recombinant VdEG1 or VdEG3 protein (100 nM). After isolation of total RNA, qRT-PCR was performed under the following conditions: an initial 95°C denaturation step for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The cotton *18S* gene, and the *N. benthamiana* and *V. dahliae EF-1α* genes were used as endogenous controls. Relative transcript levels among various samples were determined using the $2^{-\Delta\Delta CT}$ method, with three independent determinations (Livak and Schmittgen, 2001).

Generation of gene deletion mutants and complementation

The targeted gene deletion constructs were generated based on the method described previously (Liu *et al*., 2013). The fusion fragment containing two flanking sequences of the target gene and hygromycin resistance cassette was introduced into the binary vector pGKO2-Gateway. The wild-type and site-directed mutagenized genes fused to a Flag-tag were cloned into the donor vector pCT-HN for generation of complementation transformants by the *Agrobacterium*-mediated transformation method described previously (Liu *et al*., 2013). Positive gene deletion strains were verified by DNA blotting analysis using a DIG High Prime DNA Labeling and Detection Starter Kit II (Roche, Penzberg, Germany) and gene-specific DNA probes were amplified using the corresponding primers (Supporting Information Table S4). The effects of the reintroduced genes in the corresponding *V. dahliae* complementation strains were verified by Western blotting analysis using Bio-Rad Protein Assay Dye Reagent Concentrate (Cat. #500-0006; Bio-Rad, Hercules, CA, USA) and anti-FLAG (Abcam, Cambridge, UK) antibodies at 1:5,000 dilution.

Bioinformatics analysis

The Clustal X2 program was used for multiple sequence alignment of the GH12 proteins (Larkin *et al*., 2007). The secreted proteins were identified by four programs commonly used to identify protein localization, as described previously (Klosterman *et al*., 2011). Putative extracellular proteins containing a signal peptide but lacking transmembrane domains were identified as secreted proteins. Unpaired Student's *t*-tests was performed to determine statistical significance, and *P* values < 0.05 between two treatments groups were considered statistically significant.

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Conflict of Interest

The authors declare no conflict of interest.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Fig. S1. Alignment of six members of the GH12 protein family with *Verticillum dahliae* Vd991 and VdLs.17.

Fig. S2. Alignment of the GH12 protein family from the *Verticillum dahliae* Vd991.

Fig. S3. Detection of the enzymatic activities of VdEG1 and VdEG3.

Fig. S4. Detection of the electrolyte leakage and gene expression as defense responses.

Fig. S5. Analysis of the suppression activity of the CBM1 domain protein against the GH12 domain of VdEG3.

Fig. S6. Targeted gene deletion and complementation of VdEG1 and VdEG3, and their expression analysis during cotton infection.

Fig. S7. Investigation of the development of VdEG1 or VdEG3 deletion strains during infection on cotton by electron microscopy.
Fig. S8. Amino acid sequence alignment of elicitor-active peptides and CBM1 domain.
Table S1. Identification of a GH12 domain-containing protein family in *V. dahliae*.
Table S2. Predicted secretion of six GH12 domain-containing proteins in *V. dahliae*.
Table S3. Detection of the cell death-inducing activity of fungi GH12 domain-containing genes in this study.
Table S4. Information of primers used in this study.