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The novel GrCEP12 peptide from the plant-parasitic nematode *Globodera rostochiensis* suppresses flg22-mediated PTI

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Abbreviations: PTI, PAMP-triggered immunity; ROS, reactive oxygen species

The potato cyst nematode *Globodera rostochiensis* is a biotrophic pathogen that secretes effector proteins into host root cells to promote successful plant parasitism. In addition to the role in generating within root tissue the feeding cells essential for nematode development,[1] nematode secreted effectors are becoming recognized as suppressors of plant immunity.[2-4] Recently we reported that the effector ubiquitin carboxyl extension protein (GrUBCEP12) from *G. rostochiensis* is processed into free ubiquitin and a 12-amino acid GrCEP12 peptide in planta. Transgenic potato lines overexpressing the derived GrCEP12 peptide showed increased susceptibility to *G. rostochiensis* and to an unrelated bacterial pathogen *Streptomyces scabies*, suggesting that GrCEP12 has a role in suppressing host basal defense or possibly pathogen-associated molecular pattern (PAMP)-triggered immunity (PTI) during the parasitic interaction.[3] To determine if GrCEP12 functions as a PTI suppressor we evaluated whether GrCEP12 suppresses flg22-induced PTI responses in *Nicotiana benthamiana*. Interestingly, we found that transient expression of *GrCEP12* in *N. benthamiana* leaves suppressed reactive oxygen species (ROS) production and the induction of two PTI marker genes triggered by the bacterial PAMP flg22, providing direct evidence that GrCEP12 indeed has an activity in PTI suppression.

Plants are equipped with a robust immune system to defeat pathogen attack. The first layer of immunity involves the recognition of pathogen-associated molecular patterns (PAMPs) by surface-localized pattern recognition receptors and is referred to as PAMP-triggered immunity (PTI).[5] Perception of PAMPs such as bacterial flagellin or its derivative flg22 triggers numerous downstream responses, including production of reactive oxygen species (ROS), activation of mitogen-activated protein kinases, cell wall callose deposition and increased expression of defense-related genes.[6] Although the role of PTI in plant-nematode interactions remains to be elucidated, accumulating evidence suggests that plant-parasitic nematodes actively utilize their secreted effectors to manipulate PTI to enable successful infection. For example, overexpression of nematode effectors in host plants often resulted in increased susceptibility to nematode infection with some cases also to infection by other adapted plant pathogens.[3,4,7] The enhanced susceptibility to adapted pathogens is often a result of PTI suppression.[8-10] Transcriptome analysis also revealed that defense-related genes are repressed in nematode-induced feeding cells.[11-13] Most importantly, the calreticulin Mi-CRT effector from the root-knot nematode *Meloidogyne incognita* was recently shown to suppress PTI responses including callose deposition and defense gene expression mediated by the PAMP elf18,[4] providing the first direct evidence for a role of nematode effectors in PTI suppression. The GrCEP12 peptide released from *G. rostochiensis* during the parasitic interaction is a unique 12-amino acid peptide having no obvious similarity to any known peptides.[3] Interestingly, however, transgenic potato lines overexpressing *GrCEP12* showed enhanced susceptibility to *G. rostochiensis*, which is likely due to a function of GrCEP12 in PTI suppression during nematode parasitism. To evaluate this hypothesis, we used the *Agrobacterium*-mediated transient expression in *N. benthamiana* leaves to investigate whether GrCEP12 can suppress flg22-mediated PTI responses.

GrCEP12 Suppresses flg22-Mediated ROS Production in *N. benthamiana*

A ROS burst is one of the earliest PTI responses.[6] We therefore assessed whether GrCEP12 suppresses ROS production induced

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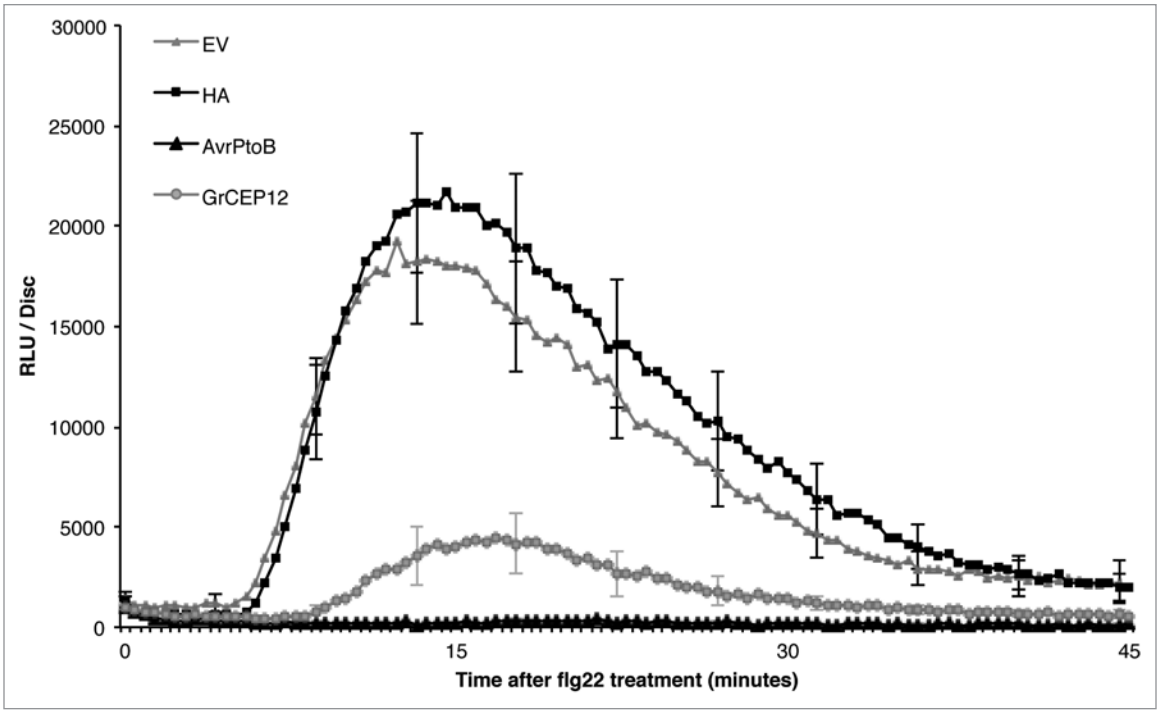

Figure 1. GrCEP12 suppresses flg22-mediated ROS production in *N. benthamiana*. *Agrobacterium tumefaciens* strain GV3101 carrying GrCEP12, AvrPtoB, HA or the empty vector (EV) construct was infiltrated into leaves of 3-wk-old *N. benthamiana* plants. Infiltrated leaf discs were collected 48 h post-agroinfiltration and assayed for ROS production as described.[14] Values indicated are average of relative luminescence units (RLUs) ± SD of 18–24 leaf discs. The experiment was repeated four times with similar results.

by flg22. *Agrobacterium tumefaciens* strain GV3101 carrying the GrCEP12 construct was infiltrated into *N. benthamiana* leaves. Forty-eight hours after infiltration, leaf discs were collected and then challenged with flg22 at a concentration of 100 nM. ROS production was measured using a luminol-based assay.[14] The bacterial effector AvrPtoB, a demonstrated suppressor of PTI,[15] as well as the empty vector (EV) and the construct expressing the 9-amino acid HA tag peptide[3] were included as positive and negative controls, respectively. We observed that, similarly to AvrPtoB, GrCEP12 dramatically suppressed flg22-induced ROS production in leaf discs when compared with the negative controls (**Fig. 1**).

GrCEP12 Suppresses flg22-Triggered Marker Gene Expression in *N. benthamiana*

One of the responses to PAMP perception is transcriptional reprogramming of plant cells.[6] *NbPti5* and *NbAcre31* genes have been demonstrated to be markers of PTI in *N. benthamiana*,[16] we therefore evaluated whether GrCEP12 affects the expression of these two marker genes after flg22 treatment. *A. tumefaciens* strain GV3101 carrying GrCEP12 was infiltrated into *N. benthamiana* leaves and leaf discs were collected 24 h post-agroinfiltration. After soaking overnight in water, leaf discs were incubated with 100 nM flg22 or distilled water for 30 min. mRNA was isolated from treated leaf tissue and gene expression was determined by quantitative RT-PCR.[3] In this assay, we also included AvrPtoB as well as the EV and the HA constructs as positive and negative controls, respectively. Both *NbPti5* and *NbAcre31* were upregulated in leaf tissue infiltrated with the EV or the HA construct after flg22 treatment (**Fig. 2**). In contrast, flg22-induced upregulation of *NbPti5* and *NbAcre31* was significantly reduced in GrCEP12 and AvrPtoB infiltrated leaf tissues as compared with those infiltrated with the negative controls (**Fig. 2**).

Taken together, our results clearly demonstrated an activity of the GrCEP12 peptide in PTI suppression. PTI appears to be conserved across the plant kingdom. Although no nematode PAMPs have been identified to date, it is possible that signaling pathways induced by the bacterial PAMP flg22 in *N. benthamiana* overlap with signaling pathways induced by an unknown nematode PAMP(s) in potato, a host of *G. rostochiensis*. It is therefore conceivable that GrCEP12 is a genuine suppressor of PTI during *G. rostochiensis* infection of potato, consistent with the increased nematode susceptibility observed in transgenic potato overexpressing *GrCEP12*. It is worth noting that, to our knowledge, GrCEP12 represents the smallest peptide effector from plant microbial pathogens showing an activity in PTI suppression. Identifying host plant target(s) of GrCEP12 may help uncover the mechanism of PTI suppression by GrCEP12 and provide insights into the role of PTI in plant-nematode interactions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

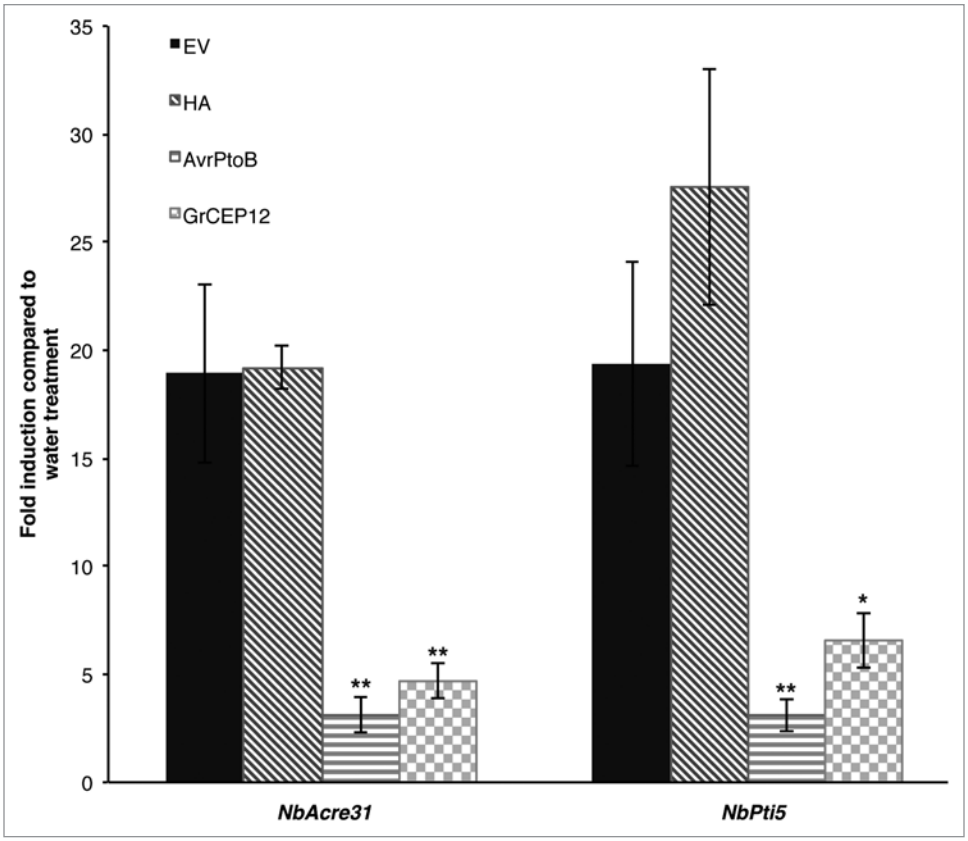

Figure 2. GrCEP12 suppresses flg22-induced expression of PTI marker genes in *N. benthamiana*. *N. benthamiana* leaf discs expressing GrCEP12, AvrPtoB, HA or the empty vector (EV) construct were collected 24 h post-agroinfiltration and soaked overnight in water. Leaf discs were then treated with 100 nM flg22 or distilled water for 30 min. mRNA was extracted from treated leaf discs and quantitative RT-PCR was conducted to determine the expression of *NbPti5* and *NbAcre31* genes in flg22-treated leaf discs relative to water-treated leaf discs. All samples were normalized against the reference gene *NbEF1*α.[17] Values are means ± SD of three independent experiments with three technical replicates of each. Asterisks indicate statistically significant difference compared with the EV control (*p < 0.05; **p < 0.01; Student's t-test).

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