Apoplastic Venom Allergen-like Proteins of Cyst Nematodes Modulate the Activation of Basal Plant Innate Immunity by Cell Surface Receptors

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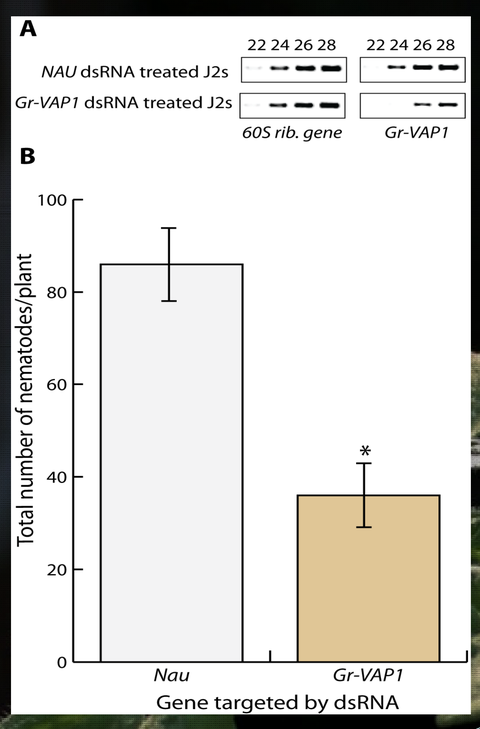
ABSTRACT

Despite causing considerable damage to host tissue during the onset of parasitism, nematodes establish remarkably persistent infections in both animals and plants. It is thought that an elaborate repertoire of effector proteins in nematode secretions suppresses damage-triggered immune responses of the host. However, the nature and mode of action of most immunomodulatory compounds in nematode secretions are not well understood. We have recently discovered that venom allergen-like proteins (VAPs) of plant-parasitic nematodes selectively suppress host immunity mediated by surface-localized immune receptors. VAPs are uniquely conserved in secretions of all animal- and plant-parasitic nematodes studied to date, but their role during the onset of parasitism has thus far remained elusive. Knocking-down the expression of *Gr-VAP1* severely hampered the infectivity of the potato cyst nematode *Globodera rostochiensis*. By contrast, heterologous expression of *Gr-VAP1* and *VAPs* from the beet cyst nematode *Heterodera schachtii* in plants resulted in the loss of basal immunity to multiple pathogens. The modulation of basal immunity by ectopic VAPs involves extracellular protease-based host defenses. Furthermore, the onset of programmed cell death was commonly suppressed by VAPs from *G. rostochiensis*, *H. schachtii*, and the root-knot nematode *Meloidogyne incognita*. Surprisingly, these VAPs only affected the programmed cell death mediated by surface-localized immune receptors. Furthermore, the delivery of VAPs into host tissue coincides with the enzymatic breakdown of plant cell walls by migratory nematodes. We, therefore, conclude that parasitic nematodes most likely utilize VAPs to suppress the activation of defenses by immunogenic breakdown products in damaged host tissue.

RESULTS

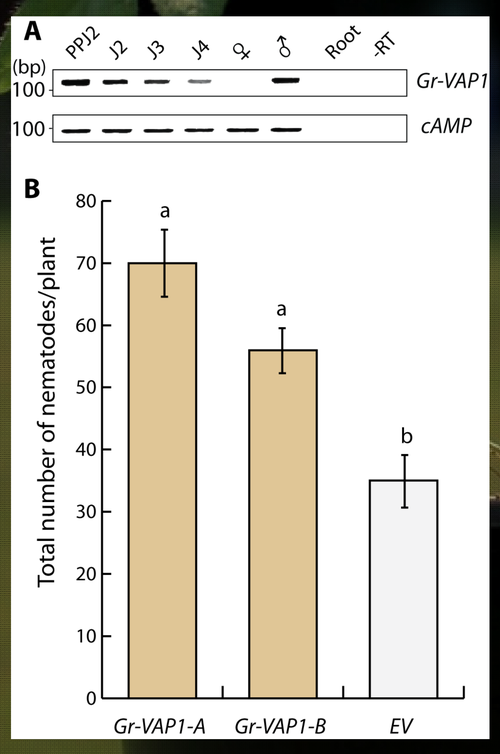
1. The venom allergen-like protein Gr-VAP1 is required for the onset of parasitism in host plants.

Fig. 1. **(A)** RNA interference specifically knocked down *Gr-VAP1* expression in pre-parasitic second stage juveniles of *G. rostochiensis*. Semi-quantitative reverse transcription-PCR of *Gr-VAP1* and a reference gene (*60S rib. gene*) in pre-parasitic second juveniles in double stranded RNA either matching the *Gr-VAP1* sequence or the sequence of the *NAU* gene of *Drosophila melanogaster* as control. Numbers indicate the cycles in the PCR. **(B)** The knockdown of *Gr-VAP1* expression significantly reduces the number of infective juveniles of *G. rostochiensis* inside roots of tomato plants (*S. lycopersicum*). Pre-parasitic second juveniles were either treated with double stranded RNA matching the *Gr-VAP1* or the *Nau* sequence. Bars represent standard error of mean of number of nematodes per plant at 7 days after inoculation over 10 replicates. Asterisk marks significance in a Student's t-test (P-value < 0.05).



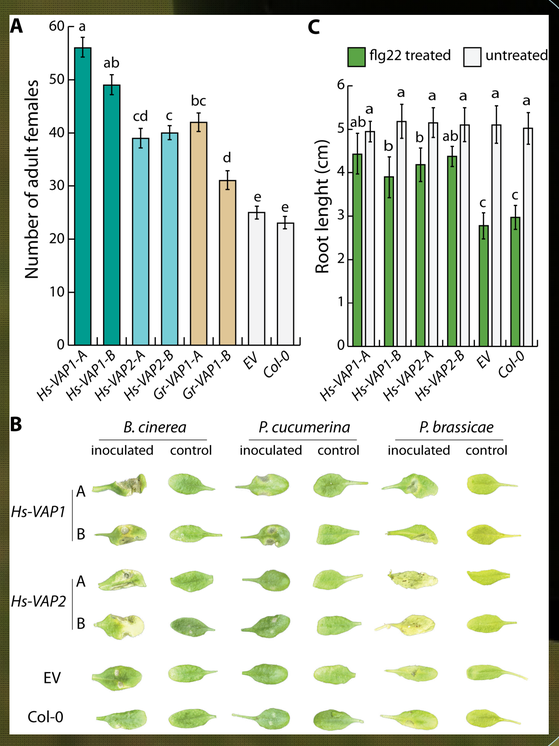
2. Apoplastic Gr-VAP1 suppresses immunity of potato plants to *G. rostochiensis*.

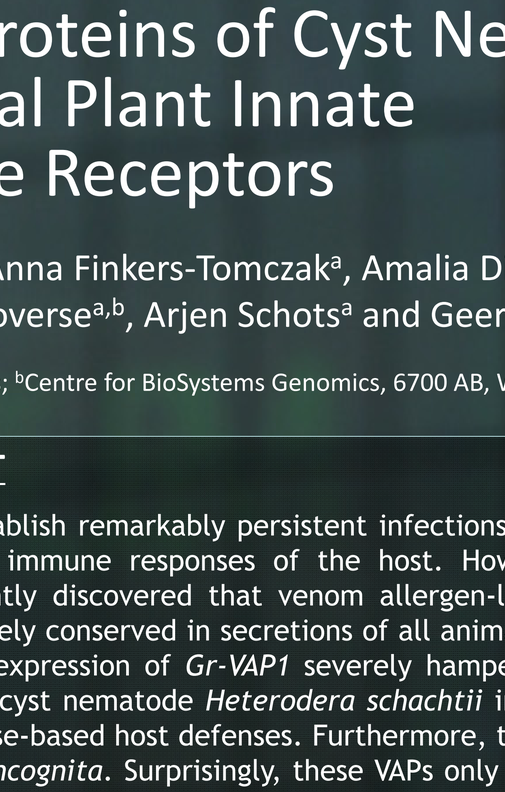
Fig. 2. **(A)** The expression of *Gr-VAP1*, as shown by semi-quantitative RT-PCR, is highly up-regulated in the migratory stages of *G. rostochiensis* (ppJ2, J2, and males (♂)), while it declines after initiation of the permanent feeding site in the sedentary juvenile stages (J3 and J4, and adult females (♀). Changes in expression of *Gr-VAP1* were assessed using the constitutively expressed *cAMP-dependent protein kinase* (*cAM*P) gene in *G. rostochiensis* as reference. Reactions using uninfected tomato roots as template (Root) and without reverse transcriptase (-RT) were included as controls. **(B)** Transgenic potato plants stably overexpressing *Gr-VAP1* in the apoplast show enhanced susceptibility to *G. rostochiensis*. The number of nematodes per plant was compared at 6 weeks post inoculation for two independent transgenic lines harboring either *Gr-VAP1* (*-A* and *-B*) or the corresponding empty vector (*EV*) line. Bars represent standard errors of the means. Different letters indicate statistically significant differences between plants (P-values < 0.05).



3. Ectopic venom allergen-like proteins suppress basal immunity in *Arabidopsis thaliana*.

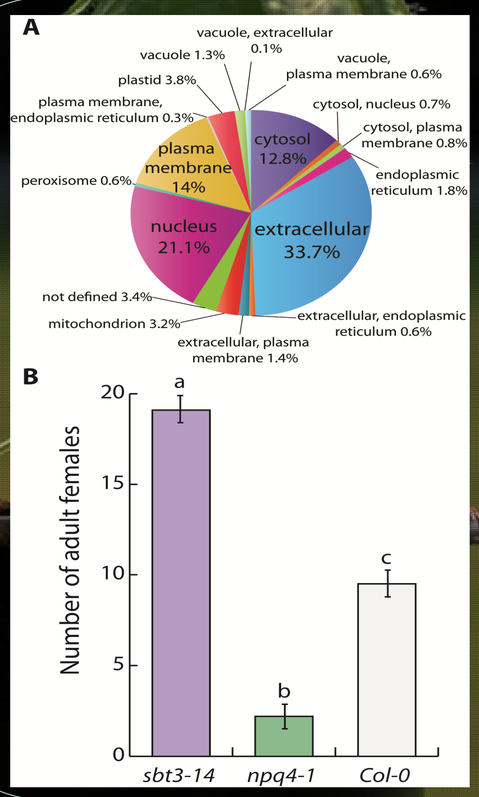
Fig. 3. **(A)** Heterologous expression of *Gr-VAP1* from *G. rostochiensis*, and *Hs-VAP1* and *Hs-VAP2* from *Heterodera schachtii* in the apoplast of transgenic Arabidopsis lines enhances their susceptibility to *H. schachtii*. Two independent lines per construct (*-A* and *-B*) were compared with corresponding transgenic empty vector (*EV*) line and wild type Col-0. Bars represent mean number of nematodes per plant with standard errors of the means. Different letters indicate statistical difference (*P*-value < 0.05). **(B)** Ectopic *Hs-VAP1* and *Hs-VAP2* enhance development of disease symptoms of pathogens in transgenic Arabidopsis. Symptoms on plants inoculated with *Botrytis cinerea*, *Plectosphaerella cucumeria*, and *Phytophthora brassicae*, or mock inoculated. **(C)** Ectopic *Hs-VAP1* and *Hs-VAP2* suppress seedling growth response of Arabidopsis to the immunogenic peptide flg22. Bars represent mean root length after 10 days in the presence or absence of 10μM flg22.





4. Ectopic venom allergen-like proteins from cyst and root-knot nematodes selectively suppress defense-related programmed cell death.

Fig. 4. **(A** and **B)** Agroinfiltration assays in *Nicotiana benthamiana* showing the transient co-expression in the apoplast of receptor-like proteins **(A)** *Cf-4* and **(B)** *Cf-9* from tomato and their cognate elicitors *Avr4* and *Avr9* from *C. fulvum* with venom allergen-like proteins from *G. rostochiensis* (*Gr-VAP1*), *H. schachtii* (*Hs-VAP1 and Hs-VAP2*), and *Meloidogyne incognita* (*Mi-VAP1*). Co-expressions with the corresponding empty binary vector (*EV*) and green fluorescent protein (*GFP*) were included as controls. Photographs were taken 7 days post infiltration. **(C** and **D)** The bars represent the mean number of events in which cell death suppression was observed for the **(C)** *Cf-4/Avr4* and the **(D)** *Cf-9/Avr9* combination in a total of 60 inoculation spots over 5 biological replicates (with standard error of the mean). Different letters indicate a significant difference (P-value < 0.05).



5. A plant cell wall-associated subtilase and non-photochemical quenching in chloroplasts regulate immunity to plant-parasitic nematodes.

Fig. 5. **(A)** Global gene expression analysis as determined by RNA-seq in 2 weeks old Arabidopsis plants overexpressing *Hs-VAP1* and *Hs-VAP2* in the apoplast. Pie chart depict percentage of products of genes significantly down-regulated by ectopic Hs-VAP1 and Hs-VAP2 in Arabidopsis, according to their predicted subcellular localization in the SUBA database. **(B)** The lack of the VAP-regulated subtilisin-like serine protease *AtSBT3.13* and the *chlorophyll-associated Photosystem II subunit S* protein in homozygous Arabidopsis mutants (*sbt3.14* and *npq4-1*, respectively) significantly alters their susceptibility to *H. schachtii*. Bars represent mean number of nematodes per plant with standard error of mean. Different letters indicate statistically significant differences between homozygous knockout mutants and corresponding wild type Arabidopsis at four weeks after inoculation (P-values < 0.05).

DISCUSSION

- Plant-parasitic nematodes deliver venom allergen-like proteins into the apoplast of host cells to suppress basal immunity mediated by surface-localized immune receptors.
- Venom allergen-like proteins may specifically suppress the immune responses triggered by plant cell wall fragments released by the enzymatic breakdown of plant cell walls during nematode migration inside host plants.
- The modulation of basal immunity by venom allergen-like proteins in plants most likely involves at least two different classes of extracellular proteases i.e. cysteine proteases and subtilisin-like serine proteases.
- Most of the genes differentially regulated by the overexpression of venom allergen-like proteins in Arabidopsis are typically associated with innate immunity and plant cell wall-associated processes.
- Ectopic Hs-VAP1 and Hs-VAP2 suppress innate immune responses in Arabidopsis, at least partly, through their regulation of PsbS which most likely reduces the formation of singlet oxygen under biotic stress

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