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The Root Lesion Nematode Effector *Ppen10370* Is Essential for Parasitism of *Pratylenchus penetrans*

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The root lesion nematode *Pratylenchus penetrans* is a migratory species that attacks a broad range of crops. Like other plant pathogens, *P. penetrans* deploys a battery of secreted protein effectors to manipulate plant hosts and induce disease. Although several candidate effectors of *P. penetrans* have been identified, detailed mechanisms of their functions and particularly their host targets remain largely unexplored. In this study, a repertoire of candidate genes encoding pioneer effectors of *P. penetrans* was amplified from mixed life stages of the nematode, and candidate effectors were cloned and subjected to transient expression in a heterologous host, *Nicotiana benthamiana*, using potato virus X–based gene vector. Among seven analyzed genes, the candidate effector designated as *Ppen10370* triggered pleiotropic phenotypes substantially different from those produced by wild type infection. Transcriptome analysis of plants expressing *Ppen10370* demonstrated that observed phenotypic changes were likely related to disruption of core biological processes in the plant due to effector-originated activities. Cross-species comparative analysis of *Ppen10370* identified homolog gene sequences in five other *Pratylenchus* species, and their transcripts were found to be localized specifically in the nematode esophageal glands by in situ hybridization. RNA silencing of the *Ppen10370* resulted in a significant reduction of nematode reproduction and development, demonstrating an important role of the esophageal gland effector for parasitism.

Keywords: nematode-plant interactions, nematode-induced plant genes, nematode effectors, transient expression using potato virus X (PVX)-based vector

Root lesion nematodes (RLN) (*Pratylenchus* spp.) are economically important pathogens that inflict significant damage and yield losses on a wide range of crops (Castillo and Vovlas 2007). *Pratylenchus penetrans* is one of the most important nematodes of the genus attacking nearly 400 species including high-value crops, grasses, and fruit trees (Castillo and Vovlas 2007). All stages of *P. penetrans*, except eggs and first-stage juveniles, are motile (Zunke 1990), and several generations of nematodes can develop during the life span of the crop. *Pratylenchus* spp. are migratory endoparasitic nematodes and their mobility throughout their life cycle causes massive damage to the root system, predisposing the roots to secondary infections by other soil-borne pathogens (Castillo and Vovlas 2007). As RLN migrate into the root of the host plant, they feed mainly from the cortex cells to gain access to the nutrients. Due to their different parasitic strategies, management approaches for controlling RLN are often ineffective and mostly rely on sanitation and the use of nematicides (Davis and MacGuidwin 2000).

Plant-parasitic nematode (PPN)-host interactions are mediated by a complex molecular dialogue, which involves the nematode secretion of different sets of proteins, called effectors (Vieira and Gleason 2019). These effectors are critical components of parasitism and are involved in all stages of nematode-host interactions. Like other PPN, *P. penetrans* effector genes are mainly expressed in the esophageal glands of the nematode and the corresponding proteins are ultimately secreted into the plant through a hollow stylet to manipulate host cell physiology and to promote infection (Vieira and Gleason 2019). Given the importance of RLN effectors in parasitism, their functional roles and mechanism of action are of particular interest for understating the molecular pathways of nematode-host interactions as well as for development of nematode control strategies.

As a first step toward elucidating the molecular basis of *P. penetrans* parasitism strategy, we have used bioinformatic analysis to identify a set of candidate effectors in the transcriptome of *P. penetrans*. Based on the presence of putative signal peptides, absence of transmembrane domains, and

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expression patterns during plant infection, we generated a catalog of predicted secreted proteins for this species (Vieira et al. 2015). Data mining of a transcriptome library originated specifically from the esophageal glands, coupled with in situ hybridization assays, validated a set of 23 candidate effector genes for *P. penetrans* (Vieira et al. 2018; Vicente et al. 2019). These encompassed a suite of genes encoding cell wall–degrading enzymes (CWDEs) and a pectin methylesterase gene, which had not been found in any nematode species prior to this study (Vicente et al. 2019). Nematode CWDEs are often implicated in the softening and degradation of the plant cell wall, allowing nematode penetration and migration within the host tissues (Smant et al. 1998). A few other genes included those frequently recognized as part of the nematode-host secretome, for instance fatty acid- and retinol-binding proteins, venom allergen-like proteins and an array of diverse classes of putatively secreted proteases involved in protection against host defenses (Vieira et al. 2018).

A prominent feature of this comparative analysis was the presence of a significant proportion of *P. penetrans* transcripts encoding putative proteins without any known function or annotation (Vieira et al. 2015). From this list of putative secreted proteins, a set of seven genes was confirmed to be expressed within the esophageal glands of *P. penetrans* (Vieira et al. 2018). The molecular mechanisms by which these effector-like proteins contribute to the parasitic behavior of the nematode and their host targets are still largely unknown (Vieira et al. 2015, 2018). Therefore, exploring the roles of these pioneer effectors of *P. penetrans* could provide an important view on the molecular mechanisms underlying RLN parasitism—a knowledge that can be useful for the development of new control measures against this resilient group of nematodes.

In this study, we cloned, sequenced, and characterized by transient expression methodology a set of seven pioneer candidate effectors of *P. penetrans*. The methodology relied on using potato virus X (PVX)-based vector, a well-established tool for high-throughput functional screening and rapid in-planta studies (Lacomme and Chapman 2008). Our main focus was to advance our knowledge on the functional roles of these pioneer effectors during parasitism of *P. penetrans*. As a result of this work, one of the core effector genes of *P. penetrans* and other *Pratylenchus* spp., designated as *Ppen10370*, was identified. Ectopic expression of *Ppen10370* in planta induced a pronounced stress response in the host, which included upregulation of a significant number of genes essential for both plant defense and for the prospective functions of the effector during parasitism. RNA-induced silencing of this gene resulted in a decreased number of nematodes recovered from infected carrot roots, suggesting its important role in the nematode parasitism.

RESULTS

Selection of *Pratylenchus penetrans* candidate effector genes and gene structure.

Our previous studies identified and validated 23 candidate effectors from *P. penetrans*, whose transcripts were specifically localized within the nematode esophageal glands (Vieira et al. 2018; Vicente et al. 2019). From this list, seven candidates were considered pioneer genes without any annotation or predictive function. These novel genes were selected for further analysis to expand their characterization and to reveal their functional roles in parasitism. The full-length genomic and cDNA sequences of each individual effector were cloned and the overall gene features were determined (Table 1). All genomic sequences revealed the presence of introns (1 to 3), with the exception of *Ppen11402* (Supplementary Fig. S1). Sequence analysis showed that the full-length cDNA clones encoded proteins with a predicted signal peptide consisting of 15 to 26 amino acid residues (Table 1), which is considered a key feature for secretion. This set of cDNAs encode for relatively small proteins, ranging from 74 to 176 amino acids (7.91 to 11.44 kDa). Noteworthy, five genes in this set display a high percentage of proline residues, which can represent up to 36.5% of the mature protein (Table 1).

Identification of homologs of candidate effector genes.

To identify potential homologs among other PPN (including different RLN species), each sequence was screened against publicly available transcriptomic and genomic datasets using BLAST analysis. Reciprocal BLASTp and tBLASTn searches allowed the identification of transcripts/genes with high identity against transcriptomes of other RLN, confirming the presence of potential homologs of at least four *P. penetrans* effector genes (Fig. 1A). No significant BLAST hits were obtained against nematodes of different genera or other organisms, suggesting that these effectors are specific to RLN.

Remarkably, significant BLAST hits against *Ppen10370* were found in all *Pratylenchus* species tested (i.e., *P. brachyurus*, *P. coffeae*, *P. thornei*, *P. neglectus*, and *P. zeae*). *Ppen10370* is part of a small family of related genes (about three sequences) in *P. penetrans*, with all of them coding for putative secreted proteins. With the exception of *P. zeae*, from which a partial transcript was recovered, all predicted proteins of the remaining RLN species possess a predicted signal peptide needed for secretion (Fig. 1B). On the basis of sequence homology analysis, the conserved amino acids between the predicted proteins among other RLN were mostly distributed at the C-terminal region, preceded by a putative predicted linker (Fig. 1B). PCR amplifications of the full-length coding sequence (CDS) of *P. neglectus* and *P. thornei* confirmed our *in silico* analysis. Since localization of transcripts in the secretory esophageal glands is often used to validate candidate effector genes with a potential role in parasitism, we investigated the localization of *P. neglectus* and *P. thornei* homologs of *Ppen10370* by in situ hybridization. Consistent with the results obtained for *Ppen10370* of *P. penetrans*, the localization of transcripts was specific to the secretory esophageal glands of *P. neglectus* and *P. thornei* (Fig. 1C). Taken together, the specific transcript localization within the esophageal glands and protein sequence similarity suggest that these effectors may share one or more similar functions in different RLN species.

Gene expression patterns of *P. penetrans* effectors during infection of different host plants.

To validate the expression profile of the seven pioneer genes during plant infection, transcript abundance was evaluated using available transcriptomic libraries of different plants infected with *P. penetrans* (Vieira et al. 2015; 2019). The analysis confirmed active transcription of this set of genes during nematode infection of alfalfa and soybean plants (Fig. 2A). To substantiate the *in silico* analysis, semiquantitative reverse transcription PCR (RT-PCR) assays were performed with independent cDNA libraries derived from nematode-infected alfalfa cv. Baker at 3 and 7 days after infection (DAI). Similar gene expression patterns were obtained for the same set of genes (Fig. 2B). These data illustrated that the temporal expression of these genes correlates with *P. penetrans* infection in roots of different host plants.

Induction of different phenotypes in planta during ectopic expression of *P. penetrans* candidate effectors.

To further dissect potential functions of the seven pioneer genes, we tested their ability to elicit phenotypic responses in the model plant *Nicotiana benthamiana* via transient expression

assay using a PVX-based vector. Each effector sequence encoding the mature protein (without the signal peptide) was amplified and cloned into the PVX-based vector (pP2C2S) (Chapman et al. 1992). For each assay, a minimum of three independent plants were tested and the PVX wild-type (WT, no insert) construct was used as a control. The severity and type of symptoms induced by the effectors varied (Fig. 3; Supplementary Fig. S2). The most pronounced phenotype occurred in plants inoculated with the recombinant virus expressing the *Ppen10370* effector gene, whose homologs were found in all *Pratylenchus* species described above. The inoculated plants were systemically infected and displayed evident vein necrosis

Table 1. Molecular characterization of the seven pioneer candidate genes of *Pratylenchus penetrans*, including their genomic and coding region features[a]

Candidate effector	Transcript length	5′ UTR	Coding sequence (nt)	3′ UTR	Poly-A	Genome (nt)	Protein (aa)	PI	MW (kDa)	Proline content[b] (%)
Ppen11402	456	26	240	177	Yes	240	79	10.58	8.79	7.8
Ppen8004	484	43	279	143	Yes	488	92	3.41	9.28	31
Ppen7984	405	13	225	156	Yes	303	74	11.44	7.91	36.5
Ppen16605	615	76	309	217	Yes	702	102	4.18	10.67	27.1
Ppen12016	663	52	390	205	Yes	476	129	9.86	12.97	24.8
Ppen10370	601	84	306	190	Yes	363	101	9.33	10.93	18.7
Ppen11230	746	6	531	209	Yes	747	176	10.94	19.63	8.6

[a] Genes are sorted based on the size of their respective predicted encoded proteins. UTR = untranslated region, PI = isoelectric point, and MW = molecular weight.
[b] In mature protein.

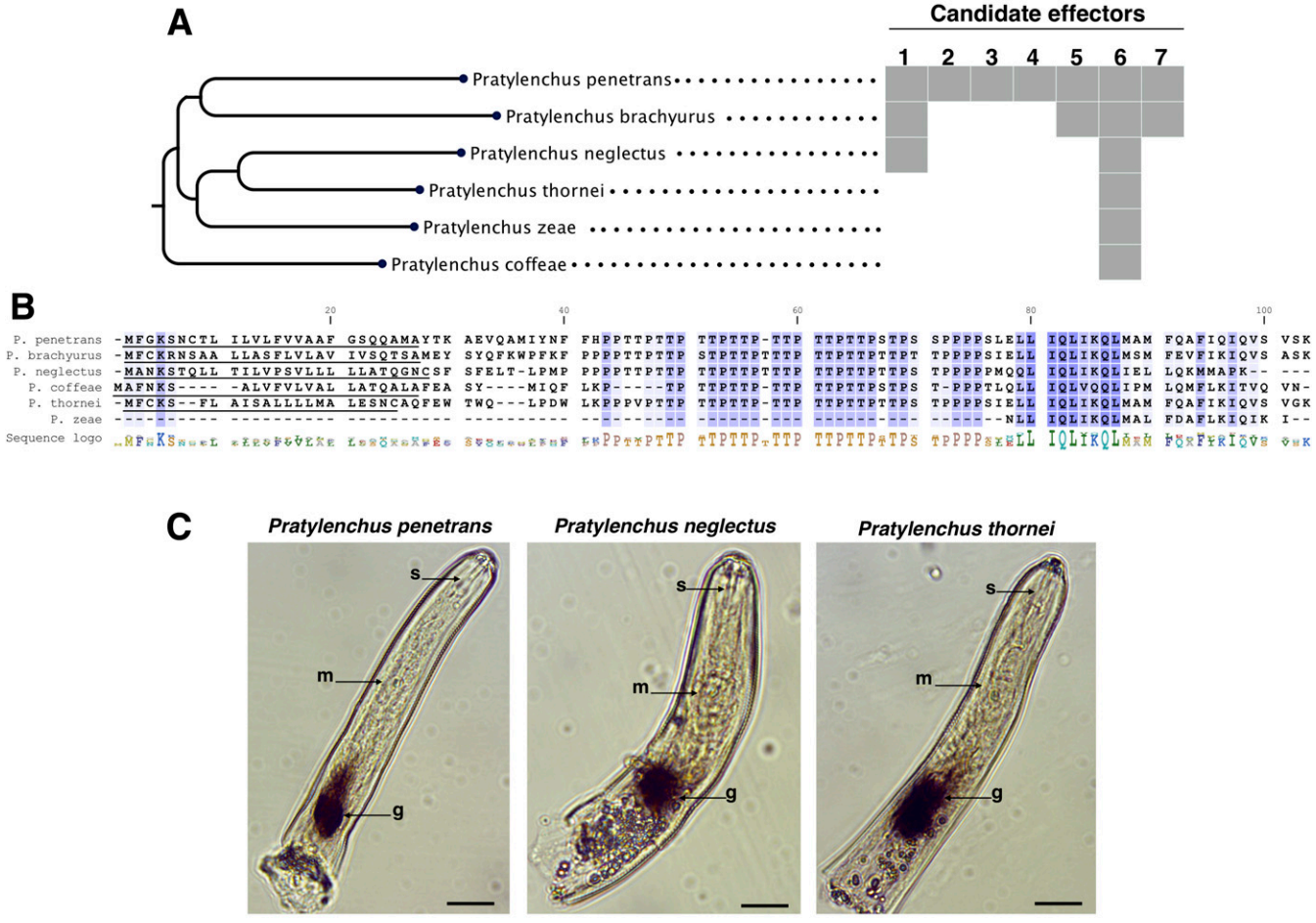

Fig. 1. Identification of homologs of candidate effector genes of *Pratylenchus penetrans* in other root lesion nematodes species. **A,** BLAST analysis against transcriptome datasets of other *Pratylenchus* species using *P. penetrans* effector candidates as query. The schematic phylogeny is based on the D2D3 region of the 28S rRNA gene: 1, *Ppen11402*; 2, *Ppen8004*; 3, *Ppen7984*; 4, *Ppen16605*; 5, *Ppen12016*; 6, *Ppen10370*; 7, *Ppen11230*. **B,** Amino acid sequence alignment of the Ppen10370 effector protein from *P. penetrans* and its putative homologs from other *Pratylenchus* species. The full-length predicted proteins were obtained from cloned coding sequences of *P. neglectus* and *P. thornei* or were predicted from the transcriptome assembly of *P. brachyurus* and *P. coffeae*. For *P. zeae* only a partial transcript was obtained. Predicted signal peptides are underlined. Conserved residues among species are indicated by dark blue shading, whereas similar residues are represented in light blue using a threshold for shading of 60% similarity. **C,** Detection of gene transcripts of *P. penetrans Ppen10370* and putative homologs of the gene from *P. neglectus* and *P. thornei* by in situ hybridization. Transcripts were detected in the nematode esophageal glands, using the corresponding antisense digoxigenin-labeled cDNA probes. g = esophageal gland, m = median bulb, and s = stylet. Scale bars = 20 µm.

on the leaves (Fig. 3A and B) and lesion-like areas on the petiole and stem of *N. benthamiana* (Fig. 3C). The plants appeared smaller in comparison with the ones infected with the empty PVX/WT vector. Expression of the *Ppen10370* gene was confirmed by RT-PCR with the effector-specific primers (Supplementary Fig. S3). Two other constructs (PVX/Ppen8004 and PVX/Ppen16605) induced small lesion-like symptoms on the leaves (Fig. 3D through I). Plants expressing the remaining genes displayed typical mosaic symptoms with dark light-green patches (Supplementary Fig. S2), similar to the phenotype induced by the PVX/WT (Fig. 3J through L). In planta expression of these nematode effectors was also confirmed by RT-PCR (Supplementary Fig. S4).

Considering that the most acute phenotypes were a result of PVX/Ppen10370 infection (Fig. 3A and B), expression assays with this construct were also performed in *N. tabacum* and tomato plants (*Lycopersicon esculentum*). In tomato plants, spot-like necrotic areas could be observed in systemically infected leaves expressing PVX/Ppen10370 (Supplementary Fig. S5A), while, in tobacco, systemically infected leaves displayed necrotic lesions (Supplementary Fig. S5B). Plants of both species inoculated with PVX/WT had typical PVX mosaic-like symptoms (Supplementary Fig. S5).

Subcellular localization of Ppen10370 in *N. benthamiana* leaves.

To gain insight into the subcellular localization of *Ppen10370*, the coding sequence of the gene without signal peptide was fused to the enhanced green fluorescent protein (Ppen10370ΔSP:eGFP) in the binary vector pK7FWG2.0 (Karimi et al. 2002) and transiently expressed in *N. benthamiana*, using agroinfiltration. Western blot assays confirmed the presence of the Ppen10370ΔSP:eGFP fusion protein in the agroinoculated leaves of *N. benthamiana* (Supplementary Fig. S6). The fusion protein was found to be localized in the endoplasmic reticulum (ER) network and surrounding the nucleus (Fig. 4A and B). Fluorescence of free eGFP used as a control was observed throughout the cytoplasm and the nucleus of *N. benthamiana* cells (Fig. 4C).

Differential gene expression analysis in plants transiently expressing PVX/Ppen10370.

To decipher a molecular basis of the *Ppen10370*-induced phenotypic abnormalities, we analyzed transcriptome responses in *N. benthamiana* plants infected with the recombinant PVX/Ppen10370 vector. For the RNA-sequencing (RNA-seq) analysis, noninoculated, systemically infected leaves showing symptoms described above were selected (Fig. 3A and B; Supplementary Fig. S3). Plants inoculated with PVX/WT were used as a control.

A total of around 268 million clean reads were generated from six RNA-seq libraries (three PVX/Ppen10370 and three PVX/WT [Supplementary Table S1]). The reads were mapped to the reference genome of PVX (GenBank number M72416.1) and to a publicly available genome of *N. benthamiana*. The average percentage of the total number of reads mapped to the corresponding genomes is shown in Supplementary Table S1. Differentially expressed genes (DEGs) unique to the PVX/Ppen10370-infected plants were depicted against the background of a wild-type PVX infection. In total, 1,086 DEGs with broad biological roles were identified in the PVX/Ppen10370-infected plants (Supplementary Table S2). Of this group, 721 DEGs were significantly upregulated (fold change > 2 and false discovery rate [FDR] < 0.05) and 365 DEGs were downregulated (Fig. 5A). A twofold higher prevalence of the upregulated genes indicated that the overall impact of *Ppen10370* lies in the activation of the host biological and cellular processes thus leading to the phenotypic changes observed in the infected plants.

Functional analyses of DEGs.

Upregulated DEGs. Gene ontology (GO) enrichment analysis of the upregulated DEG resulted in several overrepresented categories, including the most highly represented term 'response to stress' (GO:0006950) in the biological process class (Fig. 5B; Supplementary Table S3A). Several GO terms in the molecular function (MF) category were also significantly enriched (false discovery rate [FDR] < 0.05), namely, "transferase activity" (GO:

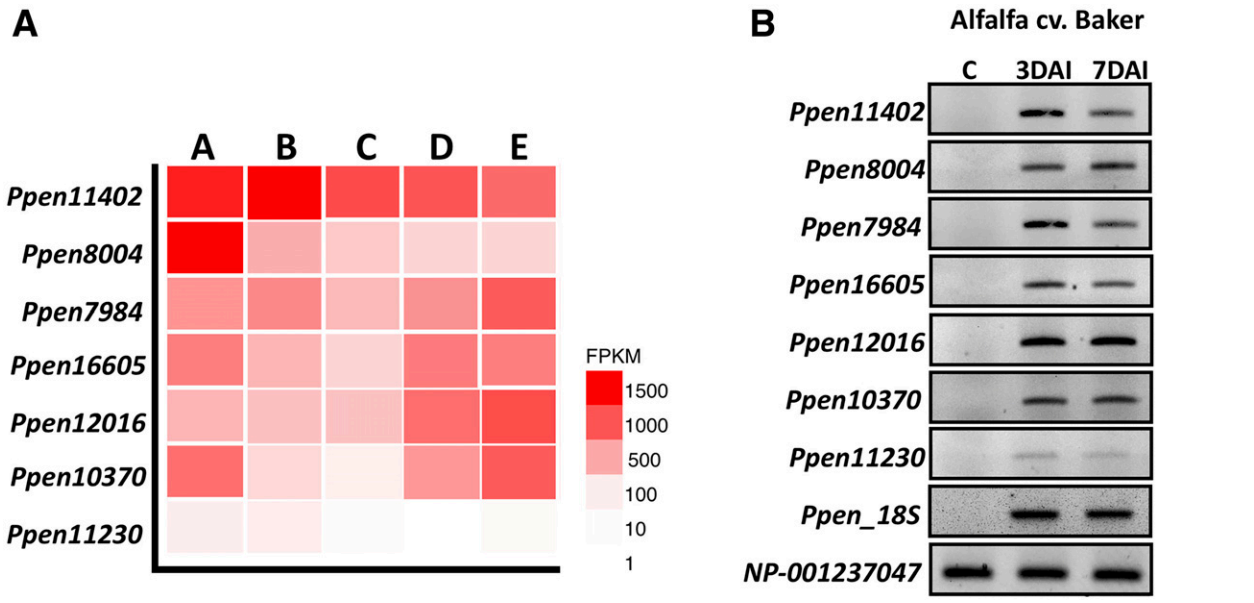

Fig. 2. Gene expression levels of *Pratylenchus penetrans* candidate effectors in planta. **A,** Expression levels were calculated using reads per kilobase of transcript per million mapped reads (FPKM) values for each effector gene, using public mRNA-seq datasets originating from **A,** nematodes only, **B and C,** soybean plants infected with *P. penetrans* at 3 and 7 days after infection (DAI), respectively (BioProject ID PRJNA304159), and **D and E,** two alfalfa cultivars, cv. Baker and MNGNR-16, respectively, infected with *P. penetrans* at 7 DAI (BioProject ID PRJNA547347). **B,** Semiquantitative reverse transcription PCR validating the expression levels of the different nematode effector genes from cDNA libraries derived from nematode-infected alfalfa roots (cv. Baker) collected at 3 and 7 DAI.

0016758), "endopeptidase activity" (GO:0004866), and "antioxidant activity" (GO:001 6209). In the cellular component class, apoplast (GO:0048046) was the only enriched GO term (Fig. 5C). To further delineate the mechanisms behind PVX/Ppen10370-induced phenotypes, we performed a manual sorting of those DEGs whose involvement may shed light on the functional activity of this particular nematode effector gene. Two most-relevant groups provisionally involved in i) host defense responses to the effector and ii) effector-originated functions and activities were apparent (Table 2). While the defense-related cluster included upregulated genes encoding proteins with well-known roles in stress response, such as NAC and WRKY transcription factors, pathogenesis-related (PR) proteins, chitinases, chloroplast proteins (Table 2), it also contained genes encoding proteins participating in detoxification, toxin removal and attenuation of oxidative stress, such as detoxification efflux carrier genes. The second group of upregulated genes presumably related to the functioning of the effector protein included 25 heat shock proteins (HSPs) and 15 ubiquitin-related proteins (URPs) exclusively upregulated in the *Ppen10370*-expressing plants. Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis reflected these observations indicating the importance of "protein processing in endoplasmic reticulum", "proteasome", and "antioxidant and detoxification-related" activities in host responses to the nematode effector (Fig. 6A; Supplementary Table S4A).

Downregulated DEGs. GO enrichment analysis of the downregulated DEGs emphasized the importance of several metabolic processes associated with the host reaction to this nematode

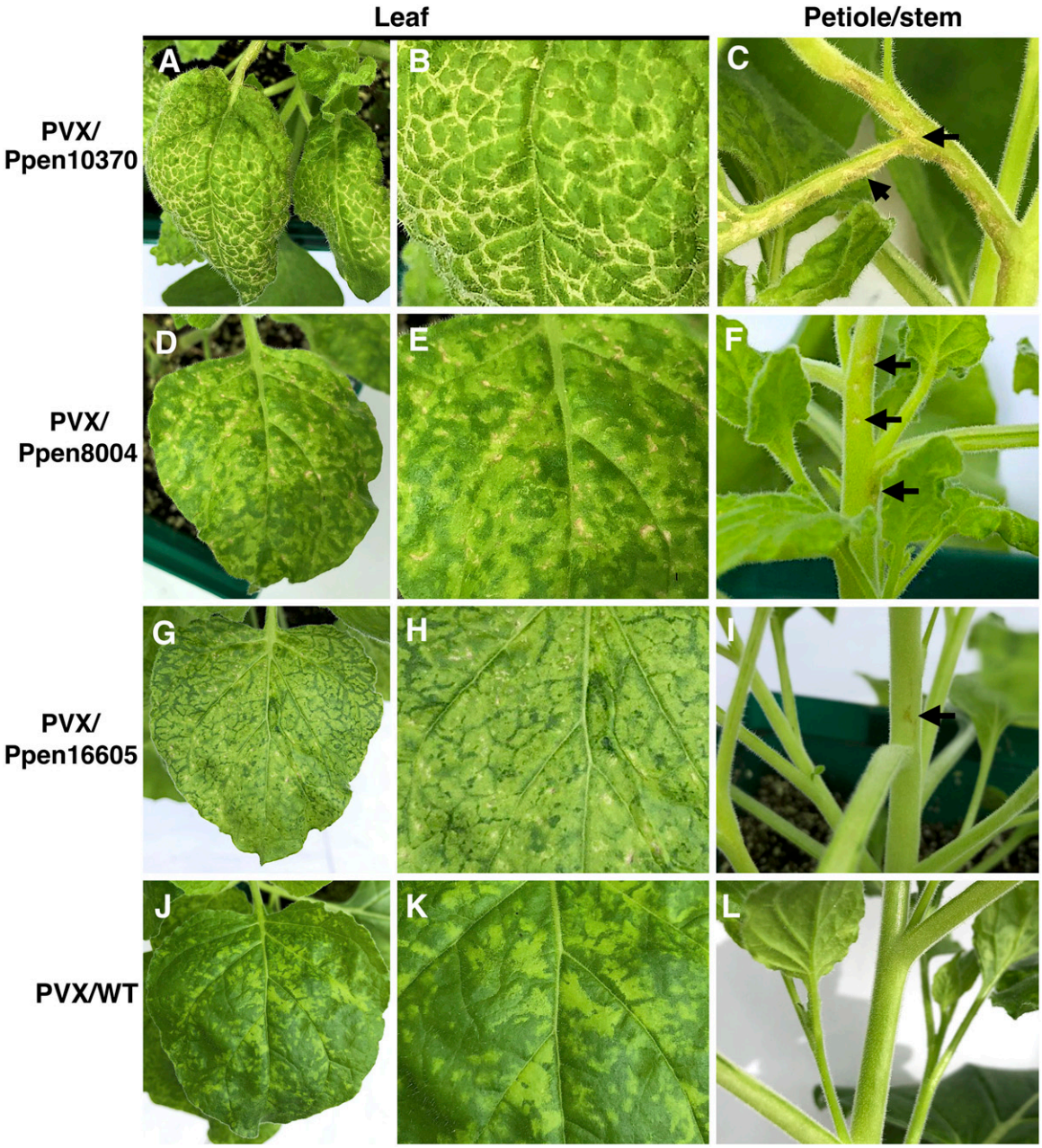

Fig. 3. Phenotypic changes in *Nicotiana benthamiana* plants induced by the infection with recombinant potato virus X (PVX) carrying genes encoding different candidate effectors of *Pratylenchus penetrans*. **A** to **C,** Expression of *Ppen10370* gene induced vein necrosis on leaves and necrosis on the petiole and stem of the infected plants (indicated by arrows). **D** to **F,** Expression of *Ppen8004* gene induced necrotic spots on leaves and stem of the infected plants (indicated by arrows). **G** to **I,** Expression of *Ppen16605* gene induced chlorosis and small-necrotic spots on leaves and lesion spots on the stem of the infected plants (indicated by arrows). **J** to **L,** Typical mosaic-like symptoms induced by PVX-based vector without insert (PVX/WT) on leaves of *N. benthamiana* plants. No lesion-like symptoms were observed. All photos were taken 14 days after inoculation.

effector gene, particularly genes in the MF domain associated with "glucosyltransferase activity" (GO:0035251) and "sucrose synthase activity" (GO:0016157) (Fig. 5B; Supplementary Table S3B). Individual sorting of the DEGs that could be redundant or unnoticed during GO and KEGG analyses resulted in the following groups of DEGs that are likely to be involved in response to the effector (Table 3). A large number of downregulated photosynthesis-related genes is consistent with the general effect of the virus infection on the host plants (Zhao et al. 2016), which appeared to be further enhanced by the expression of *Ppen10370*, leading to leaf chlorosis and extensive veinal necrosis. Other promising downregulated DEGs included several *PR* genes, such as *PR1* (Niben101Scf23606g00005.1) and *PR5* (Niben101Scf08044g00001.1), and leucine-rich repeat (LRR) receptor-like serine/threonine kinase genes. The outcome of KEGG analysis was more generalized, resulting in a large group of genes involved in main metabolic pathways (Fig. 6B; Supplementary Table S4B). DEGs involved in terpenoid backbone biosynthesis were also overrepresented in KEGG pathways. Terpenoids are important secondary metabolites with diverged functions in plant growth, development, and protection against environment (Pichersky and Raguso 2018).

Quantitative real-time PCR (qPCR) confirmation of the transcriptomic data.

Expression patterns identified by computational analysis of RNA-seq–generated data were further confirmed by qPCR assays with RNA extracted from PVX/Ppen10370 plants. A set of 25 genes was selected from the pathways most affected by the expression of the *Ppen10370* (Table 4). Overall, the results obtained by qPCR and RNA-seq were consistent and followed similar expression patterns.

Silencing of the *Ppen10370* gene hampered nematode development.

Based on the same reasoning that transient expression of *Ppen10370* led to the pronounced phenotypes in *N. benthamiana*, thus indicating its important role in the parasitism, we conducted a gene-silencing assay to directly assess its potential contribution to host parasitism. It was accomplished by soaking mixed nematode stages (juveniles and adults) in a solution containing a 212-bp double-stranded RNA (dsRNA) fragment targeting the *Ppen10370* gene. As control treatments, nematodes were soaked in a solution with a GFP-derived dsRNA fragment or in a buffer solution only. qPCR analyses showed a significant reduction of the relative transcript abundance (approximately 60%, $P < 0.05$) in nematodes silenced with the *Ppen10370* dsRNA fragment as compared with the expression levels in both control treatments (Fig. 7A). Soaking nematodes in dsRNA solution targeting the *Ppen10370* gene did not result in any changes in their movement or survival rates, as observed after 24 h of soaking. When *Ppen10370* dsRNA-treated nematodes were placed on carrot disks and allowed to develop for a period of 6 weeks, a significantly reduced number of nematodes ($P < 0.05$) was recovered from the carrot disks compared with the number of nematodes recovered from the control treatments (Fig. 7B), suggesting that this gene is required for successful establishment of parasitism.

DISCUSSION

In this work, we cloned the full-length protein-coding sequences of seven pioneer effectors based on their potential involvement in parasitism. An in-depth investigation into the biological functions of *Ppen10370*, which, based on the results of this work, were assumed to play important roles in parasitism, was performed by transient expression in a model host, utilizing phenotypical evaluation, high-throughput sequencing, differential gene expression analysis, and dsRNA silencing. As a result, potential biological roles of this specific candidate effector in parasitism were proposed and molecular mechanisms of their impact on host plants were revealed.

Recently, we applied a PVX-based transient expression approach for the characterization of different CWDEs that function as effector proteins of *P. penetrans* (Vicente et al. 2019; Vieira and Nemchinov 2020). As a result of that investigation, the biological roles of an expansin-like effector in nematode parasitism were proposed and the putative cellular targets of the protein were identified (Vieira and Nemchinov 2020). In continuation of this work, we have further explored this methodology toward deciphering functions of pioneer effector genes (i.e., without a putative function or annotation) of *P. penetrans*, which presently remain poorly understood. Of the seven candidate effectors screened in this work, expression of three effectors resulted in the induction of distinct phenotypes not observed in plants infected with PVX/WT. Based on the phenotypic observations, we proceeded to an in-depth characterization of the *Ppen10370* gene, which presented the most distinct phenotypes when ectopically expressed in planta.

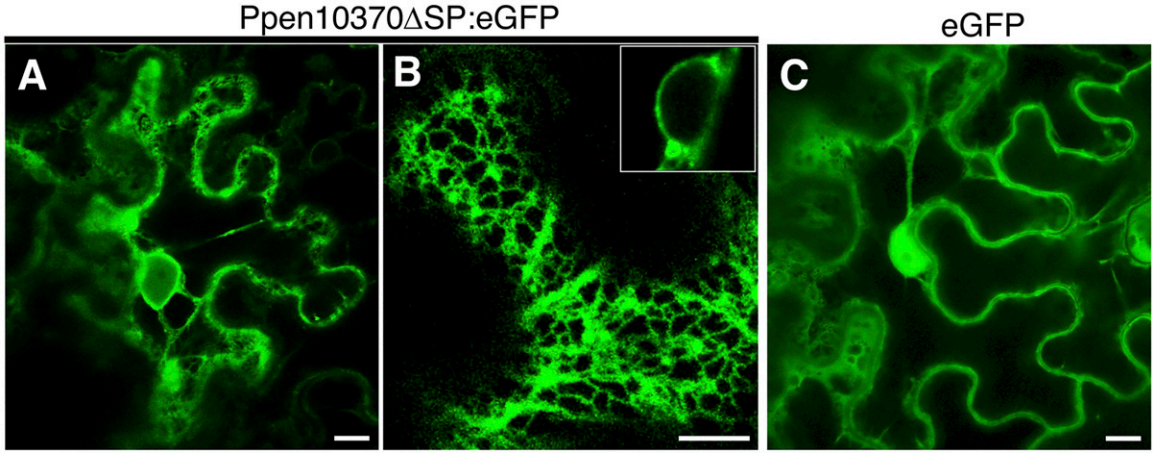

Fig. 4. Subcellular localization of Ppen10370 in *Nicotiana benthamiana* leaves. **A** and **B,** Fluorescent signal produced by the fusion Ppen10370ΔSP:eGFP protein and detected in the endoplasmic reticulum (ER) network and surrounding the nuclear membrane of *N. benthamiana* leaf cells. The insert in B shows ER around the nucleus. **C,** Localization of the free enhanced green fluorescent protein (eGFP) in the cytoplasm and nucleus of *N. benthamiana* leaves. For confocal laser scanning microscopy, leaf samples were taken 48 h postinoculation. Scale bar = 5 μm.

With the exception of RLN, the predicted protein encoded by the *Ppen10370* gene did not show any similarity to other proteins deposited in the publicly available datasets at the time of this investigation. *In silico* analysis of this particular gene resulted in the identification of additional homolog sequences in the available transcriptome and genomic data sets for five other RLN species. The occurrence of *Ppen10370* homologs in several species of the genus *Pratylenchus*, expression and localization of the gene transcripts within their secretory esophageal glands, and gene silencing results supported the idea that this gene might be a core feature related to the parasitic mode of life in this group of nematodes.

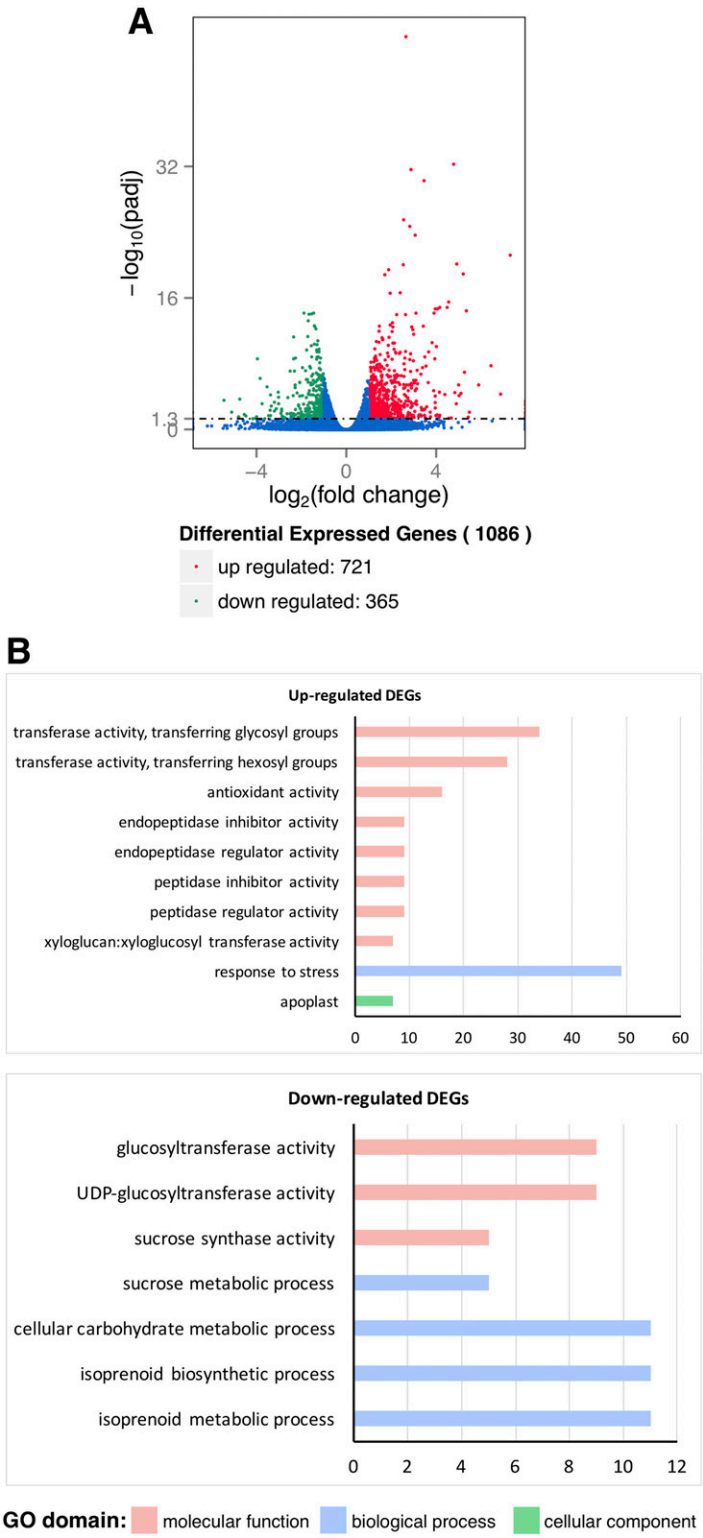

Fig. 5. Gene expression profiling of *Nicotiana benthamiana* plants inoculated with RNA transcripts derived from the PVX/Ppen10370 recombinant vector and from the PVX-based vector without insert (PVX/WT). **A,** Volcano plot representation shows the total number of differentially expressed genes (DEGs) significant between PVX/Ppen10370 and PVX/WT. **B**, Functional gene ontology (GO) enrichment analysis of DEG sets identified between plants inoculated with PVX/Ppen10370 and PVX/WT. The enriched GO terms (false discovery rate < 0.05) are plotted in relation to the number of DEGs.

Transient expression of *Ppen10370* in *N. benthamiana* plants followed by high-throughput sequencing and analysis of gene expression changes offered unique insights into the biological activity of this protein and suggested that *Ppen10370* could have toxic activities similar to those associated, for example, with Nep 1-like (necrosis and ethylene-inducing peptide1) proteins produced by many bacterial, fungal, and oomycete species (Ottmann et al. 2009). Interestingly, the Nep1-like protein, which is presumably involved in the interaction with Nep 1 as well as in a plant defense signaling pathway, was upregulated more than twofold in the PVX/*Ppen10370* plants (Supplementary Table S3).

The second group of upregulated genes related to the ectopic expression of *Ppen10370* included HSPs and URPs. It is noteworthy, that these genes were previously found to be upregulated during transient expression of another candidate effector from *P. penetrans*, *Pp-EXPB1* (Vieira and Nemchinov 2020). HSPs are responsible for protein folding, assembly, translocation, and degradation under stress and normal conditions (Park and Seo 2015). Twenty-five HSPs solely upregulated in *Ppen10370*-expressing plants suggested that a large-scale protein maintenance and stabilizing activity was induced by the expression of this nematode effector in plants. Although no supporting evidence could presently be found in the literature, hypothetically, the effector could initiate the same activity during *P. penetrans* parasitism to promote assembly and sustainment of the nematode-secreted proteins. Ubiquitin proteins play important roles in plant biology, not only related to protein modification and degradation systems but also to plant hormone signaling pathways (Bartel and Citovsky 2012). The ubiquitin-protein ligases that were found to be activated by *Ppen10370* in this study function as auxin and jasmonate receptors (Bartel and Citovsky 2012). Recently, the

Table 2. Selected proteins encoded by genes upregulated in response to transient expression of the *Ppen10370* effector gene of *Pratylenchus penetrans* in *Nicotiana benthamiana* plants

Name	Relevant functional roles	No. of genes
Heat shock proteins	Protein assembly and degradation (Park and Seo 2015)	25
Chloroplastic proteins	Photosynthesis; defense signaling (Nomura et al. 2012; Serrano et al. 2016)	22
NAC transcription factors	Plant defense (Nuruzzaman et al. 2013; Pandey and Somssich 2009)	15
Glutathione S-transferases	Detoxification; attenuation of oxidative stress (Gullner et al. 2018)	13
Ubiquitin-related proteins	Protein regulation and degradation; immune signaling (Varshavsky 2008; Marino et al. 2012)	12
WRKY transcription factors	Stress response (Pandey and Somssich 2009)	7
Receptor-like serine threonine kinases	Signal perception; pathogen recognition (Afzal et al. 2008)	7
Auxin-responsive proteins	Developmental processes; stress signaling (Kazan and Manners 2009)	6
Chitinase proteins	Plant defense; response to plant-parasitic nematode (PPN) (Grover 2012; Sato et al. 2019)	5
MYB transcription factors	Plant defense (Dubos et al. 2010)	4
Pathogenesis-related proteins	Defense responses (Breen et al. 2017)	4
Proteinase inhibitors	Resistance against PPN (McPherson and Harrison 2001)	4
Detoxification-related proteins	Removal of toxins; reduction of oxidative stress (Blokhina et al. 2003; Gillet et al. 2017)	4
ABC transporters	Plant growth and development; detoxification; defense (Hwang et al. 2016)	4
Resistance proteins	Resistance pathways (Jones and Dangl 2006; Takken et al. 2006)	1
Histone deacetylase 6	Regulation of gene expression; responses to stress (Vijayapalani et al. 2018)	1
Hypersensitive-induced reaction protein 4	Plant defense reactions (Li et al. 2019)	1
Nep1-interacting protein	Plant defense and cell death (Bailey 1995; Ottmann et al. 2009; Qutob et al. 2006; Zhang et al. 2012)	1

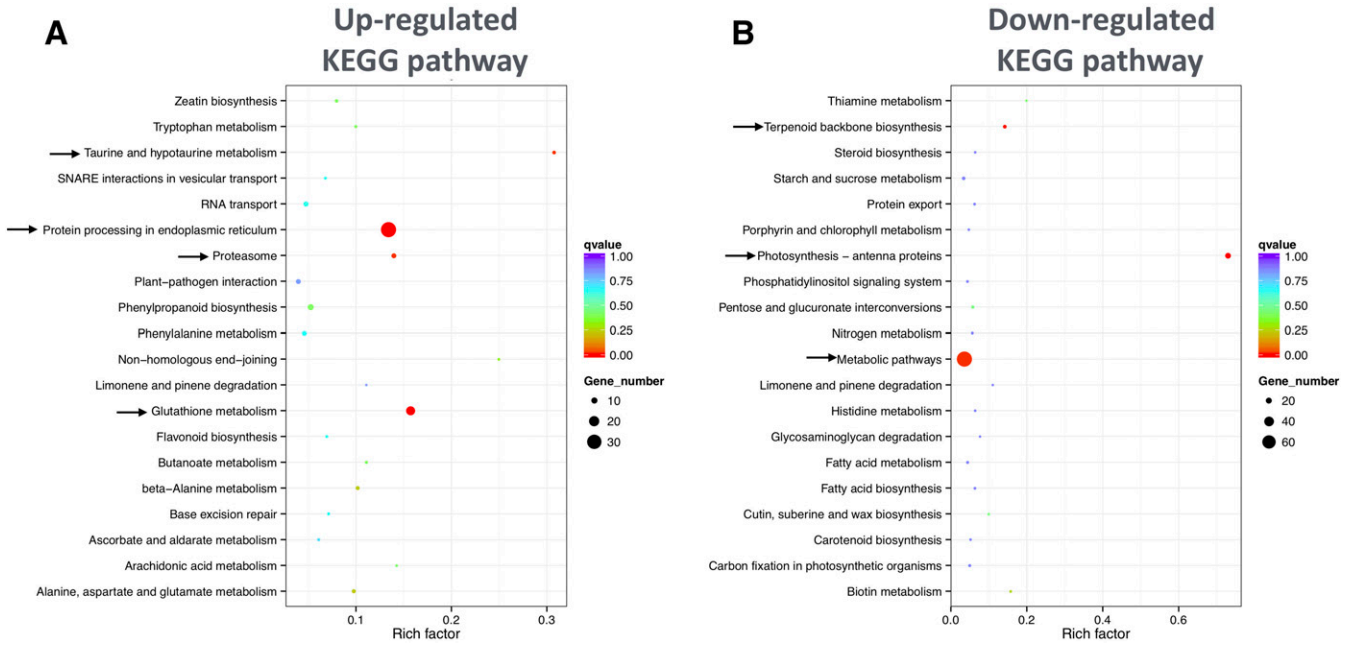

Fig. 6. Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment scatter plot analysis showing the total number of genes in **A,** the most represented upregulated and **B,** downregulated pathways. The colored dots correspond to the *q* value and the dot size indicates the number of differentially expressed genes mapped to the reference pathways. Arrows indicate the significantly enriched KEGG pathways (*q* values < 0.05).

potato cyst nematode effector RHA1B was shown to be a ubiquitin ligase that was able to manipulate the host ubiquitination pathway to promote parasitism by suppressing the plant immune system (Kud et al. 2019). Other reports suggest that pathogens use their effector proteins to hijack the host ubiquitin system or otherwise possess E3 activity to ubiquitinate host defense–related factors for degradation (Kud et al. 2019; Magori and Citovsky 2011; Üstün and Börnke 2014). Based on the number of highly upregulated ubiquitin-associated genes identified in this work, the *Ppen10370* mode of action during the parasitism of *P. penetrans* could potentially be related to one of these scenarios. Detection of the Ppen10370ΔSP:eGFP fusion protein in the ER of *N. benthamiana* cells partially supports this conclusion; ubiquitin modification systems might be involved in regulation and clearance of the ER-localized proteins (Guerra and Callis 2012).

Genes downregulated during *Ppen10370* expression are likely to be involved in the phenotypic characteristics of the infected plants and in the suppression of host defense reactions. Downregulation of chloroplast genes was previously recorded during overexpression of another RLN effector, an expansin-like protein Pp-EXPB1 in *N. benthamiana* plants (Vieira and Nemchinov 2020). A large-scale downregulation of photosynthesis-related genes may also constitute an adaptive response to biotic stress by means of redirecting host resources to immediate defense needs (Bilgin et al. 2010). Downregulation of a cluster of genes encoding LRR receptor-like serine/threonine kinases, one of the central components of the plant immune system and pathogen recognition (Afzal et al. 2008), could be in line with the potential *Ppen10370* activity related to ubiquitination of host defense–related factors for degradation. Defects in plant growth and appearance and venation patterning observed in PVX/Ppen10370-infected plants were likely due to the downregulation of several genes encoding auxin-related proteins. Decreased activities of cellulose synthases, integral membrane proteins whose sole function is production of the biopolymer cellulose, and polygalacturonases, pectin-modifying enzymes, indicate involvement in the processes of deconstruction of the plant cell wall.

In summary, based on the results obtained in this study and, particularly, on the gene expression analysis, we propose that function of the *Ppen13070* candidate effector protein is pertinent to the diversion of the host ubiquitin system to promote parasitism of *P. penetrans*. This function appears to be critical for the nematode. RNA silencing experiments demonstrated that knocking down this gene hampered nematode development. Therefore, although the detailed mechanisms of the *Ppen13070* action remain to be determined, our research provided an insight into its complex interaction with the host, which is likely aimed at manipulating host defense–related factors to ferry them for degradation. Validating the putative roles of the orthologs genes from other RLN could provide additional evidence on the importance of *Ppen13070* for parasitism across different species of this genus. The identification of the core effectors like *Ppen13070* that are critical for RLN parasitism is essential to achieve specific control measures against this group of plant-parasitic nematodes.

MATERIALS AND METHODS

Nematodes and plants.

Pratylenchus penetrans NL 10p RH collected in Beltsville, MD, U.S.A., was maintained and multiplied in vitro in roots of corn (*Zea mays* L. cv. Iochief) growing in Murashige and Skoog basal medium containing vitamins and 3% (wt/vol) sucrose (pH 5.8) that was solidified with 1.5% (wt/vol) agar (Sigma-Aldrich). Nematodes were extracted from infected corn roots as described by Vieira et al. (2015). *N. benthamiana* plants were grown in a greenhouse for 4 to 6 weeks at approximately 25°C with a photoperiod of 14 h of light and 10 h of dark.

Amplification and cloning of nematode candidate effectors.

Primers were designed for PCR amplification of both genomic DNA and complementary DNA (cDNA) of the predicted candidate effector sequences (Supplementary Table S5). DNA extraction was performed from mixed stages (eggs, juveniles, females, and males) of *P. penetrans*, using the PureLink genomic DNA mini kit (Invitrogen), following manufacturer instructions. Total RNA was extracted from mixed life stages of *P. penetrans* as well as *P. neglectus* and *P. thornei*, using the RNeasy plant mini kit (Qiagen), according to manufacturer instructions. RNA was then treated with RNase-Free DNase before RT. First-strand cDNA was synthesized using the iScript first-strand synthesis kit (Bio-Rad), following manufacturer instructions. Each PCR product was cloned into pCR II-TOPO vector (Thermo Fisher Scientific), followed by verification using automated Sanger sequencing in both directions (Macrogen Corp.).

Sequence analysis.

The cloned cDNA and genomic sequences were aligned using the MUSCLE tool (Edgar 2004), and gene schematics were generated with the Exon-Intron Graphic Maker (available online). The nucleotide and translated amino acid sequences were searched for homologs using BLAST analyses against the National Center for Biotechnology Information (NCBI) nonredundant nucleotide and protein databases. Sequence homology was also analyzed against publicly available nematode genomes (WormBase). Local tBLASTn searches were performed against the transcriptomes of *P. brachyurus*, *P. coffeae* (Haegeman et al. 2011), *P. neglectus*, *P. thornei*, *P. zeae* (Fosu-Nyarko et al. 2016). For *P. brachyurus* (BioProject PRJNA551772), *P. neglectus* and *P. thornei* (BioProject PRJNA512537 [Okubara et al. 2019]) de novo assemblies were generated using the corresponding raw reads deposited at the NCBI Sequence Read Archive (SRA). Protein sequence analysis was conducted using

Table 3. Selected proteins encoded by genes downregulated in response to transient expression of the *Pratylenchus penetrans Ppen10370* effector gene

Name	Relevant functional roles	No. of genes
Chloroplastic proteins	Photosynthesis-related; adaptation to biotic stress (Bilgin et al. 2010; Zhao et al. 2016)	66
Receptor-like serine/threonine kinase	Signal perception; pathogen recognition (Afzal et al. 2008)	14
Auxin-related proteins	Developmental processes; stress signaling (Ljung 2013)	6
bHLH transcription factors	Plant development, stress response; homeostasis, metabolism (Feller et al. 2011)	5
Cellulose synthase	Cellulose synthesis (Richmond 2000)	5
Sucrose synthase-like proteins	Disruption of sugar metabolism (Stein and Granot 2019)	4
Subtilisin-like proteases	Plant-pathogen recognition and immune priming (Figueiredo et al. 2014)	4
Polygalacturonase	Pectin-modifying enzymes (Hadfield and Bennett 1998)	4
QUIRKY proteins	Plant organ development and morphology (Fulton et al. 2009)	2
Sieve element occlusion protein	Wound sealing of the phloem (Ernst et al. 2012)	2

SignalP 4.0 for prediction of protein signal peptides (Petersen et al. 2011) and CLC Main Workbench (v.8.0) software for determination of the protein molecular mass and theoretical isoelectric points. Predicted functional domains were scanned using Pfam and InterproScan software packages (EMBL-EBI).

Differential expression of *P. penetrans* candidate effector genes.

Transcriptome data of *P. penetrans*–infected plants were extracted from the BioProjects PRJNA304159 and PRJNA547347 of the NCBI SRA (Vieira et al. 2015, 2019). The expression of the candidate effector genes among the different transcriptome libraries was calculated as reads per kilobase of transcript per million mapped reads values. Semiquantitative RT-PCR analyses were performed using total RNA extracted from the nematode-infected roots of alfalfa cv. Baker at 3 and 7 DAI, following the same methodology as described by Vicente et al. (2019). The following genes were used as references: *NP_001237047* for alfalfa (Postnikova et al. 2013) and *18S* rRNA gene for *P. penetrans* (Supplementary Table S5).

Table 4. Differential gene expression profile (log_2-fold change) for 25 selected genes of potato virus X (PVX)/Ppen10370 versus PVX/WT (wild type, no insert) infected leaves of *Nicotiana benthamiana*[a]

Gene_ID	Blast Swiss Prot	RNA-seq	qPCR
Niben101Scf09044g01012.1	P14170|OSMO_TOBAC Osmotin	5.11	6.89
Niben101Scf07491g00003.1	P08252|CHI1_TOBAC Endochitinase	4.07	5.2
Niben101Scf01015g01002.1	P09762|WIN2_SOLTU Wound-induced protein WIN2	3.56	4.12
Niben101Scf04053g02006.1	P11670|PRB1_TOBAC Basic form of pathogenesis-related protein 1	3.46	3.99
Niben101Scf02349g03002.1	P12437|PERX_SOLTU Suberization-associated anionic peroxidase	3.07	4.63
Niben101Scf08196g01001.1	P25317|GSTXA_TOBAC Probable glutathione S-transferase	2.97	3.51
Niben101Scf00046g02008.1	Q6NQK2|NAC8_ARATH NAC domain-containing protein 8	2.41	3.88
Niben101Scf05123g02015.1	Q8BKD6|R144B_MOUSE E3 ubiquitin-protein ligase	2.30	3.21
Niben101Scf05528g01009.1	Q94F47|UBC28_ARATH Ubiquitin-conjugating enzyme	2.26	4.95
Niben101Scf01294g05002.1	Q6R567|RMA1_CAPAN E3 ubiquitin-protein ligase	2.21	4.12
Niben101Scf01400g00014.1	P13046|PRR1_TOBAC Pathogenesis-related protein R major form	2.15	3.61
Niben101Scf01773g08011.1	Q6R567|RMA1_CAPAN E3 ubiquitin-protein ligase	1.95	4.76
Niben101Scf15364g00004.1	Q9CA23|UFM1_ARATH Ubiquitin-fold modifier 1	1.55	1.09
Niben101Scf04801g01004.1	Q8GYT9|SIS3_ARATH E3 ubiquitin-protein ligase	1.42	7.50
Niben101Scf01681g07003.1	F4JKK0|SUD1_ARATH Probable E3 ubiquitin ligase	1.38	2.25
Niben101Scf08130g00016.1	Q03662|GSTX1_TOBAC Probable glutathione S-transferase	1.34	2.28
Niben101Scf12160g00012.1	Q810L3|CHFR_MOUSE E3 ubiquitin-protein ligase	1.15	2.45
Niben101Scf07528g00003.1	Q6R567|RMA1_CAPAN E3 ubiquitin-protein ligase	1.11	3.22
Niben101Scf00352g02004.1	Q8LPN7|RNG1L_ARATH E3 ubiquitin-protein ligase RING1-like	1.08	1.34
Niben101Scf23606g00005.1	Q40374|PR1_MEDTR Pathogenesis-related protein PR-1	–1.05	–2.23
Niben101Scf27265g00006.1	Q9SS87|SEOB_ARATH Protein SIEVE ELEMENT OCCLUSION B	–1.65	–1.97
Niben101Scf13600g01004.1	P27492|CB21_TOBAC Chlorophyll a-b binding protein 16, chloroplastic	–1.78	–2.01
Niben101Scf00887g01011.1	Q9FX32|SUS6_ARATH Sucrose synthase 6	–1.94	–2.75
Niben101Scf08044g00001.1	P28493|PR5_ARATH Pathogenesis-related protein 5	–3.12	–4.01
Niben101Scf12954g02010.1	Q948T9|RBOHB_SOLTU Respiratory burst oxidase homolog protein B	–4.80	–4.03

[a] As detected by RNA-sequencing (RNA-seq) data and validated by quantitative real-time PCR (qPCR).

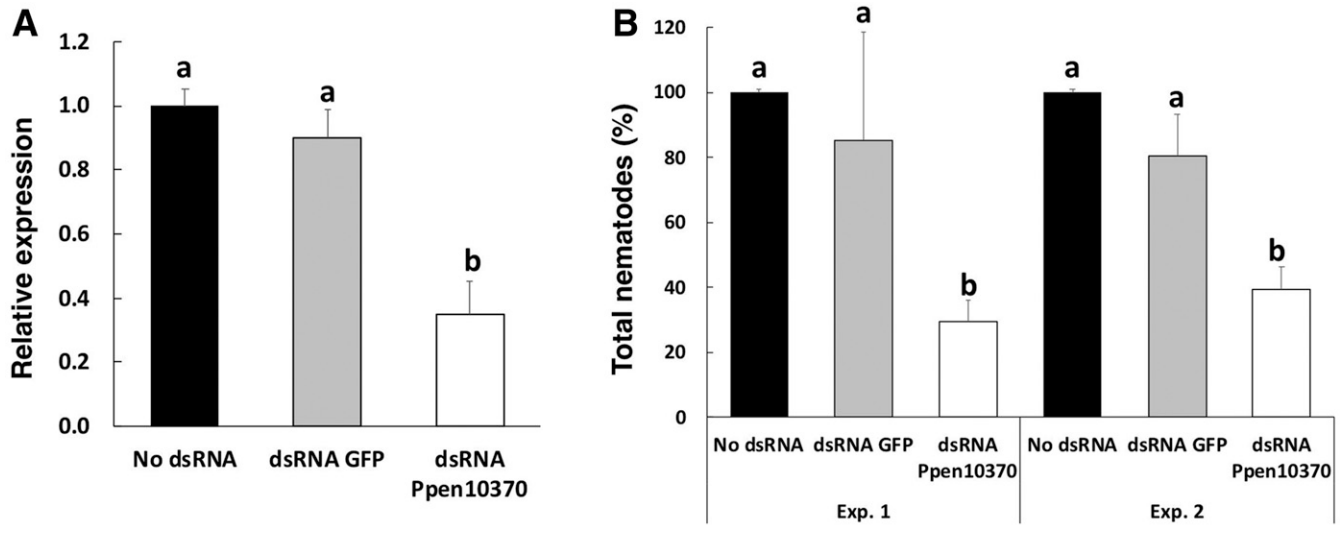

Fig. 7. Silencing of the *Pratylenchus penetrans Ppen10370* gene diminished nematode development. **A,** Relative abundance of *Ppen10370* transcripts determined by reverse transcription-quantitative PCR after nematode soaking RNA interference silencing assays. For each treatment, the gene silencing effect was evaluated 24 h after soaking (no double-stranded [ds]RNA corresponds to the soaking solution only; dsRNA GFP; and dsRNA Ppen10370). **B,** Total number of nematodes recovered from carrot disks after 6-weeks is presented as relative percentage against the treatment with soaking solution only (no dsRNA) in two independent assays. Error bars represent the standard error of the mean.

Generation of PVX-based constructs and expression in planta.

To express each individual candidate effector in planta, *Eco*RV-flanked PCR products of transcripts encoding the mature protein (without signal peptide) were cloned into the pCR II-TOPO vector (Thermo Fisher Scientific) according to the manufacturer directions and were verified by automated Sanger sequencing. The corresponding plasmids were digested with the *Eco*RV restriction enzyme, were gel-purified, and were subcloned into the *Eco*RV-linearized PVX-based vector pP2C2S (obtained from D. Baulcombe, Sainsbury Laboratories, Norwich, U.K.). The integrity of all PVX clones was verified by automated sequencing. The pP2C2S plasmids were linearized with *Spe*I, and capped transcripts were generated from the cDNA clones using the Ambion T7 mMessage machine kit (Thermo Fisher Scientific). Transcripts of each individual PVX construct were mechanically inoculated into fully expanded leaves of *N. benthamiana*, *N. tabacum*, and *Solanum lycopersicum* plants. Transcripts were also produced from pP2C2S plasmids without inserts ("empty" PVX vector) and were inoculated into plants to serve as controls representing a wild-type PVX infection (PVX/WT). Plants inoculated with buffer were used as negative ("healthy") controls. Inoculated plants were monitored daily for symptoms. The inoculation experiments included all sets of plants (a minimum of three) of each variant repeated three times. At 14 DAI, the leaves were photographed, were collected, were snap-frozen in liquid nitrogen, and were stored at –80°C until RNA extraction.

Western blotting.

Western blot assays were performed, essentially as described in Nemchinov and Natilla (2007), with leaves from *N. benthamiana* plants infected with PVX/Ppen10370 and PVX/WT. Membranes were probed with mouse monoclonal anti-GFP antibodies (Roche Applied Science) followed by phosphatase-labeled affinity purified antibody to mouse immunoglobulin G (Kirkegaard and Perry Laboratories, Inc.). Reactions were developed with a 1-Step NBT/BCIP phosphatase substrate (Thermo Fisher Scientific).

Subcellular localization in plant cells.

The coding sequence of *Ppen10370* effector gene without signal peptide was amplified by PCR using specific primers (Supplementary Table S5) and was cloned into the Gateway donor vector pCR8/GW/TOPO (Thermo Fisher Scientific). The sequences were then subcloned, using Gateway LR clonase (Thermo Fisher Scientific), into the GFP fusion vector pK7FWG2.0 (Karimi et al. 2002; provided by J. T. Jones of The James Hutton Institute, Dundee, U.K.), as a C-terminal fusion (Ppen10370ΔSP:eGFP). Competent cells of *Agrobacterium tumefaciens* EHA105 were transformed by the corresponding recombinant pK7FWG2.0 plasmid and were plated on Luria Broth (LB) agar containing rifampicin and kanamycin at a concentration of 50 µg/ml, following the protocol of Hammond et al. (2019). After incubation at 28°C, the transformed *A. tumefaciens* was grown in 5 ml of liquid LB overnight at 28°C at 250 rpm in a shaking incubator. The bacteria were then centrifuged for 10 min at 4,000 × *g* and were resuspended in 2 ml of the infiltration medium (10 mM morpholineethane sulfonic acid, 10 mM $MgCl_2$, pH5.7, 2 mM acetosyringone). After incubation at room temperature for 4 h, the culture was adjusted to an optical density at 600 nm = 0.05 and was infiltrated into three to four leaves of *N. benthamiana* plants using a needleless syringe. Infiltrated leaf patches were mounted in water and images were taken with an inverted confocal microscope (Axiovert 200 M, LSM510 META; Zeiss). GFP expression was detected after excitation with a 488-nm wavelength laser and emissions collected with a 499 to 550 nm beam path.

RNA-seq experiments.

For Illumina RNA-seq experiments, total RNA extraction was collected using the RNeasy plant mini kit (Qiagen) following manufacturer instructions. Three biological replicates (five pooled leaves from each of the three plants represented one replicate) were used for PVX/Ppen10370-infected plants. PVX/WT-infected plants were used as control. The purity and quantity of the samples were verified with a NanoDrop spectrophotometer (ThermoFisher Scientific). RNA-seq was performed by Novogene USA. cDNA libraries were generated using a poly (A) selection method, and paired-end reads (2× 150 bp) were obtained on the Illumina HiSeq 2500 platform.

Read mapping and functional analysis.

The paired-end reads were mapped onto the available genome sequence of *N. benthamiana* downloaded from the Sol Genomics Network (Bombarely et al. 2012) and to the representative genome of PVX (NCBI accession number M72416.1), using the RNA-seq analysis module in CLC Genomics Workbench (v.12) software (Qiagene). Gene annotations were based on BLASTX hits with the Swiss-Prot database. The DESeq2 package from Bioconductor in the R statistics suite (R version 3.4.0) was used to estimate the sample quality and expression levels of the genes. Genes with a fold change >2 and a FDR <0.05 were counted as DEGs. Part of the computational analyses related to GO and KEGG enrichment was performed by the RNA-seq provider Novogene USA. Significant GO and KEGG enrichment terms were detected by Fisher's exact test (FDR < 0.05).

Verification of transcriptome data by qPCR.

qPCR was performed with 25 selected genes to confirm the transcriptomic data. Primers were designed using the online Realtime PCR tool (Integrated DNA Technologies Inc) (Supplementary Table S5). cDNA for qPCR analyses was synthesized using the iScript cDNA synthesis kit (Bio-Rad), following manufacturer instructions, with the same RNA samples that were used for RNA-seq. Amplification was conducted with a CFX96 real-time system machine (Bio-Rad), with three biological replicates, using the following parameters: 95°C for 10 min (1 cycle) followed by 95°C for 10 s and 60°C for 45 s (40 cycles). The $2^{-\Delta\Delta Ct}$ method was used for the analysis of relative expression (Livak and Schmittgen 2001). The *L23* and *PP2A* genes (Liu et al. 2012) from *N. benthamiana* were used as internal reference genes to determine relative expression values.

Nematode soaking silencing assays by RNA interference.

For the RNA interference (RNAi) silencing assay, mixed stages of *P. penetrans* were soaked in dsRNA matching 212 bp of the coding sequence of the *Ppen10370* effector gene. As control, dsRNA matching a 715-bp fragment of GFP sequence was used. dsRNA sequences were synthesized with the Ambion MEGAscript RNAi kit (Thermo Fisher Scientific) by in vitro transcription, using *Ppen1037*- or GFP-specific PCR products as a template. The RNAi soaking assay followed methodologies previously applied for other *Pratylenchus* species (Joseph et al. 2012; Tan et al. 2013). Approximately 1,500 mixed stages (juveniles and adults) of *P. penetrans* were incubated in 200 µl of RNAi soaking solution composed of M9 buffer, 50 mM octopamine (Sigma-Aldrich), 3 mM spermidine (Sigma-Aldrich), and 1 mg/ml (150 µl) of the corresponding dsRNA construct. As control, nematodes were soaked with dsRNA-GFP or with the soaking solution only. Nematodes were then

incubated for 24 h at 28°C in the dark. After incubation, an aliquot of the soaked nematodes (approximately 220 nematodes) was washed with M9 buffer solution and was used for RNA extraction as described above and, consequently, for qPCR analyses, in order to validate *Ppen10370* gene silencing. To test the effect of RNAi on nematode infectivity, nematodes were then inoculated into sterilized carrot discs and were maintained for six weeks at 25°C. A minimum of six carrot discs were used per treatment (*Ppen10370*-dsRNA, dsRNA-GFP, and soaking solution only, respectively). After six weeks, nematodes were extracted from the carrot discs and were counted. Two independent biological experiments were performed. Statistically significant differences between treatments ($P < 0.05$) were determined by a one-way analysis of variance and pos hoc comparisons using Tukey's test.

ACKNOWLEDGMENTS

We thank I. Zasada for providing nematodes of *Pratylenchus neglectus* and *P. thornei.*

AUTHOR-RECOMMENDED INTERNET RESOURCES

EMBL-EBI InterPro:
https://www.ebi.ac.uk/interpro
NCBI databases: https://www.ncbi.nlm.nih.gov
Realtime PCR tool:
https://www.idtdna.com/scitools/Applications/RealTimePCR
Sol Genomics Network:
https://solgenomics.net/organism/Nicotiana_benthamiana/genome
WormBase: https://wormbase.org
WormWeb Exon-Intron Graphic Maker: WormWeb.org

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