The *Globodera pallida* Effector GpPDI1 Is a Functional Thioredoxin and Triggers Defense-Related Cell Death Independent of Its Enzymatic Activity

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ABSTRACT

The plant-parasitic nematode *Globodera pallida* is an obligate biotroph that only reproduces on select species in the *Solanum* family. The establishment of the feeding site, the syncytium, involves secretion of effectors into the plant cell to combat the plant defense response and facilitate transformation of root cells into the syncytium. Despite the important predicted roles of effectors in the plant–pathogen interactions, the functionality of *G. pallida* effectors is largely unknown. In this study, we identified and characterized a *G. pallida* effector protein disulfide isomerase (GpPDI1). GpPDI1 contains two thioredoxin domains that function together to reduce disulfide bonds, as manifested by the nullification of enzymatic

activity when either domain is absent. The transcript of *GpPDI1* is localized in the dorsal gland of the nematode during the J2 stage. In addition, GpPDI1 can trigger defense-related cell death in *Nicotiana benthamiana* and tomato (*Solanum lycopersicum*) leaf tissue and localizes in the plant host cell's cytoplasm and nucleus when transiently expressed in plant cells. Significantly, the ability of elicitation of cell death is not dependent on the enzymatic activity of GpPDI1 or correlated with the subcellular distribution of GpPDI1, suggesting that a nondisulfide reducing function or structural feature of GpPDI1 is responsible for the recognition by the host immune system to elicit cell death.

Plant-parasitic nematodes (PPNs) are obligate biotrophs that are considered a threat to global food security. *Globodera pallida* is an economically important pest of potato and has been suggested to cause up to 80% yield loss if left uncontrolled (Greco et al. 1982). *G. pallida* has five distinct life cycle phases (egg, second stage juveniles [J2], J3, adult, and cyst), beginning when a host plant's root exudate stimulates hatching of the eggs (Byrne et al. 2001; Farnier et al. 2012). The J2s emerge from the eggs and travel toward the source of the exudates where they penetrate the root, travel intracellularly, and establish a syncytium (Lilley et al. 2005). A syncytium is a feeding site originating from a single cell and incorporating up to 200 adjacent cells, which provides the nematode with nutrients to complete its lifecycle (Mejias et al. 2019; Sobczak and Golinowski 2011). Once the syncytium is established, the nematode progresses through several molts until it reaches the adult stage (Lilley et al. 2005). Depending on the size of the syncytium, the nematode will differentiate into either a male or female (Fuller et al. 2008). The males leave the root to find a mate while the females remain near the root; once fertilized, the female dies, and the body forms the protective cyst that encapsulates up to 500 eggs (Lilley et al. 2005).

A plant's battle against pathogen invasion begins when its transmembrane proteins, termed pattern recognition receptors (PRRs), perceive the commonly conserved pathogen associated molecular patterns (PAMPs) of the pathogen (Toruño et al. 2016). This leads to the activation of a basal defense system, termed PAMP-triggered immunity (PTI), and is typically characterized by the production of reactive oxygen species (ROS), mitogen-activated protein kinases, and activation of hormone-induced signaling (Ali et al. 2018; Sato et al. 2019). To overcome PTI, pathogens secrete proteins termed effectors into the plant cell to interfere with PTI signaling, presumably by a variety of mechanisms including degradation of PRRs, inhibition of the kinase cascade, and detoxification of the ROS burst (Hogenhout et al. 2009; Vieira and Gleason 2019; Waetzig et al. 1999). To counteract virulence activities rendered by the pathogen effectors, resistant plants have evolved a second defense system termed effector-triggered immunity (ETI). ETI is mediated by recognition of certain effectors by the plant's immune receptors, also generally called resistance (R) proteins (Jones et al. 2016; Sato et al. 2019). ETI is usually associated with a rapid, localized cell death, termed hypersensitive response (HR) to arrest the growth and/or colonization of pathogens (Sato et al. 2019; Toruño et al. 2016). HR is particularly effective at preventing the spread of biotrophic pathogens because the pathogen cannot feed on a dead cell and spread to neighboring cells (Spoel and Dong 2012). In the case of plant–PPN interactions, HR traps the nematode inside the cell preventing it from feeding and maturing to a reproductive stage (Sato et al. 2019; Waetzig et al. 1999).

Although effectors of PPN have not been studied as extensively as those of other pathogens, a secretion peptide has been identified at the N-terminus of PPN effectors (Thorpe et al. 2014). The secretion peptide is crucial to the function of effectors because the protein must exit the pathogen to work in the plant cell. In fact, PPNs use their stylet to pierce the host cell and inject effectors, cell wall degrading enzymes, and a variety of other virulence factors to initiate a syncytium (Lilley et al. 2005; Sato et al. 2019). In addition,

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G. pallida specifically expresses and stores effectors in the dorsal and subventral esophageal glands until secreted out of the stylet (Lilley et al. 2005; Vieira and Gleason 2019).

Thioredoxin domain-containing proteins are ubiquitous in most organisms with many different functions but the active site motif, CysXXCys, is highly conserved across organisms (Hanschmann et al. 2013; Li et al. 1996). The protein disulfide isomerase (PDI) family possesses two thioredoxin domains. The N-terminal active site initiates the oxidation by attaching to the nucleophilic region of the target, whereas the C-terminal active site reduces the disulfide bond in the target (Hanschmann et al. 2013). Pathogen PDIs play an important role in the host-pathogen interactions by protecting the pathogen from harmful ROS molecules produced by the host when it recognizes a PPN (Sato et al. 2019; Stolf et al. 2011). A PDI identified in *Phytophthora parasitica* has been shown to be essential to pathogen survival and enhanced virulence upon overexpression (Meng et al. 2015). It was recently shown that a structurally similar PDI from *Heterodera schachtii* impacts the development and structure of the syncytium and is induced during the early infection stages and in the presence of H₂O₂ (Habash et al. 2017). This research was corroborated with *Meloidogyne graminicola* PDI, which was also found to be induced during H₂O₂ stress and exhibited characteristic oxidase/isomerase activity (Tian et al. 2019). This suggests the PDI effector functions in protecting the nematode from harmful ROS compounds that are produced by the plant cell during the infection stages.

Despite previous research confirming the aforementioned characteristics of PDI effectors, the function of the enzymatic activity has only been examined once (Tian et al. 2019). In this study, we have identified a novel *G. pallida* effector, GpPDI1, that is an enzymatically active thioredoxin domain containing PDI. We found GpPDI1 can trigger strong defense-related cell death when transiently expressed in *N. benthamiana* and tomato leaves. Significantly, our functional and cellular analysis of GpPDI1 indicated the ability of GpPDI1 to elicit cell death is not dependent on its enzymatic activity or correlated to its subcellular distribution in plant cells, suggesting a structural feature or other element of GpPDI1 is recognized by the plant immune system to activate defense-related cell death signaling.

MATERIALS AND METHODS

Insulin reduction assay. Bovine insulin (0.13 mM) (Sigma-Aldrich, St. Louis, MO, U.S.A.) was placed into a salt buffer (2 mM EDTA and 0.1 M KPO₄) followed by 0.33 mM DTT to initiate the reaction. Recombinant protein (20 μM) was added and the reaction mixture was incubated in a cuvette at room temperature. During incubation, absorbency at 650 nm was measured using a spectrophotometer (ThermoFisher, Wilmington, DE, U.S.A.) every 30 s for 80 min. A Student's *t* test was performed to find significance using three biological replicates.

Plasmid constructs. *GpPDI1* was cloned from the cDNA of *G. pallida*, which was isolated from RNA collected from freshly hatched J2s using the SuperScript IV First-Strand Synthesis System (Invitrogen, Carlsbad, CA, U.S.A.). The amplified gene was cloned into the plant expression vector pBTEX to fuse with a N-terminal HA epitope tag under the control of the cauliflower mosaic virus (CaMV) 35S promoter. Individual colonies were screened by PCR for insertions and then the selected colony was confirmed by sequencing. See Supplementary Table S2 for primers used.

In situ hybridization assay. Digoxigenin (DIG)-labeled sense and antisense single-stranded cDNA were synthesized using a PCR DIG probe Synthesis Kit (Roche Applied Science, Indianapolis, U.S.A.). Freshly hatched J2 *G. pallida* nematode sections were probed following the protocol of de Boer et al. (1998). The stained nematode sections were observed using a stereomicroscope (Leica Microsystems, Wetzlar, Germany). See Supplementary Table S2 for primers used.

Agrobacterium mediated transient expression assay and western blotting assay. *Agrobacterium tumefaciens* GV2260 strain expressing HA-epitope tagged proteins were syringe-infiltrated into *N. benthamiana* or *S. lycopersicum* leaves using various concentrations for optimum protein expression. Leaf tissue was collected 36 h after infiltration and stored in -80°C until further use. *N. benthamiana* leaf tissue was ground with liquid nitrogen and resuspended with 300 μl of protein extraction buffer (50 mM Tris-HCL [pH 7.5], 150 mM NaCl, 5 mM EDTA, 2 mM DTT, 10% glycerol, 1% polyvinylpolypyrrolidone, 1 mM PMSP, and 1× plant protease inhibitor cocktail [Sigma-Aldrich]) and then centrifuged at 15,000 rpm/4°C for 12 min. Samples were fixed in 1× sodium dodecyl sulfate (SDS) loading dye and loaded on a 10% SDS-PAGE gel followed by transferring to PVDF membrane. The membrane was incubated with primary antibodies and secondary antimouse antibody (Sigma-Aldrich). Protein signal was detected with ECL Prime (GE Healthcare, Chicago, IL, U.S.A.).

For subcellular localization assay, *A. tumefaciens* strain GV2260 containing N-terminal GFP fused with Δ^{sp}GpPDI1, GpPDI1^{Δ1-143}, or Δ^{sp}GpPDI1^{Δ144-51} were injected into the leaves of 4- to 6-week-old wild type (WT) or *SGT1*-silenced *N. benthamiana* leaves. At 48 h PI, the epidermal cell layer was examined using a UV-microscope (Leica Microsystems, Wetzlar, Germany) to capture the GFP signal.

Virus induced gene silencing. Constructs for virus-induced gene silencing (VIGS) of *SGT1* (GenBank accession number AF516180) were made using tobacco rattle virus (TRV) as previously described (Peart et al. 2002). Two-week-old *N. benthamiana* seedlings were infiltrated with *A. tumefaciens* containing TRV₁ with *TRV2::00* or *TRV2::SGT* at optical density (OD)₆₀₀ = 0.2 for each strain. Four to six weeks later, the plants were used for transient expression assays.

RESULTS

Identification of *G. pallida* GpPDI1 effector. As previously shown, a list of 128 putative effectors were identified for *G. pallida* that are orthologous to previously identified effectors from various PPN (Thorpe et al. 2014). Based on the predicted enzymatic activity, several effector candidates were selected for further investigation due to their potential thioredoxin enzymatic activity. Transcripts were cloned from cDNA collected from J2 nematodes and introduced into a transient expression vector for further testing. All of the putative effectors we selected for further investigation (GPLIN_000990400, GPLIN_000574800, and GPLIN_001205000) had a high level of amino acid similarity with a partial sequence of the *H. glycines* esophageal gland cells putative secretory protein hsp3 (AF273730.1) and were closely related in amino acid sequence to HsPDI (KU948160) as determined by EMBOSS Needle (Madeira et al. 2019). Upon cloning and transient expression in *N. benthamiana* leaf tissue, GPLIN_000990400 triggered a very strong cell death response and for that reason, we decided to focus only on this protein, which we termed GpPDI1 due to its similarity in sequence to HsPDI and PpPDI1. Amino acid alignment of GpPDI1 with PDIs from other organisms showed highly conserved active domains which all have the same catalytic motif (CysGlyHisCys) (Supplementary Fig. S1). As expected, the highest level of sequence similarity among PDIs was within nematodes (>50% identity). It is generally accepted that nematode effectors possess an essential N-terminal signal peptide (SP) and the encoding genes are predominantly expressed in the glands of the parasitic stage(s) (Lilley et al. 2005; Win et al. 2012). Analysis of GpPDI1 protein structure predicted by SignalP identified a typical SP of 22 amino acids located at the N-terminus of GpPDI1 (Armenteros et al. 2019). Using PROSITE, two typical thioredoxin domains were identified at the N-terminal and C-terminal (Fig. 1A) (de Castro et al. 2006). It is common for PDIs to contain an ER retention signal, often represented by the motif KDEL at the C-terminus of proteins in eukaryotes (Stolf et al. 2011). GpPDI contains

the HTEL motif at the C-terminus, which was proven to localize protein in the ER in mammalian system but has not been tested in a plant system (Raykhel et al. 2007). This is similar to PpPDI, which has an ER retention signal motif of HEEL but differs from HsPDI and MgPDI, which lack a known ER retention signal.

Analysis of the *G. pallida* genome by Ensembl identified only seven putative paralogs of GpPDI1. However, due to the incompleteness of the genome, several genes identified were fragments and upon further examination there was only one highly similar paralog identified. This paralog (GPLIN_000574800) also contained two thioredoxin domains and a signal peptide but lacked an ER retention signal.

To determine the specific localization of *GpPDI1* transcripts in the nematode and its role in *G. pallida* parasitism, an in situ hybridization assay was performed. The digoxigenin (DIG)-labeled single-strand antisense cDNA probe of *GpPDI1* specifically hybridized in the dorsal gland of pre-J2 *G. pallida*, whereas the sense probe of *GpPDI1* did not show any nonspecific hybridization (Fig. 1B). Thus, the in situ results suggest *GpPDI1* is highly upregulated in the dorsal gland during the pre-J2 stage.

GpPDI1 is an enzymatically active thioredoxin. Since GpPDI1 was predicted as a thioredoxin protein, we determined the enzymatic activity by the insulin reduction assay, which is commonly used to verify thioredoxins based on the ability to reduce the disulfide bonds in insulin by separating the alpha and beta chains (Li et. al 1996; Park et. al 2009). It is believed that the mature nematode effector proteins lack the N-terminal SP, thus we used the GpPDI1 construct without the SP in our study (referred as

Δ spGpPDI1 hereafter). Δ spGpPDI1 was cloned into the *Escherichia coli* expression vector to generate the MBP epitope-tagged construct and the recombinant MBP- Δ spGpPDI1 protein was isolated from *E. coli* strain BL21. Insulin from bovine pancreas (Sigma-Aldrich) was used as the substrate to test GpPDI1 reductase ability. The reduction of insulin was measured at Abs 650 nm. As shown in Figure 2A, Δ spGpPDI1 reduced insulin at a very high rate at the end of 80 min ($Abs_{650} = 1.3592$) compared with control MBP ($Abs_{650} = 0.0605$) with statistical significance ($P < 0.01$), indicating GpPDI1 is a functional thioredoxin protein. To further determine the significance of the thioredoxin domains of GpPDI1, a series of GpPDI1 truncations with the deletion of one or both thioredoxin domains were generated and referred to as GpPDI1 Δ 1-143, Δ spGpPDI1 Δ 340-510, and GpPDI1 Δ 1-143, Δ 340-510 (Fig. 2B to F). Their enzymatic activity was tested, and the results showed if either thioredoxin domain is absent the reductase ability is dramatically reduced to a level similar to that of the negative control MBP (Fig. 2A). This suggests both thioredoxin domains of GpPDI1 are indispensable for its enzymatic activity.

GpPDI1 induces defense-related cell death in *N. benthamiana* and tomato. Leaf transient expression of nematode effectors has been used to study the function of effectors in plant-nematode interactions; in particular, it is used to investigate their role in suppression and/or elicitation of defense signaling (Fuller et al. 2008). Thus, to determine the possible role that GpPDI1 has in plant defense signaling, Δ spGpPDI1 was transiently expressed in model plant *N. benthamiana* leaves, with parallel expression of GFP as a negative control. As shown in Figure 3A, transient

Fig. 1. A, The schematic diagram of the functional domains of the GpPDI1 effector. **B,** *GpPDI1* is expressed in the dorsal glands. In situ hybridization of sections of *Globodera pallida* in the second stage juveniles stage shows that *GpPDI1* is expressed in the dorsal glands (top panel), whereas the sense probe did not give any detectable signal (bottom panel). Stylet (S), dorsal gland (DG), and bar set at 20 μ m.

Fig. 2. A, Insulin reduction assay indicating GpPDI1 requires both N-terminal and C-terminal thioredoxin domains for the reductase activity. Insulin (0.13 mM) was incubated with 20 μ M Δ spGpPDI1 or truncation proteins; MBP was used as a negative control. Values represent average of three biological replicates, and * indicates $P < 0.01$ using Student's *t* test ($n = 3$). **B to G,** Schematic diagram of GpPDI1 proteins and derived truncations used in this study.

expression of $\Delta^{sp}GpPDI1$, but not GFP, triggered strong cell death at 24 h postinfiltration. To further verify whether GpPDI1 can trigger cell death in a known host plant of *G. pallida*, the experiment was repeated in tomato (*S. lycopersicum* 'Alisa Craig') and similar results were observed with cell death occurring in tissue transiently expressing $\Delta^{sp}GpPDI1$ in less than 24 h postinfiltration (Fig. 3B). Due to the similar defense response, the remaining experiments were carried out in *N. benthamiana*. In theory, when cell death occurs upon transient expression of a pathogenic protein in leaf tissues, it can point to one of two things: defense signaling is triggered, initiating the HR pathway or the cell death is a result of general cellular imbalance due to simple overexpression of the protein. To test these possibilities, we examined whether the GpPDI1-triggered cell death is dependent on *SGT1*, a well-known defense signaling component essential for the defense-related cell death signaling in *N. benthamiana* (Peart et al. 2002). It has been shown that when *SGT1* is silenced in *N. benthamiana*, most R protein-mediated defense-related response (such as HR) is repressed; however, nondefense response cell death is unaffected by

SGT1 silencing (Peart et al. 2002). *Agrobacterium*-mediated transient expression of $\Delta^{sp}GpPDI1$ was carried out in *N. benthamiana* leaves in which expression of the *SGT1* gene had been suppressed by VIGS. Pto^{Y207D}, an auto-active form of a tomato resistance protein that elicits *SGT1*-dependent HR cell death in *N. benthamiana*, was included as a positive control (Rathjen et al. 1999). As shown in Figure 3C, neither $\Delta^{sp}GpPDI1$ nor Pto^{Y207D} triggered cell death in the *SGT1*-silenced *N. benthamiana* leaves. Taken together, these results indicate that overexpression of GpPDI1 can activate the

Fig. 3. GpPDI1 triggers defense-related cell death when transiently expressed in *Nicotiana benthamiana* leaves. **A**, *N. benthamiana* leaves were hand-infiltrated with *Agrobacterium* harboring $\Delta^{sp}GpPDI1$ or green fluorescent protein (GFP) constructs with an inoculum at optical density (OD)₆₀₀ = 0.2 and 0.05, respectively. Images were taken 2 days postinfiltration. The experiment was repeated five independent times with two replicates each time, all with similar results. **B**, Hypersensitive response observed on *Solanum lycopersicum* leaves transiently expressing $\Delta^{sp}GpPDI1$ or GFP (OD = 0.2). Image taken 24 h postinfiltration. **C**, The GpPDI1-triggered cell death is dependent on *SGT1*. Similar transient experiments were conducted on the nonsilenced (*TRV2::00*) and *SGT1*-silenced (*TRV2::SGT1*) *N. benthamiana* leaves. Note that the cell death elicitor construct Pto^{Y207D} was included to serve as a positive control, indicating the silencing of the *SGT1* gene.

Fig. 4. The enzymatic activity of GpPDI1 is dispensable for triggering Hypersensitive response-cell death. **A**, *Nicotiana benthamiana* leaves were hand-infiltrated with *Agrobacterium* harboring the epitope-tagged GpPDI1 (HA- $\Delta^{sp}GpPDI1$), GpPDI1 truncation (HA-GpPDI1 Δ^{1-143} , HA- $\Delta^{sp}GpPDI1\Delta^{340-510}$, or HA- $\Delta^{sp}GpPDI1\Delta^{144-510}$), or GFP (HA-GFP) construct with an inoculum at optical density (OD)₆₀₀ = 0.05 for the GFP construct and OD₆₀₀ = 0.2 for GpPDI1 constructs. Photographs were taken 2 days after injection. **B**, Western blotting analysis indicating all tested proteins were expressed and accumulated normally in plant cells. Proteins were detected using the anti-HA antibodies, and the Coomassie brilliant blue (CBB) staining indicated equal loading of protein samples.

defense-related cell death signaling and suggest that GpPDI1 may play a role in host defense responses against *G. pallida*.

The enzymatic activity of GpPDI1 is dispensable for triggering cell death in plant cells. Given the fact that GpPDI1 is a functional reductase and can trigger defense-related cell death when transiently expressed in leaves, investigation of which portion of the GpPDI1 protein is related to elicitation of cell death can shed light on how the plant recognizes the effector as an elicitor to activate defense responses. In particular, we can identify whether plant recognition is dependent on its enzymatic activity. To this end, the GpPDI1 truncations used for the enzymatic assay (Fig. 2B to E and G) were cloned into the plant expression vector to examine whether they can trigger cell death when transiently expressed in *N. benthamiana* leaves. When GpPDI1 Δ 1-143, which does not possess the reductase activity, was tested, it did not trigger HR cell death, suggesting the N-terminal fragment of 1 to 143 aa is essential for elicitation of cell death. However, expression of the 1 to 143 aa fragment (Δ spGpPDI1 Δ 144-510) alone did not cause cell death, indicating it is not sufficient for cell death induction. To further address whether this is due to loss of the enzymatic activity in GpPDI1 Δ 1-143, the truncation Δ spGpPDI1 Δ 340-510 was examined and the results show it triggered HR-cell death just as efficiently as the enzymatically active Δ spGpPDI1 did (Fig. 4A). These results

suggest the reductase activity is not necessary for GpPDI1 to trigger defense signaling. Moreover, Western blotting analysis was conducted to verify the protein expression to eliminate the possibility that the inability of Δ spGpPDI1 Δ 144-510 and GpPDI1 Δ 1-143 to trigger cell death was due to the absence of protein expression, and it was found that all truncation proteins were expressed and accumulated in plant cells (Fig. 4B). Taken together, our results indicate that the C-terminus (341 to 510 aa) of GpPDI1 is dispensable for triggering defense-related cell death, whereas the N-terminus (1 to 143 aa) is essential but not sufficient to trigger cell death. More importantly, the ability of the reductase-defective Δ spGpPDI1 Δ 340-510 to elicit defense-related cell death also suggests there is no correlation between disulfide reductase activity and cell death induction.

GpPDI1 localizes in the nucleus and cytoplasm when expressed in *N. benthamiana* cells. It has been shown that PPNs secrete effectors into a host cell where they localize to specific subcellular compartments to perform distinct activity (Toruño et al. 2016; Varden et al. 2017). We sought to determine the subcellular localization of GpPDI1 when expressed in plant cells. In particular, we asked whether the ability of GpPDI1 to activate defense signaling is related to its subcellular localization. To this end, the active intact Δ spGpPDI1 and inactive GpPDI1 Δ 1-143 and Δ spGpPDI1 Δ 144-510 were cloned into a plant expression vector with a N-terminal enhanced green fluorescent protein (eGFP) tag under the control of CaMV35S promoter. Since Δ spGpPDI1 expression in WT *N. benthamiana* induced strong cell death (Fig. 3 A), *SGT1*-silenced *N. benthamiana* plants were used to perform the subcellular localization assays. As shown in Figure 5, the fluorescence signal of all three GFP-GpPDI1 fusion proteins (GFP- Δ spGpPDI1, GFP-GpPDI1 Δ 1-143, and GFP- Δ spGpPDI1 Δ 144-510) were found in the cytoplasm and nucleus which resembles the subcellular distribution of the free GFP signal. These results suggest GpPDI1 does not localize to any specific subcellular compartment when ectopically expressed as a GFP-fusion protein in *N. benthamiana* leaves. The two GpPDI1 truncations (GFP-GpPDI1 Δ 1-143 and GFP- Δ spGpPDI1 Δ 144-510) were also expressed in the WT *N. benthamiana* leaves and the results showed the same localization pattern as in *SGT1*-silenced *N. benthamiana* leaves (Supplementary Fig. S2), which verifies that *SGT1*-silenced plants do not have an effect on the localization assay. Taken together, our results suggest there is no correlation between the ability of GpPDI1 to trigger defense signaling and its subcellular distribution in plant cells.

DISCUSSION

This study identifies and characterizes the effector GpPDI1 from *G. pallida* that is a functional thioredoxin. GpPDI1 captured our interest because it had a predicted enzymatic function that has only recently been implicated in plant–pathogen interactions (Habash et al. 2017; Meng et al. 2015; Tian et al. 2019). PPNs, including *G. pallida*, spend the majority of their life cycle inside host tissue, which requires numerous chemicals and proteins to establish parasitism (Lilley et al. 2005; Sato et al. 2019). GpPDI1 was first identified as a potential effector sharing homology with a partial sequence of *H. glycines* esophageal gland cell 3 (AF27.3730.1) (Cotton et al. 2014; Wang et al. 2001). It has been demonstrated that the transcripts of some effectors of PPNs localize in the dorsal and subventral glands where the effectors are expressed and stored until secreted out during infection (Lilley et al. 2005; Vieira and Gleason 2019). As expected, the transcript *GpPDI1* was predominantly localized in the dorsal gland, suggesting it may play a role in plant–*G. pallida* interactions.

Agrobacterium-mediated transient expression of effectors in *N. benthamiana* leaves has been used to study the potential function of PPN effectors. In particular, the ability of effectors to cause defense-related cell death via transient expression suggests the defense signaling is activated due to the recognition of the effector by the plant immune system. We found GpPDI1 can cause strong

Fig. 5. Subcellular localization of GpPDI1 and the derived truncations when expressed in plant cells. *Agrobacterium* culture harboring the GFP- Δ spGpPDI1, GFP-GpPDI1 Δ 1-143, or GFP- Δ spGpPDI1 Δ 144-510 construct under the control of the cauliflower mosaic virus (CaMV) 35S promoter was hand-injected into *SGT1*-silenced *Nicotiana benthamiana* leaves with an inoculum of optical density (OD)₆₀₀ = 0.5. At 48 h postinfiltration, the epidermal leaf tissues were collected and subjected to microscopy with a confocal laser scanning microscope. The experiment was completed five independent times with similar results. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI). Differential interference contrast (DIC) images of the same view were aligned underneath the GFP signal images. Scale bars = 50 μ m.

cell death when transiently expressed in *N. benthamiana* leaves and silencing of the *SGT1* gene in *N. benthamiana* completely abolished the GpPDI1-triggered cell death (Fig. 3C). Together, these results suggest GpPDI1 can function as an elicitor to be perceived by the immune system, thereby activating defense signaling despite the immune receptor and related immune signaling components have not been identified. This response was also observed in the leaf tissue of a known host of *G. pallida*, tomato. Consistent with these findings, we were unable to generate transgenic potato plants overexpressing GpPDI1 after repeated attempts. We believe this is most likely because expression of GpPDI1 in the transformed potato cells promotes cell death that terminates generation of transgenic explants.

It is generally thought that pathogen effectors are recognized by plant immune systems via their structural features or enzymatic functions. In fact, it was previously suggested that there might be a correlation between the enzymatic activity of putative PDI effectors of plant pathogens and the incidence of cell death caused by transient expression of these effectors (Meng et al. 2015). To differentiate these possibilities, we determined the ability of GpPDI1 truncations to elicit cell death in *N. benthamiana* leaves. We found the N-terminal thioredoxin domain is necessary but not sufficient to elicit a cell death response; whereas the truncation eliminating the C-terminal thioredoxin domain still retains the ability to trigger cell death despite it no longer possesses the enzymatic activity (Fig. 4A). However, it should be noted that the loss of enzymatic function may be due to misfolding of the proteins due to truncations. It appears that there is no correlation between the enzymatic activity of GpPDI1 and its ability to trigger cell death, suggesting a nondisulfide reducing function or structural feature of GpPDI1 is responsible for the recognition by the host immune system to elicit defense-related cell death. These findings are consistent with the transient expression assays of PpPDI1 truncations performed by Meng et al. (2015), which also found that the N-terminal of PpPDI1 is required to initiate the cell death response while the C-terminal is dispensable.

Taking into account the lack of correlation between enzymatic activity and the occurrence of cell death, we asked whether HR cell death is linked to subcellular localization of GpPDI1 when expressed in plant cells. The subcellular localization assay using GFP-fusion constructs showed Δ SPGpPDI1 and the truncations do not localize to any specific subcellular compartment when expressed as GFP-fusion protein regardless if they possess the ability to elicit cell death. These results suggest the subcellular distribution of GpPDI1 may not have a direct impact on its function to elicit cell death. However, given the fact GpPDI1 and its truncations were found to be distributed throughout the plant cell, we cannot completely rule out the possibility that the observed subcellular localization is due to overexpression from the strong CaMV 35s promoter which may result in artifact subcellular localization (Moore and Murphy 2009). It is also important to note that the subcellular results are contradictory to what was found for MgPDI and HsPDI, both of which localized in the apoplastic space (Habash et al. 2017; Tian et al. 2019). There is a possibility that under normal conditions, GpPDI is secreted into the apoplastic space and never enters inside the cell. However, there is another possibility, that HsPDI and MgPDI perform different functions than GpPDI1 which would also explain why there was no observed cell death during transient expression of HsPDI and MgPDI. Nevertheless, we found that when GpPDI1 is expressed in plant cells it elicits strong defense-related cell death independent of its thioredoxin activity, which suggests that a structural feature of GpPDI1 may be recognized by the plant immune system thereby activating the defense signaling.

Upon examination of the three PDIs that were previously described, there appears to be contradicting characteristics which include ability to trigger cell death, subcellular localization and the presence of an ER retention signal. The first described plant

pathogen PDI, PpPDI1, has the same characteristics as GpPDI1 which include trigger cell death, and has an ER retention signal (Meng et al. 2015). In contrast, HsPDI and MgPDI do not trigger cell death, lack an ER retention signal, and localize in the apoplastic space of the plant cell (Habash et al. 2017; Tian et al. 2019). Based on this information, we are compelled to make the argument that there are two different types of plant pathogen PDIs and therefore perform different functions. This idea was reinforced when examining PDIs from several different nematodes. Using GpPDI1 to BLAST search for other PDIs from *G. rostochiensis*, *H. glycines*, and *M. graminicola*, two PDI genes were identified from each species. These were placed into respective groups based off the presence of an ER retention signal. If there was an ER retention signal, they were placed into group 1, those lacking an ER retention signal belonged to group 2. ClustalO alignment showed that the protein sequence of these PDIs were more similar among the PDI groups than if the PDIs were from the same species (Supplementary Figs. S3 and S4).

Nematodes need to prevent the buildup of ROS molecules to make the host cell more habitable and inhibit defense signaling (Gillet et al. 2017; Mejias et al. 2019). It is not known if detoxification of ROS is to prevent downstream cell signaling or to make the host more habitable to the parasite or both (Sato et al. 2019). Previous studies on mammalian thioredoxin proteins have shown that these proteins are used to detoxify ROS molecules and are conserved across many species (Hanschmann et al. 2013). It has also been found that HsPDI and MgPDI are essential for their nematode's successful infection as determined by infection assays and both *HsPDI* and *MgPDI* genes are induced upon the exposure to H₂O₂ (Habash et al. 2017; Tian et al. 2019). Although our experiments were conducted in a way only a single effector was examined and it was in a nematode-free system without the presence of other effectors, we speculate that in the natural plant–nematode coevolution process, GpPDI1 was originally used by *G. pallida* to detoxify ROS produced by plant cells upon nematode invasion. To overcome this effector-conferred parasitism-related activity, plants have evolved the immune system to recognize GpPDI1 to trigger ETI (HR) to arrest *G. pallida*'s colonization, which has been observed with other nematode effectors such as ascaroside (Vieira and Gleason 2019). However, due to such a strong selection pressure on *G. pallida*, we speculate that it is possible the nematode has further evolved an unknown mechanism to interfere with this ETI-type defense to successfully parasitize host plants. Future studies could include determination of the role of GpPDI1 in the parasitism of *G. pallida*. Since we were not able to generate transgenic potato plants expressing GpPDI1 due to its lethality, examining the susceptibility of transgenic potato plants expressing the RNA-interfering (RNAi) *GpPDI1* construct may be an approach to determine the virulence activity of GpPDI1. Several publications have demonstrated that when PPNs invade transgenic host plant roots expressing RNAi-effector constructs, the nematodes could uptake dsRNA of the effector genes produced in the plant cells, resulting in silencing of the target effector genes (Fuller et al. 2008; Hogenhout et al. 2009). Thus, using this transgenic-based RNAi approach to silence GpPDI1 in *G. pallida* would allow us to determine the virulence activity of GpPDI1 in *G. pallida* parasitism.

Accession numbers: GPLIN_000990400 (GpPDI1), AF516180 (SGT1), NP_001307158.1 (Pto), and GPLIN_000054100 (GpEtf-1). Accessions for *G. pallida* sequences can be found at Wormbase.

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