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Original Research Article

Polygalacturonase-inhibiting proteins as an exogenously applied natural solution for prevention of postharvest fungal infections

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A B S T R A C T

Polygalacturonase inhibiting proteins (PGIPs) are plant proteins involved in the inhibition of polygalacturonases (PGs), cell-wall degrading enzymes often secreted by phytopathogenic fungi. Previously, we confirmed that PGIP2 from *Phaseolus vulgaris* (PvPGIP2) can inhibit the growth of *Aspergillus niger* and *Botrytis cinerea* on agar plate. In this study, we further validated the feasibility of using PGIP as an environmental and ecological friendly agent to prevent fungal infection post-harvest. We found that application of either purified PGIP (full length PvPGIP2 or truncated tPvPGIP2_5–8), or PGIP-secreting *Saccharomyces cerevisiae* strains can effectively inhibit fungal growth and necrotic lesions on tobacco leaf. We also examined the effective amount and thermostability of PGIP when applied on plants. A concentration of 0.75 mg/mL or higher can significantly reduce the area of *B. cinerea* lesions. The activity of full-length PvPGIPs is not affected after incubation at various temperatures ranging from −20 to 42 °C for 24 h, while truncated tPvPGIP2_5–8 lost some efficacy after incubation at 42 °C. Furthermore, we have also examined the efficacy of PGIP on tomato fruit. When the purified PvPGIP2 proteins were applied to tomato fruit inoculated with *B. cinerea* at a concentration of roughly 1.0 mg/mL, disease incidence and area of disease had reduced by more than half compared to the controls without PGIP treatment. This study explores the potential of PGIPs as exogenously applied, eco-friendly fungal control agents on fruit and vegetables post-harvest.

1. Introduction

Undernourishment and food security are currently a dire problem worldwide, especially in underdeveloped countries, with an estimated 8.9 % of the world population suffering from hunger (FAO, 2020). This percentage is projected to grow, as the human population has more than doubled between 1960 and 2009 and reached 7 billion recently, and is further expected to reach 9.2 billion by 2050 [1]. Working with global food systems to improve sustainability in the food chain and increase nutritional availability are major agendas that need to be addressed for our growing population [2]. The current average intake of fruits of vegetables is lacking, and is especially insufficient in developing countries [3]. Even in the United States, only approximately 1 in 10 adults meet the required intake of fruits and vegetables, citing high cost and limited availability, which may have been worsened during COVID-19 pandemic that began in 2019 [4].

Numerous studies have shown that consumption of fruits and vegetables are highly beneficial to human health, as they are a rich source of fiber, nutrients, and phytochemicals that protect against diseases [5]. However, due to the high water content, reduction in hardness during ripening, and temperature changes that can affect flexibility, most fruits and vegetables tend to be perishable and susceptible to mechanical damages during harvesting and transport [6]. When the produce is damaged and bruised, they are more susceptible to spoilage from microorganisms, including pathogenic fungi [7]. Every year, roughly 20 % of all crops are lost due to pathogenic fungi, with an additional 10 % loss after harvest [8]. Postharvest diseases and decay from pathogenic fungi causes major reductions in the quality, shelf life, and market value of the fruits and vegetables [9]. In fact, out of all the food groups, fruits and vegetables are one of the largest contributors to economic loss in the industry [10], with the final consumer never seeing 10–15 % of the crop in developed countries and 20–40 % in developing countries due to the postharvest losses [11]. Improving disease durability of the produce will likely help increase productivity, thus alleviating some costs to the agricultural sector and ultimately the consumer, helping to feed the growing population.

Currently, the most effective way of controlling pathogenic fungi in field, horticulture, and postharvest crops is through the use of chemical and synthetic fungicides [12,13]. These fungicides are so effective at plant protection that they are considered vital for food security [14]. Due to changes in climate and increasing levels of fungicide resistance, the use of fungicide is predicted to increase [15]. Though effective,

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chemical and synthetic fungicides have been shown to have negative impacts on human health [16]. For example, some chemical pesticides are known to act as neurotoxins and cause degenerative diseases and fetal defects, while increased exposure to others have carcinogenic effects on humans [17]. Questions have been raised regarding the sustainability of these pesticides especially when applied to vegetables and fruits, and a more eco-friendly approach is necessary [18]. A more socially and environmentally acceptable way to bolster food security through the control of fungal pathogens will be needed.

In recent years, biocontrol agents for fungal control have become a promising alternative to synthetic fungicides. Plants have evolved numerous antifungal mechanisms to cope with the threat of disease from fungal pathogens. Plant proteins and peptides such as chitinases, defensins, and lectins proteins have antifungal properties that distinguish them as attractive candidates for development as a pest control strategy in the agricultural sector [19,20]. One group of plant proteins that may be promising are the polygalacturonase inhibiting proteins (PGIPs), which inhibit polygalacturonases (PGs), cell wall degrading enzymes secreted by a multitude of pathogenic fungi [21]. PGIPs primarily inhibit PGs through competitive inhibition, which then slows the hydrolysis process of the cell wall from the PGs [22]. PGIPs contain a highly conserved leucine rich repeat (LRR) region that may be responsible for interacting with the active site of PGs [23]. Numerous studies have found that overexpression of PGIPs in genomically modified plants and crops have increased their resistance towards pathogenic fungi [24]. Due to the uncertainty with public perception on GMOs, utilizing PGIPs as an exogenously applied pest control agent may be more appealing, especially given their potentially shorter development and approval process compared to the long development time of transgenic plants [25].

Nicotiana benthamiana, also known as tobacco, is a popular model plant that has been extensively used by scientists for years to study plant virology, genomics, pathogens, and more [26]. It can be genetically transformed and regenerated efficiently, and is susceptible to a large number of plant-pathogenic agents, allowing it to serve as a host plant in many pathogen-host studies [27]. As food security increasingly becomes an issue with our growing population, sustainable methods of disease control in the agricultural sector are needed. The goal of this study is to investigate the effects of the PGIP proteins *in vitro* and *in planta* on *N. benthamiana* plants to better estimate their potential as exogenously applied, eco-friendly fungal agents. PvPGIP2 from *Phaseolus vulgaris*, one of the best characterized and most efficient PGIPs reported to date [28], was found to only require the regions between LRR5-LRR8 to retain similar levels of PG interaction as the full length version in our previous study [25]. Here, to verify if the truncation process was specific to PvPGIP2 or if it could be more broadly applied, GmPGIP3 from *Glycine max* was investigated *via* yeast two-hybrid (Y2H) and pectin agar assays to ascertain its minimal size required for PG interaction. Further studies on the exogenous application of PvPGIP2 were also conducted. Engineered PvPGIP2-secreting yeast strains and purified PvPGIP2 proteins were exogenously applied to phytopathogenic fungi on pectin agar, detached leaf assays, and postharvest tomato fruits. Both the full-length and truncated form, tPvPGIP2_5–8, were utilized and successfully reduced the expansion of necrotic lesions and growth of pathogenic fungi. Our results validated the potential of using PGIPs as an exogenously applied, eco-friendly fungal-control agent against pathogenic fungi especially in the postharvest sector.

2. Materials and methods

2.1. Plasmid Construction

All PvPGIP2, truncated tPvPGIP2_5–8, and PG (AnPG2, BcPG1, BcPG2, and FmPG3) plasmids were previously made according to the "Plasmid Construction" section found in Ref. [25]. The GmPGIP3 and its truncations for this study were created using the same protocol. The GmPGIP3 gene was ordered from Twist Bioscience and codon optimized for *S. cerevisiae*. Using the Gateway system, GmPGIP3 was cloned with a destination vector containing an AD domain. This LR reaction was completed using the instructions found from the Invitrogen Gateway LR Clonase II Enzyme Mix product sheet (Fischer Scientific, catalog #11789020). Truncated versions of GmPGIP3 were made using various primers as seen in Table S1 that ensured the protein would not be spliced in the middle of the β-sheets. Using primers that flanked different LRR regions allowed us to create numerous GmPGIP3 length combinations. After following Gibson assembly instructions according to the protocol "Gibson Assembly Cloning" by Addgene, truncated versions of the GmPGIP3 from the full length GmPGIP3 with the primers and inserted into a vector with the AD domain. The DNA sequences of the genes and the primers used in this study is listed in Tables S2 and S3, respectively.

2.2. GmPGIP3 Y2H growth curve assay

Plasmids encoding the full length GmPGIP3 or PvPGIP2 and plasmids encoding one of the PGs (AnPG3, BcPG1, BcPG2, or FmPG3) were co-transformed into *S. cerevisiae* strain PJ69-4A. The genotypes of the yeast strains used in this study are listed in Table S3. Standard protocols were used to complete the yeast transformations [29]. 500 μL of the overnight stock cultures were grown in -LT YNB with 5 % dextrose. After 16 h at 30 °C with orbital shaking at 250 RPM. 10 μL of the yeast expressing both AD-PGIPs and BD-PGs was added to 290 μL -HTL YNB and grown for 4 days. A plate reader was used to measure the OD at 600 nm every 24 h. The average OD across the 3 biological replicates was then plotted. The same procedure was applied for the truncated versions of GmPGIP3 Data was collected using the Gen5™ Data Analysis Software (BioTek Instruments) and compared to the full-length PvPGIP2 as a control.

2.3. Isolation and purification of PvPGIP proteins

To obtain purified PvPGIP proteins for pectin and plant assays, we transformed *S. cerevisiae* CENPK2.1D with plasmids containing either full-length PvPGIP2 or truncated PvPGIP2_5–8, both tagged with His. After growing for 4 days, a colony was selected to grow in yeast extract peptone dextrose (YPD) for 24 h 200 μL seed culture was then transferred to a 500 mL flask of -T yeast nitrogen base (YNB) and grown with shaking in an incubator at 30 °C for 48 h. The yeast cells were then spun down at 3000 rpm for 5 min and supernatant discarded. This was then resuspended in 1 mL of chilled lysis buffer (50 mM pH 7.5 HEPES-KOH, 300 mM KCl, 2.5 mM dithiothreitol [DTT]) and lysed in a cell disrupter twice at 30 s each time, with a 2-min cooldown period in between. Afterwards, the solution was spun at 15,000 rpm at 4 °C for 30 min to clarify the lysate. The lysate was then placed into a tube containing 2 mL of Ni-NTA resin and rotated at 4 °C for 2 h. This was then placed into a HisPur Ni-NTA Column (Thermo Fischer Scientific, catalog #PI88225) and allowed to elute *via* gravity. The column was washed twice with 5 mL of cold wash buffer each time (50 mM pH 7.5 HEPES-KOH, 300 mM KCl, 10 mM imidazole, 2.5 mM DTT), then finally eluted with 3 mL of cold elution buffer (50 mM HEPES-KOH pH 7.5, 300 mM KCl, 250 mM imidazole, 2.5 mM DTT). After obtaining purified PvPGIP proteins as stated in the protocol above, the proteins were concentrated in centrifugal filter units. Proteins were placed into a 3kDA filtration unit (Thermo Fischer Scientific, catalog #UFC900308) and spun in a centrifuge at 4 °C at 5000 RPM for 30 min. The liquid that pooled under the filter was discarded, and 10 mL of protein storage buffer (20 mM sodium phosphate, 25 mM NaCl, 10 mM DTT, pH 7.4) was added to the top of the filter unit. The solution was spun down once more under the same conditions. The resultant concentration of the protein was typically roughly 0.9 mg/mL to 1.1 mg/mL. The concentration of the purified PvPGIP proteins obtained was estimated by comparing the sample to a standard curve made using BSA and the Bradford Assay from Bio-Rad according to the manufacturer's specifications. The presence of

PGIPs was also confirmed *via* Western blot, using a standard protein detection protocol, the "Protein Detection Technical Handbook" from Thermo Fischer Scientific.

2.4. Materials, growth, and infections of N. benthamiana

N. benthamiana seeds were generously provided by Prof. Wenbo Ma's lab and were utilized throughout the course of this study. The seeds were grown in soil (Miracle-Gro Potting Mix) in a growth chamber for 5 weeks. The temperature was set at 24 °C and humidity at 70 %. Long-day conditions were utilized (16 h light to 8 h dark photoperiod cycle). Leaves located within the central region of the plants (between 2 inches from the bottom and 2 inches from the top) were harvested using a set of scissors sterilized with 90 % ethanol. The leaves were then soaked in a DI water solution containing 10 % bleach and 1 % Tween 20 for 10 min, then rinsed with deionized (DI) water 4 times for 20 s each time. Wet leaves were air dried in a fume hood before being placed in petri dishes containing 1 % agarose (VWR, catalog #97062-250) for the plant pathology assays.

B. cinerea isolate ECC-0165 was obtained from *Prunus persica* in Fresno, California and *A. niger* isolate ATCC 16888 was obtained from ARS Culture Collection (NRRL). Colonies were maintained on fresh potato flake agar plates and incubated at 22 °C. Once colonies had sufficient sporulation, sterile DI water was added to the plates and the surface of the colonies was scraped with a cell spreader. The liquid was filtered through cheesecloth to exclude hyphal fragments, and a hemocytometer was used to quantify the spore concentration of the resultant liquid. The suspension was diluted with sterile DI water to a concentration of 2.5×10^6 spores/mL for usage in the fungal pathogen assays.

2.5. Determining the effective concentration of PvPGIPs

After obtaining the concentrated PvPGIP proteins as stated in the protocol above, dilutions were made using protein storage buffer to create separate aliquots of PvPGIP protein solutions with concentrations of roughly 1.0, 0.75, 0.5, 0.25, or 0.125 mg/mL each. *N. benthamiana* leaf material with *B. cinerea* inoculations were made according to the protocol above. Inoculated leaves were thoroughly sprayed on both sides (2–3 sprays per leaf, equivalent to ~500 μL) with one of the concentration titrations of PvPGIP2s, then placed onto petri dishes containing 1 % agar. Dishes were kept covered in a fume hood at room temperature and progression of disease was monitored for 3 weeks. Photos were taken at the end of each week and analyzed using Fiji (Image J) to quantify the amount of necrotic tissue present. Three replicates (n = 4 leaves each) of each treatment was generated for analysis Statistical differences were measured using a one-way ANOVA test to obtain P-values.

2.6. Comparing the efficacy of PvPGIP proteins and PvPGIP-secreting yeast

The *B. cinerea* inhibition efficacy of yeast secreting PGIPs using the *Ost1* signal was compared to the application of purified PvPGIP2 proteins on the detached tobacco leaves. *S. cerevisiae*, strain CENPK2.1D, was transformed with a centromeric high copy plasmid containing *Ost1*-PvPGIP2, a created from the protocol found in Ref. [25]. Purified PvPGIP proteins were obtained as mentioned in the protocol above. According to previous investigations, the titer of a protein of 166 residues in size fused with the prepro-α signaling factor from yeast is ~6.5 mg/L [30], whereas the *Ost1* signal peptide is approximately 10–20 times more efficient at protein secretion [31]. Thus, the titer of PvPGIP2 found in the yeast medium using the *Ost1* signal peptide is roughly between 0.065 and 0.130 mg/mL, and the concentration of the purified PGIP proteins typically falls between 0.9 mg/mL to 1.1 mg/mL. The detached leaf assay was performed comparing leaves treated with *Ost1*-PvPGIP2 secreting yeast, PvPGIP2 protein, Ost1-tPvPGIP2_5–8 secreting yeast, tPvPGIP2_5–8 protein, *B. cinerea* inoculation only (negative control), *B. cinerea* with yeast containing an empty vector (negative control), and no treatment. Leaf material and inoculation was performed as stated in the protocol above. Once the leaves were inoculated with *B. cinerea*, they were thoroughly sprayed on both sides with one of the treatments then set onto 1 % agar petri dishes and covered in a fume hood at room temperature. The inhibition effect of PvPGIP-secreting yeast is mainly caused by proteins secreted during its growth on the plant. Disease progression was monitored over the course of 3 weeks and photographs were taken for analysis on Fiji (ImageJ) for quantification of diseased tissue. Three replicates (n = 4 leaves each) of each treatment was generated for analysis. Statistical differences were measured using a one-way ANOVA test to obtain P-values.

2.7. Testing the thermostability of PvPGIPs

The temperature stability of exogenously applied PvPGIP2 proteins was evaluated on detached leaf assays. Full length PvPGIP2 and truncated tPvPGIP2_5–8 proteins were incubated at −20 °C, 4 °C, 22 °C, and 42 °C for 24 h. These temperatures were chosen to simulate storing the proteins at temperatures found in freezer, refrigerator, at room temperature, and outside on a hot day. Leaf material and PvPGIP proteins were obtained as described in the protocols above. PvPGIP proteins were placed into microfuge tubes and incubated at their temperatures in a heat block. After incubating for 24 h, the proteins were removed from the heat block and added to a spray bottle. Leaves were inoculated with roughly 20 μL of suspensions containing the spores of *B. cinerea*, then thoroughly sprayed with the PvPGIP2 proteins until coated on both sides, and air dried in a fume hood. Leaves were sprayed once with roughly 20 μL of suspensions containing the spores of *B. cinerea*, then left in a covered petri dish in a fume hood at room temperature for observation. Sets of leaves that contained only *B. cinerea* inoculation and leaves without any treatment or inoculation were used as controls. The percentage of infected leaf tissue was measured 3 weeks post-inoculation using Fiji (ImageJ) software. Three replicates (n = 4 leaves each) of each treatment was generated for analysis. Statistical differences were measured using an ANOVA test to obtain P-values. Next, to test if the amount of time PvPGIP2 proteins were incubated at 42 °C had an effect, the proteins we were incubated at 42 °C for 12 h, 24 h, and 48 h, then sprayed onto the detached leaves from *N. benthamiana* treated with *B. cinerea* until the leaves were completely coated. The controls consisted of leaves without any treatment, and leaves treated with only water and *B. cinerea*. The leaves were observed over a period of 3 weeks at room temperature and the percentage of infected leaf tissue was measured at 7-, 14-, and 21-days post-inoculation using the Fiji (ImageJ) software. Similar to the previous experiment, 3 replicates (n = 4 leaves each) of each treatment were generated for analysis.

As we were unable to consistently induce *A. niger* infection into the *N. benthamiana* leaves, the effect of temperature on the full length PvPGIP2 protein was tested on pectin plate assays for A. *niger*. The pectin medium contains 4g potato flakes (Bob's Red Mill Potato Flakes), 1g citrus pectin (Fischer Scientific, catalog #AAJ6102122), and 2.5g agarose (VWR, catalog #97062-250) into 200 mL of DI water the medium. Once the pectin plates solidified, PvPGIP2 proteins were liberally sprayed onto the pectin plates then let air dry at room temperature in a fume hood. As with the detached leaf assays, PvPGIP2 protein was incubated at 42 °C for 12, 24, and 48 h before application. Once dry, the plates were spotted with 2 μL of *A. niger* spores in 4 locations and left covered in the dark at room temperature. The plates were observed over the course of 5 days and the growth radius of the *A. niger* was visually compared.

2.8. Materials, growth, and infections of cherry tomatoes

PvPGIP activity was tested in post-harvest tomato fruits. The cherry tomatoes (Goodwin's Organic Market) used in this assay were well-

formed without visible blemishes or bruising and had comparable firmness and maturity to each other. The ripe tomatoes were sterilized in a 10 % bleach solution for 10 min, rinsed with DI water 3 times, then pat dried with a paper towel. A sterile pipette tip was used to puncture the tomato at a depth of roughly 3 mm, 6 times on the blossom-end of each tomato. Fruits were inoculated with 2 μL of the *B. cinerea* spore suspension described above and 2 μL of either protein storage buffer (20 mM sodium phosphate, 25 mM NaCl, 10 mM DTT, pH 7.4), purified tPvPGIP2_5–8 protein, or purified full length PvPGIP2 protein at each site. A control with no inoculum or treatment introduced to the wound sites was utilized as well for comparison. Fruits were incubated for 5 days at 22 °C in enclosed, but not airtight, containers and monitored for fungal growth. Each treatment was kept in its own container. Three replicates (n = 4 fruit each) of each treatment was generated. Photos of

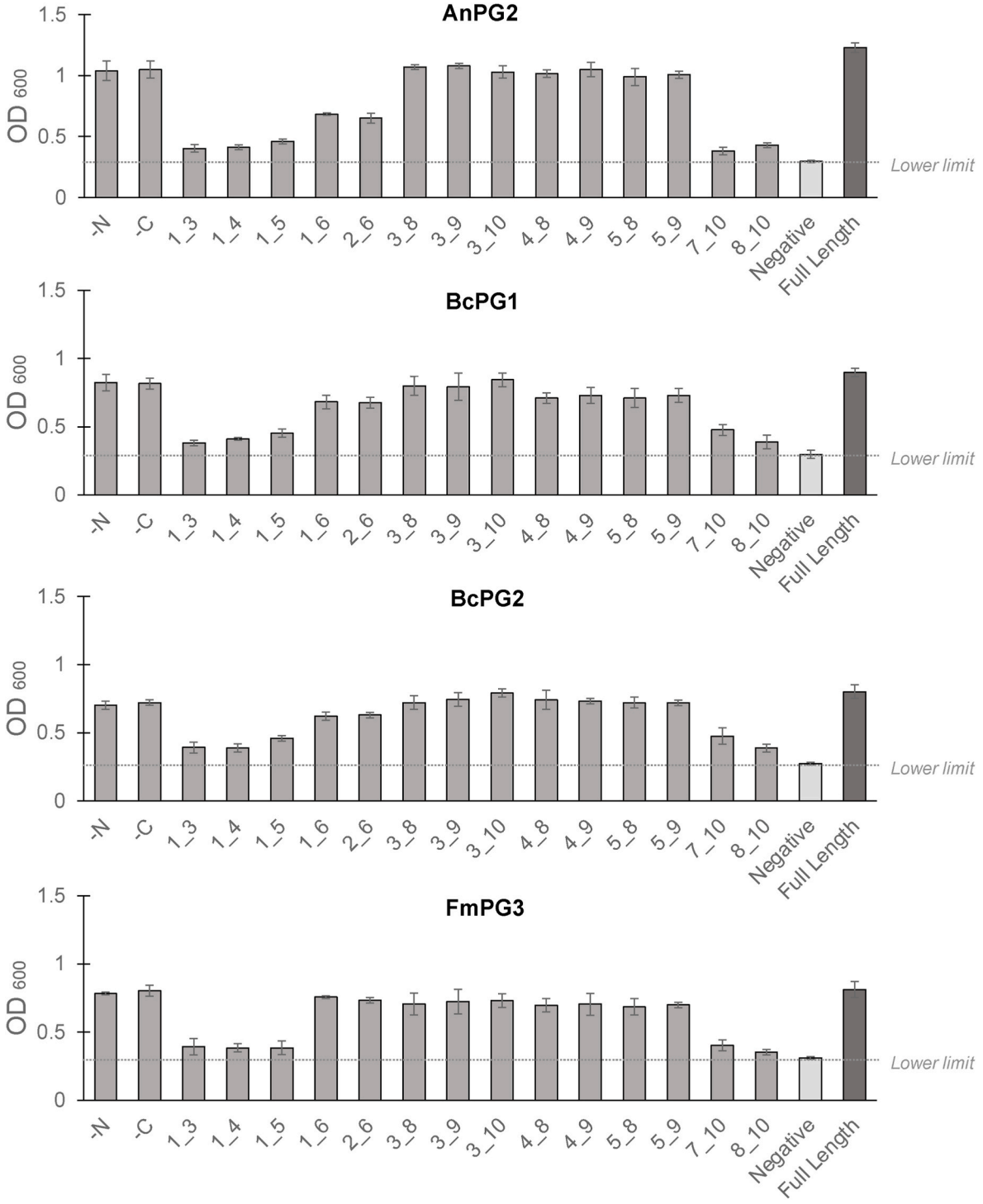

Fig. 1. Truncation of GmPGIP3 partially retains the inhibitory activity towards AnPG2, BcPGs, and FmPG3 as determined by Y2H. Growth curves of yeast strains harboring AnPG2, BcPG1, BcPG2, and FmPG3 with truncated GmPGIP3s lacking the N terminus, C terminus, tGmPGIP3_1–3, tGmPGIP3_1–4, tGmPGIP3_1–5, tGmPGIP3_1–6, tGmPGIP3_2–6, tGmPGIP3_3–8, tGmPGIP3_3–9, tGmPGIP3_3–10, tGmPGIP2_4–8, tGmPGIP3_4–9, tGmPGIP3_5–8, tGmPGIP3_5–9, tGmPGIP3_7–10, and tGmPGIP3_8–10 **(a)** AnPG2, **(b)** BcPG1, **(c)** BcPG2, and **(d)** FmPG3 were measured, and the data summarized from the 48 h point. The lower limit represents the negative control, yeast harboring BD-BcPG2 and AD-HAB1, a protein not known to interact with GmPGIP3.

the fruit were taken at day 5 and analyzed using Fiji (ImageJ) software for analysis on disease incidence and progression.

3. Results

3.1. Assessing the PG interactions of GmPGIP3 protein

Our previous research validated the use of yeast two hybrid (Y2H) assays to estimate the strength of the interactions between PGs and PGIPs [25]. Using a Y2H assay, it was found that truncating both PvPGIP1 and PvPGIP2 down to only the region contained between LRR5 to LRR8 retained a similar level of interaction with the tested PGs from the pathogenic fungi *Aspergillus niger* (AnPG2) and *Botrytis cinerea* (BcPG1, BcPG2) as their full-length counterparts [25]. The region contained between LRR5 to LRR8 is roughly one-third the size of a full-length PvPGIP. This previous research led us to hypothesize that truncating PGIPs is not unique to only those from *P. vulgaris*. Here, Y2H assays were conducted to ascertain if PGIP3 from *Glycine max* (GmPGIP3) would display greater levels of interaction than PvPGIP2 (Fig. S2). Yeast harboring Gal4 AD-GmPGIP3 and any BD-PG overall grew slower and to a lower cell density at the stationary phase compared to PvPGIP2 (Fig. S2). Minimal PG-PGIP interaction was observed when the yeast expressed Gal4 AD-GmPGIP3 and BD-FmPG3. These results are similar to what was found in previous studies utilizing different assays [32,33], and allowed us to compare GmPGIP3 and PvPGIP2 levels of PG interaction for a more direct assessment.

Interactions of PGs with truncated versions of GmPGIP3 (Fig. 1) were then evaluated using the Y2H system. Truncations containing the regions LRR1-3, LRR1-4, LRR1-5, LRR7-10, and LRR8-10 resulted in a near complete loss of inhibitory activity, with truncations at LRR1-6 and LRR2-6 experienced a moderate loss of activity. The other truncated versions of GmPGIP3, including those lacking certain portions (LRR3-8, LRR3-9, LRR3-10, LRR4-8, LRR4-9, LRR5-9, no N terminus, and no C terminus), maintained inhibitory activity similar to the full-length GmPGIP3. Notably, like PvPGIP2, the region in GmPGIP3 from LRR5 – LRR8 is smallest truncation possible to still retain inhibitory levels similar to the full-length version of GmPGIP3. This implies that the ability to truncate the PGIPs to this particular region is not unique to PvPGIP2. Taken together, our results suggest that the regions outside of LRR5 to LRR8 in PvPGIP2 and GmPGIP3 may not be essential for the interaction with PGs. We also noted that the various truncated forms of PGIPs displayed distinct patterns of interaction with different PGs. For example, the N-terminus part of GmPGIP3 seems to play a more important role in the interaction with FmPG3 compared with other PGs. Not only that, but different parts of the PGIPs may be responsible for interacting with different PGs, suggesting a potential avenue for using PGIPs in future research: broader substrate specificity could be achieved through shuffling multiple PGIPs or using a cocktail of PGIPs.

Lastly, an *in vitro* fungal spot assay was performed to validate the inhibitory activity of GmPGIP3 against *A. niger*. Yeast strains were constructed utilizing the *Ost1* secretion signal sequence [34] and fused to PvPGIP2 and GmPGIP3. *S. cerevisiae* CEN.PK2-1D was transformed with plasmids harboring *Ost1*-PvPGIP2 or *Ost1*-GmPGIP3 and spread (roughly 1.2×10^6 cells) onto agar plates amended with 0.5 % citrus pectin (weight/volume). 2 μL of *A. niger* (5×10^5 conidia/mL) was spotted into 4 locations and the growth of fungi was monitored over the course of 5 days. Natamycin, an antifungal agent, was utilized as a positive control at a concentration of 100 mg/mL. Water or yeast strain harboring an empty vector was utilized as negative controls. Plates containing PGIPs delayed sporulation of *A. niger* by 1–2 days compared to the negative controls (Fig. 2), which showed fungal growth at all sites of inoculation on the second day. On days 4 and 5, the difference in growth was visually assessed between the PGIP-treated plates and the negative controls. Both negative controls displayed a wider radius of *A. niger* growth, as well as a taller hyphal height compared to the PGIP-treated plates. Though PGIPs exhibited lower efficacy at fungal

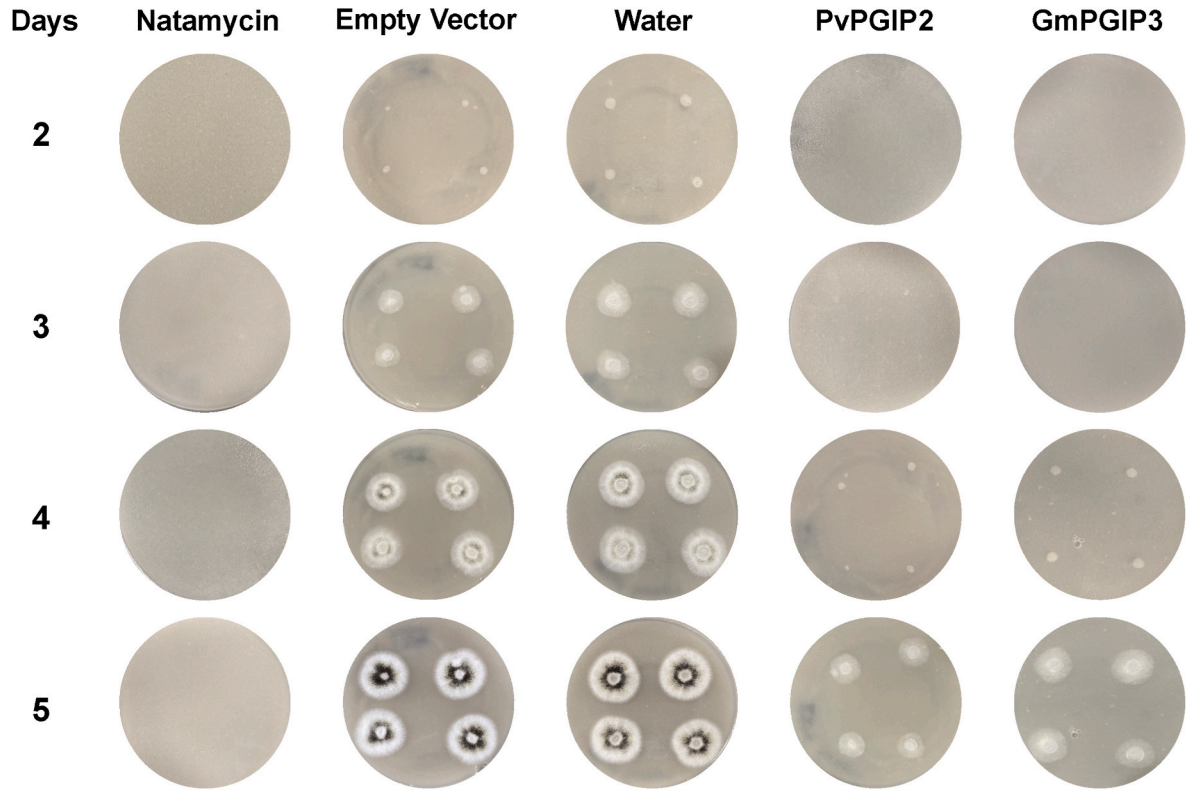

Fig. 2. Fungal spot assay comparing the efficacy of PvPGIP2 and GmPGIP3 with *A. niger*. *A. niger* was spotted onto potato agar plates treated with either natamycin (positive control), empty vector yeast (negative control), water (negative control), PvPGIP2 secreting yeast, or GmPGIP3 secreting yeast. Fungal growth was observed over the course of 5 days.

inhibition compared to natamycin, plates treated with PvPGIP2 and GmPGIP3 showed similar levels of *A. niger* growth which is reduced by nearly half. The results of this fungal spot assay suggest that, like PvPGIP2, GmPGIP3 can be applied exogenously to inhibit the growth *A. niger,* likely by impeding its ability to utilize pectin as its carbon source. The results are consistent with the Y2H assays and demonstrates that while GmPGIP3 is not more effective than PvPGIP2 at inhibiting certain PGs and pathogenic fungi within our tested assays, its ability to interact with PGs and reduce the growth of *A. niger* should still be considered for future applications of PGIPs against pathogenic fungi.

3.2. Determining the concentration of PvPGIP2 proteins for inhibiting B. cinerea in plants

To obtain purified proteins for use in pectin and detached leaf assays, *S. cerevisiae* CENPK2.1D, was used to expressed PvPGIP2 or tPvPGIP2_5–8 proteins by fusing the proteins with 6 × His-tag (Fig. S3). Titrations of PvPGIP2 at different concentrations were sprayed onto *N. benthamiana* leaves treated with *B. cinerea* and observed over the course of 3 weeks. To avoid dehydration of the detached leaves, the leaves were incubated on 1 % agar plates. 2–3 sprays (equivalent to ~500 μL) per leaf of roughly 1.0, 0.75, 0.5, 0.25, and 0.125 mg/mL were used to treat the detached leaves at room temperature and compared with the negative control, leaves containing only the *B. cinerea* spores and no PGIP treatment (Fig. 3a).

At the highest concentrations of ~1.0 mg/mL and ~0.75 mg/mL, only a limited area of necrotic lesion was visually seen at the end of the third week, with an average area of disease at 3.4 % (±2.25 %) and 3.03 % (±3.33 %) respectively as analyzed by Fiji (ImageJ, Fig. 3b). When the concentration of PGIP is ~0.5 mg/mL or lower, infection and leaf degradation is seen by week 2, with substantial and widespread damage to the leaves visible by week 3. An inverse relationship between the

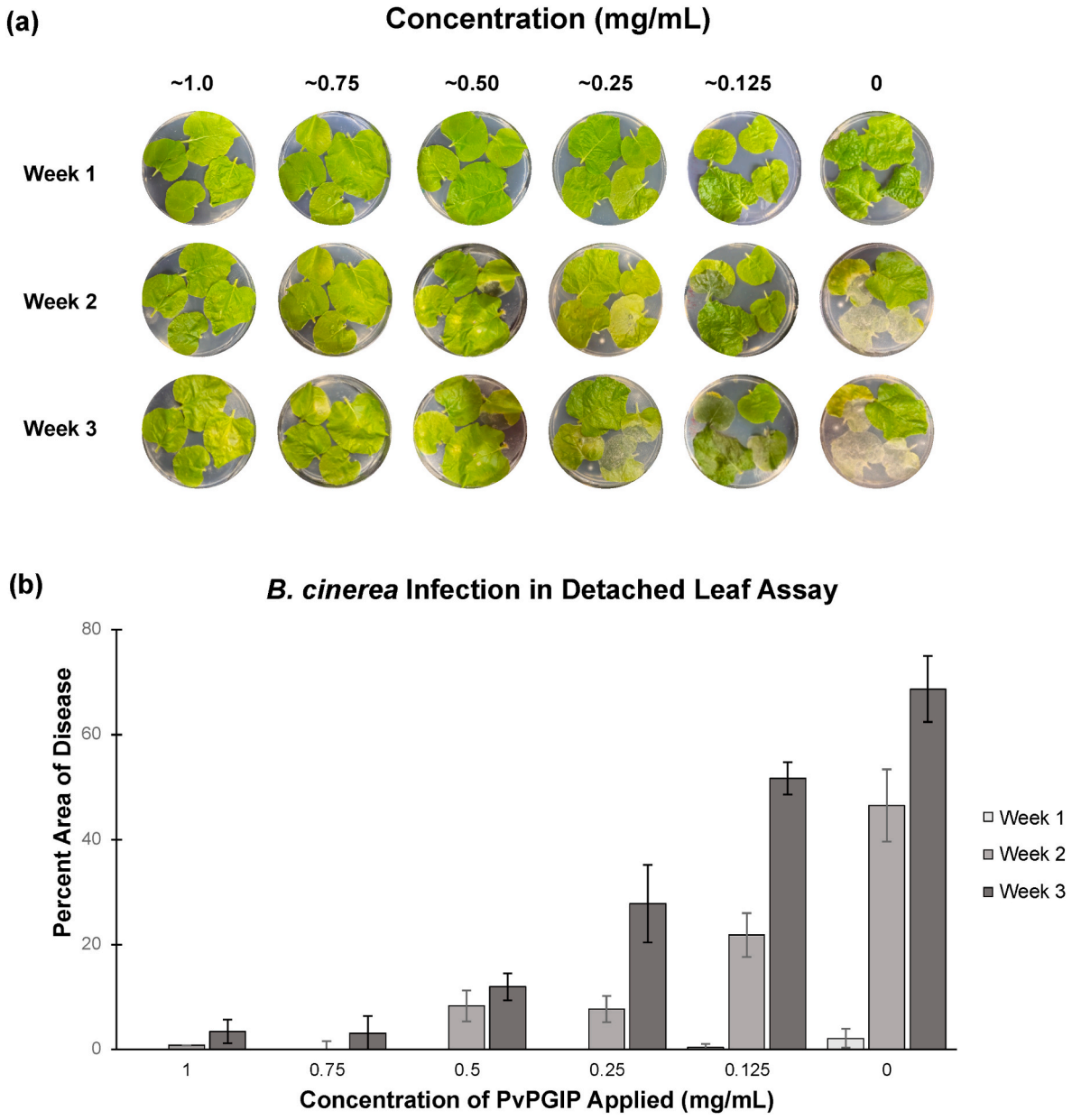

Fig. 3. Determining the dose of PvPGIP2 for inhibiting *B. cinerea* growth on detached leaf. (a) Detached leaf assay utilizing *N. benthamiana* leaves on 1 % agar, spotted with 5 μL of *B. cinerea* spores (2.5×10^6 spores/mL) and treated with 500 μL of roughly 1.0, 0.75, 0.50, 0.25, and 0.125 mg/mL of PvPGIP2 proteins. The PvPGIP2 protein treatments were compared to the negative control of leaves containing *B. cinerea* only. Each plate contained 4 leaves used as biological replicates, and each treatment was repeated three times. **(b)** Quantification of the leaf area covered by necrotic tissue using the image analysis software Fiji (ImageJ).

concentration of PvPGIP2 applied and resultant area of diseased and necrotic tissue was found, where concentrations of 0.5, 0.25, and 0.125 mg/ml of PvPGIP2 applied had 11.90 % (±2.59), 27.77 % (±7.39 %), and 51.67 (3.08 %) of their total leaf area afflicted by disease respectively. However, even at the lowest concentration of PvPGIP2 applied, it still conferred a small, but significant degree of protection against *B. cinerea* (*P value* = 0.001). Leaves treated with ~0.125 mg/mL of PvPGIP2 had reduced levels of infection compared to the negative control, which had an average of 51.67 % (±3.08 %) compared to the negative control's 68.7 % (±6.37 %) area of necrotic tissue. This trial was repeated 3 times (n = 3 to 4 leaves each) for each treatment. Photos were taken of the leaves each week to monitor progress on disease progression. The visual observations were confirmed by quantifying the area of disease on the leaves using the Fiji (ImageJ), an open source, image-processing software. The percent area of disease was graphed over time, which demonstrated that there was a difference between leaves with PvPGIP2 applied, and those without (Fig. 3b).

The efficacy of purified PvPGIP2 on *A. niger* was also tested. However, due to difficulties with consistently inoculating the *N. benthamiana* plants with *A. niger*, where *A. niger* grows faster on the agar plate than on the leaves, *in planta* assays were not conducted. Instead, a simplified fungal spot assay was completed to validate if a concentration of ~1.0 mg/mL of purified PvPGIP2 proteins could slow and reduce the growth of *A. niger* (Fig. S4). Plates containing 0.5 % citrus pectin (weight/volume) were spotted in 4 locations with 2 μL of *A. niger* (5 x 10^5 conidia/mL). 200 μL of the purified PvPGIP2 proteins (~1 mg/mL) was applied to the plates and fungal growth was monitored over the course of 6 days. Similar to results we previously found utilizing PvPGIP2-secreting yeast [25], the purified PvPGIP2 protein was able to delay onset of sporulation by 1–2 days compared to the negative control, and also decreased the

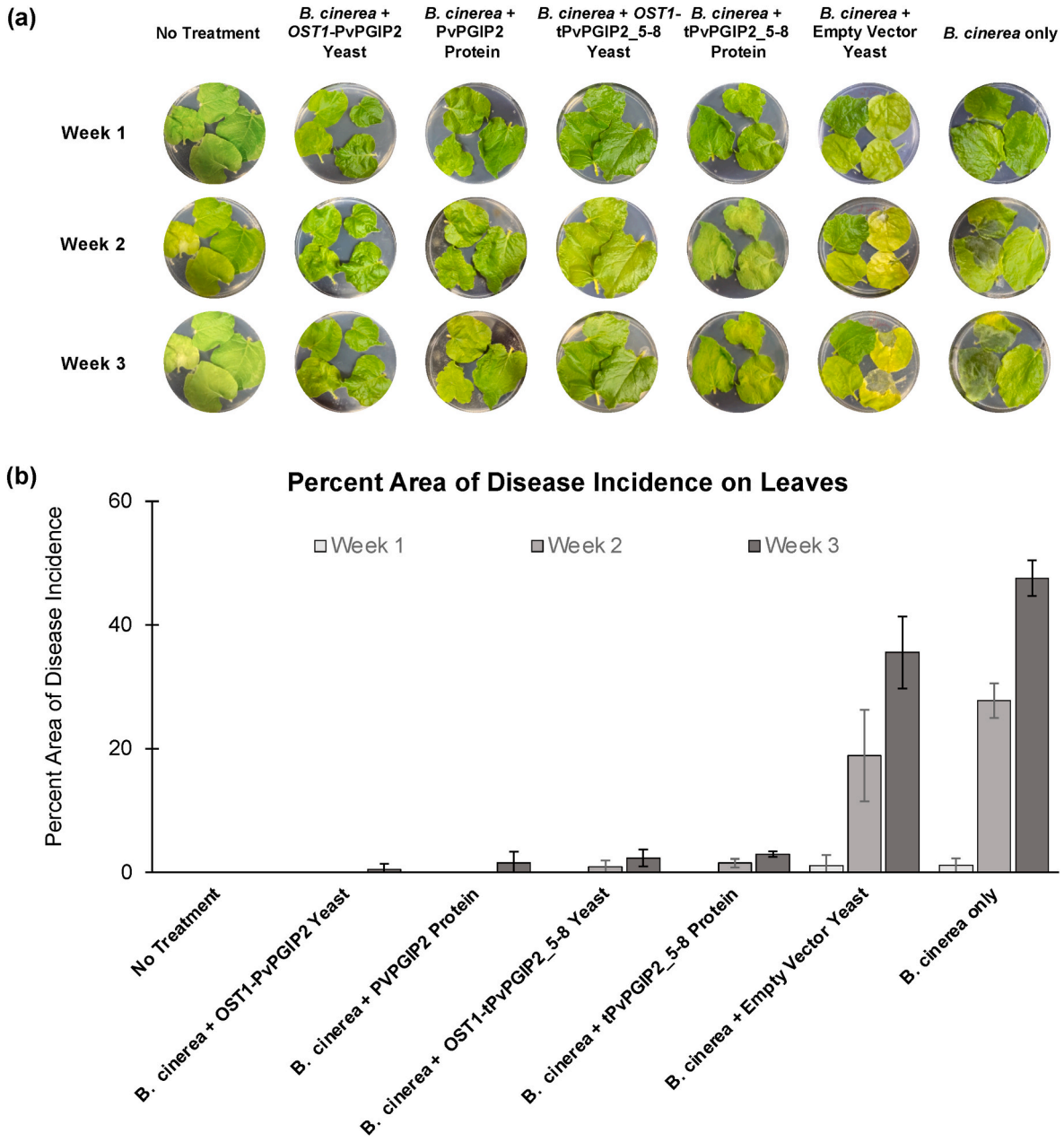

Fig. 4. Comparing the efficacy of PGIP-secreting yeast and purified PGIP proteins on a detached leaf assay with *B. cinerea*. (a) Detached leaf assay utilizing *N. benthamiana* leaves on 1 % agar, spotted with 5 μL of *B. cinerea* spores (2.5 × 10^6 spores/mL) and treated with *Ost1*-PvPGIP2 secreting yeast, PvPGIP2 protein, Ost1-tPvPGIP2_5–8 secreting yeast, tPvPGIP2_5–8 protein, yeast containing an empty vector (negative control), *B. cinerea* only (negative control), and no treatment nor inoculum. **(b)** Quantification of the leaf area covered by necrotic tissue using the image analysis software Fiji (ImageJ).

growth rate of *A. niger*. The radius of the fungal colonies treated with PvPGIP2 proteins was approximately less than half that of those without treatment. The data from the *in planta* and *in vitro* assays combined demonstrates the potential of using purified PvPGIP2 proteins as an exogenously applied fungal control tool.

3.3. Comparing the efficacy of PvPGIP2-secreting yeast and purified PvPGIP2 proteins in plants

After verifying the amount of PvPGIP2 (~100 mg/m^2 on leaf) necessary to inhibit *B. cinerea* growth, we compared the *B. cinerea* inhibition efficacy of yeast secreting PGIPs using the *Ost1* signal and purified PGIP proteins on the detached tobacco leaves (Fig. 4). The titer of PGIP in the yeast medium using the Ost1 signal peptide is roughly 0.065–0.130 mg/mL, which is much lower than the effective concentration of purified PGIP proteins when applied on *N. benthamiana* (>0.75 mg/mL). However, yeast would continuously produce the PGIPs, while the concentration of the applied purified PGIP proteins would remain static. A detached leaf assay was performed comparing leaves infected with *B. cinerea* with no treatment, or treated with PvPGIP2-secreting yeast, PvPGIP2 protein, tPvPGIP2_5-8-secreting yeast, tPvPGIP2_5–8 protein, or yeast containing an empty vector (negative control) Backspace. Each treatment contained 3–4 biological replicates, and each treatment was repeated 3 times. Both negative controls showed notable *B. cinerea* infection with visible necrotic lesions and browning at the second week, with the *B. cinerea*-only leaves displaying a greater severity of disease progression compared to the empty vector yeast treatment (Fig. 4). This may be due to the yeast acting as a physical barrier towards infection. Lesions began at the site of infection and spread rapidly throughout the week. Meanwhile, the plates containing either PvGIP2-secreting yeast or PvPGIP2 proteins did not show any sign of infection on the leaves, even after three weeks had passed. Instead, a minute amount of fungal growth was found on the agar, indicating the presence of fungi. However, this finding further implies the effectiveness of the treatment with either PGIP or PGIP-secreting yeast in safeguarding the leaves against fungal infection. Both the yeast and protein treatments had comparable results, regardless of using full length or truncated versions of PvPGIP2.

Quantification of the areas of necrotic lesion was analyzed using the Fiji (ImageJ) software (Fig. 4), which verified our visual findings that the PGIP-treated tobacco leaves had significantly reduced levels of fungal infection compared to the negative controls, containing less than 10 % of the total infected area found in either negative control by the third week. By the third week, *OST1*-PvPGIP2 secreting yeast, *OST1*-tPvPGIP2 secreting yeast, PvPGIP2 protein, and tPvPGIP2_5–8 protein had an average percent area of infection at 0.50 % (±0.867 %), 2.33 % (±1.78 %), 1.57 % (±1.37 %), and 2.97 % (±0.41 %) respectively, while the empty vector yeast and *B. cinerea*-only treatments had 35.7 % (±12.2 %) and 47.6 % (±2.91 %) respectively. Leaves that contained no *B. cinerea* or any treatment had an expected 0 % area of lesions detected. Tobacco leaves inoculated with *B. cinerea* and treated with PvPGIP proteins or PvPGIP-secreting yeast were not statistically different from one another (*P value* = 0.158), indicating that they likely have similar levels of inhibiting disease progression. Likewise, the negative controls, leaves treated with empty vector yeast with *B. cinerea* and B. *cinerea*-only, were also not statistically different from one another (*P value* = 0.172). All the PvPGIP treatments were statistically different from the negative controls (*P value* = 3.49E-10), which can be inferred that treatment with both full-length and truncated PvPGIPs, regardless of purified forms or from plasmid-transformed yeast, will result in a substantial reduction in *B. cinerea* disease incidence.

3.4. Evaluating the thermostability of PvPGIPs

The thermostability of both the full length PvPGIP2 and tPvPGIP2_5–8 was evaluated. PvPGIPs were held at −20 °C, 4 °C, 22 °C, and 42 °C for 24 h. After incubation, the samples were sprayed onto detached leaves from *N. benthamiana* spotted with *B. cinerea* (Fig. 5). The leaves were compared to the controls of leaves treated with only *B. cinerea* and leaves without any treatment, then observed over a period of three weeks to monitor disease progression. After 3 weeks, the area of disease was analyzed using Fiji (ImageJ; Fig. 5). It was found that there was no significant difference in the percent area of infected tissue found between any of the temperatures for full length PvPGIPs (*P value* = 0.974), with an average percent area of infected tissue found to be 4.03 % (±0.704 %). However, for tPvPGIP2_5–8, while no difference was found for the tPvPGIP2_5–8 treated at −20 °C, 4 °C, and 22 °C (*P value* = 0.782), with an average percent area of infected tissue found to be 5.14 % (±0.851 %), a substantial difference was detected when it was treated at 42 °C (*P value* = 0.003), and the average percent area of disease found was 14.7 % (±4.69 %). No significant difference was found between the full-length or truncated PvPGIPs between the −20 °C–22 °C range (*P value* = 0.239). However, despite the reduction in efficacy for the truncated PvPGIP2 when it is heat treated to 42 °C for 24 h, its application still results in significant inhibition of *B. cinerea* infection compared to no PvPGIP treatment (*P value* = 4.4183 × 10^{-8}). Leaves inoculated with the pathogenic fungi that lacked PvPGIPs had an average of 70.09 % (±6.26 %) coverage of infected tissue, which is more than triple the disease found from the heat-treated truncated tPvPGIP2_5–8.

Next, the PvPGIPs were incubated for different time periods at 42 °C to study how their efficacies against the pathogenicity of *B. cinerea* would be affected over time. Full-length PvPGIP2 and truncated tPvPGIP2_5–8 purified proteins were incubated at 42 °C for 12 h, 24 h, and 48 h, then sprayed onto detached leaves from *N. benthamiana* treated with *B. cinerea*. The leaves were then compared to leaves treated with only *B. cinerea* and leaves without treatment (Fig. 6). Both the full length and truncated PvPGIP2s delayed visible onset of *B. cinerea* infection by at least one week regardless of how long the proteins were incubated for at 42 °C. Both full-length and truncated PvPGIP2s had an average area of disease at less than 0.5 % while the leaves treated with only *B. cinerea* had an average of 3.62 % (±2.36 %). Both the full length and truncated PGIPs demonstrate great efficacy at delaying *B. cinerea* growth for two weeks at all temperature incubations, with the full-length PvPGIPs showing an average of 1.44 % (±1.14 %) and tPvPGIP2_5–8 displaying 4.91 % (±3.44 %) of the total leaf area covered by necrotic lesions compared to the 36.08 % (±7.166 %) from the control lacking any PGIP treatment. During week 3, the full-length PvPGIP2 protein that had been treated for 48 h displayed highly visible signs of necrotic lesion expansion on all leaves, with an average area of disease at 12.86 % (±4.57 %), while those treated for only 12 or 24 h accumulated 3.27 % (±2.19 %) and 5.60 % (±1.83 %) respectively. Meanwhile, tPvPGIP2_5–8 had 3.03 % (±1.43 %), 9.63 % (±3.43 %), and 27.27 % (±4.51 %) for 12, 24, and 48-h heat treatment respectively. Despite the decrease in efficacy in PvPGIPs treated at 42 °C for 48 h, all treatments with full-length or truncated PvPGIPs show a significant reduction in *B. cinerea* infection compared to the negative control, which resulted in 70.15 % (±7.39 %) of the total leaf area covered in necrotic tissue at the end of 3 weeks (*P value* = 1.259E-10).

As we were unable to consistently induce *A. niger* infection into the *N. benthamiana* leaves, the effect of temperature on the full length PvPGIP2 protein was tested on pectin plate assays for A. *niger* (Fig. S5). As with the detached leaf assays, PvPGIP2 protein was incubated at 42 °C for 12, 24, and 48 h before it was liberally sprayed onto pectin plates that were spotted with 2 μL of *A. niger* spores (5 × 10^5 conidia/mL) in 4 locations. The plates were observed over the course of five days. The results show that PvPGIP2 still retains some inhibitory activity against *A. niger* at all temperatures tested and treated for. Treating the protein at 42 °C for 24 and 48 h appears to slightly decrease PvPGIP2′s fungal-inhibiting activity compared to treatment at 12 h, which had similar results to PGIPs left at room temperature. All heat treatments resulted in a reduction of lesion expansion by greater than 50 %

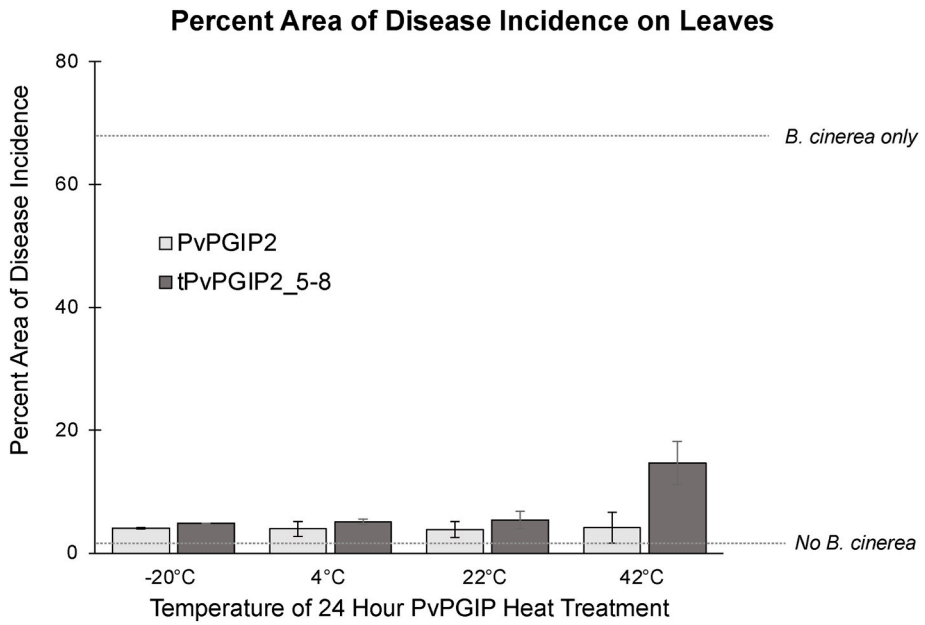

Fig. 5. Comparing the efficacy of PvPGIP2 and tPvPGIP2_5–8 proteins treated at different temperatures for 24 h on a detached leaf assay with *B. cinerea*. A detached leaf assay was performed comparing leaves inoculated with *B. cinerea* and treated with PvPGIP2 and tPvPGIP2_5–8 proteins treated at −20 °C, 4 °C, 22 °C, and 42 °C for 24 h. The lower limit consists of leaves that lacked any treatment and were not inoculated with *B. cinerea*, while the upper limit control are leaves without treatment and inoculated with *B. cinerea*. Each treatment contained 3–4 leaves as biological replicates and was repeated 3 times. Quantification of the area of disease was analyzed using Fiji (ImageJ).

compared to the negative control, which only had *A. niger*. Though prolonged incubation at 42 °C may slightly reduce the efficacy of PvPGIP2 against *A. niger,* PvPGIP2 still retains a notable amount of inhibitory activity.

4. Examining PvPGIP2 activity on tomato fruit

Furthermore, we evaluated the effectiveness of PGIPs on fruit to validate their application in postharvest crops. The efficacy of purified full-length PvPGIP2 and truncated tPvPGIP2_5–8 proteins was assessed against the growth of *B. cinerea* on post-harvest cherry tomato fruits (Fig. 7). The fruits were sterilized and wounded 6 times at the blossom end. They were then inoculated with 2 μL of a *B. cinerea* spore suspension (2.5×10^6 spores/mL) at each wound site as well as 2 μL of treatments. The treatments were either protein storage buffer (used as a control), purified tPvPGIP2_5–8 protein (~1 mg/mL), or purified PvPGIP2 protein (~1 mg/mL). An additional control with wounding, but no inoculum nor treatment was used for comparison. After 5 days, disease incidence and development were analyzed for each treatment and photos of the tomatoes were taken to quantify the percent area of fungal growth using Fiji (ImageJ; Fig. 7). Disease incidence was calculated as the average number of wounding sites that showed fungal sporulation, and area of infection was calculated using Fiji. It was found that wounding-only resulted in little to no disease. When inoculated with the controls *B. cinerea* only or *B. cinerea* with storage buffer, there was a 100 % (±0 %) rate of disease incidence after 5 days, showing fungal growth and necrotic tissue in 24 % (±6.3 %) and 26 % (±5.4 %) of the visible area, respectively. In contrast, both full-length and truncated PvPGIP2s had a reduction in incidence, with an average of 32 % (±12 %) and 50 % (±19 %) disease incidence, and area of disease to be 5.8 % (±2.1 %) and 13 % (±3.9 %) respectively. The application of purified tPvPGIP2_5–8 results in greater disease incidence and greater lesion expansion and hyphal growth compared to the full-length PvPGIP2 (*P value* = 0.014). It's important to note that while the truncated PvPGIPs are less effective than the full-length PvPGIPs, they still manage to reduce the area of disease and disease incidence by half compared to no treatment at all, which represents a significant reduction. Both PvPGIP treatments have a statistically significant effect on reducing *B. cinerea* growth in the tomato fruit (*P value* = 9.5814E-20), suggesting that application of the purified PvPGIPs has a protective effect against the necrotic pathogen and can retard its growth and disease progress.

5. Discussion

The agricultural industry is constantly under threat from pathogenic fungi. Outside of major economic losses, up to 10–15 % and 20–40 % of fruits and vegetables in developed and developing countries respectively never make it to the consumer due to postharvest disease and decay, and 8.9 % of the population suffer from hunger and food management [1]. Though the development of chemical fungicides has substantially helped with food security in the past century and continues to be a reliable solution to treating and preventing fungal diseases, chemical fungicides carry negative impacts on human health and the environment. GM crops have also greatly contributed to increasing crop yield and disease resistance, but concerns over their environmental impact and negative stigma have led some of the public to reject the usage of GMOs. The risks of these fungal control methods have made it increasingly necessary to seek alternative, sustainable approaches to protect our crops.

Our previous research demonstrated that the interactions between PGs and PGIPs can be monitored using a yeast two hybrid system (Y2H) [25], and truncating both PvPGIP1 and PvPGIP2 down to only the region contained between LRR5 to LRR8 retained a similar level of interaction with the tested PGs from the pathogenic fungi *Aspergillus niger* (AnPG2) and *Botrytis cinerea* (BcPG1, BcPG2) as their full-length counterparts [25]. The region between LRR5 to LRR8 is roughly one-third the size of a full-length PvPGIP. Here, we demonstrated that the ability to truncate PGIPs and still remain effective is not unique to only PvPGIP2. GmPGIP3 was also successfully truncated down to one-third its size to the region between LRR5 – LRR8 while retaining fungal inhibition against *B. cinerea* and *A. niger* in Y2H and pectin plate assays. Like tPvPGIP2_5–8, tGmPGIP3_5–8 had strong interactions with AnPG2 and BcPG1, with moderate or low interactions with BcPG2 and FmPG3 in the Y2H assay. The similarities in the results of tGmPGIP3_5–8

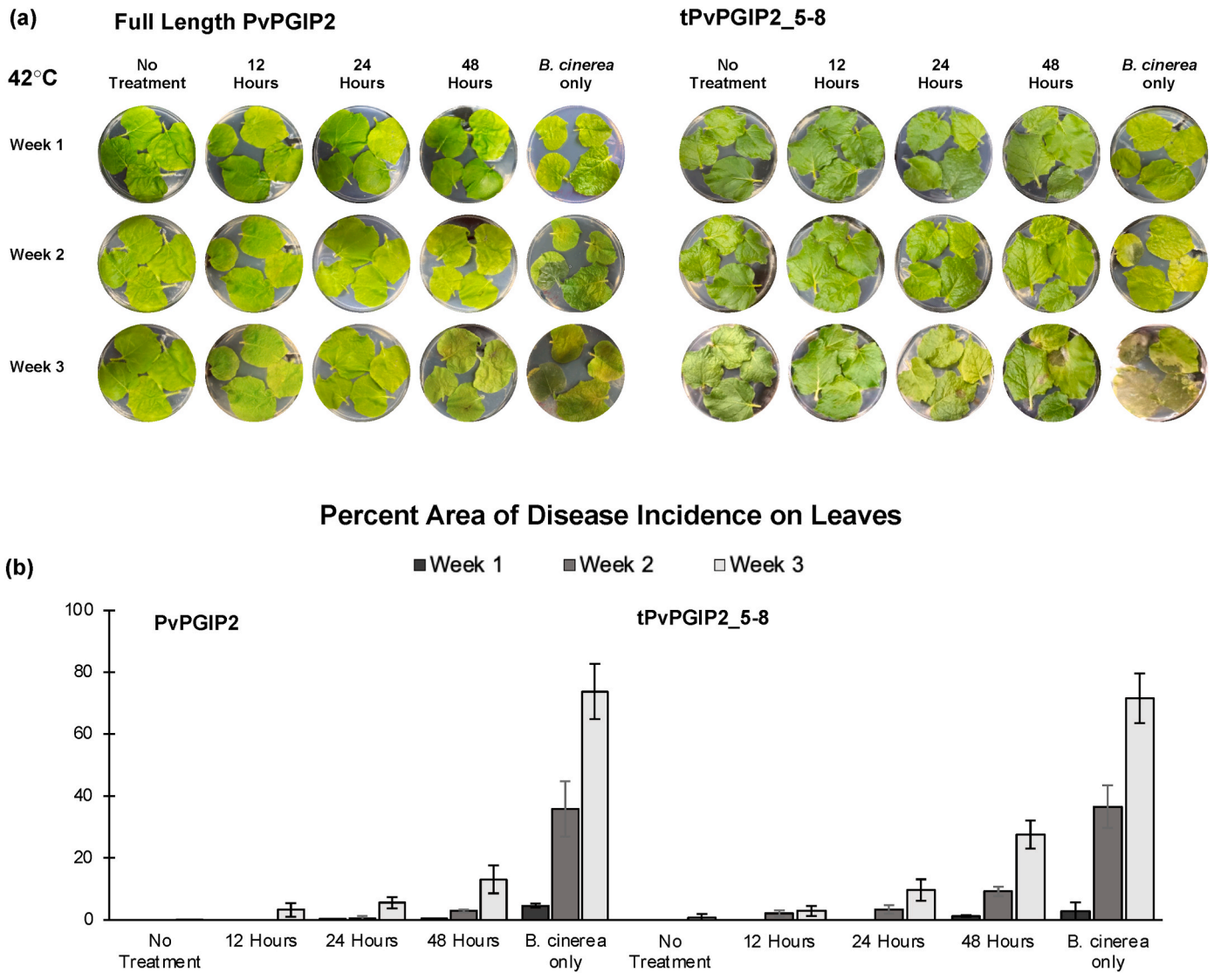

Fig. 6. Comparing the efficacy of PvPGIP2 and tPvPGIP2_5–8 proteins treated at 42 °C **for different lengths of time on a detached leaf assay with *B. cinerea*.** **(a)** Detached leaf assay utilizing *N. benthamiana* leaves on 1 % agar, spotted with 5 μL of *B. cinerea* spores (2.5×10^6 spores/mL) and treated with PvPGIP2 and tPvPGIP2_5–8 proteins treated at 42 °C for 12, 24, and 48 h. The controls are leaves that lacked any treatment and were not inoculated with *B. cinerea* (negative), while the upper limit control are leaves without treatment and inoculated with *B. cinerea* (positive). Each treatment contained 3–4 leaves as biological replicates and was repeated 3 times. **(b)** Quantification of the leaf area covered by necrotic tissue using the image analysis software Fiji (ImageJ).

to tPvPGIP2_5–8 is not unexpected, as these PGIP proteins have highly conserved regions and the two homologous proteins have an 88 % sequence similarity despite hailing from two different genus [33]. The region between LRR5 to LRR8 houses the optimal docking area (ODA) for PvPGIP2 [35], and though GmPGIP3 does not yet have a crystal structure elucidated, it can be inferred from this data that LRR5 to LRR8 may also be the location of the ODA for GmPGIP3. The ODA is where a high propensity of the PGIP protein surface residues physically interact with the active site of the PGs for competitive inhibition, and is a key component for recognition of PGs [36]. Our data suggests that areas exclusive of the ODA likely play a relatively minor role in PG interaction and recognition, though these other LRRs may still be important for thermostability, plant immune response, or other functions we are not yet aware of.

Additional research into PGIPs from other plant species may be beneficial for targeting pathogenic fungi that secrete different PGs. For example, CaPGIP1 and CaPGIP2 from peppers (*Capsicum annum* L.) have shown inhibition against PGs from *Alternaria alternata*, a common pathogenic fungus found on fruits around the world, and *Colletotrichum nicotianae*., a phytopathogenic fungi commonly found in tobacco seed beds [37]. Likewise, PGIPs from leeks (*Allium ampeloprasum* L.) have shown efficacy against a wide range of *Fusarium* species [38]. There are a large number of PGIPs found to inhibit PGs that PvPGIP2 has not been known to affect [39], and better understanding the ODAs and the amino acids involved in the PG active-site interactions of these different PGIPs could lead to better engineering of PvPGIP2 for a broader spectrum of activity and improved efficacy against PGs. For example, truncated forms of PGIPs may potentially have a lower cost of production compared to their full-length counterparts, as smaller proteins may potentially be easier to engineer and produce [40]; and thus creating a cocktail of truncated PGIPs for exogenous application on crops could allow for a more effective and broad-spectrum fungal control agent. However, though PGIPs are naturally occurring in all plants [41], and no negative effects are currently known [39], it is not yet known if truncating PGIPs may change the way they interact with the environment or if they may impact beneficial fungi.

In addition, we also confirmed that the level of fungal inhibition seen by PGIPs is likely dose dependent. An inverse relationship between the

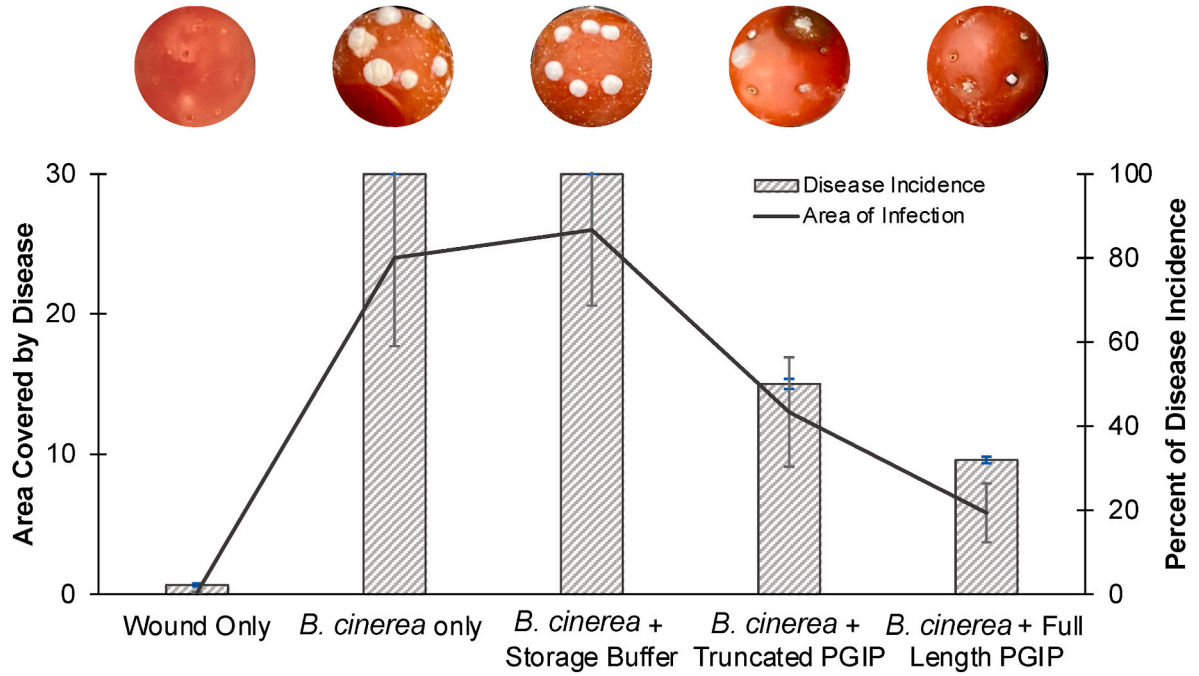

Fig. 7. A tomato fruit assay comparing disease incidence and area of disease caused by B. cinerea. Tomatoes were wounded without inoculation (negative control), inoculated with *B. cinerea* only (positive control), *B. cinerea* + storage buffer (positive control), *B. cinerea* + tPvPGIP2_5–8 protein, and *B. cinerea* + full-length PvPGIP2 protein. Each treatment contained 4 tomato fruit as biological replicates and was repeated 3 times. Quantification of the area of disease was analyzed using Fiji (ImageJ).

concentration of PGIP and the amount of fungal infection was seen, with the highest concentrations displaying minimal disease at the end of the third week and increasing areas of necrotic lesions as PvPGIP2 concentrations decreased. However, it appears that even at the lowest concentration of PvPGIP2 tested, it still confers some degree of protection against *B. cinerea* compared to the negative control. PvPGIP2 should still be further engineered to inhibit PGs at lower concentrations through structure-based rational design, random mutagenesis, and directed evolution. In addition to PvPGIP2, other plant proteins may be more effective as an antifungal tool due to their efficacy at lower doses. For example, the antifungal plant protein chitinase, from *Trichosanthes dioica,* was found to completely inhibit *Trichoderma* species at 15 μg/mL and 60 % or 30 % of *A. niger* at 30 and 15 μg/mL respectively [42]. Likewise, osmotin-like protein from *Solanum nigerum* can inhibit growth of *Fusarium solani f. sp. glycines*, *Macrophomina phaseolina*, *Collectrichum glaesporioides*, and *Collectrichum gossypii* var. *cephalosporioides* with concentrations between 0.1 μg/μL to 0.3 μg/μL [43]. On top of decreasing the necessary concentration for fungal inhibition, it will be important to develop a productive and cost-effective method of mass-producing PGIP, possibly through improving microbial factories for improved secretion or metabolism [44]. In addition to purified protein, we also validated the efficacy of PvPGIP2-secreting yeast strains as a more time and cost-efficient method of controlling fungal infections on plants, though the public may be apprehensive in regard to consuming produce sprayed with yeast.

Our prior results demonstrated that full-length and truncated PvPGIP2s retained their fungal-inhibiting abilities at room temperature, but the effects of a higher temperature were yet unknown. The purified PvPGIP2 proteins were incubated at −20 °C, 4 °C, 22 °C, and 42 °C for 24 h, then sprayed onto detached tobacco leaves inoculated with *B. cinerea*. 20 °C and 4 °C were chosen to simulate common freezer and refrigerator conditions, 22 °C for room temperature, and 42 °C was chosen to simulate a hot day, as the proteins would theoretically be used in the agricultural setting with variable weather. While our results did not find any significant differences in the area of infected tissue between any of the temperatures for full-length PvPGIPs when treated for 24 h, tPvPGIP2_5–8 did show a difference when treated at 42 °C with a significant decreased disease reduction efficacy. This suggests that regions outside LRR5 to LRR8 may be necessary for structural integrity and thermostability, though not directly involved in PG-inhibition. While no significant effect on full-length PvPGIP2 was seen when treated at 42 °C for 24 h, prolonged treatment for 48 h at 42 °C did reduce the efficacy of *B. cinerea* inhibition for the full-length PvPGIP2 by week 3. Overall, freezing, refrigerating, and keeping the PGIPs at room temperature had little to no effect on either the full-length of truncated tPvPGIP_5–8 both *in vitro* against *A. niger* and *in planta* against *B. cinerea*. Although the truncated form may lose some functionality in hot weather after 24 h and the full-length after 48 h, application of tPvPGIP2_5–8 still reduces the area of diseased tissue compared to the negative control. This suggests that exogenously applied full-length and truncated PvPGIP proteins can be kept at a wide range of temperatures and still reduce *B. cinerea* disease progression in tobacco leaves. However, further studies in field trials and a variety of weather conditions against different pathogenic fungi will still be necessary to better access the thermostability of PvPGIP2. Additionally, storage and application procedures for PGIPs will need to be optimized.

B. cinerea is a necrotrophic fungal pathogen that can infect over 200 species [45], and tomatoes (*Solanum lycopersicum*) are one such fruit that is susceptible to *B. cinerea* infection [46]. Application of both full-length PvPGIP2 and truncated tPvPGIP2_5–8 to wounded cherry tomatoes inoculated with *B. cinerea* reduced disease incidence and the degree of fungal expansion compared to the negative controls. Despite the easier access to additional carbon sources and sugars within the tomato, the PGIPs were still able to reduce growth of pathogenic fungi. In the future, other postharvest fruit should be tested. Fruits with waxy or tough exteriors may not benefit as readily from exogenous application of PGIPs. In our experiments, wounding of the tomatoes was a requirement for infection to occur. Without punctures, the tomato would soften and overripen at room temperature before disease could occur on even the negative controls, which prevented the assessment of PGIPs on PGs. This suggests that further testing of PGIPs should be done on soft fruits that lack a waxy skin, such as berries, or developing fruit still attached to the

parent plant, as this would give the fungi and PGIPs more time to work before overripening. Alternatively, application of PGIPs on wounded fruit may give insight on how damaged fruits treated with PGIPs may help reduce the likelihood of disease spreading to healthy fruits. Post-harvest diseases can cause up to 20 % of fruit loss in commercial storage conditions [47], and reducing the growth rate of pathogenic fungi on these fruits could reduce the loss of crops.

6. Conclusion

In this study, GmPGIP3 was successfully truncated to roughly one-third its size, containing only LRR5 to LRR8, much like the truncated PvPGIP2 that was previously established. This shows that truncation of PGIPs is not unique to PvPGIP2, and these smaller proteins still retain similar levels of PG-interaction as their full-length counterparts. Additionally, both full-length and truncated PvPGIP2 were tested *in-planta* on detached tobacco leaves, successfully reducing the expansion of necrotic lesions caused by *B. cinerea*. Both yeast-secreted and purified protein versions of PvPGIP2 showed favorable results in decreasing the percent area of diseased tissue. Furthermore, the full-length purified proteins were effective after being incubated at −20 to 42 °C for 24 h, and still retained some functionality even after incubation for 48 h at 42 °C. The truncated tPvPGIP2_5–8 was effective at reducing the area of necrotic tissue compared to the negative controls from −20 to 22 °C, and had reduced efficacy at 42 °C. Both full-length and truncated PvPGIP2s reduced *B. cinerea* disease incidence and area of disease in postharvest tomato fruit. These key findings suggest that there are many more PGIPs that can still be truncated in proximity to their ODA and retain functions similar to their full-length versions. PvPGIP2 is effective at a range of temperatures and can inhibit *B. cinerea* growth *in planta* and *A. niger in vitro*. This *in-planta* data is one step closer to validating the efficacy of these proteins for exogenous application on plants against PGs.

Currently, eco-friendly solutions for crop protection and fungal control are limited compared to chemical fungicides. Naturally occurring plant proteins such as PGIPs have untapped potential that can be further explored for utilization in the agricultural industry. Though additional research is needed to improve production of PGIPs for mass production, this plant protein can be an eco-friendly and sustainable way of controlling fungal diseases. It is broad spectrum, more socially acceptable than GMOs, and can be exogenously applied to both plants and postharvest fruits. This study showcases that the application of PvPGIP2s can reduce disease incidence and progression of pathogenic fungi. While more studies are needed to improve its yield and prepare PGIPs for mass production, the engineering of PGIPs is one step closer to developing an eco-friendly pest control tool for a greener future. This work not only validates the efficacy of PGIP in combating fungal infections in plants but also highlights the potential of using plant-derived antifungal proteins as alternative strategies to tackle diverse fungal infections in agriculture [20].

Additional information

Competing financial interests: The authors declare that there is no conflict of interest regarding the publication of this article.

CRediT authorship contribution statement

Tiffany Chiu: designed the experiments, performed the experiment and analyzed the results, wrote the manuscript. **Yanran Li:** conceived the project, designed the experiments, analyzed the results, and wrote the manuscript.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.synbio.2024.04.002.

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