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Engineering of Polygalacturonase Proteins as a Novel and Eco-Friendly Pest Control Strategy

A Dissertation submitted in partial satisfaction
of the requirements for the degree of

Doctor of Philosophy

in

Genetics, Genomics, and Bioinformatics

by

Tiffany Mann-shan Chiu

March 2023

Dissertation Committee:
Dr. Yanran Li, Chairperson
Dr. Hailing Jin
Dr. Alexander Putman

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2023

The Dissertation of Tiffany Mann-shan Chiu is approved:

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Committee Chairperson

University of California, Riverside

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ABSTRACT OF THE DISSERTATION

Engineering of Polygalacturonase Proteins as a Novel and Eco-Friendly Pest Control Strategy

by

Tiffany Mann-shan Chiu

Doctor of Philosophy, Graduate Program in Genetics, Genomics, and Bioinformatics
University of California, Riverside, March 2023
Dr. Yanran Li, Chairperson

Fungal pathogens induce a variety of diseases both in plants and post-harvest food crops, resulting in significant crop losses for the agricultural industry. Although the usage of chemical-based fungicides is the most common way of controlling these diseases, they damage the environment, have the potential to harm human and animal life, and may lead to resistant fungal strains. As such, there is an urgent need for diverse and effective agricultural fungicides that are environmentally- and eco-friendly. In plants, polygalacturonase-inhibiting proteins (PGIPs) play critical roles for resistance to fungal disease by inhibiting the pectin-depolymerizing activity of endopolygalacturonases (PGs), one type of enzyme secreted by pathogens that compromises plant cell walls and leaves the plant susceptible to disease.

Here, the interactions between PGIPs from *Phaseolus vulgaris* (PvPGIP1 and PvPGIP2) and *Glycine max* (GmPGIP3), and PGs from *Aspergillus niger* (AnPG2), *Botrytis cinerea* (BcPG1, BcPG2), and *Fusarium moniliforme* (FmPG3) were reconstituted through a yeast two hybrid (Y2H) system to investigate the inhibition efficiency of various

PvPGIP1 and 2 truncations and mutants. Investigation on the sequence-function relationships of PvPGIP utilizing a combination of site directed mutagenesis and a variety of peptide truncations suggests that LRR5 could have the most essential structural feature for the inhibitory activities. This suggests it may be a possible target for the future engineering of PGIP with enhanced activity.

We found that tPvPGIP2_5-8 and tGmPGIP3_5-8, which contains LRR5 to LRR8 and is only one-third the size of the full-length peptide, exhibits the same level of interactions with AnPG and BcPGs as the full-length PvPGIP2 and GmPGIP3 via Y2H. On pectin assays, application of both full length PvPGIP2 and tPvPGIP2_5-8 secreting yeast or purified PGIP proteins clearly slows down the growth of *A. niger* and *B. cinerea*. On plant assays utilizing detached leaves from *N. benthamiana*, application of both full length and tPvPGIP2_5-8 secreting yeast or purified proteins reduced the growth and infection rate of *B. cinerea.* Additionally, PvPGIP2 remains thermostable up to 42◦C and retains its inhibitory activity against *B. cinerea* on pectin assays and *in planta* assays, delaying growth of the pathogenic fungi by up to one week.

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Introduction

1.1 Problem Statement: Conventional Pesticide and Fungal Control

Pathogenic fungi have long been a consistent source of devastation for the agricultural industry[1]. Approximately 16% of the world's crops are lost to microbial diseases, with an estimated 70-80% of these losses caused by fungi [2]. The range of tactics used to manage fungal pathogens include host resistance breeding [3] and plant protection[4,5]. Transgenic technology has led to the development of crops with desirable traits, such as improved flavor[6], increased yield[7], and superior disease resistance[8] compared to non-modified crops. Notably, the use of transgenic crops permits for a significant reduction in the quantity of phytosanitary product applied to the field[9]. However, the public is often apprehensive about the safety of genetically modified organisms (GMO) and has difficulty accepting genetically modified (GM) crops[10]. For example, some consumers believe that GM crops carry more risks than benefits and are willing to pay a premium for foods labeled non-GMO[11]. Likewise, since 2001, the EU has placed a *de facto* moratorium on approvals of GMOs[12]. Another major concern includes the potential that transgenic crops could damage the ecosystem in unpredictable ways. GMOs can invade ecosystems due to an increase in stress tolerance, causing wild plants to become weeds through horizonal gene transfer[13], or produce toxic substances to pests that may affect nontarget organisms[14]. Recently, increases in pest resistance towards GM crops have also posed problems to the durability of current transgenic crops[15].

Aside from the usage of transgenic plants, plant protection has been used in fungal

control, greatly reducing the loss of crops while improving yields[16]. For example, the United States agricultural industry applies over 108 million pounds of fungicides, costing roughly $880 million annually, but in turn, gains $12.8 billion due to the increased production value from the control of plant diseases[17]. Although fungicides can efficiently reduce yield loss and improve food security, concerns about their non-target effects may affect their availability or utility in the future. Fungicides with relatively higher potential for mammalian toxicity may have limits placed on their use to mitigate exposure of applicators and field workers[18], and to ensure residue on harvested commodities remains below safe levels[19,20]. Fungicides may also negatively impact non-target organisms in agroecosystems, such as soil microbiota involved in nutrient cycling or plant symbionts[21], or insect symbionts[22]. Under certain conditions, fungicides may also be transported off-site following application via runoff or leaching and negatively impact aquatic organisms[23]. In addition to non-target effects,, the development of fungicide resistance in pathogen populations threatens to erode the efficacy of fungicides against several important pathogen[24]. A more socially and environmentally sustainable method to control fungal plant pathogens is needed.

Thus, it is necessary to seek alternative antifungal agent candidates to use as conventional fungicides. These alternative candidates should be environmentally friendly and potentially have fewer negative non-target impacts than conventional fungicides. Plants have evolved diverse mechanisms to defend against fungal infections, as summarized in Figure 1.1, with one important route utilizing the secretion of proteins to delay fungal infection or inhibit fungal growth. These plant antifungal proteins are

promising candidates since they are generally nontoxic to humans and antagonistic microorganisms, and most importantly, have evolved for hundreds of millions of years as defenses against specific phytopathogenic fungi[25].

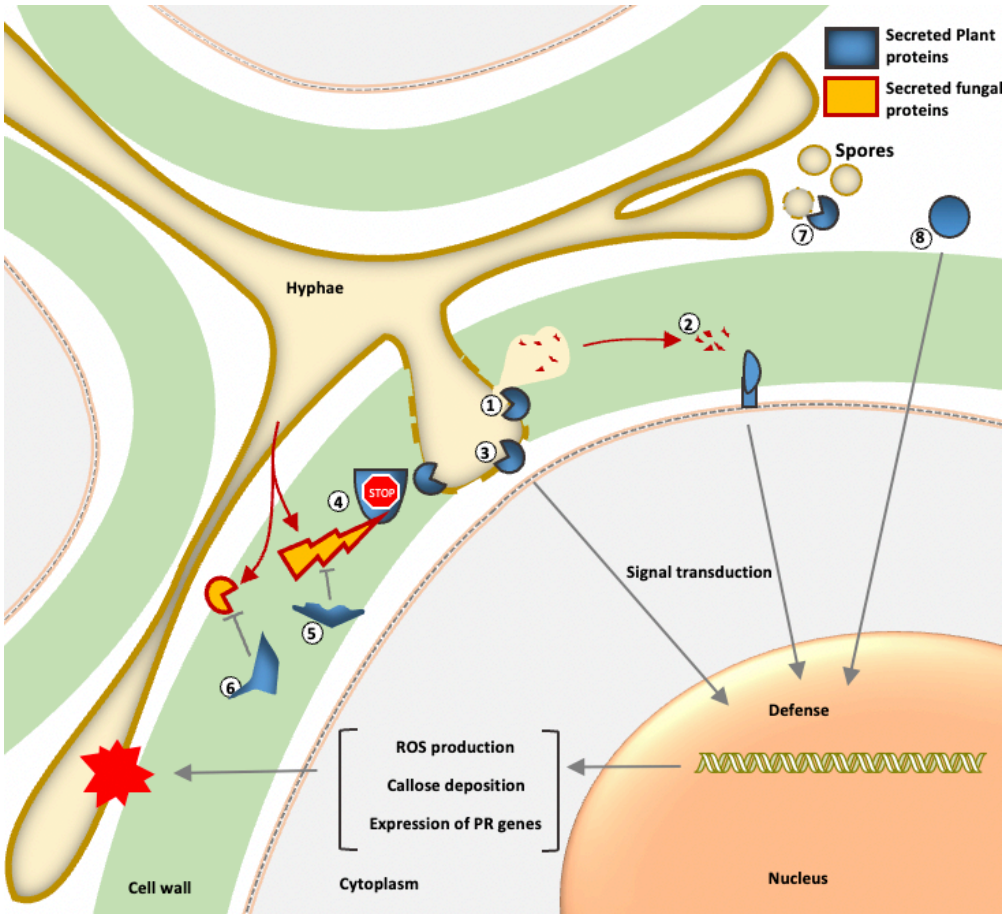

Figure 1.1 Mode of actions of secreted plant antifungal proteins with potential agricultural applications. 1) Secreted antifungal proteins reduce fungal hyphae growth by compromising the fungal cell wall and membrane integrity, leading to potential cytoplasmic leakages[26]. 2) Antifungal protein activity generates residues considered as microbe-associated molecular pattern molecules that can be recognized by plant receptors to stimulate plant immune response[27]. 3) Plant antifungal proteins, upon interacting with the target, directly stimulate plant immune response[28]. 4) Plant secreted proteins protect antifungal proteins from cleavage by fungal protease[29]. 5) Plant secreted proteins actively inhibit fungal protease[30]. 6) Inhibition of fungal cell wall hydrolase by plant secreted inhibitors[31]. 7) Spore degradation or reduction of germination rate by secreted plant antifungal proteins[32]. 8) Small secreted peptides enhance the efficacy of plant defense[33].

1.2 Plant Defense Using PGIPs Against Fungal Pathogens

The plant cell wall is the first barrier a plant pathogen must overcome on its path towards establishing a parasitic relationship[34]. Cell wall-degrading enzymes including polygalacturonases (PGs) play an important role in the pathogenicity of many pathogenic fungi[35]. To combat PGs, plants synthesize PG inhibiting proteins (PGIPs), which are highly conserved glycoproteins that contain leucine-rich repeat (LRR) regions and are located in plant cell walls[36,37]. Their primary role is to inhibit polygalacturonases (PGs), enzymes secreted by insects and fungal pathogens that degrade the plant cell walls and leave it vulnerable for infection[38]. Through competitive or noncompetitive inhibition, PGIPs slow the hydrolysis process of PGs[39–42]. PGIPs slows the hydrolysis process catalyzed by PGs through both competitive or noncompetitive inhibition, which requires PGIP to bind to the PGs either in or distant to the active site, respectively[40,41,43,44]. Additionally, PGIPs may play an additional role in plant protection is by reducing the rate at which the plant oligosaccharides are broken down[42]. Oligosaccharides, specifically, oligogalacturonides, are endogenous elicitors that may be accumulated in response to the degradation of pectin and help trigger plant defense[45], such as the production of reactive oxygen species[46] and the accumulation of phytoalexins[47].

A structural investigation of PGIPs indicates that the central LRR domain is flanked by N- and C-terminal cysteine rich regions, and the residues involved in interacting with PGs are located in the concave surface between sheets B1 and B2[48], which are highly conserved across different PGIPs[49] (Figure 1.2). These conserved regions are also known to be involved in the structural integrity of PGIPs[43]. It is believed that the central LRR

pocket is responsible for recognizing the amino acids present at the active site of many PGs[50]. This specificity allows PGIPs to differentiate fungal PGs from endogenous plant PGs[51]. Prior studies indicate that overexpression of either endogenous or heterologous PGIPs in various plants enhanced their resistance towards fungal infection[52]. Although the development of genetically modified organisms (GMOs) with anti-fungal properties are common approaches to tackling phytopathogenic properties, a lengthy and challenging development cycle and FDA approval process is often required before GMOs are made available for public use. In comparison, using the naturally derived PGIP proteins as an exogenously applied pest control agent may be more ecofriendly, easier for the general public to accept, and may potentially require a shorter development and approval process.

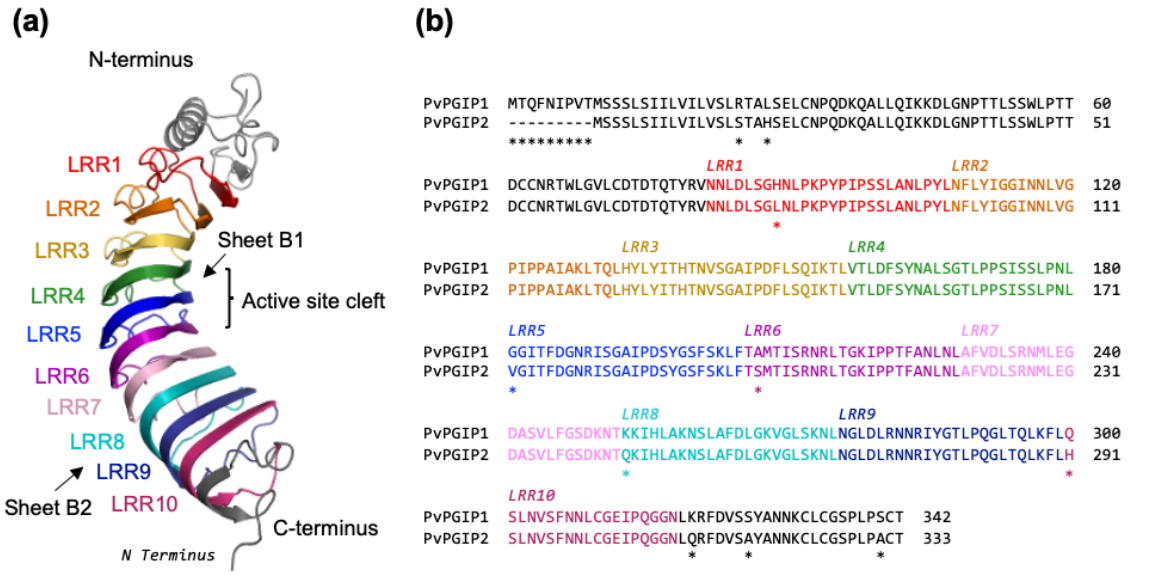

Figure 1.2 Structure and protein sequence of PvPGIPs. (a) Ribbon structure of PvPGIP2. The structure is readapted from the previously published PvPGIP2 (DB accession 10GQ)[43]. **(b)** Amino acid sequences of PvPGIP1 and PvPGIP2. LRR domains are color coded. Sheet B1 is engaged with binding to PGs. LRRs are color coded and differences in residues between PvPGIP1 and PvPGIP2 are shown with astricks.

1.3 PGIP2 from *Phaseolus vulgaris*

Though there are numerous PGIPs studied for their potential in fungal protection, the best characterized PGIP is isoform 2 of PGIP from the common bean, *Phaseolus vulgaris* (PvPGIP2) (Figure 1.2a), which exhibits inhibitory activity toward a number of fungal PGs[53,54]. Despite differing from isoform 1, PvPGIP1, by only 10 residues (Figure 1.2b), PvPGIP2 confers resistance against a greater known number of fungal PGs[38,55]. Expression of PvPGIP2 in transgenic plants resulted in increased resistance to fungal infections against *Alternaria citri, Aspergillus flavus*, *Aspergillus niger*, *Botrytis cinerea*, *Claviceps purpurea*, and *Fusarium graminearum*[56–59].

Here, we investigate the potential of using engineered PvPGIP2 as a fungal growth inhibitor. We utilized *Saccharomyces cerevisiae* as a microbial expression system, which has benefits such as the lack of background PGIP activity and faster growth than plants [60]. Our investigations highlight the potential of engineered PGIPs as a steppingstone towards developing exogenous or heterologous antifungal agents to inhibit the growth of phytopathogenic fungi as an environmentally and economically friendly approach.

Chapter 2

Using a Y2H system to characterize and engineer PGIP in yeast

2.1 Introduction

PGIPs can physically inhibit PGs via competitive[61,62] and noncompetitive inhibition[40,41], so we tested whether the Y2H system could be used to interrogate the binding between the two proteins. The crystal structure of PvPGIP2 shows a large, negatively charged pocket on the inner LRR concave surface believed to bind to the positively charged residues on the active site of PGs[43]. This physical contact between the two proteins led us to believe that Y2H can be a reasonable approach to observe PG-PGIP interactions, which were mainly monitored by surface plasmon resonance (SPR)[51,54,63]. However, unlike Y2H, SPR requires expression and purification of each component. Although Y2H has rarely been used to estimate PG-PGIP activity, it was adopted in one previous investigation to conclude that a PGIP family protein, the carrot antifreeze protein, does not exhibit PGIP activity[64].

We chose four PGs that play important roles in infection by their corresponding pathogens: PG2 from *Aspergillus niger* (AnPG2), PG1 and PG2 from *Botrytis cinerea* (BcPG1 and BcPG2), and PG3 from *Fusarium moniliforme* (updated name: *F. verticillioides*; FmPG3). *A. niger, B. cinerea, and F. moniliforme* all exhibit widespread impact on food crops. *Aspergillus niger* is one of the most significant causes of postharvest decay of many common crops, such as onions[65], grapes[66], peanuts[67], and maize[9]. Due to its wide range of pH tolerance and quick growth, *A. niger* is considered to be one of the most significant fungi associated with postharvest decay[69]. *Botrytis cinerea* is a necrotrophic

fungus with a wide host range of over 200 species, resulting in an economic loss upwards of $10 billion globally every year[70]. For example, it is considered the most economically significant pathogen of strawberry worldwide[71]. *F. moniliforme*[72] can produce mycotoxins, secondary metabolites that are toxic to both humans and animals[73]. It is one of the most significant fungal pathogens to infect corn[74]. Previous investigations indicate that PvPGIP2 inhibits the function of AnPG2[54,62,75] and BcPG1[54,61,76], weakly inhibits FmPG3[51,54], and possibly inhibits BcPG2[54]. In contrast, PvPGIP1 only inhibits the activity of AnPG2[54,75], weakly inhibits BcPG1[54] and does not inhibit FmPG3[54,55]. The activity of PvPGIP1 against BcPG2 is currently unknown.

2.2 Methods

The PGIP (PvPGIP1 and PvPGIP2) and PG (AnPG2, BcPG1, BcPG2, and FmPG3) genes were ordered from Twist Bioscience and codon optimized for *S. cerevisiae.* These pTwist genes were cloned using the Gateway system, with a destination vector containing either an AD domain for the PGIPs or a BD domain for the PGs. The LR Reaction was done using the guidelines and instructions found in the Invitrogen Gateway LR Clonase II Enzyme Mix product sheet (Fischer Scientific). Truncated versions of the PGIPs were made that ensured the protein would not be spliced in the middle of the β-sheets. The structure of the sheets was checked using the protein 3D imaging software, PyMOL. Primers were made for the N and C terminus, as well as for each LRR region to create a multitude of possible PGIP length combinations. Primers were designed according to the protocol "Gibson Assembly Cloning" from Addgene. Truncated versions of PGIPs were cloned from the full length PGIPs using these primers and underwent Gibson assembly

with a vector containing the AD domain. *Ost1*-PvPGIP1 without the stop codon was synthesized from Twist Bioscience and cloned into pDONR221 through Gateway BP reaction to generate pENTR-*Ost1*-PvPGIP1, which was then converted into yeast expression plasmids through Gateway LR reaction with pAG414-GPD-ccdb-HA (centromeric low copy number plasmid, CEN/ARS) or pAG424-GPD-ccdb-HA (high copy number plasmid, 2μ)[77]. Similarly, the low and high copy versions of the PGIPs used for our pectin plate assays were created through similar strategies using Gateway Cloning. The plasmids used to compare expression levels of different PGs were constructed through Gibson assembly.

S. cerevisiae strain PJ69-4A was transformed with plasmids encoding full length PvPGIP1 or PvPGIP2 and strain PJ69-4α was transformed with plasmids encoding one of the PGs (AnPG2, BcPG1, BcPG2, FmPG3). The yeast transformations were carried out using standard protocols[78]. 500μL of overnight stock cultures of these samples were grown at 30°C with orbital shaking at 250 RPM in -T or -L YNB respectively with 5% dextrose. After 16 hours, 10μL of each PGIP and PG containing yeast was mated together in YPD for 24 hours to create a diploid yeast strain expressing both AD-PGIPs and BD-PGs. A 1/60 dilution of each culture was made into -LT for approximately 48 hours. Finally, a 1/300 dilution of the mated yeast in the -LT was then made into 3mL of -HTL YNB. Each sample had 3 biological replicates. A plate reader was used to measure the OD at 600nm every 24 hours for 4 days. The average OD across the 3 biological replicates were then plotted. The same procedure was applied for the truncated versions of the PvPGIPs. Data was collected using the Gen5^{TM} Data Analysis Software (BioTek Instruments).

To verify that our PvPGIP protein was being secreted, samples were prepared by using the protein secretion assay as follows. *S. cerevisiae* strain PJ69-4A, was transformed containing either the plasmid with full length PvPGIP1 with the HA tag (PvPGIP1-HA), or the full length PvPGIP1 with the HA tag and preOST1 secretion signal (preOST1-PvPGIP1-HA). An empty vector yeast was used as a negative control. The yeast was grown for 72 hours in 2mL of -T YNB in a shaker at 30°C at 250 rpm. 200µL of media was pipetted into 1.5mL tubes and 10µL of 1mg/ML BSA was added to each tube. 20µL of 0.15% deoxycholate was added and samples were immediately vortexed then allowed to sit at room temperature for 10 minutes. 20µL of 1M trichloroacetic acid was added to each sample, vortexed, then placed on ice for 30 minutes to precipitate. Samples were centrifuged for 30 minutes at 4°C at 22,000g. After centrifugation, the supernatant was aspirated out and resultant pellet was washed with 200µL of ice-cold acetone once. Pellet was left to air dry at room temperature for 30 minutes, then was treated with 32.5µL DI water, 12.5µL of LDS buffer, and 5µL of reducing agent. Samples were mixed by pipetting and heated to 70°C for ten minutes. After heating, the tubes were centrifuged at room temperature for five minutes and supernatant was used to load into a gel. An 8% tris-glycine gel was used for the SDS-PAGE which was made according to the protocol found in the Surecast Handcast System Quick Reference sheet by Thermo Fisher Scientific. The SDS-PAGE was run at 150V for one hour in tris-glycine running buffer with a ladder (Fisher Scientific, catalog #50491514). A protein transfer with a PVDF membrane (Fisher Scientific, catalog #ISEQ0010) followed using the Protein Transfer Technical Handbook provided by Thermo Fisher Scientific. Once finished, the membrane was removed and

incubated at room temperature for 2 hours in 1x TBST + 5% milk. This was washed twice with 1x TBST for 5 minutes each time, then incubated in 10mL of 1x TBST + 3% milk + 2µL of antibody (Anti-HA tag antibody HRP ab1190 from Abcam) overnight at 4°C. A standard protein detection protocol was then used according to the Protein Detection Technical Handbook from Thermo Fisher Scientific.

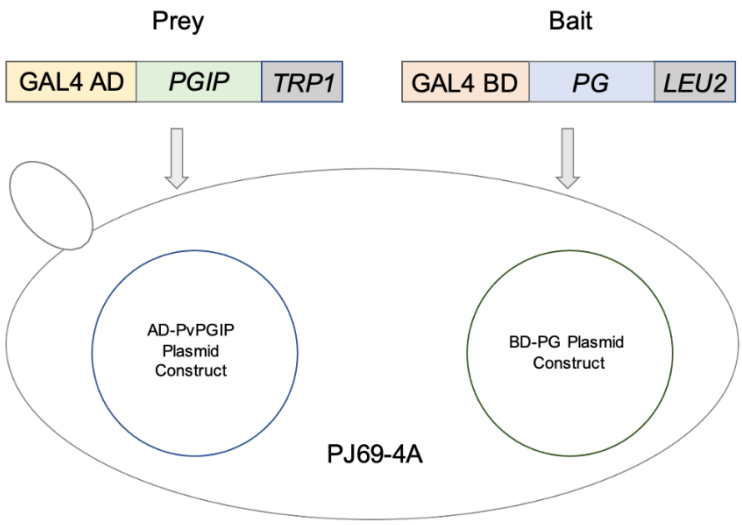

Figure 2.1 Plasmid constructs of the Y2H system to test PGIP-PG interaction. PG genes were fused to the GAL4 binding domain (GAL4-BD), and PvPGIP1 and PvPGIP2 were fused to the GAL4activation domain (GAL4-AD). The bait and prey plasmid constructs, each containing *LEU2* and*TRP1* gene respectively, were co-transformed into a Y2H reporter strain *S. cerevisiae* PJ69-4A.

2.3 Functional Estimation of PG/PGIP Interactions and PGs in Using Y2H System

In our experiments, PGs were fused to the GAL4 binding domain (GAL4-BD), and PvPGIP1 and PvPGIP2 were fused to GAL4 activation domain (GAL4-AD). The bait and prey constructs, each containing *leu2* and *trp1* gene respectively, were co-transformed into a Y2H reporter strain *S. cerevisiae* PJ69-4A[79] (Figure 2.1). PJ69-4A encodes *his3* gene regulated by the *GAL4* promoter, and interaction between the Gal4-AD and Gal4-BD

fusion proteins results in growth of yeast cells in synthetic dropout (SD) medium lacking histidine (H), tryptophan (T), and leucine (L). PG-PGIP interactions were estimated using OD_{600} of Y2H yeast strains at stationary phase. Yeast harboring Gal4 AD-PvPGIP2 and Gal4 BD-AnPG2 was grown in -LT and used as a positive control, while yeast harboring Gal4 AD-HAB1, a protein not known to interact with PGs, and Gal4 BD-BcPG2 was grown in -HTL media and used as the negative control.

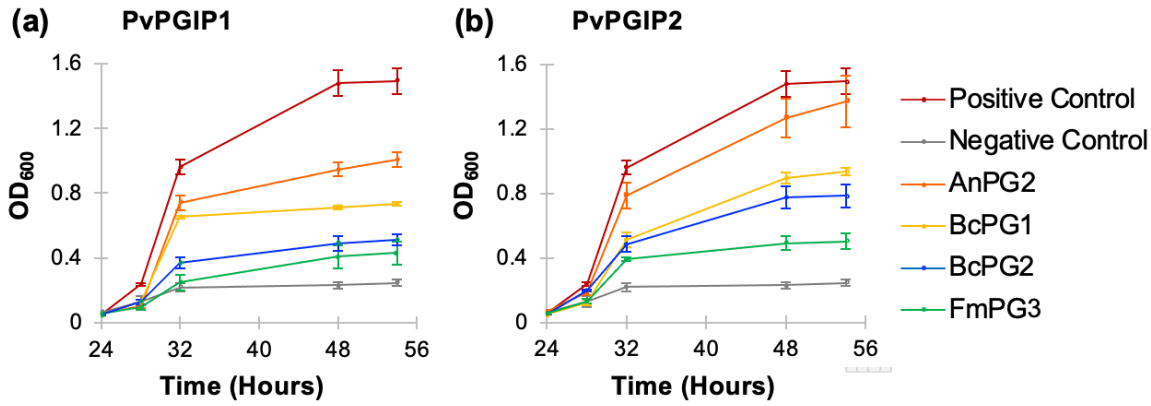

Figure 2.2 Verification of PG-PGIP interactions using the Y2H system. Growth curves of Y2H yeast strains were grown in yeast synthetic dropout media lacking histidine, tryptophan, and leucine (-HTL) harboring various PGs with **(a)** full length AD-PvPGIP1, and **(b)** full length AD-PvPGIP2.The positive control is a yeast strain harboring BD-AnPG2 with AD-PvPGIP2 and grown in yeast synthetic dropout media lacking leucine and tryptophan (-LT), while the negative control is a yeast strain harboring BD-BcPG2 with AD-HAB1, a protein not known to interact with PGs, grown in -HTL dropout media. The curves represent the mean values of three biological replicates, and the error bars represent the standard deviation of the replicates.

Yeast harboring Gal4 AD-PvPGIP1 and Gal4 BD-AnPG2 grew at a similar level to the positive control. Yeast harboring FmPG3 exhibits the slowest growth that is slightly higher than the negative control indicating little to no Y2H interaction (Figure 2.2a). With Gal4 AD-PvPGIP2, yeast containing Gal4 BD-AnPG2 or BcPG1 exhibited robust growth in -HTL medium, indicating strong PG-PGIP interactions. Yeast with Gal4 AD-PvPGIP2

harboring either Gal4 BD-BcPG2 or Gal4 BD-FmPG3 grows much slower than the positive control, which implies weak PG-PGIP interactions (Figure 2.2b) The distinct PG-PGIP interactions may be due to the different specificity of the protein recognition site at the concave surface of the PGIPs[43,61]. Additionally, the expression levels of different PGs were examined using a western blot to validate that the growth of Y2H strains is indicative of interactions rather than expression levels. As expected, BD-AnPG2, BD-BcPG1, and BD-FmPG3 were expressed at comparable levels in yeast (Figure 2.3), thus further validating the feasibility of using Y2H assay to study PG-PGIP interactions. The PG-PGIP interactions implied from Y2H assay overall are reflective of findings in previous studies[39,51,54,56,62,75,76,80] and suggests that it is feasible to use Y2H assay to functionally map PG-PGIP interactions.

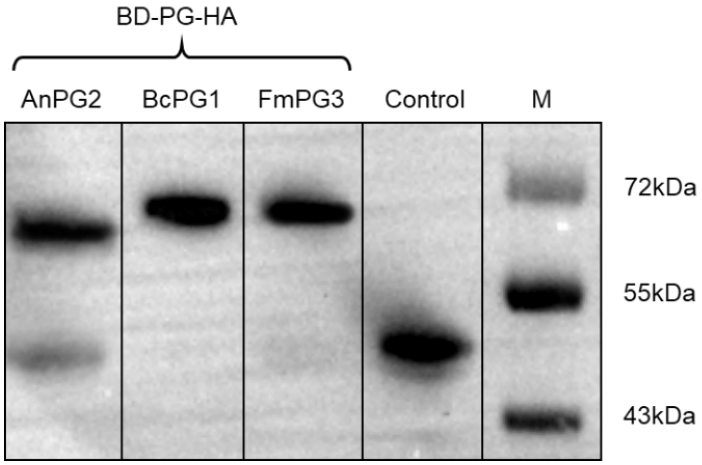

Figure 2.3 Verification of BD-PG expression levels. The expression levels of AnPG2, BcPG1, and FmPG3 were analyzed by western blot of *S. cerevisiae* containg BD-PG plasmids using anti-HA antibody. The expected size of BD-AnPG2, BcPG1, and FmPG3 are 59 kDA, 61 kDA, and 61 kDA, respectively. The control is *Ost1*-PvPGIP1-HA, which has an expected size at 45.7 kDa. M represents the protein marker (Fischer BioReagents EZ-Run Prestained *Rec* Protein Ladder, Fischer Scientific, #BP3603500). The cell lysate samples indicate the presence of the protein inside the cell and show a comparable expression level between the PGs.

2.4 Truncations of PvPGIP2 and its interactions with PGs

The modular structure of leucine-rich repeat proteins leads to the hypothesis that a truncated version of PGIP may be sufficient to efficiently inhibit PG activity. Binding of PGs to truncated versions of PvPGIP2 were tested using the Y2H system (Figure 2.4). PvPGIPs lacking the N-terminus or C-terminus exhibited similar activity levels to full length PvPGIP2, displaying comparable growth rates and final OD. This indicates that the N- and C-terminus likely do not harbor any residues integral to PG recognition. To narrow down which LRRs to truncate, we noted that a previous docking simulation of BcPG1 and PvPGIP2 found the optimal docking area of PvPGIP2 was between Val-152 and His-291, and played a vital role in the interaction with PGs[61].

Thus, PvPGIP2 was truncated to retain only LRR5 to LRR8 (residues 172–266, tPvPGIP2_5-8) which flanks the optimal docking area. tPvPGIP2_5-8 exhibited a comparable growth curve to full length PvPGIP2 in the Y2H assay, with only slight decreases in AnPG2 and BcPG1 interactions. A slightly larger truncation, tPvPGIP2_5-9 (residues 172 – 290), likewise retains similar comparable growth curves to full length PvPGIP2 and tPvPGIP2_5-8 and does not increase the OD, despite retaining an additional LRR. Further truncation of LRR5 from tPvPGIP2_5-8 to make tPvPGIP2_6-8 (residues 197 – 266) resulted in an almost complete loss of inhibitory activity to all the PGs tested. Likewise, additional truncation of tPvPGIP2_6-8 to tPvPGIP2_6-7 (residues 197 – 243) also exhibited no inhibitory activity to the tested PGs. Compared to tPvPGIP2_5-8, truncation of LRR8 from tPvPGIP2_5-8 to make tPvPGIP2_5-7 (residues 172 – 243) led to reduced interactions with AnPG2 and BcPG1 compared to tPvPGIP2_5-8. Other

truncations that contained only LRR4 to LRR5 (residues 138 – 197), LRR4 to LRR6 (residues 138 – 219), LRR1 to LRR4 (residues 73 – 172), and LRR7 – LRR10 (residues 219 - 310) resulted in a complete loss of interaction with PGs as well. These results imply that tPvPGIP2_5-8 is likely the smallest truncation to retain similar level of inhibitory activity towards AnPG2 and BcPGs, compared with full length PvPGIP2 in a Y2H assay. This is also in line with the previously established molecular docking study simulating the interactions between PvPGIP2 and BcPG1[76] that tPvPGIP2_5-8 retains clusters of residues demonstrated to be important for BcPG1 interaction.

We then measured the interactions of tPvPGIP1_5-8 and tPvPGIP2_5-8 with different PGs. The interactions of both the truncated PGIPs with different PGs mirrored that of the full length PGIPs (Figure 2.5). The growth curves for yeast harboring AnPG2 and BcPG1 had the highest stationary phase at OD_{600} among all the yeast strains and were comparable to the positive control. This again shows that the properly truncated PGIPs (here, tPvPGIP2_5-8 and tPvPGIP1_5-8) can interact with PGs in a similar capacity to their full-length counterparts. However, it is important to note that the regions flanking LRR5-LRR8 may play a role *in planta* which may not be detectable through *in vitro* assays. For example, residues outside the optimal docking area may be important to proper folding and structural integrity, and thus could be essential for keeping PGIP stable. Though different truncated versions were carefully designed to avoid cleavage within the LRR domains and thus ensure correct protein folding, certain truncations may still influence the thermostability of proteins.

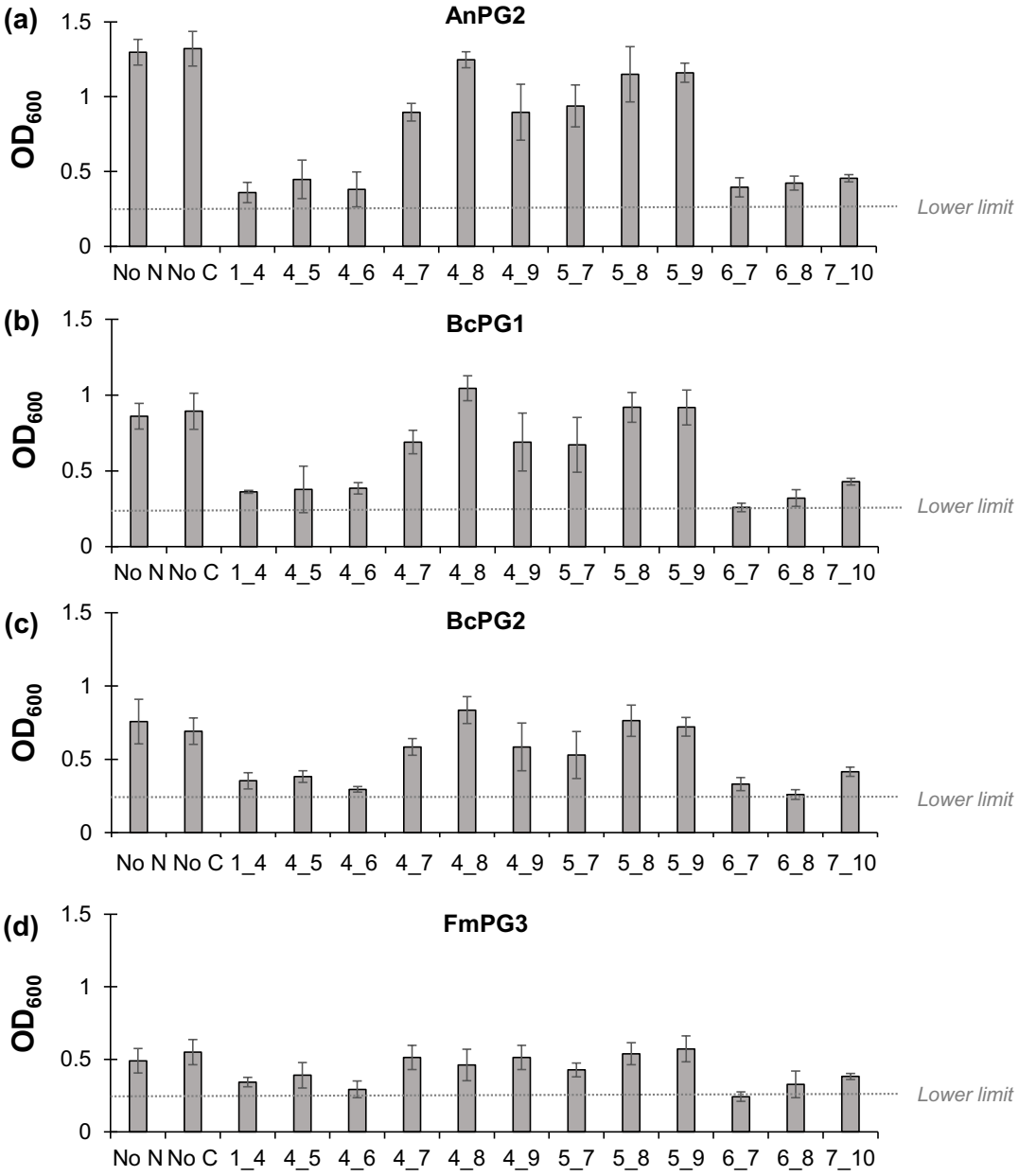

Figure 2.4 Truncation of PvPGIP2 retains the inhibitory activity towards AnPG2, BcPGs, and FmPG3 as determined by Y2H. Growth curves of yeast strains harboring AnPG2, BcPG1, BcPG2, and FmPG3 with truncated PvPGIP2s lacking the N terminus, C terminus, tPvPGIP2_4-5, tPvPGIP2_4-6, tPvPGIP2_4-7, tPvPGIP2_4-8, tPvPGIP2_4-9, tPvPGIP2_5-7, tPvPGIP2_6-7, and tPvPGIP2_6-8 with **(a)** AnPG2, **(b)** BcPG1, **(c)** BcPG2, and **(d)** FmPG3 were measured, and the data summarized from the 48 hour point. The lower limit represents the negative control, yeast harboring BD-BcPG2 and AD-HAB1 grown in -HTL dropout media. Each column represents the mean values of three biological replicates, and the error bars represent the standard deviation of the replicates.

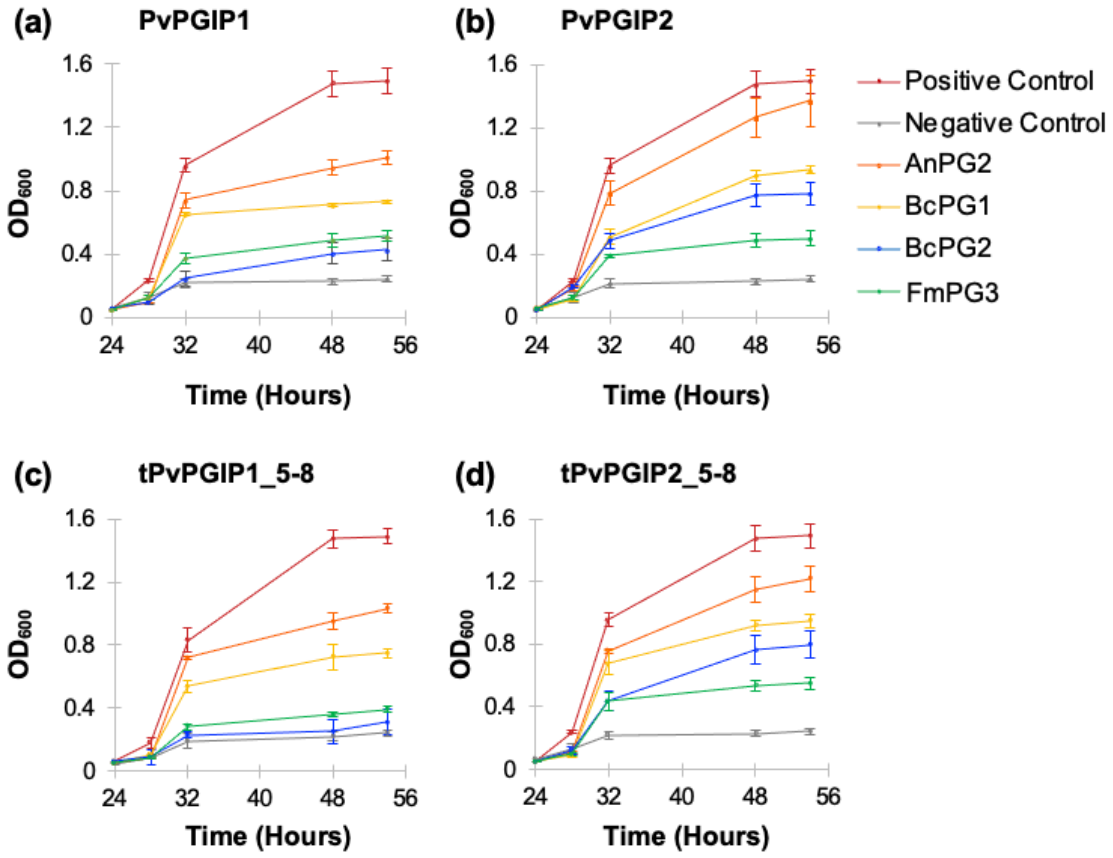

Figure 2.5 Comparing the full length and truncated PvPGIPs growth to determine PG interactions using the Y2H system. Growth curves of Y2Hyeast strains were grown in -HTL dropout media harboring various BD-PGs with **(A)** full length AD-PvPGIP1, **(B)** full length AD-PvPGIP2, **(C)** AD-tPvPGIP1_5-8, and **(D)** AD-tPvPGIP2_5-8. The positive control is a yeast strain harboring BD-AnPG2 with AD-PvPGIP2 and grown in -LT dropout media, while the negative control is a yeast strain harboring BD-BcPG2 with AD-HAB1,a protein not known to interact with PGs, grown in -HTL dropout media. The curves represent the mean values of three biological replicates, and the error bars represent the standard deviation of the replicates.

Chapter 3

Engineering tPvPGIP2 for improved efficacy against PGs

3.1 Introduction

Although PvPGIP1 and PvPGIP2 are highly similar in amino acid sequence, they exhibit distinct activities against different PGs, such as BcPG2. Deciphering the sequence-function correlation of PvPGIP1 and PvPGIP2 to PG recognition may provide insights for the engineering of PvPGIP2 for enhanced activity or spectrum. One previous docking-based study demonstrated the importance of the Val172Gly (located in LRR5) and found that replacing PvPGIP2's Val with PvPGIP1's Gly decreased inhibition activity against BcPG1[61,76]. There are ten residues that differ between the full length PvPGIP1 and PvPGIP2, but most have previously been found to have little to no effect on PG recognition, with the exception of those found within the optimal docking area[61]. Our finding that the truncated forms possess a similar activity profile to their full-length counterparts provides a unique opportunity to clearly elucidate the sequence-function correlation of PG recognition.

Given the LRR structure of PvPGIP2, with many PGIPs showing conserved sequences[54], we believed it may be possible to splice different LRRs from different PGIPs together to increase the potency of PG interaction . Different PGIPs exhibit a varying range of efficacy towards a range of PGs, and not all PGIPs have the same optimal docking area. Should a PGIP different from PvPGIP2 showcase improved PG-interaction from our Y2H assays, it would be a promising candidate for PGIP engineering. One potential PGIP is PGIP3 from soybean (*Glycine max*). Although it is in a different genus from *P. vulgaris*,

GmPGIP3 is a homologue to PvPGIP2 and shares an 88% amino acid sequence similarity[55] (Figure 3.1). GmPGIP3 is known to inhibit PGs from *S. sclerotiorum, F. moniliforme, F graminearum, B. aclada, B. cinerea, A. niger, and C. acutatum,* and overexpression in transgenic wheat results in inhibition towards the pathogenic fungi *F. graminearum, C. purpurea,* and *B. sorokiniana*[55]. Likewise, PGIP2 from lima bean (*Phaseolus lunatus*) is another potential PGIP to investigate. Among the *Phaseolus* species, *P. lunatus* has the greatest sequence difference from *P. vulgaris*, and is known to inhibit PGs from *F. moniliforme, A. niger, B. cinerea,* and *C. lupini*[81]. The high similarity between these two PGIPs with PvPGIP2 may potentially result in a greater ease of combining LRRs, while the unique differences within each protein and range of PGs they are effective against could be useful in engineering a novel protein with a broader and more effective spectrum of range against pathogenic fungi.

```
GmPGIP3      ----------MSKLSILFLLVLSFSSVLSELCNPQDKQALLQIKKDLGNPTTLSSWLLTT
PvPGIP2      ---------MSSSLSIILVILVSLSTAHSELCNPQDKQALLQIKKDLGNPTTLSSWLPTT
             ********** *   ****** * ***

GmPGIP3      DCCNRTWLGVSCDTDTQTYRVNDLDLSDLNLPKPYSIPPSIANLPYLNFLSISRTPTLIG
PvPGIP2      DCCNRTWLGVLCDTDTQTYRVNNLDLSGLNLPKPYPIPSSLANLPYLNFLYIGGINNLVG
                       *           *    *       *    *         * ***** *

GmPGIP3      QIPSAIAKLTQLRYLYITHTNVSGPIPDFLSQIKTLVTLDFSYNTLSGKLPASLSSLPNL
PvPGIP2      PIPPAIAKLTQLHYLYITHTNVSGAIPDFLSQIKTLVTLDFSYNALSGTLPPSISSLPNL
             *  *        *           *                     *   *  * *

GmPGIP3      VGITFDGNQISGAIPDSYGSFSKLFTSMTISRNRLTGKIPATFANLNLAFVDLSRNMLEG
PvPGIP2      VGITFDGNRISGAIPDSYGSFSKLFTSMTISRNRLTGKIPPTFANLNLAFVDLSRNMLEG
                    *                              *

GmPGIP3      DASVLFGTEKNTQKILLAKNMLAFDLGKVGLSKNLNGLDLRNNRIYGTLPQGLTALKFLH
PvPGIP2      DASVLFGSDKNTQKIHLAKNSLAFDLGKVGLSKNLNGLDLRNNRIYGTLPQGLTQLKFLH
                    **   *  *    *                                    *

GmPGIP3      SFNVSFNDLCGEIPQGGNMQRFDVSSYANNKCLCGSPLPPCT
PvPGIP2      SLNVSFNNLCGEIPQGGNLQRFDVSAYANNKCLCGSPLPACT
              *     *          *      *             *
```

Figure 3.1 Amino acid sequences of GmPGIP3 and PvPGIP2. LRRs are color coded and differences in residues between GmPGIP3 and PvPGIP2 are shown with astricks. Although GmPGIP3 belongs in a different genus, it still has an 88% amino acid similarity with PvPGIP2.

3.2 Methods

Prior studies show that the three residues found within the optimal docking area may have greater effect on PG recognition compared with those outside the optimal docking area between LRR5 and LRR8[61]. Three of these residues are in the truncated region of LRR5 to LRR8 (Figure 3.2a). First, to determine which of the residue(s) are essential for the inhibitory activity, we constructed six tPvPGIP_5-8 mutants that shuffled the sequences between tPvPGIP1_5-8 and tPvPGIP2_5-8. The activities of the six tPvPGIP_5-8 mutants were examined using the Y2H assay by mating PJ69-4A containing the different tPvPGIP_5-8 with the same controls as previously described. The growth of the six diploid yeast strains in -HTL medium were compared with the controls.

To confirm if various mutations at residue 172 of tPvPGIP2_5-8 enhance PG interaction, a saturated site-directed mutagenesis was performed. A modified version of splicing by overlap extension (SOE) was done[82]. Five oligonucleotides were annealed together to create tPvPGIP2_5-8 mutants with residue 172 replaced in the first oligonucleotide. The product was run on a 0.8% agarose gel, then gel purified to retrieve the DNA. The assembled DNA fragment was then inserted to the corresponding vector containing the AD-Gal domain through Gibson Assembly.

To determine if any additional mutations at sites outside of Val172 could improve PG-interaction, the GenoMorph II Random Mutagenesis kit by Agilent Technologies was used to induce random mutations on tPvPGIP2_5-8. The mutant gene was amplified and run on a PCR program as suggested by the manufacturer, then run on a 0.8% agarose gel. It was then inserted into a corresponding vector containing the AD-Gal domaining through

Gibson Assembly. The resultant plasmids were co-transformed into yeast with either Gal4 BD-BcPG2 or Gal4 BD-FmPG3 and grown on the appropriate dropout media. All colonies were washed into a tube to grow overnight in YPD, then grown for two days in dropout media, back-diluted at a ratio of 1/300 into fresh -LT YNB, and plated onto -HTL YNB. The largest colonies were selected for screening to determine their sequence. This cycle was only repeated up to three times to avoid adaptive evolution occurring on the yeast.

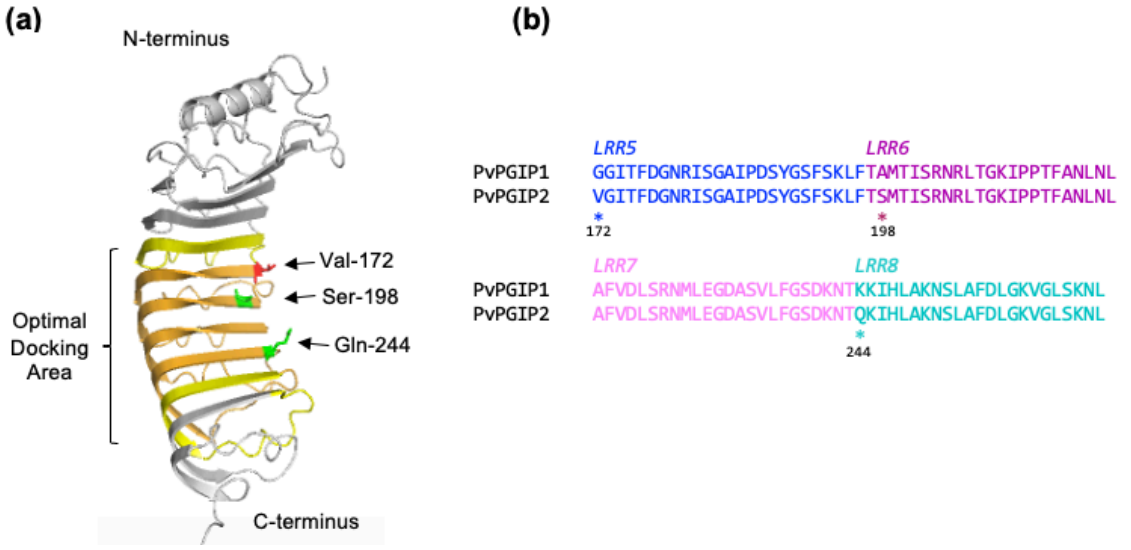

Figure 3.2 Structure and protein sequence of tPvPGIP2_5-8. tPvPGIP_5-8 containing different combinations of the three residues differing between PvPGIP1 and PvPGIP2 were tested using a Y2H system. **(a)** A color coded ribbon structure of PvPGIP2, with the optimal docking area depicted in yellow, LRR5 – LRR8 in bright orange, Val172 in red, and Ser198 and Gln244 in green. These three residues are the three amino acids that differ between PvPGIP1 and PvPGIP2 at LRR5 – LRR8, with Val172 considered the most critical of the three to PG-PGIP interaction. **(b)** The three differing residues between PvPGIP1 and PvPGIP2 are indicated by asterisks. Each mutant contains a residue from either PvPGIP1 or PvPGIP2 different at LRR5, LRR7, and LRR8, named sequentially as tPvPGIP2_111, tPvPGIP2_222, tPvPGIP2_221, tPvPGIP2_212, tPvPGIP2_211, tPvPGIP2_112, and tPvPGIP2_121.

Lastly, to decide if PlPGIP2 and GmPGIP3 were viable candidates to utilize in engineering PvPGIP2 for improved efficacy against PGs, a Y2H growth assay as described

in the previous chapter was performed, alongside various truncations to seek the smallest possible size these PGIPs could be while still maintaining the same level of PG interaction as their full-length counterparts.

3.3 Investigating Sequence-Function Correlation of PvPGIP1 and PvPGIP2

In our study, we found that that PvPGIP1 and PvPGIP2 had similar levels of interaction with BcPG1, with PvPGIP2 only displaying a slightly higher growth rate in the exponential phase. This is likely because the system lacks sensitivity, which could be due to some steric hinderance from the fused yeast reporter proteins or some post-translational modifications of the PGIP. It seems that the Y2H assay is more accurate when the PG-PGIP interactions are strong. However, Y2H is still a promising and quick strategy to illustrate the sequence-function correlation, especially when there are strong interactions between PGIP and PGs. tPvPGIP1_5-8 and tPvPGIP2_5-8 display different levels of PG interaction and contain three different residues within the optimal docking area. To determine which of the residues may be critical in PG recognition and inhibitory activity, six mutants were created that shuffled the three residues between PvPGIP1 and PvPGIP2. Each mutant contains a combination of Val172Gly (LRR5), Ser198Ala (LRR6), or Gln244Lys (LRR8), named in that order and all located within the variable region of the corresponding LRR domain[43] (Figure 3.2b), with each mutation designated as 1 or 2 depending on if the amino acid was from PvPGIP1 or PvPGIP2 respectively. The mutants are tPvPGIP_5-8 “221,”, tPvPGIP_5-8 “212,” tPvPGIP_508 “211,” tPvPGIP_5-8 “122,” tPvPGIP_5-8 “112,” and tPvPGIP_5-8_”121” (Figure 3.3). When the chimeric tPGIP_5-8 contained Val from PvPGIP2 instead of Gly from PvPGIP1 at position 172, it retained PG

interaction ability like tPvPGIP2_5-8 regardless of mutations at other sites. If residue 244 contains Gln from PvPGIP2, a slight increase in FmPG activity is found compared to pure PvPGIP1, indicating the importance of 244Gln towards the recognition of FmPG. Residue 198 was not noted to have any substantial impact on PG-PGIP interaction in our Y2H system.

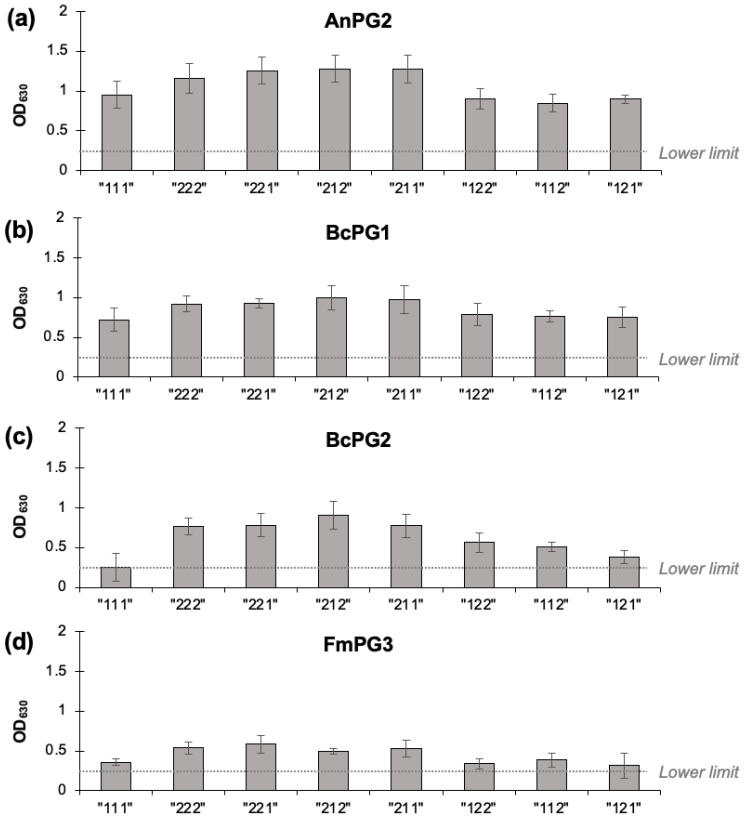

Figure 3.3 Sequence-function correlation of tPvPGIP2_5-8. Each mutant contains a residue from either PvPGIP1 or PvPGIP2 different at LRR5, LRR7, and LRR8, named sequentially as tPvPGIP2_111, tPvPGIP2_222, tPvPGIP2_221, tPvPGIP2_212, tPvPGIP2_211, tPvPGIP2_112, and tPvPGIP2_121. The OD_{600} was measured at 48 hours of yeast harboring these mutant PGIPs with **(a)** AnPG2, **(b)** BcPG1, **(c)** BcPG2, and **(d)** FmPG3. The positive control was yeast expressing BD-AnPG2 and AD-PvPGIP2 grown in -LT dropout media, while the negative control was yeast expressing BD-BcPG2 and AD-HAB1 grown in -HTL dropout media. The curves represent the mean values of three biological replicates, and the error bars represent the standard deviation of the replicates.

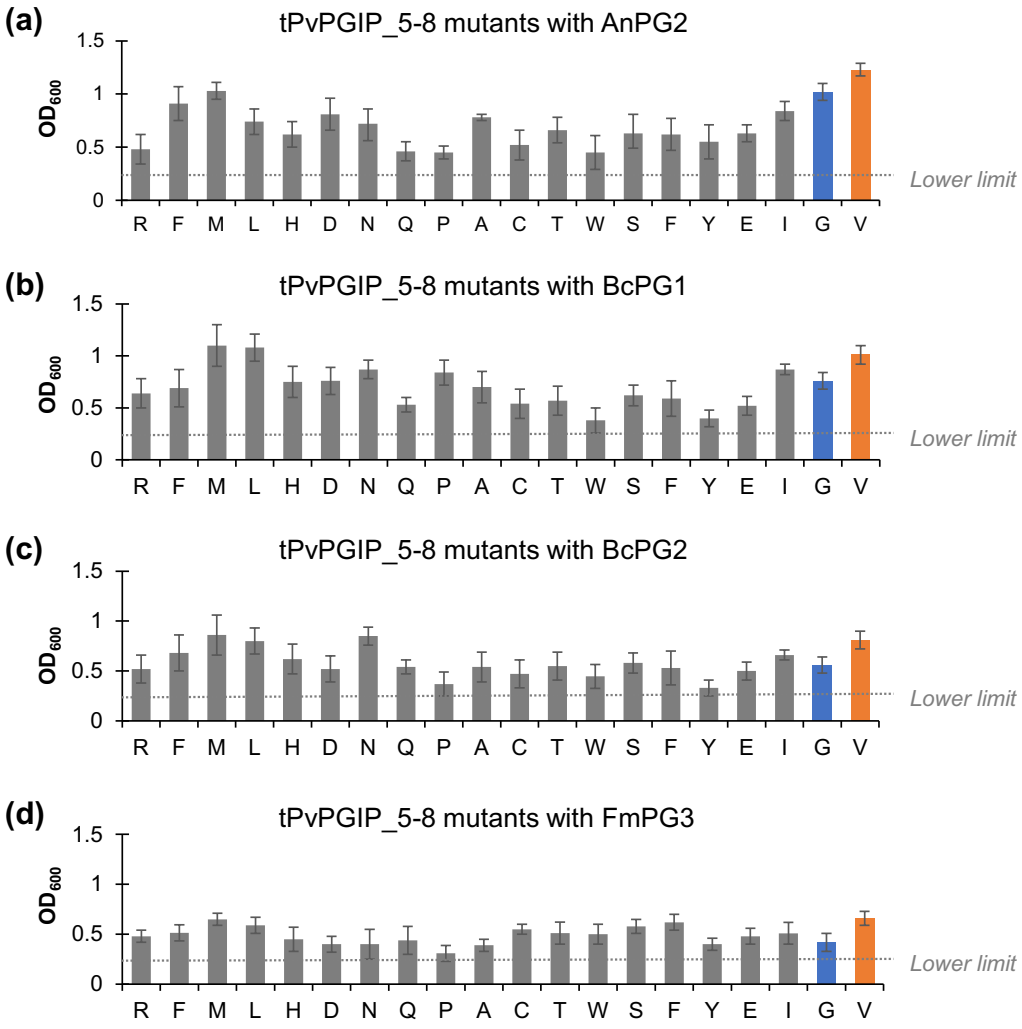

Figure 3.4 Testing of various mutations at residue 172 of PvPGIP2_5-8 with PGs using the Y2H system. After 48 hours, the OD of the yeast harboring various mutations mated to **(a)** BD-AnPG2 **(b)** BD-BcPG1, **(c)** BD-BcPG2, and **(d)** BD-FmPG3 to create diploid yeast expressing both PGIP and PGs was measured. Each column represents the mean values of five biological replicates, and the error bars represent the standard deviation of the replicates. The lower limit shows the activity level of the negative control, yeast expressing BD-BcPG2 and AD-HAB1 grown in -HTL dropout media. The wild type residues for PvPGIP1 and PvPGIP2 are G and V, respectively, and highlighted by the blue and orange bars, respectively.

3.4 Testing Site Directed Mutagenesis on Val172

We conducted saturated site-directed mutagenesis at residue 172 of tPvPGIP2_5-8. All 20 amino acids were compared using Y2H assay. While most mutants showed decreased levels of interaction with the tested PGs compared to the wild type tPvPGIP2_5-8, several mutants displayed a different inhibition profile against different PGs. tPvPGIP2_5-8^{V172M} showed a similar level of interaction with FmPG3 and BcPG2, a slightly higher interaction with BcPG1, but decreased interactions with AnPG2, compared to the wild type tPvPGIP2_5-8. In addition, tPvPGIP2_5-8^{V172L} showed slightly higher interactions than wild type tPvPGIP2_5-8 against BcPG1 but not the other three PGs. However, when comparing error bars and deviation between the samples, no amino acid substitution showed a statistically significant improvement in PG interactions compared to the wild type tPvPGIP2_5-8, indicating that natural evolution has already selected for the optimal amino acids for interacting with the tested PGs (Figure 3.4).

As no mutant demonstrated a higher level of interaction with PGs, Y2H assays were conducted on PGIP2 from *Phaseolus lunatus* (PlPGIP2), the lima bean, and PGIP3 from *Glycine max* (GmPGIP3), the soy bean. These were selected due to their similarity in structure to PvPGIP2, which we theorized would make them compatible candidates for splicing different LRR sections together. However, neither PlPGIP2 nor GmPGIP3 exhibited stronger PG interactions with the PGs tested. This may be due to a lack of sensitivity in the Y2H assay, or more likely, the PGIPs we selected were too similar to PvPGIP2, in which case, similar levels of PG-interaction are to be expected.

3.5 Mutant Library Screening

Because various mutations at residue 172 did not yield any evidence of an amino acid replacement that may result in enhanced levels of PG-interaction, we looked to induce random mutations throughout tPvPGIP2_5-8 to determine if certain mutations could result in increased PG interaction levels. However, after thousands of mutant colonies were grown and analyzed, we did not find any mutations that would significantly increase the Y2H interactions between PG and PGIP To better target which amino acids to utilize, docking models were briefly used to model tPvPGIP2_5-8 and BcPG1 interactions, but no amino acids could be clearly identified as potential targets. While it is possible that the PGIP protein has already reached its full potential in terms of PG-interaction after millions of years of evolution, we believe a more likely reason is that the screening library scope is too small and inefficient, with each batch only reaching less than 300 mutants. Due to the difficulty at scaling up the mutant library and the near infinite combinations of possible mutants, it is more likely that we simply have not yet found the potential PGIP mutants that can be further engineered for increased efficacy towards inhibiting PGs.

3.6 PlPGIP2 and GmPGIP3

Y2H assays were conducted to ascertain if either PlPGIP2 or GmPGIP3 would display greater levels of interaction than PvPGIP2 (Figure 3.5). It was found that yeast harboring either Gal4 AD-PlPGIP2 and Gal4 AD-GmPGIP3 and any BD-PG overall grew slower and to a lower OD at stationary phase compared to PvPGIP2. While both Gal4 AD-PlPGIP2 and Gal4 AD-GmPGIP3 had an appreciable growth when they harbored Gal4 BD-AnPG2, and moderate growth when they harbored Gal4 BC-BcPG1, they did not

perform better with higher OD than their PvPGIP2 counterparts. While these results do match what previous studies have found, this was the first where we were able to compare their levels of interactions with PvPGIP2 for a more direct assessment.

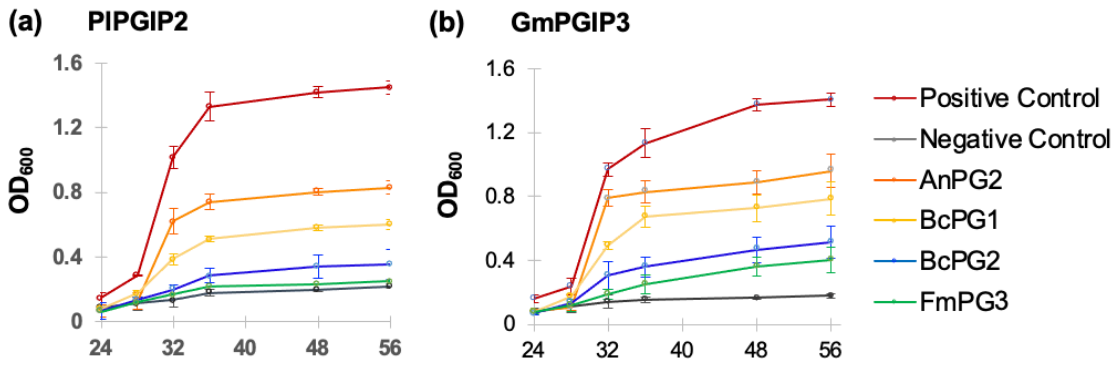

Figure 3.5 PG-PGIP Interactions using Y2H for PlPGIP2 and GmPGIP3. Growth curves of Y2H yeast strains in yeast synthetic dropout media lacking histidine, tryptophan, and leucine (-HTL) harboring various PGs with **(a)** full length AD-PlPGIP2, and **(b)** full length AD-GmPGIP3The positive control is a yeast strain harboring BD-AnPG2 with AD-PvPGIP2 and grown in yeast synthetic dropout media lacking leucine and tryptophan (-LT), while the negative control is a yeast strain harboring BD-BcPG2 with AD-HAB1, a protein not known to interact with PGs, grown in -HTL dropout media. The curves represent the mean values of three biological replicates, and the error bars represent the standard deviation of the replicates.

Binding of PGs to truncated versions of PlPGIP2 (Figure 3.6) and GmPGIP3 (Figure 3.7) was then tested using the Y2H system. In PlPGIP2, truncations containing LRR1-4, LRR1-5, LRR1-6, LRR7-10, and LRR8-10 resulted in a substantial loss in PG-PGIP interaction. Meanwhile, LRR3-5, LRR3-7, LRR4-7, and LRR4-8 showed mediocre growth, indicating a moderate level of PG-PGIP interactions. PlPGIP2 truncated to contain LRR4-9, LRR5-7, LRR5-8, LRR5-9, LRR6-8, LRR6-9, LRR6-10, no N terminus, and no C terminus had higher levels of PG interaction, but remained somewhat lower than that of

their full-length counterpart. Little to no interaction was seen with FmPG3 regardless of truncation. Though notable PG-PGIP interaction is possible in truncated PlPGIP2, these results suggest that despite its sequence similarities to PvPGIP2, PlPGIP2 is less suitable to truncations compared to PvPGIP2, as no truncation retained similar levels of PG activity as the full-length version.

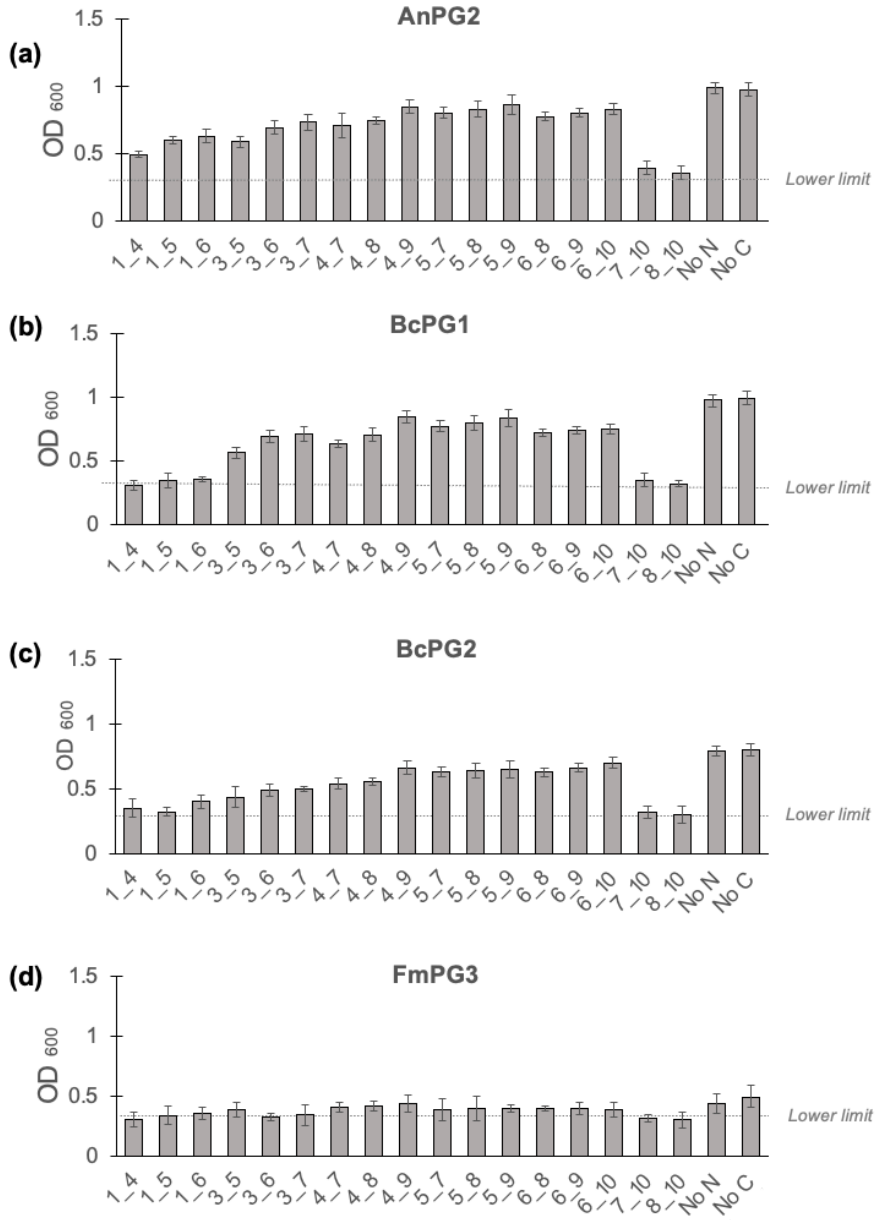

Figure 3.6 Truncation of PlPGIP2 retains some of the inhibitory activity towards AnPG2, BcPGs, and FmPG3 as determined by Y2H. Growth curves of yeast strains harboring AnPG2, BcPG1, BcPG2, and FmPG3 with truncated PlPGIP2s lacking the N terminus, C terminus, tPlPGIP2_1-4, tPlPGIP2_1-5, tPlPGIP2_1-6, tPlPGIP2_3-5, tPlPGIP2_3-6, tPlPGIP2_3-7, tPlPGIP2_4-7, tGmPGIP2_4-8, tPlGIP2_4-9, tPlPGIP2_5-8, tGmPGIP3_5-9, tPlPGIP2_6-8, tPlPGIP2_6-10, tPlPGIP2_7-10, and tPlPGIP2_8-10 with **(a)** AnPG2, **(b)** BcPG1, **(c)** BcPG2, and **(d)** FmPG3 were measured, and the data summarized from the 48 hour point. The lower limit represents the negative control, yeast harboring BD-BcPG2 and AD-HAB1.

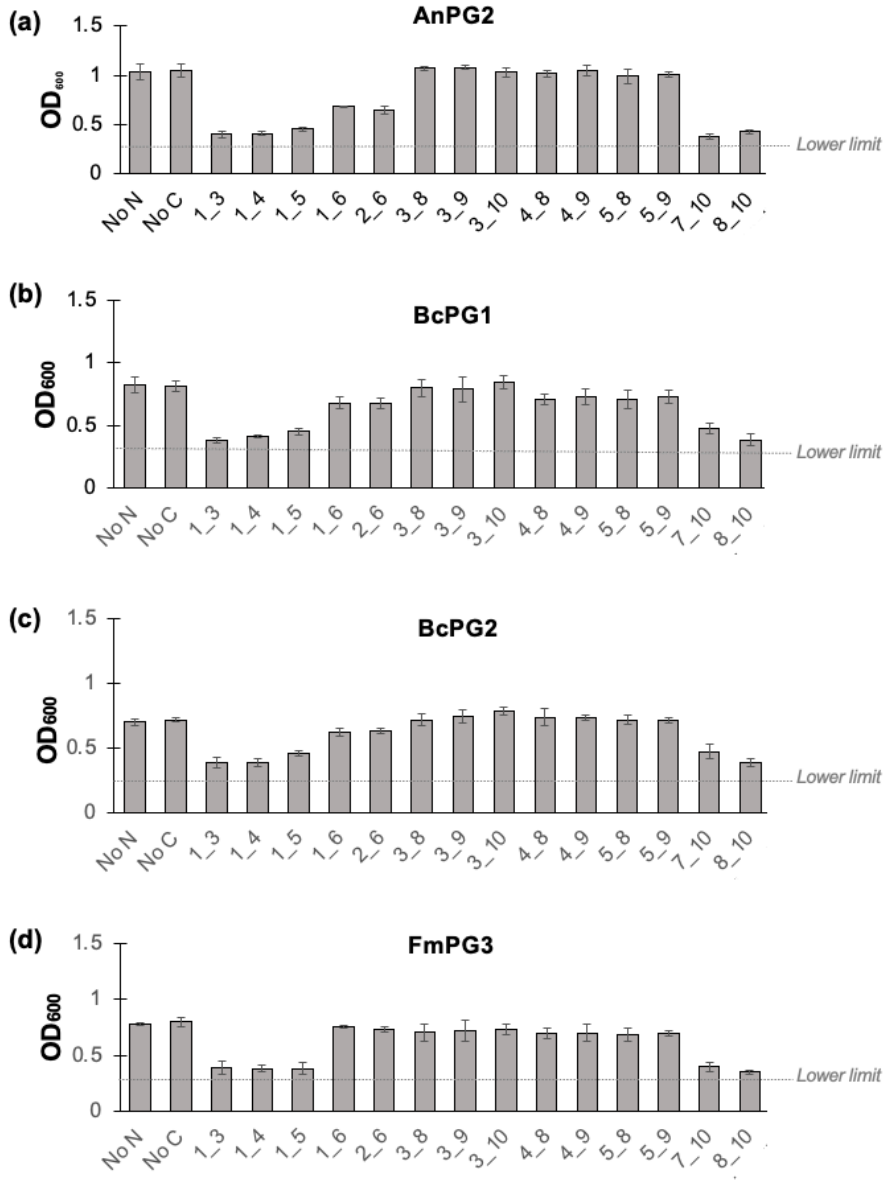

Figure 3.7 Truncation of GmPGIP3 retains the inhibitory activity towards AnPG2, BcPGs, and FmPG3 as determined by Y2H. Growth curves of yeast strains harboring AnPG2, BcPG1, BcPG2, and FmPG3 with truncated GmPGIP3s lacking the N terminus, C terminus, tGmPGIP3_1-3, tGmPGIP3_1-4, tGmPGIP3_1-5, tGmPGIP3_1-6, 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 hour point. The lower limit represents the negative control, yeast harboring BD-BcPG2 and AD-HAB1.

In GmPGIP3, 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 experiencing a moderate loss of activity. While the truncations containing LRR3-8, LRR3-9, LRR3-10, LRR4-8, LRR4-9, LRR5-9, no N terminus, and no C terminus all retained similar levels of inhibitory activity as the full-length version of GmPGIP3, 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 indicate that the regions outside of LRR5 to LRR8 in PvPGIP2 and GmPGIP3 are not essential for the interaction with PGs, and the specific function of these parts of PGIPs remain to be clearly elucidated.

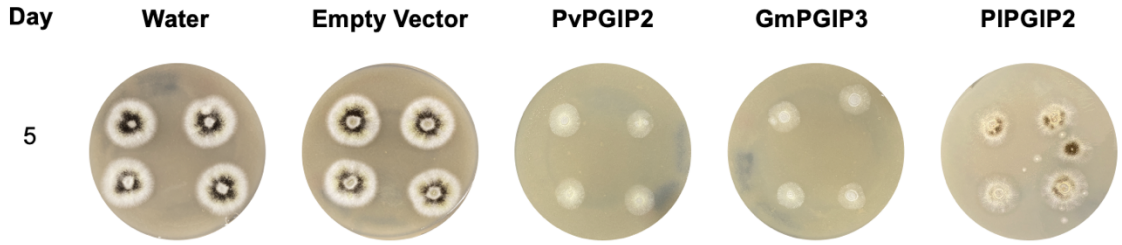

Figure 3.8 Pectin assay comparing the efficacy of PvPGIP2, GmPGIP3, and PlPGIP2 against *A. niger*. Yeast using the *Ost1* signal peptide was used to secret the three different PGIPs onto pectin plates. Water and yeast harboring an empty vector were used as negative controls.

Lastly, an in vitro assay using the protocol in Chapter 4 was performed comparing yeast secreting PvPGIP2, GmPGIP3, and PlPGIP2 with the *Ost1* signal peptide. Water and yeast containing an empty vector were utilized as negative controls. Growth of the plates was monitored over the course of five days. On day 5, the difference in growth is clear between the PGIP-treated plates and the negative controls. Both negative controls

displayed thicker, larger, and darker *A. niger* growth compared to the PGIP plates. PvPGIP2 and GmPGIP3 had near-identical levels of *A. niger* growth, with PlPGIP2 containing slightly more. These *in vitro* results further reaffirm that GmPGIP3 and PlPGIP2 are not more effective than PvPGIP2 at inhibiting certain PGs and pathogenic fungi.

Chapter 4

Studying PGIP Efficacy *In Vitro* and *In Planta* Assays

4.1 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[83]. This percentage is projected to grow, especially as the human population has more than doubled between 1960 to 2009 and reached 7 billion people, and is further expected to reach 9.2 billion by 2050[84]. 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[85]. The current average intake of fruits of vegetables is lacking, and is especially insufficient in developing countries[86]. 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[87].

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[88]. 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 with limited shelf-life, and are very susceptible to mechanical damage during harvesting, processing, and transport[89]. When the produce is damaged and bruised, they are more susceptible to spoilage from numerous microorganisms, including pathogenic fungi[90]. Every year, roughly 20% of all crops are

lost due to pathogenic fungi, with an additional 10% loss after harvest[91]. Postharvest diseases from pathogenic fungi causes major reductions in the quality, shelf life, and market value of the fruits and vegetables [92]. In fact, out of all the food groups, fruits and vegetables are one of the largest contributors to economic loss in the industry[93], with the final consumer never seeing 10-15% of the crop in developed countries and 20-40% in developing countries due to postharvest losses[94]. 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.

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[95]. It can be efficiently genetically transformed and regenerated, and is susceptible to numerous plant-pathogenic agents, allowing it to effectively serve as a host plant in many pathogen-host studies[96]. As food security increasingly becomes an issue with our growing population, sustainable methods of disease control in the agricultural sector are needed. Our goal is to study 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. While the Y2H assays have been beneficial in estimating PGIP interactions and demonstrate the ability of Y2H to be a quick and easy way to screen for protein interaction levels, the potential of PGIP proteins as an exogenously applied anti-fungal agent has not yet been studied *in vitro* or *in planta.*

4.2 Methods

The *in-planta* assays utilize *N. benthamiana* plants that have been grown to roughly 5 – 7 weeks of age in an indoor greenhouse. Their leaves were then cut and sterilized in a 10% bleach and 0.5% TWEEN solution and washed three times in DI water. The leaves were then thoroughly sprayed by either a solution containing yeast secreting PGIPs, or with purified PGIP proteins, then air dried until droplets had dried on the leaf. Leaves were placed on petri dishes containing 1% agar to help prevent dehydration of the leaves. 20μL of a suspension of $X \times 10^{Y}$ *B. cinerea* condia/mL was spotted onto each leaf and growth of the fungus was monitored over the course of three weeks on covered petri dishes at room temperature. The plates were placed unsealed inside a fume hood in the dark. The fungi used were *B. cinerea* isolate ECC-0165 obtained from *Prunus persica* in Fresno, CA and *A. niger* isolate ATCC 16888 obtained from USDA-ARS Culture Collection. A plug from an actively growing colony was transferred to fresh potato flake agar plates that were sealed with Parafilm and incubated on the lab bench for *A. niger* and not sealed and incubated for *B. cinerea*. After sufficient sporulation, sterile DI water was added to plates, and a cell spreader was used to scrape the surface of the colony. The liquid was then poured through several layers of cheesecloth to exclude hyphal fragments. The spore concentration was quantified with a hemocytometer, then the suspension was diluted to a concentration of 2.5×10^{5} spores per mL. The suspension was aliquoted into 2 mL microcentrifuge tubes with screw caps and stored at 4°C until use.

The fungal pectin assay is an *in vitro* assay that observes the effects of PvPGIP proteins on fungal growth. *S. cerevisiae* containing plasmids with PvPGIP2, preOST1-

PvPGIP2, or a negative control containing an empty vector were grown for 16 hours in -Trp Dropout YNB. Two different growth media were used in this assay: potato flake agar for *A. niger* and potato dextrose agar (PDA) for *B. cinerea*. The potato flake 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 200mL of the medium. The PDA medium contains 4.8g potato dextrose broth (VWR, catalog #90003-494), 1g citrus pectin, and 2.5g agarose in 200mL of DI water. The medium was autoclaved and 150µL was pipetted into each well of a 96 well plate, with the exception of the edge-most wells due to evaporation. The agar was then allowed to cool and solidify. 60µL of the yeast in YNB was added to each plate along with 20µL of the fungal inoculum. The yeast and inoculum were added directly on top of the wells. These plates were left in a dark room at 20°C for 4 days and observed. The wells were visually compared for their fungal growth and the OD was measured at 630nm in a plate reader using the Gen5^{TM} Data Analysis Software (BioTek Instruments). The OD correlates directly to fungal dry weight. Another fungal pectin assay was performed where the growth radius of the resultant fungal colonies was compared. The same media, potato flake agar, was made with the same protocol as above for *A. niger* but poured into petri dishes instead of 96 well plates. The yeast and fungal inoculum are also prepared the same way. For *B. cinerea*, the only difference is that 0.1mg/mL of bromophenol blue (VWR, #MFCD00013793) was added to the medium. Once the plates solidified, 200µL of natamycin, yeast, or water was spread evenly onto the plates and allowed to dry. 2µL of the *A. niger* or *B. cinerea* fungal inoculum was then spotted into four locations per plate and growth was observed over several days.

To obtain purified PGIP proteins to test on our pectin and plant assays, the plasmids containing either PvPGIP2 or tPvPGIP2_5-8 proteins with the *his*-tag were transformed into CENPK2.1D yeast. After growing for four days, a colony was selected to grow in YPD for 24 hours, 200µL was transferred to a 500mL flask of -T YNB. This was grown with shaking in an incubator at 30°C for 48 hours. Afterwards, the yeast was spun down at 3000 rpm for 5 minutes and supernatant discarded. The yeast was resuspended in 1mL of chilled lysis buffer (50 mM pH 7.5 HEPES-KOH, 300mM KCl, 2.5mM DTT) then lysed in a cell disrupter 2 times at 30 seconds each time, with a two minute cooldown period in between each time. The solution was then spun at 15,000 rpm at 4°C for 30 minutes to clarify the lysate. The lysate was then placed into a tube containing 2mL of Ni-NTA resin and rotated at 4°C for 2 hours. This was then placed into a HisPur Ni-NTA Column from Thermofischer and allowed to elute via gravity. The column was washed twice with 5mL of cold wash buffer each time (50mM pH 7.5 HEPES-KOH, 300mM KCl, 10mM imidazole, 2.5mM DTT), then finally eluted with 3mL of cold elution buffer (50mM HEPES-KOH pH 7.5, 300mM KCl, 250 mM imidazole, 2.5mM DTT). The final elution was concentrated by using a 3 kDA filtration unit and 1mL of protein storage buffer (20mM sodium phosphate, 25mM NaCl, 10mM DTT, pH 7.4). The concentration of the protein was ascertained using the Bradford Assay from Bio-Rad according to the manufacturer's specifications.

4.3 Validating the inhibitory activity of PGIPs on pectin plate assays

To validate the inhibitory activity of PGIPs to PGs, and the potential of using PGIPs exogenously to inhibit fungal growth, we constructed yeast strains that can secrete PGIPs

and monitored fungal growth on pectin in the presence of the PGIP-secreting yeast strains. The *Ost1* signal sequence, a well-documented secretion tag[97], was fused to PvPGIP1 and PvPGIP2. Since the corresponding clone of PvPGIP2 (the *OST1-PvPGIP2-HA construct* [hemagglutinin, or HA, is a general antibody epitope tag used in western blots]) was not successfully constructed after repeated trials, we used PvPGIP1 construct instead to validate the utilization of *OST1* signal sequence. The presence of PvPGIP1 in both the cell lysate and medium was verified through western blot (Figure 4.1a). Interestingly, a faint band also detected in the medium of yeast harboring PvPGIP1 without the *Ost1* signal sequence, which implies that PvPGIP1 can be natively secreted out of the cell. This is consistent with its defense function reported previously[38].

With the secretion of PvPGIP1 aided by the *Ost1* signal sequence confirmed, PvPGIP1 and PvPGIP2-secreting yeast strains without the HA tag were constructed and evaluated in an *in vitro* assay to evaluate whether PGIPs can inhibit the growth of fungi in the presence of pectin. *A. niger* or *B. cinerea* was inoculated on agar plate amended with 0.5% citrus pectin (weight/volume) in the presence of PGIP-secreting yeast (roughly 1.2 x 10^6 cells), the antifungal agent natamycin at a concentration of 100 mg/L (positive control), or wild-type yeast strains (negative control). The growth of *A. niger* and *B. cinerea* were estimated with OD readings at 630nm[98]. Both the full length PvPGIP2 and tPvPGIP2_5-8 efficiently delayed the growth of both fungi (Figure 4.1b and 4.2c). Meanwhile, when PvPGIP2 or tPvPGIP2 were expressed from high copy plasmids, lower OD readings at 630nm were detected than when expressed from low copy plasmids, which shows that the inhibitory activity of PGIP is likely dose dependent.

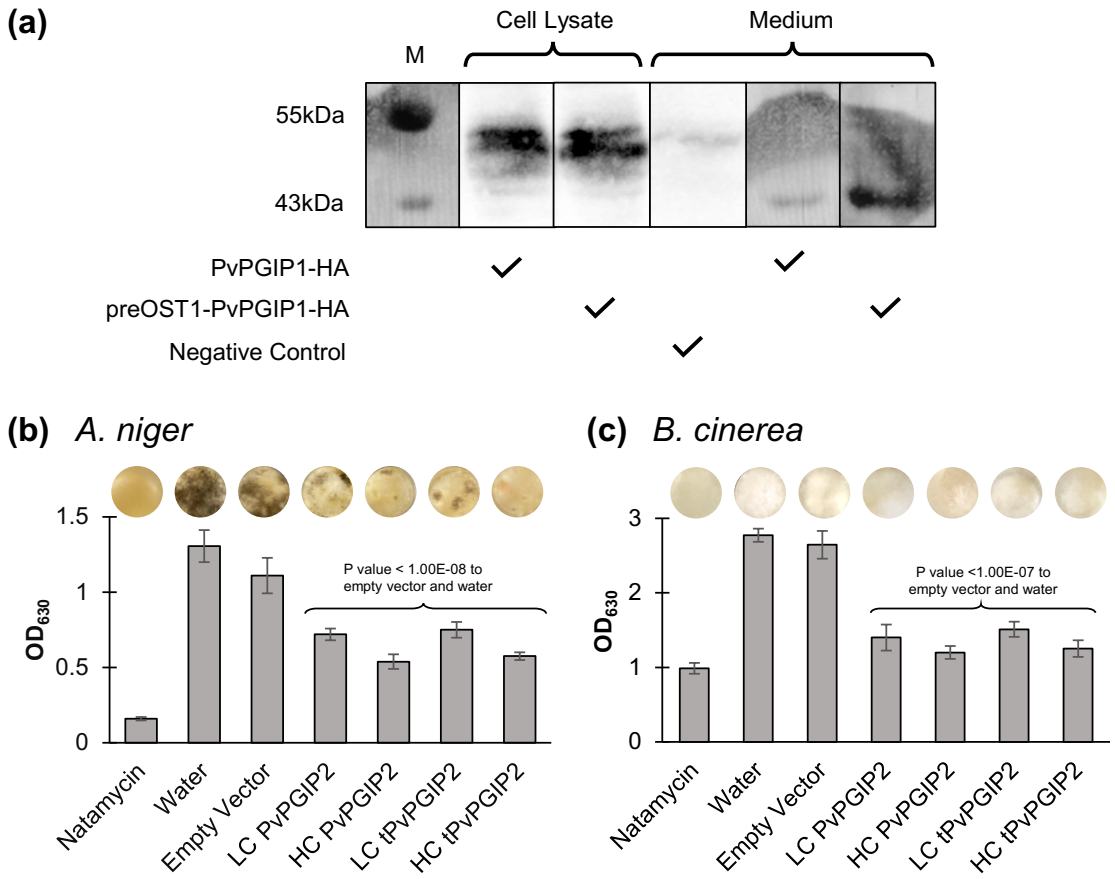

Figure 4.1 Construction of the yeast strain secreting PvPGIPs and establishment of fungal growth inhibition assay. (a) Western blot analysis of *S. cerevisiae* engineered to secrete PvPGIP1 with anti-HA antibody after three days of growth. PvPGIP1-HA has an expected size at 45.7 kDa. The proteins are running higher than the protein marker likely due to post-translational modificiation and glycoslyation of the PGIPs, which normally resulting in a larger protein size. M represents the protein marker (Fischer BioReagents EZ-Run Prestained *Rec* Protein Ladder, Fischer Scientific, #BP3603500). The cell lysate samples indicate the presence of the protein inside the cell. The medium samples indicate the presence of the secreted protein into the yeast medium. The inhibition activity of the secreted PvPGIPs was then tested on a fungal growth inhibition assay. OD readings at 630nm of **(b)** *A. niger* and **(c)** *B. cinerea* treated with either natamycin (positive control), empty vector yeast (negative control), low copy (LC) or high copy (HC) tPvPGIP2_5-8 secreting yeast, or LC or HC PvPGIP2 secreting yeast were taken after four days. At 630nm, OD readings are reported to be proportional to the dry weight of fungi[98]. Each bar represents the mean values of six biological replicates, and the error bars represent the standard deviation of the replicates. The well pictured above each bar is representative sample of the replicates. P values were obtained through a one-way ANOVA test.

To further confirm the activities of PGIPs, the PGIP- secreting yeast was spread onto the entire plate and 2µL of *A. niger* (5 x 10^5 conidia/mL) was spotted into four locations. The growth of fungi was also monitored over seven days and both full length PvPGIP2 and tPvPGIP2_5-8 delayed sporulation of both *A. niger* (Figure 4.2) and *B. cinerea* (Figure 4.3) by at least one day. Similarly, PvPGIP2 or tPvPGIP2_5-8 expressed from high copy plasmids was more effective in inhibiting the growth of *A. niger* and *B. cinerea* compared to the ones expressed from low copy plasmids. These results suggest that PvPGIP2 and tPvPGIP2, when applied exogenously, can inhibit *A. niger* and *B. cinerea* utilization of pectin and thus impede fungal growth when pectin is present as one carbon source, though with a lower efficiency than the antifungal agent natamycin. The consistency between the Y2H assay and fungal-pectin assay also demonstrates that Y2H can be utilized as a functional monitoring or screening method for PGIP-PG interactions.

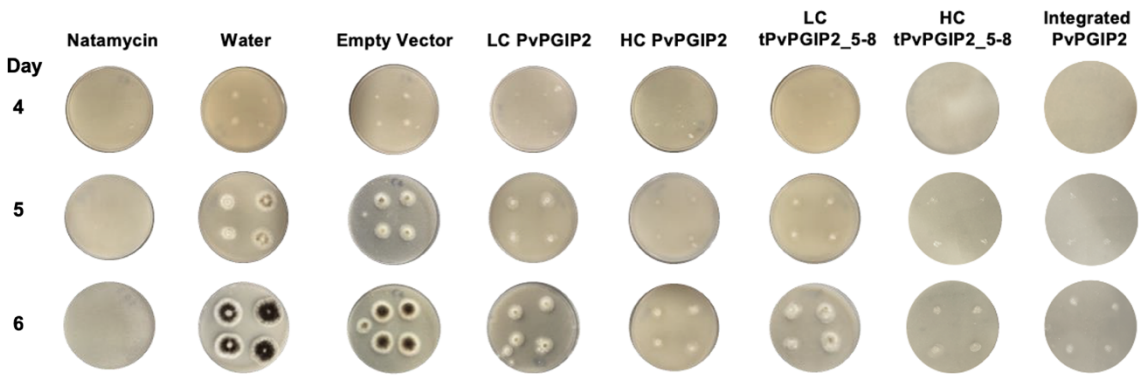

Figure 4.2 Fungal spot assay of *A. niger* and PvPGIP2. Here we test the effects of natamycin (positive control), yeast harboring an empty vector (negative control), tPvPGIP2_5-8 secreting yeast, and full length PvPGIP2 secreting yeast on *A. niger*. *A. niger* was spotted onto potato agar plates treated witheither natamycin (positive control), water (negative control), empty vector yeast (negative control), tPvPGIP2_5-8 secreting yeast, or PvPGIP2 secreting yeast. Growth was observed over the course of a week.

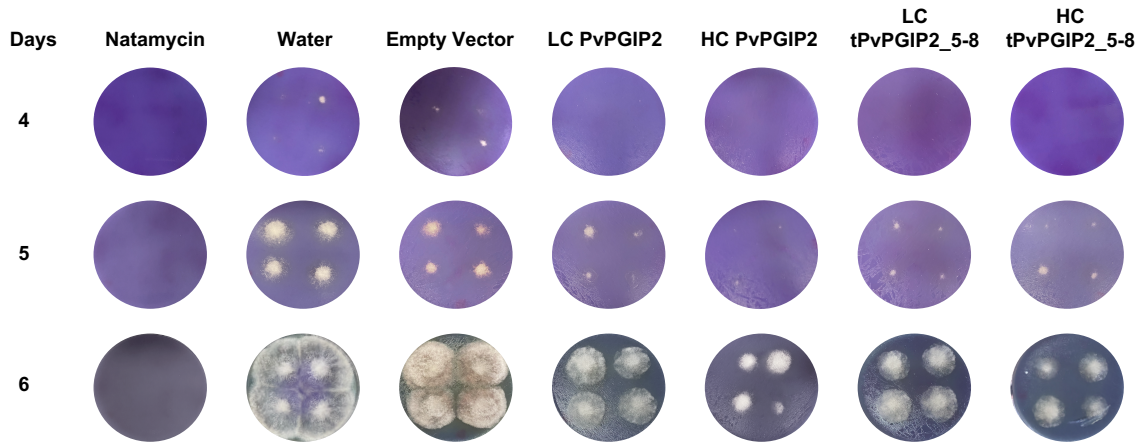

Figure 4.3 Fungal spot assay of *B. cinerea* and PvPGIP2. Here we test the effects of natamycin (positive control), yeast harboring an empty vector (negative control), tPvPGIP2_5-8 secreting yeast, and full length PvPGIP2 secreting yeast on *B. cinerea*. *B. cinerea* was spotted onto potato agar plates containing bromophenol blue for enhanced contrast and treated with either natamycin (positive control), water (negative control), empty vector yeast (negative control), tPvPGIP2_5-8 secreting yeast, or PvPGIP2 secreting yeast. Growth was observed over the course of a week.

4.4 Testing the Concentration of PvPGIPs *In Vitro* and *In Planta*

To obtain purified proteins for use in pectin and detached leaf assays, yeast was used to expressed PvPGIP2 or tPvPGIP2_5-8 proteins containing the *his*-tag, which was then eluted and concentrated for use. The presence of protein was confirmed on an SDS-PAGE (Figure 4.4a) and concentration estimated using a standard curve made using BSA and the Bradford Assay (Figure 4.4b). After concentration, the protein solution averaged roughly 0.9 mg/mL to 1.1 mg/mL.

To determine what concentration of PvPGIP2 is necessary to inhibit fungal growth, various titrations of PvPGIP2 were sprayed onto *N. benthamiana* leaves treated with *B. cinerea* and observed over three weeks. Concentrations 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 (Figure 4.4). We previously discussed that the level of fungal inhibition seen by PGIPs is likely dose dependent, which this assay further confirms. At the highest concentrations of ~1.0mg/mL and ~0.75mg/mL, no fungal infection is seen by week 3. An inverse relationship between the concentration of PGIP and the amount of fungal infection is seen on the leaves starting at week 2. When the concentration of PGIP is ~0.5mg/mL or lower, infection and leaf degradation is seen by week 2, with substantial and widespread damage to the leaves visible by week 3. However, it appears that even at the lowest concentration of PGIP tested, it still confers some degree of protection against *B. cinerea*, as the leaves treated with ~0.125mg/mL of PvPGIP2 still has reduced levels of infection compared to the negative control.

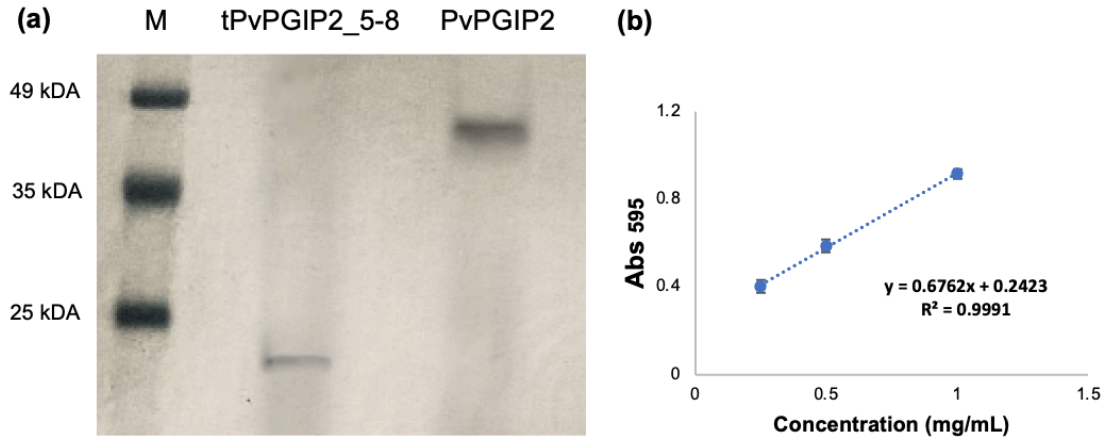

Figure 4.4 Verification of the presence of purified PGIP proteins. (a) An SDS-PAGE was used to confirm the presence of tPvPGIP2_5-8 and PvPGIP2 proteins after elution from a Ni-NTA column. **(b)** A standard curve created using BSA and the Bradford assay to estimate the concentration of PGIP proteins when measured at an absorbance of 595nm.

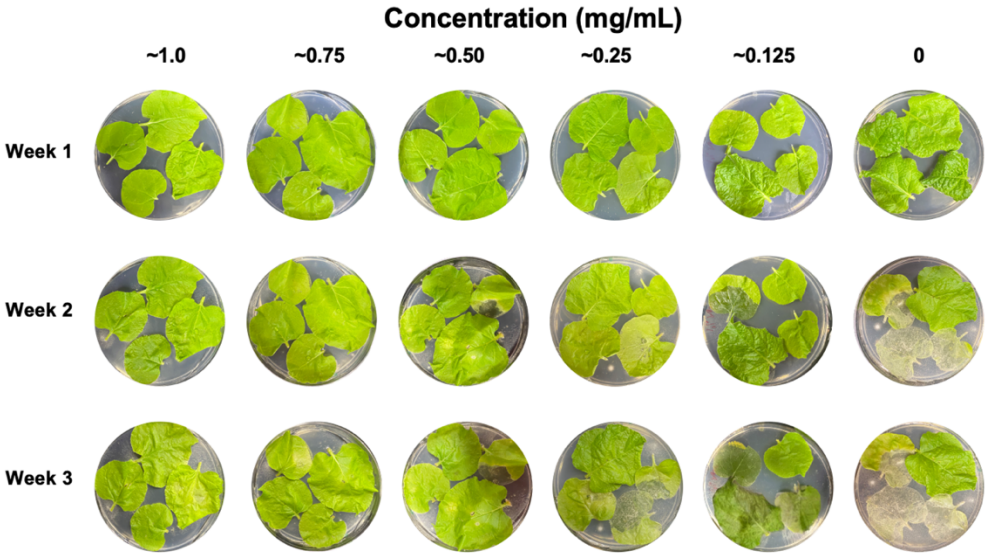

Figure 4.5 Determining the concentration of PvPGIP2 necessary to inhibit growth of B. cinerea on detached leaf assays. *N. benthanmiana* eaves spotted with *B. cinerea* spores were treated with roughly 1.0, 0.75, 0.50, 0.25, and 0.125mg/mL of PvPGIP2 proteins and compared to the negative control of leaves containing *B. cinerea* only. Each plate contained 4 leaves used as biological replicates.

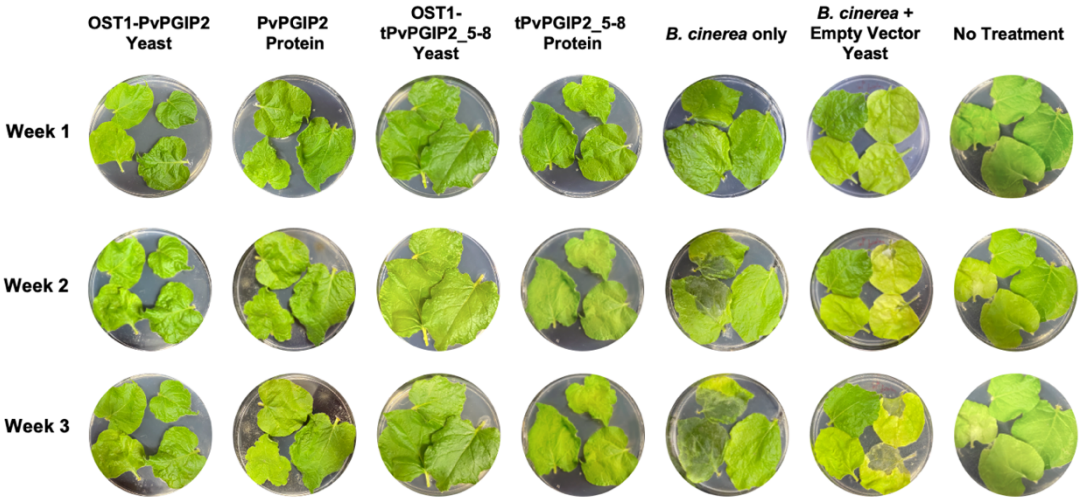

Figure 4.6 Comparing the efficacy of PGIP-secreting yeast and purified PGIP proteins on a detached leaf assay with B. cinerea. A detached leaf assay was performed comparing leaves treated with *Ost1*-PvPGIP2 secreting yeast, PvPGIP2 protein, Ost1-tPvPGIP2_5-8 secreting yeast, tPvPGIP2_5-8 protein, *B. cinerea* only (negative control), *B. cinerea* and yeast containing an empty vector (negative control), and no treatment. Each plate contained at least 3 leaves used as biological replicates.

We next compared the *B. cinerea* inhibition efficacy of yeast secreting PGIPs using the *Ost1* signal and purified PGIP proteins on the detached tobacco leaves. The titer of PGIP in the yeast medium using the Ost1 signal peptide is roughly 0.065 to 0.130 mg/mL, and the concentration of our purified PGIP proteins typically falls between 0.9 mg/mL to 1.1 mg/mL. However, despite the significantly higher concentration of purified proteins, the yeast would continuously produce the PGIPs as long as it survives, while the concentration of the applied purified PGIP proteins would remain static. A 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* only (negative control), *B. cinerea* with yeast containing an empty vector (negative control), and no treatment. Both negative controls showed notable *B. cinerea* infection at week 2, with the *B. cinerea* only leaves being more severe than the empty vector yeast plate. This may be due to the yeast acting as a physical barrier towards infection. However, none of plates containing either PGIP-secreting yeast or PGIP proteins showed any sign of infection on the leaves, even at three weeks. Instead, a minute amount of fungal growth was found on the agar, implying that the pathogenic fungi preferred to grow on the agar rather than the leaf treated with PGIPs. Both the yeast and protein treatments had comparable results, regardless of using full length of truncated versions of PvPGIP2.

4.5 Testing the Thermostability of PvPGIPs

Next, the thermostability of both the full length PvPGIP2 and tPvPGIP2_5-8 was evaluated. Our prior results demonstrated that the PGIPs retained their fungal-inhibiting abilities at room temperature, but the effects of a higher temperature were yet unknown.

PvPGIP2 purified proteins were incubated at 42°C for 12 hours, 24 hours, and 48 hours, then sprayed onto detached leaves from *N. benthamiana* treated with *B. cinerea* (Figure 4.7). The leaves were then compared to leaves treated with only *B. cinerea* and leaves without treatment, to better visualize the effects of fungal infection and natural leaf deterioration over time respectively. 42°C was chosen to simulate a hot day, as the proteins would theoretically be used in the agricultural setting with variable weather. The leaves were observed over a period of three weeks. Both the full length and truncated PvPGIPs delayed the onset of *B. cinerea* infection by at least one week regardless of how long the proteins were incubated for at 42°C. Both the full length and truncated PGIPs demonstrate great efficacy at delaying *B. cinerea* growth for two weeks, with no visible leaf damage of signs of infection visible. Meanwhile the leaves treated with only *B. cinerea* began to show infection and leaf degradation by the second week. However, at week 3, the full length PvPGIP2 protein that had been treated for 48 hours showed signs of fungal infection on all leaves. Meanwhile, tPvPGIP2_5-8 had evidence of infection on both the 24 and 48 hour-treated proteins. Both PvPGIP2 and tPvPGIP2_5-8 had no notable *B. cinerea* growth or any visible damage to the leaves at week 3 for the proteins treated for 12 hours. These results suggest that full length PvPGIP2 is thermostable at 42°C and retains its full inhibitory activity even when treated for up to 24 hours but begins to lose efficacy at 48 hours. On the other hand, tPvPGIP2_5-8 appears to lose some inhibitory activity by week 3 when the protein is treated for greater than 24 hours at 42°C. It is possible that some regions outside the LRR5-8 area may be responsible for thermostability, even if they are not directly related to PG recognition and interaction.

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* (Figure 4.6). As with the detached leaf assays, PvPGIP2 protein was incubated at 42°C for 12, 24, and 48 hours before it was liberally sprayed onto pectin plates that were spotted with 4 dots of *A. niger* spores. 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 hours appears to slightly decrease PvPGIP2's fungal-inhibiting activity compared to treatment at 12 hours, which had similar results to PGIPs left at room temperature. All heat treatments resulted in a reduction of fungal growth by greater than 50% compared to the negative control, which only had *A. niger.* This suggests that though prolonged incubation at 42°C may slightly reduce the efficacy of PvPGIP2 against *A. niger,* it still retains a notable amount of inhibitory activity.

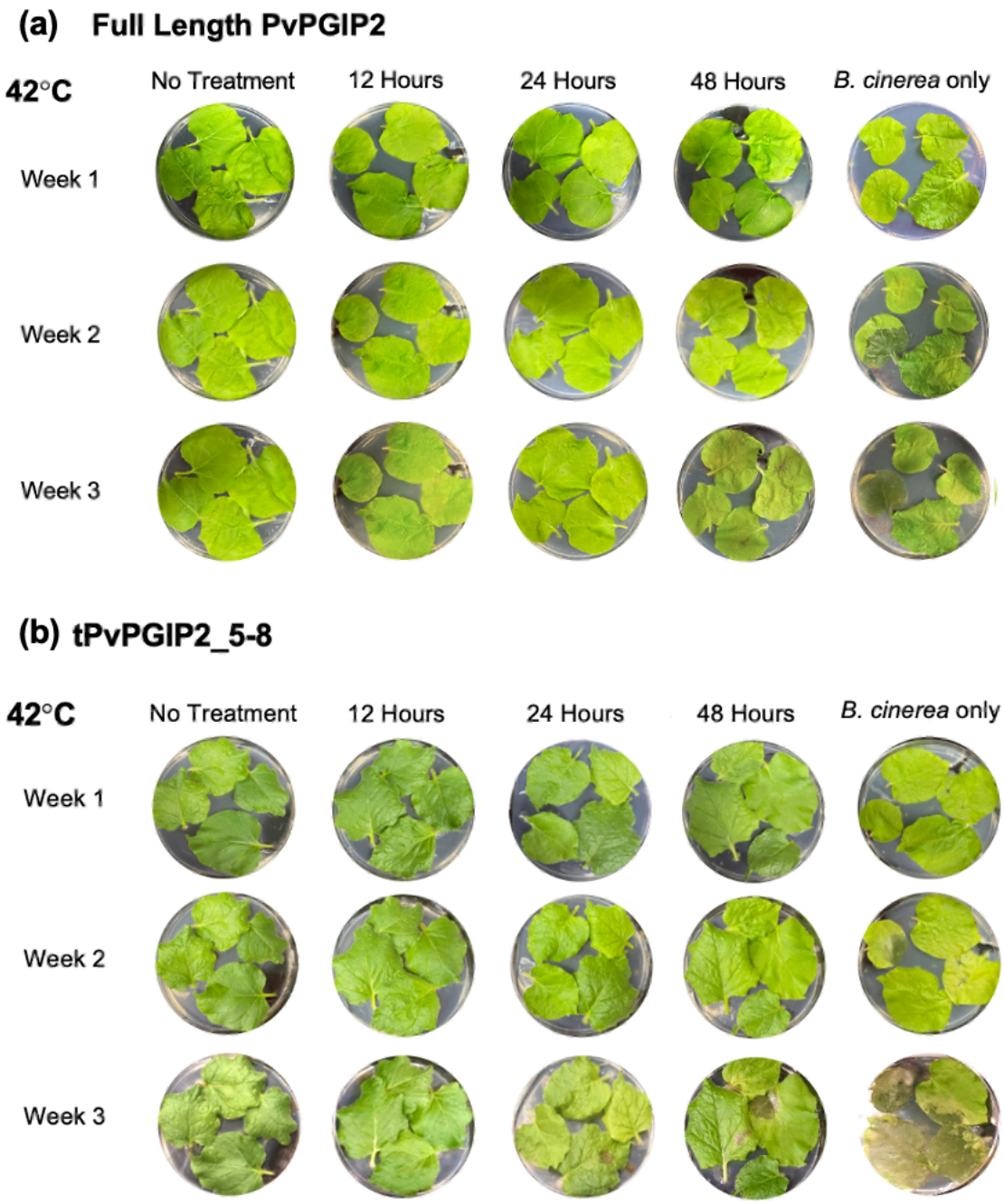

Figure 4.7 Testing the thermostability of full length PvPGIP2 and tPvPGIP2_5-8 on detached leaf assays with *B. cinerea*. Here we test the effects of PvPGIP2 (a) and tPvPGIP2_5-8 (b) incubated at 42°C for 12, 24, and 48 hours compared with the controls of leaves treated with of *B. cinerea* only (negative control) or no treatment (neither *B. cinerea* nor PGIPs). Each plate contained four leaves used as biological replicates.

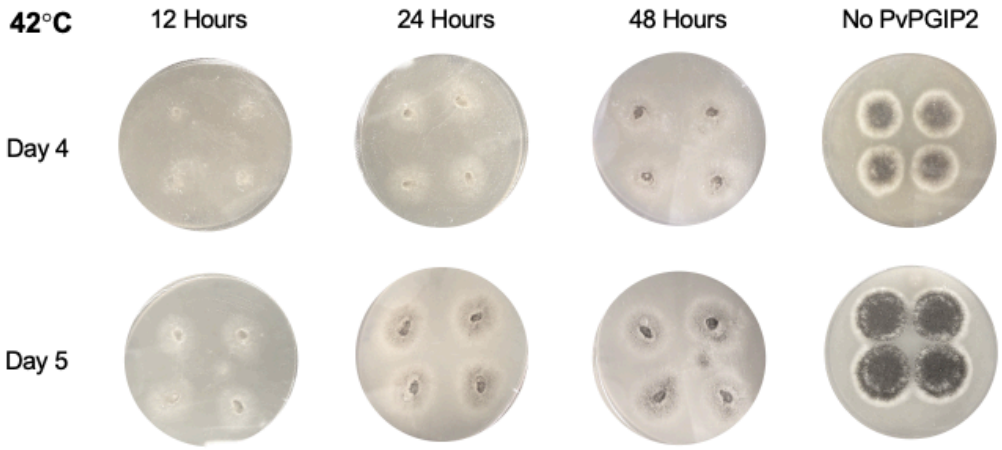

Figure 4.8 Testing the thermostability of full length PvPGIP2 on pectin plate assays with *A. niger*. Here we test the effects of PvPGIP2 incubated at 42°C for 12, 24, and 48 hours compared with the pectin plate treated with of *A. niger* only (negative control).

Chapter 5

Conclusions

5.1 Key Developments of this Dissertation

As the risk of conventional fungicides continue to be a cause for a concern, there is increased demand for more eco-friendly pest management approaches. Regulation of fungicide use has increased in many countries and regions, and consumers are wary of chemical residues on their fruits and vegetables. However, more sustainable approaches to control fungal pathogens, especially at the post-harvest stage, are still in the early stages of development. PGIPs are naturally occurring plant proteins that can slow the rate of infection from pathogenic fungi by inhibiting one of the key steps in the infection process—the degradation of cell walls via PGs. While conventional fungicides continue to be key to ensuring food security and preventing crop loss across the world, our approach for developing and engineering PGIPs is a step towards providing an alternative, eco-friendly strategy to fungal pest control. The results of this research validate the utilization of Y2H to estimate the interactions between PvPGIPs and PGs. PvPGIP1, PvPGIP2, and GmPGIP3 have the strongest interactions with AnPG2 and BcPG1 compared to BcPG2 and FmPG3, as evidenced by both higher growth rate and stationary phase in growth curves. PvPGIP2 and GmPGIP3 have greater levels of interactions with PGs compared to PvPGIP1, which supports the findings from previous studies[54,76,99,100].

In our study, we found that LRR5 resides in a critical region for recognizing PGs, and that regions of PvPGIP2 and GmPGIP3 exclusive of LRR5 to LRR8 plays a very minor role in PG recognition. The successful truncation of PvPGIP2 and GmPGIP3 to one-third

of the original size that retains a similar level of inhibition activity retained indicates the versatility of LRR proteins and the potential to engineer this group of proteins for altered recognition and activities. Further investigation utilizing a molecular docking study on the interactions of tPvPGIP_5-8s, tGmPGIP_5-8s, and PGs may aid in a more comprehensive understanding towards the engineering of PGIPs for enhanced activity and broader specificity. In this study, we were able to reconstitute the PG-PGIP interaction using a Y2H system and identified a method to secrete functional PGIP from yeast into the medium. While a trace amount of PvPGIP1 was secreted without the *Ost1* signal, utilizing the signal peptide allowed us to clearly confirm the presence of PGIP in the medium. The presence of the N-terminus domains, Gal4 AD or *Ost1* signal peptide, may have enhanced the stability and folding of PvPGIP2 and GmPGIP3 in the yeast environment. According to previous investigations, the titer of a protein 166 residues in size fused with the *prepro-α* signaling factor from yeast is ~ 6.5 mg/L[101], whereas the *Ost1* signal peptide is approximately 10 to 20 times more efficient at protein secretion[102]. Thus, the titer of PGIP in the yeast medium using the *Ost1* signal peptide is estimated to be around 65 to 130 mg/L. Based on the results from the fungal growth assay on pectin agar, the concentration of PvPGIP2, tPvPGIP2, or GmPGIP3 in the exogenous application should not be lower than this magnitude (hundreds of mg/L) to efficiently inhibit pathogenic fungal growth.

Not only was the PvPGIP2 protein effective when it was secreted by yeast onto pathogenic fungi *in vitro*, it also showed promise and reduced pathogenic fungal growth *in planta*, delaying the onset of *B. cinerea* disease by one week, as well as slowing its infection rate once the initial sporulation was seen. Both full-length PvPGIP2 and

tPvPGIP2_5-8 were successfully purified and applied onto both pectin and *N. benthamiana* leaves. A concentration of 0.75 mg/mL of either the full-length or truncated PvPGIP2 protein protected against infection by *B. cinerea* by one additional week compared to 0.5 mg/mL and lower. However, even at concentrations as low as 0.125 mg/mL, there seems to be a slight decrease in the infection rate compared to no application of PvPGIP2 at all, indicating that the efficacy of PvPGIP2 is likely dose-dependent. The PGIP proteins have also been found to retain most of their PG-inhibiting activity even after a 24-hour treatment at 42°C, with some efficacy remaining after 48 hours. We believe this could be useful as the PGIPs would remain active even during hot weather if applied to plants.

Chemical-based fungicides are known to be detrimental to the environment and may lead to resistance in pathogenic fungi[103]. Unlike chemical fungicides, the use of exogenously applied natural plant proteins with known antifungal properties can potentially be an eco-friendly and sustainable method for controlling fungal diseases. These natural plant proteins are more socially acceptable, and compared with the production of transgenic plants, are more flexible. Additionally, antifungal plant proteins offer a variety of mechanisms and tools, urgently needed to fight against the rapidly evolving fungal pathogens. These naturally occurring plant peptides are strong candidates for developing broad-spectrum, fungal-control strategies.

5.2 Recommendations for Future Research

Some challenges remain unsolved and further research is needed to develop truncated PGIP peptides into practical approach. First, there are beneficial fungi that play essential roles to promote plant growth, such as mycorrhizal fungi that mediate plant-soil

feedback[104]. However, we do not know if the application of PvPGIP proteins will affect these nonpathogenic fungi. Second, while the full length PvPGIP2 is found in nature and has not been known negatively impact the environment in any studies, it is unknown if the truncated form has unwanted targets or toxic side products from its interactions. While unlikely to be a toxic agent, *in vivo* studies are needed to test the toxicity of PGIPs before practical and large-scale applications. Additionally, it is not currently known if the truncating PGIPs has any effect towards PGs that are inhibited through a noncompetitive inhibition mode. PvPGIP2 may be the most studied PGIP, but other plant PGIPs are of interest as well. For example, CkPGIP1 from *Cynanchum komaroviz*[105] and MdPGIP1 from *Malus domestica*[106] have shown to have activity against PGs that PvPGIP2 is not yet known to have, such as those from *Rhizoctonia solani*, *Diaporthe ambigua,* and *Botryosphaeria obtusa*[105,106]. Studying how other PGIPs inhibit PGs and understanding what motifs are present that allow for that interaction may offer hints at further evolving our truncated PvPGIP2 to have a broader spectrum of activity.

As the long-term goal of this research is to develop a sustainable, alternative fungal treatment, another future direction and challenge to tackle includes improving the protein yield in the medium. *S. cerevisiae* is a well-studied model organism that is promising as a microbial host factory[107,108]. Efficient production of PGIPs by yeast will require a combination of improved secretion signal peptides and fine-tuning yeast metabolism [109]. By developing efficient synthesis, the cost of the proteins will decrease, enabling the peptides to be more viable and appealing as a product. Additionally, packaging PGIPs as a user-friendly product that can be applied with standard equipment is essential to the

practical utilization of this strategy. One method is to freeze dry the PGIP-secreting yeast for application. Previous studies have found that freeze drying naturally occurring microbiota on apples and applying a resuspended solution on postharvest crops greatly reduces fungal disease and loss of crop during shipping[110]. Furthermore, tPvPGIP2_5-8, although small compared to the full length PvPGIP2, is still much larger than most plant peptides [111] and requires correct folding to be functional. Thus, enhancing thermostability of the products would also be an important trait for efficiency, storage, and utility purposes. Most importantly, protocols will need to be developed to test the PGIPs on model plants, postharvest fruits, and seed storage to confirm if spraying the PGIPs on fruit and plant surfaces will reduce fungal infection. The *in-planta* data will be critical in validating the efficacy of these proteins, which may also imply whether PGIP applied exogenously can reach the targets (PGs) efficiently.

There are numerous innovative ways that plant proteins can be developed into tools for disease prevention in crops. One of the biggest hurdles to consider when developing these proteins is lowering the cost of production while enabling mass production. It will be necessary to explore and further optimize microbial factories and protein extraction methods before many of these natural plant proteins can be utilized readily in agricultural industry. Numerous studies showcase the efficacy of these proteins both *in vitro* and *in planta* against pathogenic fungi. The potential of using natural plant proteins exogenously to control agricultural fungal diseases remains largely untapped and need to be considered when developing future eco- and environmentally friendly antifungal agents. Here, PGIPs showcase proof of concept that plant proteins can potentially be utilized as an exogenously

applied, sustainable, fungal control agent that reduces disease incidence in postharvest crops. This research is a steppingstone towards developing an eco-friendly future where green products are an ever-growing industry.

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