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Exploring the Potential of Engineering Polygalacturonase-Inhibiting Protein as an Ecological, Friendly, and Non-toxic Pest Control Agent

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Abstract

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 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. We found that tPvPGIP2_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 via Y2H. The inhibitory activities of tPvPGIP2_5-8 on the growth of *A. niger* and *B. cinerea* were then examined and confirmed on pectin agar. On pectin assays, application of both full length PvPGIP2 and tPvPGIP2_5-8 clearly slows down the growth of *A. niger* and *B. cinerea*. Investigation on the sequence-function relationships of PGIP 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, and may be a possible target for the future engineering of PGIP with enhanced activity. This work highlights the potential of plant-derived PGIPs as a candidate for future *in planta* evaluation as a pest control agent.

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Author contributions

Y.L. and A.P. conceived of the project, Y.L., T.C., A.B., J.C., and A.P. designed the experiments, T.C. and A.B. performed the experiment and analyzed the results, Y.L., T.C., and A.P. wrote the manuscript.

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Introduction

Pathogenic fungi have long been a consistent source of devastation for the agricultural industry (De Lucca, 2007). Approximately 16% of the world's crops are lost to microbial diseases, with an estimated 70-80% of these losses caused by fungi (Moore et al., 2011). The range of tactics used to manage fungal pathogens include host resistance breeding (Melchers & Stuiver, 2000), cultural practices (Dekker, 1983), and plant protection (Möller & Stukenbrock, 2017; Wink, 1988). However, breeding for host resistance is a slow process and is ineffective for certain pathogens, while cultural practices often do not provide complete control. Plant protection has greatly reduced the loss of crops while improving yields (Morton & Staub, 2008). 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 (Gianessi & Reigner, 2006). However, the development of fungicide resistance in pathogen populations threatens to erode the efficacy of fungicides against several important pathogens (Ishii, 2006).

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 (Arcury & Quandt, 1998), and to ensure residue on harvested commodities remains below safe levels (Alavanja et al., 2004; Thabet et al., 2016). Fungicides may also negatively impact non-target organisms in agroecosystems, such as soil microbiota involved in nutrient cycling or plant symbionts (Yang et al., 2011), or insect symbionts (Wilson et al., 2014). Under certain conditions, fungicides may also be transported off-site following application via runoff or leaching and negatively impact aquatic organisms (Maltby et al., 2009). A more socially and environmentally sustainable method to control fungal plant pathogens is needed.

The plant cell wall is the first barrier a plant pathogen must overcome on its path to establishing a parasitic relationship (Hématy et al., 2009). Cell wall-degrading enzymes including polygalacturonases (PGs) play an important role in the pathogenicity of many pathogenic fungi (Kubicek et al., 2014). 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 (Adele Di Matteo et al., 2006; Hammond-Kosack & Jones, 1997). PGIPs slows the hydrolysis process catalyzed by PGs through competitive or noncompetitive inhibition, which requires PGIP to bind to the PGs either in or distant to the active site, respectively (A. Di Matteo et al., 2003; Kalunke et al., 2014; King et al., 2002; Stotz et al., 2000). Additionally, PGIPs may play a role in plant protection is by reducing the rate at which the plant oligosaccharides are broken down (Frediani et al., 1993). Oligosaccharides, specifically, oligogalacturonides, are endogenous elicitors that may be accumulated in response to the degradation of pectin and help trigger plant defense (Ferrari et al., 2013), such as the production of reactive oxygen species (Galletti et al., 2011) and the accumulation of phytoalexins (Davis et al., 2013). 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 (Caprari et al., 1996), which are highly conserved across different PGIPs (Matsaunyane et al., 2015) (Figure 1a). These conserved regions are also known to be involved in the structural integrity of PGIPs (A. Di Matteo et al., 2003). It is believed that the central LRR pocket is responsible for recognizing the amino acids present at the active site of many PGs (Helft et al., 2011). This specificity allows PGIPs to differentiate fungal PGs from endogenous plant PGs (L. Federici et al., 2001). Prior studies indicate that overexpression of either endogenous or heterologous PGIPs in various plants enhanced their resistance towards fungal infection (Rathinam et al., 2020). However, whether this approach can be utilized as a tool for plant disease prevention remains unclear due to difficulties with public perception accepting transgenic plants. 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.

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), which exhibits inhibitory activity toward a number of fungal PGs (Capodicasa et al., 2004; Leckie et al., 1999). Despite differing from isoform 1, PvPGIP1, by only 10 residues, PvPGIP2 confers resistance against a greater known number of fungal PGs (Kalunke et al., 2015; Aditi Maulik et al., 2009) (Figure 1). 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 (Haeger et al., 2020). We reproduced previously reported interactions between PvPGIP2 and various PGs in a yeast two hybrid (Y2H) system, thus validating it as an effective approach to rank PvPGIP2 activity. Y2H was then used to rank the ability of various PvPGIP2 truncations and mutants to inhibit the activity of PGs. Different truncations resulted in varied levels of interaction with PGs, showing that some LRRs may be more important for recognizing certain PGs compared to others. We found that truncating the PvPGIP2 to only the region between LRR5 and LRR8 retained similar PG interaction levels to the full length PvPGIP2. Furthermore, mutants were created with amino acid residues that differed between PvPGIP1 and PvGIP2. Our approach also showed that Val172 of PvPGIP2 is the most critical residue of those tested for recognition of fungal PGs, in agreement with previous research (Helft et al., 2011; Leckie et al., 1999; Sicilia et al., 2005). The interaction of PGIP2 variants with various PGs was demonstrated by assaying fungal growth on pectin agar, with or without the presence of engineered yeast strains. 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.

Results

Interactions between PG and PGIP can be monitored using yeast two hybrid system.

Because PGIPs can physically inhibit PGs *via* competitive (Sicilia et al., 2005; Spinelli et al., 2009) and noncompetitive inhibition (King et al., 2002; Stotz et al., 2000), 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 (A. Di Matteo et al., 2003) (Figure 1). 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) (Cervone, 1997; L. Federici et al., 2001; Leckie et al., 1999). 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 (Zhang et al, 2006).

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 due to its ability to affect many common crops, such as onions (Gherbawy et al., 2015), grapes (Logrieco et al., 2009), peanuts (Xu et al., 2015), and maize (Palencia et al., 2010). 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 (Sharma, 2012). *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 (Weiberg et al., 2013). For example, it is considered the most economically significant pathogen of strawberry worldwide (Petrasch et al., 2019). *F. moniliforme* (Pamphile & Azevedo, 2002) can produce mycotoxins, toxic secondary metabolites to both humans and animals (Schoeman et al., 2018). It is one of the most significant fungal pathogens to infect corn (Munkvold, 2003).

Previous investigations indicate that PvPGIP2 inhibits the function of AnPG2 (Leckie et al., 1999; Spadoni et al., 2006; Spinelli et al., 2009) and BcPG1 (Leckie et al., 1999; Manfredini et al., 2005; Sicilia et al., 2005), weakly inhibits FmPG3 (L. Federici et al., 2001; Leckie et al., 1999), and possibly inhibits BcPG2 (Leckie et al., 1999). In contrast, PvPGIP1 only inhibits the activity of AnPG2 (Leckie et al., 1999; Spadoni et al., 2006), weakly inhibits BcPG1 (Leckie et al., 1999) and does not inhibit FmPG3 (Leckie et al., 1999; Aditi Maulik et al., 2009). The activity of PvPGIP1 against BcPG2 is unknown. 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 (Shaffer et al., 2012) (Figure S1). 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. Growth of 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. With Gal4 AD-PvPGIP2, yeast containing Gal4 BD-AnPG2 or BcPG1 exhibited robust growth in -HTL medium, indicating strong PG-PGIP interactions (Figure 2). Yeast with Gal4 AD-PvPGIP2 harboring either Gal4 BD-BcPG2 or Gal4 BD-FmPG3 grows much slower than the positive control (Figure 2), which implies weak PG-PGIP interactions. The distinct PG-PGIP interactions may be due to the different specificity of the protein recognition site at the concave surface of the PGIPs (A. Di Matteo et al., 2003; Sicilia et al., 2005). Yeast harboring Gal4 AD-PvPGIP1 and Gal4 BD-AnPG2 grew at a similar level to the positive control (Figure 2). Yeast harboring FmPG3 exhibits the slowest growth that is slightly higher than the negative control indicating little to no Y2H interaction. The PG-PGIP interactions implied from Y2H assay overall are reflective of findings in previous studies (L. Federici et al., 2001; Luca Federici et al., 2006; Leckie et al., 1999; Manfredini et al., 2005; A. Maulik et al., 2012; Sella et al., 2004; Spadoni et al., 2006; Spinelli et al., 2009) and suggests that it is feasible to use Y2H assay to functionally map PG-PGIP interactions. Furthermore, 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 S3), thus further validating the feasibility of using Y2H assay to study PG-PGIP interactions.

Truncated forms of PvPGIP1 and 2 consisting of only LRR5 to LRR8 retain similar level of interaction with AnPG2 and BcPG1 compared to the full length PvPGIP2.

The modular structure of leucine-rich repeat proteins (Figure 1) leads to the hypothesis that a fraction 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 3). PvPGIPs lacking the N-terminus (Figure 3, S4a) or C-terminus (Figure 3, S4b) 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. (Sicilia et al., 2005). Thus, PvPGIP2 was truncated to retain only LRR5 to LRR8 (residues 172 – 266, tPvPGIP2_5-8, Figure 1c), 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 (Figure 3, S4c). A slightly larger truncation, tPvPGIP2_5-9 (residues 172 – 290, Figure 3, S4d), 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 (Figure 3, S4e). Likewise, additional truncation of tPvPGIP2_6-8 to tPvPGIP2_6-7 (residues 197 – 243, Figure 3, S4f) also exhibited no inhibitory activity to the tested PGs. Truncation of LRR8 from tPvPGIP2_5-8 to make

tPvPGIP2_5-7 (residues 172 – 243, Figure 3, S4g) led to reduced interactions with AnPG2 and BcPG1 compared to tPvPGIP2_5-8 (Figure 3, S4g). Other truncations that contained only LRR4 to LRR5 (residues 138 – 197, Figure 3, S4h), LRR4 to LRR6 (residues 138 – 219, Figure 3, S4i), LRR1 to LRR4 (residues 73 – 172, Figure 3, S4j), and LRR7 – LRR10 (residues 219 - 310, Figure 3, S4k) 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 (Manfredini et al., 2005) that tPvPGIP2_5-8 retains clusters of residues demonstrated to be important for BcPG1 interaction (Figure 1b). Taken together, our results indicate that the regions outside of LRR5 to LRR8 in PvPGIP2 are not essential for the interaction with PGs, and the specific function of these parts of PGIPs remain to be clearly elucidated.

We then measured the interactions of both 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 S2). 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) are able to interact with PGs in a similar capacity to their full length counterparts (Figure S2). 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.

Both full length and truncated PvPGIP2 can efficiently inhibit the growth of *A. niger* when applied exogenously.

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 (Fitzgerald & Glick, 2014), 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 4a). 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 (Kalunke et al., 2015).

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 (Langvad, 1999). Both the full length PvPGIP2 and tPvPGIP2_5-8 efficiently delayed the growth of both fungi (Figure 4b and 4c). 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. 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 S5) and *B. cinerea* (Figure S6) 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.

Investigation on PGIP sequence-function correlation

Although PvPGIP1 and PvPGIP2 are highly similar in amino acid sequence (Figure 1c), they exhibit distinct activities against different PGs, such as BcPG2 (Figure 2). 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 (Manfredini et al., 2005; Sicilia et al., 2005). However, 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 (Figure 2). 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. There are ten different residues 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 (Sicilia et al., 2005). Three of these residues are located in the truncated region of LRR5 to LRR8 (Figure 5a). 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 (Figure S2).

To determine which of these 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. 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 (A. Di Matteo et al., 2003), 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” (Figure 5, S7c), tPvPGIP_5-8 “212” (Figure 5, S7d), tPvPGIP_508 “211” (Figure 5, S7e), tPvPGIP_5-8 “122” (Figure 5, S7f), tPvPGIP_5-8 “112” (Figure 5, S7g), and tPvPGIP_5-8_”121” (Figure 5, S7h). 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 (Figure 5). The growth of the six diploid yeast strains in -HTL medium were compared with the controls. When the chimeric tPGIP_5-8 contained Val from PvPGIP2 instead of Gly from PvPGIP1 at position 172, it retained PG interaction ability similar to 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 impact on PG-PGIP interaction. Furthermore, we conducted saturated site-directed mutagenesis at residue 172 of tPvPGIP2_5-8 (Figure 6). 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 (Figure 6). In addition, tPvPGIP2_5-8^{V172L} showed slightly higher interactions than wild type tPvPGIP2_5-8 against BcPG1 (Figure 6b) but not the other three PGs (Figure 6). However, no amino acid substitution showed a statistically significant improvement in PG interactions compared to the wild type tPvPGIP2_5-8, which indicates that natural evolution has already selected for the optimal amino acids for interacting with the tested PGs.

Discussion

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. PGIPs are naturally occurring plant proteins that can slow the rate of infection from pathogenic fungi by inhibiting one of the methods fungi use for colonizing plants—the degradation of cell walls via PGs. The results of this research

validate the utilization of Y2H to estimate the interactions between PvPGIPs and PGs. Both PvPGIP1 and PvPGIP2 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 has greater levels of interactions with PGs compared to PvPGIP1, which supports the findings from previous studies (Bishop, 2005; D'Ovidio et al., 2004; Leckie et al., 1999; Manfredini et al., 2005).

In our study, we found that LRR5 resides in a critical region for recognizing PGs, and that regions of PvPGIP2 exclusive of LRR5 to LRR8 plays a very minor role in PG recognition. The successful truncation of PvPGIP2 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 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 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 (Kajava et al., 1995), whereas the *Ost1* signal peptide is approximately 10 to 20 times more efficient at protein secretion (Barrero et al., 2018). 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 or tPvPGIP2 in the exogenous application should not be lower than this magnitude (hundreds of mg/L) to efficiently inhibit pathogenic fungal growth.

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 (Kadowaki et al., 2018). 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 is not known to negatively impact the environment, 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 is 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 komarovii* (Liu et al., 2016) and MdPGIP1 from *Malus domestica* (Oelofse et al., 2006) 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* (Liu et al., 2016; Oelofse et al., 2006). 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 (Borodina & Nielsen, 2014; Siddiqui et al., 2012). Efficient protein production by yeast will require a combination of improved secretion signal peptides and fine-tuning yeast metabolism (Delic et al., 2013). 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 (Navarta et al., 2020). Furthermore, tPvPGIP2_5-8, although small compared to the full length PvPGIP2, is still much larger than most plant peptides (Das et al., 2020) 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. With more research, they can be enhanced to further improve their efficacy and more functionalities may be discovered. Here, PvPGIPs are proof that plant proteins can successfully be utilized as exogenously applied, sustainable, natural fungal control agent and reduce disease incidence in postharvest crops. This research is a steppingstone towards developing an eco-friendly future where green products are an ever-growing industry.

Materials and Methods

Plasmid construction

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, catalog #11789020). Truncated versions of the PGIPs were made using various primers as seen in Table S4 that ensured the protein would not be spliced in the middle of the β-sheets. This 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) (Alberti et al., 2007). 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. The strains and plasmids constructed and used in this study are listed in Table S1 and Table S2, respectively. The sequences of the genes and primers used in this study are listed in Table S3 and Table S4, respectively.

Y2H growth curve assay

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 (Truong & Gietz, 2007). 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™ Data Analysis Software (BioTek Instruments).

Verifying and qualifying secreted PvPGIP protein presence via western blot

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 sat 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.

Site-Directed Mutagenesis

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 (Horton et al., 1993). Five oligonucleotides, listed in Table S4, were annealed together to create tPvPGIP2_5-8 mutants with residue 172 replaced in the first oligonucleotide. Each PCR reaction contained 20μL of DI water, 0.5μL of 10mM dNTPs, 0.3μL of each oligonucleotide, and 0.3μL of Expand polymerase. Afterwards, an additional 0.3μL of the first and last oligonucleotide were added, along with 0.3μL dNTPs and 0.2μL Expand polymerase for another round of PCR following a standard thermal cycling program suggested by the manufacturer (Expand Long Template PCR System, Sigma-Aldrich).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. Similarly, to create the mutants seen in Figure 5, five oligonucleotides containing degenerate nucleotides at PvPGIP2 residues 172, 198, and 244 (SOE Degen A-E, Table S4) were ordered from IDT DNA and assembled using the same SOE PCR protocol as described above. The degenerate nucleotides would code for either of the residues seen at PvPGIP1 or PvPGIP2, leading to our sequence function correlation data.

Fungal pectin assay

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. The fungi used were *B. cinerea* isolate ECC-0165 obtained from *Prunus persica* in Fresno, CA and *A. niger* isolate ATCC 16888 obtained from ARS Culture Collection (NRRL). 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. 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 the medium. 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. 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™ 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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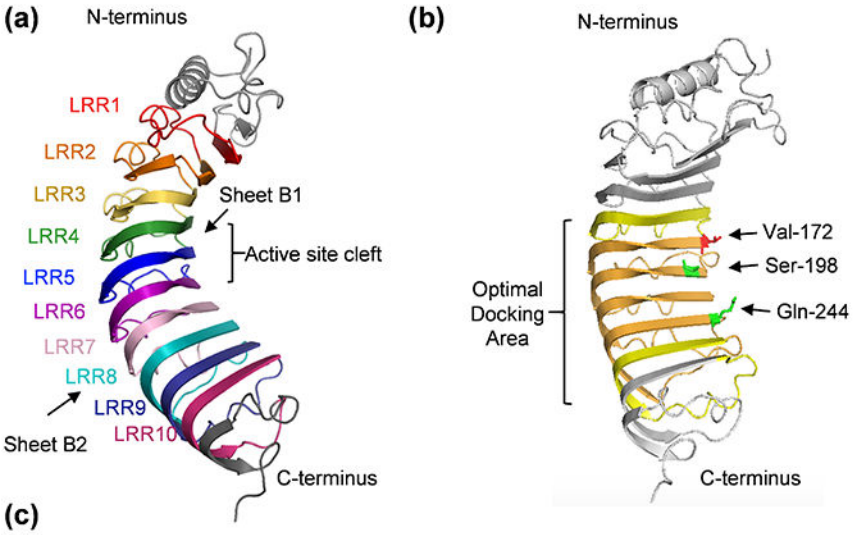

(c)

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                N Terminus
PvPGIP1         MTQFNIPVTMSSSLSIILVILVSLRTALSELCNPQDKQALLQIKKDLGNPTTLSSWLPTT      60
PvPGIP2         ---------MSSSLSIILVILVSLSTAHSELCNPQDKQALLQIKKDLGNPTTLSSWLPTT      51
                *********                 *  *

                                  LRR1                      LRR2
PvPGIP1         DCCNRTWLGVLCDTDTQTYRVNNLDLSGHNLPKPYPIPSSLANLPYLNFLYIGGINNLVG      120
PvPGIP2         DCCNRTWLGVLCDTDTQTYRVNNLDLSGLNLPKPYPIPSSLANLPYLNFLYIGGINNLVG      111
                                        *

                        LRR3                      LRR4
PvPGIP1         PIPPAIAKLTQLHYLYITHTNVSGAIPDFLSQIKTLVTLDFSYNALSGTLPPSISSLPNL      180
PvPGIP2         PIPPAIAKLTQLHYLYITHTNVSGAIPDFLSQIKTLVTLDFSYNALSGTLPPSISSLPNL      171

                LRR5                      LRR6                      LRR7
PvPGIP1         GGITFDGNRISGAIPDSYGSFSKLFTAMTISRNRLTGKIPPTFANLNLAFVDLSRNMLEG      240
PvPGIP2         VGITFDGNRISGAIPDSYGSFSKLFTSMTISRNRLTGKIPPTFANLNLAFVDLSRNMLEG      231
                *                         *

                        LRR8                      LRR9
PvPGIP1         DASVLFGSDKNTKKIHLAKNSLAFDLGKVGLSKNLNGLDLRNNRIYGTLPQGLTQLKFLQ      300
PvPGIP2         DASVLFGSDKNTQKIHLAKNSLAFDLGKVGLSKNLNGLDLRNNRIYGTLPQGLTQLKFLH      291
                        *                                                 *

                LRR10               C Terminus
PvPGIP1         SLNVSFNNLCGEIPQGGNLKRFDVSSYANNKCLCGSPLPSCT      342
PvPGIP2         SLNVSFNNLCGEIPQGGNLQRFDVSAYANNKCLCGSPLPACT      333
                                  *     *           *
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Figure 1.

Structure and protein sequence of PvPGIPs. **(a)** Ribbon structure of PvPGIP2. The structure is readapted from the previously published PvPGIP2 (DB accession 10GQ) (A. Di Matteo et al., 2003). LRR domains are color coded. Sheet B1 is engaged with binding to PGs. **(b)** 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. **(c)** The

amino acid sequences of PvPGIP1 and PvPGIP2. LRRs are color coded and differences in residues are shown with astricks.

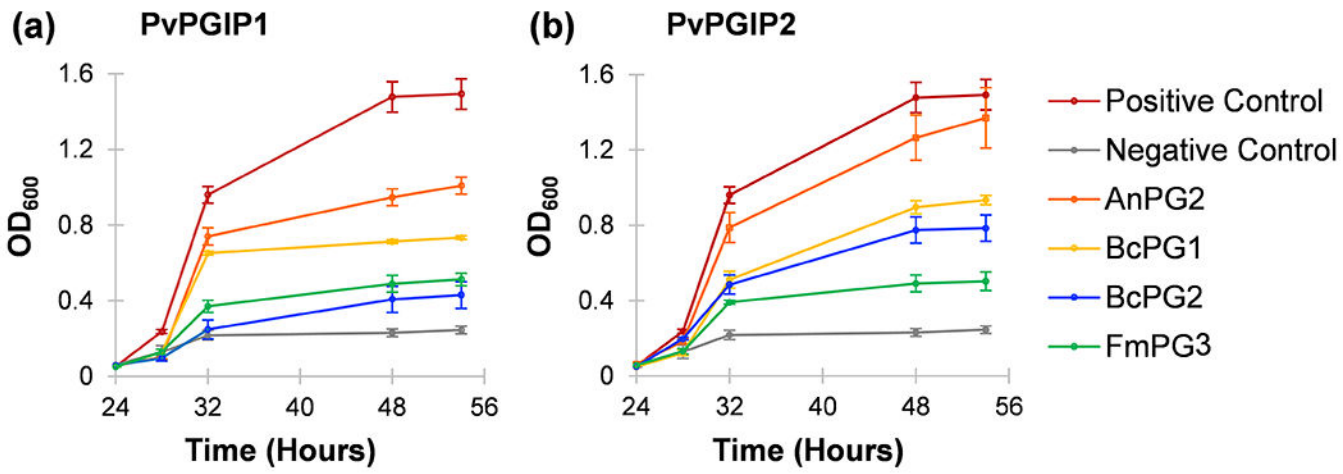

Figure 2.

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.

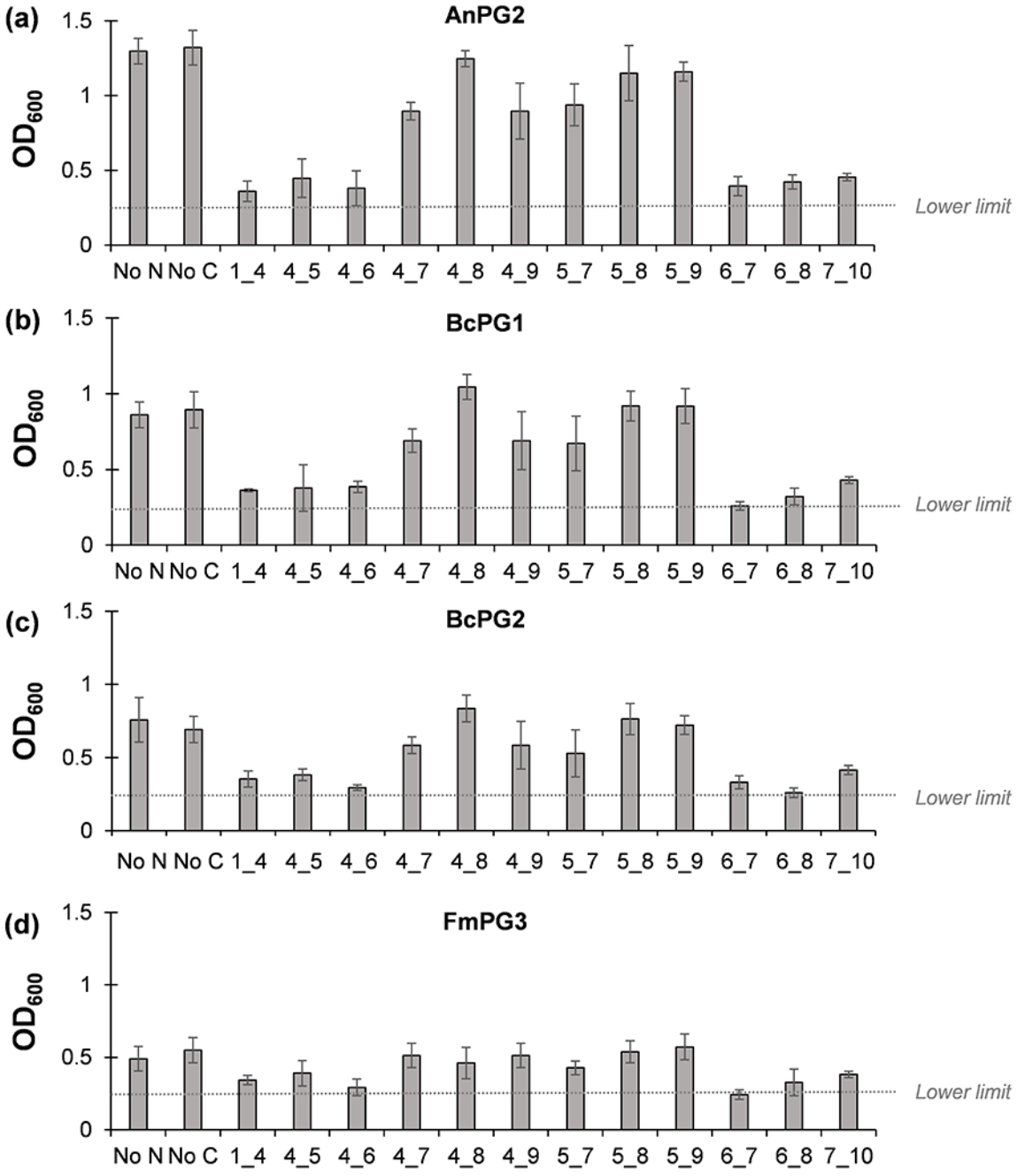

Figure 3.

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. The lower limit indicates the activity level of the negative control.

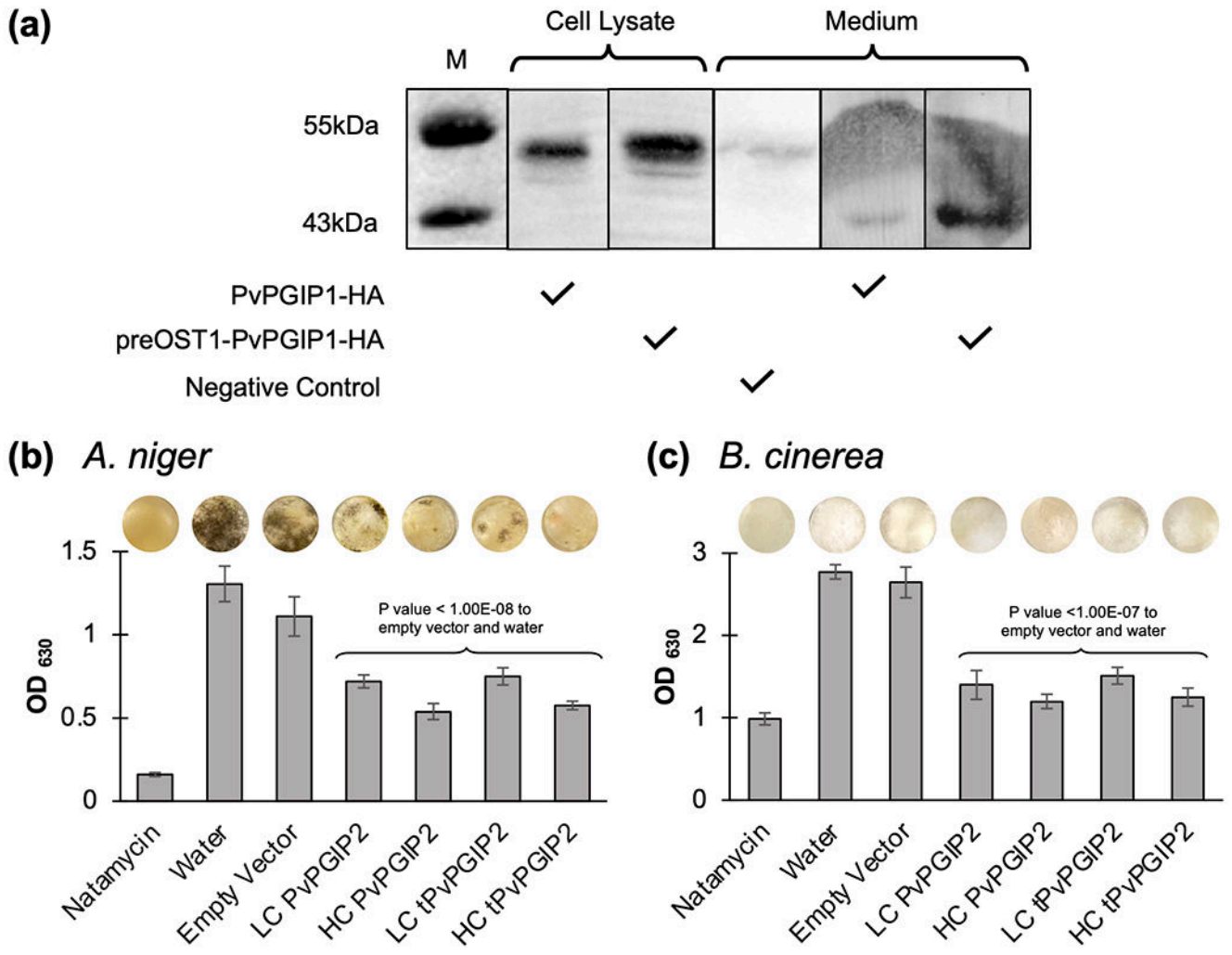

Figure 4.

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 (Langvad, 1999). 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.

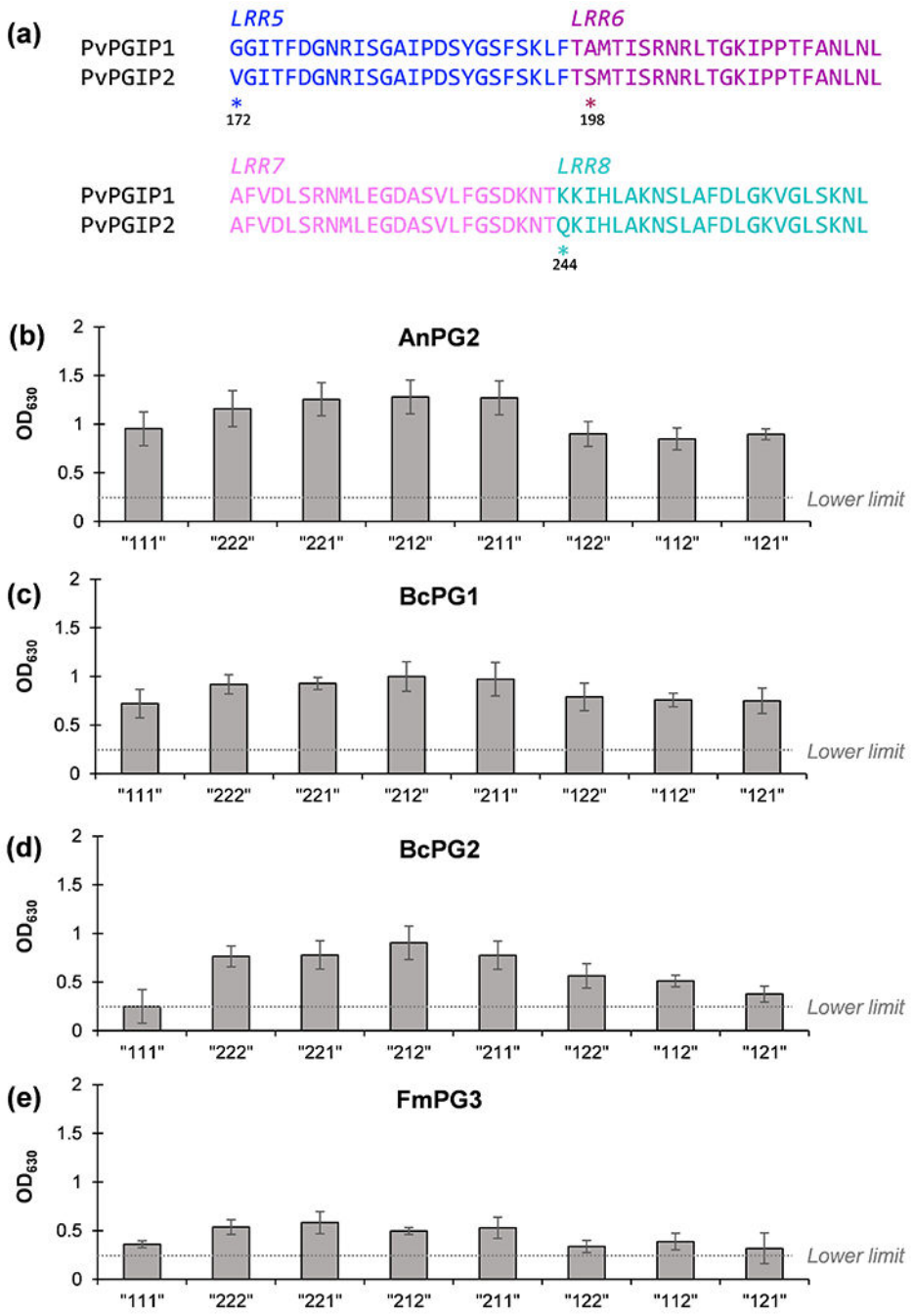

Figure 5.

Sequence-function correlation 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)** shows the three differing residues between PvPGIP1 and PvPGIP2, 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. The OD_{600} was measured at 48 hours of yeast harboring these mutant PGIPs with **(a)** AnPG2,

(b) BcPG1, **(c)** BcPG2, and 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.

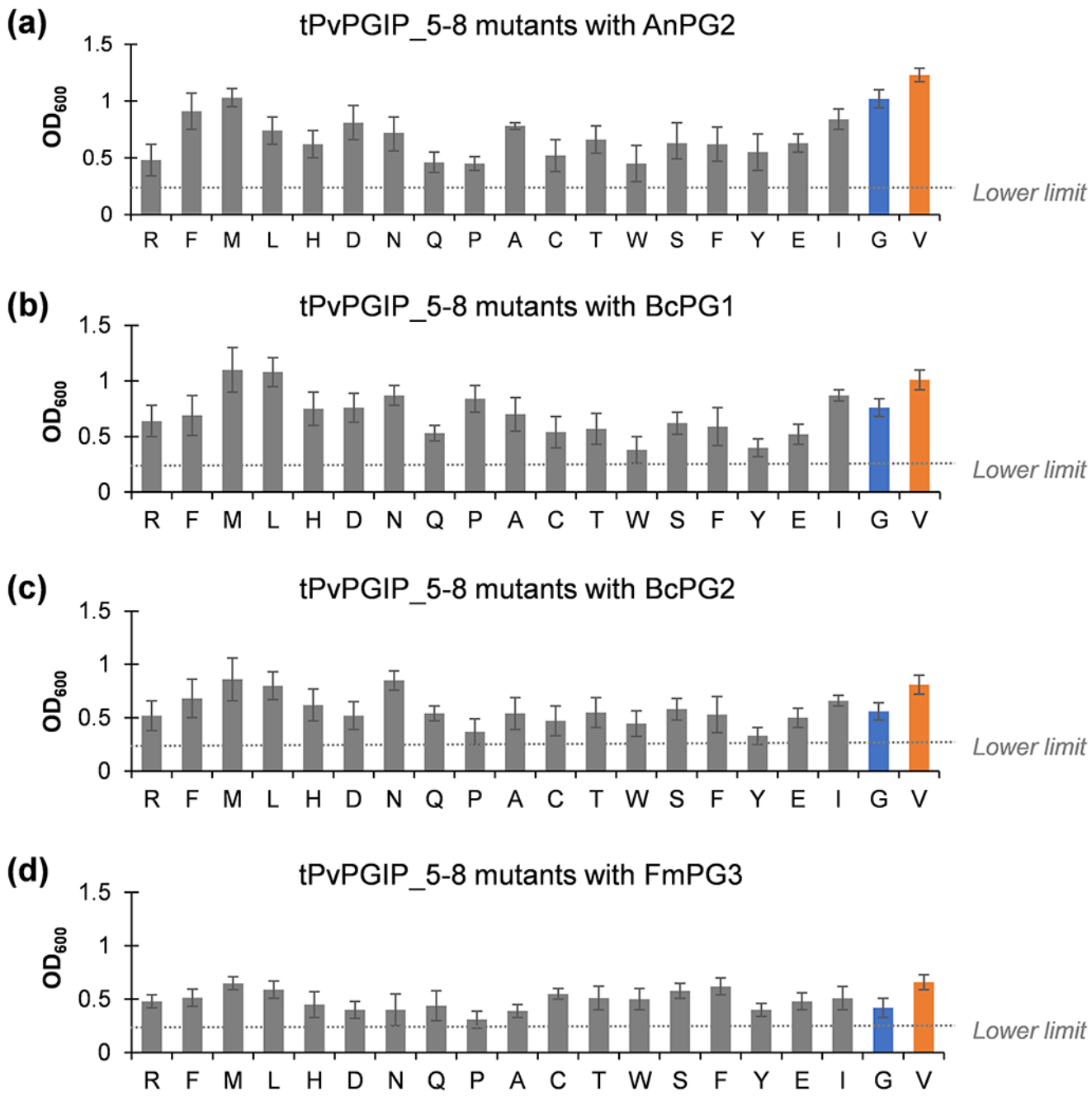

Figure 6.

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.