IDENTIFICATION OF ENDO- AND EXO-POLYGALACTURONASE ACTIVITY IN Lygus hesperus (KNIGHT) SALIVARY GLANDS

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Polygalacturonase (PG) activity found in the salivary gland apparatus of the western tarnished plant bug (WTPB, Lygus hesperus Knight) has been thought to be the main chemical cause of the damage inflicted by this mirid when feeding on its plant hosts. Early viscosity and thermal stability studies of the PG activity in L. hesperus protein extracts were difficult to interpret. Thus, it has been suggested that one or more PG protein(s) with different hydrolytic modes of action are produced by this mirid. In order to understand the quantitative complexity of the WTPB salivary PG activity, PG purification from a protein extract from salivary glands excised from L. hesperus insects was performed using affinity and ion exchange chromatography. To elucidate the qualitative complexity of the purified PGs, the digestion products generated by the PGs were separated using high performance anion exchange chromatography with pulsed amperometric detection. At least five PG proteins were detected; these differing in terms of their glycosylation, mass-to-charge ratios, and/or molecular mass. The characterization of the products generated by these PGs showed that endo- and exo-acting PGs are produced by WTPB. Although none of the PGs was purified to homogeneity, the present work provides biochemical evidence of a multiplicity of PGs that

Abbreviations: AEB, anion exchange buffer; CMFF, carboxymethyl Sepharose fast flow; CEB, cation exchange buffer; Con-A, concanavalin A; CAB, Con-A bound; CNB, Con-A not bound; ddH2O, distilled sterile water; DP, degree of polymerization; GalA, galacturonic acid; HPLC, high performance liquid chromatography; OGA, oligogalacturonides; PAGE, polyacrylamide gel electrophoresis; PEG, polyethylene glycol; PG, polygalacturonase; PGA, β-1,4-polygalacturonic acid; PGIP, polygalacturonase-inhibiting protein; PrPGIP, Phaseolus vulgaris polygalacturonase-inhibiting protein; QAE-S, quaternary ammonium ethane-Sephadex; SGE, salivary gland extract; TPB, tarnished plant bug, L. lineolaris; WTPB, western tarnished plant bug.

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degrade the pectin component of the plant tissue in different fashions. The implications of these findings affect the understanding of WTPB feeding damage and, potentially, help identify ways to control this important crop pest. © 2008 Wiley Periodicals Inc.

Keywords: Lygus hesperus; salivary gland; polygalacturonase; mirid-feeding damage

INTRODUCTION

One of the most important agricultural pest species belonging to the subfamily Mirinae (Hemiptera: Heteroptera: Miridae) is Lygus hesperus Knight (Schaefer and Panizzi, 2000). L. hesperus, the western tarnished plant bug (WTPB), ranges across Canada to southern Guatemala and is considered a key pest of cotton in California’s San Joaquin Valley (Wheeler, 2000). This mirid feeds primarily on reproductive plant parts and during the growing season follows a succession of flowering plants, both wild and cultivated (Wheeler, 2000). WTPB is polyphagous, known to feed from 150 host plants (Wheeler, 2000) and the plant damage symptoms observed upon WTPB feeding include organ abscission, deformation of developing fruits, necrosis at the feeding site, seeds with aborted embryos, and reduced vegetative growth (Strong, 1970). Phytophagous mirids are cell rupture feeders, and feeding behavioral studies carried out specifically on third instar WTPB nymphs conclude that this insect species feeds by rupturing plant cells using a mixture of laceration and maceration tactics (Backus et al., 2007).

Pectinase is a generic term for any pectin-degrading enzyme and polygalacturonase (PG) refers specifically to a pectin hydrolase whose substrate is unsubstituted α-1,4-polygalacturonic acid (PGA). Almost four decades ago it had been concluded that the injury caused by Lygus hesperus feeding was due principally to the enzymatic digestion of the plant’s tissue by PG secreted during feeding (Strong, 1970), but clear proof of this was lacking until recently (Shackel et al., 2005). Strong and Kruitwagen (1968) reported PG activity in the salivary apparatus of L. hesperus and suspected the presence of two or three PGs with either endo- or exo- or both hydrolytic modes of action. Endo-polygalacturonases degrade the PGA component of the plant cell wall randomly, generating galacturonic acid (GalA)-containing oligosaccharides of varying sizes as products. In contrast, exo-PGs target the non-reducing end of the PGA polymer to produce monosaccharide GalA as a product. Some PG proteins may exhibit combined endo- and exo-actions, producing GalA oligomers with a degree of polymerization (DP) of seven or smaller (Cook et al., 1999). So far, exo-PG activity has been found in the salivary gland of the mirid Poecilocapsus lineatus (Cohen and Wheeler, 1998). Other studies have suggested the presence of multiple PG isoforms in crude protein extracts from the tarnished plant bug (TPB, L. lineolaris) and WTPB, based on thermal stability (Agblor et al., 1994) and polyacrylamide gel electrophoresis (PAGE) analyses (Shackel et al., 2005). Moreover, recent molecular biology studies suggest the presence of a PG gene family for L. lineolaris and L. hesperus (Allen, 2007; Allen and Mertens, 2008; Celorio-Mancera et al., 2008, in press). Additionally, pathogens and insects produce digestive enzymes encoded by a family of highly polymorphic genes (ten Have et al., 2001; Zhu-Salzman et al., 2003) which may allow...
them to adapt to the challenges of plant PG-inhibiting proteins (PGIPs) (D'Ovidio et al., 2004; Ferrari et al., 2006) and diverse plant cell wall pectin substrates by qualitative and quantitative remodeling of gene expression.

It is crucial for the development of long-lived, biologically based management of insect pests of crop plants to understand the ways that insects interact with plants and plants detect and respond to insects. There are indications, as mentioned above, that PG activity in WTPB salivary glands is quite complex. In order to further understand the role of polygalacturonase in WTPB feeding damage, this complexity must be further assessed. In this study, we present the results obtained in our attempt to understand the complexity of PG activity in WTPB salivary glands and secreted during insect feeding.

MATERIALS AND METHODS

Insects

Western tarnished plant bugs were sweep net-collected from the alfalfa fields belonging to the Department of Animal Science at the University of California, Davis, within an approx. 200 m radius from coordinates: N 38° 32.038’, W 121° 47.850’, elevation 34 ft. WTPB individuals were sorted and identified (Mueller et al., 2003).

Salivary Gland Extract (SGE)

Following the salivary gland apparatus isolation (Shackel et al., 2005), batches of 300 to 400 live L. hesperus Knight insects were dissected each day over a five-day period (1800 total). The salivary gland pairs obtained each day were placed in microcentrifuge tubes with 1 ml distilled sterile water (ddH2O) on ice. Each batch of salivary glands was vortexed thoroughly, and centrifuged in an Eppendorf microfuge (5 min, 16,000 x g at room temperature, ca. 22°C). The supernatant was separated from the pellet and stored at −20°C. SGE supernatants were pooled after thawing in ice and dd H2O was added to a final volume of 5.4 ml.

Polygalacturonase Assays

Polygalacturonase activity was tested using the radial diffusion assay described previously (Shackel et al., 2005). The incubation for the radial diffusion assay was 15 h at 37°C. For the purification of SGE PGs, active fractions detected by the radial diffusion assay were further analyzed by using the 2-cyanoacetic acid method for measuring PG-generated reducing sugars (Gross, 1982) with α-D-galacturonic acid as a standard. For this assay, insect protein samples containing PG (based on the radial diffusion assay) were incubated with 0.2% PGA in 0.05 M sodium acetate (pH 5.0) at 37°C. Aliquots were taken from the incubation mixture over time for measurement of reducing sugars generated and analysis of PGA digestion products (see below). Polygalacturonase activity was tested under acidic conditions, since it has been reported that pH 5 is optimal for WTPB salivary gland PG activity (Strong and Kruijswagen, 1968).
Protein Determination

Protein concentrations were determined according to Bradford (1976), using bovine serum albumin as a standard.

Concanavalin-A-Sepharose 4B Chromatography

The SGE (5 ml) was chromatographed on concanavalin A-Sepharose 4B (Con-A, Amersham Biosciences, Uppsala, Sweden). The SGE was brought into equilibrium with the column buffer (Tris-HCl pH 7.4, 0.5 M NaCl containing 1 mM each CaCl$_2$, Mn Cl$_2$, and Mg Cl$_2$) by dialysis. The sample was applied to the column (1 cm diam., 9 cm length, 7 ml total bed volume) and the column was eluted with several bed volumes of column buffer. Then, material bound to the column was eluted with column buffer containing 1 M methyl-$\alpha$-D-mannopyranoside (Sigma, St. Louis, MO).

Chromatography on Carboxymethyl Sepharose Fast Flow (CMFF)

The Con-A column fractions containing PG activity were combined and dialyzed overnight against two changes of 4L of cation exchange buffer (CEB, 0.05 M sodium acetate, pH 5) to remove the $\alpha$-methyl mannoside. The dialyzed Con-A-purified PGs were then concentrated by dialysis against polyethylene glycol (PEG, Sigma, St. Louis, MO), molecular weight 15 to 20 KDa, using a dialysis membrane with a 6 to 8 kDa size cut-off (Spectra/Por, Rancho Dominguez, CA). Following the loading of the PG sample onto the CEB-equilibrated CMFF column (Hi-Prep 16/10, 1.6 cm diam., 10 cm length, 20 ml included volume) the column was washed with 72 ml of CEB and then eluted with a 300 ml gradient (0.0 to 1.0 M NaCl in CEB) at a flow rate of 1 ml/min that was maintained by using a peristaltic pump. Fractions of 3 mL in volume were collected and assayed for PG, and fractions corresponding to distinct PG peaks were combined, dialyzed against CEB, and chromatographed for a second time through the CMFF column under the conditions described above, but with a 400 ml gradient from 0.2 to 1.0 M NaCl.

Chromatography on Quaternary Ammonium Ethane-Sephadex (QAE-S)

The active fractions from the second CMFF step were combined and dialyzed against 2 changes of 4L of 0.02 M sodium phosphate pH 7.3 (anion exchange buffer, AEB) at 4°C, with constant stirring. The PG sample was then concentrated by dialysis against PEG, as above, and loaded onto the QAE-S column (1.6 cm diam., 10 cm length, 20 ml included volume) that had been equilibrated with AEB. The column was then eluted with 40 ml of AEB and then eluted with a 300 ml gradient from 0.0 to 1.0 M NaCl in AEB at a flow rate of 1 ml/min using a peristaltic pump.

One-Dimensional Gel Separation of SGE Proteins

In order to analyze the protein composition of the SGE through the purification process, protein samples were subjected to PAGE analysis. Aliquots of approx. 200 $\mu$L from the starting SGE and from each purification step (after pooling, dialysis and concentration) were stored at $-20^\circ$C. Protein concentration (above) was measured for all purification steps including the starting SGE. The 200 $\mu$L aliquots were thawed and protein concentration normalized in order to prepare samples for separation on a one-dimensional 12% resolving polyacrylamide gel using the Laemmli sodium dodecyl sulfate-discontinuous system (Laemmli, 1970). After electrophoresis, since some
samples contained less than 30 ng total protein, the gel was stained with silver following the manufacturer’s instructions (Silver Stain Kit, Bio-Rad, Hercules, CA).

**High Performance Anion Exchange Chromatography-Pulsed Amperometric Detection of *L. hesperus* PG digestion products.**

Oligogalacturonides (OGAs) generated during the incubation of different *L. hesperus* PGs with PGA were analyzed by anion exchange chromatography with a Dionex AL50 high performance liquid chromatography (HPLC) instrument and a Dionex CarboPAC PA1 column coupled to a Dionex pulsed amperometric detector (Dionex, Sunnyvale, CA) (Melotto et al., 1994). Samples of PGA digestion products were prepared by incubating an aliquot of the unfractionated SGE and 100 μl aliquots from the most PG-active fractions from various PG purification steps (based on the radial diffusion assay or the reducing sugar assay above) with 0.5 ml of 0.2% PGA in 50 mM sodium acetate pH 5.0. A drop of toluene was added to prevent microbial growth. The reaction mixture was incubated at 37°C for 24 hrs. The reactions were stopped by freezing the samples at −20°C. For the HPLC analysis, 200 μL of selected samples was loaded onto the column which was then eluted for 2 min with 0.1 M sodium hydroxide and 0.2 M sodium acetate, followed by a 35 min gradient of sodium acetate (0.2 M to 0.9 M) in 0.1 M NaOH, a program similar to that reported by Melotto et al. (1994). Eluant flow rate was maintained at 1 ml min⁻¹.

**RESULTS**

The salivary gland extract from 1800 adult WTPB salivary glands (34 mg total protein in the 5 ml sample) was subjected to lectin affinity chromatography on the Con-A column (Fig. 1). For this separation step the protein content in collected fractions was sufficiently high to be measured in each fraction. However, as purification continued into successive protein separation steps (i.e., CMFF and QAE-S, below) protein content

![Figure 1](image-url)  
**Figure 1.** Chromatography of 5 ml *L. hesperus* salivary gland extract (protein from 1800 pairs of glands in 5 mL) on Concanavalin A-Sepharose 4B. After the initial elution in Con-A buffer (first 35 1-ml fractions), the column was eluted with Con-A buffer containing 1 M methyl-α-D-mannopyranoside. Fractions were assayed for PG activity using the radial diffusion assay (15 μL from each fraction assayed). PG active fractions (within vertical lines) were combined for further purification (CNB = PG not bound by Con-A, fractions 7–35). Fractions in the groups identified as 1–4 represent PG proteins that are Con-A bound (CAB). They were pooled by group number and characterized further. Fractions were also assayed for protein content, this is reported as the amount of protein in the 1 ml volume fraction (●).
decreased to the point where accurate determination of protein content became impossible. Polygalacturonase activity coincident with a substantial protein peak was detected in the fractions representing proteins that were not bound by the immobilized Con-A. Presumably the PG activity in these Con-A-not-bound (CNB) fractions represents PG proteins that are not glycosylated. After the column had been eluted with 175 ml of Con-A buffer (35 1–5 ml fractions, 5 column volumes), the CNB PG activity peak tailed off and the eluting buffer was changed to Con-A buffer containing 1M methyl-\(\alpha\)-D-mannopyranoside (arrow, Figs. 1, 2). After a lag of ca. 10 fractions, PG activity and protein once again were eluted from the column and a broad, steadily dropping peak of PG and protein was eluted in the sugar-containing buffer. Flow was stopped and the column was held at 4°C overnight while the fractions representing Con-A-bound (CAB) proteins (fraction sets identified as 1–3, Fig. 1) were assayed for PG using the radial diffusion assay. Fractions in set 3 showed low amounts of activity in the assay. Because PG elution was still continuing, although with decreasing amounts of assayable activity, an additional 80 ml of methyl-\(\alpha\)-D-mannopyranoside-containing Con-A buffer was passed through the column. The fractions were assayed (fraction set 4) and a last peak of PG activity, coincident with a small protein peak, was identified. Following fraction set 4 no additional PG activity was detected.

The radial diffusion assay is, at best, only semi-quantitative. Prior to continuing the purification of the PG in the CNB fractions, their PG activity was measured using the

![Figure 2](image-url)

**Figure 2.** Distribution of PG activity in the CNB fractions of the sample of *L. hesperus* salivary gland extract separated on Concanavalin A-Sepharose 4B (Fig. 1), based on reducing sugar assays.
more quantitative reducing sugar assay (Fig. 2). This PG activity profile presented a leading-edge shoulder of PG activity that was only slightly separated from the main PG peak, suggesting the presence of more than one PG isozyme.

**Further Purification of CNB PG Activity**

The CNB PG fractions from the Con-A step were combined, dialyzed to equilibrate them with CEB, and concentrated for the cation-exchange chromatography step on the CMFF column. Following sample loading, the column was eluted with 30 ml of CEB and then a NaCl gradient (0 to 1.0 M in 300 ml CEB) was applied. This attempt at PG purification provided a further partial separation of two PG activity peaks, but the two were not fully resolved (data not shown). Fractions representing the front of the earlier PG activity peak and the trailing half of the later-eluting PG peak were pooled separately, dialyzed against CEB and concentrated. They were then chromatographed separately on CMFF, with a shallower NaCl gradient. The individual PG separation patterns were then plotted on the same set of axes (Fig. 3a), the patterns providing a clear indication that the CNB PG included two proteins with slightly differing binding affinities.

Non-overlapping CNB PG fractions from peaks a and b of the last CMFF steps were pooled separately, dialyzed against AEB and concentrated. Peaks a and b were then separately subjected to anion exchange chromatography on QAE-S. For each of these PGs, only a single activity band was eluted, this in a position indicating that it had not bound to the anion exchanger (Fig. 3b; only the run for CNB PG-a is shown).

**Further Purification of CAB PG Activity**

The CAB PG activity was divided into four sets of Con-A fractions (Fig. 1). Each set was dialyzed against CEB, concentrated, and chromatographed separately on CMFF (Fig. 4). CAB fraction sets 1 and 2, representing the greatest amount of CAB PG activity and protein, gave identical PG profiles with three partially separated, column-bound PG activity peaks (designated a, b, and c, Fig. 4a; only the separation of fraction set 1 is shown). Chromatography of CAB fraction set 3 on CMFF gave a single PG peak at a gradient NaCl concentration of 0.5 M and eluted approximately where CAB PG peak b from fraction sets 1 and 2 had eluted (data not shown). Chromatography of CAB
fraction set 4 on CMFF also gave a low PG activity peak eluting at a gradient NaCl concentration of 0.5 M (data not shown). Fractions with PG activity for both sets 3 and 4 after CMFF were pooled, dialyzed against AEB, and subjected to QAE-S chromatography. For both set 3 and 4 of the CAB PG, a single activity peak eluted in the void region indicating no interaction with the anion exchange column (data not shown).

Fractions corresponding to each of the three PG peaks that were seen upon CMFF analysis of CAB PG fraction sets 1 and 2 (Fig. 4A) were pooled from the separate CMFF runs of set 1 and set 2 (a = fractions 56 to 62, b = fractions 63 to 68, and c = fractions 69 to 75) and subjected to QAE-S chromatography separately. In each case, only one peak of PG activity was detected; its elution point indicating no interaction with the anion exchange matrix (Fig. 4B, only the run for PG peak c is shown).

**HPLC Analysis of Oligo- and Monosaccharide Products Generated During PG Action on PGA**

A control HPLC analysis of the original SGE preparation revealed (Fig. 5A) no evidence of GalA or OGAs while an analysis of the PGA substrate used (Fig. 5B) indicated, at the most, the presence of very small amounts of OGAs. HPLC analysis of the products generated during incubation of the two CNB PG activity peaks a and b...
that had been identified by cation-exchange on CMFF (Fig. 3a) with PGA revealed a prominent GalA peak, substantial amounts of OGAs with DPs of 2, 3 and 4 and much smaller peaks representing OGAs with DPs > 4 (Fig. 5c,d). Incubations of the various CAB PG activities that had been fractionated further on CMFF revealed populations of larger OGAs and relatively minor amounts of GalA (Fig. 5e–h). Fig. 6 shows the
distributions of products generated over time when the complex mixture of proteins in the SGE, including the several PGs identified in the present study, was incubated with PGA. The data indicate that relatively little monosaccharide GalA is produced when the incubation time has been relatively short, but that the DPs of products shift to primarily di- and trisaccharides and a substantial amount of GalA product as the incubation is extended.

**DISCUSSION**

The partial purification of *L. hesperus* salivary PGs resulted in the identification of at least five PG proteins. Two were not bound by Con-A, indicating either that they are not glycoproteins or that their glycosylation does not include the terminal mannosyl or glucosyl residues that are recognized by the lectin. These were partially separated during chromatography on carboxymethyl-Sepharose, indicating slight differences in mass:charge ratios and/or molecular mass. Three additional PGs were bound to Con-A

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and these glycosylated PGs were partially separated from one another, also by cation-exchange chromatography. Additionally, potentially more extensively glycosylated PGs may have eluted in the later stages of the Con-A chromatography step (i.e., fraction sets 3–4) but there was insufficient PG activity in these fractions to allow further analysis. Based on PAGE electrophoresis of the most highly purified PG proteins none of the PGs was purified to homogeneity. For example, silver staining of proteins separated by PAGE analysis indicated that the CNB PG that was eluted in the salt gradient during chromatography on CMFF (Fig. 4a, peak b) and then did not bind to the QAE-S column contained one major protein with minor contaminants (Fig. 7).

The endo- vs. exo- action of pure PG proteins can be determined by the characterization of the OGA products that are generated when PG digests PGA. Exo-PGs generate monosaccharide GalA throughout the digestion period. In contrast, endo-PGs generate mixtures of OGAs, with OGAs of relatively high DP produced early in the reaction and smaller OGAs accumulating later in the reaction as the endo-PG acts on the first OGA products of higher DP. If both types of PG are present, including PGs with combined endo-/exo-action, mixtures of GalA and OGAs will accumulate early in the reaction and increasing amounts of GalA will accumulate with time as exo-PG action on the OGA products of endo-PG action increases. Because none of the PG preparations we tested contained only a single protein, we cannot identify the type of PG present in each of the PG activity peaks that was isolated. However, it is clear that the L. hesperus salivary PG family includes both exo- and endo-PGs. Only the two CNB PGs produced an appreciable amount of monosaccharide GalA when incubated with PGA (Fig. 5c,d). Thus, these proteins behave as exo-PGs. However, these product profiles also contained a substantial amount of OGA di-, tri- and tetraGalA, indicating

Figure 7. Silver stained SDS-PAGE gel revealing protein bands in: A: Initial SGE; B: pooled CNB PG fraction; C: pooled CAB PG fraction (set 1); and D: CNB PG, peak b, Fig. 3A. The location of the high range molecular weight SDS-PAGE protein standards used is designated by their respective molecular weights on the right hand side of the picture. From highest to lowest molecular weights standard: myosin, β-galactosidase, phosphorylase and bovine serum albumin (Bio-Rad, Hercules, CA).
endo-PG action. Thus, the CNB proteins also include endo-PG or the CNB PGs are of the mixed exo-/endo-PG type (Cook et al., 1999). In contrast, the CAB PGs produced little or no monosaccharide GaA and a series of higher order OGAs during incubation with PGA (Fig. 5e–h), indicating a primarily endo-PG action. In some profiles a greater proportion of larger OGAs was detected (e.g., Fig. 5h) while in other profiles a greater proportion of smaller OGAs was detected (Fig. 5e,f). This could indicate that the mixture of L. hesperus endo-PGs contains enzymes with differing substrate specificities or reflect the fact that equal amounts of PG activity, based on reducing sugar generation, were not available for each PG protein-containing column PG peak.

The multiplicity of PG proteins indicated by our purification effort is not surprising. In their early work on WTPB salivary PG, Strong and Kruitwagen (1968) speculated that the insect produced more than one PG, with the PG complement including both endo- and exo-acting enzymes. Our data confirm this speculation. Additionally, evidence for the idea that lygus bugs contain multiple PG proteins comes from recent studies that have led to the cloning of Lygus spp. PG-encoding genes. Three L. lineolaris PG genes have been reported recently (Allen and Mertens, 2008). We (Celorio-Mancera et al., unpublished data) have provided genomic and proteomic data that demonstrate the secretion of three L. hesperus PGs into the insects’ diet and that one of these PGs has not been reported for L. lineolaris thus far. Attempts to express recombinant PGs are underway. Success in this effort will support a more detailed characterization of pure lygus bug PGs, including an exploration of the possibility that one or more of these PG(s) is of the mixed endo-/exo-type, since the way PGs degrade their substrate may have importance in terms of the interaction the insect has with the plant. There is a correlation between the cleavage mechanisms with susceptibility of PGs to inhibition by polygalacturonase-inhibiting proteins (PGIPs) (Cook et al., 1999).

Strong (1970) concluded that the principal cause of WTPB feeding damage was the biochemical action of salivary PG. Support for this conclusion was provided when Shackel et al. (2005) showed that microinjection of a partially purified PG from L. hesperus salivary protein extract, caused lygus-like damage symptoms on alfalfa florets and cotton flower buds as did the injection of a few pure fungal endo-PGs. However, whether the damage caused by the L. hesperus salivary PGs was due to the action of the exo-PG, the endo-PG or both activities is not clear. The availability of recombinant WTPB PGs would make this determination possible. Since WTPB salivary PGs are damage-causing factors, they could be an important target of strategies for limiting crop damage caused by lygus bug feeding. The expression of the gene encoding the pear fruit PGIP in transgenic tomatoes and grapevines has been shown to mitigate disease development caused by the grey mold pathogen (Botrytis cinerea) and the bacterial pathogen Xylella fastidiosa, respectively (Powell et al., 2000; Aguero et al., 2005). Polygalacturonase-inhibiting protein genes from many plant species have been cloned and their specificity in inhibiting PGs from a variety of species of plant pathogens has been described (De Lorenzo et al., 2001).

The common bean (Phaseolus vulgaris) produces a family of four PGIPs (i.e., PvPGIP1, PvPGIP2, PvPGIP3 and PvPGIP4), several of which have been shown to inhibit the PGs of fungal pathogens (Manfredini et al., 2005). In addition, PvPGIP3 and PvPGIP4 inhibit PG activity in whole insect protein extracts of the mirid bugs L. rugulipennis and Adelphocoris lineolatus (D’Ovidio et al., 2004). Subsequently, Frati et al. (2006) found that PG activity in similar whole insect protein extracts from other mirid species (L. pratensis, Orthops kalmi, and Closterotomus norvegicus) was also inhibited.
by bean PGIPs 3 and 4; however, these mirid PGs were not inhibited by *Arabidopsis thaliana* or *Glycine max* PGIPs. D’Ovidio et al. (2004) suggested that the redundancy and sub-functionalization of PGIP genes might be important for the adaptation of plant defenses to pathogenic fungi and phytophagous insects. However, this sub-functionalization does not appear to be relevant for all PGIPs. When expressed in *M. sativa*, pear PGIP inhibits WTPB PG activity (Shackel et al., 2005). The pear PGIP also inhibits fungal PGs (Stotz et al., 2000) and PG from the bacterium *X. fastidiosa* (Perez-Donoso et al., 2008, in press).

Thus, manipulation of PGIPs in crop plants may be a useful approach for controlling damage caused by WTPB and, perhaps, other insects. However, the complexity of the WTPB PGs described herein and the selective inhibition of different PGs by diverse PGIPs (discussed above) make clear that development of a strategy for WTPB damage mitigation that is based on expression of PGIP genes in crop plants requires knowledge of (1) the specific insect PGs that are damage-causing and (2) PGIPs that inhibit those PGs. The present study will be useful in devising tests of such a strategy.

**LITERATURE CITED**


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