Polygalacturonase and Polygalacturonase Inhibitor Protein: Gene Isolation and Transcription in *Glycine max* - *Heterodera glycines* Interactions

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The cell wall acts as the first line of defense during pathogen invasion. Polygalacturonases (PGs) are a class of cell-wall-modifying enzymes with precise temporal and organ-specific expression. A 350-bp fragment with high homology to PGs was identified by differential display (DD) analysis of soybean cyst nematode (SCN) race 3 resistant PI 437654 and susceptible cultivar Essex. The fragment was strongly expressed in Essex, 2 days after inoculation (DAI). Complete coding sequences of two PG cDNAs, *PG1* and *PG2*, were isolated by 3' and 5' rapid amplification of cDNA ends polymerase chain reaction (RACE PCR). PI 437654 and Essex had identical *PG1* and *PG2* sequences. A transversion from A to C created a *Pst*I restriction site in the *PG2* cDNA that was used to distinguish the two PG cDNAs by cleaved amplified polymorphic sequence (CAPS) analysis. A cDNA encoding a polygalacturonase-inhibitor protein (PGIP) that is 89% identical to the *Phaseolus vulgaris* PGIP was isolated from soybean roots by reverse transcription (RT)-PCR. Steady-state levels of PG and PGIP were investigated by RNA gel blot analysis in roots 1 to 5 DAI and in hypocotyls and leaves. Differences in the constitutive levels of PG mRNAs were observed in roots of different soybean genotypes. Steady-state levels of PG mRNAs were enhanced during compatible interactions with SCN and reduced in incompatible interactions and in mechanically wounded roots. Enhanced PGIP transcription was observed in response to mechanical wounding in both PI 437654 and Essex, but only in compatible interactions with SCN, suggesting uncoupling of PGIP functions in developmental and stress cues. Constitutive expression in incompatible interactions shows PGIP is not a factor in SCN resistance. Thus, the up-regulation of endogenous PG transcription in soybean roots early after SCN infection could facilitate successful parasitism by SCN.

Additional keywords: oligogalacturonides, open reading frame, syncytia, systemic, transduction.

Pectins, also referred to as matrix polysaccharides, are a major component of the plant cell wall and glue neighboring cells together. Pectin metabolism is crucial to many developmental processes. Plant cells undergo drastic changes in shape and developmentally regulated cell separation, during which the pectin network is systematically disassembled. A wide range of enzymes, including, *exo*- and *endo*-polygalacturonases (PGs), pectate lyase, pectin methyl esterase, and beta-galactosidase, are involved in pectin modifications (Hadfield and Bennett 1998).

In many plant developmental processes, particularly those that require cell separation, PGs cleave the $\alpha(1-4)$ linkages of D-galacturonic acid, which forms the backbone of pectins. Several PGs have been cloned in various plant species and show time- and organ-specific expression (Brown and Crouch 1990; DellaPenna et al. 1986; Slater et al. 1985; Taylor et al. 1990; Tebutt et al. 1994; Wing et al. 1989).

During pathogenesis, cell walls act as the first line of defense that phytopathogens encounter to colonize the plant tissue and obtain nutritional requirements. Plant-pathogenic bacteria and fungi have evolved a battery of pectic enzymes, primarily *endo*-PGs, to hydrolyze the plant cell walls during the early stages of pathogenesis (reviewed in Hahn et al. 1989). In the more evolved types of parasitism, such as the one between soybean cyst nematode (SCN) and soybean, the endoparasite has developed sophisticated means of exploiting the host plant. A long and stout stylet aids the nematode in penetrating several cell layers in search of food. The sessile nature of developing juveniles (J3, J4 stages) and adult females of SCN helps to limit necrotic plant reactions detrimental to nematode development. For the successful completion of the life cycle, SCN must initiate localized reorganization of the host cell's morphology, resulting in the formation of specialized feeding sites or syncytia (Jones 1981). Dissolution of walls of the cells surrounding the initial feeding site leads to the formation of multi-cellular syncytia. The cell wall dissolution is an enzymatic process, as observed in infected roots of susceptible cultivar Essex (Mahalingam and Skorupska 1996). In both SCN-compatible and SCN-incompatible soybeans, syncytia are initiated as early as 2

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days after inoculation (DAI; Mahalingam and Skorupska 1996). We report the changes in the steady-state levels of soybean *PG* transcripts that may play a role during early stages of soybean-SCN interactions.

All dicotyledonous plants have a cell-wall-localized polygalacturonase-inhibitor protein (PGIP). PGIPs are specific, high-affinity receptors for fungal *endo*-PGs (Hahn et al. 1989). Formation of the PG-PGIP complex in vitro results in an alteration of the balance between release of elicitor-active oligogalacturonides and depolymerization of oligogalacturonides into inactive molecules, favoring the accumulation of elicitor-active molecules (Cervone et al. 1989). The plant resistance genes that participate in gene-for-gene resistance—*Cf-2*, *Cf-9* of tomato, and *Xa21* of rice—show high homology to the PGIPs (Jones and Jones 1997). The receptor-like, leucine-rich, modular structure of the PGIP provides for the function of PGIP as a component of the cell-surface signaling system (De Lorenzo et al. 1994). We observed an induction of *PGIP* when there was an induction of *PG* in response to pathogen but not wounding. This induction was specific to compatible interactions, and regulatory mechanisms have yet to be identified. We tested *PGIP* expression to investigate if a general resistance gene like *PGIP* may be important in soybean response to SCN. The transcription of *PGIP* during the soybean-SCN interactions excludes PGIP as a factor in resistance response.

RESULTS

Two *PG* coding sequences were identified from soybean root cDNAs.

A 350-bp differential display (DD) fragment was amplified from cDNA generated from Essex roots infected with SCN race 3, 2 DAI (Fig. 1). This amplicon was reproducibly obtained with independent RNA isolations and at two concentrations of the template (not shown). The fragment was not observed in the root cDNAs of PI 437654. The sequence of the fragment was 343 bp long and contained the decamer sequence at both 5' and 3' ends. This fragment had an open reading frame (ORF) of 112 amino acids (aa) that showed homology to PGs from several plant, bacterial, and fungal species. The highest homology was with Arabidopsis PG (74% identity; BLAST score, 377; $P = 1.2 \times 10^{-46}$).

To obtain the full-length sequence of the soybean *PG* cDNA, the DD fragment was subjected to 5' and 3' rapid amplification of cDNA ends polymerase chain reaction (RACE PCR). Sequencing of more than a dozen clones generated by RACE PCR from PI 437654 and Essex indicated that there were two *PG* coding sequences (named *PG1* and *PG2*; Fig. 2). Both *PG1* and *PG2* cDNA sequences were identical in Essex and PI 437654. *PG1* cDNA predicted a single ORF encoding 443 aa (Fig. 3) with the start codon at nucleotide 73 and the stop codon at nucleotide 1402. *PG2* cDNA sequence revealed an ORF of 439 aa (Fig. 3) with the start codon at nucleotide 85 and the stop codon at nucleotide 1402. Apart from *PG2* being 4 aa smaller than *PG1*, there were 23 aa substitutions between *PG1* and *PG2* sequences. The molecular masses of the deduced peptide of *PG1* and *PG2* were estimated as 47.3 and 47.1 kDa, respectively. The diagnostically conserved sequence, CGPGHGIGSL, identified in all the PGs cloned to date (Petersen et al. 1996; Hadfield et al. 1998), was pres-

ent in both *PG1* and *PG2*, between positions 269 and 280 in *PG1* (Fig. 3). *PG1* and *PG2* had overall 92% nucleotide identity: (94% in the ORF, 82% in the 5' untranslated region [UTR] and 86% in the 3' UTR). A transversion from A to C created a *Pst*I restriction site in the *PG2* sequence (Fig. 2). In cleaved amplified polymorphic sequence (CAPS) analysis, the 1,400-bp amplification product from the *PG1*-specific primers, (namely *PG1*-1 as the 5' primer and *PG1*-3 as the 3' primer) lacked the *Pst*I restriction site and hence remained uncut after digestion (Fig. 4). Amplification of root cDNA with *PG2*-specific primers, namely *PG2*-1 as the 5' primer and *PG2*-3 as 3' primer, followed by restriction digestion with *Pst*I generated 1,160- and 240-bp fragments. Nonspecific combinations of primers (e.g., *PG1*-1/*PG2*-3 or *PG2*-1/*PG1*-3) failed to produce any amplification products (Fig. 4).

PG1 and *PG2* cDNAs were used as probes on DNA gel blots of PI 437654 and Essex digested with five different restriction enzymes, namely, *Dra*I, *Eco*RI, *Eco*RV, *Hind*III, and *Taq*I. Both *PG1* and *PG2* cDNAs hybridized to 4 to 10 genomic fragments, and the hybridization patterns were identical. Several weakly hybridizing bands were observed under high stringency washing conditions, indicating the presence of other *PG*-related sequences in soybean genome.

A novel *PGIP* sequence from soybean roots.

PCR amplification from genomic DNA and root cDNAs of PI 437654 and Essex with the primers for soybean *PGIP* according to Favaron et al. (1994) revealed an expected 950-bp fragment (Fig. 5). Sequences of *PGIP* clones from roots of PI

Fig. 1. Differential display of cDNAs generated from T₁₇-C anchor primer in combination with the decamer primer 5'-TCCGATGCTG-3' and separated on a 6% denaturing polyacrylamide gel. Only a portion of the autoradiogram with the differentially expressed polygalacturonase (*PG*) fragment (indicated by an arrow) is shown. Lanes 1 and 2: noninfected PI 437654 roots; lanes 3 and 4: PI 437654 roots 2 days after inoculation with race 3 soybean cyst nematode; lanes 5 and 6: noninfected roots of Essex; lanes 7 and 8: Essex roots 2 days after inoculation with race 3 soybean cyst nematode.

437654 and Essex were 100% identical and contained perfectly matching sequences for the 5' and 3' primers. The genomic clone of *PGIP* published by Favaron et al. (1994) was 77% identical at nucleotide level to the *PGIP* sequence we identified in roots (Fig. 6). At amino acid level, the sequence of *PGIP* from roots (Gm *PGIP*) was 88% identical to that of the common bean, *Phaseolus vulgaris*, (Pv *PGIP*) sequence while the *PGIP* reported by Favaron et al. (Gmc *PGIP*; Favaron et al. 1994) was 67% identical to the Pv *PGIP* (Fig. 7).

Southern blot analysis with *PGIP* cDNA showed one strongly hybridizing fragment after digestion with *EcoRI* and two strongly hybridizing fragments after *HindIII* digestion, apart from several other weakly hybridizing fragments. Only

one *HindIII* site and no *EcoRI* restriction sites are present in this *PGIP* sequence.

***PG* transcription is enhanced in compatible roots early after SCN infection.**

RNA blot hybridization with the full-length *PG* cDNA required long exposure of up to 7 days, indicating low levels of *PG* transcripts in roots. We could not differentiate between *PG1* and *PG2* by RNA blot analysis. The levels of *PG* transcripts differed between PI 437654 and Essex in the noninfected roots. In the susceptible cultivar Essex, the steady-state levels of *PG* in the noninfected roots were higher while in the resistant PI 437654 *PG* was hardly detectable, confirming ex-

PG1	ATAACA-CTTTCTCTTCAAATAATTCATTCTTCAGCTTCTTATTACTT---TGTATCTCTGTGTATATTGAAAATGAA-AA--A	80
PG2	----T..C..AC.....C.....C.....AATT.....C.C.....A..G..TT.	83
PG1	TATGAATATGAAACTAAACACCATTGCATTGATAATTGCATTGCATTTTCTGTGTGGTCTTCAAGCTGCACAGCAGCAGTGTTCAGC	168
PG2	C.....G.....T.....A.....	171
PG1-1 ->		
PG1	CACTGGAGGAAACTGAAAGCTGCTGCATCTGCTACTTCTTTTAATGTGTAGACTATGGTCTAAAGGTGATGGACATGCAGATGATA	256
PG2	C.....A.....AA.....A..G.....	256
PG2-1 ->		
PG1	CAAAGGCCTTTGAAGATGCATGGCAGCTGCTTGAAGGTGGAGGGTCAACAATGGTGTCCATCTGGTCTGTATTTTGGTGAA	344
PG2	C..A.....T.....C.....	344
PG1	ACCAATCTCTTTCTCAGGCCCTAATTTGTGAGCCAAACATGTTTTTCAGTTGGATGGCAAAATCATTGCACCTACAAGCTCTGAAGCT	432
PG2	G.....C.....	432
PG1	TGGGGCTCTGTACCTTGCAATGGCTTGAATTCTCAAACCTTAATACAATCACAATCAGAGGCAAGGTGTCTGATGGTCAAGGCT	520
PG2	A.....G.....C..AG..T.....	520
PG1	CGGTTTGGTGGACAATGACTCTCCCACTTACAATCCACAGAAGTGATGCTGGAAAGCAATGGAAGACTTCCAAGCACAAAACCAAC	608
PG2	..T.....T.....T.....	608
PG1	AGCACTCAGGTTCTATGGAAGTGATGGTGTAAACAGTGACAGGCATAACAATTCAGAATAGTCAACAAACCCATCTCAAGTTTGACTCT	696
PG2	C.....	696
PG1	TGCACAAATGTTCAAGTCTCTGGCATCAGTGTTCATCACCTGGTGATAGCCCCAACACTGATGGAATTCATTGCAAACTCCCAAA	784
PG2	T..C.....T.....A.....T..T.....	784
<- PG1-2		
PG1	ACGTGGTTCATCTATAGTAGCACACTTGCTTGGCGGATGATTGCGTCTCTATACAAACTGGTTGCTCGGACATATATGTACACAATGT	872
PG2	T.....C.....T..T.....C..TA.....	872
<- PG2-2		
PG1	GAATTGTGGACCTGGCCATGGAATCAGCATTGGAAGTCTTGGCAGAGAGAACACTAAAGCCTGTGTAGAAATGTCACCGTCCGAGAT	960
PG2	C.....C..G.....A.....C.....	960
PG1	GTTACTATCCAAAATACCCCTCACTGGTGTGAGAATTAACATGAGCAGGGTGGCTCTGGTTCCGTTCCAAAATATCATGTTCTCGAAGC	1048
PG2	T.....G.....C.....C.....C.....C..T.....	1048
PG1	TTCAAGTTTCTGGGGTTCAAACCTCCAATTCCATTGACCAATACTATTGTGACGGTGGAGGTGCAGGAATGAATCATCAGCTGTGGC	1136
PG2	T.....TG..C.....T.....C..A..T.....	1136
PG1	AGTTTCAGGCATACATTATGTGAATGTAAAGGAACATACACTAAGGAGCCTATCTATTTTGCATGTAGTGACAACTTACCTTGCACT	1224
PG2	..G.....C.....A.....C.....T.....C.....	1224
PG1	GGCATAACCCCTTGATACCATACAACCTGGAATCAGCCCAAGAACTAAAAATTCCAATGTTCCATTTTGTGGGAAGCATATGGGGAGT	1312
PG2	GG..A.....	1312
PG1	TGAAGACCACAACGGTCCCTCCTGTTGAATGTCTTCAGAGAGGAAACCCATCAAAGAAGGAGGAATCAATTCTAACATTGATTCTTG	1400
PG2	A..A.....C.....C.....T.....C.....A.....	1400
PG1	CTGAATTATCAACACAATGTCCGGGATATATAGATTATTATGATATATACAT--CATGGAAAGTTGTACTAGTTAGTTATTATGAT	1486
PG2	..A.....A.....AC.....AC.....C.....	1488
PG1	ATCTTAAATAGTTTCACATTCAATGTAATATAATGTTGGATTAGTGGATTGTAATACCGTGTGTCAAAAAGATGTTGGGGGAGAAGA	1574
PG2	T.....TC.C.....C.....-T.....C..G.....	1575
<- PG1-3		
PG1	TTTACAGCCCTATAC-TACTTAGATTGAAAAATGTATGAAGTGGAAAGAGTGATTAGCCATATGTAATATATCAATTTTTTTTCAA	1661
PG2	T.....ACC.TA.G.....CT.....A..A.....T.....AT.....-..T.....-CC...	1659
<- PG2-3		
PG1	AAAAAAAAAAAAAAAA--	1676
PG2	AA	1676

Fig. 2. Nucleotide sequences of the full-length cDNAs of *PG1* and *PG2*. The gene-specific primers used for the cleaved amplified polymorphic sequence (CAPS) analysis are underlined. Identical nucleotides between *PG1* and *PG2* sequences are indicated by dots, and deletions by dashes in the *PG2* sequence. The first start codon and the stop codon are underlined. The *PstI* recognition site present only in the *PG2* sequence is indicated. Primers used in the CAPS analysis are shown: right arrows next to the primer name indicate forward or 5' primer; left arrows indicate reverse or 3' primer.

pression in DD analysis (Fig. 8A). In the SCN-susceptible cultivars Dillon, Brim, Perrin, Lee, and Hutcheson, hybridization to *PG* transcript was distinct (data not shown). No hybridization with *PG* cDNAs was detectable in PI 438/489B (resistant to all races of SCN tested; Diers et al. 1997) and PI 209332 (resistant to races 3 and 14, susceptible to race 1).

After infection with SCN race 3, *PG* transcripts were induced in the infected roots of Essex as early as 1 DAI, reaching the highest levels 3 DAI. *PG* expression returned to the same level as in the noninfected roots by 5 DAI (Fig. 8A). In infected roots of PI 437654, a faint signal was detected 1, 1.5, 2, and 3 DAI. There was no detectable signal 5 DAI. In mechanically wounded roots, the *PG* transcripts were totally down-regulated (Fig. 8A).

In leaf RNA of PI 437654 and Essex, there was no detectable signal with *PG* cDNAs (Fig. 8B). In hypocotyls, *PG* transcript levels slightly increased after infection in Essex, while in PI 437654 they were down-regulated (Fig. 8B).

In compatible host-race combinations, Peking and PI 88788 infected with SCN race 14, transcripts were detected with the *PG* cDNAs in roots (Fig. 8C). In Essex roots infected with race 14, the steady-state levels of *PG* cDNAs were the highest. In incompatible host-race combinations, namely, Peking and PI 88788 infected with race 3, *PG* transcripts were undetectable (Fig. 8C). Interestingly, in PI 437654, which is resistant to race 14, a clear signal was detected after hybridization with the *PG* cDNAs (Fig. 8C).

***PGIP* transcription is induced in response to stress.**

Fig. 3. Deduced amino acid sequence of *PG1* and *PG2* full-length cDNAs. Deletions are indicated by dashes in the *PG2* sequence. Sequence alignments were performed with the CLUSTAL W multiple sequence alignment program (version 1.7, 1997; found on-line from the Human Genome Center, Baylor College of Medicine) and viewed with BOXSHADE (found on-line from ISREC [the Swiss Institute for Experimental Cancer Research]). Regions of identity (black) and similarity (shaded) are indicated. Dissimilar amino acids are in white boxes.

PI 437654, while in Essex no differences were detected in the amount of smaller transcript after infection. The larger transcript (2.2 kb) showed reduced expression after infection in both genotypes.

In compatible host-race interactions, i.e., Essex, Peking, and PI 88788 infected with SCN race 14, there was a stronger expression of *PGIP* than in the incompatible interaction in

Peking and PI 88788 infected with race 3 (Fig. 8C). There were no detectable differences in the abundance of *PGIP* transcript levels in PI 437654 infected with races 3 or 14 (Fig. 8C).

DISCUSSION

PG transcription differs in soybean-SCN interactions.

All the *PGs* cloned to date in plants are associated with developmental processes (Hadfield and Bennett 1998). Recently, a *Medicago sativa* *PG* gene was shown to be involved in the early stages of the *Rhizobium meliloti*-legume symbiosis (Munoz et al. 1998). For the first time, we report a plant *PG* that is differentially expressed in a plant-pathogen interaction. Detection of *PG* expression in roots of soybean required prolonged autoradiography (5 to 7 days) of RNA blot analysis, indicating low levels of *PG* transcripts. In this context, detection of a differentially expressed fragment of low abundance level demonstrates sensitivity of the DD methodology.

Extensive sequencing of the 5' and 3' RACE products (27 clones) aided in the identification of two *PG* cDNAs from soybean roots. Existence of duplicate genes in soybean is not surprising because of the polyploidization event in its ancestry. Even when the 3' UTR of the cDNAs was used for the Southern blot analysis, restriction fragment length polymorphism (RFLP) patterns with *PG1* and *PG2* cDNAs were identical (not shown), suggesting that the duplication event may have occurred recently during evolution. The two *PG* cDNAs were 92% identical at the nucleotide level and could be distinguished by CAPS analysis with *Pst*I. Although RNA blot analysis clearly showed only one band, it is possible that the

Fig. 5. Polygalacturonase-inhibitor protein (*PGIP*) gene amplification from genomic DNA and cDNAs with the primers described in Favaron et al. (1994). The amplification products were resolved on a 1.4% agarose gel. Amplification from genomic DNA of PI 437654 with the following. Lane 1: *PGIP* 5' primer alone; lane 2: *PGIP* 3' primer alone; lane 3: *PGIP* 5' and 3' primers; lane 4: Amplification from Essex DNA with *PGIP* 5' and 3' primers; lanes 5 and 6: Amplification of cDNA with *PGIP* 5' and 3' primers. Lane 5: PI 437654; lane 6: Essex; lane 7: 100-bp DNA ladder (Promega, Madison, WI).

GmcPGIP	GAGCTATGCAACCCACAAGACAAACAAACGCTACTCCAATCAAGAAAGAGCTTGGCAACCAACCCTCTTCTCTCATG	80
GmPGIP	G.C.....T.....A.....C.....	80
GmcPGIP	GCACCCAAAGACCGACTGTTGCAACAACAGCTGGGTAGGTCTCATGCGATACCGTCACCCCAACATACCGCGTCGACA	160
GmPGIP	..TT.T..CC..T..T..C.....GA.CA...C.....T.....C.....A.....A.....A.....G	160
GmcPGIP	ACCTCGACCTCTCCGAACCTAACCTCCGCAAAACCTACCTATTCCTCCCTCCGTAGGTAGCCTTCCCTGCCTCAAAATT	240
GmPGIP	T.....C.....T.....T.....A.....CC.A.....A.T.G..T..	240
GmcPGIP	CTTTACATCACCAACAACCCCAAC-ATCGTTGGCACAATACC--CA-CCACC-----A-TCACCAAACTCACCAAGCTTC	310
GmPGIP	C.....T.G..-GG.....CC.AA.C..TCA...C..CT..G...T.GCTAA.C.....C.....-----C	310
GmcPGIP	GTGAGCTCAACATCAGATACCAATATCTCCGGCCAGATACCCCATTTCTTGTCCCAATCAAAGCCCTCGGATTCCTC	390
GmPGIP	..T.T..TT.T.....CCC.....G.....CT.....AG.....T.....A..T.A.TCAC.....	390
GmcPGIP	GACCTCTCCAACAACAACCTCTCCGGCAACCTCCCTTCTCTGGCTTCTTCTCTCCCGACCTTTACGGAATCTCCTTCGA	470
GmPGIP	..TT.....T.....CC.....A.....CG...C...CT...G..G...A.T..CGT.....A.....	470
GmcPGIP	CAACAACTACATCTCCGGCCCCATCCAGACTTGTTCGCTTCCGTCTCGAAGCTGTTTACGGCGATCTCACTCTCCGGCA	550
GmPGIP	.GG...C.A.....G.....G...C.A..GC..AT.....T.....T.....GA.GA.....C...	550
GmcPGIP	ACCGCTCATCGGGAAGATTCCGTCGCTGGGGGGGAAGCCGGACATGAAGATTGTGGACTTGTCCCGGAACATGCTGGAG	630
GmPGIP	CA.....G..AC.TTC.C...T.T.A..C..GC.T.C.....T.....	630
GmcPGIP	GGTGATGCGTCGTTGTTTGGGTCGGAGAAACACACAGAGAGGATATATCTGGCAAACAACCTGTTTGGCTTTTGATTT	710
GmPGIP	A.....A.....GC...A...CT.....G..G...A..C.....C.....	710
GmcPGIP	GGGAAAGTAAGTTGTGCAAGACCTTGGGGGTTTGGATGGTGGCCATAATCTTATATATGGGACGCTTCCTAAGGGAC	790
GmPGIP	...G.....G.C.....T...A...AAC.GC.....CT.A.GA.C..C.G...T.....A...C.....	790
GmcPGIP	TTACGTCGCTTAAGGATCTGTATTATTGGATGTGAGCTACAATAATCTCTGTGGTGAGATTCTCGGGGTGGTAAATTG	870
GmPGIP	G.....TT.....C.CAG...TA.....T.....G...T.....G...A...G...CA..	870
GmcPGIP	CAAGAATTTGATGCGCTTTTGTATGCTAATAACAAGTGCTTGTGTGGCTCCCTCTCTCTCTGCACTTAA	942
GmPGIP	...CGT.....C.TA...CT.....C.....T.....	942

Fig. 6. Comparison of the nucleotide sequence of *GmPGIP* (this research) and *GmcPGIP* (Favaron et al. 1994). Identical nucleotides between *GmPGIP* and *GmcPGIP* are indicated by dots.

two *PG* cDNAs may produce similar sized transcripts that cannot be resolved by RNA blot analysis. Using the 3' UTR of the cDNA clones (approximately 250 bp), we could not differentiate between steady-state levels of *PG1* and *PG2*. However, CAPS analysis showed that both *PG1* and *PG2* are expressed in the roots of both PI 437654 and Essex. In tomato, there is only one *PG* gene per haploid genome that is expressed exclusively during fruit ripening (Bird et al. 1988). The *PG* gene, detected in flower abscission zones in tomato, was only 43% identical to the fruit *PG*, and it failed to cross-hybridize (Kalatizis et al. 1995). Similarly, the *PGs* in soybean leaves may be significantly divergent from the root *PGs*, since no signal was detectable with the root *PG* in RNA blot analysis. Both the identified *PG* cDNAs were expressed in roots and hypocotyls and produced transcripts of the same size. Although constitutively expressed, *PG* transcription was genotype dependent, as shown by different levels of *PG* transcripts in the 14 soybean genotypes tested. Furthermore, *PG* transcription was associated with host reaction to SCN. The levels of *PG* cDNAs were down-regulated in the incompatible interactions with race 3. The enhanced expression of *PG* cDNAs in compatible hosts for SCN race 14 (Peking, PI 88788) and greatly elevated levels of *PGs* in Essex infected with race 14, compared with infection with race 3, suggest that the nematode genotype may play a role in *PG* transcription.

Cellular rearrangements during the formation of the feeding site by sedentary parasitic nematodes in general and SCN in particular utilize portions of the normal plant developmental program (Bird 1996; Mahalingam and Skorupska 1996). Grundler et al. (1994) reported that the procambial cells recruited in syncytium formation by *Heterodera schachtii* in *Arabidopsis* were xylem initials and this was responsible for the observed suppression of xylem formation. However, Bird (1996) suggested that, rather than suppressing xylem formation, syncytium formation depletes the supply of xylem precursors. Bird (1996) also suggested that precocious expression of developmentally programmed cell wall dissolution, characteristic of final stages of xylem differentiation, may be responsible for syncytium formation. The identification of plant *PG* cDNAs that catalyze the hydrolysis of cell wall α (1-4) linkages of D-galacturonans, and are up-regulated as early as 1 DAI, corroborates Bird's (1996) observations and supports the hypothesis that developmentally regulated genes may be involved in syncytium formation (Mahalingam and Skorupska 1996). There was no cross-hybridization of *PG* cDNA to SCN genomic DNA, indicating that the *PG* is of soybean origin (data not shown).

The total down-regulation of *PG* transcripts in hypocotyls of PI 437654 after SCN infection indicates that the signal transduction machinery responsible for their suppression is very strong. We speculate that *PGs* are not efficient in cell wall breakdown in PI 437654 because of possible *trans*-acting factors such as the inhibitors of *PGs*, or the *cis*-acting nematode responsive sequences (if any) essential for increased expression are not functional. Further, the higher abundance of *PG* transcripts in hosts susceptible to at least one race of SCN strongly suggests that steady-state levels of *PG* may be one of the key factors for establishment of syncytium. The differences in the observed patterns of expression between compatible Essex and incompatible PI 437654 probably reside in the upstream promoter sequences of the *PG* genes since there are no differences between the two genotypes in the ORFs of *PG1* and *PG2*. Mechanically wounded roots were included in this analysis to verify whether the induction of *PG* expression was a general response to stress. The down-regulation of *PG* transcription in the wounded roots further exemplified that enhanced *PG* transcription in response to SCN infection is specific and may provide cues for manipulating response to SCN.

***PGIP* transcription differs in soybean-SCN interactions.**

We isolated a *PGIP* sequence from the root cDNA of PI 437654 and Essex that shows 72% amino acid homology to the reported *PGIP* from genomic DNA of soybean cultivar Canton (Favaron et al. 1994). To confirm that this was not due

Fig. 7. Comparison of the deduced amino acid sequences of polygalacturonase-inhibitor proteins (PGIPs). Shown are the sequences of *Phaseolus vulgaris* PGIP (PvPGIP), *Glycine max* PGIP from roots (GmPGIP, this research), and GmCPGIP (Favaron et al. 1994). Sequence alignments were performed with the CLUSTAL W multiple sequence alignment program (version 1.7, 1997; found on-line from the Human Genome Center, Baylor College of Medicine) and viewed with BOXSHADE (found on-line from ISREC [the Swiss Institute for Experimental Cancer Research]). Regions of identity (black) and similarity (shaded) are indicated. Dissimilar amino acids are in white boxes.

to sequencing errors, several additional clones from Essex and PI 437654 were sequenced. All sequenced clones (four from PI 437654 and four from Essex) were 100% identical but showed low homology to the earlier reported *PGIP*. Favaron et al. (1994) sequenced only one genomic clone of *PGIP* and confirmed its sequence by direct sequencing of the PCR-amplified product. Furthermore, the sequence of the *PGIP* we obtained is 88% identical to the common bean (*Phaseolus vulgaris*) sequence and hence we claim that our sequence represents *PGIP* of soybean. The sizes of the fragments generated from the cDNAs and genomic DNA were identical, indicating that *PGIP* is an intronless gene (Fig. 5). The presence of other weakly hybridizing fragments in Southern blot analysis indicates that there are other *PGIP*-like sequences in the soybean genome.

Fig. 8. RNA blot analysis with the polygalacturonase (*PG*) and polygalacturonase-inhibitor protein (*PGIP*) cDNA clones. Ten micrograms of total RNA was separated on a 1.2% formaldehyde-agarose gel. **A**, Temporal expression analysis of *PG* and *PGIP* cDNAs in soybean-SCN interactions. Lane 1: noninfected roots of Essex; lane 2: non-infected roots of PI 437654; lane 3: Essex; lane 4: PI 437654, 1 day after inoculation (DAI); lane 5: Essex; lane 6: PI 437654, 1.5 DAI; lane 7: Essex; lane 8: PI 437654, 2 DAI; lane 9: Essex; lane 10: PI 437654, 3 DAI; lane 11: Essex; lane 12: PI 437654, 5 DAI; lane 13: wounded roots of Essex; and lane 14: wounded roots of PI 437654. The third panel shows the methylene blue staining of the rRNA to demonstrate loading of RNA. **B**, Systemic expression analysis of *PG* and *PGIP* cDNAs in soybean-SCN interactions. Leaf: lane 1: Noninfected Essex; lane 2: Essex, 2 DAI; lane 3: noninfected PI 437654; lane 4: PI 437654, 2 DAI; Hypocotyls: lane 5: noninfected Essex; lane 6: Essex, 2 DAI; lane 7: noninfected PI 437654; and lane 8: PI 437654, 2 DAI. **C**, Race-specific responses of *PG* and *PGIP* cDNAs in SCN resistant and susceptible soybeans. Peking: lane 1: race 3; lane 2: race 14; PI88788: lane 3: race 3; lane 4: race 14; PI 437654: lane 5: race 3; lane 6: race 14; Essex: lane 7: race 3; and lane 8: race 14.

PGIPs have been reported in all the plant tissues, including the floral organs. We observed strong *PGIP* expression in roots, leaves, and hypocotyls. Based on their ubiquitous expression, *PGIPs* have been regarded as being important for general resistance to biotic and abiotic stresses and also in cell wall repair mechanisms. *PGIPs* effectively inhibit fungal *PGs* (Desiderio et al. 1997; Stotz et al. 1993). In vitro, fungal endopolygalacturonases convert polygalacturonic acid of pectins to inactive mono-, di-, and even trigalacturonic acids (English et al. 1972). It is well established that cell wall fragments or oligogalacturonides are active elicitors in the induction of plant defense responses (Nothangel et al. 1983; Walker-Simmons et al. 1984; Cervone et al. 1989). Especially linear α -(1 $\rightarrow$ 4)-linked oligogalacturonides of chain lengths between 10 and 13 are strong inducers of phytoalexins and lignification; shorter oligomers have little or no elicitor activity (Hahn et al. 1981).

PGIP is a classical resistance gene. If *PGIP* were a typical incompatibility factor in the soybean-SCN interaction, then its expression should be higher in the incompatible interactions than in the compatible interactions. However, we observed the opposite phenomenon in the soybean-SCN interactions, indicating that *PGIP* does not behave as a resistance factor. We speculate that *PG* and *PGIP* expression during normal plant development may be under mutual control at a transcriptional level. In such an instance, if the constitutive level of *PG* expression is undisturbed then the constitutive levels of *PGIP* remain unaltered. If the levels of *PG* transcripts increase (e.g., after SCN infection), then a default mechanism would trigger levels of *PGIP* as seen in the compatible interactions. We observed an induction of *PGIP* only when there was an induction of *PG* in response to pathogen but not mechanical wounding. This observed induction was not genotype specific; on the contrary, it was interaction specific. In all the compatible host-race combinations tested, we observed increases in *PG* and *PGIP* transcripts. Increased level of *PGs* implies more wall breakage, and this would eventually lead to the formation of elicitor-active oligogalacturonides that is detrimental to a compatible response. If *PGIP* would be brought into the system, it may bring equilibrium favoring the suppression of elicitor-active oligogalacturonides. Elucidation of regulatory elements of *PG* and *PGIP* transcription may provide insights into the complexity of the interaction and the subtleties of the manipulative nature of the parasite. Hence, if *PG* levels are reduced, the constitutively expressed levels of *PGIP* should remain unaltered, as observed in incompatible SCN-soybean interactions. After mechanical wounding of roots, levels of *PG* expression were undetectable, while the *PGIP* expression was strongly induced in both PI 437654 and Essex. Devoto et al. (1998) reported that the *Pgip-1* promoter of *Phaseolus vulgaris* was induced in response to wounding but not to elicitors or pathogen infection, suggesting multiple members of the *PGIP* gene family may be differentially regulated depending upon the stimuli. We speculate differential regulation of *PGIP* transcription to biotic or abiotic stresses and normal developmental processes in soybean roots. Although speculative at this juncture, the transcriptional regulation of endogenous *PG* could have been exploited by SCN to allow for successful parasitism of soybeans. The *cis*-acting elements and *trans*-acting factors controlling *PG* transcription may provide some clues for engineering resistance to SCN in soybeans.

MATERIALS AND METHODS

Plant materials.

Two soybean genotypes, differing in response to SCN, were used in DD analysis. PI 437654 is a plant introduction from China and is resistant to all known races of SCN in the U.S. (Anand 1991; Rao-Arelli et al. 1992). Essex is a commercial cultivar that is susceptible to all races of SCN (Smith and Camper 1973). Nematode populations behaving like race 3 or race 14 according to the race classification scheme (Riggs and Schmitt 1988) were used for infections in our experiments. Three-day-old seedlings of SCN resistant PI 437654 and susceptible Essex were used for inoculations with race 3 as described in Mahalingam et al. (1998). For the analysis of *PG* and *PGIP* expression in infected plants, roots were harvested 1, 1.5, 2, 3, and 5 DAI, and leaves and hypocotyls, 2 DAI. Roots, leaves, and hypocotyls of noninfected seedlings were controls. Mechanically wounded roots (harvested 24 h after piercing with a 18G needle to simulate nematode penetration) were also analyzed. Five SCN-susceptible soybean genotypes (Brim, Dillon, Hutcheson, Lee, and Perrin) and six soybean genotypes with resistance to at least one SCN race (Bedford, PI 438489B, PI 209332, PI 90763, PI 88788, and Peking) were included in the expression analysis of *PG* and *PGIP*. Roots of Peking (resistant to race 3, susceptible to race 14), PI 88788 (resistant to race 3, moderately resistant to race 14), PI 437654, and Essex were infected with races 3 or 14 and were collected 3 DAI.

Isolation of *PG* by DD.

Total RNA of PI 437654 and Essex roots, 2 DAI, and noninfected controls were isolated with the RNeasy Plant Isolation Kit (Qiagen, Chatsworth, CA). For every RNA tested, two independent samples were processed. The RNA was treated with RNase-free DNase (Promega, Madison, WI) to remove chromosomal DNA contamination. The reverse transcriptions and PCR analyses were conducted as described in Liang and Pardee (1992). With the combination of T17-C anchor primer and decamer S07: 5'-TCCGATGCTG-3' (Operon Technologies, Alameda, CA), a 350-bp fragment that was differentially expressed in susceptible Essex was identified. This band was reproducibly observed in independent samples and was isolated from the gel, cloned into the pGEM vector (Promega). The cloned fragment was sequenced with the ABI Prism Dye Terminator Cycle Sequencing Ready Reaction Kit (Perkin Elmer, Foster City, CA). Sequencing was conducted in an ABI 131 automated DNA sequencer at Clemson University. Sequences were tested for homology to other sequences with the NCBI's BLAST algorithm (Altschul et al. 1990).

5' and 3' RACE PCR to isolate full-length cDNA of *PG*.

Based on the sequence data of the isolated DD clone, two primers were synthesized (Integrated DNA Technologies, Coralville, IA) for the 3' RACE (5'-GCTTGCGGCGATGATTGCG-3'; 5'-ATGTACACAATGTGAATTGTGG-3') and two for the 5' RACE (5'-CTGTTACACCATCACTTCC-3'; 5'-ATAGAACCTGAGTGCTGTTGG-3'). Total RNA from PI 437654 and Essex was used in the RACE analysis. The cDNA synthesis and subsequent PCRs were performed according to the kit protocol (GibcoBRL, Rockville, MD). Several clones from the 5' and 3' RACE PCR were analyzed and an overlap-

ping consensus sequence of 1,676 bp was assembled. Based on this sequence, two primers (5'-ATATGAACTAAACA CCATTGC-3'; 5'-GTAAATCTTCTCCCCAACAT-3') were designed to amplify a full-length *PG* cDNA from Essex and PI 437654. The 1.5-kb fragment was isolated from a low-melting agarose gel and purified with the Wizard PCR preps (Promega), cloned into pGEM vector (Promega) and sequenced as described above.

Identification of two *PG* cDNAs in roots.

Sequencing analysis of more than 20 clones of the 1.5-kb coding *PG* sequence clearly indicated that there were two *PG* sequences, designated as *PG1* and *PG2*. CAPS analysis was used to distinguish between *PG1* and *PG2* cDNAs. Gene-specific primers were designed for the two *PG* cDNAs. For *PG1*, primers were PG1-1 (5'-GAAAGCTGCTGCATCTGCTACT-3'), PG1-2 (5'-AGAGACGCGCAATCATCGCCG-3'), and PG1-3 (5'-AAGTAGTATAGGCTTGTAATC-3'). For the *PG2* sequence: PG2-1 (5'-CAAAGCTGCCTATGCTACAACT-3'), PG2-2 (5'-TAGAGATACAGTCATCACCA-3' and PG2-3 (5'-AGCATATGGCTAAGTAAAT-3'). RT-PCR was conducted for 30 cycles at 94°C for 40 s, 59°C for 40 s, and 72°C for 2 min. The RT-PCR-generated products were digested with *Pst*I for 3 h and the digested products were separated on a 2% agarose gel.

Isolation of *PGIP* cDNA.

Three micrograms of total RNA was reverse transcribed with oligo(dT)₁₅ primer with RNase H⁻ reverse transcriptase according to the manufacturer's instructions (Gibco-BRL, Bethesda, MD). Root cDNA and genomic DNA from Essex and PI 437654 were used as templates in PCR. The primers (5'-GAGCTATGCAACCCACAAGA-3'; 5'-TTAAGTGCAGGAAGGAAGAG-3') and PCR amplification conditions were as described in Favaron et al. (1994). The products of PCRs were separated on 1.4% agarose gel. The amplified fragment (approximately 950 bp) was excised from the gel and purified with the QIAEX II gel extraction kit (Qiagen), cloned into pGEM vector, and several clones were sequenced as described earlier.

RNA and DNA blot analyses.

Total RNA from noninfected roots, SCN race 3-infected roots (1, 1.5, 2, 3, and 5 DAI), and wounded roots of PI 437654 and Essex were used to investigate the expression of *PG* and *PGIP* transcripts. To examine the systemic expression, RNA was extracted from leaves and hypocotyls (noninfected and 2 DAI) from both genotypes. Root RNA from the SCN susceptible (Dillon, Perrin, Brim, Lee, Hutcheson, and Essex) and resistant (Peking, PI 88788, PI 209332, PI 438489B, PI 90763, Bedford, and PI 437654) lines was used to investigate genotypic differences in *PG* and *PGIP* expression. RNA from Peking, PI 88788, PI 437654, and Essex, 3 DAI with SCN races 3 and 14 was used to examine race-specific responses of *PG* and *PGIP*. RNA gel blots and hybridizations were performed as described in Sambrook et al. (1989). The full-length cDNAs of *PG* (both *PG1* and *PG2*) and *PGIP* were random primed with α [³²P]dCTP (Feinberg and Vogelstein 1983) and used as probes in high stringency hybridizations.

Five restriction enzymes (*Dra*I, *Eco*RI, *Eco*RV, *Hind*III, and *Taq*I) were used for digesting genomic DNA from PI

437654 and Essex. Full-length cDNAs of *PG1*, *PG2*, and *PGIP* were used as probes for hybridizations as described in Skorupska et al. (1993).

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