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Genomic Organization and Differential Expression of Two Polygalacturonase-Inhibiting Protein Genes from *Medicago truncatula*

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Two adjacent polygalacturonase-inhibiting protein (PGIP) genes were characterized from the model legume *Medicago truncatula*. *MtPGIP1* and *MtPGIP2* were isolated from a single bacterial artificial chromosome clone identified from library-screening with cDNA probes. Ten and nine characteristic stretches of leucine-rich repeats, respectively, were identified from the predicted MtPGIP1 and MtPGIP2, showing 58% sequence identity at the amino acid level. These *MtPGIP* genes are likely present as a small gene family. Transcripts encoding MtPGIP1 were expressed highly in the flowers and at low levels in the roots and stems, whereas those encoding MtPGIP2 were not detected in any untreated organs. Inoculation of the *M. truncatula* cultivar 'Jemalong' with the pathogenic fungus *Colletotrichum trifolii* induced a hypersensitive response and the expression of both genes. The two genes were also expressed in response to the application of jasmonic acid, although mechanical wounding induced only *MtPGIP1* and salicylic acid induced neither. Abiotic stresses, such as high-salt, cold, or drought, induced the expression of *MtPGIP1*, whereas low-temperature stress induced *MtPGIP2* only. Consistent with these observations, sequence elements specific to plant defense and stress responses were identified, in varying numbers, from the putative promoter regions of the two genes. Furthermore, supportive of their putative functional roles, bacterially expressed recombinant MtPGIP1 and MtPGIP2 inhibited fungal polygalacturonase activity. Therefore, these results suggest that *MtPGIP1* and *MtPGIP2*, copies that presumably arose from duplication, are regulated by separate signaling pathways and likely play roles in response to pathogenic and environmental stresses.

Keywords: differential expression, gene family, *Medicago truncatula*, model legume, plant stress response, polygalacturonase-inhibiting protein

Medicago truncatula is a model legume suitable for molecular genetic analyses. This Mediterranean-originated annual diploid has a small genome (2n = 2x = 16; 450 Mbp/C) (Blondon et al., 1994) and a short life cycle (Barker et al., 1990), and is amenable to transformation and regeneration (Trieu et al., 2000). *M. truncatula* and *Lotus japonicus* (Handberg and Stougaard, 1992) are the two major species used for structural, comparative, and functional genome analyses of legumes (VandenBosch and Stacey, 2003). The research using these two species has elevated our understanding of fundamental physiological processes unique to legume-microbe interactions, such as nodulation (Cullimore and Denarie, 2003) and symbiotic associations with arbuscular mycorrhizae (Albrecht et al., 1999). Moreover, the resources established for structural and functional genomics, e.g., large-insert genomic libraries (Nam et al., 1999) and expressed sequence tag (EST) databases (Cannon et al., 2005) have facilitated assessments of biotic and abiotic stress responses in legume species. Microarray analysis of the pathogenic interactions between *M. truncatula* and *Colletotrichum trifolii*, one of the primary pathogenic fungi on legumes (Lenne, 1992), has revealed multiple up- or down-regulated genes for resistance responses (Torregrosa et al., 2004), including a polygalacturonase-inhibiting protein (PGIP) gene.

PGIPs are a key component of plant defense proteins, inhibiting the activity of endopolygalacturonases from many fungal pathogens (Martin et al., 2003). They hydrolyze the $\alpha(1 \rightarrow 4)$ linkages between D-galacturonic acid residues in homogalacturonans (de Lorenzo et al., 2001). PGIPs are structurally related to the products of several plant resistance genes belonging to the superfamily of leucine-rich repeat (LRR) proteins (de Lorenzo et al., 2001). The genes encoding PGIPs have been cloned from a number of mono- (Jang et al., 2003) and dicotyledonous species (Toubart et al., 1992; Yao et al., 1999; Favaron et al., 2000; Ferrari et al., 2003; Li et al., 2003). *PGIP* genes

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exist as a small gene family in certain species (Desiderio et al., 1997; Yao et al., 1999). In *Arabidopsis*, two *PGIP* genes are expressed differentially (Ferrari et al., 2003). Moreover, the individual gene products often exhibit different specificities against fungal and other pathogens (D'Ovidio et al., 2004). The defensive role of PGIPs has been demonstrated by the reduction of *Botrytis cinerea* colonizations in a transgenic tomato that constitutively expresses pear *PGIP* (Powell et al., 2000). *PGIP* genes are expressed not only in response to fungal infection, but also to elicitors, wounding, or abiotic stresses (Yao et al., 1999; Ferrari et al., 2003; Li et al., 2003), and even during floral development (Jang et al., 2003).

Our objective here was to isolate and characterize *PGIP* genes from *M. truncatula*, and to examine their expression patterns. We also used bacterially expressed recombinant proteins to assess the putative functional effects of structural differences between predicted proteins.

MATERIALS AND METHODS

Plant Material and Stress Treatments

Seeds of *M. truncatula* Gaertn cultivar 'Jemalong' (ecotype A17) were germinated in darkness at 4°C for 48 h. Seedlings were placed, in square plastic dishes (12.5×12.5×2 cm), between two layers of Whatman paper pre-moistened with Murashige Skoog (MS) media (Duchefa Biochem, Netherlands). They were then reared for 10 d at a semi-vertical position in a growth chamber at 25°C under a 14-h photoperiod. Spores of *C. trifolii* race 1 were extracted with sterile water, and 2 mL of 2×10^6 spores mL^{-1} solution was sprayed onto 15, 10-d-old seedlings in the presence of 0.01% Tween-20. The inoculated seedlings were incubated in square dishes and harvested at multiple time points.

To examine the effects of stress stimuli, 10-d-old seedlings were sprayed with a solution containing 1 mM salicylic acid (Sigma, USA) or 500 μM methyl jasmonate (Sigma) in the presence of 0.01% Silwet L-77 (Lehle Seeds, USA). For the salinity and dehydration treatments, a final concentration of 200 mM NaCl or 1 mM abscisic acid (ABA) (Sigma), respectively, was added to the MS medium. For the wounding treatment, the first and second emerging leaves of 10-d-old seedlings were cut with razor blades. Seedlings were also placed at 4°C or under 50% relative humidity to induce low-temperature or drought stress. Seedling samples were collected at various time points and immediately frozen in liquid nitrogen for RNA isolation.

BAC Library Screening and Clone Analysis

The *Bam*HI BAC library of *M. truncatula* (Park and Nam, 2006) was screened with either the *MtPGIP1* or *MtPGIP2* cDNA fragment amplified from cold-treated *M. truncatula* seedling RNA using specific primers, *MtPGIP1a* and *MtPGIP2a* (Table 1). Probes were labeled with [α-^{32}P]dCTP (New England Nuclear, USA) by the Ready-to-Go Labeling Kit (Amersha, Pharmacia, UK). Hybridization was carried out with

Table 1. Nucleotide sequences of the oligonucleotide primers used for amplifying *PGIP* and other stress-responsive genes of *M. truncatula*.

Gene	Primer[a]	
MtACTIN	gtccgtgacataaaggagaag (F)	gcacttcctgtggacaatgg (R)
MtCOR47-like	caagtacgag gaaactacagcaacc (F)	gatcatgatcagtagcagtcttagg (R)
MtPGIP1a	gcgtcaaatgcgaccttataacacac (F)	gaagataagcatacacatcaaacctatc (R)
MtPGIP1b	atggtaactatgtttggtgg agcttcaa (F)	tcacttgcatttaggaagaggtggt (R)
MtPGIP1c	gcactccatatgcatcaccaccatcatcactgcaacccacaagacaagag ggt (F)	
	gcactcgcggccgctcacttgcatttaggaagaggtggt (R)	
MtPGIP2a	ttatccattgcgacgtaacaacttct (F)	taactctttcactttggtccatccc (R)
MtPGIP2b	atggcgactatgtttggaggatctgttttg (F)	tcacttgcatttgggaagcggtgac (R)
MtPGIP2c	gcactccatatgcatcaccaccatcatcactgcaacccacaagacaagaaagc (F)	
	gcactcgcggccgctcacttgcatttgggaagcggtgac(R)	
MtPIN20	ctagctacctcaaatgatgttgagc (F)	tatactcagcttcataagtagcagc (R)
MtPR10-1	tgtcttcaactttgaggatg aaacc (F)	aatcaggatttgccaaaacgtaacc (R)

[a]F, forward; R, reverse.

the BAC colony filters, as described by Park and Nam (2006). Alternatively, the *MtPGIP1a* and *MtPGIP2a* primers were used directly for screening the BAC DNA multiplex pools by polymerase chain reaction (PCR) (Park and Nam, 2006). Plasmid DNA was isolated from positively identified BAC clones, and was analyzed by *Not*I digestion and pulsed field gel electrophoresis as described (Park and Nam, 2006). CsCl density-gradient-purified BAC DNA was used for the nucleotide sequence analysis. Sequence analysis, protein alignment, and phylogenetic tree construction were carried out with DNASTAR software and the Vector NTI program (Invitrogen, USA). Analysis of the promoter regions was facilitated by web software at http://intra.psb. ugent.be:8080/PlantCARE (Rombauts et al., 1999).

DNA Gel Blot Analysis

Genomic DNA was isolated from 8-week-old *M. truncatula* leaves according to the cetyltrimethylammonium bromide (CTAB) method (Rogers and Bendich, 1994). Twenty micrograms was digested with *Cla*I, *Eco*RI, *Hin*dIII, or *Nco*I for 24 h, then separated on 1% agarose gels in Tris-acetate (TAE) buffer. The resulting DNA fragments were transferred to Hybond N^+ nylon filters (Amersham Pharmacia). Two blots, prepared identically, were hybridized with either the *MtPGIP1* or *MtPGIP2* probe, both of which were obtained by amplifying the putative full-coding regions from cold-treated *M. truncatula* seedling RNA using the primer pair *PGIP1b* or *PGIP2b* (Table 1). Probe-labeling and hybridization were carried out as described for the BAC library screening except that hybridization was allowed to occur at 55°C. Afterward, the blots were washed sequentially with 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.5× SSC/0.1% SDS for 30 min each at 55°C, and exposed for autoradiography at -80°C for 72 h.

RNA Gel Blot Analysis and RT-PCR

Total RNA was isolated from *M. truncatula* seedlings using the TRIZOL reagent (Molecular Research, USA). Twenty micrograms of RNA was separated on a 1.3% agarose gel containing 30% formaldehyde in 1× MOPS (3-[N-morpholino]-propansulfonic acid) buffer, then transferred onto a nylon membrane. Hybridization was carried out in a buffer (5× SSPE, 2× Denhardt's solution, 0.1% SDS, 50% formamide, and 50 μg ml^{-1} salmon sperm DNA), using the full-length *MtPGIP1 or MtPGIP2* cDNA probe at 42°C for 16 h, as described by Lee et al. (2004). Afterward, the membranes were washed sequentially with 2× SSC/0.1% SDS and 1× SSC/0.1% SDS at 42°C for 30 min each, and finally with 0.1× SSC/0.1% SDS at room temperature for 30 min.

For reverse transcriptase (RT)-PCR, first-strand cDNA was synthesized from total RNA isolated from the stress-treated seedlings, as described by Kim et al. (2004). A 100-μL mixture containing 10 μg of template RNA, 500 ng of oligo(dT) primer (Amersham Pharmacia), 0.5 mM dNTPs, 10 mM DTT, 5 μL of RNase inhibitor (Amersham Pharmacia), and 5 μL of reverse transcriptase (Invitrogen) was incubated at 37°C for 1 h, heated at 95°C for 5 min, and used as template. PCR was carried out in a mixture of 10 μL that contained 0.5 μL of the first-strand cDNA, 10 pmol each of the gene-specific primers (Table 1), 0.2 μM dNTPs, and 0.2 μL of Taq DNA polymerase (Takara, Japan). Typical amplification conditions were: 30 sec at 94°C, 30 sec at 55°C, and 1 min at 72°C. To saturate the amplified fragments for each experiment, PCR cycles were adjusted from 20 to 30. After PCR, the products were analyzed by separation on a 1.5% agarose gel in TAE buffer. All experiments were repeated at least three times.

Bacterial Expression and Activity Assay of Proteins

The bacterial expression plasmid pMS107 (Surette and Stock, 1996) was used to overexpress MtPGIPs in *Escherichia coli*. Full-length cDNAs encoding the two MtPGIPs were amplified from the first-strand cDNA preparations that had been synthesized from cold-treated seedling RNA. Two specific primer pairs, *MtPGIP1c* and *MtPGIP2c*, contained overhangs for the *Nde*I (forward) or *Not*I (reverse) restriction site plus the codons for six His residues (forward) or a translation stop (reverse) (Table 1). The predicted N-terminal sequences of the recombinant proteins were MHHHHHHCNPQDKRV... (MtPGIP1) and MHHHHHHCNPQDKKA... (MtPGIP2). Amplified cDNA fragments were initially inserted into another expression plasmid, p604, a derivative of pET15b (Novagen, Germany) (K.-H. Jung, unpublished), and subsequently transferred into pMS107 by digestion with *Xba*I and *Not*I. The resulting vector constructs were introduced into *E. coli* strain BL21 (Invitrogen). Cells were grown at 37°C overnight, then induced with 200 μg mL^{-1} of isopropyl-β-D-thiogalactopyranoside (IPTG). Lysates were prepared by sonication and pelleting. Afterward, the supernatant was mixed with Ni^{+2}-NTA Agarose (Qiagen, Germamy), and the mix-

ture was incubated with agitation at 4°C for 4 h. The protein-agarose mixture was applied to a Glass Econo-Column (Bio-Rad, USA) and washed several times with 25 mM imidazole in sonication buffer (50 mM Tris-Cl, 300 mM NaCl, pH 7.5). Proteins were eluted with 250 mM imidazole and dialyzed with polygalacturonase assay buffer (20 mM sodium acetate, pH 5.0) (Desiderio et al., 1997), using a Centricon-10 microconcentrator (Millipore, USA). Protein quantity was determined by the method of Bradford (1976), and the proteins were analyzed by SDS-PAGE (Laemmli, 1970) and Coomassie Blue staining. Immunoblotting was performed with anti-6× His monoclonal antibodies (Clontech, USA) and goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, USA) as the primary and secondary antibodies, respectively. PGIP activities were measured by monitoring changes in the amount of reducing sugars that were produced by *Aspergillus niger* pectinase (Sigma), using the *p*-hydroxybenzoic acid hydrazide (PAHBAH) assay (York et al., 1985). Measurements were repeated three times and standard errors were calculated.

RESULTS

Isolation and Sequence Analysis of Two *MtPGIP* Genes

To isolate the *MtPGIP* genes from a BAC library of *M. truncatula*, we designed two oligonucleotide primer pairs, *MtPGIP1a* and *MtPGIP2a* (Table 1), from two tentative consensus sequences, TC108159 and TC106828, in the expressed sequence tag (EST) database of *M. truncatula* (http://www.tigr.org/tdb/tgi/mtgi). The cDNA fragments encoding partial peptides of MtPGIP1 and MtPGIP2 were amplified from cold-treated *M. truncatula* seedling RNA, and were used as radiolabeled probes to screen the *Bam*HI BAC library filters. Three BAC clones (B19J07, B54O09, and B62N18) were identified positively with either the *MtPGIP1* or the *MtPGIP2* cDNA probe (Table 2). When the same primers were directly used for screening the multiplex BAC DNA pools by PCR (Park and Nam, 2006), identical results were obtained, indicating that the two *MtPGIP* genes are present in a single BAC clone.

Nucleotide sequences of the full coding regions for *MtPGIP1* and *MtPGIP2* were determined from the positive BAC clone B19J07. The two genes lacked introns and encoded proteins of 342 and 321 amino acids, respectively. Alignment of the two deduced polypeptides with *Arabidopsis* PGIP1 (At5g06860) revealed that MtPGIP1 is 58% and 59% identical to MtPGIP2 and AtPGIP1, respectively, and MtPGIP2 is 46% identical to AtPGIP1 (Fig. 1). Like all previously reported plant PGIPs, MtPGIP1 and MtPGIP2 each consist of a signal peptide, an amino terminal domain, a conserved LRR domain with the consensus sequence of xLxxLxLxxNxLxGxlPxxLGxLx (de Lorenzo et al., 2001), and a carboxyl terminal domain. Interestingly, MtPGIP1 had 10 canonical tandem imperfect LRRs, whereas MtPGIP2 had only 9 imperfect LRRs, in which the N-terminal half of the first LRR was disrupted by the insertion of a putative octapeptide (TTPFPNPE). Moreover, the deletion of a single LRR (5th-6th repeat) in MtPGIP2 apparently reduced the effective length of the LRR to less than nine. Despite these structural irregularities, however, both proteins shared additional structural features that are common to plant PGIPs, such as the conserved cysteine residues in the amino and carboxyl termini flanking the LRR domain (not shown).

Comparison of the MtPGIP1 and MtPGIP2 with other plant PGIPs showed that they are more distantly related to each other than are most other PGIPs within a single species (Fig. 2). This indicates that the genes encoding these two MtPGIPs likely diverged more extensively than the corresponding paralogs of *Arabidopsis* (Ferrari et al., 2003) and *Brassica napus* (Li et al., 2003). Nonetheless, the clade with MtPGIPs belongs to a larger phylogenetic group that contains PGIPs from many dicotyledonous species including legumes, separate from a monocotyledonous out-group, OsPGIP (Jang et al., 2003).

Genomic Organization and Tissue-Specific Expression of *MtPGIP* Genes

Database searches further revealed that the two *MtPGIP* genes are tandemly located in a sequenced BAC clone AC157891 (http://www.ncbi.nlm.nih.gov;

Table 2. Identification of BAC clones containing *PGIP1* and *PGIP2* genes of *M. truncatula*.

Probe	No. of hits	Positive clones (insert size in kbp)
MtPGIP1	3	B19J07 (110), B54O09 (145), B62N18 (180)
MtPGIP2	3	B19J07 (110), B54O09 (145), B62N18 (180)

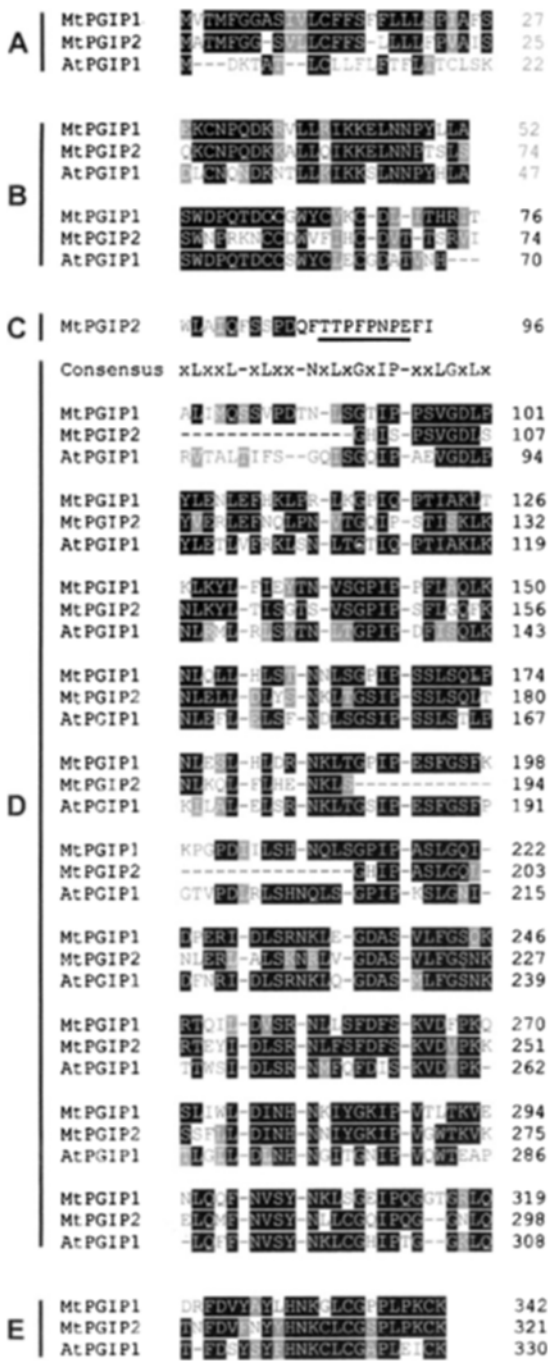

Figure 1. Sequence features of MtPGIPs compared with *Arabidopsis* homolog (AtPGIP1; At5g06860). Predicted amino acid sequences of three PGIPs were aligned by Clustal method (Higgins and Sharp, 1988). Typical PGIP domains are shown as A: signal peptides, B: presumed N-terminal regions, C: N-terminal half of first LRR of MtPGIP2 disrupted by putative octapeptide insertion, D: LRR domains, and E: C-terminal regions. Identical and conserved residues are shaded in black and gray, respectively. Putative octapeptide insertion is underlined. Consensus sequence for LRRs is indicated.

web-posted March 24, 2005). The coding regions of these two genes are 7,755 nucleotides apart (from the translation stop codon of *MtPGIP1* to the start codon of *MtPGIP2*). The presence of two such homologous genes in a narrow chromosomal region indicates that *MtPGIP1* and *MtPGIP2* likely descended from the duplication of a single progenitor gene.

To estimate the copy number of *PGIP* genes in the *M. truncatula* genome, DNA gel blot analysis was carried out with either the *MtPGIP1* or *MtPGIP2* full-length cDNA probe (Fig. 3). Hybridization identified DNA fragments of the sizes equivalent to those predicted from our sequence information. The hybridization signals from one gene tended to overlap with those from the other gene. For example, the 3.7-kbp (*Eco*RI), 4.8-kbp (*Hin*dIII), and 9.5-kbp (*Nco*I) bands detected with *MtPGIP1* had equivalents with *MtPGIP2*. Likewise, the 6.5-kbp (*Eco*RI), 3.6-kbp (*Hin*dIII), and 16-kbp (*Cla*I) bands detected with *MtPGIP2* had equivalents with *MtPGIP1*. These results indicate that cross-hybridization occurred at low intensities between the two *MtPGIP* genes. The total number of hybridized bands ranged from two to five, including a few unidentifiable from the sequence information. Assuming that all of these bands represent homologous genes, it is estimated that a small gene family containing two to five *PGIPs* is present in *M. truncatula*.

RNA gel blot analysis was carried out to examine the expression patterns of the *MtPGIP* genes in various plant organs (Fig. 4). Transcripts encoding MtPGIP1 were abundant in the flowers, but accumulated only to low levels in the roots and stems, and even less in the leaves and hypocotyls. By contrast, the transcripts encoding MtPGIP2 were not detected in any unstressed organs. Although RT-PCR analysis using primers specific to *MtPGIP2* for an extended number of cycles revealed that the transcripts encoding MtPGIP2 were indeed present in the stems and roots (data not shown), it is likely that the *MtPGIP* genes normally are not expressed appreciably in any organs except the flowers.

Expression of *MtPGIP* Genes in Response to Fungal Infection and Stress-Related Stimuli

To assess the expression of *MtPGIP* genes in defense against pathogens, *M. truncatula* seedlings were inoculated with spores of the pathogenic fungus *C. trifolii* (Torregrosa et al., 2004). Characteristic of the hypersensitive response (HR), these seedlings exhibited localized necrotic spots on young emerging leaves at 3 to 4 d post-inoculation (not shown). Such a symp-

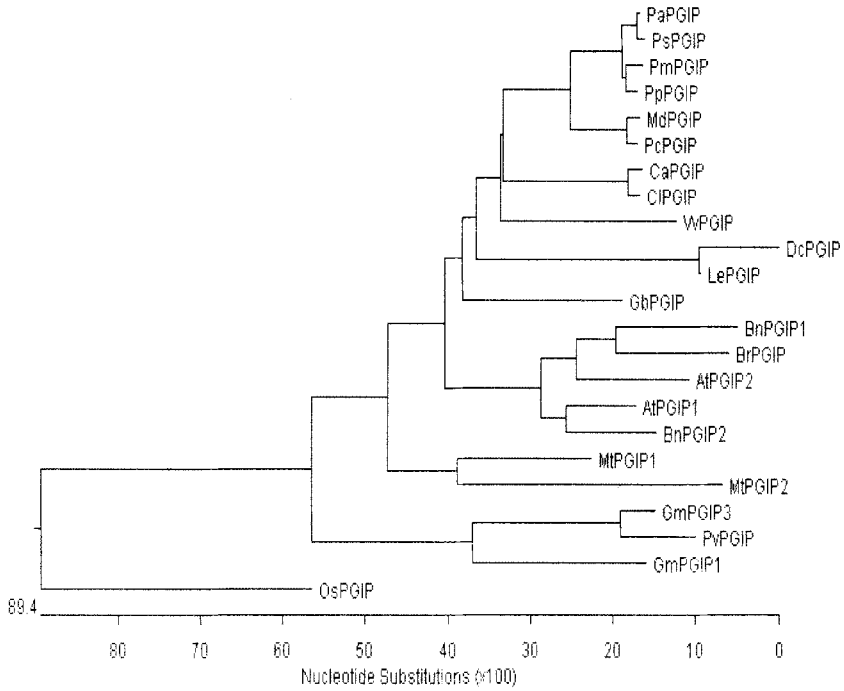

Figure 2. Phylogenetic relationship of MtPGIPs with other plant PGIPs. Full-length PGIP sequences were compared by multiple alignment using Clustal method; their evolutionary relationship was deduced. Proteins include: AtPGIP1 (*Arabidopsis thaliana*, AF229249), AtPGIP2 (*A. thaliana*, AF229250), BnPGIP1 (*Brassica napus*, AF529291), BnPGIP1 (*B. napus*, AF529693), BrPGIP (*Brassica rapa*, AY964100), CaPGIP (*Citrus aurantifolia*, AB071020), ClPGIP (*Citrus latipes*, AB071017), DcPGIP (*Daucus carota*, AY081214), GbPGIP (*Gossypium barbadense*, AY279357), GmPGIP1 (*Glycine max*, AF130974), GmPGIP2 (*G. max*, X78274), LePGIP (*Lycopersicon esculentum*, L26529), MdPGIP (*Malus domestica*, U77041), OsPGIP (*Oryza sativa*, Q8GT95), PaPGIP (*Prunus americana*, AY883418), PcPGIP (*Pyrus communis*, L09264), PmPGIP (*Prunus mume*, AY903223), PpPGIP (*Prunus persica*, AY903219), PsPGIP (*Prunus salicina*, AY986899), PvPGIP (*Phaseolus vulgaris*, X64769), and VvPGIP (*Vitis vinifera*, AF499451).

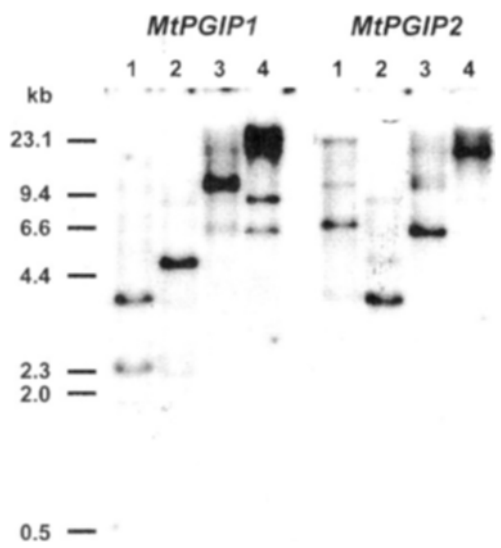

Figure 3. Genomic DNA gel blot analysis of *MtPGIP* genes. Genomic DNA (20 μg per lane) digested with *Eco*RI, *Hin*dIII, *Nco*I, or *Cla*I was separated by agarose gel electrophoresis, blotted and hybridized with radiolabeled full-length *MtPGIP1* or *MtPGIP2* cDNA probe. Lanes 1, *Eco*RI; 2, *Hin*dIII; 3, *Nco*I; 4, *Cla*I. Molecular size standards are shown at left.

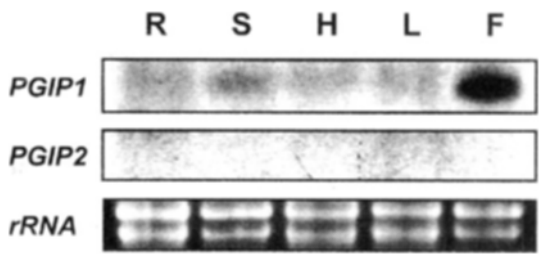

Figure 4. Tissue-specific expression patterns of *MtPGIP* genes. Total RNAs isolated from various *M. truncatula* organs were separated on formaldehyde-agarose gel and blot was hybridized with radiolabeled full-length *MtPGIP1* or *MtPGIP2* cDNA probe. Ethidium bromide-stained rRNA bands are shown as equal loading controls. R, roots; S, shoots; H, hypocotyls; L, young leaves; F, flowers.

tom is associated with the expression of *MtPR10-1* (Gamas et al., 1998), an HR marker of plant defense responses (Martin et al., 2003). As demonstrated by our semi-quantitative RT-PCR (Fig. 5), *MtPR10-1* was induced at 6 h post-inoculation, and strongly expressed until 96 h. Concomitantly, *MtPGIP1* was induced at 6 h post-inoculation, and continued to be

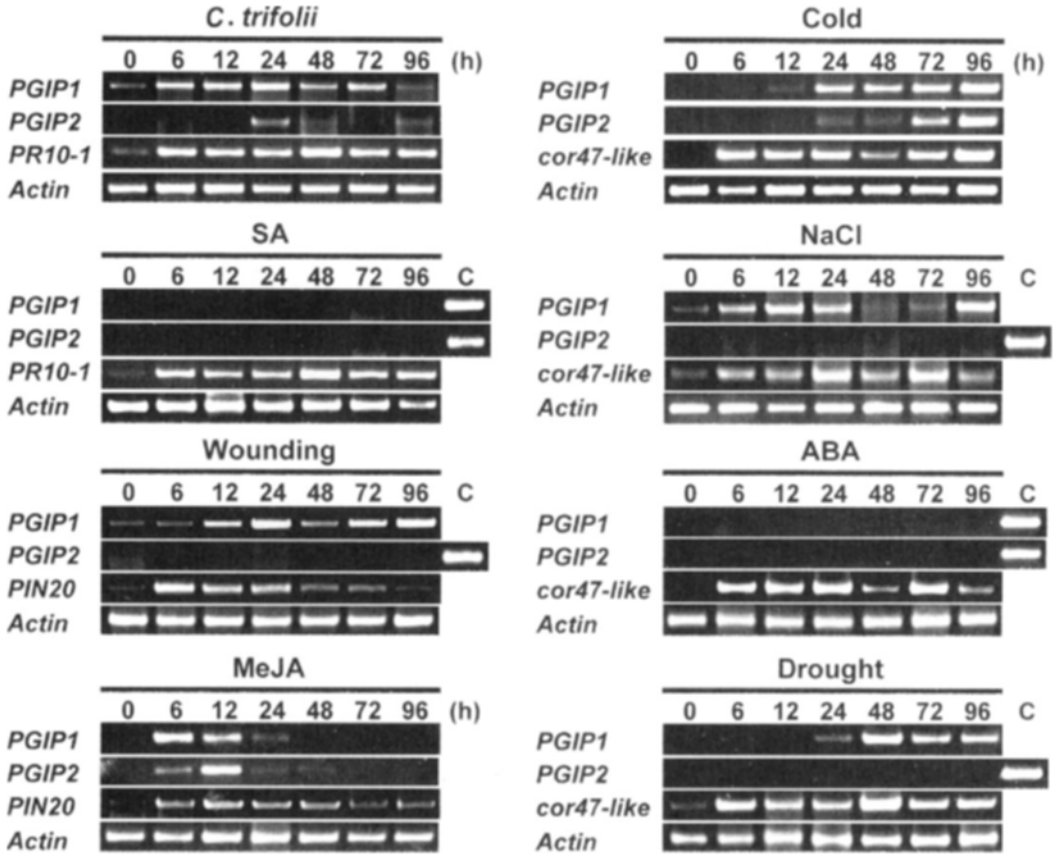

Figure 5. Differential expression of *MtPGIP1 and MtPGIP2* genes in response to *C. trifolii* and various stress stimuli. Total RNAs isolated from *M. truncatula* seedlings at different time points after each treatment were analyzed by semi-quantitative RT-PCR using *MtPGIP1a* and *MtPGIP2a* gene-specific primers (Table 1). Primers specific to *MtPR10-1*, *MtPIN20*, and *MtCOR47-like* genes were used as stress-responsive markers and primers specific to *MtACTIN* gene were used as internal control. ABA, abscisic acid; MeJA, methyl jasmonate; SA, salicylic acid; C, genomic DNA (30 ng). Comparison of band intensities is meaningful within a single data set.

expressed at high levels until over 72 h. In contrast, *MtPGIP2* was expressed only at moderate levels at 24 h post-inoculation, and remained low over the testing period.

We also examined the expression patterns of *MtPGIP* genes in response to various stress-related stimuli. Seedlings sprayed with salicylic acid (SA) failed to induce either gene, although the same treatment induced the expression of *MtPR10-1* (Fig. 5). Application of jasmonic acid (JA) induced early expression of both *MtPGIP1* and *MtPGIP2*, which lasted until 48 h. Mechanical wounding induced relatively strong expression of *MtPGIP1*, but not *MtPGIP2*. Seedling responses to JA application or wounding were verified by the expression of *MtPIN20*, a proteinase inhibitor marker (AF526372). Salinity (NaCl) and water stress (drought) induced the expression of *MtPGIP1* exclusively, with drought eliciting a markedly slower induction. Low-temperature stress (cold) induced comparatively late expression of both *MtPGIP1* and *MtPGIP2*. Finally, the desiccation hormone ABA failed to induce either gene. All four abiotic stresses (cold, salinity, ABA, and drought) induced the expression of the *Mtcor47-like* gene, a marker specific to such environmental conditions (Welin et al., 1994).

To elucidate the apparent differences in expression patterns for *MtPGIP1* and *MtPGIP2*, nucleotide sequences (AC157891) of the promoter regions were analyzed for the presence of putative *cis*-acting regulatory elements (Table 3). Putative elements containing the binding sites for Myb-related transcription factors (Jin and Martin, 2000) were identified from both promoters, including the type II MYB consensus sequence (MBSII), a versatile element responsive to a wide range of environmental stimuli such as wounding, elicitors, and pathogens (Rushton and Somssich,

Table 3. Putative *cis*-acting regulatory elements identified in the promoter regions of *MtPGIP1* and *MtPGIP2* genes.

Category	*cis* Element[a]	*MtPGIP1*		*MtPGIP2*	
		Sequence[b]	Position[c]	Sequence[b]	Position[c]
Myb sites	MBSII	accaacc	-509/-503	accaacc	-1307/-1301
	[a(a/c)c(a/t)a(a/c)c]	tttggt	-843/-848[d]	accaac	-1333/-1328
	MYBR (tggttag)	cataacca	-118/-125[d]	ctaacct	-55/-61[d]
				tggtaag	-1252/-1246
				taacca	-1574/-1579[d]
Myc site	MYCR (cacatg)	cacatg	-296/-292		
Pathogen response	HSRE (taaaatnttng)	taaaatatctg	-441/-431	taaaatattgg	-132/-122
		taaaatctcag	-1601/1591	taaaatattct	-723/-713
				taaaatatttg	-997/-987
	LS4 (ttgact)	agtcaa	-950/-955[d]	agtcaa	-1038/-1043[d]
		agtcaa	-1019/-1024[d]	agtcaa	-1193/-1198[d]
		ttgact	-1278/-1273	agtcaa	-1785/-1790[d]
Wound induction	PINIIK (aagcgtaagt)	aagcgtaagt	-248/-257[d]		
Jasmonate response	GCC-box (agccgcc)			agccggc	-1371/-1365
	G-box [(g)cacgt(t)g]	cacgtg	-619/-614	gcacgtt	-86/-80
		cacgtg	-80/-75		
Cold induction	LTRE (gccgac)			ccgac	-1898/-1902[d]
	ICEr2 (actccg)			actccg	-1895/-1900[d]
Water deficit, cold	DRE (taccgacat)	atgtcggat	-1945/-1953[d]		

[a]Consensus sequences are in parentheses.
[b]Sequences are shown from the 5′ to the 3′ end.
[c]Position of the *cis* element with respect to the translation start site (5′ end/3′ end).
[d]Sequence on the complementary strand.

1998), as well as MYBR, an element responsive to abiotic stress conditions, e.g., water deficit and ABA (Abe et al., 1997). Another abiotic stress-responsive MYB-related element, MYC (Abe et al., 1997), was found only in the *MtPGIP1* promoter. Two elements associated with the pathogen response - HSRE (Pontier et al., 2001) and LS4 (Eulgem et al., 2000) - were present in multiple copies in both promoters. Interestingly, PINIIK, a wound-inducible element from the potato proteinase inhibitor II K promoter (Doares et al., 1995), was detected only in *MtPGIP1*. Two light-responsive elements were also associated with the methyl jasmonate response (Brown et al., 2003; Xu and Timko, 2004). Whereas the G-box was present in both promoters, GCC-box was found only in *MtPGIP2*. Cold- and water deficit-responsive elements were present unilaterally: LTRE (Baker et al., 1994) and ICEr2 (Zarka et al., 2003), only in *MtPGIP2*, whereas DRE (Liu et al., 1998), only in *MtPGIP1*. Finally, an additional ABA-responsive element, ABRE (PyACGTGGC) (Yamaguchi-Shinozaki and Shinozaki, 2005), and an SA-responsive element, LS7 (ACGTCA) (Mahalingam et al., 2003), were absent from both promoters.

Inhibition of Polygalacturonase Activities by Bacterially Expressed MtPGIPs

To investigate whether the two *MtPGIPs* encode functional proteins, the MtPGIP1- or MtPGIP2-encoding regions without signal peptides were introduced into a bacterial expression plasmid that contained six His codons following the translation start site. SDS-PAGE analysis revealed that bacterial cells carrying the expression constructs synthesized proteins equivalent in size to the recombinant MtPGIP1 (35.94 kD) and MtPGIP2 (33.96 kD) (Fig. 6A). These proteins were purified from bacterial lysates using a Ni^{+2}-agarose affinity column (Fig. 6A). To verify the identity of the purified proteins, immunoblotting was carried out with anti-6×His monoclonal antibodies. These antibodies bound to proteins of ~35-kD for both PGIP1 and PGIP2 as the primary antigens (Fig. 6B), indicat-

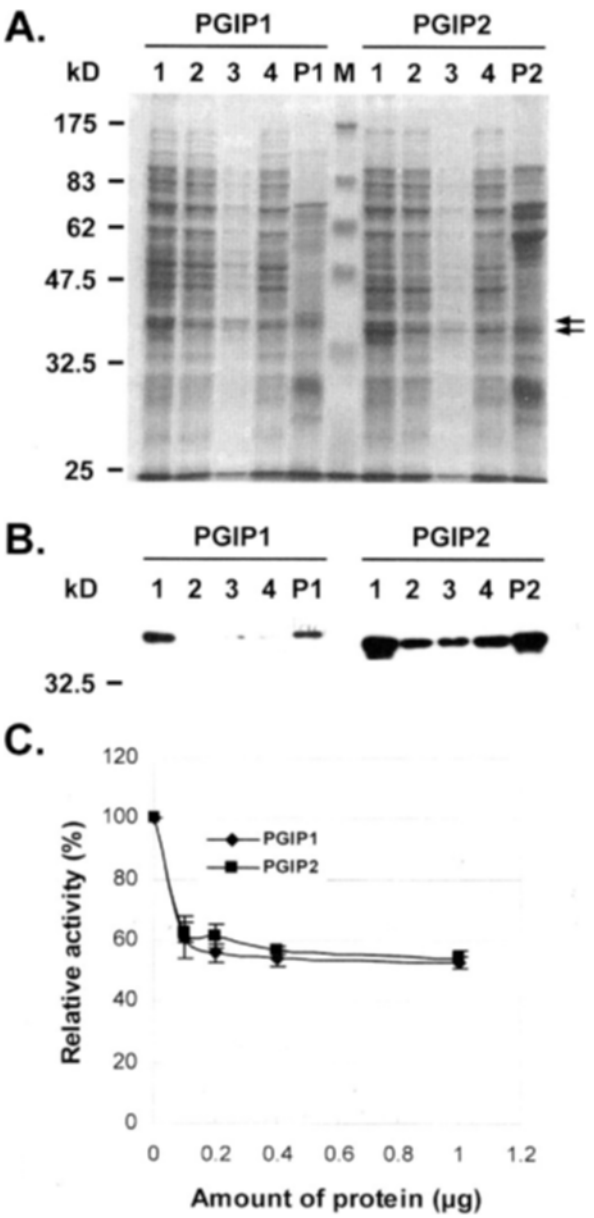

Figure 6. Activity assay of bacterially expressed MtPGIP1 and MtPGIP2. **(A)** Recombinant MtPGIP1 and MtPGIP2 carrying six His tags were synthesized in *E. coli* and isolated by affinity chromatography. Samples taken from each purification step were analyzed by SDS-PAGE and Coomassie Blue staining. Lanes 1, cell lysate; 2, supernatant; 3, pellet; 4, flow-through; P1, concentrated PGIP1 eluate; P2, concentrated PGIP2 eluate; M, protein size standards. Arrows indicate protein bands equivalent in size to MtPGIP1 (36 kD) and MtPGIP2 (34 kD). **(B)** Immunoblot analysis of PAGE-separated proteins in **(A)**. Anti-6×His monoclonal antibodies were used to detect recombinant proteins. **(C)** Inhibition of polygalacturonase activity by MtPGIP1 and MtPGIP2. Relative activities of MtPGIPs to decrease the amount of reducing sugars in fungal polygalacturonase reaction mixture were calculated from absorbance measurements at 410 nm. Bars indicate errors.

ing that the recombinant His-tagged MtPGIPs were correctly synthesized from the engineered vector constructs.

To demonstrate enzymatic activity by the recombinant MtPGIPs, each protein concentrate was added to a polygalacturonase (PG) reaction mixture that contained glycans and PAHBAH as the substrate and a dye indicating the presence of reducing sugars. This addition of protein concentrates (at 0.1 µg) effectively inhibited PG activity (Fig. 6C). As increasing amounts of the concentrates were added, absorbance dropped gradually, indicating a corresponding decrease in the amount of reducing sugars (Fig. 6C). Apparently, the inhibitory activities differed little between MtPGIP1 and MtPGIP2. These results indicate that the recombinant MtPGIP1 and MtPGIP2 are most likely functional inhibitors of PG activity.

DISCUSSION

We have shown that two *PGIP* genes are located adjacent to each other at a single chromosomal locus in *M. truncatula*. Similar examples are found in other plant species. In *Arabidopsis*, the *PGIP1* and *PGIP2* genes are tandemly located at a chromosomal location, but the distance between them is closer than that between *MtPGIPs* (Ferrari et al., 2003). Four bean *PGIP* genes also comprise a gene cluster at a single locus (D'Ovidio et al., 2004). These cases can be explained by gene duplication, one of the evolutionary mechanisms underlying how new genes arise from an old progenitor, thereby creating a gene family (Hulbert et al., 2001). Duplication is particularly common in plant disease resistance loci, as alleles tend to acquire novel specificities against newly generated races of pathogens (Hulbert et al., 2001). Indeed, each product of those four clustered bean *PGIP* genes displays unique activities against several fungal and pathogen races (Desiderio et al., 1997), although the two *Arabidopsis* PGIPs exhibit indistinguishable activities (Ferrari et al., 2003). Therefore, it would be necessary to examine the specificities of our two *MtPGIP* gene products against several fungal pathogens that infect *M. truncatula*.

It is intriguing that our *MtPGIP* genes were not highly expressed in any unstressed organs but the flowers. Such expression patterns are in contrast to those from other plant species, in which *PGIP* genes are expressed constitutively in many organs in the absence of any extraneous cues (Desiderio et al., 1997; Yao et al., 1999; Ferrari et al., 2003; Li et al.,

2003). Nonetheless, a high-level expression in the flowers suggests that *MtPGIP1* may play roles in floral development, as has been proposed for a rice *PGIP* gene (Jang et al., 2003).

The transcripts encoding MtPGIPs were expressed in response to various pathogenic and environmental stress stimuli. This indicates that *MtPGIP* expression is regulated by multiple signal transduction pathways that modulate plant responses to biotic and abiotic challenges. Notably, both *MtPGIP1* and *MtPGIP2* were induced by JA, yet neither was expressed in response to SA. These findings are consistent with those for *Arabidopsis* (Ferrari et al., 2003) and *B. napus* (Li et al., 2003), but contrast with those reported from bean (Toubart et al., 1992). Despite the central importance of SA in plant defense response, SA-independent pathways have been characterized extensively (Pieterse and van Loon, 1999). Moreover, separate JA-dependent and SA-dependent defense response pathways operate in response to distinct microbial pathogens (Thomma et al., 1998). Thus, it is likely that the *MtPGIP* genes are regulated by SA-independent and JA-dependent signaling pathways against fungal pathogens.

Here, mechanical wounding induced the expression of *MtPGIP1*, but not that of *MtPGIP2*. Similar results were obtained from our salinity and drought stress treatments. Such differential *PGIP* expression patterns have also been observed when *Arabidopsis* or *B. napus* leaves are treated with elicitors (Ferrari et al., 2003) or mechanically wounded (Li et al., 2003), respectively. Preferential expression of certain alleles over the others in a particular resistance gene family takes place as a means of specifically recognizing a pathogen or a pathogenic race, thereby conferring a selective advantage (Hulbert et al., 2001). Likewise, a different mode of expression in response to abiotic stimuli can occur through the activation of a particular subset of transduction pathways, which results in the adjustment of plants to changing environments and better survival rates. Thus, *MtPGIP* expression in response to abiotic stresses also likely involves multiple layers of regulation and signaling.

Our analysis of *MtPGIP* promoters further sheds light on possible clues for the differential expression patterns of these two genes. The *cis*-acting elements for pathogen and JA responses as well as MYB-related elements are found in both promoters, whereas those for wounding and water-deficit responses, including MYCR, are present only in *MtPGIP1*. These differences provide at least a partial explanation for the expression of both genes upon pathogen inoculation and JA treatment, but of only *MtPGIP1* in response to wounding, salinity, or drought stresses. Moreover, the exclusive presence of the cold-responsive *cis*-acting elements LTRE and ICEr2 in *MtPGIP1* seems to underlie the subtle differences observed in expression patterns between *MtPGIP1* and *MtPGIP2* under our low-temperature conditions. Similarly, the absence of induction for either gene in response to SA or ABA might be attributed to the lack of any corresponding responsive elements in either promoter.

Despite their proposed duplicated nature, the two *MtPGIP* genes encode proteins that are substantially divergent from each other. Only about 200 amino acids are identical between MtPGIP1 and MtPGIP2. This similarity is significantly lower than that between *Arabidopsis* PGIP1 and PGIP2, which differ in 70 amino acids (Ferrari et al., 2003), and is even lower than that found between bean PGIP1 and PGIP2, which differ only in eight amino acids (D'Ovidio et al., 2004). The apparent divergence between these two MtPGIPs is likely due to structural aberrations that result from the insertion and deletion of two discrete peptide fragments in the LRR domain of MtPGIP2 (Fig. 1). To verify the impact of such structural modifications on protein function, we synthesized His-tagged recombinant MtPGIPs from bacteria and examined their ability to inhibit PG activity. Here, both recombinant MtPGIP1 and MtPGIP2 prevented the production of reducing sugars in the PG assay mixture, indicating that their ability as PG inhibitors was preserved. Despite this apparent functional conservation, it is possible that MtPGIP2 may have lost or acquired novel properties necessary for PG binding (Federici et al., 2001) because of those structural changes introduced into the LRR. Therefore, the availability of the recombinant MtPGIPs raises a possibility for assessing their specificities against PGs from various fungi and other pathogens.

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