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Molecular relatedness of the polygalacturonase-inhibiting protein genes in *Eucalyptus* species

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Abstract Plants produce polygalacturonase-inhibiting proteins (PGIPs) as part of their defense against disease. PGIPs have leucine-rich motifs, a characteristic shared by many proteins involved in plant resistance against pathogens. The objective of this study was to clone and analyse the partial sequences of the *pgip* genes from five selected commercially important *Eucalyptus* species. Genomic DNA from *E. grandis*, *E. urophylla*, *E. camaldulensis*, *E. nitens* and *E. saligna* was isolated from young leaves and used as the template in PCR reactions. Primers PC1, previously described, and Per3, developed in this study, were used in a degenerate PCR reaction to amplify a pgip fragment. A PCR fragment of 909 bp was amplified from each *Eucalyptus* spp., cloned and sequenced. The *Eucalyptus pgip* genes were highly conserved (98–100% identity). Analysis of the deduced amino-acid sequences revealed high similarities (44–94%) with other known PGIPs. In general, PGIPs have high homologies within genera as is the case in the genus *Citrus*. These observations strengthen the belief that PGIP plays an important role in plants.

Keywords *Eucalyptus* · Leucine-rich repeats · PGIPs · Signal transduction · Resistance

Introduction

The genus *Eucalyptus* is an economically important source of wood and fibre in many parts of the world. During the course of the past decade, there has been an increasing demand for wood, given diminishing petrochemical reserves and the desire to preserve old-growth forests. One of the major challenges facing forestry industries is the loss of plantation stands due to diseases. Currently, foresters rely on fungicides and breeding for disease resistance, to reduce losses due to disease. However, the use of fungicides is environmentally undesirable, while breeding for resistance is a tedious and time-consuming process. Considerable effort is being focused on improving disease tolerance in *Eucalyptus*. Manipulation of the expression of tree defense genes has potential in this regard.

Polygalacturonase-inhibiting proteins (PGIPs), first described by Weurman (1953), are leucine-rich repeat (LRR) proteins that are associated with cells of all dicotyledonous plants that have been studied (De Lorenzo and Cervone 1997). They are also present in at least one monocotyledonous (leek) plant (Favaron et al. 1997). They have been shown to effectively and specifically bind to and inhibit fungal endopolygalacturonases, which are important fungal virulence factors (Cervone et al. 1989). There are two factors that suggest that PGIPs have a role in the plant defense system. Firstly, the inhibition of the polygalacturonase (PG) activity of several pectolytic fungi by pear PGIP is inversely proportional to the ability of those fungi to colonise pears (Powell et al. 1994). Secondly, in tissues where PGIP occurs in low amounts, they can be induced by wounding. PGIPs are also pathogen-induced (Bergmann et al. 1994; Devoto et al. 1997).

The *pgip* gene sequences have been reported for bean, soybean, apple, pear, raspberry, tomato and kiwifruit (Toubart et al. 1992; Johnston et al. 1993; Stotz et al. 1993; Favaron et al. 1994; Stotz et al. 1994; Simpson et al. 1995; Yao et al. 1995). PGIPs are thermolabile glycoproteins with a molecular mass of around 44 kDa. When de-glycosylated, the mass is around 34 kDa, with N-linked glycosylation accounting for the 10 kDa difference. PGIPs are mostly encoded by a single open reading frame of about 1,000 base pairs. They contain a signal peptide that is processed through the endomembrane system for targeting to the apoplast.

Purified PGIPs show differential inhibition against several fungal PGs. Tomato PGIP, for example, inhibits

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Table 1 GenBank accession numbers of sequences used in this study

Species	Sequence description	Genebank accession number
Actinidia deliciosa	*pgip*	Z49063
Citrus iyo	*pgip* A	AB016205
Citrus iyo	*pgip* B	AB016206
Citrus jambhiri	*pgip* A	AB013397
Citrus jambhiri	*pgip* B	AB015198
Citrus sinensis	*pgip*	Y08618
Citrus sp cv sannumphung	*pgip* A	AB015356
Citrus sp cv sannumphung	*pgip* B	AB015643
Citrus unshiu	*pgip*	AB016204
Eucalyptus camaldulensis	*pgip*	AF159168
Eucalyptus grandis	*pgip*	AF159167
Eucalyptus nitens	*pgip*	AF159171
Eucalyptus saligna	*pgip*	AF159170
Eucalyptus urophylla	*pgip*	AF159169
Fortunella margarita	*pgip*	AB020529
Glycine max	*pgip*	X78274
Lycopersicon esculentum	*pgip*	L26529
Malus domestica	*pgip*	U77041
Phaseolus vulgaris	*pgip*	X64769
Poncirus trifoliata	*pgip*	AB020528
Prunus armeniaca	*pgip*	AF020785
Pyrus communis	*pgip*	L09264

PGs from *Glomerella cingulata*, but not from *Botrytis cinerea* (Stotz et al. 1994). In bean, PGIPs with different specificities have been observed, which shows that PGIPs are encoded by a family of genes (Desiderio et al. 1997; Leckie et al. 1999).

PGIPs belong to the LRR protein superfamily and, therefore, may be involved in signalling defense messages to the rest of the plant when a pathogen attack does occur (Jones and Jones 1997). The leucine-rich repeats within PGIPs may play an important role in the interactions between resistance proteins and other important ligands (Powell et al. 1994). PGIPs are also evolutionarily related to several plant resistance proteins that participate in gene-for-gene resistance (Jones and Jones 1997).

Overexpression of PGIPs in plants could potentially be exploited to improve resistance to pathogens (Lafitte et al. 1994; Powell et al. 1994; Burger et al. 1997; Labavitch et al. 1997). Powell and co-workers (1994) expressed a pear PGIP in *Botrytis cinerea*-susceptible tomatoes and the transgenic tomatoes were reported to be resistant to *Botrytis* infection.

PGIP-PG interaction can be utilised to provide a simple and elegant system to investigate the molecular recognition at the level of the plant cell wall. A major goal in plant pathology is to understand the molecular basis of plant-pathogen interactions and, as a first step toward that goal, isolation and cloning of responsible genes is vital. The objective of this study was to clone and analyze the *pgip* gene from selected *Eucalyptus* species and to infer the sequence relatedness in the species.

Materials and methods

Sample collection, DNA extraction and PCR amplification

One gram of fresh, young leaves was collected from 2 year-old plants of *Eucalyptus grandis*, *Eucalyptus camaldulensis*, *Eucalyptus*

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      N  P  D  D  K  K  V  L  L  Q  I  K  K  A  F  G  D  P      18
GGAATTCAATCCGGACGACAAGAAGGTCCTCCTACAAATCAAGAAAGCCTTCGGCGACCCC   61

 Y  V  L  A  S  W  K  S  D  T  D  C  C  D  W  Y  C  V  T  C    38
TATGTCTTGGCCTCATGGAAATCAGACACCGACTGTTGTGATTGGTACTGCGTCACCTGT   121

 D  S  T  T  N  R  I  N  S  L  T  I  F  A  G  Q  V  S  G  Q    58
GACTCAACCACAAACCGCATCAACTCCCTCACCATCTTTGCCGGCCAGGTATCCGGCCAA   181

 I  P  A  L  V  G  D  L  P  Y  L  E  T  L  E  F  H  K  Q  P    78
ATCCCCGCCCTAGTTGGAGACTTGCCGTACCTTGAAACCCTTGAATTCCACAAGCAACCC   241

 N  L  T  G  P  I  Q  P  A  I  A  K  L  K  G  L  K  F  L  R    98
AATCTCACTGGCCCAATCCAACCCGCCATTGCCAAGCTCAAAGGACTCAAGTTTCTCAGG   301

 L  S  W  T  N  L  S  G  S  V  P  D  F  L  S  Q  L  K  N  L   118
CTCAGCTGGACCAACCTCTCAGGCTCTGTCCCTGACTTCCTCAGCCAACTCAAGAACCTC   361

 T  F  L  D  L  S  F  N  N  L  T  G  A  I  P  S  S  L  S  Q   138
ACATTCCTCGACCTCTCCTTCAACAACCTCACCGGCGCCATCCCCAGCTCGCTTTCTCAG   421

 L  P  N  L  N  A  L  H  L  D  R  N  K  L  T  G  H  I  P  K   158
CTCCCAAACCTCAACGCTCTTCATCTAGACCGCAATAAGCTCACAGGTCATATTCCGAAA   481

 S  F  G  Q  F  I  G  N  V  P  D  L  Y  L  S  H  N  Q  L  S   178
TCGTTTGGGCAGTTCATTGGCAACGTTCCAGACCTGTATCTCTCCCACAACCAGCTCTCG   541

 G  N  I  P  T  S  F  A  Q  M  D  F  G  K  H  R  L  S  R  N   198
GGCAACATTCCAACCTCATTTGCCCAGATGGACTTCGGCAAGCATAGACTATCACGGAAC   601

 K  L  E  D  A  S  V  I  F  G  L  N  K  T  T  Q  I  V  D  L   218
AAGCTCGAGGACGCATCAGTGATATTTGGGCTGAACAAGACAACCCAGATTGTGGACCTA   661

 S  R  N  L  L  E  F  N  L  S  K  V  E  F  P  T  S  L  T  S   238
TCCAGGAACTTGCTGGAATTTAATCTGTCAAAGGTGGAGTTTCCGACAAGCTTGACCTCA   721

 L  D  V  N  H  N  K  I  Y  G  S  I  P  V  E  F  T  Q  L  N   258
CTGGATGTAAACCACAATAAGATCTACGGGAGTATCCCAGTGGAGTTTACCCAACTGAAT   781

 F  Q  F  L  N  V  S  Y  N  R  L  C  G  Q  I  P  V  G  G  K   278
TTCCAGTTCCTGAACGTGAGCTACAACAGGCTGTGTGGTCAGATTCCAGTGGGCGGAAAG   841

 L  Q  S  F  N  E  Y  S  Y  F  H  N  R  C  L  C  G  A  P  L   298
TTGCAAAGCTTCAACGAGTATTCTTATTTCCATAACCGATGCCTGTGTGGTGCACCCCTC   901

CACACTGC  909
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Fig. 1 The nucleotide sequence and predicted amino-acid sequence of a putative mature polygalacturonase-inhibiting protein of *E. grandis* (data for *E. camaldulensis*, *E. urophylla*, *E. nitens* and *E. saligna* are not shown, see accession numbers indicated in Table 1). Putative N-glycosylation sites are *underlined* and cysteine residues are indicated in *open boxes*. A leucine-rich repeat motif is indicated in *bold print*. The nucleotide sequence has been submitted to GenBank (accession No. AF159167)

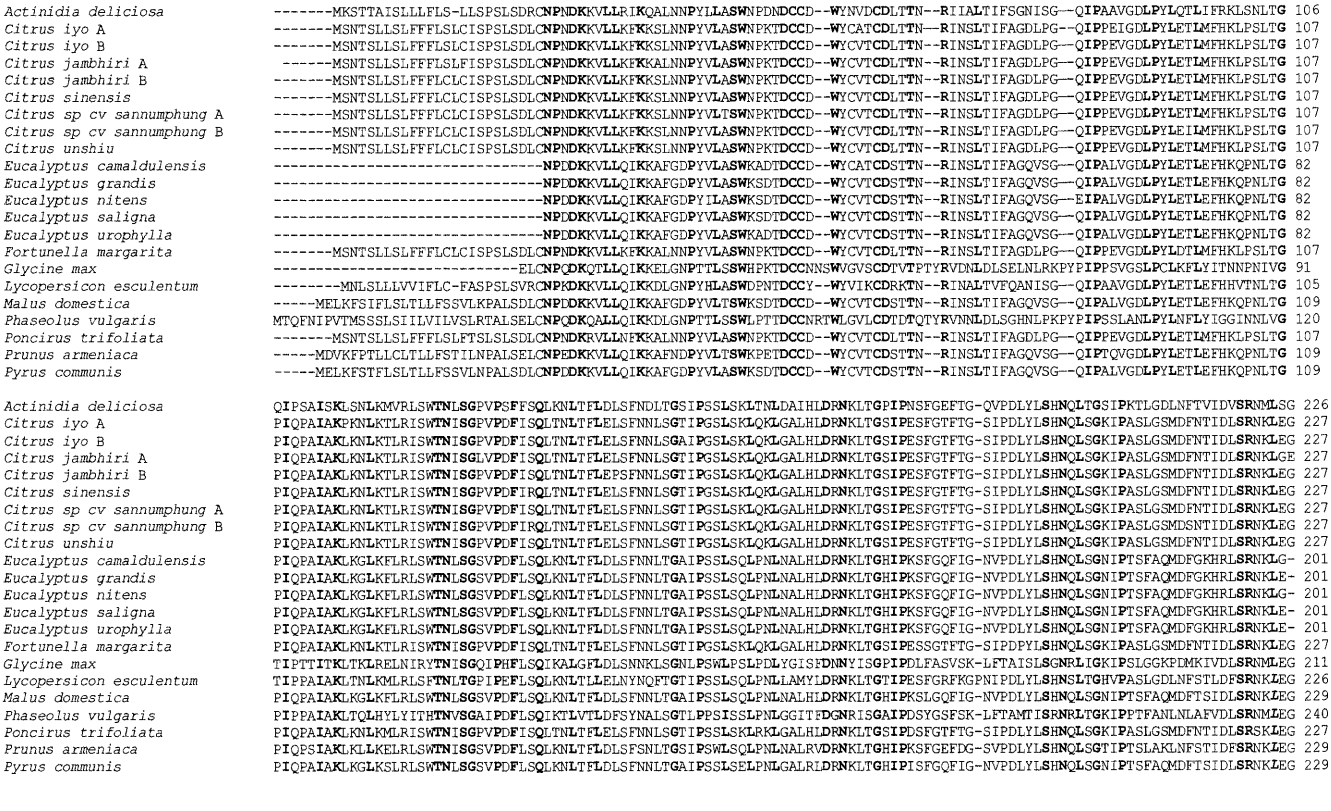

Fig. 2 Amino-acid alignment of the PGIP peptide sequences. The conserved amino acids are indicated in *bold print*

nitens, *Eucalpytus urophylla* and *Eucalyptus saligna.* The leaves were frozen in a sterile plastic bag for 20 min at –20°C. Mid-ribs of the leaves were removed with a sterile razor blade. The rest of the leaf tissue was cut into 1–2 mm strips. Genomic DNA was isolated from the leaves using the Nucleon Phytopure kit (Amersham Life Science, UK) as recommended by the manufacturers.

Polymerase chain reactions were done in a HYBAID Omnigene TR3 CM220 (UK) thermocycler. In all the PCR reactions, the following reaction mixture was used: oligonucleotide primer PC1 (5'-GGAATTCAAYCCNGAYGAYAARGT-3', Stotz et al. 1993) (0.12 pmol/µl), oligonucleotide primer Per 3 (5'RCANWSNG GNARNGGNGCNCCRCANARRCA-3' (designed in this study by inspection of the C-termini of aligned published peptide PGIPs), (4 pmol/µl), *Eucalyptus* template DNA (25 ng), 1 mM dNTPs, 2.5 mM $MgCl_2$, 10×PCR buffer, and 5 units of *Taq* polymerase enzyme (Roche Diagnostics, Germany) in 100 µl. PCR was performed for 30 cycles (one cycle=1 min at 94°C, 2 min at 58°C, and 2 min at 72°C). The reactions had an initial denaturation step of 3.5 min at 94°C and a final elongation step of 7 min at 72°C. The PCR products were separated on a 1% agarose gel stained with ethidium bromide (Sambrook et al. 1989) and visualised under a UV transluminator. The degenerate PCR amplifications were repeated several times in independent conditions with water controls to ensure that amplifications were authentic and not artifacts.

Cloning

All DNA manipulations were done according to the standard protocols in Sambrook et al. (1989). After separating the degenerate PCR products on a 1% agarose gel, a *Eucalyptus* PCR fragment of about 900 bp was purified from the gel with Qiagen columns (Qiagen, Germany) and cloned into the polylinker region of the pGEM-T-Easy vector (Promega). Ligation was done at 4°C for 16 h. Ligation mixtures were transformed into competent *Escherichia coli* (JM109) cells (Promega, UK). Transformants were screened on LB-ampicillin plates using the blue/white phenotype.

Sequencing and analysis

Plasmid DNA was isolated from recombinant bacterial clones by the alkaline-lysis method (Sambook et al. 1989) and further purified

Table 2 Comparison of the percentage similarity of the pgip polypeptide sequences considered in this study

Percentage similarity

Fm																					
Pt	95																				
Ci	97	95																			
Cb	98	96	98																		
Cu	98	95	98	99																	
Cs	98	95	98	98	99																
Cg	97	94	97	98	99	98															
Cj	98	95	98	98	99	99	98														
Cc	96	95	97	97	97	99	96	97													
Pa	70	70	70	71	70	71	70	70	70												
Md	72	72	72	73	72	73	71	72	72	83											
Ce	98	95	98	98	99	99	99	99	97	70	72										
Pv	44	44	43	44	44	44	44	44	43	46	47	44									
Le	63	62	62	62	63	63	62	63	62	64	64	63	47								
Gm	45	46	44	45	45	45	45	45	45	46	45	45	67	43							
Ad	67	67	67	68	68	67	67	67	67	67	69	67	44	64	45						
Pc	72	73	72	74	73	73	72	73	73	83	97	73	47	64	45	70					
Eg	74	75	74	75	75	74	74	74	75	83	95	74	46	65	44	69	94				
Ec	73	74	73	74	74	73	73	73	74	82	94	73	46	65	44	68	93	98			
Eu	73	74	73	74	74	74	73	74	74	82	94	74	46	65	44	68	93	99	98		
Es	74	75	74	75	75	74	74	74	75	83	95	74	46	65	44	69	94	100	98	99	
En	73	74	73	74	74	74	73	74	74	82	95	74	46	65	44	69	94	99	98	98	99
	Fm	Pt	Ci	Cb	Cu	Cs	Cg	Cj	Cc	Pa	Md	Ce	Pv	Le	Gm	Ad	Pc	Eg	Ec	Eu	Es

Fm *Fortunella margarita*, Pt *Poncirus trifoliata*, Ci *Citrus iyo* A, Cb *Citrus iyo* B, Cu *Citrus unshiu*, Cs *Citrus sp. cv sannumphung* A, Cg *Citrus sp. cv sannumphung* B, Cj *Citrus jambhiri* A, Cc *Citrus jambhiri* B, Pa *Prunus armeniaca*, Md *Malus domestica*, Ce *Citrus sinensis*, Pv *Phaseolus vulgaris*, Le *Lycopersicon esculentum*, Gm *Glycine max*, Ad *Actinidia deliciosa*, Pc *Pyrus communis*, Eg *Eucalyptus grandis*, Ec *Eucalyptus camaldulensis*, Eu *Eucalyptus urophylla*, Es *Eucalyptus saligna* and En *Eucalyptus nitens*

over Qiagen columns. Recombinant plasmid clones were confirmed by restriction enzyme digestion with *Eco*R1. Five recombinant clones from each *Eucalyptus* species were sequenced using the BIG Dye terminator cycle sequencing kit with an ABI Prism model 377 sequencer (Perkin-Elmer). T7 and SP6 primers were used for forward and reverse sequencing of the double-stranded plasmid template.

The DNA sequences were used in BLASTX searches to look for homologous polypeptide sequences (http://www.ncbi.nlm.nih.gov). Peptide sequence alignment for the five *Eucalyptus* species was performed using the CLUSTAL (EBI) database (http://www2.ebi.uk). Other PGIP peptide sequences were included for comparison purposes. The computer programme PAUP (Swofford 1998) was used to draw dendrograms using sequence data obtained from this study and from GenBank (Table 1). The phylogram showing the phylogenetic relatedness of plant species using PGIP sequences was compared to a phylogram produced using the partial ribosomal RNA sequences of the same plants available in GenBank.

Results

Cloning of the *Eucalyptus pgip* gene

To determine the partial nucleotide sequence of the *Eucalyptus pgip* gene, degenerate primers were used and the amplified products were cloned and sequenced. The *E. grandis pgip* DNA sequence (909 bp) was translated into a 298 amino-acid polypeptide with a single open reading frame (see Fig. 1). In all the *Eucalyptus* spp. included in the study, the sequenced fragments were also 909 bp (data not shown). A single open reading frame for the *Eucalyptus* spp. was observed and this is consistent with most of the reported PGIPs (Toubart et al. 1992; Stotz et al. 1993, 1994).

Seven putative N-glycosylation sites (Asn-X-Ser/Thr) were found in the *E. grandis* PGIP polypeptide sequence (Fig. 1) and they are comparable to those found in pear PGIP (Stotz et al. 1993). In all the *Eucalyptus* PGIPs, the peptide sequence of the putative N-glycosylation sites are the same, which has also been observed in *Citrus* spp. (Fig. 2) in positions where the glycosylations sites are conserved. Interestingly, most of the N-glycosylation sites in the majority of the PGIPs are conserved. This observation suggests important implications for the function of PGIPs.

The *E. grandis* putative mature polypeptide has seven cysteine residues which are in both the N- and C- termini of the polypeptide (Fig. 1). The cysteine residues may have implications in the function or stability of PGIPs. In bean and pear PGIPs, the cysteine residues are conserved (Stotz et al. 1993). The putative mature *Eucalyptus* PGIP has a hydrophilic character as revealed by the distribution of the basic, acidic and hydrophilic amino acids (58.2%) on the entire polypeptide.

Polypeptide sequence alignment of the *Eucalyptus* PGIPs (see Fig. 2) revealed high homologies with other PGIPs. Similarity between the *Eucalyptus* spp. alone was between 98 and 100%, while it was 44–94% when compared to other plant species (see Table 2). PGIPs are generally conserved within species, for example in *Citrus* spp. the identity is between 96 and 99% at the peptide level (Table 2) and 97–99% at nucleotide level.

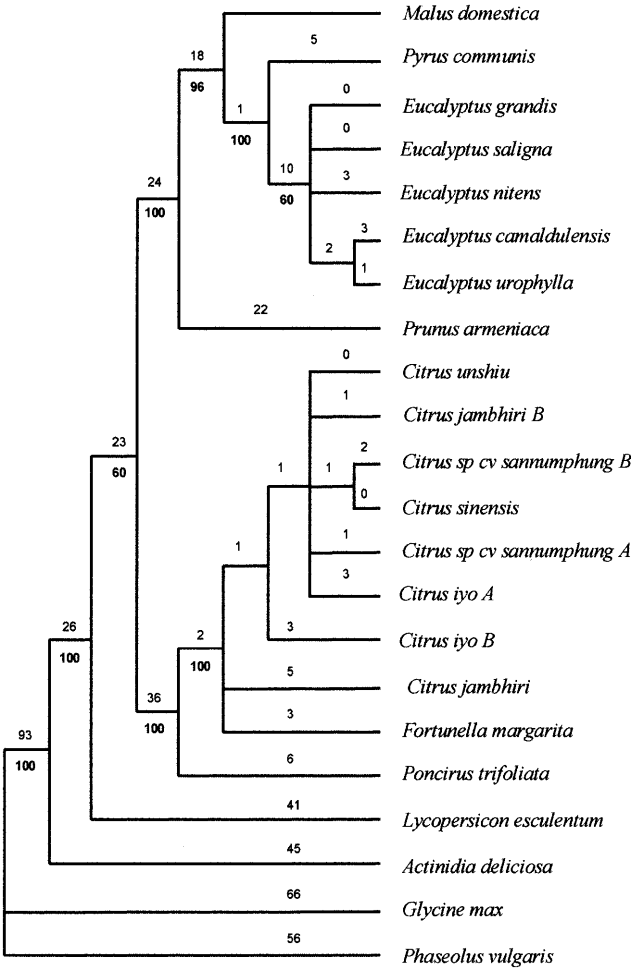

Fig. 3 A dendrogram produced with a heuristic analysis from aligned sequences of pgip polypeptides using PAUP. Bootstrap values (%) based on 1,000 replications and branch lengths are indicated above and below the branches respectively

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Eucalyptus camaldulensis        YCATCDSTTNRINSLTIFAGQVSG
Eucalyptus grandis              YCVTCDSTTNRINSLTIFAGQVSG
Eucalyptus nitens               YCVTCDSTTNRINSLTIFAGQVSG
Eucalyptus saligna              YCVTCDSTTNRINSLTIFAGQVSG
Eucalyptus urophylla            YCVTCDSTTNRINSLTIFAGQVSG

Eucalyptus camaldulensis        QIPALVGDLPYLETLEFHKQPNLT
Eucalyptus grandis              QIPALVGDLPYLETLEFHKQPNLT
Eucalyptus nitens               EIPALVGDLPYLETLEFHKQPNLT
Eucalyptus saligna              QIPALVGDLPYLETLEFHKQPNLT
Eucalyptus urophylla            QIPALVGDLPYLETLEFHKQPNLT

Eucalyptus camaldulensis        GDASVIFGLNKTTQIVDLARNLLE
Eucalyptus grandis              EDASVIFGLNKTTQIVDLSRNLLE
Eucalyptus nitens               GDASVIFGLNKTTQIVDLSRNLLE
Eucalyptus saligna              EDASVIFGLNKTTQIVDLSRNLLE
Eucalyptus urophylla            EDASVIFGLNKTAQIVDLARNLLE
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Fig. 4 The leucine-rich repeat (LLR) structure of *Eucalyptus* PGIPs. Nonsynonymous substitutions are indicated in *bold print* while the *boxed portion* depicts the β-strand/β-turn region showing the general xxLxLxx consensus

Evolutionary relationships between *Eucalyptus* PGIPs and other PGIPs

In this study, we report on the cloning and sequence analysis of mature *pgip* genes from five *Eucalyptus* spp. A heuristic search was done on the manually aligned amino acids and DNA sequences of the 22 PGIPs used in this study, and a dendrogram was obtained (Fig. 3). The *Eucalyptus* PGIP sequences form a well-supported clade (bootstrap support 100%). The dendrogram produced from PGIP data show that *Eucalyptus* PGIP sequences are more closely related to those of stone and pome fruits. Within the *Eucalyptus* clade, the branches have low bootstrap support. This indicates that these species are very closely related and that the PGIP gene sequences are not variable enough to allow resolution at the species level.

Discussion

Five PGIP polypeptides from *Eucalyptus* spp. have been cloned and sequenced and are very closely related to each other. It remains necessary to determine whether there is more than one PGIP gene in the *Eucalyptus* genome as is the case in other plants such as *Phaseolus vulgaris* (Desiderio et al. 1997).

Eucalyptus PGIPs, like all other PGIPs, fall into the category of the leucine-rich repeat class of proteins. The 24 amino-acid motif, LxxLxxLxxLxLxxNxLxGxIPxx, shown in Fig. 1 is conserved in all PGIPs sequenced thus far. This may suggest an important role of PGIPs in recognition and signal transduction in plant defense (Jones and Jones 1997). Eight amino-acid positions are different on the peptide sequences of the *Eucalyptus* spp. PGIPs. With respect to *E. grandis*, all the differences are due to non-synonymous substitutions. Of the eight substitutions, only one occurs in the β-strand/β-turn region of the LLR structure (Fig. 4). The S221 residue in *E. grandis* PGIP is changed to A221 in *E. camaldulesis* and *E. urophylla* PGIPs, it remains invariant in *E. nitens* and *E. saligna*. This may have consequences in the ability of the different PGIPs to interact with their ligands, endopolygalacturonases. In *P. vulgaris*, it has been shown that even only one substitution is sufficient to alter the interaction capacity of PGIPs and their ligands (Leckie et al. 1999). Leckie et al. (1999) showed that bean PGIP-2, which has a Q253, has the capacity to interact with endoPGs of *Aspergillus niger* and *Fusarium moniliforme*, while PGIP-1 which lacks it can only interact with the endoPG of *A. niger*. Three of the non-synonymous substitutions occur in the outside the LLR region while the other four occur in the region contiguous with the xxLxLxx motif of the LLR (Fig. 4). In all the *Eucalyptus* spp. PGIPs there are no synonymous substitutions. It must however be mentioned that the presence of a family of *pgip* genes in each of the *Eucalyptus* spp. can not be ruled out. According to Leckie et al. (1999), variations in the LLR structure influence recognition specificities, and

in this study the LLRs are almost 100% identical. This strongly suggests that the PGIPs described in this study may have very similar recognition specificities to endoPGs except for the S221-A221 switch in the solvent exposed area on the β-strand/β-turn region.

The similarity of the PGIPs in the *Eucalyptus* spp. confirms that they are species of the same genus; similarly, in *Citrus* species PGIPs also form a distinct clade (Fig. 3). The sequence conservation across the 22 individuals suggests a conservation of the PGIP functional role in the plant defense system in those plant species. *Eucalyptus* PGIPs were shown to be close to those of the pome and stone fruit PGIPs. This suggests relatedness in the evolution of PGIPs in the two groups of plants. This may have implications when considering the type and diversity of pathogens that can infect these plants.

Distinguishing *E. grandis* and *E. saligna* taxonomically using morphological features is a difficult task. It is, therefore, not surprising that the mature PGIP sequences of these two species show a 100% identity (see Table 2). The relevance of the small differences in the amino acids of the other *Eucalyptus* spp. could be investigated for recognition specifities with surface plasmon resonance studies (Leckie et al. 1999).

In the PGIP phylogram, *Prunus armeniaca* groups away from the rest of the stone fruit sequences. *P. armeniaca* PGIP is encoded by a gene with an intron. Thus, when this sequence is included in the analysis, *P. armeniaca* clusters away from other pome and stone fruits.

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