MOLECULAR PLANT-PATHOGEN INTERACTIONS WITH SPECIAL REFERENCE TO *EUCALYPTUS GRANDIS* POLYGALACTURONASE-INHIBITING PROTEINS AND FUNGAL POLYGALACTURONASES

by

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DECLARATION

I, the undersigned hereby declare that the thesis submitted herewith for the degree, Philosophiae Doctor, to the University of Pretoria, contains my own independent work. This work has hitherto not been submitted for any degree at any other University faculty.
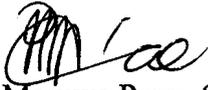
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PREFACE

Fungal pathogens such as *Cryphonectria cubensis*, *Botryosphaeria dothidea*, *Phytophthora cinnamomi* and *Coniothyrium zuluense* are the cause of high mortalities in South African *Eucalyptus* plantations. South African plantations cover about 1.5 million hectacres of plantation area. As a result, the amount of fibre obtained from plantations can be greatly reduced; affecting the economies of paper, pulp and timber industries. Exact figures of the extent of losses due to fungal diseases only have not been determined.

Currently, the method of control of pathogens is by standard cultural practices, use of chemicals and selecting resistant *Eucalyptus* clones obtained through traditional breeding. However, chemicals are expensive and often not environmentally desirable, while classical breeding is time-consuming. At this stage no specific resistance genes have been characterised Genetic engineering for disease resistance could potentially provide a cheaper and environmentally more acceptable means of disease control.

Prior to implementing a genetic engineering strategy for disease control, it is necessary to fully understand mechanisms of pathogenicity and resistance. We postulated that fungal polygalacturonases have a role in pathogenicity and their plant inhibitors have a role in resistance, thus the objective of this study was to gain an understanding of the interaction between fungal endopolygalacturonases (endoPGs) and their plant counterparts, polygalacturonase-inhibiting proteins (PGIPs). Primarily, this was done through studies of and number of *Eucalyptus* spp. and fungus *Cryphonectria cubensis*, which is pathogenic to it. The endoPG gene of *Fusarium circinatum*, an important pitch canker pathogen of pines was also studied.

In this thesis, a review of the interactions between endoPGs and plant PGIPs in host defence is presented in Chapter 1. The current state of knowledge on the predicted role of endoPGs in pathogenicity and that of PGIPs in defence and development is presented. The

chapter describes some of the concepts of signal transduction systems in pathogenicity and resistance linked to PGIP-PG interactions.

In Chapter 2, the molecular characterisation of PGIPs from selected, commercially important *Eucalyptus* spp. is presented. The species included in the study are *E. grandis*, *E. nitens*, *E. saligna*, *E. camaldulensis* and *E. urophylla*. The relatedness of PGIPs in these species and with other PGIPs is also discussed in this chapter.

Chapter 3 deals with the molecular characterisation of the endoPG gene of *C. cubensis*. The copy number of the *C. cubensis* endoPGs was determined. A comparison of the *C. cubensis* endoPG molecular structure and that of its close relative, *Cryphonectria parasitica,* is presented in this Chapter.

Chapter 4 of the thesis deals with endoPGs from *Cryphonectria cubensis*. The patterns of endoPG production in isolates of *C. cubensis* with varying levels of pathogenicity were investigated. A unique hypovirus transfected isolated was included in this study. An interaction between the hypovirus and polygalacturonase production is established for this fungus.

In Chapter 5, the qualitative effects of PGIP extracts from clonal lines of *Eucalyptus* (TAG5 and ZG14) on PGs from *Eucalyptus* pathogens are considered. The pathogens *C. cubensis*, *Coniothyrium zuluense*, *Botryosphaeria dothidea* and *Phytophthora cinnamomi* were included in the investigation.

Molecular characterisation of the important pine pathogen *Fusarium circinatum* endoPGs is presented in Chapter 6. The genome copy number of *F. circinatum* endoPG was determined. The relatedness of *F. circinatum* endoPG with those of other *Fusarium* spp. is also considered.

The thesis consists of independent chapters that deal with the characterisation of elements associated with the interaction of fungal pathogens and the plant cell wall, that is, endoPGs from *C. cubensis* and *F. circinatum* including PGIPs from *Eucalyptus* spp. Chapters Two and Six have already been published. However, repetition of some of the facts in these individual Chapters has been unavoidable. A uniform style has been adopted in the entire thesis.

LITERATURE REVIEW:

FUNGAL ENDOPOLYGALACTURONASES AND THEIR

INHIBITORS

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FUNGAL ENDOPOLYGALACTURONASES AND THEIR INHIBITORS

Introduction

Plants are susceptible, at all stages of growth and development, to attack by various types of disease-causing microorganisms. Thus, a major focus within the discipline of plant biology is the study of the interactions between plants and pathogens (Walton, 1997). Development of novel strategies to control diseases is the objective of this discipline (Lawton, 1997; Lamb *et al.*, 1992). An area of importance in our laboratory is the study of the factors involved in the interaction between fungal pathogens and *Eucalyptus* plants especially, endopolygalacturonases (endoPGs) and polygalacturonase-inhibiting proteins (PGIPs), respectively.

Phytopathogenic fungi have a general strategy of invading possible host plants. Immediately after attachment to plant surfaces, they secrete a mixture of cell wall degrading enzymes (CWDEs) (Cervone *et al.*, 1990, 1997; Whitehead *et al.*, 1995; Anderson, 1989). These CWDEs break down the polysaccharide-rich cell wall, not only to gain entry to the interior of the cell, but also to produce simple sugar nutrients for sustenance and growth (Cervone *et al.*, 1997; Kombrink and Somssich, 1995). There is reasonable consensus among researchers that these enzymes contribute to pathogenicity, but the exact role of these enzymes remains obscure (Walton, 1997).

The external cuticle and cell walls of plants, present a structural barrier that an invading fungal pathogen must circumvent. Primary cell walls comprise of cellulose, hemicelluloses and pectin, the latter being a major constituent (Carpita and Gibeaut, 1993). EndoPGs are among the first CWDEs that are secreted by pathogens (Albersheim and Anderson, 1971). Secretion of these endoPGs forms part of a targeted cell wall degradation programme of phytopathogenic fungi (Xu and Mendgen, 1997). EndoPGs cleave pectic chains, which comprise pectin and release shorter

chain sugars called oligogalacturonides (OGAs) (Johnston *et al.*, 1993; Bergmann *et al.*, 1994; De Lorenzo *et al.*, 1994; Darvill and Albersheim, 1984).

While physical barriers such as the cuticle and cell walls play an important role in defending the plant from pathogen invasion, the plant also elicits defensive biochemical processes (Lamb *et al.*, 1989; Mills *et al.*, 1996; Podila *et al.*, 1989). Plant cell walls contain proteins that effectively inhibit endoPG-mediated cell wall degradation. These proteins are known as PGIPs, and they belong to a super-family of proteins that contain leucine-rich repeats (Jones and Jones, 1997).

When fungal PG degrades the cell wall pectin, initially long chain OGAs are released. These are eventually degraded to shorter chains (De Lorenzo *et al.*, 1994). Longer OGAs (10-14 monomer units) have an elicitor activity, which activates plant defence responses. However, the shorter chains have little or no elicitor activity. Accumulation of elicitor-active OGAs due to the action of PGIPs protects the plant (De Lorenzo and Cervone, 1997; Cervone *et al.*, 1989).

The Plant Cell Wall

The plant cell wall is composed of complex polysaccharides, phenolics and structural proteins that maintain the cell and tissue integrity. In addition, it protects the cell from the hostile external environment (Showalter, 1993; Stafford 1991; Walton 1994). Pectin is the major matrix in the middle lamella and the primary cell wall with a backbone composed of alternating homogalacturonans and rhamnogalacturonans (Varner and Lin, 1989; Carpita and Gibeaut, 1993; Glinka and Protsenko, 1998). The plant epidermis is, therefore, the first line of defence that must be breached by fungal pathogens that penetrate directly, that is, without using wounds, stomata, or other natural openings (Walton, 1997; Agrios, 1988). In order to penetrate and metabolise the cell wall, most microorganisms produce a broad spectrum of CWDEs (Albersheim and Anderson 1971; Collmer and Keen 1986; Walton, 1994). CWDEs that degrade pectin polymers have been

the best studied, probably because they are the first to be secreted by pathogenic fungi during the penetration process (Albersheim and Anderson, 1971).

General Fungal Invasion Strategy

Plants make use of a preformed defence system such as the cell wall, resident pathogen repellents like polyphenols and tannins for protection against pathogens. They also utilise induced defence systems like the hypersensitive responses for this purpose (Walton, 1997). Nonetheless, pathogens have evolved mechanisms for entering and colonising plant tissues (Lawton, 1997). In order to ensure their survival, pathogens must adapt to the prevailing apoplastic or cellular conditions, overcome the existing physical or biochemical defences, employ mechanisms of obtaining plant nutrients and must circumvent the plant's inducible defence responses (Lawton and Lamb, 1987; Cook *et al.*, 1999).

Fungal pathogens must penetrate their hosts in order to establish infection (Walton, 1997, Schäfer, 1994). Although some penetrate through natural openings such as stomata or wounds, many fungi invade by direct penetration through the plant surface (Agrios, 1988). Penetration of the plant cell can be achieved by mechanical forces or by enzymatic degradation (Schäfer, 1994). In cases where penetration is not by mechanical force, enzymatic degradation is usually used. Even after penetration, CWDEs are utilised for derivation of nutrients from the plant cell wall (Walton, 1994). Therefore, for survival the pathogen must be able to recognise, become associated with, exploit the nutrient reserves of, and combat defence responses of its host (Herron *et al.*, 2000).

Secretion of CWDEs is generally the way that pathogens circumvent the physical barrier that is presented by the plant cell wall (Walton, 1994). Most CWDEs are glycoside hydrolases that degrade cellulose and pectate matrices by the addition of water to break the glycoside bond (Herron *et al.*, 2000). The release of CWDEs has previously been shown to occur in a time scale where some enzymes are secreted much earlier than others (Albersheim and Anderson, 1971).

CWDEs that are secreted by pathogenic fungi during penetration include endo-ß-1,4-glucanases, ß-glucosidases, ß-galactosidases, endo/exo-polygalacturonic acid lyases, endo/exopolygalacturonases, pectin lyases and pectin methylesterases (Collmer and Keen, 1986; Agrios, 1988; Walton, 1997). Each of these enzymes has a dedicated role in degrading the plant cell wall. EndoPGs are the first of these CWDEs to be secreted during penetration and have thus received considerable attention from plant pathologists (Herron et al., 2000; Cervone et al., 1989; Dixon and Lamb, 1990).

Fungal pathogens express sets of genes involved in establishing infection while other novel genes are expressed in the plant as part of the host response (Carry et al., 1995; Green et al., 1995; Dean and Timberlake, 1989; Karr and Albersheim, 1970; Bateman and Beer, 1965). Examples of fungal pathogens whose endoPGs have been characterised include those from *Sclerotinia sclerotiorum*; *Fusarium moniliforme*; *Fusarium oxysporum*; *Cryphonectria parasitica* (Reymond et al., 1994; Caprari et al., 1993; Di Pietro and Roncero, 1998; Gao et al., 1996). Studies of the role of endoPGs and other CWDEs are still necessary for different fungal pathogens, as the roles tend to be different among different pathogens.

EndoPGs are specifically inhibited by PGIPs (De Lorenzo and Cervone, 1997). This PGIP-PG interaction has two important consequences. Firstly, it blocks invasion of the fungal pathogen by halting hyphal ingress into the plant. This is achieved by inhibiting the endoPGs from degrading the cell wall barrier (Walton, 1994). Secondly, it allows transmission of defence signals to cells that are proximal to infected cells, thus allowing the plant to protect itself. This is achieved by triggering defence responses such as the production of phytoalexins and other toxic secondary metabolites (Cervone et al., 1997; Ryan and Farmer, 1991).

The Role of EndoPGs in Pathogenesis

The ability of a plant to respond defensively against an invading pathogen depends on its perception (recognition) of the pathogen. This information then must be transmitted from the infected cells to adjacent plant cells (Cervone *et al.*, 1997). These cells then elicit biochemical changes that act cooperatively to limit invasion of the pathogen (De Lorenzo and Cervone, 1997; De Lorenzo *et al.*, 1994). EndoPGs degrade the pectin structure of the plant cell wall. In this process they weaken the cell wall and allow the fungal pathogen to invade the rest of the plant tissue (Cervone *et al.*, 1989). However, the link between secretion of endoPGs and pathogenicity is not absolute. In some cases the link has been clearly demonstrated (Weeds *et al.*, 1999; Ten Have *et al.*, 1998) and in others, it appears to be less significant (Gao *et al.*, 1996; Scott-Craig *et al.*, 1990). This implies that investigations on the role of individual endoPGs should be carried out in a case-by-case manner and generalisations for different plant pathogen interactions should be made with great care.

The first molecular evidence to show that endoPGs have an important function in degradation of cell walls was first reported by Caprari and co-workers (1996). In their study, site-directed mutagenesis was used to show that histidine 234 residue of the endoPG of *Fusarium moniliforme* is critical for enzymatic and macerating activity, and not for binding to PGIP. This histidine residue is conserved in endoPGs. These results focussed on the specific function of a single amino acid residue (H234) in degrading the pectic constituent of the plant cell.

EndoPGs have been reported to have two opposing roles in fungal pathogenesis (Cervone *et al.*, 1997). Firstly, they are utilised by fungi as efficient tools of aggression. This is achieved by degrading the plant cell wall structure allowing a pathogen to penetrate its host. Secretion of additional endoPGs results in further penetration and enhances the survival of the pathogen in the host (De Lorenzo and Cervone, 1997; Cervone *et al.*, 1989). Secondly, they act as potential pre-elicitors of plant defence signal molecules. This is an advantage to the host plant, since the plant then defends itself from the invading pathogen (Cervone *et al.*, 1997). EndoPGs initiate the

production of elicitors for signal transduction known as oligogalacturonides (OGAs) from degradation of homogalacturonan polymer of pectin (Cervone *et al.*, 1989, 1997). Degradation of pectin by endoPGs in the presence of PGIPs gives rise to the production of elicitor-active OGAs of 10-15 residues in size. Furthermore, the presence of PGIPs increases the residence time of such molecules to act as signalling molecules. The early production of these endoPGs is compatible with both roles (De Lorenzo and Cervone, 1997; Cervone *et al.*, 1997).

EndoPGs from different fungal species are different in their substrate degradation capacity and they also differ in their susceptibility to PGIP inhibition (Cook *et al.*, 1999). This observation has two implications. Firstly, it means that successful pathogens should have the capacity to degrade the pectin walls of their hosts more rapidly than non-virulent pathogens. Secondly, they should secrete endoPGs that are not easily inhibited by the host PGIPs.

The pathogenicity of fungi like *Botrytis cinerea* on tomatoes and apples is controlled by the rate of endoPG production by that fungus (Ten Have *et al.*, 1998; Weeds *et al.*, 1999). In more pathogenic isolates of *B. cinerea*, higher levels of endoPGs are produced than in less pathogenic isolates. This has important implications in designing control measures against this pathogen. Genetic transformation of plants with a superior PGIP that strongly inhibits these endoPGs in *B. cinerea* would be an attractive option.

In *Leptosphaeria maculans*, the causal agent of blackleg of canola, endoPGs are secreted at the initiation of disease. EndoPGs from *L. maculans* are inhibited by extracts from the stems of canola (Annis and Goodwin, 1997). Resistant canola cultivars have higher inhibition efficiency to the endoPGs from *L. maculans* than the susceptible cultivars. This shows that the PGIPs in canola interact with the endoPGs of *L. maculans* to give rise to resistance to blackleg.

The pectic fragments obtained after digestion of bean cell walls with endoPGs of the bean pathogen *Colletotrichum lindemuthianum*, resulted in differential elicitation of defence responses in bean seedlings (Nuss *et al.*, 1996; Boudart *et al.*, 1998). *C. lindemuthianum* secretes endoPGs to degrade the cell walls of the host. Boudart and co-workers (1998) showed that between two

near isogenic lines of bean, resistant and susceptible to *C. lindemuthianum*, the pectic fragments from the resistant lines elicited higher levels of pathogen-related (PR) proteins than those from the susceptible lines. Sizes of fragments that are produced from endoPG digestion of the pectic polymer determine the extent of defence signal elicitation. In the presence of the PGIP inhibitors, the length of the OGAs is between 10-15 residues, which are elicitor-active (Lafitte *et al.*, 1984). This is a classical example of an interaction between endoPGs and PGIPs that results in triggering PR proteins whose function is to enhance resistance of the host plant against a pathogen.

Oligogalacturonides (OGAs) in Signal Transduction

Plant cell walls are a first line of defence in plant-pathogen interactions (Esqueré-Tugayé *et al.*, 2000). OGAs play an important role in this interaction (Cervone *et al.*, 1989; Esqueré-Tugayé *et al.*, 2000). Plant cell walls participate in what can be thought of as a molecular "communication" between the host and the pathogen. In the absence of PGIPs, endoPGs degrade plant cell walls to small absorbable sugars that are utilised as nutrient by the fungus. The extent of degradation is controlled by the presence of PGIPs that counteract the action of endoPGs. The action of PGIPs on endoPGs results in elicitor-active OGAs (Hahn *et al.*, 1989; Cervone *et al.*, 1989). OGAs are the signal molecules that trigger different forms of cell responses in PGIP-PG interactions.

OGAs have been shown to trigger production of the antifungal diterpene known as casbene in caster bean seedlings (Jin and West, 1984). Soybean cell walls were partially digested with endoPGs of the fungus *Rhizopus stolonifer* and the pectic fragments (OGAs) were tested for elicitor activity. The resultant OGAs elicited activity of the casbene synthase gene in castor bean seedlings. This demonstrates the ability of OGAs to trigger the expression of defence responses.

In plants, OGAs can act as signal molecules in growth and development (Altamura *et al.*, 1998). They can stimulate the pericycle cell wall thickening. Furthermore, they can induce mitotic

divisions that lead to stoma formation in the epidermal cells of tobacco leaf explants. Additionally, transcriptional activation of defence genes and accumulation of hydrogen peroxide can be induced by OGAs (Altamura *et al.*, 1998). This shows that OGAs are multifunctional molecules in cell signalling processes.

Although there are some examples of the involvement of OGAs in signal transduction in plant cells, their role in post-infection defence has not yet been fully elucidated. The fact that the bean PGIP-1 promoter was activated by wounding and not by OGAs may suggest that OGAs are not intermediate signal molecules to wound response (Devoto *et al.*, 1997). The mechanism of action of OGAs as signalling molecules has not been shown. In addition, the accumulation of these OGAs (typical 10-15 residues) has also not been shown *in planta* (Cervone *et al.*, 1997; Johnston *et al.*, 1993). However, the key proteins that give rise to elicitor-active OGAs in PGIP-PG interactions are PGIPs.

Molecular Structure, Biochemical and Physical Properties of PGIPs

PGIPs are cell wall associated glycoproteins that specifically bind to and inhibit fungal endoPGs. Furthermore, they have a reversible, saturable high affinity receptor characteristic for fungal endoPGs. PGIPs can be competitive or non-competitive inhibitors of endoPGs depending on the origin of their endoPGs (Stotz *et al.*, 2000; Glinka and Protsenko, 1998; Ramanathan *et al.*, 1997). Most PGIPs have a mean apparent molecular mass of 43 kDa. The mean apparent molecular mass after chemical deglycosylation is 34 kDa, indicating that carbohydrates constitute about 20 % of the mass (Cervone *et al.*, 1987; Johnston *et al.*, 1993; Stotz *et al.*, 1993; Toubart *et al.*, 1992).

PGIPs characterised thus far have a conserved N-terminal region. They are encoded by intronless genes of about 1 kb in size except raspberry and peach PGIPs that have one intron (see Table 1). In bean, PGIPs are localised in the heterochromatin region in the embryonic suspensor cells of bean (Frediani *et al.*, 1993). In some species PGIPs are encoded by more than one gene

(Table 1). Currently, PGIPs have been characterised from at least 20 plant species from both dicots and monocots (Table 1).

PGIPs are relatively heat stable and have a leucine-rich periodic tandem repeat. A leucine-rich repeat (LRR) is present in other signal molecules and resistance (R) gene products. The presence of cysteine residues is thought to have a role in the heat stability and function of PGIPs (Stotz *et al.*, 1993). PGIPs belong to the LRR super-family of proteins (Cervone *et al.*, 1997). This suggests the PGIPs may have a role in molecular signalling and plant resistance (De Lorenzo *et al.*, 1994; Jones and Jones 1997). The receptor-like structure of PGIP provides a molecular basis for the proposed role of PGIP as a secreted receptor component of the cell-surface signalling system involved in recognition events between plant and fungi. There is however, not sufficient evidence to prove this hypothesis. The structure of PGIPs is specialised for interactions with other macromolecules, because of the presence of LRR domain (De Lorenzo *et al.*, 1994; Kobe and Deisenhofer, 1995). Due to their molecular structure, two roles of PGIPs have been proposed. They are firstly believed to be involved in plant defence and secondly, in plant development.

Role of PGIPs in Plant Defence

PGIPs are constitutively expressed in plants but their distribution is organ specific. For example, in pears, young leaves have higher levels of PGIP than ripe fruits (Abu-Goukh and Labavitch, 1983, Abu-Goukh *et al.*, 1983a). The fact that PGIPs are expressed in the epidermis of plant cells supports the view that they have an important role in host resistance and that they are one of the first plant gene products that the pathogen must evade (Bergmann *et al.*, 1994). The level of expression also varies according to the developmental stage of the plant (Devoto *et al.*, 1997). *In planta*, the level of PGIPs is low but it is increased after induction by wounding,

elicitors and fungal infection (Bergmann *et al.*, 1994). It is therefore likely that PGIPs play a crucial role in defence.

The mechanism of action of PGIPs in plant defence is still unclear. It has been suggested that PGIPs do not contribute directly to plant defence. Instead, they are thought to retard endoPG-mediated degradation of the cell wall pectin resulting in the formation of elicitor-active OGAs within the infection site (Cervone *et al.*, 1989). Furthermore, De Lorenzo and co-workers (1994) proposed that bean PGIP might be represent a recognition system that determines host specific resistance. However, there is evidence that endoPGs of three races of *Colletotrichum lindemuthianum* are inhibited by PGIPs from four cultivars of bean to the same extent, regardless of cultivar interaction (De Lorenzo *et al.*, 1990). This casts doubt on the view that, bean PGIP may represent a recognition system, since different cultivars of bean showed similar inhibition activity. If the PGIPs were a recognition system for endoPGs they would have been expected to display differences.

Sharrock and Labavitch (1994) showed that immature pear fruits were more resistant to *Botrytis cinerea* than mature fruits. This was attributed to higher levels of PGIPs present in immature pears. They concluded that PGIPs have a role in defence of the pear fruit against *B. cinerea*. However, PGIPs did not prolong the half-life of elicitor-active OGAs since the immature pears were not infected by *B. cinerea*. This cast doubt regarding the role of PGIPs in increasing the retention time of elicitor–active OGAs to induce PR-proteins. In addition, it highlights the need to clarify the role of PGIPs in plant defence. It must also be recognised that the mechanism of action of PGIPs may be different amongst species of plants.

PGIPs are thought to provide protection to leek plants against pectinolytic fungal decay (Favaron *et al.*, 1997). PGIPs from *Allium porrum* L have activity against endoPGs from *Sclerotinia sclerotiorum* and *Botrytis aclada* (Favaron *et al.*, 1997). These studies show that there exists a possibility of using PGIPs for protection of leek plant against endoPG-mediated

tissue degradation. Interestingly, PGIPs of *Allium porrum* are the only examples from monocotyledonous plants that have been well characterised.

Evidence that PGIPs have a role in plant defence has been shown by two transformation experiments. Transgenic tomatoes expressing a pear PGIP were shown to be more resistant to *B. cinerea* than the non-transgenic tomatoes (Powell *et al.*, 1994). Similarly, when the bean PGIP-1 was expressed in maize, the transgenic maize showed increased tolerance to the cob-rot pathogen *Stenocarpella maydis* considerably and that bean PGIP-1 inhibits polygalacturonases from *Stenocarpella maydis* (Burger *et al.*, 1997; Berger *et al.*, 2000).

Role of PGIPs in Plant Development and other Environmental Stress Factors

There is evidence to suggest a role of PGIPs during of plant development and in response to stress. The PGIPs in apple are developmentally regulated and activated by wounding and fungal infection (Yao *et al.*, 1999). In apple fruits there was a differential expression of PGIPs between different stages of fruit maturity. More PGIP transcripts were shown in mature infected fruits than in immature fruit. The same phenomenon has been reported in pears (Abu-Goukh *et al.*, 1983a, b). In apples, PGIPs were induced by cold storage, wounding and by infection by *Botrytis cinerea* and *Penicillium expansum*. These observations suggest that PGIPs may have multiple roles during development and stress response (Yao *et al.*, 1999).

Meyer and co-workers (1999) reported on an anti-freeze protein from carrot, which had high homology to PGIPs. This gene is known to have a role in cold acclimation (Meyer *et al.*, 1999). This shows that the ability of plants to constantly adapt to stress is the result of the proteins like PGIPs that contain LRRs. The hypervariability of the LRR domain of the PGIP gives rise to multifunction of the protein (Desiderio *et al.*, 1997).

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By serendipity, Desiderio and co-workers (1997) showed that PGIPs have a role in growth and development. Transgenic expression of a bean PGIP in tomato plants resulted in transgenic plants being larger than normal tomato plants, suggesting a developmental role for PGIPs (Desiderio *et al.*, 1997). However, it must be noted that in bean, PGIPs occur in multicopy genes (Frediani *et al.*, 1993) so that over-expression of one of these genes may not necessarily result in increased defence against pathogens as was intended by Desiderio and co-workers (1997). This also showed that PGIPs have different functions and different endoPG specificities are present in bean.

PGIPs as Receptors in Defence Signalling

Plants lack a vertebrate-type immune system, and instead, have a preformed and/or an inducible defence capability. The inducible defence utilises the presence of LRRs in specialised proteins to act as signal receptors (Capodicasa *et al.*, 1999; Hammond-Kossack and Jones, 1997; Hammond-Kossack *et al.*, 1998). Experimental evidence to show that PGIPs can function as receptors during signal transduction has not yet been reported. The only evidence available is implied from structural analysis.

A key feature in most resistance genes (R) is the presence of the LRR motif in the gene products. PGIPs contain LRRs, therefore, are evolutionarily related to several R genes that participate in gene-for-gene resistance (Jones and Jones, 1997). Many R genes, such as *Cf-9*; *Cf-2*; *Xa*21, including PGIPs, have been isolated from tomato, flax, tobacco, rice and arabidopsis (Devoto *et al.*, 1998; Dixon *et al.*, 1998, 2000; Jones and Jones 1997; Stotz *et al.*, 2000). These all contain the LRR motif (Leech *et al.*, 2000). This LRR has been implicated in the signal transduction pathway that coordinates multifaceted plant defence responses (Leech *et al.*, 2000; Jones and Jones, 1997; Stotz *et al.*, 2000).

A LRR is a versatile binding-protein motif that is used in protein-protein interactions and cell signalling. The surface-exposed parallel beta-sheet and non-globular shape of the LLR structure is responsible for the protein binding (Kobe and Deisenhofer, 1993, 1994). It is used in molecular recognition in various processes including signal transduction, cell adhesion, cell development, DNA repair, RNA processing, ligand binding and enzyme inhibition (Kobe and Deisenhofer, 1995). So far, PGIPs are the only example of LRR proteins in plants whose ligand, the endoPG, is known. The flanking regions of LRR are conserved and are likely to have a role in providing stability to the proteins that belong to the LRR super-family (Marino et al., 2000).

Warren and co-workers (1998) have shown that a mutation within the LRR domain of the Arabidopsis disease resistance gene RPS5, partially suppresses multiple bacterial and downy mildew resistance genes. This illustrates the importance of LRRs in plant defence against pathogens. The molecular recognition of pathogens by plants is often characterised by gene-for-gene relation that requires a specific plant resistance R gene and a corresponding pathogen avirulence (avr) gene. The pathogen should be able to initiate signal transduction to activate defence, as well as have the capacity to evolve new avr gene specificities rapidly. The LRR characteristic of the R gene allows a plant to transmit defence signals from infection sites to the rest of the plant (Hammond-Kossack and Jones, 1997; Hammond-Kosack et al., 1998). PGIPs may play role as cell receptors by virtue of the presence of the LRR in their structure.

Signalling Systems in Secretion of Cell Wall Degrading Enzymes (CWDEs)

PGIPs, as part of a signalling cascade system should function co-operatively with other components within that signalling pathway for host defence (Lamb et al., 1989). Since PGIPs interact with endoPGs, which are a member of CWDEs, the identification of genetic elements that regulate the expression of CWDEs, like endoPGs, is an attractive approach to gain a better understanding of signalling cascades in plant-pathogen interactions. At the moment a few such

genetic elements have been studied, even though a link with PGIPs has not been proved (Celenza and Carlson, 1986; Tokunari *et al.*, 2000).

Secretion of CWDEs by fungi, and hence pathogenesis is regulated by proteins such as SNF1 (SNF = sucrose nonfermenting) (Celenza and Carlson, 1986). SNF1 is a protein kinase that phosphorylates nuclear DNA transcriptional repressors such as creA (creA = carbon catabolite repressible enzymes in *Aspergillus nidulans*) in fungi (Arst and McDonald, 1975; Tokunari *et al.*, 2000). Phosphorylation of creA inhibits its binding to the promoters of the gene it represses (Ronne, 1995; Ruijter and Visser, 1997). The role of SNF1 gene in CWDE expression and virulence in maize has been shown using a gene replacement technique (Tokunari *et al.*, 2000). The production of CWDEs is under catabolite repression (glucose) in pathogenic fungi. The SNF1 gene is required for expression of catabolite-repressed genes when glucose is limiting, since it allows derepression of genes like those for invertases (Tokunari *et al.*, 2000).

In the maize pathogen *Cochliobolus carbonum*, homologues of the SNF1 protein kinases are required for biochemical processes important in pathogenesis (Tokunari *et al.*, 2000). By targeted gene disruption of the SNF1 gene in *C. carbonum*, it was shown that penetration is the single most important step at which SNF1 is required. The specific processes controlled by SNF1 include the ability to degrade polymers of the plant cell wall and to uptake and metabolise sugars produced. Clearly, SNF1 is required for secretions of CWDEs and an association with PGIPs in host-pathogen signalling can be envisaged during control of pathogenicity (Tokunari *et al.*, 2000).

G-Protein Linked Signalling Pathway

G-protein linked signalling pathway has a role in secretion of CWDEs and hence pathogenesis. A G-protein linked signal transduction system is disrupted by a hypovirus in *Cryphonectria parasitica* (Rigling and van Alfen, 1991; Powell and van Alfen, 1987). Nuss (1996) reported that the hypovirus infection of the chestnut blight pathogen, *C. parasitica*,

resulted in reduced virulence (hypovirulence). Hypovirulence was associated with a reduction in expression of CWDEs such as laccases, cutinases, cellobiohydrolases and endoPGs.

Targeted disruption of two G–proteins subunits of *C. parasitica* caused a marked reduction in fungal growth rate, loss of virulence and reduced transcriptional induction of the laccase gene, reminiscent of hypovirus induced phenotypic traits (Gao and Nuss, 1996; Chen *et al.*, 1996a, b). Hypovirulence-associated viruses interfere with *C. parasitica* metabolism by perturbing the IP3-calcium second messenger systems (Larson *et al.*, 1992). Kasahara and Nuss (1997) also reported that the disruption of the G-protein, beta-subunit gene reduces virulence. These results constitute molecular evidence that pathogenicity is under control of a complex signalling system that may involve PGIPs and other cell signalling systems.

Cell Density-Dependent Signalling: Quorum Sensing

Quorum sensing is a species-specific cell-cell communication that enables a pathogen to recognise when it has reached a specific threshold after which, it should proceed with further steps in the infection process (Schell, 1996; Bassler, 1999). In *Ralstonia solanacearum* a ubiquitous bacterial phytopathogen, quorum sensing is needed to express genes that control the production of virulence factors such as plant CWDEs by the pathogen (Schell, 1996). Similarly, in *Pseudomonas aeroginosa*, a human and plant pathogen, biofilm synthesis and virulence are co-ordinated by quorum sensing (Parsek and Greenberg, 2000). Knowledge of proteins and exact processes involved in quorum sensing is vital in developing control strategies for increased resistance against plant pathogens.

Conclusions

The review has attempted to put together most of the body of information regarding the function of PGIPs and endoPGs. Several examples of isolated PGIPs are known and each of them has a differential inhibition capacity and specificity to endoPGs. The mode action of PGIPs

involves a stage of balancing the concentration of oligogalacturonides. However, it remains to be shown that oligogalacturonides can be detected *in planta*. This review showed that PGIPs have a defence role and in some cases a crucial role in growth and development of a plant. The role of PGIPs in different plant species must be investigated individually.

From the pathogen side, this review has shown that endoPGs have an important role in determining the pathogenicity of some selected fungal species. The fact that endoPG-mediated degradation of plant cell wall give rise to elicitor active oligogalacturonides means that endoPGs have a crucial fungal pathogenesis and cell-cell signalling. However, in some fungal species the connection between pathogenicity and secretion of endoPGs is not clear. Therefore, there is need for case-by-case studies on the exact role of these enzymes in pathogenesis and cell-cell signalling.

In specific cases, if a full understanding of the role of endoPGs in pathogenesis and PGIP interaction is achieved, it becomes easy to design novel strategies of enhancing disease resistance. Additionally, it becomes possible to delineate cell-cell signalling pathways, which at the moment are only known as models.

The aim of this project was to characterise the PGIPs of *Eucalyptus* species and well as to characterise the endoPGs from important forestry pathogens, *Cryphonectria cubensis* and *Fusarium circinatum*. *C. cubensis* is a filamentous fungus that causes a disease known as Cryphonectria canker on *Eucalyptus* plants while *F. circinatum* is also a filamentous fungus that that causes the pitch canker disease on pines.

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TABLES

Table 1. PGIPs from a variety plant species

Plant Species	Group	Copy Number	Putative Role	Accession Number	Reference
Allium porrum	monocot	ND	defence	none	Favaron *et al.*, 1997
Actinidia deliciosa	dicot	ND	ND	Z49063	Simpson *et al.*, 1995
Citrus iyo	dicot	2	ND	AB016205	Kazuya (unpublished)
Citrus jambhiri	dicot	2	ND	AB013397	Kazuya (unpublished)
Citrus sinensis	dicot	ND	ND	Y08618	Mayer (unpublished)
Citrus sp cv sannumphung	dicot	2	ND	AB015356	Kazuya (unpublished)
Citrus unshiu	dicot	ND	ND	AB016204	Kazuya (unpublished)
Eucalyptus camaldulensis	dicot	>1*	defence	AF159168	Chimwamurombe *et al.*, 2001
Eucalyptus grandis	dicot	>1*	defence	AF159167	Chimwamurombe *et al.*, 2001
Eucalyptus nitens	dicot	>1*	defence	AF159171	Chimwamurombe *et al.*, 2001
Eucalyptus saligna	dicot	>1*	defence	AF159170	Chimwamurombe *et al.*, 2001
Eucalyptus urophylla	dicot	>1*	defence	AF159169	Chimwamurombe *et al.*, 2001
Fortunella margarita	dicot	ND	ND	AB020529	Kazuya (unpublished)
Glycine max	dicot	>1	defence/development	X78274	Favaron *et al.*, 1994
Lycopersicon esculentum	dicot	2	defence	L26529	Stotz *et al.*, 1994
Malus domestica	dicot	>2	defence/development	U77041	Yao *et al.*, 1999
Phaseolus vulgaris	dicot	>5	defence/development	X64769	Toubart *et al.*, 1992
Poncirus trifoliata	dicot	ND	ND	AB020528	Kazuya (unpublished)
Prunus armeniaca	dicot	ND	ND	AF020785	Puehringer (unpublished)
Prunus mahaleb	dicot	ND	ND	AF263465	Zhang and Zhang (unpublished)
Pyrus communis	dicot	2	defence/development	L09264	Stotz *et al.*, 1993
Rubus idaeus	dicot	>2	defence/development	None	Ramanathan *et al.*, 1997
Solanum tuberosum	dicot	ND	defence	none	Machinandiarena *et al.*, 2001

ND=not determined, * unpublished data

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

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CHAPTER 2

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

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*.

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 defence system. Firstly, the inhibition of 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, Favaron *et al.*, 1994, Yao *et al.*, 1995, Stotz *et al.*, 1993, Johnston *et al.*, 1993, Stotz *et al.*, 1994, Simpson *et al.*, 1995). PGIPs are thermolabile glycoproteins with a molecular mass of around 44 kDa. When deglycosylated, the mass is around 34 kDa, with N-linked glycosylation

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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 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 proteins superfamily and, therefore, may be involved in signalling defence 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), not withstanding the fact that there are other resistance mechanisms that occur without utilising the LRRs. 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 (Powell *et al*, 1994, Lafitte *et al.*, 1994, Labavitch *et al.*, 1997, Burger *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 to that goal, isolation and cloning of responsible genes is vital. The objective of this study was to clone and analyse 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 *E. grandis*, *E. camaldulensis*, *E. nitens*, *E. urophylla* and *E. saligna*. The leaves were frozen in a sterile plastic bag for 20 minutes 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 (United Kingdom) 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 C-termini of aligned published peptide PGIPs), (4 pmol/μl), *Eucalyptus* template DNA (25 ng), 1 mM dNTPs, 2.5 mM MgCl$_2$, 10X 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 and 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 artefacts.

Cloning

All DNA manipulations were done according to 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 pGEM-T-Easy vector (Promega). Ligation was done at 4 °C for 16 hours. Ligation mixtures were transformed into competent *E. 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 (Sambrook *et al.*, 1989) and further purified 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 the GenBank (Table 1). The phylogram showing 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 Figure 1). In all the *Eucalyptus* spp included in the study, the sequenced fragments were also 909 bp (results not shown). A single open reading frame for the *Eucalyptus* spp is 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 (Figure 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 (Figure 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 in 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 (Figure 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 Figure 2) revealed high homologies with other PGIPs. Similarity between the *Eucalyptus* spp. alone was between 98-100 %, 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 96-99 % at peptide level (Table 2) and 97-99 % at nucleotide level.

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 (Figure 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 figure 1 is conserved in all PGIPs sequenced thus far. This may suggest an important role of PGIPs in recognition and signal transduction in plant defence (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. 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 *Phaseolus 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 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 the xxLxLxx motif of the LLR. 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 the 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 PGIP DNA sequences between *Eucalyptus* spp. is similar to that observed in other genera (*e.g. Citrus*) (Figure 3). There is however, a high degree of sequence conservation between known PGIP DNA sequences, this suggests conservation of PGIPs functional role in the plant defence 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 is a difficult as they are morphologically similar. 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. *Prunus 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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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

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

	Fm	Pt	Ci	Cb	Cu	Cs	Cg	Cj	Cc	Pa	Md	Ce	Pv	Le	Gm	Ad	Pc	Eg	Ec	Eu	Es
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

PERCENTAGE SIMILARITY (column span heading); SPECIES (row axis label)

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*.

FIGURES

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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  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
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CACACTGC   909
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Figure 1

The nucleotide sequence and predicted amino acid sequence of a putative mature polygalacturonase

inhibiting protein of *Eucalyptus grandis* (results for *E. camaldulensis*, *E. urophylla*, *E. nitens* and *E.*

saligna are not shown, see accession numbers which are 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).

```
Actinidia deliciosa          -------MKSTTAISLLLFLS-LLSPSLSDRCNPNDKKVLLRIKQALNNPYLLASWNPDNDCCD--WYNVDCDLTTN--RIIALTIFSGNISG--QIPAAVGDLPYLQTLIFRKLSNLTG 106
Citrus iyo A                 -------MSNTSLLSLFFFLSLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCD--WYCATCDLTTN--RINSLTIFAGDLPG--QIPPEIGDLPYLETIMFHKLPSLTG 107
Citrus iyo B                 -------MSNTSLLSLFFFLSLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCD--WYCVTCDLTTN--RINSLTIFAGDLPG--QIPPEVGDLPYLETIMFHKLPSLTG 107
Citrus jambhiri A             ------MSNTSLLSLFFFLSLFISPSLSDLCNPNDKKVLLKFKKALNNPYVLASWNPKTDCCD--WYCVTCDLTTN--RINSLTIFAGDLPG--QIPPEVGDLPYLETIMFHKLPSLTG 107
Citrus jambhiri B            -------MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCD--WYCVTCDLTTN--RINSLTIFAGDLPG--QIPPEVGDLPYLETIMFHKLPSLTG 107
Citrus sinensis              -------MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCD--WYCVTCDLTTN--RINSLTIFAGDLPG--QIPPEVGDLPYLETIMFHKLPSLTG 107
Citrus sp cv sannumphung A   -------MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLTSWNPKTDCCD--WYCVTCDLTTN--RINSLTIFAGDLPG--QIPPEVGDLPYLETIMFHKLPSLTG 107
Citrus sp cv sannumphung B   -------MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCD--WYCVTCDLTTN--RINSLTIFAGDLPG--QIPPEVGDLPYLEIIMFHKLPSLTG 107
Citrus unshiu                -------MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKSLNNPYVLASWNPKTDCCD--WYCVTCDLTTN--RINSLTIFAGDLPG--QIPPEVGDLPYLETIMFHKLPSLTG 107
Eucalyptus camaldulensis     ----------------------------NPDDKKVLLQIKKAFGDPYVLASWKADTDCCD--WYCATCDSTTN--RINSLTIFAGQVSG--QIPALVGDLPYLETLEFHKQPNLTG 82
Eucalyptus grandis           -----------------------------NPDDKKVLLQIKKAFGDPYVLASWKSDTDCCD--WYCVTCDSTTN--RINSLTIFAGQVSG--QIPALVGDLPYLETLEFHKQPNLTG 82
Eucalyptus nitens            ----------------------------NPDDKKVLLQIKKAFGDPYILASWKSDTDCCD--WYCVTCDSTTN--RINSLTIFAGQVSG--EIPALVGDLPYLETLEFHKQPNLTG 82
Eucalyptus saligna           -----------------------------NPDDKKVLLQIKKAFGDPYVLASWKSDTDCCD--WYCVTCDSTTN--RINSLTIFAGQVSG--QIPALVGDLPYLETLEFHKQPNLTG 82
Eucalyptus urophylla         ----------------------------NPDDKKVLLQIKKAFGDPYVLASWKADTDCCD--WYCVTCDSTTN--RINSLTIFAGQVSG--QIPALVGDLPYLETLEFHKQPNLTG 82
Fortunella margarita         -------MSNTSLLSLFFFLCLCISPSLSDLCNPNDKKVLLKFKKALNNPYVLASWNPKTDCCD--WYCVTCDLTTN--RINSLTIFAGDLPG--QIPPEVGDLPYLDTIMFHKLPSLTG 107
Glycine max                  --------------------------ELCNPQDKQTLLQIKKELGNPTTLSSWHPKTDCCNNSWVGVSCDTVTPTYRVDNLDLSELNLRKPYPIPPSVGSLPCLKFLYITNNPNIVG 91
Lycopersicon esculentum      --------MNLSLLLVVIFLC-FASPSLSVRCNPKDKKVLLQIKKDLGNPYHLASWDPNTDCCY--WYVIKCDRKTN--RINALTVFQANISG--QIPAAVGDLPYLETLEFHHVTNLTG 105
Malus domestica              -----MELKFSIFLSLTLLFSSVLKPALSDLCNPDDKKVLLQIKKAFGDPYVLTSWKSDTDCCD--WYCVTCDSTTN--RINSLTIFAGQVSG--QIPALVGDLPYLETLEFHKQPNLTG 109
Phaseolus vulgaris           MTQFNIPVTMSSSLSIILVILVSLRTALSELCNPQDKQALLQIKKDLGNPTTLSSWLPTTDCCNRTWLGVLCDTDTQTYRVNNLDLSGHNLPKPYPIPSSLANLPYLNFLYIGGINNLVG 120
Poncirus trifoliata          -------MSNTSLLSLFFFLSLFTSLSLSDLCNPNDKRVLLNFKKALNNPYVLASWNPKTDCCD--WYCVTCDLTTN--RINSLTIFAGDLPG--QIPPEVGDLPYLETIMFHKLPSLTG 107
Prunus armeniaca             -----MDVKFPTLLCLTLLFSTILNPALSELCNPEDKKVLLQIKKAFNDPYVLTSWKPETDCCD--WYCVTCDSTTN--RINSLTIFAGQVSG--QIPTQVGDLPYLETLEFHKQPNLTG 109
Pyrus communis               -----MELKFSTFLSLTLLFSSVLNPALSDLCNPDDKKVLLQIKKAFGDPYVLASWKSDTDCCD--WYCVTCDSTTN--RINSLTIFAGQVSG--QIPALVGDLPYLETLEFHKQPNLTG 109
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Figure 2

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

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Actinidia deliciosa          QIPSAISKLSNLKMVRLSWTNLSGPVPSFFSQLKNLTFLDLSFNDLTGSIPSSLSKLTNLDAIHLDRNKLTGPIPNSFGEFTG-QVPDLYLSHNQLTGSIPKTLGDLNFTVIDVSRNMLSG 226
Citrus iyo A                 PIQPAIAKPKNLKTLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGSIPESFGTFTG-SIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEG 227
Citrus iyo B                 PIQPAIAKLKNLKTLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGAIPGSLSKLQKLGALHLDRNKLTGSIPESFGTFTG-SIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEG 227
Citrus jambhiri A            PIQPAIAKLKNLKTLRISWTNISGLVPDFISQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGSIPESFGTFTG-SIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLGE 227
Citrus jambhiri B            PIQPAIAKLKNLKTLRISWTNISGPVPDFISQLTNLTFLEPSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGSIPESFGTFTG-SIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEG 227
Citrus sinensis              PIQPAIAKLKNLKTLRISWTNISGPVPDFIRQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGSIPESFGTFTG-SIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEG 227
Citrus sp cv sannumphung A   PIQPAIAKLKNLKTLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGSIPESFGTFTG-SIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEG 227
Citrus sp cv sannumphung B   PIQPAIAKLKNLKTLRISWTNISGPVPDFIRQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGSIPESFGTFTG-SIPDLYLSHNQLSGKIPASLGSMDSNTIDLSRNKLEG 227
Citrus unshiu                PIQPAIAKLKNLKTLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGSIPESFGTFTG-SIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEG 227
Eucalyptus camaldulensis     PIQPAIAKLKGLKFLRLSWTNLSGSVPDFLSQLKNLTFLDLSFNNLTGAIPSSLSQLPNLNALHLDRNKLTGHIPKSFGQFIG-NVPDLYLSHNQLSGNIPTSFAQMDFGKHRLSRNKLG- 201
Eucalyptus grandis           PIQPAIAKLKGLKFLRLSWTNLSGSVPDFLSQLKNLTFLDLSFNNLTGAIPSSLSQLPNLNALHLDRNKLTGHIPKSFGQFIG-NVPDLYLSHNQLSGNIPTSFAQMDFGKHRLSRNKLE- 201
Eucalyptus nitens            PIQPAIAKLKGLKFLRLSWTNLSGSVPDFLSQLKNLTFLDLSFNNLTGAIPSSLSQLPNLNALHLDRNKLTGHIPKSFGQFIG-NVPDLYLSHNQLSGNIPTSFAQMDFGKHRLSRNKLG- 201
Eucalyptus saligna           PIQPAIAKLKGLKFLRLSWTNLSGSVPDFLSQLKNLTFLDLSFNNLTGAIPSSLSQLPNLNALHLDRNKLTGHIPKSFGQFIG-NVPDLYLSHNQLSGNIPTSFAQMDFGKHRLSRNKLE- 201
Eucalyptus urophylla         PIQPAIAKLKGLKFLRLSWTNLSGSVPDFLSQLKNLTFLDLSFNNLTGAIPSSLSQLPNLNALHLDRNKLTGHIPKSFGQFIG-NVPDLYLSHNQLSGNIPTSFAQMDFGKHRLSRNKLE- 201
Fortunella margarita         PIQPAIAKLKNLKTLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGTIPGSLSKLQKLGALHLDRNKLTGSIPESSGTFTG-SIPDPYLSHNQLSGKIPASLGSMDFNTIDLSRNKLEG 227
Glycine max                  TIPTTITKLTKLRELNIRYTNISGQIPHFLSQIKALGFLDLSNNKLSGNLPSWLPSLPDLYGISFDNNYISGPIPDLFASVSK-LFTAISLSGNRLIGKIPSLGGKPDMKIVDLSRNMLEG 211
Lycopersicon esculentum      TIPPAIAKLTNLKMLRLSFTNLTGPIPEFLSQLKNLTLLELNYNQFTGTIPSSLSQLPNLLAMYLDRNKLTGTIPESFGRFKGPNIPDLYLSHNSLTGHVPASLGDLNFSTLDFSRNKLEG 226
Malus domestica              PIQPAIAKLKGLKFLRLSWTNLSGSVPDFLSQLKNLTFLDLSFNNLTGAIPSSLSQLPNLNALHLDRNKLTGHIPKSLGQFIG-NVPDLYLSHNQLSGNIPTSFAQMDFTSIDLSRNKLEG 229
Phaseolus vulgaris           PIPPAIAKLTQLHYLYITHTNVSGAIPDFLSQIKTLVTLDFSYNALSGTLPPSISSLPNLGGITFDGNRISGAIPDSYGSFSK-LFTAMTISRNRLTGKIPPTFANLNLAFVDLSRNMLEG 240
Poncirus trifoliata          PIQPAIAKLKNLKMLRISWTNISGPVPDFISQLTNLTFLELSFNNLSGTIPSSLSKLRKLGALHLDRNKLTGSIPDSFGTFTG-SIPDLYLSHNQLSGKIPASLGSMDFNTIDLSRSKLEG 227
Prunus armeniaca             PIQPSIAKLKLLKELRLSWTNISGSVPDFLSQLKNLTFLDLSFSNLTGSIPSWLSQLPNLNALRVDRNKLTGHIPKSFGEFDG-SVPDLYLSHNQLSGTIPTSLAKLNFSTIDFSRNKLEG 229
Pyrus communis               PIQPAIAKLKGLKSLRLSWTNLSGSVPDFLSQLKNLTFLDLSFNNLTGAIPSSLSELPNLGALRLDRNKLTGHIPISFGQFIG-NVPDLYLSHNQLSGNIPTSFAQMDFTSIDLSRNKLEG 229
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Figure 2 continued

54

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Actinidia deliciosa       DISFMFGSNKTIQIVDFSRNKFQFDLSKVVFPQSLTSLDLNHHNKIYGSLFVGLTKLD-LQYLNVSYNRLCGHIPTGGKLQGFDQTSYFHNRCLCGAPLPDCK 327
Citrus iyo A              DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPQSLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYTEYFHNRCLCGAPLER-- 327
Citrus iyo B              DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPESLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYTEYFHNRCLCGAPLER-- 327
Citrus jambhiri A         DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPQSLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYTEYFHNRCLCGPPLER-- 327
Citrus jambhiri B         DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPQSLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYTEYFHNRCLCGAPLER-- 327
Citrus sinensis           DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPQSLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYTEYFHNRCLCGAPLER-- 327
Citrus sp cv sannumphung A DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPQSLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYTEYFHNRCLCGAPLER-- 327
Citrus sp cv sannumphung B DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPQSLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYTEYFHNRCLCGAPLER-- 327
Citrus unshiu             DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPQSLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYTEYFHNRCLCGAPLER-- 327
Eucalyptus camaldulensis  DASVIFGLNKTTQIVDLARNLLEFNLSKVEFPTSLTSLDVNHHNKIYGSIFVEFTQLN-FQFLNVSYNRLCGQIPVGGKLQSFNEYSYFHNRCLCGPPL---- 298
Eucalyptus grandis        DASVIFGLNKTTQIVDLSRNLLEFNLSKVEFPTSLTSLDVNHHNKIYGSIPVEFTQLN-FQFLNVSYNRLCGQIPVGGKLQSFNEYSYFHNRCLCGAPL---- 298
Eucalyptus nitens         DASVIFGLNKTTQIVDLSRNLLEFNLSKVEFPTSLTSLDVNHHNKIYGSIPVEFTQLN-FQFLNVSYNRLCGQIPVGGKLQSFNEYSYFHNRCLCGAPL---- 298
Eucalyptus saligna        DASVIFGLNKTTQIVDLSRNLLEFNLSKVEFPTSLTSLDVNHHNKIYGSIPVEFTQLN-FQFLNVSYNRLCGQIPVGGKLQSFNEYSYFHNRCLCGAPL---- 298
Eucalyptus urophylla      DASVIFGLNKTAQIVDLARNLLEFNLSKVEFPTSLTSLDVNHHNKIYGSIPVEFTQLN-FQFLNVSYNRLCGQIPVGGKLQSFNEYSYFHNRCLCGAPL---- 298
Fortunella margarita      DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPESLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYTEYFHNRCLCGAPLER-- 327
Glycine max               DASVLFGSEKHTERIYLANNLFAFDLGKVRLSKTLGVLDGGHNLIYGTLPKGLTSLKDLYYLDVSYNNLCGEIPRGGKLQEFDASLYANNKCLCGSPLPSCT 313
Lycopersicon esculentum   DVSFLFGKNKTSQVIDLSRNLLEFDISKSEFAESLISLDLNHHNRIFGSLPPGLKDVP-LQFFNVSYNRLCGQIPQGGTLQSFDIYSYLHNKCLCGSPLPKCK 327
Malus domestica           DASVIFGLNKTTQIVDLSRNLLEFNLSKVEFPTSLTSLDINHHNKIYGSIPVEFTQLN-FQFLNVSYNRLCGQIPVGGKLQSFDEYSYFHNRCLCGAPLPSCK 330
Phaseolus vulgaris        DASVLFGSDKNTKKIHLAKNSLAFDLGKVGLSKNLNGLDLRNNRIYGTLPQGLTQLKFLQSLNVSFNNLCGEIPQGGNLKRFDVSSYANNKCLCGSPLPSCT 342
Poncirus trifoliata       DASFLFGLNKTTQRIDVSRNLLEFNLSKVEFPESLTNLDLNHHNKIFGSIPAQITSLENLGFLNVSYNRLCGPIPVGGKLQSFGYKEYFHNRCLCGAPLER-- 327
Prunus armeniaca          DASMIFGLNKTTQIVDLSRNLLEINLSNVEFSKSLTSLDLNHHNKITGGIFVGLTQVD-LQFLNVSYNRLCGQIPVGGKLQSFDSSTYFHNRCLCGAPLPSCK 327
Pyrus communis            DASVIFGLNKTTQIVDLSRNLLEFNLSKVEFPTSLTSLDINHHNKIYGSIPVEFTQLN-FQFLNVSYNRLCGQIPVGGKLQSFDEYSYFHNRCLCGAPLPSCK 327
```

Figure 2 continued
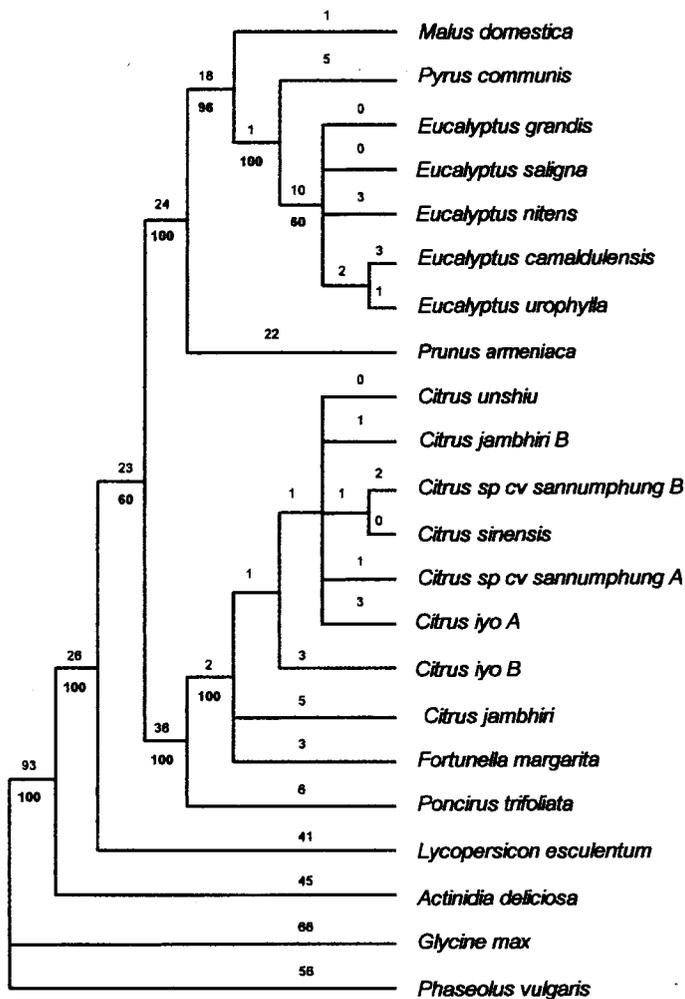
Figure 3

A dendrogram produced with a heuristic analysis from aligned DNA sequences of PGIPs using PAUP. Bootstrap values (%) based on 1000 replications and branch lengths are indicated above and below branches respectively.

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

Eucalyptus camaldulensis      QIPALVGDLPYLETLEFHKDPNLT
Eucalyptus grandis            QIPALVGDLPYLETLEFHKDPNLT
Eucalyptus nitens             EIPALVGDLPYLETLEFHKDPNLT
Eucalyptus saligna            QIPALVGDLPYLETLEFHKDPNLT
Eucalyptus urophylla          QIPALVGDLPYLETLEFHKDPNLT


Eucalyptus camaldulensis      GDASVIFGLNKTTQIVDLARNLLE
Eucalyptus grandis            EDASVIFGLNKTTQIVDLSRNLLE
Eucalyptus nitens             GDASVIFGLNKTTQIVDLSRNLLE
Eucalyptus saligna            EDASVIFGLNKTTQIVDLSRNLLE
Eucalyptus urophylla          EDASVIFGLNKTAQIVDLARNLLE
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Figure 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.

This chapter has been submitted for publication to *DNA Sequence* as:

Molecular analysis of an endopolygalacturonase gene from *Cryphonectria cubensis*, a *Eucalyptus* canker pathogen

Chimwamurombe PM, Wingfield BD, Botha A-M, Wingfield MJ

CHAPTER 3

Molecular analysis of an endopolygalacturonase gene from *Cryphonectria cubensis*, a *Eucalyptus* canker pathogen

ABSTRACT

Cryphonectria cubensis is an important fungal pathogen that causes a serious canker disease of plantation grown *Eucalyptus* worldwide in the tropics and Southern hemisphere. Fungal cell wall degrading enzymes are important during the early stages of interaction of the fungus with *Eucalyptus*. To improve our understanding of the molecular regulation of the interaction of *Eucalyptus* and *C. cubensis*, the relevant genes involved in this interaction should be identified, cloned and studied. As part of this larger objective, the aim of this study was to clone the endopolygalacturonase gene of *C. cubensis*. *Cryphonectria cubensis* was grown on a medium supplemented with *Eucalyptus* cell wall extracts. Degenerate primers were designed to amplify part of the endoPG gene from *C. cubensis* genomic DNA. The resulting sequence was used to design specific primers for use in inverse PCR to amplify the entire endoPG gene of *C. cubensis* (*ccen-1*). The endoPG sequence of *C. cubensis* has 93 % amino acid sequence similarity to that of the chestnut blight pathogen, *Cryphonectria parasitica*.

INTRODUCTION

The plant cell wall is a major barrier that fungal pathogens must circumvent. To do this, fungi secrete a complex of cell wall degrading enzymes (CWDEs) that degrade the complex polymers making up cell walls (Mendgen *et al.*, 1996; Sexton *et al.*, 2000). CWDEs have a specific role in penetration and digestion of plant cell walls to release nutritive sugars that support pathogen growth. Individually, or as a group, CWDEs are potential virulence and pathogenicity determinants in fungal-plant interactions (Walton, 1994).

Pectin is one of the main constituents of plant cell walls. Pectin is comprised of alternating homogalacturonans and rhamnogalacturonans (Carpita and Gibeaut, 1993). To penetrate the pectin defence barriers, fungal pathogens secrete a variety of pectinases. These pectinases include exo/endopolygalacturonases and pectic lyases. Endopolygalacturonases (endoPGs) are secreted earlier than other cell wall degrading enzymes and are thus considered to be important in initiating pathogenesis (Albersheim and Anderson, 1971).

Cryphonectria cubensis is an ascomycetous fungus that causes a serious disease known as Cryphonectria canker on *Eucalyptus* in plantations in the tropics and subtropics (Wingfield, 1999). This disease has seriously damaged seedling stands as well as clonal plantations, and is considered to be one of the more important threats to *Eucalyptus* propagation (Wingfield, 1999). Understanding role of endoPGs in pathogenesis could lead to design of novel ways to protect *Eucalyptus* from endoPG–mediated damage. In this study we thus report on the molecular characterisation of an endopolygalacturonase gene from *Cryphonectria cubensis.*

MATERIALS AND METHODS

Fungal culture and growth conditions

An isolate of *C. cubensis* known to highly pathogenic and which is routinely used in disease screening trials (Van Heerden and Wingfield, 2001) was selected for use in this study. This isolate (CMW2113) is maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) at the University of Pretoria. The fungus was grown for 5 days on malt extract agar plates at 25 °C in the dark. Five plugs (4 mm each) of actively growing margins of the cultures were inoculated into liquid medium. The medium contained: 0.5 g yeast extract (Merck); 1.0 g NaOH, 3.0 g $_{DL}$-Malic acid; 2.0 g NH_4NO_3; 1.0 g KH_2PO4; 0.1 g $MgSO_4$ and was supplemented with 1 % w/v *Eucalyptus* cell wall extracts as a carbon source in a litre (Errampalli and Kohn, 1995). Cell extracts were prepared as outlined by Mwenje and Ride (1997). The culture was incubated, with shaking, at 100 rpm at 25 °C for 10 days. Mycelium was harvested by filtering through Whatman No. 1 filter paper and freeze-dried for storage. All the enzymes used in this study were obtained from Roche Biochemicals (Switzerland) unless stated otherwise.

DNA extraction and degenerate PCR amplification

Freeze-dried fungal mycelium was ground to a fine powder under liquid nitrogen, using a mortar and pestle and sterile glass beads. Genomic DNA was isolated as previously outlined by Chimwamurombe *et al* (2001). All standard DNA techniques were done according to Sambrook *et al* (1989).

Degenerate primers cc2 (5'-TAY AAR GAR TGG GAR GGN CYN CTN ATH-3')

and cc4 (5'-NSW NCC RAT NSW NAG NCC TRG NCC NCC-3') were designed from

conserved regions of fungal endoPG genes to amplify part of the endoPG gene. In the PCR

amplifications the following reaction mixture was used: oligonucleotide primer cc2 (0.6 pmol/

µl), oligonucleotide primer cc4 (0.6 pmol/µl), *C. cubensis* template DNA (25 ng), 1 mM

dNTPs, 1.5 mM $MgCl_2$, 10X PCR buffer, and 5 units of Taq polymerase enzyme in 50 µl.

PCR was performed for 30 cycles (one cycle=1 min at 94 °C, 1.5 min at 62 °C and 1.5 min at

72 °C). The reactions had an initial denaturation step of 4 min at 94 °C and a final elongation

step of 5 min at 72 °C. The PCR products were separated on a 1 % agarose gel stained with

ethidium bromide and visualised under a UV transluminator. PCR fragments were purified

from agarose gel and cloned into pGEM-T-Easy vector (Promega). Ligation mixtures were

used to transform competent *E. coli* (JM109) cells.

Sequence analysis, Southern hydridization and inverse PCR

Sense and antisense strands of six candidate clones from degenerate PCR cloning were

sequenced using the dideoxy-DNA chain-termination method on the ABI-377 Prism

Automated sequencer (Perkin Elmer, USA) using the BigDye Terminator Cycle Sequencing

Reaction Kit (Perkin Elmer, USA). The sequences were used in BLASTX searches and they

were aligned to other published sequences in the GenBank.

Genomic DNA of *C. cubensis* (10 µg) were separately digested for 16 h with

restriction enzymes, *Eco*RI, *Hind*III, *Eco*RV, *Bam*HI and *Pst*I and blotted to nylon

membranes (Roche Diagnostics, Switzerland). A 593-bp *C. cubensis* endoPG PCR fragment

obtained from the degenerate PCR was DIG-labeled (Roche Diagnostics, Switzerland) and

used to probe the southern blots using a hybridisation temperature of 68 °C using the manufacturer's specifications.

To amplify the remainder of the endoPG gene, a modified inverse PCR technique described by Ochman *et al.* (1990), was used. From the southern blots, it was known that a 3.5 kb fragment was generated with *Hind*III digestion (Figure 2). The circularised template DNA was generated from genomic *Hind*III fragments (about 8 μg), which were excised from the gel and purified and was then self-ligated with T4 TDNA ligase overnight. Specific primers icpg1 (5'-ACC GTC GAT AAC ATG TCC-3') and icpg2 (5'-GGC GGT TCT TGC ACC GGA-3') were designed from the 593-bp *C. cubensis* endoPG DNA fragment. In the inverse PCR reactions the following reaction mixture was used: oligonucleotide primer icpg1 (0.6 pmol/ μl), oligonucleotide primer icpg2 (0.6 pmol/μl), circularised *C. cubensis* template DNA (5 ng), 1 mM dNTPs, 1.5 mM MgCl$_2$, 10X PCR buffer, and 5 units of Taq polymerase enzyme in 50 μl. PCR was performed for 30 cycles (one cycle=0.5 min at 94 °C, 0.5 min at 50 °C and 2 min at 72 °C). The reactions had an initial denaturation step of 4 min at 94 °C and a final elongation step of 5 min at 72 °C. Inverse PCR fragments were purified from the agarose gel and cloned into pGEM-T-Easy vector. Ligation mixtures were used to transform competent *E. coli* (JM109) cells. Plasmid DNA was isolated from five candidate clones and was used in cycle sequencing with the BigDye kit (Perkin-Elmer, USA). The sequences were compared to the 593 bp fragment in order to identify the clone containing regions of over and hence gene of interest. The sequence data for *Cryphonectria cubensis* endopolygalacturonase (*ccen-1*) has been deposited in the Genbank database (accession number is AF360316).

RESULTS

A 593 bp fragment was obtained from degenerate PCR amplification and this had high homology to published endoPG sequences as revealed by BLASTX searches. The remaining 5'- and 3'- portions of the *C. cubensis* endoPG (*ccen-1*) were determined by sequencing the inverse PCR products. An additional 1335 bp fragment was sequenced from the inverse PCR products and this sequence contained regions overlapping regions with the previously sequenced 593 bp fragment. The complete ccen-1 is encoded by a single open reading frame of 1320 bp (Figure 1) that is interrupted by two introns that are 64 bp and 145 bp in size (see summary in Table 1). A putative TATA signal was observed at position 194 on the DNA sequence, although no CAAT signals were observed. Southern blot analysis of the *C. cubensis* genome using the 593 bp *ccen-1* fragment showed that there are at least two homologues of the ccen-1 gene (Figure 2). At least two hybridization bands were observed on the DNA blot of the genomic DNA that was completely digested with five different restriction enzymes.

Ccen-1 polypeptide is comprised of 369 amino acids that have a predicted molecular mass of 37.4 kDa and a calculated pI value of 6.42. There are two potential N-linked glycosylation sites. These are $^{218}NIT^{220}$ and $^{300}NGS^{302}$ (Figure 1) and they are conserved in their relative position in the Cryphonectria parasitica endoPG (enpg-1) (Gao *et al.*, 1996). The mature ccen-1 peptide commences at A^{32} (Von Heijne, 1986). Characteristic peptide endoPG signatures $^{226}CXGGHGXSIGSVG$ and ^{263}RIK were observed on the predicted ccen-1 sequence (Rambosek and Leach, 1987). Ccen-1 has 93% peptide sequence similarity to the *C. parasitica* endopg (Figure 3) and the DNA sequences of these endoPGs have 87 % identity. Web-based SOPMA to for secondary structure prediction model showed that ccen-1 belongs to the ß-structural proteins like most pectolytic enzymes (data not shown). ß-structural proteins are those proteins that make a barrel fold made of a single beta-sheet that twists and

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coils upon itself so, in most cases, the first strand in the beta sheet hydrogen bonds to the last strand. This shape has a structural signifance, to for example, enzyme activity.

DISCUSSION

In this study, we have successfully cloned and analysed an endopolygalacturonase gene from *C. cubensis*. To achieve this we used degenerate PCR and a modified inverse PCR technique. The modification involved cutting out a specified region of *Hind*III genomic digests using information obtained from Southern blot analysis. This allows enrichment of circularised template DNA the contained the region of interest and minimises getting smears during PCR, thus few PCR optimisation reactions are done. The ccen-1 gene has at least two homologues in the genome of *C. cubensis* and has a high sequence similarity to that of *C. parasitica* (enpg-1) (Gao *et al.*, 1996). High sequence similarity in endoPGs in related fungi not surprising and has been reported previously in fungi (Chimwamurombe *et al.*, 2001; Posada *et al.*, 2000).

A comparison of *C. cubensis* and *C. parasitica* endoPGs revealed some interesting features. Our results have shown that the introns in the endoPG of *C. cubensis* are 64 bp and 145 bp in size, compared to those of *C. parasitica* that are 61 bp and 62 bp, respectively. The typical introns splices sites, GTPuNGT...NPuCTPuACN...PyAG, are conserved in *C. cubensis* as in other filamentous fungi (Rambosek and Leach, 1987). It would thus be possible to distinguish between the two fungi based on the size of the second intron. In addition, the endoPG peptide signature sequence CXGGHXSIGSVG show that the endoPG of *C. cubensis* lacks the sixth residue of the conserved motif because of a 3 bp deletion in its coding sequence. It was also observed that the two N-linked glycosylation sites are conserved in their relative sites on the endoPGs of *C. cubensis* and *C. parasitica* (Figure 3). This means that these enzymes have may similar glycosylation patterns.

The C-terminal portions of the endoPGs of *C. cubensis* and *C. parasitica* have no amino acid variations. However, variations exist in the N-terminal and middle domains. This may have implications in the substrate specificity of these enzymes as well as their differential ability to interact with polygalacturonase-inhibiting proteins (PGIPs).

We showed that *C. cubensis* endoPG exists as a multicopy gene; therefore the sequence data for the remaining members of this endoPG family are still to be determined. Furthermore, the role of the *C. cubensis* endoPG (ccen-1) in causing diseases, could subsequently be delineated by a targeted-gene disruption approach, although the endoPG in *C. parasitica* has been shown not to be a significant factor in pathogenicity (Gao *et al.*, 1996). However, careful interpretation of this finding (Gao *et al.*, 1996) is required since only one of the endoPG homologues was disrupted and it is possible that the other homologues may be more relevant.

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Table 1. DNA sequence features of *C. cubensis* endoPG (*ccen-1*)

Feature	From	To	Comments
Putative TATA boxes	56	60	Two putative TATA sites
	194	197	
Intron 1	476	538	Size compares to that of *C. parasitica*
Intron 2	668	813	Size is twice that in *C. parasitica*
Exon 1	244	475	Compares to *C. parasitica*
Exon 2	539	667	Compares to *C. parasitica*
Exon3	814	1564	Compares to *C. parasitica*

FIGURES

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1    ccacacccatgagctacatgcctgacgggatgcgtctcacaatggccatttttcatataa    60
61   agaaggcaaacctgctgtcatcaaataatcattgaactgtcaagtcaacactcaagaaac   120
121  attctttcggttaatctgtccatacttaccagcatccttgtcctcggaacaagatattca   180
181  cttcttacctgtgctatagaacacgacctagcttcttgcctctctttctagaatcctacc   240

241  acaATGCTTTCACTCGTTCTGCTCGCGGCCCTGCTGCCCCTGATACAGGCTGTTCCAGCA   300
         M  L  S  L  V  L  L  A  A  L  L  P  L  I  Q  A  V  P  A      19
301  CCTGCTGTGACACCTGCCGCCCAATTGGTAGATAGGGCCAGTGGCTCTTGTACCTTCACG   360
       P  A  V  T  P  A  A  Q  L  V  D  R  A  S  G  S  C  T  F  T    39
361  GATGCTGCCGCTGTCTCAAAGTCCAAGACGTCCTGTGCCACGATCACTCTCAGCAACATT   420
       D  A  A  A  V  S  K  S  K  T  S  C  A  T  I  T  L  S  N  I    59
421  GCGGTTCCTTCTGGCACCACGCTCGACCTTACCAAGCTGAACTCTGGGACTAAGgtgatt   480
       A  V  P  S  G  T  T  L  D  L  T  K  L  N  S  G  T  K         77
481  agacccatacttcttgtcgctgtttggagtcacttcaaactcaccgtccttcaaacagGT   540
                                                                 V    78
541  CATTTTCGAAGGCACTACTTCGTTCGGCTACAAGGAGTGGGAGGGTCTGCTGATTTCGGT   600
       I  F  E  G  T  T  S  F  G  Y  K  E  W  E  G  L  L  I  S  V    98
601  ATCTGGCACAGATATTGAGGTGACCGGAGCCTCTGGACATGTTATCGACGGTAACGGCGC   660
       S  G  T  D  I  E  V  T  G  A  S  G  H  V  I  D  G  N  G  A   120
661  GGCATGGgtgagtggccttctcacgtttgattttcctgacagtccttttggggaagaaa   720
       A  W                                                        122
721  gaaagcccacgatacttttgcagcctcccttgaatcattctgtcacatcctttcaatagt   780
781  ccgattgcaaagtctgaccgcaaaaaatatttagTGGGATGGTGAAGGCAGCAACGGCGG   840
                                          W  D  G  E  G  S  N  G  G  129
841  CAAGACCAAGCCGAAGATGTTCTATGCCCACTCCTTGAAGCAGTCCACCATCAGCGGACT   900
       K  T  K  P  K  M  F  Y  A  H  S  L  K  Q  S  T  I  S  G  L   149
901  GAACGTCAAGAACACTCCAGTTCAGTTCATGAGCATCAACTCAGCCACCGACCTCAACAT   960
       N  V  K  N  T  P  V  Q  F  M  S  I  N  S  A  T  D  L  N  I   169
961  CATTAATGTGAAGATGGACAACTCAGCCGGTGCCAGCAAGGGCCACAACACGGACGCCTT  1020
       I  N  V  K  M  D  N  S  A  G  A  S  K  G  H  N  T  D  A  F   189
1021 TGACGTTGGCTCCTCTGAGAACATCTACATCTCAGGGGCAGTTATCAACAACCAGGATGA  1080
       D  V  G  S  S  E  N  I  Y  I  S  G  A  V  I  N  N  Q  D  D   209
1081 TTGCTTGGCTATCAACTCGGGAACCAACATCACTTTCACTGGCGGTTCTTGCACCGGAGG  1140
       C  L  A  I  N  S  G  T  N  I  T  F  T  G  G  S  C  T  G  G   229
1141 CCACGGCTCAATCGGGTCTGTGGGCGGCCGCTCCGACAATACTGTCAAGACCGTCTCCAT  1200
       H  G  S  I  G  S  V  G  G  R  S  D  N  T  V  K  T  V  S  I   249
1201 CACCACCTCCAAGATCATCACCACCTCGCAGAACGGCGTGCGTATCAAGACTGTCTACGA  1260
       T  T  S  K  I  I  T  T  S  Q  N  G  V  R  I  K  T  V  Y  D   269
1261 CGCGGCTGGTTCCGTGTCCGACGTTCCTTACTCTGGAATCACACTAAGCGGCATTACGAA  1320
       A  A  G  S  V  S  D  V  P  Y  S  G  I  T  L  S  G  I  T  N   289
1321 CTATGGCATCGTCATTGAGCAGGATTACGAGAACGGCAGCCCCACTGGAACCCCTACGAC  1380
       Y  G  I  V  I  E  Q  D  Y  E  N  G  S  P  T  G  T  P  T  T   309
1381 CGGCGTGCCCATCACCGGCCTTACTGTATCCAAGGTTACTGGTTCTGTCGCGTCATCTGC  1440
       G  V  P  I  T  G  L  T  V  S  K  V  T  G  S  V  A  S  S  A   329
1441 GACTGATGTTTACATTCTCTGTGGGAAGGGGTCTTGCTCTGGCTGGAAGTGGTCAGGCAA  1500
       T  D  V  Y  I  L  C  G  K  G  S  C  S  G  W  K  W  S  G  N   349
1501 CAGCGTGACCGGTGGAAAGAAGAGCTCGAGCTGCAAGAATATCCCAAGTGGCGCTTCCTG  1560
       S  V  T  G  G  K  K  S  S  S  C  K  N  I  P  S  G  A  S  C   369
1561 CTGAttcaataatagctttgaggctggagagcaagggtttcaaaacaaccatagacagtt  1620
1621 cgtggtgcctcaaacatgatgctatgagtggcacacttctattcttgatgtttatattct  1680
1681 aattgttaaaaataaaaaagcgctggacatttcgctccaatcaaccgtgtggacaattat  1740
1741 cttgcgttgtgtggctgttccagaatctggtgcactataagtatgatcgtatgaatttat  1800
1801 ttgtgccgttgatgacttcgggactggactcggccagcttggagctaagaagggaaaagt  1860
1861 aggttagcatactctataaaaagtcgacctttttgcacttgttgaagcttcataaaattgt  1920
1921 agctagta                                                       1928
```

Figure 1.

Figure 1.

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The nucleotide sequence and predicted amino acid sequence of the *C. cubensis* endoPG. The start codon is indicated in bold, while the TATA signals are in small case and underlined. Introns are in small case and found within the coding region. The underlined amino acids indicate the characteristic motifs of endopolygalacturonases. The mature endoPG peptide begin at ala[32] indicated in bold.
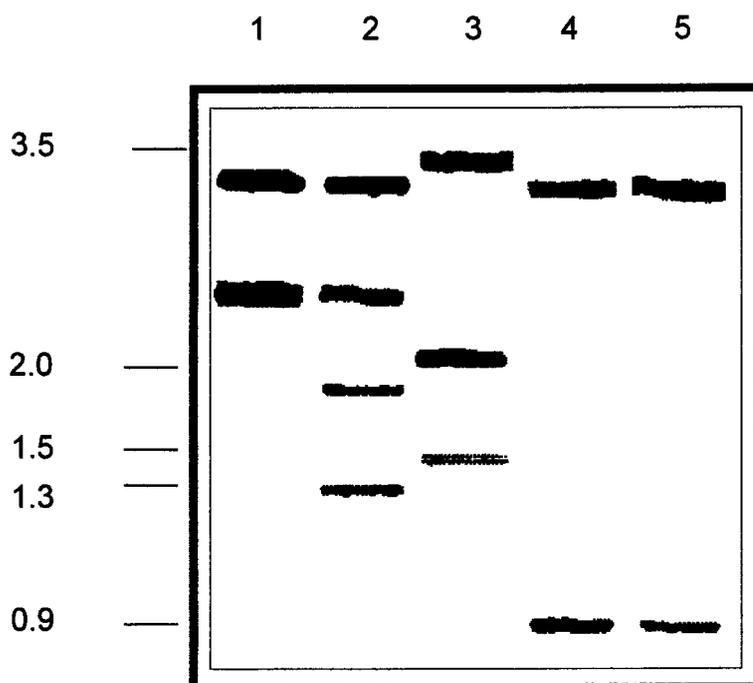
Figure 2

Southern blot analysis. The genomic DNA was digested with enzymes *Eco*RI (1), *Eco*RV (2), *Pst*I (3) *Bam*HI (4) and *Hin*dIII (5). Band sizes were estimated from a DNA marker (λDNA digested with *Eco*R1 and *Hin*dIII)

```
C.cubensis      MLSLVLLAALLPLIQAVPAPAVTPAAQLVDRASGSCTFTDAAAVSKSKTSCATITLSNIA 60
C.parasitica    MFSTLLLAALLPLIQAAPAPAVTPAAHLEDRASKSCTFTDAAAVSKSKASCATITLNNIA 60
                *  *   ********** ******** *  **** ************** ****** ***

C.cubensis      VPSGTTLDLTKLNSGTKVIFEGTTSFGYKEWEGLLISVSGTDIEVTGASGHVIDGNGAAW 120
C.parasitica    -PSGTTLDLTKLNSGTKVIFAGTTSFGYKEWEGPLISVSGTDIEVTGASGHVIDGNGAAW 119
                ******************** ************ **************************

C.cubensis      WDGEGSNGGKTKPKMFYAHSLKQSTISGLNVKNTPVQFMSINSATDLNIINVKMDNSAGA 180
C.parasitica    WDGEGSNGGKTKPKMFYAHSLKQSTIHNLKVKNTPVQFMSINSATDLNVIDVTMDNSAGA 179
                *************************  * ****************** * * *******

C.cubensis      SKGHNTDAFDVGSSENIYISGAVINNQDDCLAINSGTNITFTGGSCTGGHG-SIGSVGGR 239
C.parasitica    SKGHNTDAFDVGSSENIYISGAVINNQDDCLAINSGTNITFTSGSCTGGHGLSIGSVGGR 239
                ****************************************** ******** ********

C.cubensis      SDNTVKTVSITTSKIITTSQNGVRIKTVYDAAGSVSDVPYSGITLSGITNYGIVIEQDYE 299
C.parasitica    SDNTVKTVSITNSKIIN-SQNGVRIKTVYDATGSVSDVTYSGITLSGITNYGIVIEQDYE 298
                *********** ****  ************* ****** **********************

C.cubensis      NGSPTGTPTTGVPITGLTVSKVTGSVASSATDVYILCGKGSCSGWKWSGNSVTGGKKSSS 359
C.parasitica    NGSPTGTPTTGVPITGLTVSKVTGSVASSATDVYILCGKGSCSGWKWSGNSVTGGKKSSS 358
                ************************************************************

C.cubensis      CKNIPSGASC 369
C.parasitica    CKNIPSGASC 368
                **********
```

Figure 3

An amino acid sequence alignment of the endoPGs from *C. cubensis* and *C. parasitica*. The N-linked glycosylation sites are indicated in bold. Asterisks indicate similar amino acids between the two endoPGs.

CHAPTER FOUR

This chapter has been submitted for publication to *Physiological and Molecular Pathology* as:

CHAPTER 4

Production of polygalacturonases in isolates of *Cryphonectria cubensis* of differing pathogenicity

ABSTRACT

Cryphonectria cubensis causes a serious stem canker disease on *Eucalyptus* in tropical and subtropical parts of the world. Previously, it was shown that isolates of *C. cubensis* display varying levels of pathogenicity. The aim of this study was to consider whether a relationship exists between the production levels of polygalacturonases and pathogenicity in isolates of *C. cubensis*. Seven isolates of *C. cubensis* known to have differing levels of pathogenicity were assessed for polygalacturonase production using agarose gel diffusion and reducing sugar assays. Our results showed that the levels of polygalacturonase production are not significantly different in natural isolates of *C. cubensis* that have different pathogenicity levels. However, in one hypovirulent isolate, CMW6009, which has been artificially transfected with hypovirus CHV1-713 from *Cryphonectria parasitica*, a delay of six days in the production of polygalacturonases was observed. We conclude that polygalacturonases probably have a minor role in determining the pathogenicity of the *C. cubensis*. Furthermore, the hypovirus CHV1-713 that causes hypovirulence in *C. cubensis*, has a major role in controlling pathogenicity and its mechanism of action may involve disruption of the production of polygalacturonases and other cell wall degrading enzymes.

INTRODUCTION

The first contact of microbial plant parasites with their host occurs at the plant cell wall surface. During penetration and colonisation, the pathogen secretes plant cell wall degrading enzymes such as endopolygalacturonases. These enzymes are primarily involved in the nectrotrophic stage of pathogenesis (Walton, 1994). Many cell wall degrading enzymes have been reported from fungal pathogens (Bourdart *et al.*, 1998; Centis *et al.*, 1997; Dixon and Lamb, 1990; Fraissinet-Tachet *et al.*, 1995; Le Cam *et al.*, 1994; Wattad *et al.*, 1995). There are some reports that specifically associate the production of endopolygalacturonases in pathogens with pathogenesis (Durrands and Cooper, 1988; Walton, 1994; Le Cam *et al.*, 1994; Ten Have *et al.*, 1998).

Endopolygalacturonases (endoPGs) have been reported to have two opposing roles in fungal pathogenesis. Firstly, they are utilised by fungi as agents involved in disease development and secondly, they are thought to act as potential defence signal molecules (Cervone *et al.*, 1997). The early timing of the production of these endoPGs is consistent with both roles. EndoPGs initiate the production of elicitors for signal transduction. These elicitors are known as oligogalacturonides. They are produced from degradation of homogalacturonan polymer of pectin (Cervone *et al.*, 1989). Degradation of pectic polymers by endoPGs in the presence of polygalacturonase-inhibiting proteins (PGIPs), gives rise to elicitor-active oligogalacturonides. It also increases the residence time of such molecules to act as defence signal molecules (Cervone *et al.*, 1997, Cervone *et al.*, 1989).

Host defence responses include increased production of PGIPs, production of chitinases and glucanases, phytoalexin production, stimulation of the phenylpropanoid pathway, superoxide peroxidations and hypersensitive responses (Cervone *et al.*, 1997). These events halt the progress of disease in an infected plant. The efficacy of plant defence responses

to a pathogen depends strongly on the extent and speed of the onset of the defence signals (Dixon and Lamb, 1990).

Cryphonectria cubensis is a well-known and important canker pathogen of *Eucalyptus* spp. (Hodges, 1980; Wingfield *et al.*, 1989). This pathogen is most important where susceptible *Eucalyptus* spp. are grown in tropical and subtropical countries (Hodges, 1980; Wingfield *et al.*, 1989). Isolates of *C. cubensis* have been shown to display different levels of pathogenicity in both greenhouse and field inoculation trials (Van Heerden and Wingfield, 2001).

In another study, the hypovirus CHV1-713, isolated from the chestnut blight pathogen *Cryphonectria parasitica* (Nuss, 1996) was shown to reduce pathogenicity in a highly virulent South African *C. cubensis* isolate, namely CMW2113 (Van Heerden *et al*, 2001). Furthermore, the transfected isolate produced a bright yellow orange mycelium compared to the white mycelium of the non-transfected isolate.

The economic importance of *Eucalyptus* has justified studies on pathogens such as *C. cubensis*. Of particular interest is a need for knowledge pertaining to infection. In this regard, we have considered the role of cell-wall degrading enzymes, such as polygalacturonases, in pathogenesis. In this study, we report on the production of polygalacturonases *in vitro* by isolates of *C. cubensis* known to have different levels of pathogenicity.

MATERIALS AND METHODS

Fungal Isolates

Seven South African isolates of *Cryphonectria cubensis*, (CMW2113, CMW6103, CMW6106, CMW6097, CMW6087, CMW6111 and CMW6009) were grown at 25 °C on malt extract agar (MEA; 2 % w/v malt extract, 2 % w/v agar) plates for 6 days. The cultures are maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Isolates CMW2113, CMW6103 and CMW6106 have been shown to have high levels of pathogenicity and isolates CMW6087, CMW6097, and CMW6111 are known to have low pathogenicity (Van Heerden and Wingfield, 2001). Isolate CMW6009 represents the hypovirus transfected form of the highly pathogenic isolate CMW2113, which is routinely used in field screening trials to select disease tolerant planting stock. This isolate has been transfected with the hypovirus CHV1-713 from *Cryphonectria parasitica* (Van Heerden *et al.*, 2001). The transfected isolate has subsequently also been shown to be hypovirulent.

Polygalacturonase production in *C. cubensis*

To induce production of polygalacturonases *in vitro*, five mycelial plugs (4 mm^2) were taken from the actively growing margins of cultures on 2 % MEA. These plugs were grown in minimum salts liquid medium (100 ml). The medium contained: 0.5 g yeast extract (Merck); 1.0 g NaOH, 3.0 g $_{DL}$-Malic acid; 2.0 g NH$_4$NO$_3$; 1.0 g KH$_2$PO4; 0.1 g MgSO$_4$ and supplemented with 0.5 % w/v polygalacturonic acid (PGA) (Sigma Chemical Company) as a carbon source in a litre of sterile distilled water (Errampalli and Kohn, 1995). Cultures were incubated with shaking at 100 rpm at 25 °C in the dark for ten days. Samples from the culture vessels were collected on each of the ten days. Mycelium was separated by suction filtration through Whatman No. 113 filter paper using a Buchner funnel. The filtrates were then filter-

sterilised through 0.22-micron disposable syringe filters (Millipore, USA) and stored at 4 °C. All samples were assayed for polygalacturonase activity in triplicate.

Cup-plate agarose diffusion assay

Production of polygalacturonase was assessed by a modified agarose diffusion assay described by Dingle *et al.* (1953). The assay medium contained 0.5 % ammonium oxalate, 0.2 % sodium azide and 1.0 % Type II agarose (Sigma Chemical Co.) dissolved in 100 ml of 0.2 M potassium phosphate buffer (adjusted to pH 5.3). PGA (0.01 %) was used as substrate. The medium was transferred to Petri dishes (20 ml per plate). A 4 mm cork borer was used to punch three wells 2.5 cm apart in the solid gel. Each well was filled with 30 µl of endoPG standard, blank control and the filtrate. The plates were incubated overnight at 30 °C. After incubation, the gel was developed by flooding the plates with 10 ml of 0.05 % ruthenium red (Sigma Chemical Co., USA) for 2 h at 25 °C. Excess dye was removed by washing the plates several times with distilled water.

A distinct clear zone on the stained agarose gel indicated PG activity. Two diameter readings (at right angles to each other) of the zones were taken from duplicate plates and the average value was calculated. Each isolate was independently tested three times. Production of polygalacturonases was calibrated against a dilution series of *Aspergillus niger* endoPG (418 unitsml⁻¹, Sigma Chemical Co., USA, one unit equals the amount of enzyme required to catalyse the production of a reducing sugar per minute). Assays without enzyme served as controls. The mean diameter readings were compared for all the isolates and for each day. Differences in the ability of isolates to produce polygalacturonases were analysed using Tukey 's multiple comparison method from SAS software (SAS Statistical Software, USA).

Reducing sugar assay

Polygalacturonase activity in the different filtrates was determined by measuring the reducing-end groups using the p-hydroxybenzoic acid hydrazide (PAHBAH) method (York *et al.*, 1985). This measurement was done to confirm the outcome of agarose gel diffusion assays. The PAHBAH assays were calibrated against a dilution series of D-galacturonic acid. The activity of polygalacturonases were determined by incubating 50 µl of the different samples in a 1 ml solution containing 0.25 % w/v PGA and 40 mM sodium acetate (pH 5.0) for 1 h at 30 °C. This reaction was terminated by addition of 1.5 ml freshly prepared 5 % PAHBAH. The sample tubes were boiled for 10 minutes and cooled to room temperature before taking absorbance readings at 410 nm using a spectrophotometer (Pharmacia LKB. Ultrospec III, Sweden). The assays were performed in triplicate. Statistical analyses of data were similar to those for the agarose diffusion assays. One unit of enzyme activity was defined as the amount of enzyme producing 1 µmol of reducing group per minute at 30 °C in 40 mM of sodium acetate (pH 5.0).

RESULTS

Cup-plate agarose diffusion assay

All the isolates of *C. cubensis* produced polygalacturonases. The maximum polygalacturonase activity was reached at different times, although the amounts produced were statistically similar (F=6.21, DF=179, P<0.0001) (Table 1) for all except the transfected isolate CMW6009. The transfected isolate showed a delay of 6 days before polygalacturonases were produced. This was considerably longer than for the remaining isolates (Figure 1). The weakly pathogenic isolate CMW6087 behaved differently. It displayed a gradual increase in polyagalacturonase production until 8 days, which was different to he behaviour of other isolates.

Reducing sugar Assay

No significant differences were observed in the production of polygalacturonases for naturally occurring *C. cubensis* using the reducing sugar assay (F=6.21, DF=179, P<0.0001). However, isolate CMW6009 showed a delayed production of polygalacturonases. A similar trend had been observed with agarose diffusion assays described above. The mean units of PG activity in all the isolates were 0.2 $\mu molml^{-1}min^{-1}$ (see Table 1). PG production during the course of this study is illustrated in Figure 2.

DISCUSSION

Results of this study show that there are no significant differences in the ability of *C. cubensis* isolates that differ in pathogenicity to produce polygalacturonases *in vitro*. However, the hypovirulent isolate CMW6009 displayed an obviously delayed production of polygalacturonases. These results are consistent with those of previous studies where production of polygalacturonases in pathogens has not been tightly linked to varying levels of pathogenicity (Scott-Craig *et al.*, 1990; Gao *et al.*, 1996; Di Pietro and Roncero, 1998). Our findings also suggest that polygalacturonases are not an important determinant in the pathogenicity of *C. cubensis*.

In a previous study (Chimwamurombe *et al.*, 2001), we observed that the DNA sequences of polygalacturonase-inhibiting proteins (PGIPs) in selected *Eucalyptus* species have very similar amino acid sequence. This suggests a low diversity of the PGIPs in the host plant. A high variability and diversity of PGIPs suggests that the host plant is constantly under evolutionary pressure to adapt to a greater diversity of endoPGs (Leckie *et al.*, 1999; Stotz *et al.*, 2000). Since the PGIPs of *Eucalyptus* have low diversity, it can be expected that the endoPGs of the pathogen, *C. cubensis*, would also have a low diversity. Furthermore, this suggests that there are few isoforms of polygalacturonases in *C. cubensis*.

The role of endopolygalacturonases in pathogenicity is known to differ for different fungal species (Cleveland and Cotty, 1991; Gao *et al.*, 1996; Ten Have *et al.*, 1998; Scott-Craig *et al.*, 1990). In some species, there is a strong correlation between the production of endopolygalacturonases and pathogenicity, while in others there is no such relationship (Cleveland and Cotty, 1991; Di Pietro and Roncero, 1998; Walton and Cervone, 1990). In *C. parasitica*, a close relative of *C. cubensis*, targeted disruption of the endopolygalacturonase (enpg-1) gene resulted in no reduction of pathogenicity on American chestnuts (Gao *et al.*, 1996). This implies that the role of endoPGs in the pathogenicity of *C. parasitica* is minor.

However, the results of Gao and co-workers (1996) require careful interpretation. Only one isoform of the endoPG was disrupted in their study and this did not result a reduction in pathogenicity. The possibility that other isoforms may still be functional must be considered, because fungi can produce different isoforms of endoPGs at the onset of infection (Fraissinet-Tachet *et al.*, 1995; Yao and Koller, 1995). In the present study, all the possible isoforms of polygalacturonases were collectively assayed (including exopolygalacturonase activity). We thus feel confident in our suggestion that polygalacturonases are not a determinant in the pathogenicity of *C. cubensis.*

There are reports that show that polygalacturonases are involved in the pathogenicity of *Botrytis cinerea* and *Aspergillus flavus* (Ten Have *et al.*, 1998; Cleveland and Cotty, 1991). The invasiveness of *A. flavus* in wounded cotton bolls was also found to be closely associated with the production of specific fungal polygalacturonases (Cleveland and Cotty, 1991). In *B. cinerea*, production of an endopolygalacturonase (Bcpg1), was responsible for its high level of virulence on tomatoes and apples (Ten have *et al.*, 1998). Therefore, even though endoPGs appear not to be involved in the pathogenicity of *C. cubensis*, such relationships must be investigated individually from different pathogens.

Results of this study showed that the *C. parasitica* hypovirus (CHV1-713) results in a decrease in the production of polygalacturonases in *C. cubensis*. This provides support for the view that the virus could be useful in biological control of Cryphonectria canker. *In planta*, the isolate has reduced pathogenicity on *Eucalyptus grandis* (Van Heerden *et al.*, 2001). In *C. parasitica*, the same hypovirus has been reported to decrease the accumulation of enzymes such as laccases, cutinases, cellobiohydrolases and polygalacturonases and it is associated with hypovirulence (Nuss, 1996). Therefore, it is not surprising that a reduction in polygalacturonase production is also observed in *C. cubensis*. This decrease in

polygalacturonse may contribute to the reduced virulence although it is unlikely to be the sole cause of the reduced pathogencity observed in *C. cubensis*.

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Table 1. Polygalacturonase production in isolates of *C. cubensis* differing in pathogenicity[A].

Isolate number	Relative Pathogenicity	No. of days to attain maximum activity	Units of PG activity[a] ($\mu mol\, ml^{-1} min^{-1}$)
CMW2113	high	8	0.19
CMW6103	high	4	0.18
CMW6106	high	7	0.20
CMW6087	low	8	0.19
CMW6097	low	5	0.21
CMW6111	low	5	0.19
CMW6009	low	8	0.20

[a] values are an average of three repeats, and not significantly different (F=6.21, DF=179, P<0.0001).

[A] Agarose diffusion method of Dingle *et al.*, 1953 was used to determine these units of acitivity of polygalacturonases.

FIGURES
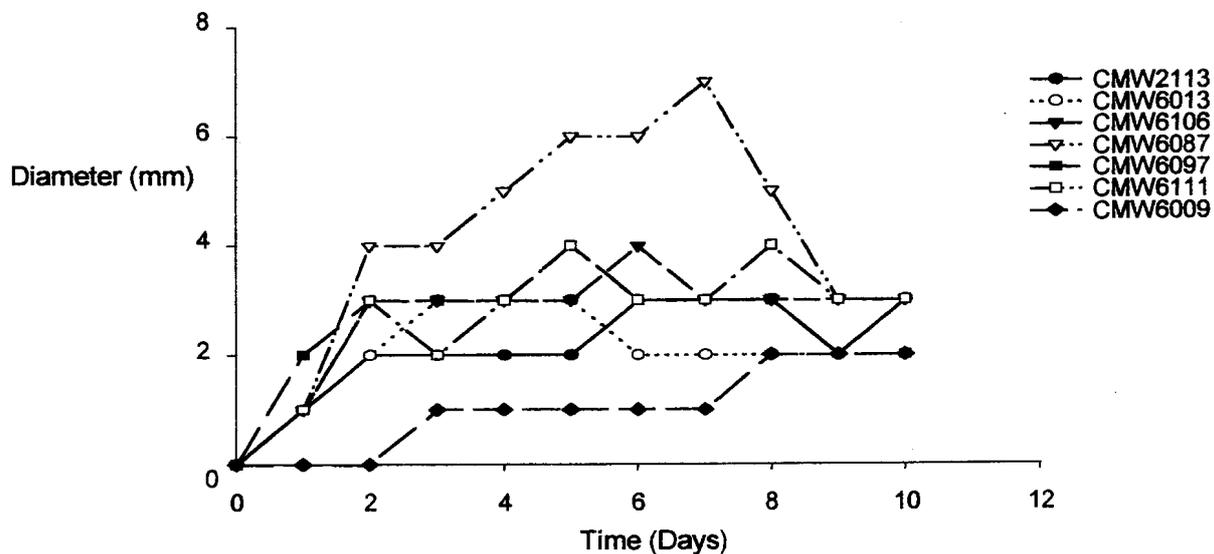
Figure 1

Graphical representation of the trend of polygalacturonase production by seven isolates of *C. cubensis* in minimum salts medium for 10 days. Diameter values are in mm and are averages of three repeats. (Standard error bars are omitted to reduce clumsiness)
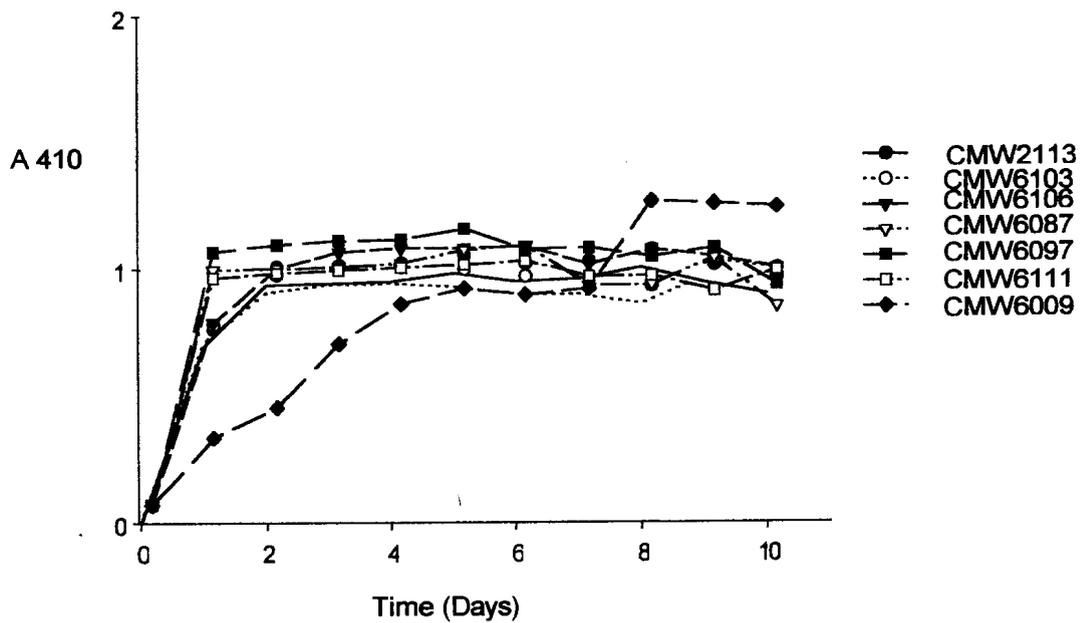
Figure 2

The trend of production of polygalacturonases for seven isolates of *C. cubensis* cultured for 10 days in minimum salts medium. Absorbance values (A_{410}) are averages of three repeats. There is a characteristic slow rise in absorbance values for isolate CMW6009. The other six isolates have statistically identical rates of production of polygalacturonases. (Standard error bars are omitted to reduce clumsiness).

CHAPTER FIVE

This chapter has been submitted for publication to *Journal of Phytopathology* as:

Inhibition of polygalacturonases from four tree pathogens by stem extracts from two *Eucalyptus grandis* **clones**

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CHAPTER 5

Inhibition of polygalacturonases from four tree pathogens by stem extracts from two *Eucalyptus grandis* clones.

ABSTRACT

Eucalyptus grandis is an important exotic forest plantation species in many parts of the world. Diseases caused by fungal pathogens are an important constraint to the productivity of *Eucalyptus* plantations. Fungi secrete cell wall degrading enzymes (CWDEs) as part of their colonisation strategy on plants. Endopolygalacturonases (endoPGs) constitute some of the important CWDEs. The aim of this study was to detect and compare the amounts of polygalacturonases produced by group of different *Eucalyptus* pathogens. A further objective was to determine the inhibition levels of polygalacturonase-inhibiting proteins (PGIPs) associated with a disease tolerant and a susceptible *Eucalyptus* clone on endoPGs from the fungal pathogens. Extracts of the polygalacturonases were collected from selected isolates of the *Eucalyptus* canker pathogens *Cryphonectria cubensis*, *Coniothyrium zuluense*, *Botryosphaeria dothidea* and the root pathogen *Phytophthora cinnamomi*. Polygalacturonase production and inhibition by PGIPs was measured by reducing sugar assays. We found that *B. dothidea* and *C. zuluense* produced more endoPGs than *C. cubensis* and *P. cinnamomi*. In most cases PGIPs from the generally disease tolerant clone were equally as effective as those from the susceptible clone in their capacity to inhibit the endoPGs. An exception was for *C. zuluense*, where PGIPs from the resistant clone were considerably more effective than those from the susceptible clone. We conclude the PGIP extracts from *Eucalyptus* selectively inhibit endoPGs from different species. The tolerance of TAG5 clones to *C. zuluense* could be linked to the ability of its PGIPs to inhibit endoPGs from *C. zuluense*

INTRODUCTION

Pectic polysaccharides are a major component of primary cell walls of most plants (Carpita & Gibeaut, 1993). The breakdown of pectin structures is one of the first functions of fungal pathogens during infection (Albersheim & Anderson, 1971; Favaron, *et al.*, 1997; Walton, 1994, 1997). This is achieved by secretion of pectin degrading enzymes such as polygalacturonases, especially endopolygalacturonases (endoPGs). There are also reports that specifically associate the production of endoPGs by pathogens with pathogenesis (Ten Have *et al.*, 1998; Walton, 1997).

Plants possess systems that prevent the enzymatic breakdown of pectin. Susceptibility of pectin to degradation by endoPGs can be affected by the formation of calcium bridges across pectin chains (Favaron *et al.*, 1997). Formation of such bridges result in decreased rates of degradation of pectin by endoPGs. Furthermore, plants possess proteins that inhibit the action of these enzymes and these are known as polygalacturonase-inhibiting proteins (PGIPs). These proteins reduce the rate of hyphal penetration into plants and allow plants to mobilise other defence systems to halt disease development (Cervone *et al.*, 1989; De Lorenzo & Cervone, 1997; Stotz *et al.*, 2000).

EndoPGs have been reported to have dual and opposing roles in fungal pathogenesis. Firstly, they are utilised by fungi as agents involved in disease development and secondly, they are thought to act as potential defence signal molecules. The early production of these endoPGs is consistent with both roles (Cervone *et al.*, 1997). It has been shown that PGIPs favour the formation of elicitor-active oligogalacturonides from pectin degradation by endoPGs (Cervone *et al.*, 1989).

PGIPs possess structural characteristics of leucine rich repeats (LRRs) similar to those of signal transduction molecules (De Lorenzo *et al.*, 1994; Jones & Jones, 1997). They can be induced by salicylic acid, wounding and fungal pathogens. This illustrates the importance of

PGIPs in general host defence responses to stress (Bergmann *et al.*, 1994; Nuss *et al.*, 1996; Favaron *et al.*, 1997). The host defence responses include production of chitinases and glucanases, phytoalexin production, lignification and hypersensitive responses (Cervone *et al.*, 1997).

The economic importance of *Eucalyptus* in commercial plantation forestry worldwide has justified studies on pathogens that damage this tree species. Some of the most important stem and root pathogens of plantation eucalyptus include *Cryphonectria cubensis*, *Botryosphaeria dothidea*, *Phytophthora cinnamomi* and *Coniothyrium zuluense* (Van Heerden and Wingfield, 2001; Van Zyl, 1999; Smith *et al.*, 1994, 1996; Linde *et al.*, 1999). *C. cubensis* causes Cryphonectria canker; *B. dothidea* causes die back and cankers in plantations in temperate areas , *C. zuluense* causes a stem canker disease in the tropics and subtropics and *P. cinnamomi* is a well known root pathogen on *Eucalyptus* in many parts of the world (Van Heerden and Wingfield, 2001; Fischer *et al.*, 1993; Van Zyl, 1999; Smith *et al.*, 1994, 1996; Linde *et al.*, 1999).

The role of cell-wall degrading enzymes, such as polygalacturonases, in pathogenesis is important because they are involved in the first step towards a pathogenic interaction of the fungus and the host. Similarly, knowledge of PGIPs is valuable due to the role that they play in combating the action of the endoPGs produced by fungal pathogens. Knowledge of the role of polygalacturonases could lead to the design of novel PGIPs that have a broad specificity to inhibit endoPGs from different fungal pathogens. The aim of this study was, therefore, to detect and compare the amounts of polygalacturonases produced by the fungal pathogens. Our subsequent aim was to assess the inhibition of these enzymes by extracts from two *Eucalyptus grandis* clones that have varying disease tolerance levels.

MATERIALS AND METHODS

Fungal Species

Isolates used in this study were specifically chosen from other studies on the pathogens of interest and where they had been selected for their high levels of pathogenicity. Thus isolates CMW2113, CMW7218, CMW7217 and CMW2100 were chosen for the pathogens *C. cubensis*, *B. dothidea*, *P. cinnamomi* and *C. zuluense*, respectively (Van Heerden and Wingfield, 2001; Slippers (personal communication); Linde *et al.*, 1999; Van Zyl, 1999). The cultures are all maintained in the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI), University of Pretoria. Prior to testing, the pathogens were grown on a 2 % malt extract agar (2g malt extract, 2g agar, 100 ml water) in the dark.

Plant Material

Tissue culture microplants of *E. grandis* clones, TAG5 and ZG14 were used in this study. TAG5 is known to be reasonably tolerant to diseases and clone ZG14 is known to be highly susceptible to *B. dothidea*, *C. zuluense* and *C. cubensis* in pathogenicity tests. They are also respectively tolerant and susceptible to damage by insects such as termites (Van Zyl, 1995).

Eucalyptus cuttings were grown on modified solid MS medium at pH 5.8 (Murashige & Skoog, 1962) at 24 °C under fluorescent lights with 16-hour light and 8-hour dark cycles. 16h/day. Modifications to the MS media included the addition of vitamins (10 mll^{-1} of 1000X stock), sucrose (4 gl^{-1}), Benzylaminopurine (0.2 mgl^{-1}), Naphthalene acetic acid (0.01 mgl^{-1}). The 1000X vitamin stock consisted of the following: myo-inositol (10 gl^{-1}), thiamine-HCl (67.3 mgl^{-1}), nicotinic acid (246.2 mgl^{-1}), pyridoxine-HCl (61.69 mgl^{-1}), calcium panthothenic acid (47.65 mgl^{-1}), biotin (4.9 mgl^{-1}), choline (13.96 mgl^{-1}), riboflavin (37.64 mgl^{-1}), ascorbic acid (17.61 mgl^{-1}) and glycine (2 mgl^{-1}) (Sigma Chemical Company).

Production of polygalacturonases

Polygalacturonases were induced *in vitro* by taking five mycelial plugs (4 mm^2) from the actively growing margins of each of the fungi and inoculating these into minimum salts liquid medium (100 ml) in Erlenmeyer flasks. The medium contained: 0.5 g yeast extract (Merck); 1.0 g NaOH; 3.0 g $_{DL}$-Malic acid; 2.0 g NH$_4$NO$_3$; 1.0 g KH$_2$PO4; 0.1 g MgSO$_4$ and supplemented with polygalacturonic acid (PGA) (5g/l) (Sigma Chemical Company) as a carbon source in a litre of sterile distilled water (Errampalli and Kohn, 1995). The cultures were incubated in the dark with shaking (100 rpm) at 25 °C for 10 days (when polygalacturonase production levels were highest). Samples from the culture vessels were collected on each of these days. Mycelium was separated by suction filtration with a Buchner funnel. The filtrates were then filter-sterilised through 0.22-micron disposable syringe filters (Millipore, USA) and stored at 4 °C. All samples were assayed for polygalacturonase activity in three independent repeats. Protein concentrations were determined by measurement of absorbance values at 280 nm using bovine serum albumin as a standard. Equal amounts of protein were used in the assays.

Production of PGIP extracts

To induce PGIPs, the plantlets were removed from the culture bottles and salicylic acid (50 mM) was sprayed onto the stems. The plantlets were then placed back on to solid MS medium aseptically. The culture vessels containing the treated plantlets were covered with aluminium foil. Control plants were not subjected to the treatment with salicylic acid. After 24 hr, PGIPs were extracted with salt washes using a protocol modified from Salvi and co-workers (1990). The plants were finely ground using a mortar and pestle for 4 min in cold 4 mM sodium acetate buffer (pH 5.2) containing 500 mM sodium chloride (2 ml buffer/g plant tissue) at 4 °C. The suspension was vacuum filtered through a Whatman No. 113 filter paper

and the filtrates were centrifuged at 13000 g for 30 min. The supernatant was dialysed (membrane: molecular weight cut off 8000) against 5 litres of 20 mM sodium acetate (pH 5.0) at 4 °C overnight. The sample was centrifuged at 13 000 g for 30 min and the supernatant was concentrated by placing it in a dialysis bag and covering it with several changes of polyethylene glycol powder (Mwt 6000) until the volume was reduced four times. This PGIP extract was dialysed for 24 h with 5litres of 20 mM sodium acetate. The PGIP extract was then centrifuged for 30 min at 13 000 g and the supernatant was stored at 4 °C. Protein concentration of the various PGIP extracts were determined by measurement of absorbance values at 280 nm using bovine serum albumin as standard. Equal amounts of protein were used in the assays.

Enzymatic activity of fungal endoPGs

Polygalacturonase activity in the different filtrates was determined by measuring the reducing-end groups using the p-hydroxybenzoic acid hydrazide (PAHBAH) method (York *et al.*, 1985). The activity of each polygalacturonase extract was determined by incubating 50 µl of extract in a 1 ml solution containing PGA (25g/l) and 40 mM sodium acetate (pH 5.0) for 1 h at 30 °C. This reaction was terminated by addition of 1.5 ml of freshly prepared PAHBAH (50g/l). The sample tubes were boiled for 10 minutes and cooled to room temperature before taking absorbance readings at 410 nm using a spectrophotometer (Pharmacia LKB. Ultrospec III, Sweden). The assays were repeated three times. Statistical analyses of data were done using SYSTAT software (SYSTAT, 1997). Assays without enzyme served as controls. The absorbance readings were compared for all the species and for each repeat. Differences in the ability of species to produce polygalacturonases were analysed using analysis of variance and Tukey 's multiple comparison method.

Inhibition of fungal polygalacturonases by PGIP extracts

To determine the inhibition capacity of extracts from ZG14 and TAG5, a modified PAHBAH assay was used (York *et al.*, 1985). In this assay, equal concentrations of PGIP extracts and equal concentrations of polygalacturonase extract were used to enable comparisons of inhibition levels. The activity of each PGIP extract, including an untreated control to rule out artefact inhibition, was determined by incubating 50 µl of PGIP extract and 50-µl polygalacturonase extract. The mixture was incubated at 25 °C for 20 min. From this mixture, 80 µl was added to a 1 ml solution containing PGA (25g/l) and 40 mM sodium acetate (pH 5.0) and then incubated for 1 h at 30 °C. One hour was chosen because it was the time taken for optimum polygalacturonase activity to be reached. The reaction the mixture was boiled for 10 min and placed on ice immediately. Thereafter, 250 µl of sterile distilled water and 750 µl of freshly prepared PAHBAH (50g/l) were added. The tubes were boiled for 10 minutes and cooled to room temperature before taking absorbance readings at 410 nm using a spectrophotometer (Pharmacia LKB. Ultrospec III, Sweden). The assays were repeated three times. The readings at time (t=0 min) were used as blanks. Statistical analyses of data were done using SYSTAT (SYSTAT, 1997). Assays with PGIP extracts from untreated plants and those without PGIPs were used as controls.

RESULTS

Production of polygalacturonases

C. zuluense and *B. dothidea* produced more polygalacturonases than *C. cubensis* and *P. cinnamomi* (F=6400.972, df=3, P=0.0001). *C. zuluense* and *B. dothidea* had similar levels of production of polygalacturonases and the same trend was observed for *C. cubensis* and *P. cinnamomi* (Figure 1). Production of polygalacturonases was recorded as absorbance values at

wavelength 410 nm. A higher absorbance value corresponded to more polygalacturonase enzyme produced. The maximum absorbance was reached after 60 min for all four fungal pathogens (data not shown).

Inhibition of polygalacturonases by PGIP extracts

Polygalacturonases from *C. zuluense* and *B. dothidea* were inhibited to the same extent by PGIPs extracts from TAG5 (F=18.797, df=11, P=0.001). Polygalacturonases from *C. cubensis* and *P. cinnamomi* were inhibited more strongly than those from the other two pathogens (Figure 2). The polygalacturonases from *C. cubensis* and *B. dothidea* were inhibited similarly while those from *P. cinnamomi* were most inhibited by the PGIPs from ZG14.

TAG5 and ZG14 PGIPs inhibited *P. cinnamomi* polygalacturonases at approximately the same level. The same was observed for *C. cubensis*. Interestingly, TAG5 PGIPs inhibited *C. zuluense* polygalacturonases more than those from ZG14 (F=17.58, df=11, P=0.001) (Figure 2.). An opposite result was observed for the polygalacturonases extracted from *B. dothidea*.

DISCUSSION

In this study, we have shown that the generally resistant *Eucalyptus grandis* clone TAG5, produces PGIP extracts that have a greater capacity to inhibit the polygalacturonases of *C. zuluense* than the generally susceptible clone ZG14. These results suggest that PGIPs are involved in the ability of TAG5 to protect itself against endoPG–mediated damage from *C. zuluense*. Similar observations have been made from the leek plants (*Allium porrum* L) (Favaron *et al.*, 1997). In addition, our results suggest that TAG5 produce PGIPs that are

specifically able to inhibit the polygalacturonases produced by *C. zuluense*. Similarly, the results of this study also suggest that *C. zuluense* requires polygalacturonases as part of its suite of characteristics linked to pathogenicity on *Eucalyptus*. Similar findings have been reported for *Aspergillus flavus* on cotton (Cleveland and Cotty, 1991) and *Botrytis cinerea* infection of tomatoes and apples (Ten Have *et al.*, 1998).

The inhibition of polygalacturonases of *C. zuluense* by TAG5 and ZG14 PGIP extracts is particularly intriguing. We have previously observed that in *Eucalyptus* species there is high amino acid sequence similarity amongst PGIPs (Chimwamurombe *et al.*, 2001) and those of TAG5 and ZG14 are identical. However we observed what seems to potentially be a differential PGIP specificity to inhibit *C. zuluense* PGs. Differential glycosylation of PGIPs of identical amino acids sequences has previously been reported (Leckie *et al.*, 1999). We, therefore suggest differential glycosylation patterns in the β-strand/ β-turn motif of the PGIPs of the TAG and ZG14 *Eucalyptus* clones could explain our observations. Differential glycosylation is believed to interfere with the ligand binding capacity of PGIP and hence specificity to polygalacturonases (Leckie *et al.*, 1999).

C. cubensis and *P. cinnamomi* endoPGs were equally inhibited by TAG5 and ZG14 PGIP extracts. Although there are many factors that control pathogenicity of fungi, our results tempt us to suggest that PGs are potentially not a major factor in the pathogenicity of either fungus since we have previously observed that isolates of *C. cubensis* with varying pathogenicity produce similar amounts of endoPGs (Chimwamurombe *et al.*, submitted). Therefore, it is not surprising that *C. cubensis* endoPGs were inhibited equally by extracts of both disease tolerant and disease susceptible clones. If endoPGs were a major component of the pathogenicity determinants of *C. cubensis* on *Eucalyptus*, then differences in the inhibition by the two *Eucalyptus* PGIP extracts should be detected.

PGIP extracts from ZG14 inhibited the polygalacturonases from *B. dothidea* to a greater extent than those from TAG5. This observation was intriguing as an opposite result was expected. There are two possible reasons for this observation. Firstly, it is known that *B. dothidea* is an endophyte and latent pathogen of *Eucalyptus* (Smith *et al.*, 1994, 1996), which only leads to disease after trees have been stressed. The tissue culture plants that were used in this study were already under tissue culture stress and thus the TAG5 plants, which are naturally tolerant to infection *B. dothidea*, could have been stressed by the study conditions. Another possible explanation for the contradictory results found with *B. dothidea* could be that the PGIPs from ZG14 have greater specificity for polygalacturonase of *B. dothidea* than those from TAG5. Thus ZG14 PGIPs would inhibit *B. dothidea* polygalacturonases more that those from TAG5. This would then imply that polygalacturonases are not required for the pathogenicity of *B. dothidea*. This would be consistent with reports from *Cochliobolus carbonum* on a maize (Scott-Craig *et al.*, 1990) and *Cryphonectria parasitica* on chestnuts (Gao *et al.*, 1996) where endoPG disruption studies showed that the pathogenicity of these fungi is not dependent on endoPGs.

The overall results of this study have shown that polygalacturonases are not limiting factors for the pathogenicity of *C. cubensis*, *P. cinnamomi* and *B. dothidea* while they are required for the pathogenicity of *C. zuluense* on *Eucalyptus* plants. TAG5 and ZG14 PGIP extracts have different capacities to inhibit polygalacturonases from *C. zuluense* and this, at least in part, explains the tolerance of TAG5 against polygalacturonase-mediated damage by *C. zuluense* in field experiments (Van Zyl, 1999). It is clear from these results that expression of TAG5 PGIPs may improve the tolerance of ZG14, and potentially could provide an option to reduce endoPG-mediated damage of ZG14 plants by *C. zuluense*.

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FIGURES
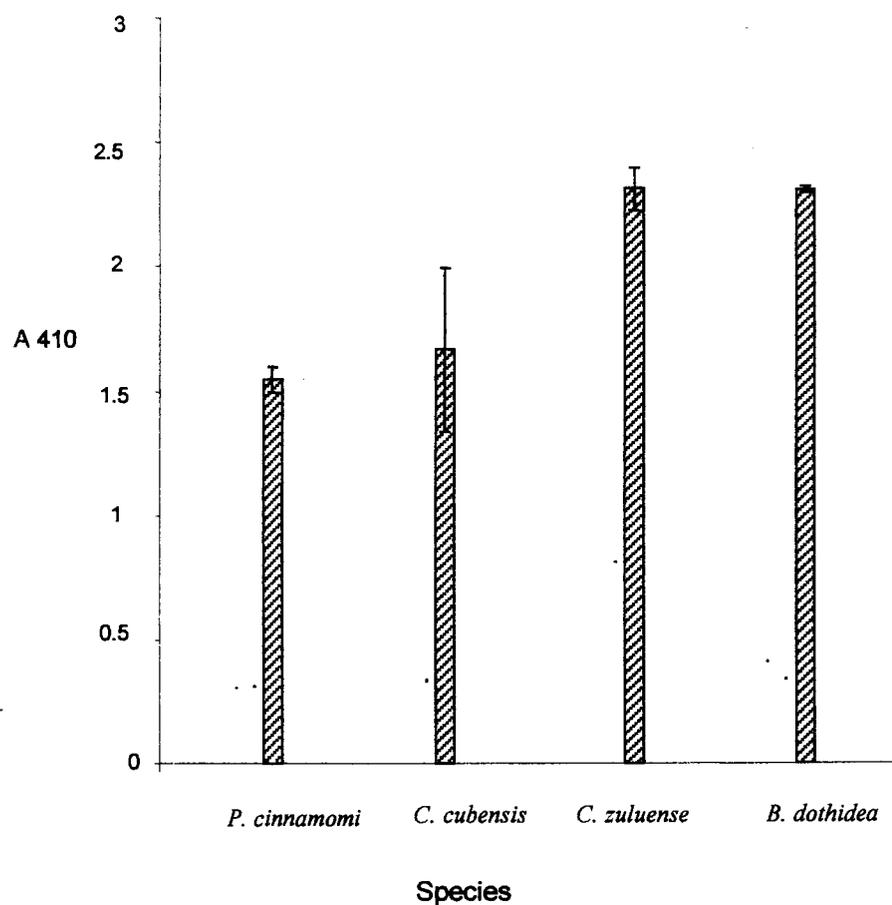
Figure 1

Graphical representation of the production of polygalacturonase from fungal species at t= 60 min. The graph indicates data points for the maximum polygalacturonases for each of the fungal species. The bars indicate the standard error of the means of three replicates.

108
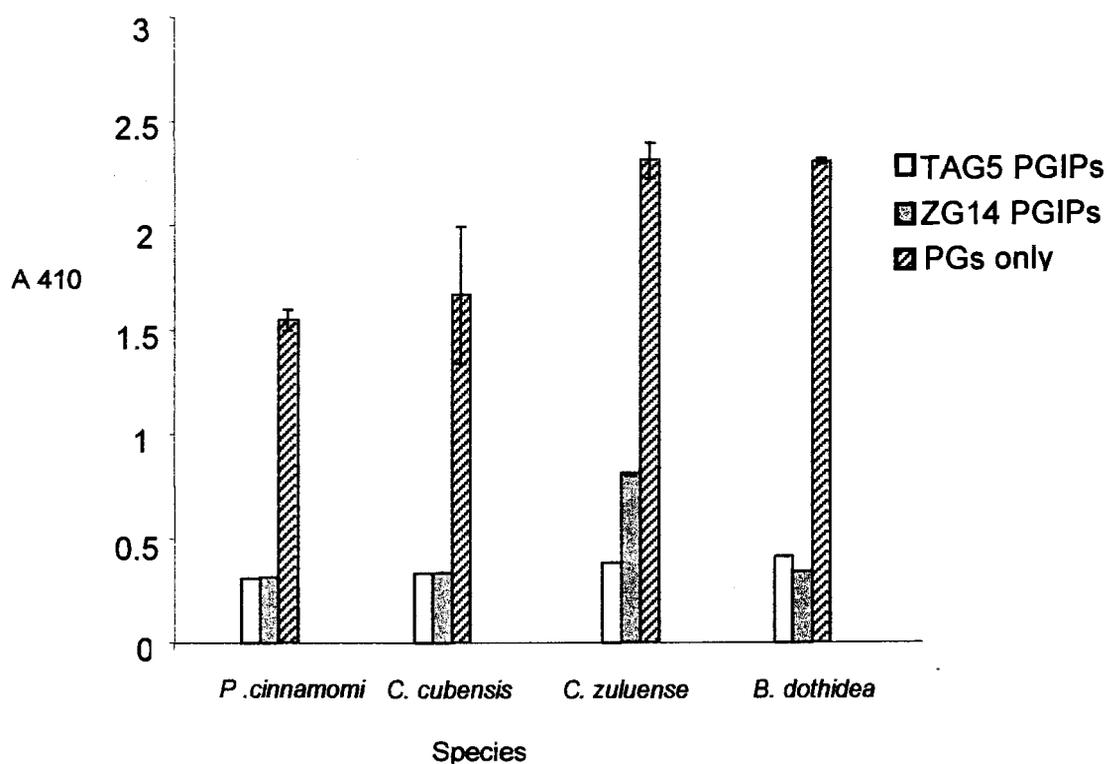
Figure 2

Comparing the levels of inhibition of fungal polygalacturonases by PGIPs from by TAG5 and ZG14 at t=60 min. The absorbance values of the untreated samples were subtracted from those of the salicylic acid-treated samples. The bars indicate the standard error of the means of three replicates.

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Cloning and sequence analysis of the endopolygalacturonase gene from the pitch canker fungus, *Fusarium circinatum*

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CHAPTER 6

Cloning and sequence analysis of the endopolygalacturonase gene from the pitch canker fungus, _Fusarium circinatum_

ABSTRACT

The fungus _Fusarium circinatum_ causes pitch canker disease on mature pine trees and root rot and damping-off of pine seedlings. Endopolygalacturonases (endoPGs) play a major role during penetration of plants by fungi. Digestion of the pectic polysaccharides in the plant primary cell walls is one of the earliest functions of endoPGs during infection. The research objective was to clone and characterize the gene encoding endopolygalacturonase in _F. circinatum_. A 970-bp DNA fragment was cloned using degenerate PCR amplification from _F. circinatum_ DNA. Sequence data for this fragment were used to design specific primers for use in genome walking to amplify and sequence the remaining portion of the _F. circinatum_ endoPG gene (_Fcpg_). The amino acid sequence predicted from this gene showed 90 % and 87 % similarity to _Fusarium oxysporum_ and _Fusarium moniliforme_ endoPGs, respectively. Southern hybridisation showed that _F. circinatum_ contains only one copy of this endopolygalacturonase gene.

INTRODUCTION

Endopolygalacturonases (poly[1,4-α-D-galacturonide] glycanohydrolase, EC 3.2.1.15) are the first cell wall degrading enzymes that are produced by fungal pathogens during infection (Centis *et al.*, 1996; Cervone *et al.*, 1989). Therefore, endopolygalacturonases (endoPGs) play a role in the establishment of fungal pathogens. Several features of the endoPGs indicate a multiple role for them during pathological and physiological events characterized by targeted degradation of the cell wall (Hahn *et al.*, 1989). EndoPGs digest plant cell walls to produce small oligosaccharides that are assimilated by the fungus. They also release oligogalacturonides that function as elicitors for plant defense responses (Cervone *et al.*, 1989). The levels of elicitor-active oligogalacturonides increase *in vitro* upon interaction with plant cell proteins, called polygalacturonase-inhibiting proteins (PGIPs). This may trigger the hypersensitive response in incompatible plant–pathogen interactions (Hahn *et al.*, 1989; Cervone *et al.*, 1989).

The fungus *Fusarium circinatum* is the causal agent of an important disease known as pitch canker on mature pine trees. In South Africa, it causes root rot and damping off in pine seedlings (Viljoen and Wingfield, 1994). The role of endoPGs in the pathogenicity of *F. circinatum* has never been considered. The aim of this study was to clone and sequence the gene that encodes for endoPGs in *F. circinatum*. This forms part of a concerted effort to expand the base of knowledge pertaining to the physiology and biology of the pitch canker fungus.

MATERIAL AND METHODS

Fungal culture, growth conditions and DNA isolation

F. circinatum, isolate MRC6213, was obtained from the culture collection of the Forestry and Agricultural Biotechnology Institute (FABI) and was cultured in malt extract broth at 25 °C in a 250-ml conical flask for 10 days without shaking. Fungal mycelium was harvested by filtration and freeze-dried. Total genomic DNA was isolated from the fungal mycelia according to the protocol of Raeder and Broda (1985).

PCR Amplification and Cloning

DNA manipulations were done according to standard protocols in Sambrook *et al.* (1989). All PCR reactions were done in a Hybaid Omnigene (UK) thermocycler. Degenerate primers FPG1 (5'-GA(CT)AA(CT)GA(CT)TT(CT)(GA)A(CT)CC(GCT)AT(CT)-3') and FPG2 (5'-CA(AGCT)GT(AG)TT(AGCT)GT(AGC)GG(AG)TA(AG)TT(AG-3') (Arie *et al.*,1998), were used to amplify endoPG fragments. The PCR products were cloned into the polylinker region of pGEM-T-Easy vector (Promega, UK).

Plasmid DNA was isolated from recombinant bacterial clones and sequenced using SP6 (5'-TAATACGACTCACTATAGGG-3') and T7 (5-'TATTTAGGTGACACTATAG-3') primers with an ABI Prism model 377 sequencer (Perkin-Elmer Inc, USA). The sequence was used to design *F. circinatum* endoPG-specific primers FcPG1 (5'-GGGGGAAAATACTTTGGG-3'), FcPG2 (5'-GGTATATTCTGTGTGGTG-3'), FcPG3 (5'-CCACTAATGACAATGGGG-3') and FcPG4 (5'-GGTGGTGGTAAGACTAGC-3').

Genome Walking and Sequence Analysis

Genome walking protocol was modified from Siebert *et al.* (1995). To amplify the regions on both the 5' and 3' ends of the partial *Fcpg* sequence, genomic DNA (3 µg) of *F. circinatum* was digested for 24 h with enzyme *Hin*dII (20 units). The digested fragments were resuspended in 20 µl of water. Ten µl of the digested fragments were ligated to 5 µl adaptors (50 pmol/µl) for 16 h at 10 °C. The structure of adaptors is illustrated in Figure 1. Ligated DNA was purified over Qiagen PCR clean-up columns and diluted 100-fold.

A primary PCR reaction was performed with primer pairs AP1 (5'-GATCATAATACGACTCACTATAGGG-3'), FcPG1 or AP1, FcPG2. A secondary PCR reaction was performed in the same way as the primary PCR reaction using primer pairs AP2 (5'-AATAGGGCTCGGGCGG-3'), FcPG3 or AP2, FcPG4 for 20 cycles. (genome-walking strategy on Figure 1). Distinct PCR fragments were purified from agarose gels and were cloned into pGEM-T-Easy vector. Plasmids with inserts were sequenced with T7 and SP6 primers. Sequences were analyzed with Sequence Navigator (Perkin-Elmer) and were used to draw UPGMA trees using computer package PAUP. Fcpg DNA and peptide sequence alignments were produced using the CLUSTAL program (http://www.2.ebi.ac.uk). Secondary structure prediction plots were obtained using a web based SOPMA tool (http://www.expasy.ch/tools/#secondary/).

Genbank accession number: The sequence data for *Fusarium circinatum* endopolygalacturonase was deposited at the Genbank database under accession number AF207825.

RESULTS

Cloning and sequence analysis of *Fcpg*

The sequence of a PCR fragment of *F. circinatum* genomic DNA obtained with primers FcPG1 and FcPG2 was found to be 970 bp in size and 85 % identical to that of the endoPG of *F. oxysporum*. By genome walking towards the 5' and 3' ends, distinct fragments of about 500 bp and 300 bp, respectively were amplified. Sequence analysis of the cloned fragments showed that they had overlapping stretches with the previously sequenced 970-bp endoPG fragment. In total, 1561 bp of DNA sequence was obtained (data not shown). This part of the genome contained the full coding region for an endoPG. This was supported by the high similarities with other *Fusarium* endoPGs, *i.e.*, 90 % and 87 % similar to *F. oxysporum* and *F. moniliforme*, respectively (Di Pietro and Roncero, 1998; Caprari *et al.*, 1993b).

F. circinatum endoPG is encoded by a gene of 1332 bp in length and from sequence comparison with published endoPG sequences of *Fusarium* spp., *Fcpg* has four introns. Intron sizes were consistent with those of most filamentous fungi, between 49 bp and 85 bp (Rambosek and Leach, 1987). Some putative promoter motifs were present in the 5' non-coding region.

The peptide sequence of Fcpg has 374 amino acids and a molecular mass of 38.8 kDa with a theoretical pI of 6.63. There are two potential N-glycosylation sites of the Fcpg peptide sequence while the endoPGs of *F. oxysporum* and *F. moniliforme* have 3 and 4 sites, respectively. These two N-glycosylation sites are conserved in all the three *Fusarium* spp. The characteristic motifs, CXGGHGXSIGSVG and RIK (Centis *et al.*, 1996) for fungal endopolygalacturonases were also observed in the predicted Fcpg peptide sequence. Secondary structure prediction plots showed that the Fcpg belongs into β-structural proteins class (results not shown).

Relatedness of *Fusarium* endopolygalacturonases

An unweighted pair group method with arithmetic averages (UPGMA) tree was produced using the endoPG peptide sequence of the three *Fusarium* spp. and *Cryphonectria parasitica* as outgroup (Figure 2). The tree shows that the *F. circinatum* endoPG sequence is more closely related to that of *F. oxysporum* than it is to *F. moniliforme*.

DISCUSSION

In this study, we report on the nucleotide sequence of *Fcpg*, the gene that encodes for the endopolygalacturonase in the pitch canker fungus *F. circinatum*. *Fcpg* occurs as a single copy in the genome (data not shown). This is consistent with previous studies on *F. oxysporum* and *F. moniliforme* (Capari *et al.*, 1993a; Di Pietro and Roncero, 1998). The *F. moniliforme* endoPG enzyme has four different isoforms (Capari *et al.*, 1993b). These are derived from differential glycosylations of one polypeptide. The fact that Fcpg has two potential N-glycosylations compared to four in *F. moniliforme* endoPG suggests that the number of isoforms in *F. circinatum* may be fewer than those in *F. moniliforme*.

The peptide sequence of Fcpg has an interesting characteristic in the signal peptide region. The endoPG signal peptide sequences of *F. moniliforme* and *F. oxysporum* are very different (Di Pietro and Roncero, 1998). Alignment of endoPG signal peptide sequences shows that the Fcpg peptide has two motifs PSSSLQERD and AIAALPAA. Remarkably, the PSSSLQERD motif is present in *F. moniliforme* endoPG and is absent in *F. oxysporum* endoPG. The AIAALPAA motif is present in *F. oxysporum* and is absent in *F. moniliforme* endoPG. Fcpg possesses both of these motifs. It is therefore possible to distinguish between the three *Fusarium* species using only the signal peptide sequence. This may have implications in the evolution of endoPGs in *Fusarium* spp.

We have cloned and sequenced the endopolygalacturonase gene of *Fusarium circinatum* using degenerate PCR and an elegant technique of genome walking. This gene has been shown to occur only once in the genome and is closely related to the endoPGs of other *Fusarium* spp.

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FIGURES

Adaptor: 5' CTA ATA CGA CTC ACT GGG CTC GAG CGG CGG CCC GGG CAG GT 3'
3'H$_2$N-CCG GTC CA-P 5'
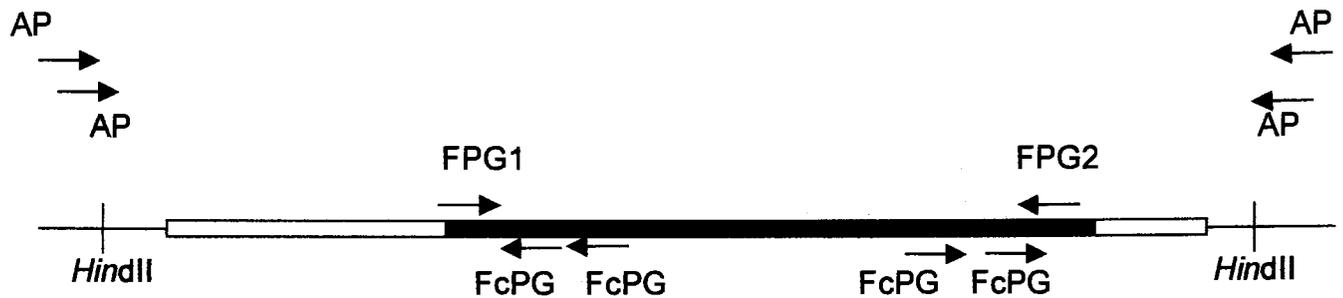
Figure 1

Genome-walking Strategy. The shaded box represents a 970-bp fragment of *F. circinatum* endoPG, and the open box depicts an unknown endoPG region to be sequenced. The restriction enzyme *Hin*dII cleaves in the region outside the endoPG coding region. Arrows point to the direction of amplification. Names of primers are indicated. Primers AP1, AP2 bind in the adaptor region.
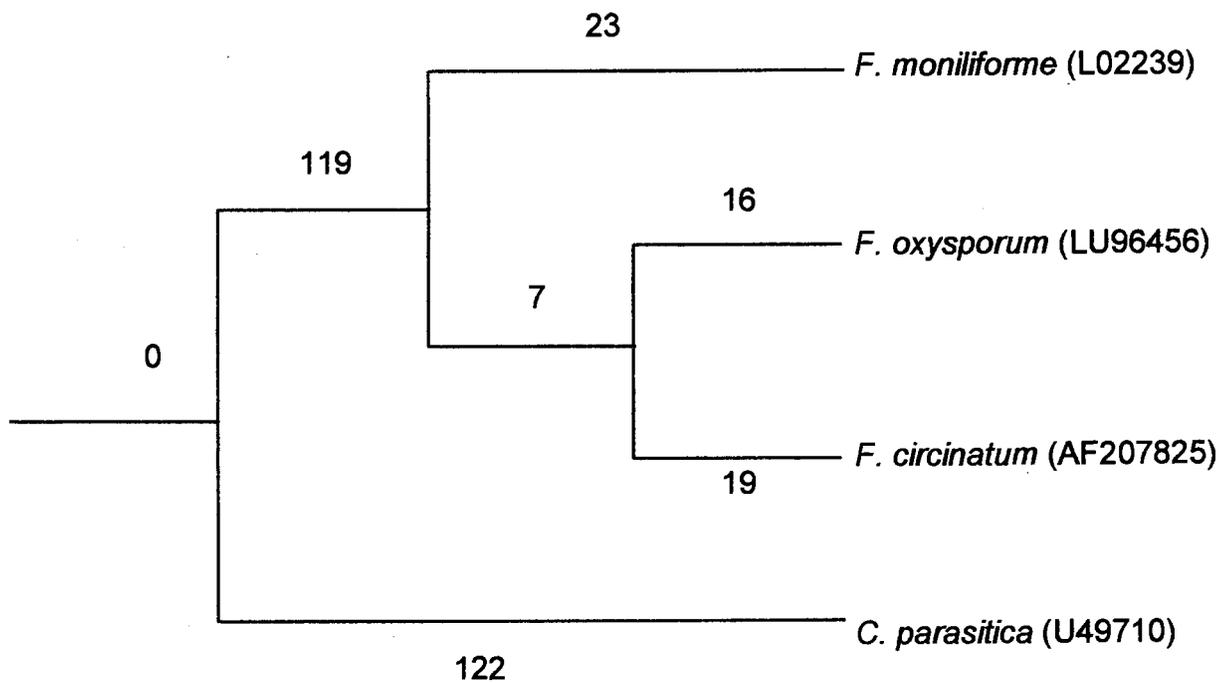
Figure 2

A UPGMA tree produced from an alignment of peptide sequences of fungal endopolygalacturonases. The number of differences is indicated.

SUMMARY

Phytopathogenic fungi parasitise plants for survival. Knowledge of the interactions between plants and these fungi is of importance in designing control strategies. In this thesis the interactions of *Eucalyptus* polygalacturonase-inhibiting proteins (PGIPs) and fungal polygalacturonases (PGs) were characterised. About thirty years of research on the interaction between PGIPs and PGs was reviewed in Chapter 1.

The gene sequences of the mature PGIP peptides from selected *Eucalyptus* species were determined in Chapter 2. The PGIP genes have very high similarities among themselves, signifying conservation of the function of PGIPs in this genus.

Molecular characterisation of the endoPG gene of *C. cubensis* was presented in Chapter 3. This endoPG gene occurs more than two times in the genome of *C. cubensis*. The gene sequences of the other endoPGs remain to be determined.

EndoPG production by *C. cubensis*, a *Eucalyptus* canker pathogen, was presented in Chapter 4. It was found that polygalacturonases probably have a minor role in determining the levels of pathogenicity in different *C. cubensis* isolates. However, the hypovirus CHV1-173 has a role in reducing polygalacturonase production in infected hypovirulent *C. cubensis* isolates.

Chapter 5 dealt with the interaction between *Eucalyptus* PGIPs and endoPGs from different fungal pathogens. *Coniothyrium zuluense* and *Botryosphaeria dothidea* produced more PGs than *C. cubensis* and *Phytophthora cinnamomi* in liquid culture. Disease tolerant *Eucalyptus* TAG5 clones produced PGIPs that are more effective in inhibiting endoPGs from *C. zuluense* than the susceptible *Eucalyptus* ZG14 clones.

In Chapter 6 the gene sequence of the endoPG of *Fusarium circinatum* was determined. This fungus causes the pitch canker disease in pine trees. This endoPG gene occurs as a single copy and is related to those of other *Fusarium* spp.

The results presented in this thesis add to the current scientific knowledge pertaining to the role of PGIPs and PGs in pathogenic interactions, especially, between *Eucalyptus* PGIPs and fungal endoPGs. Interaction of *Eucalyptus* PGIPs and fungal endoPGs was demonstrated; therefore it is possible to genetically engineer *Eucalyptus* for more disease tolerant *Eucalyptus* plants using PGIP genes, especially, against *Coniothyrium zuluense*. This means that more research is needed to identify genes that will give a more global protection against many fungal pathogens.

OPSOMMING

Plantpatogeniese fungi voer 'n bestaan deur op plante te parasiteer. Dit is dus van groot belang om die interaksies tussen plante en hierdie fungi te verstaan. Kennis van hierdie onderwerp help met die voorkoming en beheer van fungale infeksies wat oesverliese beperk. In hierdie tesis word die interaksie tussen *Eucalyptus* poligalakturonase-inhiberende proteiene (PGIPe) en *Cryphonectria cubensis* poligalakturonases (PGe) bestudeer. *Fusarium circinatum* PGe is ook op vlak van DNA-basisvolgorde gekarakteriseer.

Hoofstuk een is 'n opsomming van 30 jaar se navorsing op die interaksie tussen plant PGIPe en fungi PGe. Verskillende aspekte van beide PGIPe en PGe se biologiese rolle moet egter nog opgeklaar word. Sommige aspekte van sel-sel seining, gekoppel aan selwand-afbrekende ensieme en patogenesiteit, word ook aangeraak.

In Hoofstuk twee word die geenbasisvolgordes van volwasse PGIP-peptiede van uitgesoekte kommersiële *Eucalyptus* spesies bepaal. Hierdie volgordes dui daarop dat daar groot ooreenkomste tussen die verskillende PGIP-gene is. Dit is 'n aanduiding van die konservering van funksie vir PGIPe in hierdie genus. Dit bevestig ook die mening dat PGIPe 'n bepaalde rol het in plantverdediging.

Hoofstuk 3 handel oor die produksie van endoPGe deur *C. cubensis* wanneer dit op *Eucalyptus* selwandekstrakte gegroei word. Die geenvolgorde van een van hierdie endoPGe is bepaal. Hierdie geen kom meer as tweekeer in die fungusgenoom voor. Afskeiding van hierdie endoPGe bevestig dat hulle tydens vroeë infeksie benodig word. Die ander endoPGe se geenvolgordes moet nog bepaal word.

Die produksie van endoPG deur *C. cubensis*, ook 'n *Eucalyptus* patogeen, is in Hoofstuk vier bestudeer. Hierdie studie is gedoen om lig te werp op die rol wat endoPGe speel in plantpatogenisiteit speel. Poligalakturonases slegs 'n klein rol tydens die

patogenisiteitsbepaling van verskillende *C. cubensis* isolate. Daar word ook gewys dat die

hipovirus CH1-173 'n groot invloed op die verlaging in poligalakturonase produksie in *C.*

cubensis isolate het. Hierdie hipovirus verlaag ook die virulensie van beide *C. cubensis* en *C.*

parasitica. Die verlaging in virulensie kan 'n weg volg wat selwandafbrekende-ensieme, soos

endoPGe, se produksie stop.

Hoofstuk vyf bestudeer die interaksie wat tussen *Eucalyptus grandis* PGIPe en fungale

Pge plaasvind. Al die fungi wat hier gebruik is, is patogenies op *Eucalyptus* en sluit

C.cubensis, Coniothyrium zuluense, Botryosphaeria dothidea en *Phytophthora cinnamomi* in.

Daar word gewys dat *C. zuluense* en *B. dothidea* meer PGe produseer as *C. cubensis* en *P.*

cinnamomi wanneer hulle in vloeibare medium gegroei word. Meer effektiewe inhibering van

C. zuluense endoPGe vind deur PGIPe vanaf bestande *Eucalyptus* TAG5-klone plaas.

Poliglakturonase-inhiberende proteïene vanaf vatbare *Eucalyptus* ZG14-klone lewer minder

inhibering. Daar is gevind dat *Eucalyptus* PGIPe, endoPGe van verskillende patogeniese

sepsis, in verskillende grade inhibeer. Dit bevestig die nut van individuele raming van die

kwalitatiewe-en kwantitatiewe effekte van PG-PGIP-interaksies tydens 'n

bestandheidsrespons.

Die endoPG-basispaarvolgorde van *F. circinatum* is in Hoofstuk 6 bepaal. Hierdie

fungus is die patogeen wat "pitch canker"-siekte in dennebome veroorsaak. Die endoPG-geen

kom ook as 'n enkelkopie in die genoom voor en is naby verwant aan die endoPGe wat in *F.*

moniliforme (=F. vertcilliloides) en *F. oxysporum* aangetref word.

Die werk wat in hierdie tesis weergegee word, het bygedra tot die kennis oor die rol

van PGIPe en PGe tydens patogeniese interaksies, veral met betrekking tot die proteïene wat

deur *Eucalyptus* en *C. cubensis* geproduseer word. Dit blyk dat die PGIPe van *Eucalyptus*

spesies naby verwant is en nie gebruik kan word om vir bestandheid in teelprogramme te toets

nie. As daar individueel na die endoPGe gene wat in *F. circinatum* voorkom gekyk word, blyk

dit nie asof hulle 'n groot rol in patogenesiteitsbepaling speel nie. Aangesien interaksie tussen *Eucalyptus* PGIPe en *C. cubensis* endoPG voorkom, sal dit wel moontlik kan wees om met genetiese manipulasie plante te produseer wat meer bestandheid toon.

APPENDICES

APPENDIX A: Gene sequence of *Fusarium circinatum* endopolygalacturonase used in Chapter 6

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                   gacgatataaaccaatggaaccggcgcttattaaaagtgtgtgataccc      -49
cgagggatcactcacattattattatcatttcccacccaccacccacccacccgttatag       0
ATGGTTCGGAACATTGCTCTCGCGGCTTTGCTCCCAGCTGCCTGGGCTCTGCCTTCAAGC      60
 M   V   R   N   I   A   L   A   A   L   L   P   A   A   W   A   L   P   S   S     20
TCTgtaagccaaacagacccttcaacaacaattcaatcactaatgaactgaaatacagCT     120
 S                                                                  21
CCAGGAACGAGATGCTTGCACCGTGACTGACTACTCTGGCCTCGCCACCGCTGTCTCATC     180
 L   Q   E   R   D   A   C   T   V   T   D   Y   S   G   L   A   T   A   V   S   S    42
CTGCACAAACATCGTGCTCAAAGGTTTCCAAGTCCCGACTGGCAAGCAACTTGATCTTTT     240
 C   T   N   I   V   L   K   G   F   Q   V   P   T   G   K   Q   L   D   L   F     62
CAAGCTCAAGGCTGGTACAACCGTTACCTTCAAGGGCACAACTgtaagcaacttcctttg     300
 L   K   A   G   T   T   V   T   F   K   G   T   T                   76
ttctttctgaggtctactgaagctgaatgtcttagACCTTTGCCACCACTGCCGATAACG     360
                                       T   F   A   T   T   A   D   N      84
ACTTTAACCCCATTGTCATTAGTGGAAGTGGCATCACCATCACTGGTGCATCTGGCCATG     420
 D   F   N   P   I   V   I   S   G   S   G   I   T   I   T   G   A   S   G   H     104
TCATCGATGGCAATGGCCAAGCATATTGGGATGGCCAAGGTTCCAACAACGCAGACAACC     480
 V   I   D   G   N   G   Q   A   Y   W   D   G   Q   G   S   N   N   A   D   N     124
CAAAGtattttcccccaatacctgctgcagaggctatactgacttataaaggCCCGATC     540
 P   K                                                           P   D    126
ACTTCATCGTTGTTAAGAAGGTTACCGGTACCTCAAAGATTACAAACCTCAACATCCAGA     600
 H   F   I   V   V   K   K   V   T   G   T   S   K   I   T   N   L   N   I   Q     146
ACTGGCCCGTTCACTGCTTCGACATCACCGGCAGTTCAGATCTGACCATCTCGGGGCTCA     660
 N   W   P   V   H   C   F   D   I   T   G   S   S   D   L   T   I   S   G   L     166
TTCTCGATAACAGAGCAGGCGACAAGCCCAATTCCAAGAGCGGTGGTTTGCCCGCTGCGC     720
 I   L   D   N   R   A   G   D   K   P   N   S   K   S   G   G   L   P   A   A   H    189
ATAACAGCGACGGTTTCGACATCTCGTCCTGTGACCACGTCACGCTGGATAACAACCACG     780
 N   S   D   G   F   D   I   S   S   C   D   H   V   T   L   D   N   N   H   V     209
TTTATAACCAGGATGACTGTGTTGCCGTCACCTCTGGCACAAACATCGTCGTTTCTAACA     840
 Y   N   Q   D   D   C   V   A   V   T   S   G   T   N   I   V   V   S   N   M     229
TGTACTGCTCCGGTGGTCATGGTCTCAGCATCGGTTCTGTCGGCGGAAAGAGCACCAATG     900
 Y   C   S   G   G   H   G   L   S   I   G   S   V   G   G   K   S   T   N   V     249
TCGTGAATGGTGTCCAGTTCTTGAACTCACAAATTGTGAACAGTGAGAATGGATGCCGCA     960
 V   N   G   V   Q   F   L   N   S   Q   I   V   N   S   E   N   G   C   R   I    269
TCAAGTCCAACTCTGGCACAACTGGCACGgtcagtaaacactcaagtcaccttattatgt    1020
 K   S   N   S   G   T   T   G   T                                278
agctaggctaactgatgatcagATCAACAACGTTACCTACCAAAACATTGCTCTTACCAA    1080
                        I   N   N   V   T   Y   Q   N   I   A   L   T   N    291
CATCAGCAAGTACGGTGTTGATGTCCAGCAGGACTACCTCAACGGCGGCCCTACTGGAAA    1140
 I   S   K   Y   G   V   D   V   Q   Q   D   Y   L   N   G   G   P   T   G   K    311
GCCCACCAACGGCGTCAAGATTAGCAACATCAAATTCACCAAGGTCACTGGCACGGTGGC    1200
 P   T   N   G   V   K   I   S   N   I   K   F   T   K   V   T   G   T   V   A    331
TAGCTCAGCTCAGAATTGGTATATTCTGTGTGGTGATGGTAGCTGCTCTGGATTTACTTT    1260
 S   S   A   Q   N   W   Y   I   L   C   G   D   G   S   C   S   G   F   T   F    351
CTCTGGCAACGCCATTACTGGTGGTGGTAAGACTAGCAGCTGCAACTATCCTACCAACAC    1320
 S   G   N   A   I   T   G   G   G   K   T   S   S   C   N   Y   P   T   N   T    371
CTGTCCCTCTTAAttagggaaatttaaacatcatgattgatagacccattgataatcatt    1380
 C   P   S                                                        374
gtttcatgttagttcattcccccgaggaggtagttaggtttgtttaaatatattttatat    1440
ctgactgggtt                                                      1451
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