ISOLATION AND CHARACTERIZATION OF A POSSIBLE POLYGALACTURONASE-INHIBITING PROTEIN FROM WHEAT

By

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■ LIST OF ABBREVIATIONS ■

A

ABS	absorbance
Api	apiose (see Appendix B)
Ara	arabinose (see Appendix B)
avr	avirulence gene

B

BCIP	5-bromo-4-chloro-3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
bp	base pair(s)

C

CWDE	cell wall degrading enzyme

D

Da	Dalton
Dha	2-keto-3-deoxy-D-lyxo-heptulosaric acid (see Appendix B)
dicot(s)	dicotyledon(s)
DP	Degree of polimerization

E

ECL	enhanced chemi-luminescence
EDTA	ethylene-diaminetetraacetic acid
EPG	endopolygalacturonase

F

FDEGEN	DNA fragment as amplified by degenerate primers
FSTOTZ	DNA fragment as amplified by patented primers
Fuc	fucose (see Appendix B)

G

g	centrifugal force
g	gram
Gal	galactose (see Appendix B)
Glc	glucose (see Appendix B)

H

h	hour(s)
h.p.i.	hours post inoculation
h.p.t.	hours post treatment
ha	hectare(s)
HG	homogalacturonan
HPLC	high performance liquid chromatography
HR	hypersensitive reaction

K

kb	kilobase(s)
Kdo	2-keto-3-deoxy-manno-octulosonic acid (see Appendix B)

M

µCi	microCurie
$\mu Em^{-2}s^{-1}$	microEinstein per square meter per second
µJ	microJoule
µM	micromolar
m	meter
M	molar
MALDI-TOF MS	Matrix-assisted laser desorption-ionization time of flight mass spectrometry
mg	milligram(s)
min.	minute(s)
mL	milliliter(s)
mM	millimolar
monocot(s)	monocotyledon(s)

N

NBT	nitroblue tetrazolium

O

ORF	open reading frame

P

PCR	polymerase chain reaction
PGA	polygalacturonic acid
pgip	polygalacturonase-inhibiting protein (DNA)
PGIP	polygalacturonase-inhibiting protein (amino acid)
PGIP-I	antibody generated in a rabbit against purified bean PGIP
PGIP-II	antibody raised in a rabbit against synthetically produced peptide corresponding to 11 amino acids of the N-terminal sequence of bean PGIP

PMSF	phenylmethanesulfonylfluoride
PR	pathogenesis related
PVP	polyvinylpyrrolidone

R

R	resistance gene
RG-I/II	rhamnogalacturonan-I/II
Rha	Rhamnose (see Appendix B)
rpm	revolutions per minute

S

SA	salicylic acid
SAR	systemic acquired resistance

T

TBST	Tris buffered saline with Tween-20
TCA	trichloroacetic acid

U

U	unit(s)

V

$V.cm^{-1}$	volt per centimeter
v/v	volume per volume

W

w/v	weight per volume
WGA	wheat germ agglutinin

X

Xyl	Xylose (see Appendix B)

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■ PREFACE ■

This study focuses on active biochemical mechanisms of resistance in plants to fungal pathogens. More specifically, the occurrence of a polygalacturonase-inhibiting protein (PGIP) in wheat is investigated. Following a general introduction, the manuscript is divided into a number of different chapters. The first chapter is a literature review to serve as background on the cell wall carbohydrates, cell wall degrading enzymes and PGIP. The subsequent chapters are each dedicated to a different aspect of the investigation, each with its own aim, materials, methods, results and discussion. This format facilitates future publications, but unavoidably creates duplication of certain aspects. To put everything into perspective, the final chapter is dedicated to a general discussion and conclusion. Literature references, appendix with additional data, as well as summaries in both English and Afrikaans conclude the thesis.

INTRODUCTION

The nature of plants has mystified scientific minds for centuries and continues to be the subject of extensive investigation. In addition to their indispensable contribution to the sustainability of life, plants have contributed significantly to our knowledge of science. One of the biggest contributions came from pea plants and an Austrian monk, Gregor Mendel who, in 1866, established the fundamentals of genetics. Based on his laws of segregation and independent assortment of genes, plant breeders have developed and improved many crop plants of economic significance.

Wheat[1] arrived in South Africa during the middle of the 17th century as European immigrants settled in the Cape. One of the first ventures of Jan van Riebeeck after his arrival at the Cape in 1652 was to sow wheat on the site of present-day Cape Town. Through the years that followed, wheat production expanded gradually as the early pioneers settled in new areas (Du Plessis, 1933). Today, wheat is produced in large parts of the country, the most important production areas being the southwestern parts of the Western Cape with its reliable winter rainfall and the eastern Free State in the summer rainfall interior.

In South Africa, the estimated 45 million people mainly use wheat for human consumption. With the final statistics[2] for 2001/02 not yet available, approximately 957 250 ha of wheat were planted which will produce an estimated 2.32 million tons of wheat. During the 2000/01 season an estimated 934 000 ha of wheat was planted which produced an estimated 2.35 million tons of wheat. For the 1999/2000 season, an amount of 3.1 million tons of wheat were available for local consumption, which included carry-over stock from the previous season as well as 624 000 tons of imported wheat. The total domestic commercial demand for this period was just over 2.5 million tons, with shortages being reported in the local wheat market as of June 2000. Looking back at the production figures for the last 20 years, data indicate an average human consumption of 6% more than the available 2.06 million tons produced per year.

Any biotic or abiotic stress factor, accompanied by a poor harvest, could have important economic implications for wheat production in South Africa. Depending on conditions during a particular cropping season, the demand for wheat could thus

[1] See Appendix A
[2] Directorate Statistical Information of the South African National Department of Agriculture

easily exceed the supply, leading to shortages, a common challenge in all crop-producing countries.

Considering biotic stress factors, infectious diseases pose the biggest threat to wheat production in South Africa. In infectious diseases of susceptible host plants, a series of successive events, leading to the development of disease and perpetuation of the pathogen, occurs. The primary events in such a disease cycle are inoculation, penetration, establishment of infection and colonization of the host, and growth, reproduction, dissemination and survival of the pathogen (Parry, 1990; Lucas *et al.*, 1992).

The principle biological agents that cause wheat diseases are fungi, viruses, bacteria and nematodes with all of them being parasitic and causing infectious diseases transmissible from plant to plant. According to Wiese (1987), the actual number of wheat diseases is unknown, but nearly 200 have been described of which 50 are considered to be of economic importance.

Fungal pathogens attach themselves to the surface of a host plant through mechanisms that include the secretion of enzymes altering the adhesion properties of the cutin in the leaves (Nicholson and Epstein, 1991). Following adhesion, pathogens enter their hosts either by penetrating the intact plant surface through wounds or natural openings, such as stomata, hydathodes, or through areas of the plant where the external protective layers are especially thin or completely absent, e.g. glands and nectaries (Dickenson and Lucas, 1977; Schäfer, 1994).

The substances manufactured by the plant cell is of particular interest to fungal pathogens, as these parasites have acquired the ability to not only live off them, but depend on it for their survival (Agrios, 1988; Schäfer, 1994). Many of these substances, however, are contained in the protoplast of the plant cell and gaining access to them forces the pathogen to breach the protective cell wall surrounding the cell. Once inside the cell the pathogen uses nutrients from the host for survival, resulting in the deterioration and ultimate death of the plant cell, often followed by death of the whole plant or parts thereof (Staples, 2000).

A number of fungal pathogens exhibiting this destructive nature are of economic importance to the wheat farming community in South Africa (Scott, 1990). These include[3] *Puccinia* spp., the causal agents of leaf, stem and stripe rusts, *Septoria* spp., *Gaeumannomyces graminis* var. *tritici*, *Tilletia* spp., *Ustilago tritici*, *Pseudocercosporella, Erysiphe graminis* f. sp. *tritici* and *Fusarium* spp.

Although seasonal in occurrence, leaf rust, caused by *Puccinia triticina*, is an important disease of wheat in South Africa. It is usually most severe on spring wheat grown in the winter rainfall areas of the Western Cape (Pretorius *et al.*, 1987). Recently Boshoff (2000) emphasized the importance of leaf rust in this region by recording losses as high as 78% in naturally infected experimental plots. The wheat leaf rust fungus primarily attacks the leaf blades. Once on the leaf, the spores germinate, forming infection structures that penetrate the stomata and subsequently colonize the host (Schäfer, 1994). Plants infected with, and showing a compatible reaction to leaf rust, typically exhibit small, round, orange-red pustules of about 1 to 2 mm in diameter on the upper surface of the leaves. Infection with rust not only destroys the plant cells, but also deprives the plant of its nutrients. This causes the leaves to dry out and, in doing so, destroys the major photosynthetic area of the plant (Knott, 1989; Lucas *et al.*, 1992).

In reaction to the presence and activities of this pathogen, the plant produces chemical substances in its own defense that interfere with the advance or existence of the pathogen (Agrios, 1988; Kemp *et al.*, 1999). This natural defense mechanism is a topic of considerable interest, as a better understanding of the processes involved could lead to improved disease resistance, with subsequent far-reaching effects on both the economy and the environment. These biochemical processes are often quite complex, and through its protective nature serve as the plant equivalent to the human immune system.

Plants often respond to fungal infection by means of a hypersensitive reaction (HR), which is characterized by the localized cell death surrounding the site of infection, induction in metabolic processes, as well as the production of antimicrobial substances to confine the dissemination of the pathogen (Fritig *et al.*, 1998). The accumulation of pathogenesis-related (PR) proteins accounts for the major

[3] Personal communication: Prof. ZA Pretorius, Department of Plant Pathology, University of the Free State, RSA

quantitative change in protein composition during the HR. These PR proteins either directly combat the pathogen by damaging fungal structures (chitinases and β-1,3-glucanases) (Fritig *et al.*, 1998), or indirectly by acting on the hydrolytic enzymes released by the fungus (polygalacturonase-inhibiting protein) (De Lorenzo *et al.*, 2001).

One of the first barriers a plant pathogenic fungus encounters is the polysaccharide-rich plant cell wall. As mentioned, all pathogenic fungi need to breach this barrier to gain access to the plant cell and its nutrients, and therefore secrete a number of enzymes capable of degrading cell wall polymers. Among these enzymes is endopolygalacturonase (EPG), when left unattended will cause wall degradation and plant maceration through its hydrolytic action on galacturonic acid in pectin, one of the major constituents of the primary cell wall (De Lorenzo *et al.*, 2001). To protect the cell wall, endopolygalacturonase activity is controlled by cell wall-localized polygalacturonase-inhibiting proteins (PGIPs), which are critically important in limiting fungal colonization by acting as inhibitors and regulators of PG activity (De Lorenzo *et al.*, 2001).

These defense proteins and cell wall guardians are associated with cells of all dicotyledonous plants studied (De Lorenzo and Cervone, 1997), and according to De Lorenzo *et al.* (2001), it is also present in leek and onion, two monocotyledonous plants. Other than this, no credible evidence exists on the presence, expression and possible role of PGIP in economically important monocotyledonous plants such as cereals.

Through the purification of PGIP from wheat, showing specificity for its ligand, examining the expression in the plant in reaction to outside stimuli, and by cytologically pinpointing the protein in the plant cell, this study aims to provide the first evidence for the presence and expression of PGIP in *Triticum aestivum*.

CHAPTER 1

■ THE PLANT CELL WALL

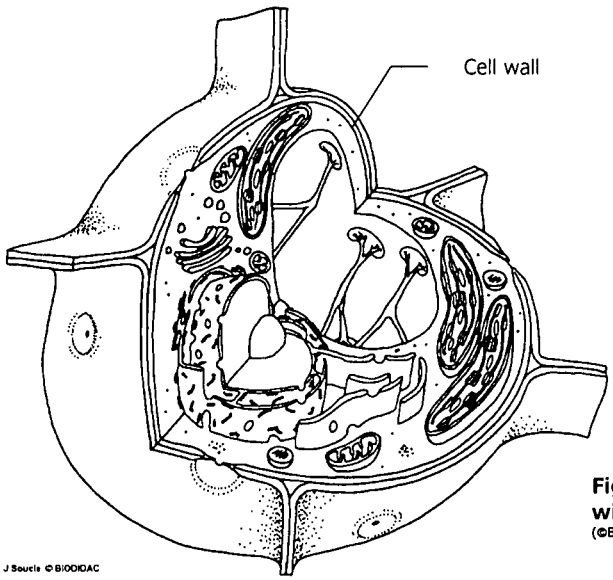


Fig. 1.1. Illustration of a plant cell with the cell wall indicated.
(©BIODIDAC)

The cell walls of plants are fundamentally involved in many aspects of plant biology including the morphology, growth and development of cells and the interactions between plant hosts and their pathogens. Cell walls are semi-rigid structures surrounding the cytoplasmic membrane of each cell (Fig. 1.1) (Albersheim, 1976), which, upon merging with the walls of adjacent cells, give the tissue physical coherence and strength (Esau, 1960). In doing so, it acts as both the skin and the skeleton of plants, protecting the cell from invasion by viral, bacterial, and fungal pathogens. These pathogens, through the secretion of enzymes, degrade the components of the wall (Albersheim, 1965).

The walls of growing plant cells are called primary cell walls and are composed of approximately 90% polysaccharide and 10% proteins, in the form of glycoproteins (Mcneil *et al.*, 1984), which often serve as the first line of defense against the outside world. Pathogens thus encounter a large array of difficult to degrade, differently linked glycosyl residues and non-carbohydrate substituents during attempts to penetrate and degrade plant cell walls (Hahn *et al.*, 1989).

Research spanning more than three decades has led to the general understanding that plant cell walls are composed of cellulose, the hemicelluloses xyloglucan and arabinoxylan, and the pectic polysaccharides homogalacturonan, rhamnogalacturonan I and rhamnogalacturonan II (Albersheim *et al.*, 1996).

■ CELLULOSE

Cellulose is probably the best known of all plant cell wall polysaccharides, consisting of linear 1,4-linked β-D-glucans which account for about 20%-30% of the dry mass of most primary cell walls, and effectively providing much of the cell's mechanical strength (Keon *et al.*, 1987; Albersheim *et al.*, 1996). These β-glucan chains are combined together to form microfibrils through intra- and inter-chain hydrogen bonds as well as through hydrophobic interactions (Fig. 1.2). The resulting highly ordered and partially crystalline microfibrils provide much of the tensile strength of the primary wall (Rose *et al.*, 2000).

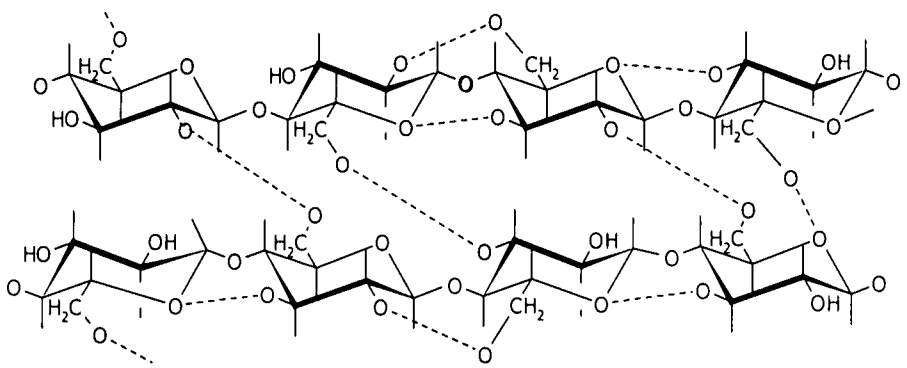


Fig. 1.2. The 1,4-linked β-D-glucosyl residues that associate to form cellulose. Adjacent glucosyl residues are rotated 180° relative to each other. The formation of intra- and inter-molecular hydrogen bonds ( ) between the glucan chains is in large part responsible for the rigid structure of cellulose (Rose *et al.*, 2000).

■ XYLOGLUCAN

Xyloglucan is a hemicellulose that is present in both dicotyledonous (dicots) and monocotyledonous (monocots) cell walls, with far larger amounts present in the walls of dicots (~20%) than monocots (~2%) (Darvill *et al.*, 1980). As with cellulose, the xyloglucans also have a backbone of 1,4-linked β-D-glycosyl (Glc*p*)[4] residues (Fig. 1.3). Approximately 75% of these residues have substitutions of α-D-xylosyl (Xyl*p*), β-D-galactopyranosyluronic acid (Gal*p*A) as Gal*p*-(1,2)-α-D-Xyl*p*, and α-L-fucosyl(Fuc*p*)-(1,2)-β-D-Gal*p*-(1,2)-α-D-Xyl*p* at C-6 (Rose *et al.*, 2000) with the Gal*p* residues often being *O*-acetylated at C-6. Variations in the structural composition of the xyloglucans between different plants have been observed, e.g. monocotyledonous plants have both β-1,3- and β-1,4-glycosyl residues in the xyloglucan backbone (Keon *et al.*, 1987), while fucose has not been detected in the xyloglucans of Poaceae (Carpita, 1996) or Solanaceae (York *et al.*, 1996; Rose *et al.*, 2000).

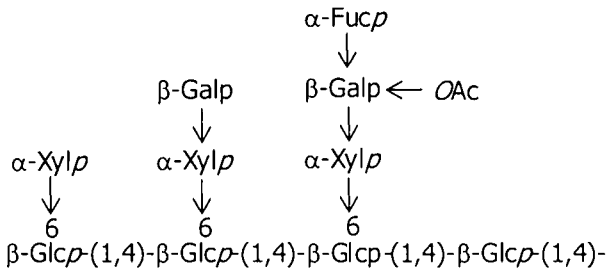


Fig. 1.3. A partial structure of a xyloglucan. The 1,4 linked β-D-glucan backbone is substituted at C-6 with mono-, di- and trisaccharides in a regular pattern (Rose *et al.*, 2000).

Arabinoxylans constitute the major hemicellulose in the primary cell walls of monocots and are found in smaller amounts in the primary cell walls of dicots (Darvill *et al.*, 1980). All known arabinoxylans consist of a backbone of 1,4-linked β-D-Xyl*p* and contain rabinofuranosyl (Ara*f*) residues linked to C-2 and/or C-3 which, in turn, may contain ester-linked phenolic acids such as ferulic acid (Ishii, 1997) acting as potential sites for cross-linking (Fig. 1.4). The xylan backbone can also contain β-D-Glc*p*A or 4-*O*-Me β-D-Glc*p*A, in which case it is called glucuronoarabinoxylan (Rose *et al.*, 2000).

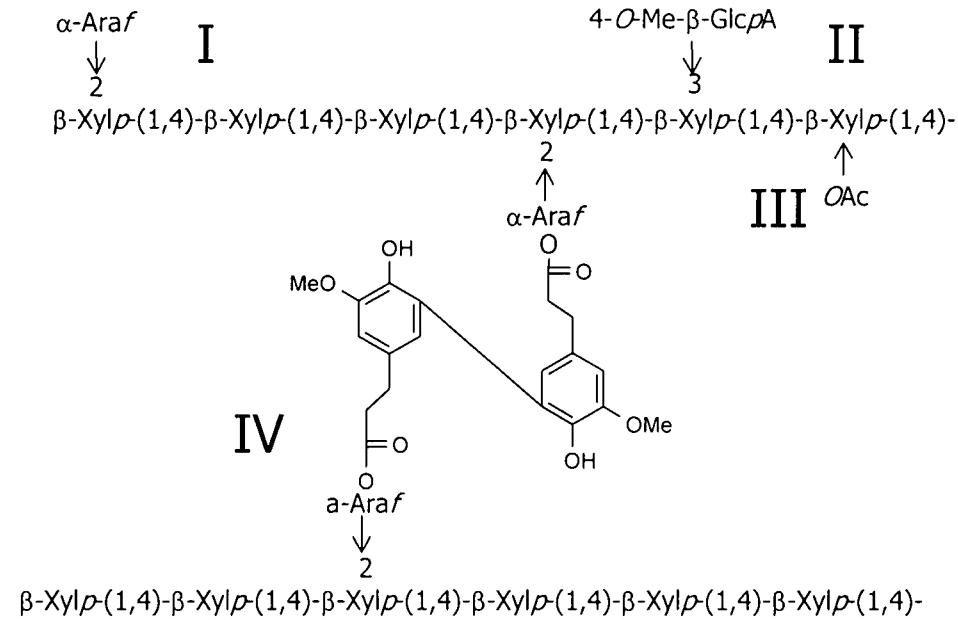


Fig 1.4. A partial structure of a glucuronoarabinoxylan. The 1,4-linked β-D-xylan backbone is substituted with arabinosyl (I) and (4-*O*-Me) glucuronosyl residues (II), and *O*-acetyl groups (III). Some of the arabinosyl residues are substituted with ester-linked phenolic acids such as ferulic acid. These ferulic acid residues may be oxidatively coupled and thereby form inter- and intra-molecular cross-links (IV) (Rose *et al.*, 2000).

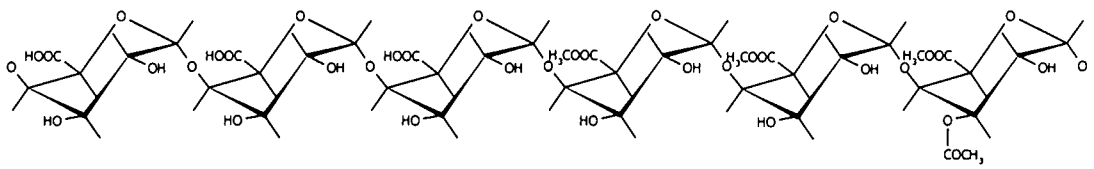
[4] See Appendix B

■ PECTIC POLYSACCHARIDES

This class of structural polysaccharides of the cell wall is of primary significance to this study. Three pectic polysaccharides that form pectin have been isolated from the primary cell walls of plants (O'Neill *et al.*, 1990). They form a matrix that coexists with the cellulose and hemicellulose in the cell wall. These are 1) homogalacturonan, 2) rhamnogalacturonan I (RG-I) and 3) rhamnogalacturonan II (RG-II) (O'Neill *et al.*, 1990; Albersheim *et al.*, 1996).

■ HOMOGALACTURONANS (HG)

Homogalacturonans are homopolymers consisting predominantly of 1,4-linked α-D-galactosyluronic acid residues in which some of the carboxyl groups are methyl esterified (Fig. 1.5) (O'Neill *et al.*, 1990; Voragen *et al.*, 1995). HGs tend to be insoluble under certain well-defined conditions (McNeil *et al.*, 1984), which supplies the gel forming characteristics that are widely employed in the food industry. Methyl-esterified forms of homogalacturonan appear, according to Marty *et al.* (1995), to be concentrated in specific regions of the primary cell wall such as the middle lamella of tobacco cells. In barley, a monocot, esterified and unesterified pectin was located in the cell corners and middle lamella, while unesterified pectin was also detected at the outer portions of the epidermal cell walls adjacent to the cuticle (Clay *et al.*, 1997). Shibuya and Iwasaki (1978) also found strong evidence for the presence of HG in rice endosperm, another monocot.



O-acetylated Gal*p*A

Fig. 1.5. A partial structure of homogalacturonan. In primary cell walls between 50 and 80% of Gal*p*A are esterified.

■ RHAMNOGALACTURONAN I (RG-I)

Rhamnogalacturonan I comprises a group of closely related pectic polysaccharides that contain a backbone of the repeating disaccharide (1,4)-α-D-Gal*p*A-(1,2)-α-L-Rha*p* with some of the Gal*p* residues being *O*-acetylated on C-2 and/or C-3, and no evidence of methyl-esterification (Fig. 1.6) (O'Neill *et al.*, 1990; Rose *et al.*, 2000). Between 20-80% of the rhamnosyl (Rha*p*) residues are, depending on the plant

source and the method of isolation, substituted at C-4 with neutral and acidic oligosaccharide side chains (Fig. 1.6). The length of these side chains may range from a single glycosyl residue to more than twenty glycosyl residues and are composed of linear and branched α-L-Ara*f* and β-D-Gal*p* reisdues, which could differ depending on the plant source (O'Neill *et al.*, 1999). While most work on RG-I has been performed in dicots, RG-I has also been identified in onion (a monocot) (Ishii, 1982). Primary cell walls also contain arabinan, galactan and two forms of arabinogalactan: type I with a 1-4 linked β-D-galactan backbone, and type II arabinogalactan with a 1-3 linked β-D-galactan backbone (Stephen, 1983).

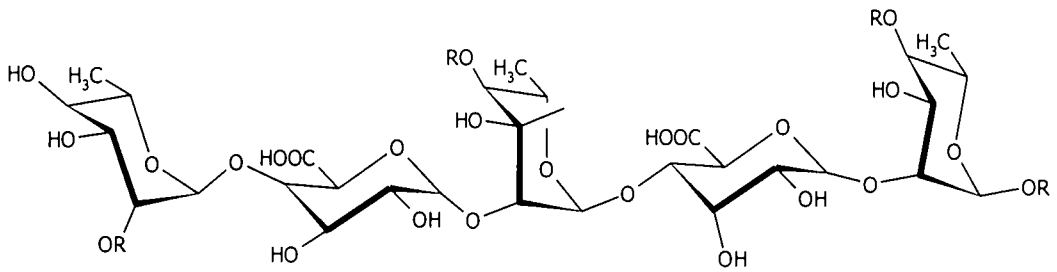


Fig. 1.6. The backbone of RG-I is composed of the repeating disaccharide –4)-α-D-Gal*p*A-(1,2)-α-L-Rha*p*-(1-. Some of the Gal*p*A residues are often *O*-acetylated (Azadi *et al.*, 1995).

■ RHAMNOGALACTURONAN II (RG-II)

Rhamnogalacturonan II is structurally very different from rhamnogalacturonan I. RG-II is a low molecular mass (~5-10 kDa) complex pectic polysaccharide (11 different sugars in more than 20 different linkages)(O'Neill *et al.*, 1990), which, unlike RG-I, does not have a backbone of the repeating disaccharide -4)-α-D-Gal*p*A-(1,2)-α-L-Rha*p* (Rose *et al.*, 2000), but instead shows a high level of similarity to that of homogalacturonan (Fig. 1.7). Its backbone consists of a highly conserved structure composed of at least seven 1,4-linked α-D-galactosyluronic acid (Gal*p*A) residues, some of which may be methyl esterified (Albersheim *et al.*, 1996), and which can be released from primary cell walls by endo-α-1,4-polygalacturonase (EPG) digestion (O'Neill *et al.*, 1990). Two of the backbone Gal*p*A residues are substituted at C-3 with two structurally different disaccharides, while two structurally different octasaccharides are attached to C-2 of two other backbone Gal*p*A residues (Fig. 1.7) (Rose *et al.*, 2000). These attached side chains, apparently, sterically prevent endopolygalacturonase from cleaving the backbone, explaining why intact RG-II is released from cell walls by this enzyme (Albersheim *et al.*, 1996). Like RG-I,

most work has been performed in dicots, however RG-II has been described in cell walls from oats (a monocot) (Darvill *et al.*, 1978).

Recently, a new discovery in cell wall chemistry showed that RG-II could exist as a dimer cross-linked by a specifically located borate ester (Fig. 1.7V) (O'Neill *et al.*, 1996). Although its function has not been determined, boron is an essential microelement for plant growth. Studies of plants suffering from boron deficiency reveal disorganized cell expansion and the formation of cell walls with abnormal morphology (Loomis and Durst, 1992; Welch, 1995). Boron is believed to form borate-diol esters that covalently cross-link cell wall pectic polysaccharides (Loomis and Durst, 1992; Ishii and Matsunaga, 1996). This may provide a partial explanation for how the network of three types of pectic polysaccharides are covalently connected and cross-linked (Albersheim *et al.*, 1996), as described below.

■ PECTIN CONTENT OF PLANT CELL WALLS

Exactly how the pectic polysaccharides interact to form pectin remains a mystery as all information on how HG, RG-I and RG-II are linked together in the wall is lost when they are solubilized by chemical or enzymatic treatments for analysis (O'Neill *et al.*, 1999). Information about the exact structure of pectin is therefore very speculative. It is proposed that the borate ester cross-linking of RG-II may generate a macro-molecular pectin complex in the wall, if RG-II, HG and RG-I are covalently linked together (O'Neill *et al.*, 1996). This link is supported, in part, by the high level of similarity between the backbones of HG and RG-II (O'Neill *et al.*, 1999).

It has been reported that HGs contain, in addition to methyl esters, other unidentified esters that may cross-link HG to wall polymers, while unsubstantiated reports have HG and RG-I covalently linked to cellulose, xyloglucan, and/or structural glycoproteins (O'Neill *et al.*, 1999).

Pectin is present in the primary cell walls of all seed-bearing plants and is located particularly in the middle lamella (Carpita and Gibeaut, 1993). Pectins are major components of the cell walls of dicotyledons (~35%)(Darvill *et al.*, 1980), and, although abundant in the primary walls of non-graminaceous monocotyledons (e.g. onion, garlic, lemna and sisal), pectic polysaccharides account for relatively less of

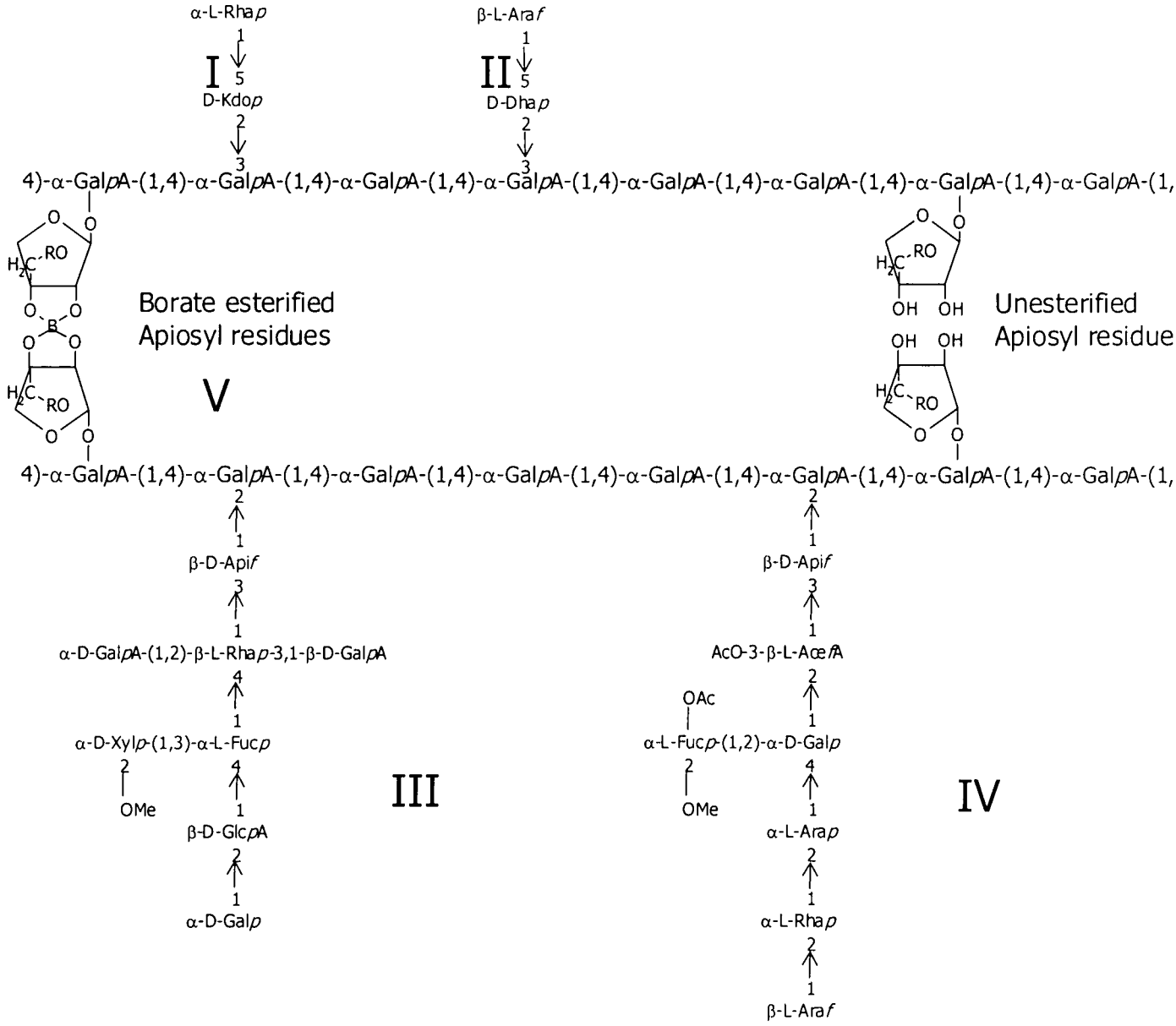


Fig. 1.7. A partial structure of rhamnogalacturonan II. The backbone of RG-II is composed of 1,4 linked α-D-Gal*p*A residues. Four side chains (I-IV) are known to be attached to the backbone, although their locations relative to one another are not known. A single 1:2 borate-diol ester is believed to cross-link two RG-II monomers (Rose *et al.*, 2000).

the primary wall in the monocotyledonous graminaceae (e.g. barley and wheat) (Jarvis *et al.*, 1984; Bacic *et al.*, 1988).

The pectin content of wheat and other cereals has been shown to be approximately 1% of the dry straw weight (Bonner, 1950; Moerschbacher *et al.*, 1999), while Darvill *et al.* (1980) hypothesized the pectin content in monocot cell walls, in general, to be around 3.5%.

These low levels have led to the pectic polysaccharides of the primary cell walls of monocots not being studied as extensively as those of the dicots.

■ CELL WALL DEGRADING ENZYMES (CWDE'S) AND THEIR ROLE IN PLANT DISEASE

■ PLANT CWDE'S

Important roles are proposed for the endogenous cell wall degrading enzymes in plant developmental processes, which include fruit ripening, leaf and fruit abscission, and pod dehiscence (Giovannoni, 1998). The abscission of bean leaves, for example, is accompanied by the synthesis of cellulase (Lewis and Varner, 1970). Also, the process of maturation in tomato is preceded by the action of pectin methylesterase, followed by endopolygalacturonase (EPG) hydrolysis (Ebbelaar *et al.*, 1996) playing a significant role in tissue deterioration in later stages of the fruit ripening process (Hadfield and Bennet, 1998). All these processes require a reduction in cell-cell adhesion and this may be regulated in part by the enzymatic modification and fragmentation of pectin and other cell wall polysaccharides (Giovannoni, 1998).

■ PATHOGENIC CWDE'S

Unknown to him, Heinrich Anton De Bary, in the middle of the nineteenth century, made the first important contribution towards the unraveling of the understanding of plant diseases. He postulated that an extracellular "enzyme", of undetermined nature, was involved in the infection of plant tissue by a parasitic fungus. He envisaged the enzyme's action resulting in the swelling and softening of the plant cell wall, and the protoplast becoming detached from it (Byrde, 1982).

It has been argued that the production of enzymes capable of degrading cell wall polymers plays an important role in the penetration phase of the pathogen life cycle. Depolymerization of the wall components would facilitate passage through the cell wall and allow access to nutrients both within the wall and underlying cells (Scott-Craig *et al.*, 1998). Work done by Howard and co-workers (1991) confirmed that softening of the cell wall facilitates penetration, as the fungus would otherwise rely solely on mechanical turgor force for penetration.

Numerous CWDEs produced by phytopathogenic fungi have been investigated (see Table 1.1). Most of the research, however, has centered around the pectin degrading enzymes (pectinases) as they are typically produced first, in the largest amounts, and are the only CWDE capable of macerating plant tissue and killing plant cells on their own (Cooper, 1983).

■ THE PECTINASES

Fungi produce different types of pectinases that are classified by their substrates, type of bond cleavage and mode of action on the pectin polymer. Under these criteria pectinases can be divided into hydrolases and lyases.

■ HYDROLYASES

Hydrolyases cleave glycosidic bonds through hydrolysis. Two types of hydrolases have been identified: 1) *Exo-glycanases* (e.g. exo-polygalacturonase) typically release a glycosyl residue from the terminal non-reducing end of a polymer compared to 2) *endo-polyglycanases* (e.g. endo-polygalacturonase) that hydrolize internal glycosidic bonds, generating oligosaccharide fragments (O'Neill *et al.*, 1999).

A third type of hydrolase activity has recently been discovered which might, without investigators knowing it, have had an influence on results obtained from endo- and exo-glycanases. This third type exhibits both an endo- and exo-glycolytic acyivity (Cook *et al.*, 1999). Two pectin hydrolases have been identified; viz. polygalacturonase and rhamnogalacturonase.

Table 1.1
Examples of cell wall-degrading enzymes (CWDEs) cloned from fungi (adapted from Annis and Goodwin, 1997).

Substrate	Enzyme	*Fungus*
Callose	Exo-β-1,3-glucanase	*Cochliobolus carbonum*
Cutin	Cutinase	*Nectria haematococca*
		Magnaporthe grisea
		Alternaria brassicicola
		Colletrotrichum capsici
		Colletrotrichum gloeosporioides
Cellulose	Endo-1,4-β-glucanase	*Macrophomina phaseolina*
		Fusarium oxysporum
	Cellobiohydrolase	*Fusarium oxysporum*
Disaccharides	β-glucosidase	*Sclerotinia sclerotiorum*
	β-galactosidase	*Sclerotinia sclerotiorum*
Pectin/pectate	Exopolygalacturonase	*Aspergillus tubigensis*
	Endopolygalacturonase	*Sclerotina sclerotiorum*
		Cochliobolus carbonum
		Fusarium moniliforme
		Colletrotrichum lindemuthianum
		Cryphonectria parasitica
		Aspergillus niger
		Aspergillus flavus
		Aspergillus oryzae
		Aspergillus parasiticus
		Aspergillus tubigensis
		Aspergillus aculeatus
	Rhamnogalacturonase	*Glomerella cingulata*
	Pectin lyase	*Aspergillus niger*
		Glomerella cingulata
	Pectate lyase	*Nectria haematococca*
		Aspergillus nidulans
	Pectin methylesterase	*Aspergillus niger*
Xylan	Xylanase	*Cochliobolus carbonum*
		Magnaporthe grisea

■ Polygalacturonase (PG)

This enzyme degrades unesterified 1,4 linked α-D-galactosyluronic acid polymers (Fig. 1.8) (Rexová-Benková and Markovič, 1976).

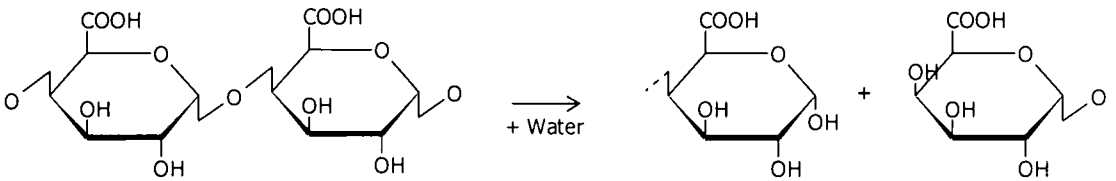


Fig. 1.8. A simplified example of the hydrolytic action of polygalacturonase (endo/exo) (Pilnik and Voragen, 1991).

■ Rhamnogalacturonase (RHG)

Cleaves the bond between the alternating galactose and rhamnose residues in rhamno-galacturonan (Suykerbuyk *et al.*, 1995). Kofod *et al.* (1994) described two recombinant rhamnogalacturonan-cleaving enzymes secreted by *Aspergillus aculeatus* (rRGase A and rRGase B) that fragment the backbone of partially debranched RG-I in an endo fashion. These two enzymes initially believed to be rhamnogalacturonases have since been shown to be an endohydrolase, that cleaves the -4)-α-D-Gal*p*A-(1,2)-α-L-Rha*p* linkage, and an endolyase, that cleaves the -2)-α-L-Rha*p*-(1,4)-α-D-Gal*p*A linkage, respectively (Azadi *et al.*, 1995).

■ LYASES

Lyases fragment acidic polysaccharides by a β-elimination reaction that generates oligosaccharides containing a Δ 4,5-unsaturated residue at the terminal non-reducing end (O'Neill *et al.*, 1999). Three pectin-degrading lyases have been identified; they are pectate lyase, pectin lyase and rhamnogalcturonan lyase.

■ Pectate Lyase (PL) [EC 4.2.2.2]

Degrades unesterified pectate (Fig. 1.9) (Rexová-Benková and Markovič, 1976; O'Neill *et al.*, 1999)

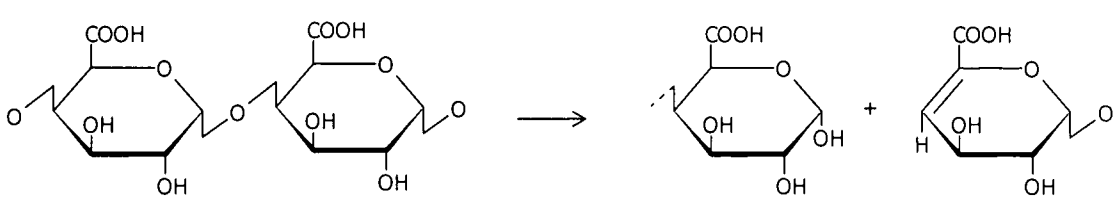


Fig. 1.9. A simplified example of the degradation of unesterified pectate through β-elimination (Pilnik and Voragen, 1991).

■ Pectin Lyase (PNL) [EC 4.2.2.10]

Breaks esterified pectin polymers apart (Fig. 1.10) (Rexová-Benková and Markovič, 1976; O'Neill *et al.*, 1999).

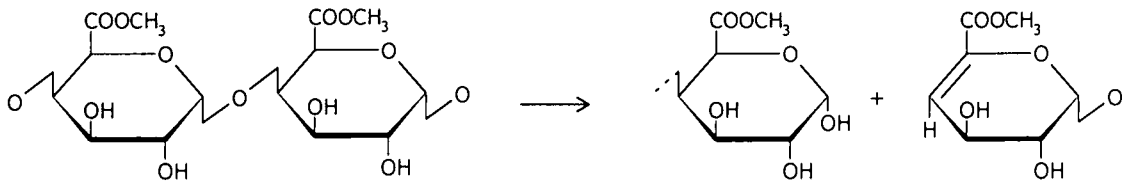


Fig. 1.10. A simplified example of the degradation of esterified pectin through β-elimination (Pilnik and Voragen, 1991).

■ Rhamnogalacturonan lyase

See rhamnogalacturonase.

■ Pectin modifying enzymes

A number of pectin modifying enzymes have been characterized, including *Pectin methyl esterases* [EC 3.1.1.11] which release methanol from a methyl esterified Gal*p*A - ($COOCH_3$ → -COOH + CH_3OH). *Rhamnogalaturonan acetyl esterases* that release acetic acid from an *O*-acetylated Gal*p*A – (-C-$OOCCH_3$ → -C-OH + CH_3COOH), and *O-feruloyl esterases* that release ferulic acid (O'Neill *et al.*, 1999), are two more examples.

Of all these enzymes, endopolygalacturonase (EPG) is the most important one for this study.

■ ENDOPOLYGALACTURONASE (EPG) (EC 3.2.1.15)

Endopolygalacturonase is the first polysaccharide-degrading enzyme secreted by phytopathogenic fungi during plant infection (English *et al.*, 1972; Jones *et al.*, 1972). Apart from fungi (Cervone *et al.*, 1987a; Robertsen, 1987; Caprari *et al.*, 1993a), endopolygalacturonases have been shown to be present in plants (Moshrefi and Luh, 1984), bacteria (Barash *et al.*, 1984) and insects (Shen *et al.*, 1996).

According to Pilnik and Voragen (1991), endogenous endopolygalacturonases have been found in numerous plant fruiting bodies (e.g. apple, avocado, banana, citrus, cherries, mango, papaya and pear) as well as vegetative and other tissues (Devoto

et al., 1998; Salvi *et al.*, 1990; Hong and Tucker, 2000). The enzyme is hypothesized to contribute to the developmental regulation of fruit softening (see above), which is typically involved in the ripening process and is accompanied by the disintegration of the middle lamella (Wakabayashi, 2000). It is usually present at low concentration until fruits begin to ripen upon which the concentration dramatically increases (Spencer, 1965). The plant's endogenous PGIP (see below) has no inhibitory effect on its endogenous EPG[5] (Cervone *et al.*, 1990).

The endopolygalacturonases from a number of different fungi (Cervone *et al.*, 1987a; Robertsen, 1987; Caprari *et al.*, 1993a) appear to share only 20% homology with bacterial and plant PGs. Fungal PG genes are, with some exceptions, approximately 60-65% similar to each other (Bussink *et al.*, 1991; Kitamoto *et al.*, 1993; Reymond *et al.*, 1994; Centis *et al.*, 1996) and contain highly conserved domains within an eighty residue region that may contain the active site and/or be involved in binding of the substrate (Bussink *et al.*, 1991; Caprari *et al.*, 1993b; Kitamoto *et al.*, 1993; Reymond *et al.*, 1994). Various characterized EPGs were found to be glycoproteins (Cervone *et al.*, 1986) with an optimum pH around 5.0 and, following deglycosylation, having molecular weights in the order of 33 kDa to 36.2 kDa (Cervone *et al.*, 1987a; De Lorenzo *et al.*, 1987; Robertsen, 1987; Annis and Goodmin, 1997).

Fungal endopolygalacturonases may have two opposing functions during fungal attack of plant tissues. On the one hand, EPG are pathogenicity factors disrupting plant cell walls allowing fungal colonization of the plant tissue while also providing nourishment for the fungus (Cervone *et al.*, 1989). On the other hand, EPG generates potential elicitors, which may activate plant defense responses by releasing plant cell wall fragments that signal the plant to defend itself (Cervone *et al.*, 1989; Salvi *et al.*, 1990).

■ ELICITORS AND THEIR ROLE IN PLANT DEFENSE

The result of any plant-pathogen interaction depends on, and is the result of, complex cascades of recognition, attack and defense reactions at the plant-pathogen interface (Klarzynski *et al.*, 2000). Within minutes following pathogen recognition, a

[5] Personal communication: Dr. CW Bergmann, Complex Carbohydrate Research Center, University of Georgia, USA

variety of events take place in the host. These include ion fluxes across the plasma membrane, cascades of phosphorylation and dephosphorylation, and the production of reactive oxygen species (Dixon *et al.*, 1994). Within hours these events are followed by a broad spectrum of metabolic modifications that include the production of defense-specific chemical messengers such as salicylic acid (SA) or jasmonates, and the accumulation of components with antimicrobial activities such as phytoalexins and the induction of pathogenesis-related (PR) proteins (Kombrink and Somssich, 1995).

Studies of plant-microorganism interactions yielded the first evidence that oligosaccharides could act as regulatory molecules, acting as biological signals that activate these antimicrobial activities (Darvill *et al.*, 1992). The active components in these extracts are known as elicitors (Dixon and Lamb, 1990) and can be divided into two classes of regulatory oligosaccharides (oligosaccharins); those originating from the cell wall of the microorganism (glucans, chitins and chitosans) and those from the plant cell wall (Côté *et al.*, 1998).

Plant cell wall 1,4-linked α-D-galactosyluronic acid oligomers with a degree of polymerization (DP) between 10 and 15 elicit plant defense responses which include phytoalexin accumulation in soybean (Hahn *et al.*, 1981), production of antimicrobial shikonins in suspension-cultured *Lithospermum erythrorhizon* cells (Tani *et al.*, 1992), lignin accumulation in cucumber (Robertsen, 1986) and induction of the PR proteins β-1,3-glucanase and chitinase in parsley and tobacco, respectively (Davis and Hahlbrock, 1987; Broekaert and Peumans, 1988). Shorter oligomers generally show much less biological activity, however oligosaccharides with a DP as low as 2 have been shown capable of inducing proteinase inhibitors in tomato (Farmer *et al.*, 1991). Furthermore, dimer and trimer galacturonides from wheat leaves have the ability to suppress disease resistance (Moerschbacher *et al.*, 1999).

Glucan elicitors from fungal cell walls, released through hydrolytic enzymes (e.g. β-1,3-glucanases) released by infected plants, have been shown to induce phytoalexins in numerous plants (Cline *et al.*, 1978; Sharp *et al.*, 1984; Gunia *et al.*, 1991; Klarzynski *et al.*, 2000). These oligosaccharins consist primarily of glucans containing 3-, 6-, and 3,6-linked β-glycosyl residues (Hahn and Albersheim, 1978) and are structurally very similar to the carbohydrates in mycelial walls (Bartnicki-

Garcia, 1968). Inui *et al.* (1997) found that glucan oligomers with more than 4 residues stimulated chitinase activity, while oligomers with more than 6 residues elicited phenyl ammonia-lyase (PAL) activity in rice.

Oligosaccharide fragments may be derived from chitin (Fig. 1.11I) through the action of chitinase. Chitin is a linear structural polysaccharide of the cell walls of many fungi (Ruiz-Herrera, 1991), and is composed of 1,4-linked β-D-*N*-acetylglucosaminyl residues. Oligosaccharides of chitosan (Fig. 1.11II), its de-*N*-acetylated derivative, have also been shown to elicit defense responses in some plants (Côté *et al.*, 1998). Chitosan-derived oligosaccharides elicit phytoalexin accumulation in pea pods (Hadwiger and Beckman, 1980), induce defense related proteinase inhibitors in tomato and potato leaves (Walker-Simmons *et al.*, 1983, Peña-Cortes *et al.*, 1988), and turn on production of the defense related β-1,3-glucan, callose, in suspension-cultured parsley (Conrath *et al.*, 1989) and *Catharanthus roseus* (Kauss *et al.*, 1989). Also, chitin and chitosan derived oligosaccharides were shown to induce defense related cell wall lignification of pine cells (*Pinus elliottii*) (Lesney, 1990).

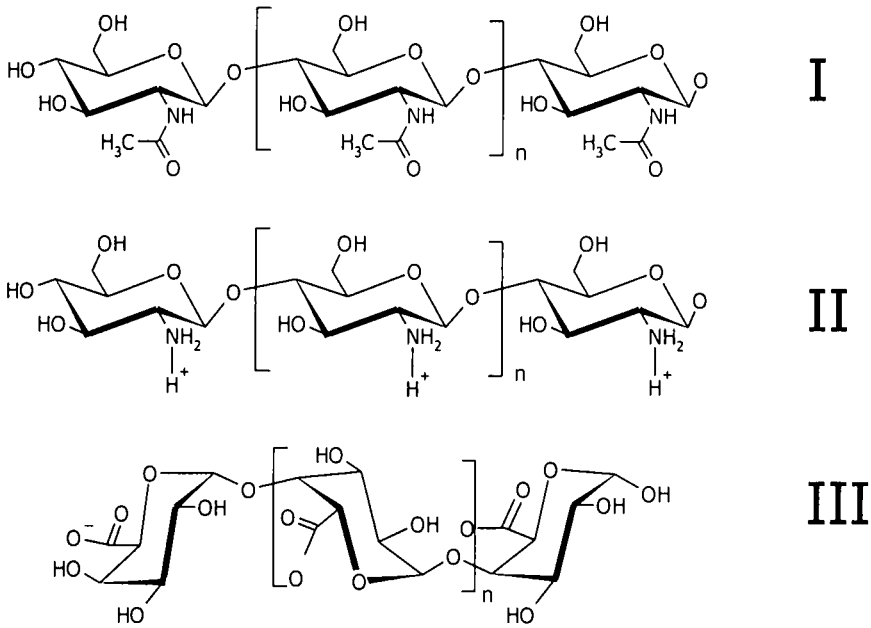


Fig. 1.11. Structures of oligosaccharide elicitors involved in plant-pathogen interactions. (I) Chitin oligoglucoside, (II) Chitosan oligosaccharide, (III) Oligogalacturonic acid (Côté *et al.*, 1998).

These oligosaccharides generally must have a DP of more than four to induce any biological response (Darvill *et al.*, 1992). This is true for wheat, where purified oligomers of GlcNAc with a DP of ≥ 7 are able to elicit only peroxidase activity, while chitosans of intermediate degrees of acetylation are able to successfully induce both peroxidase and phenylalanine-lyase activity (Vander *et al.*, 1998).

■ POLYGALACTURONASE-INHIBITING PROTEIN (PGIP)

In 1971, Albersheim and Anderson published an article on the observation that proteins extracted from the cell walls of Red Kidney bean tissue completely inhibit fungal polygalacturonase activity. Since then, the occurrence of polygalacturonase-inhibiting proteins (PGIPs) has been reported and they have been purified from a variety of dicotyledonous plants, with calculated sizes for the peptide backbone of the mature protein ranging from 34 kDa to 37 kDa[6]. The PGIPs purified from fruits e.g. oranges, apricot, apple, citrus, prunus and tomato are in the order of 36 kDa, with pear having the smallest fruit PGIP at 34 kDa. Soybean (*Glycine max*) and bean (*Phaseolus vulgaris*) PGIP are both approximately 34 kDa. Due to glycosylation these sizes are often mistakenly recorded as being much larger. Examples are the 44 kDa inhibitor initially purified from pear (Abu-Goukh *et al.*, 1983), the 54 kDa inhibitor from oranges (Barmore and Nguyen, 1985), the 42 kDa inhibitor, as determined by SDS-PAGE, from bean (Cervone *et al.*, 1987b) and the 42 kDa sized inhibitor from soybean (Favaron *et al.*, 1994). A more accurate molecular weight determination through matrix-assisted laser desorption ionization mass spectrometery (MALDI-MS), revealed the glycosylation bean PGIP to be approximately 37 kDa[7].

The isoelectric points of these purified inhibitors are quite diverse. Calculated pI values from isolated PGIP's range from 6.23 (pear), 6.93 (apricot), 6.98 (apple), 7.99 (prunus), 8.00±0.50 (oranges), 8.32 (soybean), 8.69 (tomato), 8.96 (bean), and 8.24 & 9.02 (*Arabidopsis* PGIP 1 and 2 respectively).

As early as 1975, Albersheim and Anderson-Prouty concluded that no endopolygalacturonase inhibitors are present in the cell walls of monocotyledons,

[6] http://www.expasy.ch/sprot
[7] Personal communication: L. Stanton, Complex Carbohydrate Research Center, University of Georgia, USA

contemplating the role of another degradative enzyme in the breakdown of monocotyledonous plant's primary cell walls (Albersheim and Anderson-Prouty, 1975). This could probably be seen as a reason why so little work on the role of PGIP in monocotyledonous plants has been published to date. However, 18 years after this statement by Albersheim and Anderson-Prouty, it was proved that extracts from monocotyledonous *Allium cepa* and *Allium porrum* were able to inhibit polygalacturonases from various fungal pathogens (Favaron *et al.*, 1993). Four years later this inhibitor was identified when two inhibitor isoforms of 39 kDa and 42 kDa were isolated from *Allium porrum* (Favaron *et al.*, 1997).

Despite these findings, and based on the lack of published work, PGIP has never been found in the economically important cereals. In the mid 1990's two publications reported on PGIP in wheat (Zheng *et al.*, 1994; Zhou *et al.*, 1995), however, these results could not be verified.

■ LOCATION OF PGIP

Endopolygalacturonase inhibitors have, from their first examination, been shown to be closely associated with plant cell walls (Albersheim and Anderson, 1971). Early work on susceptible and resistant beans (*Phaseolus vulgaris*) showed that 50-70% of the endopolygalacturonase inhibitor in hypocotyls appeared to be ionically bound to the cell wall, while the remainder was solublized from tissue homogenates (Lafitte *et al.*, 1984). Both fractions had an approximate size of 46 kDa, and appeared from their elution profiles to be the same protein. Lafitte *et al.* (1984) also found that 23-40% more EPG was found bound to the cell walls of the resistant bean, effectively making the plant more resistant. Removal of the inhibitor from the cell wall made the cell wall more susceptible to pathogen degradation.

Salvi and coworkers (1990) found PGIP activity in the intercellular spaces (apoplast) of the roots, leafs, cotyledons, flowers, stem, seeds and embryos of bean plants. The highest levels were recorded in the vegetative apex, while the roots exhibited the lowest PGIP levels (Salvi *et al.*, 1990). These results, obtained through vacuum infiltration of the apoplast of bean tissue, were convincing evidence that PGIP is cell wall associated and extracellular. These experiments also showed the tissue specific expression of PGIP in the plant.

■ INTERACTION BETWEEN PGIP AND PG

By exploiting the affinity of PGIP for PG under certain pH and ionic strength conditions, Cervone *et al.* (1987b) have shown that PGIP retards the PG-catalyzed hydrolysis of its substrate, sodium polypectate. It was concluded that this inhibition resulted from the formation of a PG-PGIP complex, as a change in pH or ionic strength reversed the complex formation, and in effect, the inhibition of EPG (Cervone *et al.*, 1987b).

Beyond this understanding, the exact mode of interaction is still unclear. Enzyme kinetics have been used to provide some answers. Johnston *et al.* (1993) have proposed non-competitive inhibition, i.e. binding of an inhibitor to a site on the PG molecule different from the active site, for the PG-PGIP interaction in raspberry fruits, while competitive inhibition was observed between pear PGIP and the PG produced by *Botrytis cinerea* (Abu-Goukh *et al.*, 1983).

Recent work at the CCRC, University of Georgia, USA using deuterium exchange mass spectrometery, fluorescence, and a model of PGIP based on its membership in the LRR family of proteins (see below), provided evidence that for the bean PGIP/*A. niger* II PG combination, the PGIP binds on a side of the PG opposite to that of the substrate binding cleft. The PGIP is thought to prevent a conformational change in the EPG brought about by binding of the pectate substrate in this cleft (Bergmann *et al.*, 2001)

■ SPECIFICITY OF PGIP

From its discovery 30 years ago, it has been observed that a level of specificity exists between PGIP and its ligand, EPG. It was first noticed that the 50 kDa purified inhibitor from Red Kidney bean (*Phaseolus vulgaris* cv. Red Kidney) clearly distinguished between polygalacturonases secreted by different species of pathogenic fungi (Albersheim and Anderson, 1971). Working on apples infected with an assortment of apple fungal pathogens, Brown (1984) purified a 45 kDa glycoprotein inhibitor from infected apples. The properties of this protein confirmed Albersheim and Anderson's observation that when the endopolygalacturonase (EPG) produced by one pathogenic species was not at all inhibited, the same EPG could be completely inhibited by inhibitors from a different apple cultivar. Testing the

inhibition properties of four PGIPs from three different plants further confirmed this observation. Cook *et al.* (1999) showed that PGIP from two bean cultivars tested were able to inhibit the hydrolytic EPG activity from six fungi, while those from pear and tomato could only inhibit four of the six. All PGIPs were incapable of inhibiting a PG from a wood rot fungus.

Recent work has provided a partial explanation for this specificity. Polygalacturonase-inhibiting proteins belong to the large family of leucine-rich repeat (LRR) proteins. The LRR is a versatile structural motif implicated for many protein-protein interactions and involved in many different cell functions such as receptor dimerization, domain repulsion, regulation of adhesion and binding events (Buchanan and Gay, 1996). In plants LRR proteins play a relevant role in both development and defense, where specificity of recognition is a fundamental prerequisite. To date the majority of resistance genes cloned encode proteins classified as NBS-LRR proteins as they contain a nucleotide-binding site (NBS) and a leucine-rich repeat (LRR) domain (Ellis and Jones, 1998). The mature PGIP is characterized by the presence of 10 repeats, each derived from modifications of a 24 amino acid LRR (De Lorenzo *et al.*, 2001). The LRR element in PGIP matches the extracytoplasmic consensus GxIPxxLxxLxxLxxLxLxxNxLx (De Lorenzo *et al.*, 1994) found in the products of many *R* genes like the *Cf* resistance genes of tomato, which confer resistance to different races of the fungus *Cladosporium fulvum* (Hammond-Kosack and Jones, 1997), and *Xa21* of rice, which confers resistance to *Xanthomonas oryzae* pv. *oryzae* (Wang *et al.*, 1996).

The amino acids of PGIP that determine specificity and affinity for fungal PGs are internal to the conserved xxLxLxx motif (where L indicates a conserved leucine or other aliphatic residue and x represents any amino acid) (Dodds *et al.*, 2001), which is predicted to form a β-sheet/β-turn structure (Kobe and Deisenhofer, 1994) where the x residues are exposed to the solvent and are available for interactions with potential ligands (Jones and Jones, 1997). Nucleotide substitutions leading to amino acid variations do not occur randomly along the LRR-coding sequence, but occur preferentially within this xxLxLxx motif (Leckie *et al.*, 1999).

The following example can be considered: it was found that two members of the *pgip* gene family (*pgip-1* and *pgip-2*) of *Phaseolus vulgaris* cv. Pinto encode for two

proteins with only eight amino acids difference between them (Leckie *et al.*, 1999). The two proteins exhibit distinct specificities: PGIP-1 is not able to interact and inhibit the PG from *F. monoliforme*, while PGIP-2 is. Through site-directed mutagenesis in the xxLxLxx motif, Leckie and co-workers could cause a loss of affinity for *F. monoliforme* PG. In addition, they were able to turn the PG from *F. monoliforme* into a ligand of PGIP-1 by substituting a single crucial amino acid in the PGIP-1 backbone (Leckie *et al.*, 1999). Through this work, strong evidence was provided that variations in the predicted solvent-exposed β-sheet/β-turn structure of an LRR protein may have an effect on the functional significance and discriminatory ability for recognition of a specific ligand (Leckie *et al.*, 1999).

■ STRUCTURE AND EXPRESSION OF PGIP

In 1992, Toubart and coworkers were the first to clone the gene coding for PGIP in *Phaseolus vulgaris* through a combination of PCR with degenerate primers, and hybridization techniques. Work done since on *P. vulgaris* has shown that PGIP is encoded by a gene family, comprising at least five members and possibly as many as fifteen, likely to be clustered in a single complex locus (Frediani *et al.*, 1993). These genes typically code for protein products comprising a signal peptide for translocation into the ER with a mature polypeptide of 300-315 amino acids containing several potential glycosylation sites (De Lorenzo *et al.*, 2001).

Expression of PGIP in *P. vulgaris* is regulated during normal plant development with PGIP activity present at low levels in all tissue, but notably abundant in pistils and pods (Salvi *et al.*, 1990). Transcripts of *pgip* are also present at low levels in most bean tissues, with higher levels observed in pods, and in etiolated hypocotyls (Devoto *et al.*, 1997). Accumulation of *pgip* mRNA and PGIP levels have been demonstrated in suspension-cultured bean cells following addition of elicitor-active oligogalacturonides or fungal glucan, and in bean hypocotyls in response to wounding or treatment with salicylic acid (Bergmann *et al.*, 1994; De Lorenzo *et al.*, 2001). Rapid induction of *pgip* transcripts has also been associated with the establishment of an incompatible interaction manifested by the appearance of a hypersensitive reaction in bean infected with *Colletotrichum lindemuthiamum* (Nuss *et al.*, 1996). These observations suggest that, as for many other defense genes, the regulatory mechanism of the *pgip* gene must include specific developmental

cues, with environmental stress and pathogen signals superimposed on them (Lois *et al.*, 1989; Wingender *et al.*, 1990; Lorbeth *et al.*, 1992).

In an attempt to analyze the regulation of *pgip*, Devoto *et al.* (1998) studied only one of the *pgip* members, specifically the promoter region of the *pgip1* gene cloned by Toubart *et al.* in 1992. Different *pgip-1*-constructs were transfected into tobacco protoplasts, microbombarded to bean and tobacco leaves, or transformed into tobacco plants. The results showed that the promoter only responded to cell wounding, while no response to oligogalacturonides, fungal glucan, salicylic acid, cryptogein, nor pathogen infection was observed. The study also showed that the region from nucleotide (nt) +1 to +27 regulates higher expression of *pgip1* in protoplasts. This was contrary to the induction in PGIP levels, as noted above, found by Bergmann *et al.* (1994) and Nuss *et al.* (1996), which, according to Devoto *et al.* (1998) were a collective result of the whole *pgip* family, and not only *pgip1*.

■ ROLE OF PGIP IN DISEASE RESISTANCE

Plants possess multiple mechanisms to protect themselves against pathogen attack. Specific pathogen recognition mechanisms usually lead to a hypersensitive response (HR), keeping the pathogen isolated from the rest of the plant through localized cell and tissue death appearing as necrotic lesions at the site of infection (De Wit, 1997; Fritig *et al.*, 1998). The formation of necrotic lesions, either as part of the hypersensitive response, or as symptom of disease caused by a necrotizing virulent pathogen, is associated with the co-ordinated induction of an integrated set of defense responses. These include cell wall rigidification, synthesis of phytoalexins, and accumulation of PR proteins (Pieterse and Van Loon, 1999). These local responses can often trigger non specific resistance throughout the plant, known as systemic acquired resistance (SAR), that provides significant and durable protection against challenge infection by a broad range of pathogens (Sticher *et al.*, 1997).

The HR and SAR are triggered in a number of ways, of which two will be of importance to this study. First, through specific plant-pathogen recognition that is genetically governed by interactions between the product of a disease resistance (*R*) gene in the plant and the product of a corresponding phytopathogen avirulence (*avr*) gene (Fritig *et al.*, 1998). Secondly, through the chemical induction of the disease response (as mentioned above) by treating the plants with chemical activators such

as SA and its structural analogs that mimics the SAR (Ryals *et al.*, 1996; Sticher *et al.*, 1997). The resulting resistance effect is visualized by the accumulation of the PR proteins that represent the major quantitative change in protein composition that occurs during the HR (Fritig *et al.*, 1998).

To date, 11 PR protein families have been characterized from various plant species and classified according to similarities, ranging from PR-1 to PR-11. These include proteins with β-1,3-glucanase- and endochitinase activities (as discussed before), as well as proteinases and peroxidases, and according to Van Loon *et al.* (1994), an unclassified PR protein family includes α-amylase and polygalacturonase inhibiting protein (PGIP).

It has since its discovery been suggested, due to its direct interaction with invading pathogens, that PGIP might play an important role in some form of disease resistance (Albersheim and Anderson, 1971; Lafitte *et al.*, 1984). For a long time it was believed that PGIP plays no role in active defense as no evidence could be found that inhibitor levels increased following fungal infection (Lafitte *et al.*, 1984). However, quite recently, Bergmann *et al.* (1994) proved the opposite by showing an increase from basal levels of PGIP transcripts in *Phaseolus vulgaris* following fungal attack. Besides confirming these results, Devoto *et al.* (1997) also showed PGIP expression to be developmentally regulated with stage- and organ specific expression. Furthermore, this inhibitor's role in disease resistance was underlined, when it was shown that PGIP accumulates to its highest levels around infection sites (Bergmann *et al.*, 1994; Devoto *et al.*, 1997).

Today, with more evidence in hand, the role of PGIP and PG in disease resistance can be summarized through the following four observations (Powell *et al.*, 2000):

- PGs are produced early in plant-fungus interactions and in doing so, contribute to the expansion of the infection site (Ten Have *et al.*, 1998).
- PGIPs can inhibit some of these destructive fungal PGs *in vitro* (Yao *et al.*, 1995; Sharrock and Labavitch, 1994).
- The PGs inhibited *in vitro* by PGIPs produce small quantities of polygalacturonic acid oligomers (Cervone *et al.*, 1989) that have the ability to

induce some defense responses when applied exogenously to plant cells (Darvill *et al.*, 1992).

- PGIPs share with plant resistance gene products a structural motif; the leucine-rich repeat (LRR) sequence motif (De Lorenzo *et al.*, 1994; Stotz *et al.*, 1994), necessary for genotype-specific pathogen resistance (Staskawicz *et al.*, 1995; Baker *et al.*, 1997).

CHAPTER 2

DEFENSE RELATED PROTEIN SYNTHESIS IN WHEAT FOLLOWING LEAF RUST INFECTION, WITH AN EMPHASIS ON THE POSSIBLE EXPRESSION OF POLYGALACTURONASE-INHIBITING PROTEIN (PGIP)

Proteins involved in the natural defense mechanisms of plants are induced following pathogenic attack. In an attempt to shed more light on this mechanism in wheat the newly synthesized peptides following fungal infection were visualized and, through the use of anti-PGIP antibodies, a subset of the induced polypeptides were further investigated to determine whether PGIP is expressed as part of the defense reaction.

■ MATERIALS:

■ CHEMICALS

TRAN^{35}S-LABEL^{TM} was obtained from ICN pharmaceuticals, Inc[8]. Cyclohexamide and chloramphenicol were Sigma[9] and USB[10] products respectively. Nitrocellulose and the ECL detection kit were purchased from Amersham. Endo-polygalacturonase from *Aspergillus niger* was obtained from Megazyme International Ireland Limited[11]. Polygalacturonic acid (PGA) was an ICN Biochemicals Inc[12] product. Sodium metaperiodate was from Sigma. All other chemicals used were of analytical grade.

■ PLANT MATERIAL

Wheat (*Triticum aestivum* L.) plants resistant [Thatcher/*Lr34*] and susceptible [Thatcher] to *Puccinia triticina* were supplied by the Department of Plant Pathology, University of the Free State, Bloemfontein.

■ ANTIBODIES

Antibodies against wheat germ agglutinin (WGA) for the detection of chitinases (Nagel, 1995; Trillas *et al.*, 2000) were obtained from Sigma. Anti β-1,3-glucanase was produced in a rabbit against purified wheat β-1,3-glucanase, and received from Dr X-M Qian[13]. An antibody (PGIP-I) generated in a rabbit against purified PGIP from

[8] Radiochemicals Division, Irvine, California, USA
[9] Sigma Chemical Company, St. Louis, Missouri, USA
[10] Amersham International place, Buckinghamshire, England
[11] Megazyme, Wicklow, Ireland
[12] ICN Biochemicals Inc, Aurora, Ohio, USA
[13] Xiao Mei Qian, Ph.D thesis, Department of Botany and Genetics, University of the Free State, RSA

Phaseolus vulgaris was received from Dr. CW Bergmann[14]. As secondary antibody a goat anti-rabbit IgG–horseradish peroxidase conjugate was used (Amersham).

■ METHODS:

■ WHEAT GENOTYPES AND GROWING CONDITIONS

Thatcher and Thatcher/*Lr34* plants were grown in a sterilized soil:peatmoss mixture (1:1 v/v), in an environment free from leaf rust, at continuous 20-25°C. Cool-white fluorescent tubes, emitting 120 $\mu Em^{-2}s^{-1}$, provided a 14 hour (h) daylength.

■ INOCULATION

For protein synthesis experiments, selected leaves were inoculated four weeks after planting. A different set of plants, used for PGIP expression experiments, was inoculated 10, 17 and 24 days after planting by spraying the whole plant with freshly harvested spores of *Puccinia triticina* pathotype UVPrt13, suspended in distilled water with a drop of Tween-20 (approx. 65000 spores/ml). Control plants were sprayed with distilled water/Tween-20 only. Inoculated and control plants were allowed to dry for 1 h before placement in the dark in a dew-simulation chamber at 18-20°C for 12 h. Plants were then transferred to the greenhouse.

■ ADMINISTERING PROTEIN SYNTHESIS INHIBITORS AND ^{35}S-METHIONINE

One-centimeter segments were excised from selected-sprayed leaves at 144 hours post inoculation (h.p.i). The segments were exposed to 10 µCi ^{35}S-methionine in 50 µl of 100 mM MES buffer, pH 6.0, containing 0.5% Tween-20, for 3 h at room temperature in the absence or presence of cyclohexamide (0.025 mg ml^{-1}) and chloramphenicol (0.2 mg ml^{-1}), respectively (Van der Westhuizen & Botha, 1993). Segments were placed under light vacuum to aid in the successful uptake of inhibitors and isotope. To ensure adequate inhibition of protein synthesis, leaf segments were pretreated for 1 h with the respective inhibitor prior to addition of ^{35}S-methionine.

[14] Complex Carbohydrate Research Center, University of Georgia, USA

EXTRACTION OF TOTAL WATER SOLUBLE PROTEINS

Total water-soluble proteins were extracted with 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM 2-mercaptoethanol, 2 mM PMSF (phenylmethanesulfonylfluoride) and 2 mM EDTA (ethylene-diaminetatraacetic acid) from leaves ground to a fine powder in liquid nitrogen.

INFILTRATION OF THE LEAVES AND EXTRACTION OF APOPLASTIC FLUID FOR PGIP ASSAY

PGIP located in the intercellular spaces near the cell wall was extracted through vacuum infiltration (Rohringer *et al.* 1983; Salvi *et al.*, 1990). Leaves from each treatment were sampled at 0, 24, 48, 72, 96, and 144 h.p.i. The cut ends of the leaves were washed in water to remove intracellular contamination due to mechanical wounding. The leaf pieces were vacuum-infiltrated for 5 min. with a 50 mM sodium acetate buffer (pH 5.0) containing 0.3 M NaCl (Salvi *et al.*, 1990). To obtain the apoplastic fluid, leaves were centrifuged at 500 *g* with their tips pointing upwards. The apoplastic fluid was collected, protein concentration determined, assayed for activity, and the required amount for immunoblotting (20 μg in synthesis study, and 6 μg in expression study) precipitated and frozen at -20°C for later use.

DETERMINATION OF PROTEIN CONCENTRATION AND ^{35}S-METHIONINE INCORPORATION

The concentration of extracted proteins was determined according to Bradford (1976) using the protein assay reagent from Bio-Rad[15] with gamma globulin as standard. A microplate reader (Bio-Rad Model 3550) was used for this purpose as described by Rybutt and Parish (1982).

To determine total incorporated methionine, a known volume of plant extract was spotted onto a glass microfibre filter (Whatman, GF/C), boiled for 5 min. in TCA, transferred to cold TCA, washed three times with cold, 100% acetone and dried. Incorporated methionine was counted in Insta Gell II scintillation cocktail (Packard Radiomatic) on a Beckman scintillation counter.

[15] Bio-Rad Laboratories, Hercules CA, USA

■ ASSAY FOR PGIP ACTIVITY

PGIP activity was measured as an inhibition of *A. niger* EPG activity by intercellular proteins of constant concentration over an incubation period of 1 h at 37°C in 50 mM NaAc (pH 5.0). The inhibition was quantified by spectrophotometrically measuring at 500 nm the release of reducing end-groups (Nelson, 1944; Somogyi, 1952) from polygalacturonic acid with respect to time.

PGIP activity for one sampling point was calculated using four data sets with three repeats of absorbancies each. Each data set incorporated 1) a positive control that consisted solely of enzyme (EPG) and substrate (PGA), while 2) the negative control only consisted of PGA. The 3) background sugar data set was composed of PGA and plant extract and the 4) assay reaction included PGA, EPG and plant extract. These values were incorporated into the following equation to calculate PGIP activity:

$$[ABS_{Positive\ control} - ABS_{Negative\ control}] - \{[ABS_{Assay\ reaction} - ABS_{Negative\ control}] - [ABS_{Background\ sugar\ reaction} - ABS_{Negative\ control}]\}$$

A standard curve (not shown) relating the amount of D-galacturonic acid equivalents to absorbance (A_{500}) was employed for quantifying enzyme activity. The formation of D-galacturonic acid was a linear function of the enzyme (EPG) and the polygalacturonic acid substrate added to the reaction. The formation of reducing sugars in this assay, hereafter referred to as PGIP activity, is expressed in µmole D-galacturonic acid h^{-1} mg^{-1} protein extract.

This formula subtracts the influence of reducing sugars in the plant extracts from the assay reaction. The resulting value showed, where applicable, an increase in PGIP activity as a positive trend when plotted, compared to the otherwise negative trend when expressed as a decrease in PG activity.

■ SDS-PAGE AND IMMUNOBLOTTING

Polypeptides were resolved on 15% polyacrylamide gels with a 6% stacking gel (ratio of acrylamide to N,N'-methylene-bisacrylamide, 100:1) according to Laemmli (1970). Peptides were visualized with Coomassie brilliant blue R-250, and the gels were dried under vacuum. ^{35}S-labeled proteins on the gel were visualized by exposure to X-ray film for two weeks at -80°C.

Six micrograms of apoplastic proteins per sampling stage, as determined through an extract dilution series to be the optimum for recognition by the antibody (results not shown), were loaded onto the SDS-PAGE gel for each immunoblot in the expression study. Separated polypeptides were transferred to nitrocellulose membranes using a Bio-Rad Trans-Blot SD semi-dry electrophoretic transfer cell in a transfer buffer containing 25 mM Tris (pH 8.3), 190 mM glycine and 10% (v/v) methanol (Towbin *et al.*, 1979).

Following transfer, the nitrocellulose membranes were treated with sodium metaperiodate to disrupt the carbohydrate moieties of PGIP, which have the potential for non-specific cross-reactions (Bergmann *et al.*, 1994). Thus, for the antibody raised to purified PGIP the membrane was washed with TBST (20 mM Tris-HCl, pH 7.6; 137 mM NaCl; 0.1% (v/v) Tween-20) (twice for 10 min. each), followed by 50 mM sodium acetate (once for 10 min.), then incubated in a sodium-meta-periodate solution (2 $mg.ml^{-1}$ sodium-meta-periodate in 50 mM sodium acetate for 30 minutes) and neutralized with Tris (1M Tris, pH 8.0; 3 washes of 20 min. each) followed by a 10 min. wash in TBST.

The nitrocellulose membranes were blocked in TBST containing 5% (w/v) fat free milk powder. They were then incubated for 1 h in primary antibody diluted (1:1000) in TBST containing 5% (w/v) milk powder. The filters were washed with TBST and incubated in a 1:2000 dilution of the secondary antibody, in 5% (w/v) fat free milk powder. The antigens were visualized by enhanced chemi-luminescence (ECL) using the Amersham ECL-detection kit as described by the manufacturer.

■ RESULTS:

Methionine is the first amino acid to be incorporated into newly synthesized peptides during protein synthesis. Therefore, radioactively labeled methionine was used to visualize newly synthesized proteins following rust infection in resistant and susceptible wheat plants.

In plants, protein synthesis occurs in both the cytoplasm and the organelles (chloroplasts and mitochondria), resulting in a protein profile that consists of the cumulative production of all newly synthesized peptides from the cytoplasm and organelles. To selectively visualize proteins synthesized in the cytoplasm from those

produced in the organelles, the cells were exposed to chloramphenicol and cyclohexamide. Chloramphenicol is an antibiotic that inhibits protein synthesis by inhibiting chain elongation in the organelles without affecting synthesis in the cytoplasm, while cyclohexamide inhibits synthesis by blocking translocation in the cytoplasm.

Presented in Fig. 2.1 and Fig. 2.3 (20μg per well) are the total protein profiles obtained by SDS-PAGE of uninfected (control) and infected susceptible and resistant wheat plants, treated with cyclohexamide and chloramphenicol.

The Coomassie brilliant blue stained protein profiles of the infected and uninfected susceptible line (Thatcher) and the profile of the infected and uninfected resistant line (Thatcher/*Lr34*), appear almost similar (Fig. 2.1 & 2.3; lanes 1-3 and 4-6 respectively). A diffuse ±37.0 kDa band is induced following infection in both susceptible and resistant plants, and a ±45.0 kDa band expressed at higher levels in the resistant plants, appear to be the only visible differences using this staining method.

From the autoradiograph of the newly synthesized polypeptides in the susceptible plant (Fig 2.2), two bands appear following fungal infection. These ±24.5 kDa and ±37.0 kDa bands are apparently actively synthesized following infection, while a ±72.5 kDa band disappears from the profile upon infection.

The autoradiograph of the resistant plant showed that the ±37.0 kDa and ±72.5 kDa bands observed in the infected susceptible plant were present in both the uninfected and infected resistant plants (Fig. 2.4).

Treatment with the different inhibitors was successful (Fig. 2.2 & Fig. 2.4). The uptake of cyclohexamide into the cells was highly efficient, visible in the total suspension of protein synthesis in the cytoplasm. Only two bands are visible in the ±52.0 kDa region, due to uninhibited protein synthesis in the organelles (Fig. 2.2; 2.4). Treatment and subsequent uptake of chloramphenicol was equally successful judging from the absence of the organelle synthesized bands in the ±52.0 kDa region, compared to the presence thereof in the control. No difference could be

observed in the organelle synthesized protein profiles between the rust infected and uninfected cyclohexamide treated samples.

Immunoblotting of the transferred and sodium-meta-periodate treated protein extracts revealed at least six identifiable bands. A single band at ±36.5 kDa was revealed with the antibodies against β-1,3-glucanase (Fig. 2.5), while the anti-WGA revealed two bands at ±56.0 kDa and ±62.5 kDa respectively (Fig. 2.6). The PGIP-I antibody revealed numerous bands of which only three, due to their induction following infection and their molecular weights falling within the range of known PGIPs, are considered to be of interest; at ±37.0 kDa, ±42.0 kDa and ±44.0 kDa (Fig. 2.7).

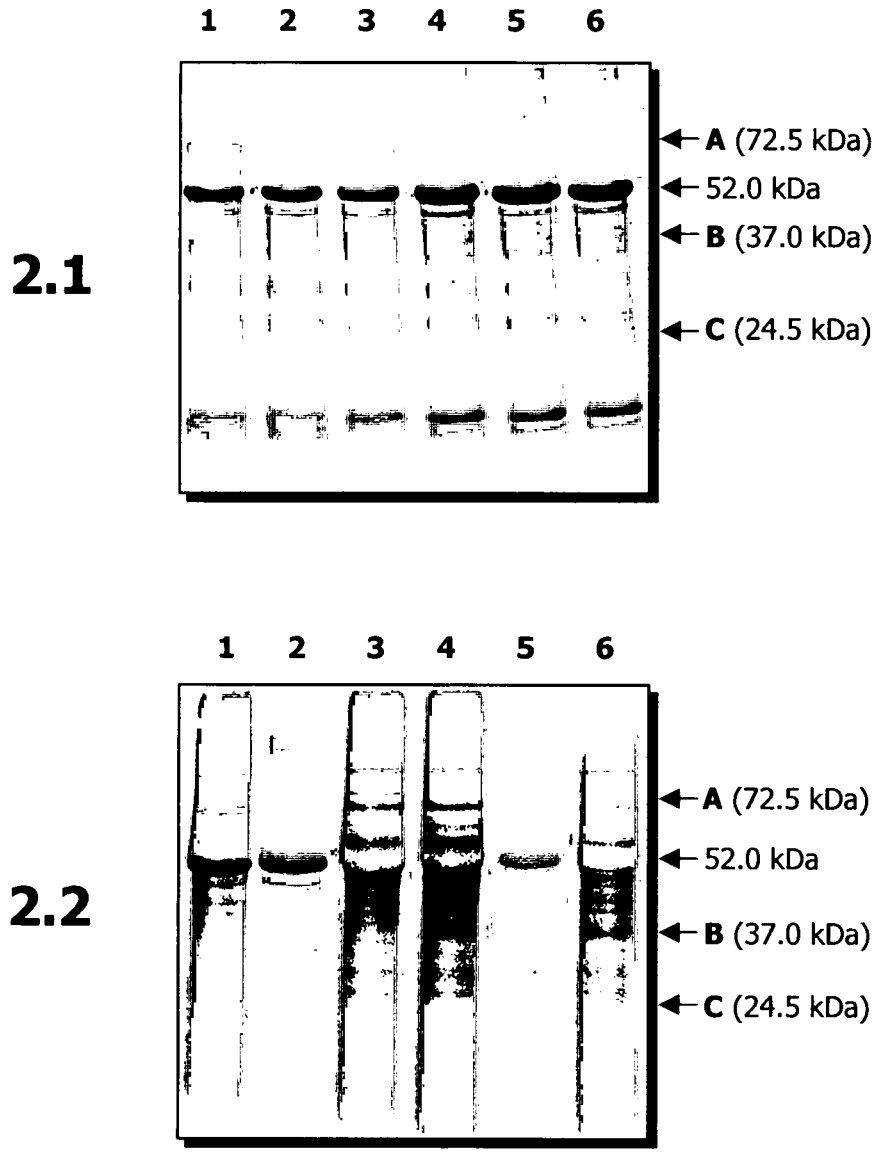


Fig. 2.1. SDS-PAGE separation and, Fig. 2.2, corresponding autoradiograph of methionine labeled susceptible wheat extract. Lane 1) Uninfected positive control. **Lane 2)** Uninfected, cyclohexamide treated plants. **Lane 3)** Uninfected, chloramphenicol treated plants. **Lane 4)** Leaf rust infected positive control. **Lane 5)** Leaf rust infected, cyclohexamide treated plants. **Lane 6)** Leaf rust infected, chloramphenicol treated plants. A, B and C indicate bands of interest.

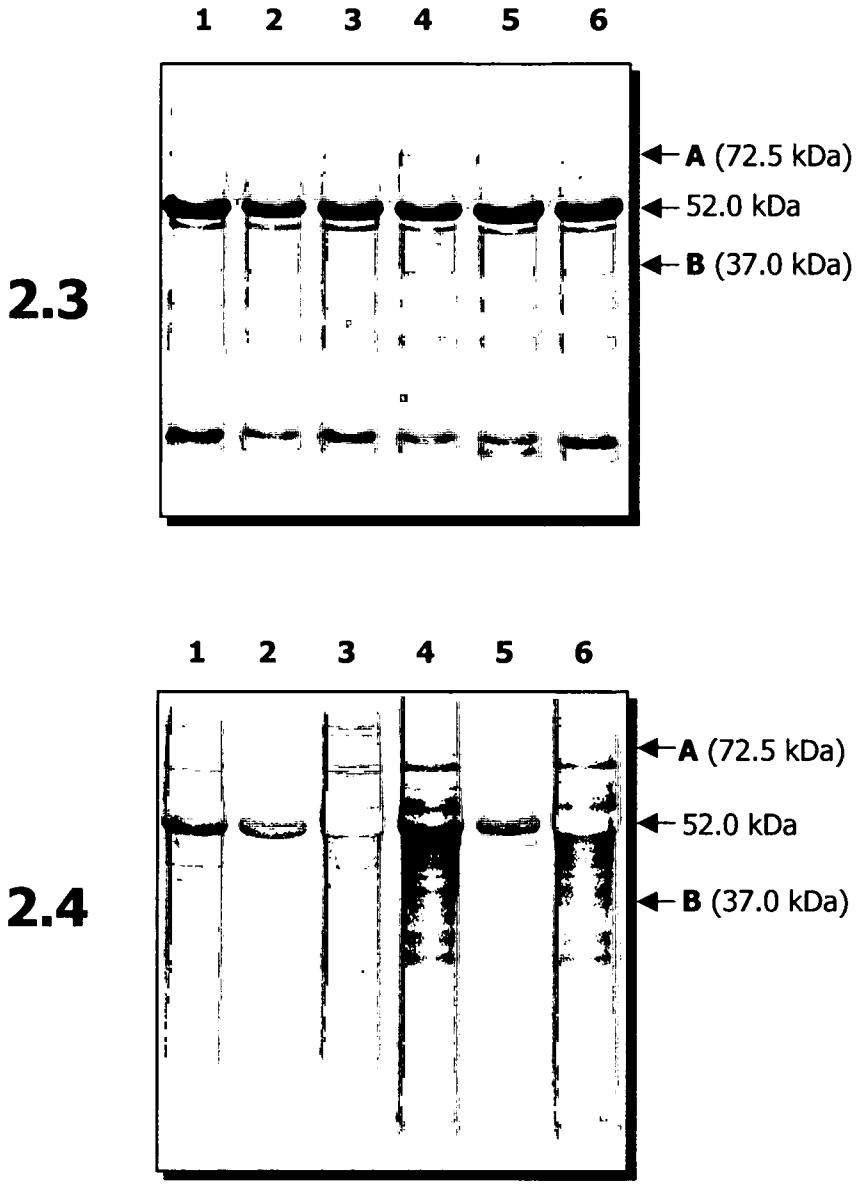
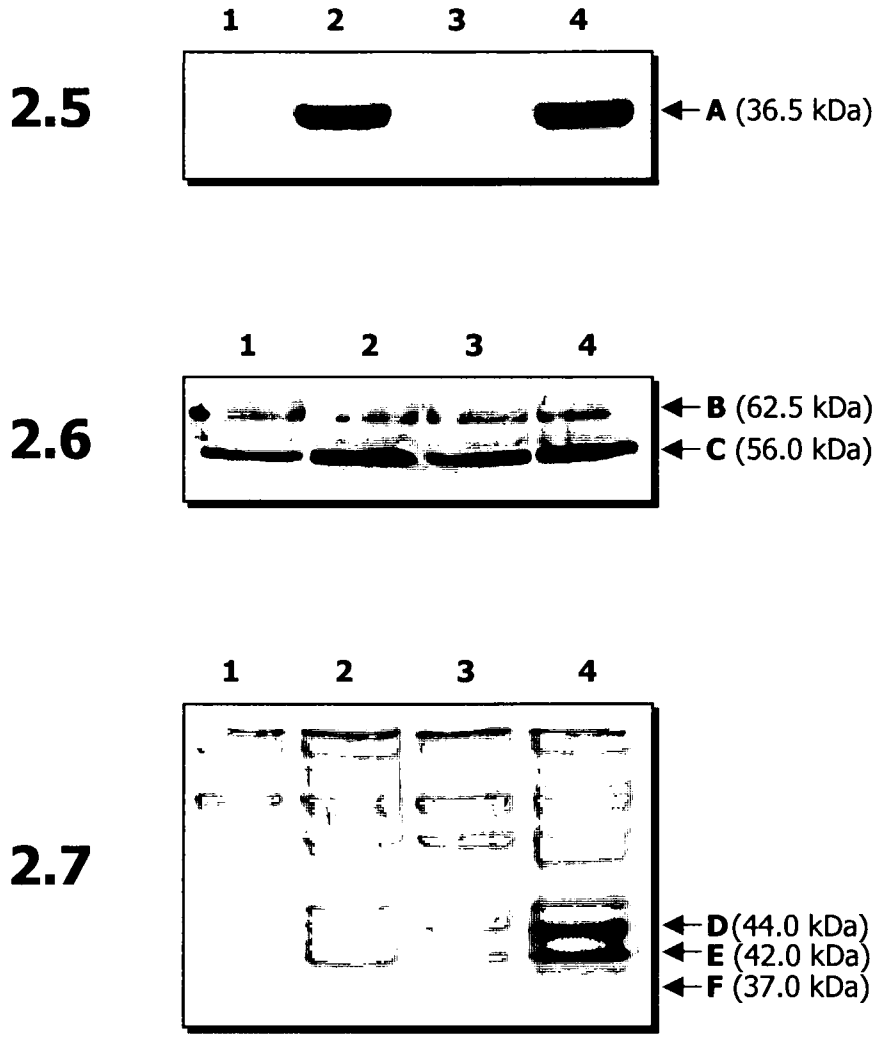


Fig. 2.3. SDS-PAGE separation and, Fig. 2.4, corresponding autoradiograph of methionine labeled resistant wheat extract. Lane 1) Uninfected positive control. **Lane 2)** Uninfected, cyclohexamide treated plants. **Lane 3)** Uninfected, chloramphenicol treated plants. **Lane 4)** Leaf rust infected positive control. **Lane 5)** Leaf rust infected, cyclohexamide treated plants. **Lane 6)** Leaf rust infected, chloramphenicol treated plants. A and B indicate bands of interest.



Immunoblot analysis of 20 μg total protein extract from uninfected and rust infected wheat plants to aid in identifying unknown synthesized bands. Fig. 2.5. Probed with anti-β-1,3-glucanase. **Fig. 2.6.** Probed with anti-WGA. **Fig. 2.7.** Probed with PGIP-I. **Lane 1)** Uninfected susceptible wheat extract. **Lane 2)** Infected susceptible wheat extract. **Lane 3)** Uninfected resistant wheat extract. **Lane 4)** Infected resistant wheat extract.

■ PGIP ACTIVITY

Motivated by the fact that the immunoblot indicated the possible presence of PGIP in wheat, the expression of this inhibitor in susceptible and resistant wheat following rust infection was further investigated. This approach was justified by the expectation of an induction of the potential inhibitor, as expected from all PR proteins, and measurable in activity assays with a corresponding change in the observed immunoblot bands.

The general trend observed was different to the expected expression, with the uninfected plants exhibiting in almost all the cases, except in the resistant plant (Fig. 2.8B) where no difference was observed, a higher inhibition level than the infected samples. From the inhibition assay profile of the one-week-old plants (Fig. 2.8A), the uninfected plants clearly exhibited higher inhibition levels compared to the infected plants. Inhibition levels drastically increased in both infected and uninfected control plants from 96 h.p.i. in the susceptible line and 72 h.p.i. in the resistant line. Although the control plants were not infected, their sampling times are also referred to as 'hours post inoculation' (h.p.i.).

The two-week-old plant's inhibitor assay profile (Fig. 2.8B) again showed higher inhibition levels in the uninfected susceptible plants compared to the infected control. As mentioned above, the resistant line showed no difference in inhibition between infected and uninfected plants.

In the three-week-old wheat plants the trend of higher inhibition levels for the uninfected plants were taken to new highs as inhibition levels peaked from 48 h.p.i. in the susceptible plants (Fig. 2.8C) and 24 h.p.i. in the resistant plants, with levels in the infected plants remaining considerably lower.

As recorded in these three repeats the trend of lower PGIP activity following infection was different from the expected expression. The general trend of the infected treatment being lower than the uninfected control was considered meaningful in this preliminary investigation, rather than differences between individual sampling points.

■ SDS-PAGE AND IMMUNOBLOTTING

Immunoblotting with the PGIP-I antibodies revealed a large number of bands. Most of these were dismissed as being possible cross-reactions with the carbohydrates in the structure of PGIP (Bergmann *et al.*, 1994). Treatment with sodium-meta-periodate removed most of these false PGIP bands. The remaining bands were analyzed further. Bands were considered important if it showed either an induction or reduction in intensity, or it remained present during a substantial part of the sampling stages.

Three bands fell into these criteria, i.e. ±66.0 kDa; ±36.0 kDa and ±24.5 kDa.

The **66.0 kDa** band appeared to be present, although at different intensities, over the entire three-week sampling period (Fig. 2.9 – 2.14). The medium-sized **36.0 kDa** band was expressed from week 2 onwards following infection in the susceptible line, but constitutively expressed in both the uninfected and infected resistant plants (Fig. 2.11, 2.12). This band was considered absent during week 1 (Fig. 2.10), although traces could be observed in all the resistant plants from week 2 (Fig. 2.12, 2.14). The **24.5 kDa** band showed a clear reduction in band intensity in all the infected and uninfected plants from high levels present at 0 h.p.i. to lower levels at 144 h.p.i. (Fig. 2.9 - 2.14).

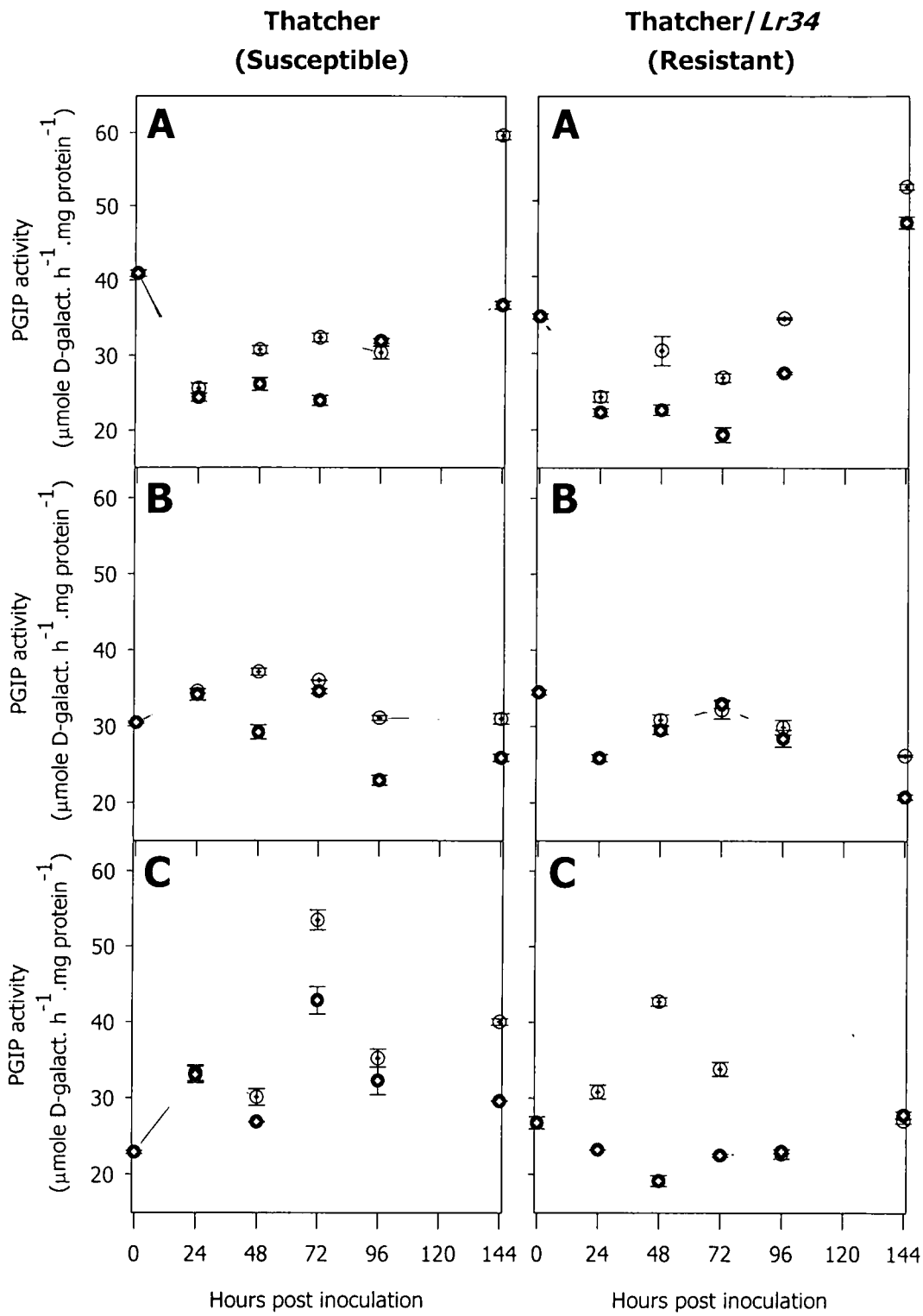
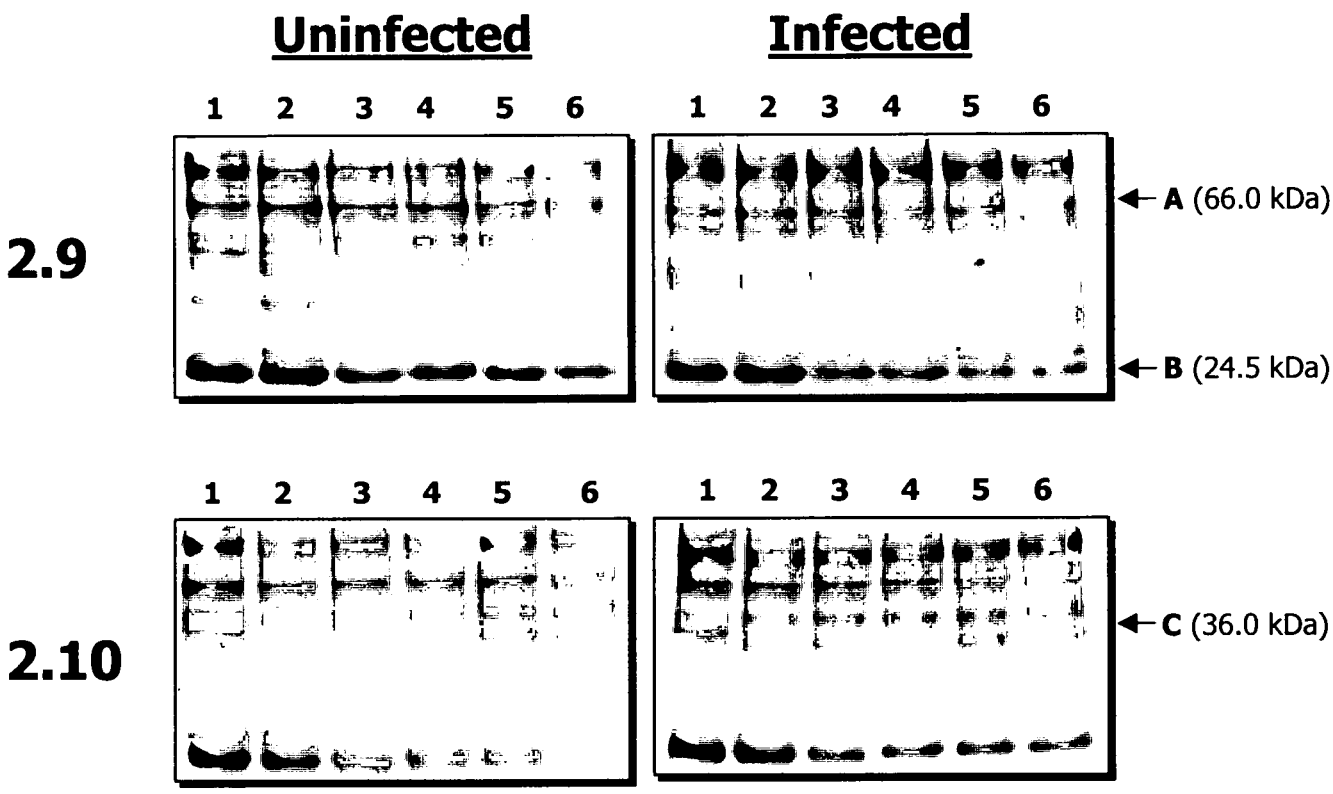
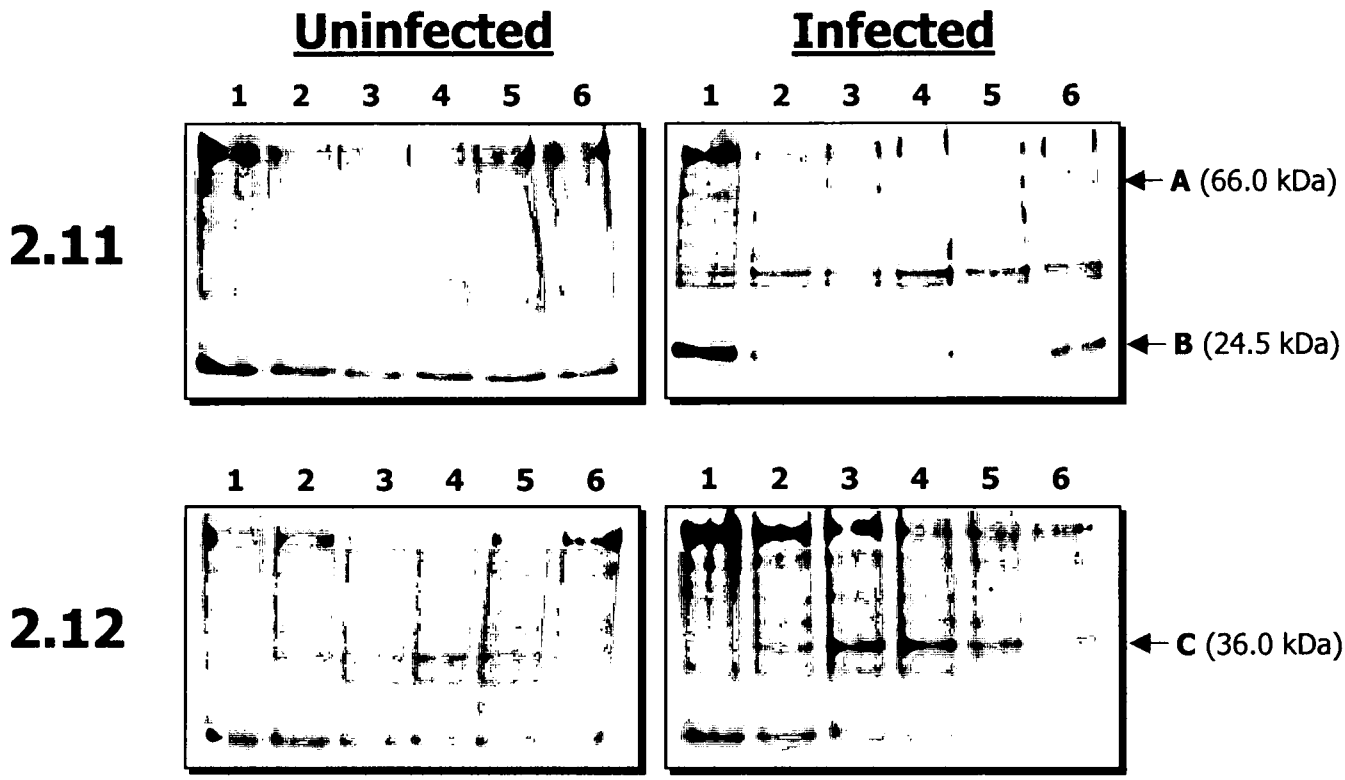


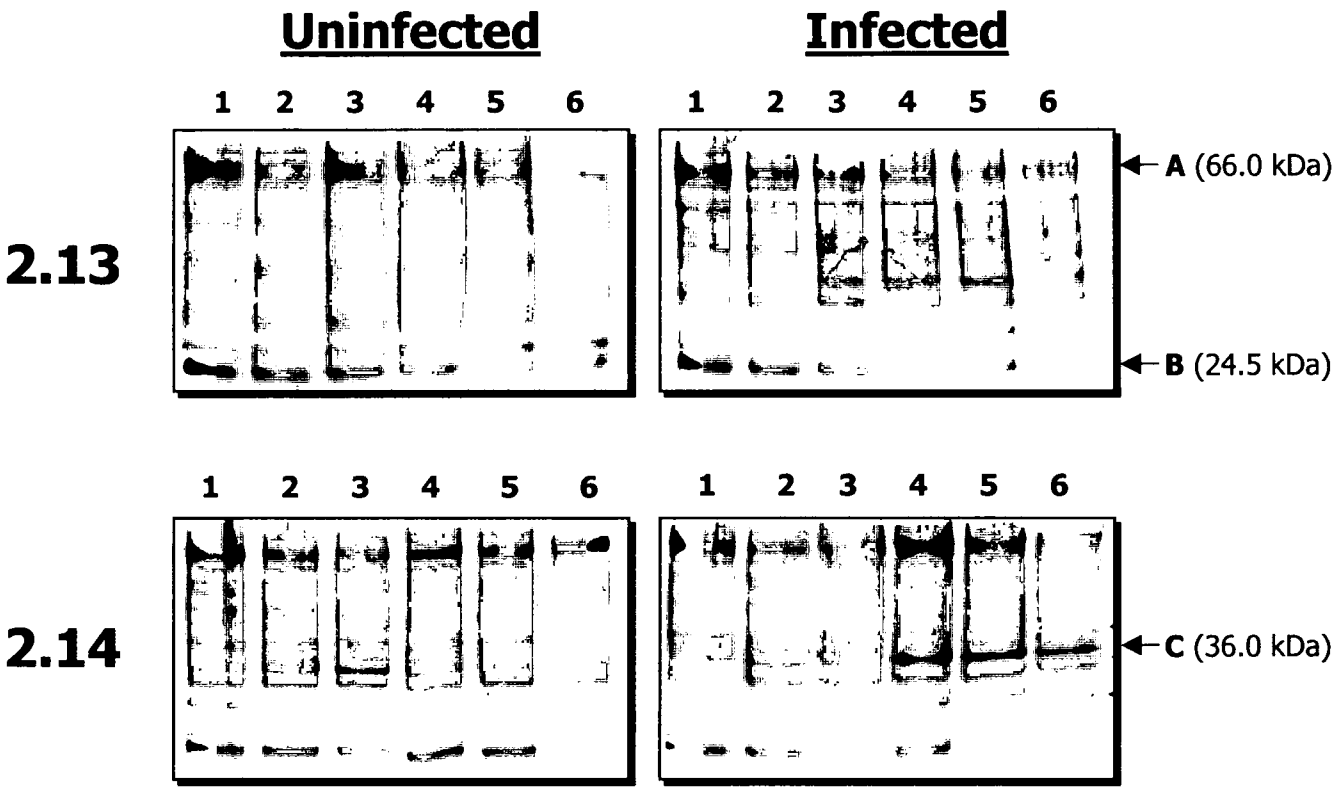
Fig. 2.8. Inhibition of *A. niger* endopolygalacturonase (expressed as PGIP activity) by intercellular protein extracts of leaf rust infected (-●-) and uninfected (-O-) susceptible and resistant wheat cultivars over a period of 144 hours. A) One week-old seedlings. **B)** Two week-old seedlings. **C)** Three week-old seedlings. PGIP activity refers to the formation of reducing sugars in the assay and is expressed in µmole D-galacturonic acid h^{-1} mg^{-1} protein extract. Error bars represent the standard deviation (n=3).



Immunoblot analysis of 6 μg intercellular proteins from leaf rust infected and uninfected susceptible and resistant one-week-old wheat plants over a period of 144 hours. Fig. 2.9 Susceptible Thatcher wheat. **Fig 2.10.** Resistant Thatcher/*Lr34* wheat. **Lane 1)** 0 hours post inoculation (h.p.i.). **Lane 2)** 24 h.p.i. **Lane 3)** 48 h.p.i. **Lane 4)** 72 h.p.i. **Lane 5)** 96 h.p.i. **Lane 6)** 144 h.p.i.



Immunoblot analysis of 6 μg intercellular proteins from leaf rust infected and uninfected susceptible and resistant two-week-old wheat plants over a period of 144 hours. Fig. 2.11 Susceptible Thatcher wheat. **Fig 2.12.** Resistant Thatcher/*Lr34* wheat. **Lane 1)** 0 hours post inoculation (h.p.i.). **Lane 2)** 24 h.p.i. **Lane 3)** 48 h.p.i. **Lane 4)** 72 h.p.i. **Lane 5)** 96 h.p.i. **Lane 6)** 144 h.p.i.



Immunoblot analysis of 6 μg intercellular proteins from leaf rust infected and uninfected susceptible and resistant three-week-old wheat plants over a period of 144 hours. Fig. 2.13 Susceptible Thatcher wheat. **Fig 2.14.** Resistant Thatcher/*Lr34* wheat. **Lane 1)** 0 hours post inoculation (h.p.i.). **Lane 2)** 24 h.p.i. **Lane 3)** 48 h.p.i. **Lane 4)** 72 h.p.i. **Lane 5)** 96 h.p.i. **Lane 6)** 144 h.p.i.

■ DISCUSSION:

It has been well documented that the invasion of a host plant by a pathogen triggers a wide range of defense responses (Sachs and Ho, 1986; Bowles, 1990). In this regard, wheat is no exception (Kemp *et al.*, 1999). These defense mechanisms invariably begin through activation of gene transcription in the plant, leading to mRNA production and terminating in the formation of new proteins. It was the intention of this preliminary study to investigate the extent of induction of new proteins, to identify a subset of them, and shed more light on a correlation of protein expression with disease development.

One method for searching for potential defense mechanisms is to investigate differences in the total protein profiles of uninfected and infected plants, as well as between susceptible and resistant plants. The Coomassie stained protein profiles in this work revealed the presence of a ±37.0 kDa band induced following infection in both resistant and susceptible wheat lines. The autoradiograph of the susceptible line clearly showed new synthesis of a ±37.0 kDa band in the infected plants. In comparison, resistant plants showed an elevated level of protein synthesis of the ±37.0 kDa band in both the uninfected and infected plants.

This work did not reveal many newly synthesized peptides following rust infection. Identification of these peptides through immunoblotting was inconclusive, as the antibodies used identified a peptide in the 36.0-37.0 kDa range to be either a β-1,3-glucanase or a possible PGIP. The role of glucanases in wheat-pathogen interactions is well known and has been studied extensively (Kemp *et al.*, 1999), however this is not the case, as pointed out before, for PGIP in wheat.

As pathogenesis-related (PR) proteins are pathogen inducible, susceptible and resistant wheat plants were infected with leaf rust at different plant stages to examine those proteins, identified by immunoblot (±44.0 kDa, ±42.0 kDa and ±37.0 kDa), expressed in response to fungal infection. Expression was investigated by measuring the inhibitory activity and by means of immunological studies using polyclonal antibodies.

As the name describes, PGIP is a protein with the potential to inhibit polygalacturonase activity. PGIP activity in a plant is therefore, for this study, seen

as an inverse indication of the *in vitro* endopolygalacturonase activity, which means that an increase in PGIP activity is equal to a decrease in the EPG activity of the assay reaction. For this experiment a resistant wheat line was used that contained the leaf rust resistance gene *Lr34*, characterized by providing resistance to adult wheat plants. The expression of the possible wheat PGIP was therefore measured over a period of three weeks, as the plant grew older, compared to its uninfected control, aiming to determine whether resistant wheat plants exhibited a different expression profile compared to susceptible wheat.

In the experiment, the uninfected control plants reflected the constitutive background inhibitor levels that accompany the development process and, additionally, serve to contrast the infected plant's reaction to infection. PR proteins typically react to fungal infection by a significant increase in activity levels compared to the uninfected control. Interestingly, the general trend revealed infected plants exhibiting lower inhibitor levels when compared to the uninfected control in both resistant and susceptible lines. From the results it would thus appear as if wheat plants exhibit their highest level of endopolygalacturonase inhibition when not challenged, with less endopolygalacturonase inhibition, or alternatively an increase in endopolygalacturonase activity, present in fungal infected wheat plants. An increase in EPG activity can only be achieved in one, or a combination of three ways. Firstly, through lower inhibitor concentrations, secondly, through the induction of EPG activity and thirdly, through the increase in EPG concentration.

Considering the immunoblots for a possible explanation, the PGIP-I antibody revealed three bands. A ±24.5 kDa, ±36.0 kDa and ±66.0 kDa band appeared (Fig. 2.9 – 2.14), of which the 36.0 kDa band can be assumed to be the same as the 37.0 kDa band (Fig. 2.7F) previously observed. This band being the only one of the three to have a size that fall within the general accepted range of molecular weights for PGIP, first appeared in the infected two-week-old plants at 24 h.p.i, with minor traces visible in the uninfected control plants from 72 h.p.i. The intensity of the band is quite pronounced in the three-week-old infected susceptible and resistant plants.

With the immunoblots confirming the induction of PGIP levels, and the 'reducing sugar reaction' (in the formula used to calculate inhibition activity), ruling out the

presence of an EPG in the plant extract that would artificially increase the EPG concentration in the assay, an explanation for the recorded lower inhibitor activity seems to point at either an induction in EPG activity or the suppression of PGIP activity, as will be discussed later. Additionally, the observed reduction in PGIP activity could be explained by less free extractable inhibitor being available in the plant due to its preoccupation with the EPG, which will result in less available PGIP for assay purposes, resulting in lower recorded inhibition values. Furthermore, an explanation could also be found in the genetics of the plant itself. With the control plants being susceptible and therefore not able to resist infection, and the resistant plants conferring resistance only in adult plants, an explanation could be that none of the sampling stages were mature enough and therefore being unable to react by increasing PGIP levels following fungal infection.

In conclusion, these preliminary results indicate that wheat plants react to fungal infection, which, when compared to the control plants, can be seen in inhibition assays with *A. niger* EPG. These assay profiles are, however, inconclusive and serve as motivation to steer future research into finding convincing evidence for, 1) a possible induction in EPG or suppression of PGIP activity, 2) a possible deficit of accessible PGIP in rust infected plants that could negatively reflect on assay results, and 3) the lower PGIP activity by using resistant wheat lines that confers resistance to wheat seedlings instead of adult wheat plants. Due to the specificity, as pointed out before, which exists between PGIP and EPG, one additional objective could be set, 4) to investigate the specificity of wheat PGIP to the *A. niger* EPG used, and if need be, to find a more suitable EPG.

CHAPTER 3

EXTRACTION AND PURIFICATION OF PGIP FROM WHEAT

From the literature it is clear that varying levels of specificity exist between PGIPs and the EPGs that they will potentially inhibit. This was pointed out as a potential problem during the investigation into the expression of PGIP in wheat (see previous chapter). This chapter describes an attempt to extract and purify PGIP from wheat and use the partially purified fractions to detect inhibitable EPGs.

Ideally, a PGIP-EPG investigation occurs between a purified EPG from a known fungal pathogen and the plant that is under investigation. The EPG is traditionally obtained by growing the fungus on a medium containing pectin or PGA as its sole source of carbon. The fungus then releases pectin-degrading enzymes into the media, from which endopolygalacturonase is harvested. Wheat leaf rust is an obligate parasite and thus not readily cultured on artificial media. Fasters *et al.* (1993) developed a method for the successful cultivation of stem rust in a liquid medium containing various carbohydrates as carbon source. Although optimized for the development of fungal tissue this method needs further optimization for the production of large amounts of EPG isoforms with adequate activity for inhibition assays. Due to time constraints the large-scale production of *Puccinia triticina* EPG for this study was excluded, and required that an alternate EPG be identified through previously optimized protocols.

■ MATERIALS:

■ PLANT MATERIAL

Wheat seeds (*Triticum aestivum*, winter wheat, cultivar unknown) were grown under greenhouse conditions for four weeks until harvesting of foliage and stems.

■ ANTIBODIES

A more specific antibody (PGIP-II) generated in a rabbit against a peptide corresponding to residues 10-21 of *Phaseolus vulgaris* PGIP was received from Dr. CW Bergmann[16] (Bergmann *et al.*, 1994). This polyclonal antibody has been successfully used by James & Dubery (2001) to purify PGIP from cotton.

[16] Complex Carbohydrate Research Center, University of Georgia, USA

As secondary antibody an alkaline phosphatase conjugated goat anti-rabbit antibody (Sigma)[17] was used.

■ METHODS:

■ PGIP EXTRACTION AND PURIFICATION

Four hundred grams of leaf and stem tissue were homogenized in a blender with 750 mL of acetone. The pulp was filtered through a nylon cloth, squeezed dry, re-blended with 500 mL of acetone and allowed to completely dry out. The dried pulp was soaked in extraction buffer (20 mM NaAc pH 6.0, 1M NaCl, 1% [w/v] Polyvinylpyrrolidone (PVP)) and allowed to stir for 12 h.

The material was centrifuged at 14000 *g* for 45 min. followed by centrifugation of the supernatant for a further 45 min. at 18000 *g*. The clear supernatant was subsequently dialyzed against 4000 mL of 20 mM NaAc pH 6.0 over a period of three days with frequent changes. The dialyzed sample was centrifuged for 45 min. at 18000 *g*, the supernatant was recovered and concentrated down to 200 mL on an Amicon stirred cell with a PM10 membrane. Following concentration, the sample was centrifuged and the supernatant was diluted 1:1 with an equal amount of 2x Con A buffer (200 mM sodium acetate pH 6.0, 2 M NaCl, 2 mM $CaCl_2$, 2 mM $MgCl_2$, 2 mM $MnCl_2$).

The PGIP was purified from the extract by cation exchange HPLC using a HiTrap-S column (Pharmacia)[18] at a flow rate of 0.7 $mL.min^{-1}$. The column was equilibrated with buffer A (20 mM NaAc pH 5.0) and eluted with buffer B (20 mM HEPES pH 7.5, 0.3 mM NaCl), using a linear gradient of $1\%.min^{-1}$. The 0.5 mL fractions (ConA-bound-HiTrap-bound) were analyzed by inhibition assay (see below) and immunoblotting for the presence of PGIP.

Fractions exhibiting high levels of inhibition were further purified by size exclusion on a Superdex 75[19] column. Samples demonstrating inhibitory activity were loaded in 200 μL aliquots and eluted from the column with a 50 mM NaAc (pH 5.0), 300 mM

[17] Sigma Chemical Company, St. Louis, MO, USA
[18] Pharmacia LKB Biotechnology Inc. Piscataway, NJ, USA
[19] Amersham Biosciences. Piscataway, NJ, USA

NaCl buffer and collected in different fractions, which were analyzed for EPG inhibition.

■ PGS

Endopolygalacturonases from different fungal sources were used. Endopolygalacturonase from *Cochliobolus sativus* and *Fusarium moniliforme* were produced and purified to homogeneity as described (Clay *et al.*, 1997; Caprari *et al.*, 1993a). *Aspergillus niger* PGI and PGII were obtained from J Visser[20]. *Colletotrichum lindemuthianum* PG was produced and purified to homogeneity as described by English *et al.* (1972) and modified by Cook *et al.* (1999). *Cryphonectria parasitica* PG was obtained from L Shain[21]. *Aspergillus niger exo* PG was obtained from R Pressey[22]. *Postia placenta* PG was obtained from C Clausen[23]. Where possible, all PGs were analyzed for purity as decribed by Cook *et al.* (1999).

■ PGIP ACTIVITY ASSAY

PGIP activity was measured as indicated in Chapter 2, but modified so that the inhibition of EPG activity was measured as a change in activity of an appropriate EPG (as previously determined), by spectrophotometrically (410nm) measuring the release of reducing sugars by the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (York *et al.*, 1985) from polygalacturonic acid (PGA) at room temperature.

A positive control was included that consisted solely of enzyme (EPG) and substrate (PGA), while the negative control only consisted of PGA. Inhibition levels were calculated as indicated by the formula in Chapter 2 and expressed as a percentage, with 100% inhibition having no reducing sugars after assay with an absorbance value equal to that of the negative control, and 0% inhibition showing no change in absorbance from that of the positive control.

■ PROTEIN GEL ELECTROPHORESIS

Fractions exhibiting inhibitory activity were separated by SDS-PAGE as described by Laemmli (1970). Polyacrylamide gels (12%) were silver stained (Shevchenko *et al.*, 1996)

[20] Wageningen, The Netherlands
[21] University of Kentucky, KY, USA
[22] USDA, Athens, GA, USA
[23] US Forestry service laboratory, Madison, WI

■ IMMUNOBLOTTING

Separated polypeptides were transferred to nitro-cellulose membranes using a mini trans-blot electrophoretic transfer cell with transfer buffer (25 mM Tris, pH 8.3, 190 mM glycine and 10% [v/v] methanol) at a constant amperage of 350 mA (Towbin *et al.*, 1979).

Following transfer, the nitrocellulose membrane was blocked in TBST (20 mM Tris pH 7.6, 135mM NaCl, 0.1% Tween-20) containing 1% (m/v) BSA followed by incubation for 1 h in primary antibody (PGIP-II, 1:200) diluted in TBS containing 1% (w/v) BSA. The membrane was washed with TBS and incubated in a 1:2000 dilution of the secondary antibody in 1% (w/v) BSA. The antigens were visualized by NBT and BCIP.

■ MATRIX-ASSISTED LASER DESORPTION-IONIZATION TIME OF FLIGHT MASS SPECTROMETRY (MALDI-TOF MS)

Samples of interest were analyzed on a Hewlett Packard MALDI-TOF mass spectrometer. The matrix used was a saturated 50% water and 50% acetonitrile solution of sinapinic acid. Equal volumes of sample and matrix (approx. 0.5 μL) were loaded on to the probe and dried under vacuum. The laser energy ranged from 7 to 12.5 μJ. Since the sample was in a buffer containing 20 mM sodium acetate, 100 mM sodium chloride, and 20 mM HEPES, the protein was dried on the probe tip and the salt washed out of the protein with 1 μL water before adding the matrix to obtain a stronger signal.

■ RESULTS:

The proteins bound to the ion exchange column were eluted and collected in different fractions (Fig. 3.1A). Every other fraction was assayed for endopolygalacturonase (EPG) inhibitor activity against PGs from *A. niger* EPGI and EPGII, *C. sativus*, *C. lindemuthianum*, *A. niger* Exo-PG, *P. placenta*, *F. moniliforme*, *C. parasitica* and a bacterial polygalacturonase. Of all nine polygalacturonases assayed, the partially purified wheat extract was able to successfully inhibit only EPG from *C. sativus* (Fig. 3.2, see arrow). The inhibition profile of the eluted proteins with the EPG from *C. sativus* revealed 35 fractions with inhibition (fractions 11-45)

containing a potential wheat PGIP (Fig. 3.1B). Apart from these fractions from the ConA-bound and HiTrap-bound columns, no fraction of the other eluents (ConA-bound-HiTrap-unbound; ConA-unbound-HiTrap-bound; ConA-unbound-HiTrap-unbound) had any *C. sativus* EPG inhibitory activity.

Eight of these fractions exhibiting the highest levels of inhibition (every other fraction from 27-41) were separated on a 12% SDS-PAGE and silver stained (Fig. 3.3). They revealed a number of bands per fraction, of which any one could be responsible for the observed inhibition. When probed with the highly specific PGIP-II antibody, it recognized three bands in fraction 37 (Fig. 3.5). MALDI-TOF mass spectrometry indicates the sizes of these three proteins as approximately 44500 Da, 36100 Da and 33300 Da, respectively (Fig. 3.4C).

Fraction 37 was loaded onto a Superdex 75 size exclusion column in an attempt to further separate the sample (Fig. 3.6A). The different fractions resulting from this separation were again assayed for inhibition. Inhibition of *C. sativus* EPG inhibition peaked at fraction 33 (Fig. 3.6B) which corresponded with the peak in absorbance observed in the eluted protein profile from the size exclusion column (Fig. 3.6A, see arrows).

Loading and running alternating fractions 29 to 35 of the Superdex separation on a SDS-PAGE, followed by silver staining thereof, revealed a faint band in fraction 33, the fraction containing the highest level of inhibition (Fig. 3.7). Probing it during an immunoblot with the PGIP-II antibody revealed a faint band (Fig. 3.8).

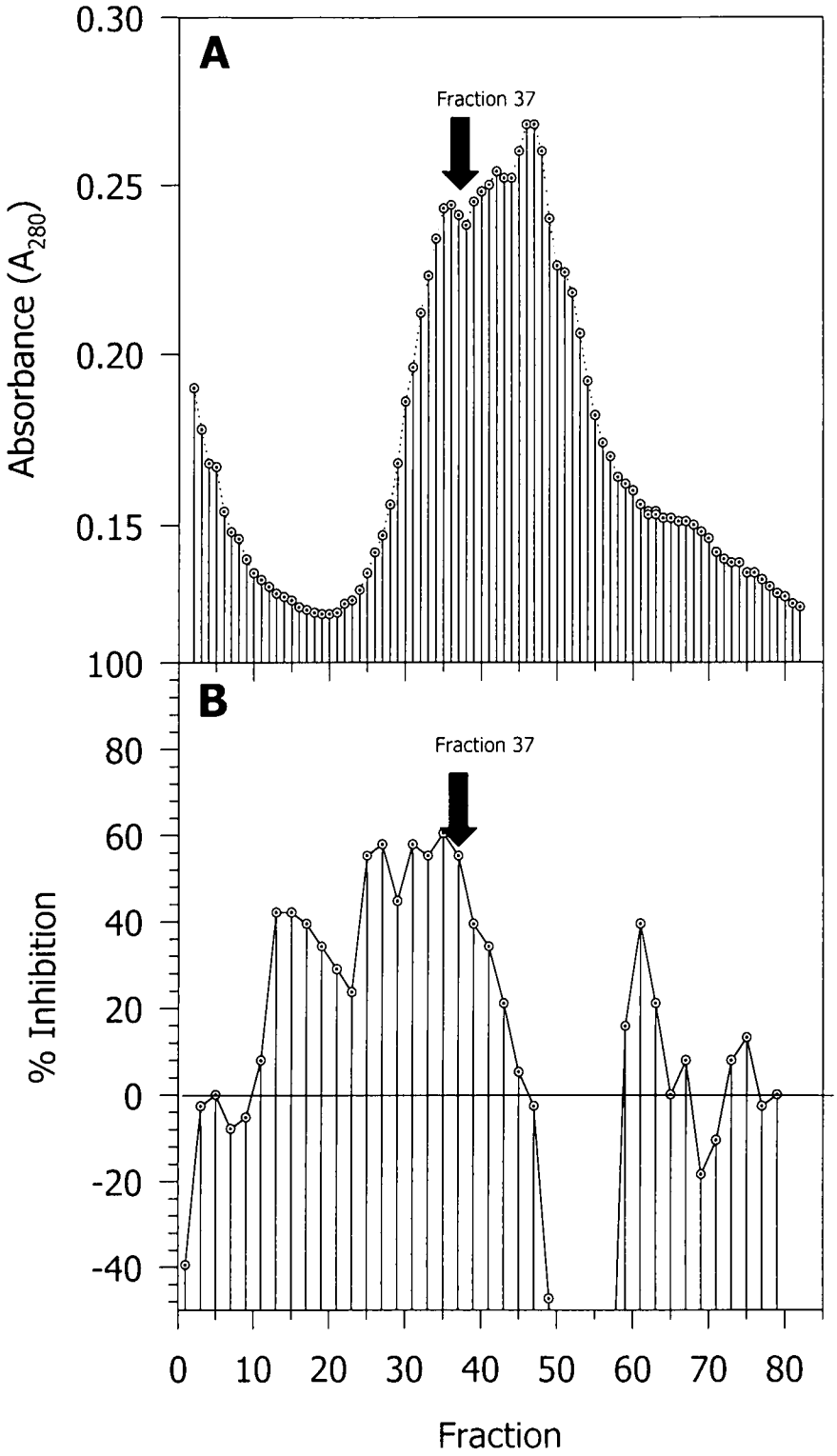


Fig. 3.1. A) Elution profile of proteins bound to HiTrap column. **B)** Inhibition of *C. sativus* EPG by eluted proteins.

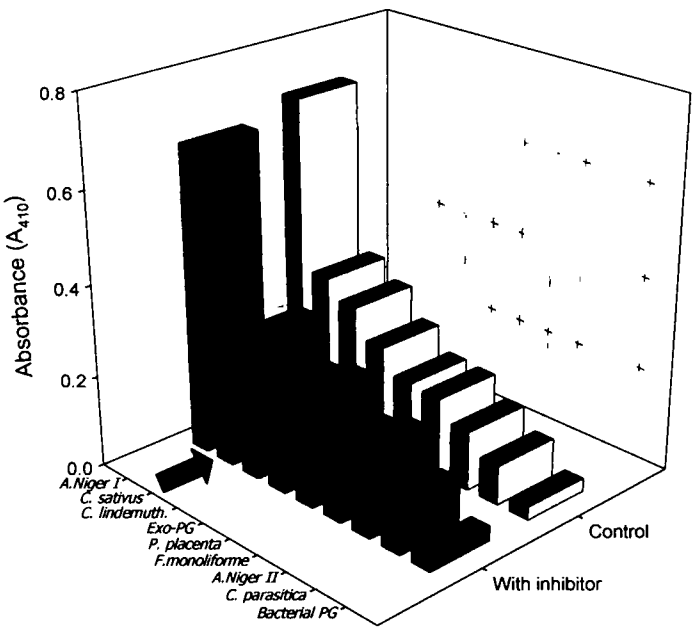


Fig. 3.2. Inhibition of different EPGs by purified wheat fraction 37 as well as uninhibited control. Sources for the EPGs are (from back to front); *A. niger* PGI, *C. sativus*, *C. lindemuthianum*, *A. niger* Exo-PG, *P. placenta*, *F. monoliforme*, *A. niger* PGII, *C. parasitica* and bacterial polygalacturonase.

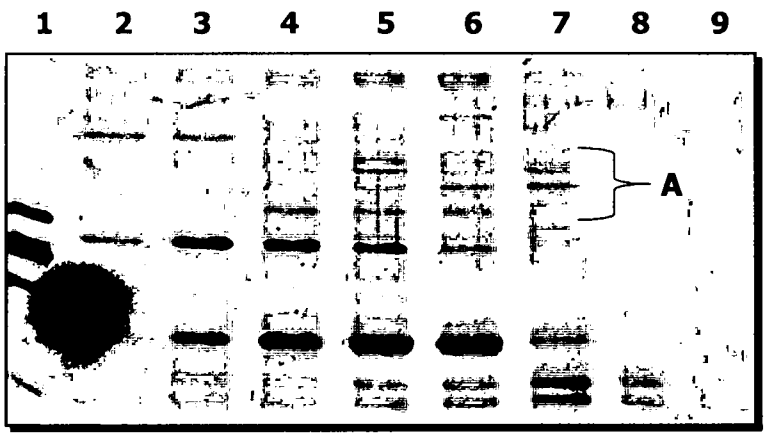
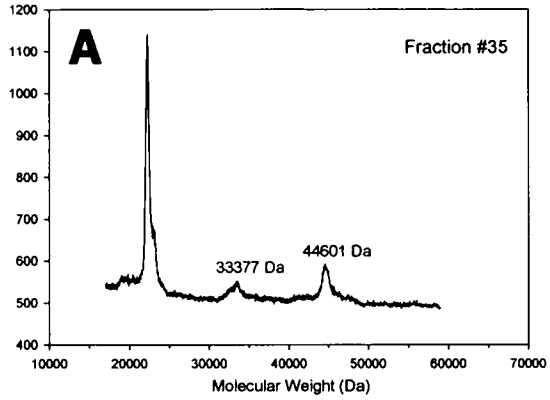
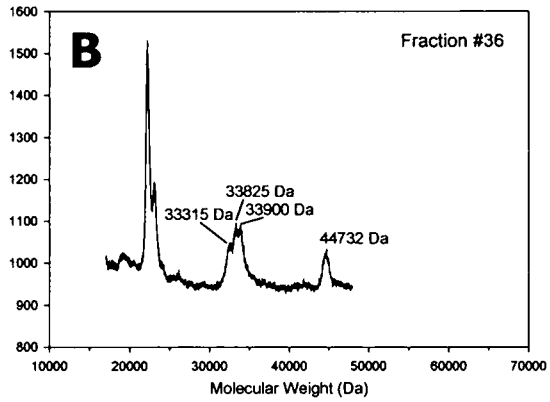
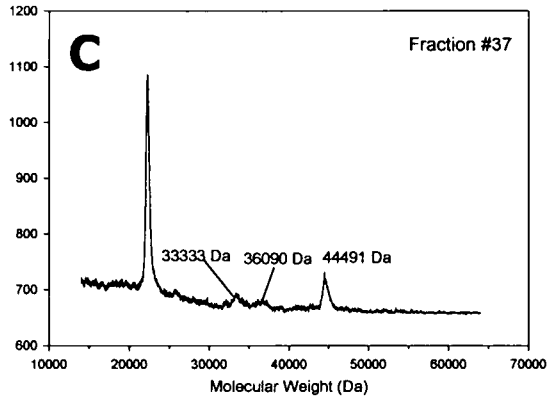
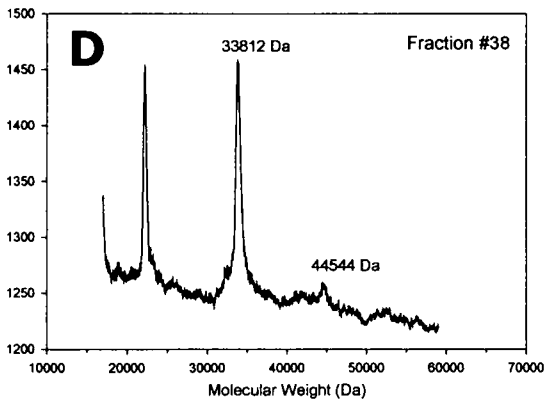


Fig. 3.3. Silver stained SDS-PAGE of fractions from the HiTrap ion exchange column. Lane 1) Pinto bean control. **Lane 2)** Fraction 27. **Lane 3)** Fraction 29. **Lane 4)** Fraction 31. **Lane 5)** Fraction 33. **Lane 6)** Fraction 35. **Lane 7)** Fraction 37. **Lane 8)** Fraction 39. **Lane 9)** Fraction 41. Bands of interest are indicated by the letter A (see below).









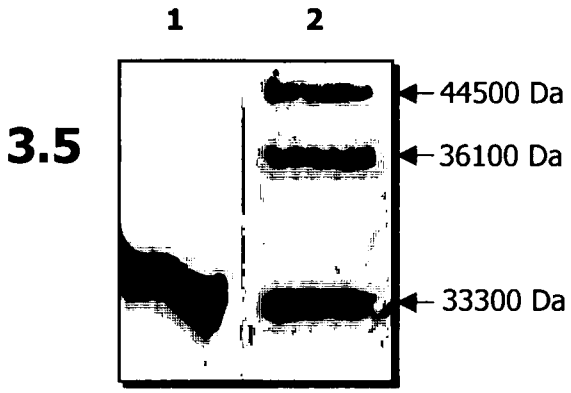


Fig. 3.4. MALDI-TOF Mass spectrometry profiles of HiTrap ion-exchange column fractions in the 20kDa to 60kDa region. A) Fraction 35. **B)** Fraction 36. **C)** Fraction 37. **D)** Fraction 38. **Fig. 3.5. Immunoblot with PGIP-II of two samples. Lane 1)** Pinto bean control. **Lane 2)** Fraction 37.

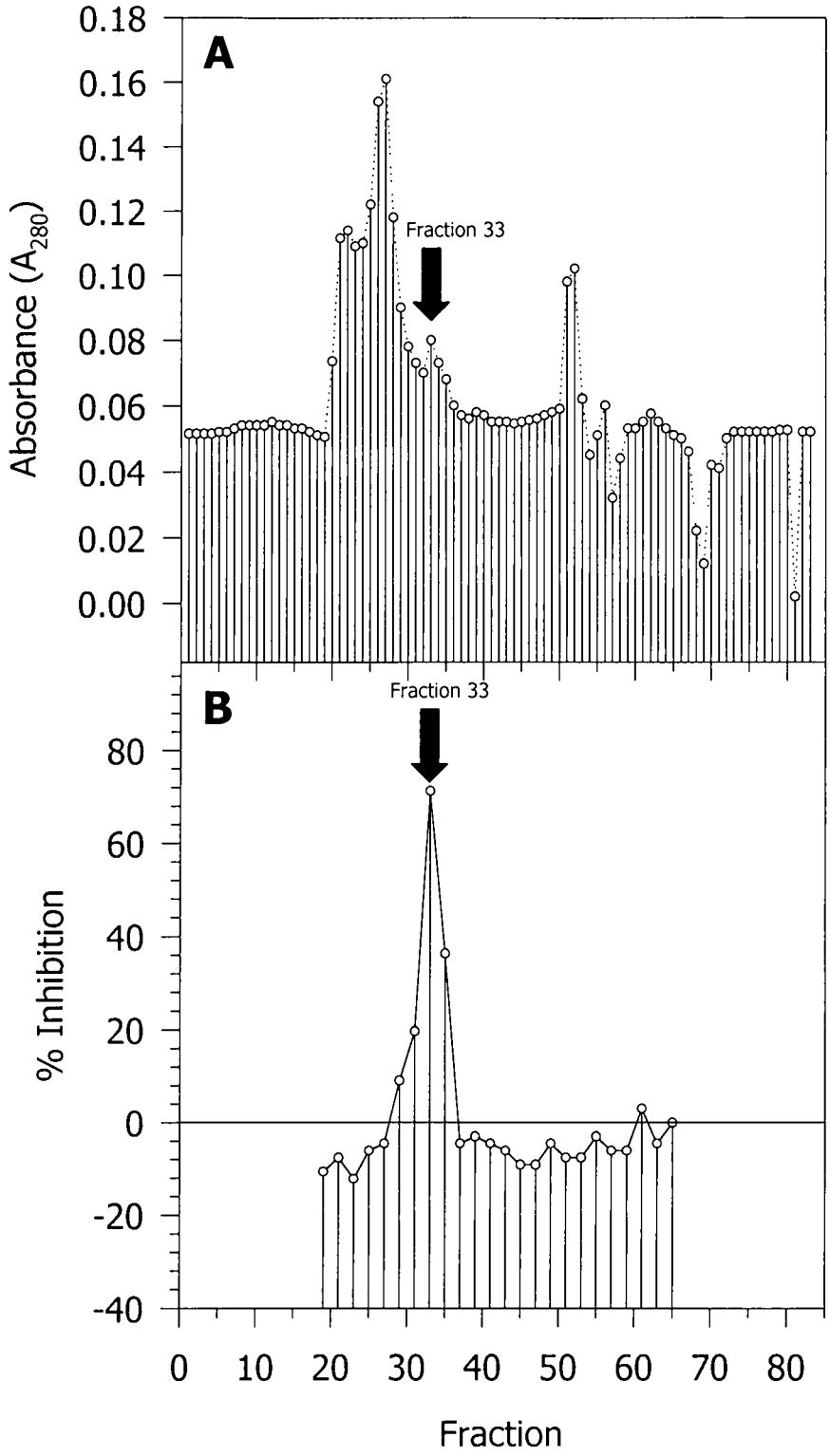


Fig. 3.6. A) Elution profile of proteins bound to Superdex size exclusion column. **B)** Inhibition of *C.sativus* EPG by eluted fractions from the Superdex size exclusion column.

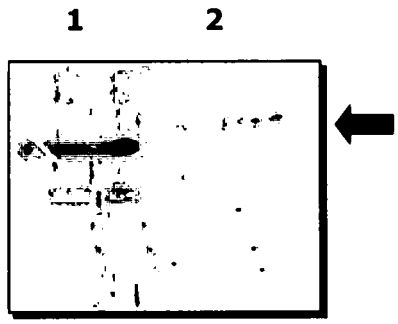


Fig. 3.7. Silver stained SDS-PAGE of fraction 33 off the Superdex size exculsion column. Lane 1) Pinto bean PGIP control. **Lane 2)** Fraction 33.

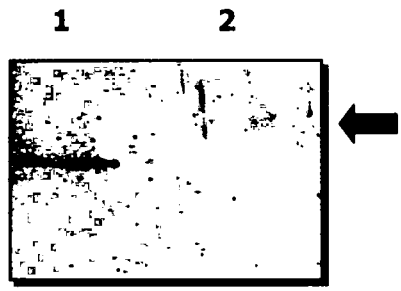


Fig. 3.8. Immunoblot with PGIP-II of fraction 33. Lane 1) Pinto bean PGIP control. **Lane 2)** Fraction 33.

■ DISCUSSION:

Partially purified wheat PGIP was assayed against nine polygalacturonases for inhibition, but was successful against only EPG from *C. sativus*, a wheat pathogen. This proves not only that the observed inhibition is the result of a selective biological inhibitor that does not inhibit EPGs other than an EPG from a cereal pathogen, but also rules out the possibility of it being a protease. This provided concrete evidence for the presence of a PGIP in wheat, and illustrates the specificity of wheat PGIP for its ligand.

Obtaining further information on the size of wheat PGIP was an objective that proved to be extremely difficult. As discussed earlier, wheat contains about 10 times less pectin in the plant cell wall, when compared to dicotyledonous plant cells, and thus a correspondingly low level of PGIP in the tissue of wheat plants will not be extraordinary. In addition, in dicotyledonous plants, vegetative leaf tissue contains substantially less PGIP than fruit or seed (Salvi *et al.*, 1990).

Our findings confirmed extremely low levels of PGIP in wheat leaves. HPLC fractions containing peak levels of PGIP were still many times less than PGIP levels observed in, for instance, fractions from beans. An attempt to sequence the purified protein through Edman degradation failed due to the low PGIP concentration. In spite of this low PGIP levels, further characterization was possible, albeit much more cumbersome.

Separation by SDS-PAGE of those HPLC fractions exhibiting the highest PGIP activities revealed a number of bands per fraction; any of which could be wheat PGIP. The average dicotyledonous PGIP is about 35-40 kDa in size, therefore emphasis was placed on bands between 20 kDa and 60 kDa. Fraction 37, which exhibited the highest activity, contained bands with sizes of 33 kDa, 36 kDa and 44 kDa as determined by MALDI-TOF mass spectrometry. These three bands were also the only bands recognized in fraction 37 during immunoblotting with the PGIP-II antibody.

Further separation of fraction 37 by Superdex 75 size exclusion column revealed one band, at approximately 36 kDa, following SDS-PAGE and silver staining of fraction 33. Not only did fraction 33 off the Superdex column exhibit the highest PGIP activity, but also the 36 kDa band was positively identified by immunoblot to be wheat PGIP.

CHAPTER 4

INHIBITION OF *COCHLIOBOLUS SATIVUS* EPG BY WHEAT PGIP IN REACTION TO CHEMICAL AND FUNGAL STIMULI

Pathogenic infection and salicylic acid (SA) treatment induce proteins that are associated with the natural defense reactions of the host plant, including PGIP expression (Fritig *et al.*, 1998; De Lorenzo *et al.*, 2001). Salicylic acid has been shown to induce certain *pgip* genes in *Phaseolus vulgaris* (Bergmann *et al.*, 1994), as well as *A. thaliana* (De Lorenzo *et al.*, 2001).

In this experiment wheat plants will either be infected with the leaf rust pathogen, or will be treated with the chemical elicitor, salicylic acid. This will assist in determining whether wheat reacts to outside stimuli through the induction of activity and protein levels of PGIP.

■ MATERIALS:

■ CHEMICALS

Endopolygalacturonase, from *Cochliobolus sativus*, was obtained from Dr. CW Bergmann[24]. Polygalacturonic acid (PGA) was an ICN Biochemicals Inc product. Nitrocellulose and the ECL detection kit were purchased from Amersham and salicylic acid from BDH Chemicals. Laminarin and PAHBAH (*p*-hydroxybenzoic acid hydrazide) were purchased from Sigma. All chemicals used were of analytical grade.

■ PLANT MATERIAL

Wheat (*Triticum aestivum* L.) lines Thatcher/*Lr29* and Thatcher were supplied by the Department of Plant Pathology, University of the Free State, Bloemfontein. In the seedling stage Thatcher is susceptible to leaf rust, whereas the near-isogenic Thatcher/*Lr29* line is resistant. The *Lr29* gene for resistance to *Puccinia tricina* was originally transferred to bread wheat from *Thinopyrum ponticum* and its resistant phenotype is characterized by a typical hypersensitive response[25] (McIntosh *et al.*, 1995).

[24] Complex carbohydrate Research Center, University of Georgia, USA
[25] See Appendix C

■ ANTIBODIES

Anti-PGIP antibodies (PGIP-II) were received from Dr. CW Bergmann[1] (Bergmann *et al.*, 1994). As secondary antibody a goat anti-rabbit IgG–horseradish peroxidase conjugate was used (Amersham[26]).

■ METHODS:

■ WHEAT GENOTYPES AND GROWING CONDITIONS

Seeds of Thatcher and Thatcher/*Lr29* were grown in a sterilized soil:peatmoss mixture (1:1 v/v), in an environment free from leaf rust, at 20-25°C until inoculation. Cool-white fluorescent tubes, emitting 120 $\mu Em^{-2}s^{-1}$, provided a 14 h daylength.

■ INOCULATION WITH RUST

Seedlings were inoculated 14 days after planting by spraying the whole plant with freshly harvested spores of pathotype UVPrt9, suspended in distilled water with a drop of Tween-20. Control plants were sprayed with distilled water/Tween-20 only. Inoculated and control plants were allowed to dry for 1 h before placement in the dark in a dew-simulation chamber at 18-20°C for 12 h. Plants were then transferred to the greenhouse. The experiment was repeated in an independent study.

■ TREATMENT WITH SALICYLIC ACID

Seedlings were treated 14 days after planting by spraying the whole plant with a saturated solution (50 mM) of SA in distilled water with a drop of Tween-20 and adding 25 mL of the solution, without the Tween-20, to the soil. Control plants were sprayed with distilled water and Tween-20 only. Treated and control plants were immediately transferred to the greenhouse. The experiment was repeated with 10 mM SA.

■ INFILTRATION OF THE LEAVES AND PREPARATION OF APOPLASTIC FLUID

Leaves from leaf rust inoculated and control plants were sampled at 0, 24, 48, 72 and 120 hours post-inoculation (h.p.i.), and those from SA treated plants at 0, 6, 12, 24 and 72 hours post-treatment (h.p.t.). PGIP in the intercellular spaces of wheat leaves was extracted through vacuum infiltration (Rohringer *et al.* 1983; Salvi *et al.*, 1990). The cut ends of the leaves were washed in water to remove intracellular contamination due

to mechanical wounding by scissors. The leaf pieces were then subjected to vacuum-infiltration for 5 min. with 50 mM sodium acetate buffer (pH 5.0) containing 300 mM NaCl (Salvi *et al.* 1990). To obtain the apoplastic fluid, leaves were centrifuged at 500 *g* with their tips pointing upwards. The apoplastic fluid was collected, protein concentration determined, assayed for β-1,3-glucanase (in SA treated plants) and PGIP activity. Fifty micrograms of protein was precipitated for immunoblotting and stored at -20°C for later use.

■ PROTEIN DETERMINATION

The concentration of extracted proteins was determined according to Bradford (1976) using the protein assay reagent from Bio-Rad[27] with gamma globulin as standard. A microplate reader (Bio-Rad Model 3550) was used for this purpose as described by Rybutt and Parish (1982).

■ ASSAY FOR PGIP ACTIVITY

PGIP activity was measured at each sampling time and expressed as percentages of *C. sativus* EPG inhibition as described in Chapter 3.

■ ASSAY FOR β-1,3-GLUCANASE ACTIVITY

The colorimetric assay of ß-1,3-glucanase was done according to Fink et al. (1988) but modified by spectrophotometrically (410nm) measuring the release of reducing sugars through the PAHBAH (*p*-hydroxybenzoic acid hydrazide) procedure (York *et al.*, 1985) from laminarin as substrate at 37°C for 15 minutes.

A standard curve relating the amount glucose equivalents to absorbance was employed for determination of enzyme activity. The formation of glucose was a linear function of enzyme concentration extracted from the apoplastic fluid (not shown). ß-1,3-glucanase activity was expressed as mM glucose.μg $prot^{-1}$. h^{-1}, respectively.

[26] Amersham International place, Buckinghamshire, England
[27] Bio-Rad Laboratories, Hercules CA, USA

■ SDS-PAGE AND IMMUNOBLOTTING

Fifty micrograms of apoplastic proteins per sampling stage, as determined through an extract dilution series to be the optimum for recognition by the antibody (results not shown), were separated by SDS-PAGE. This was followed by transfer to Hybond C nitrocellulose membrane (Amersham) and probing of the membrane with the PGIP-II antibody (1:50), as previously described.

■ RESULTS:

■ PGIP ACTIVITY FOLLOWING LEAF RUST INFECTION

Leaf rust inoculation was highly effective, with the fungus rapidly developing and colonizing the sprayed leaves[28]. Surprisingly, the sampled leaf stages demonstrated a decrease in PGIP activity from 24 h.p.i. onwards (Fig. 4.1A, B). This trend was confirmed when the experiment was repeated (Fig. 4.1C, D).

■ PGIP ACTIVITY FOLLOWING SALICYLIC ACID TREATMENT

The spraying of wheat leaves with 50 mM SA was evaluated by first recording the plant's response in terms of β-1,3-glucanase activity to this chemical inducer (Fig. 4.2A, B). Glucanase activity increased continuously from 6 h post treatment in both the susceptible and resistant wheat lines, up to the last sampling stage at 72 h.p.t., and constantly remained higher than in the untreated plants (Fig. 4.2A, B).

PGIP activity in the treated susceptible plants drastically increased from 6 h.p.t. to 24 h.p.t., while in the resistant line the effect was not as pronounced. PGIP activity in the treated resistant plants showed an increase from 6 h.p.t. to 12 h.p.t. followed by a drop in activity, to remain at elevated levels higher than in the control plants. The observation that PGIP levels remain higher in the treated plants than the untreated controls was seen in both the susceptible and resistant lines (Fig 4.2C, D).

The trend was confirmed when the experiment was repeated with 10 mM SA (Fig. 4.3). PGIP activities peaked in both SA treated wheat lines at 6 h.p.t. followed by a similar drop in activity to that previously recorded, while remaining elevated in the treated plants as compared to the untreated plants. The β-1,3-glucanase activities confirmed

[28] See Appendix C

the uptake and recognition of the chemical inducer in a manner similar to that seen in the previous experiment.

■ IMMUNOBLOTTING

All immunoblots were loaded with an equal amount (50 μg) of precipitated apoplastic protein. The peptide that was used for antibody production has a lower potential for antigenicity, therefore all immunoblots probed with the specific PGIP-II antibody (see Chapter 3) required using eight times more apoplastic proteins, than the immunoblots probed with the less specific PGIP-I antibody (see Chapter 2). Significantly longer incubation times also had to be incorporated into the protocol.

While infected wheat plants, in general, showed a reduction in PGIP activity, the immunoblot (Fig. 4.4) revealed the presence of a ±34.5 kDa band present from 48 h.p.i. onward in the uninfected control plants, probably due to constitutive expression or glasshouse stress, and exhibiting a low level of induction from 24 h.p.i. onwards in the leaf rust infected plants (Fig. 4.4).

Plants treated with 50 mM SA also confirmed the recognition of the chemical inducer with the induction of a ±34.5 kDa band. In both the untreated and treated susceptible plants (Fig. 4.5) the band was visible from 12 h.p.i. onwards with the expression of a ±33.0 kDa band following treatment with SA.

The immunoblot profile of the resistant plants (Fig. 4.6) again showed the presence of a ±34.5 kDa band in the untreated control plants with an induction, visible in the treated plants as an increase in band intensity from 12 h.p.t. up to 72 h.p.t., as well as showing that a ±33.0 kDa band was expressed.

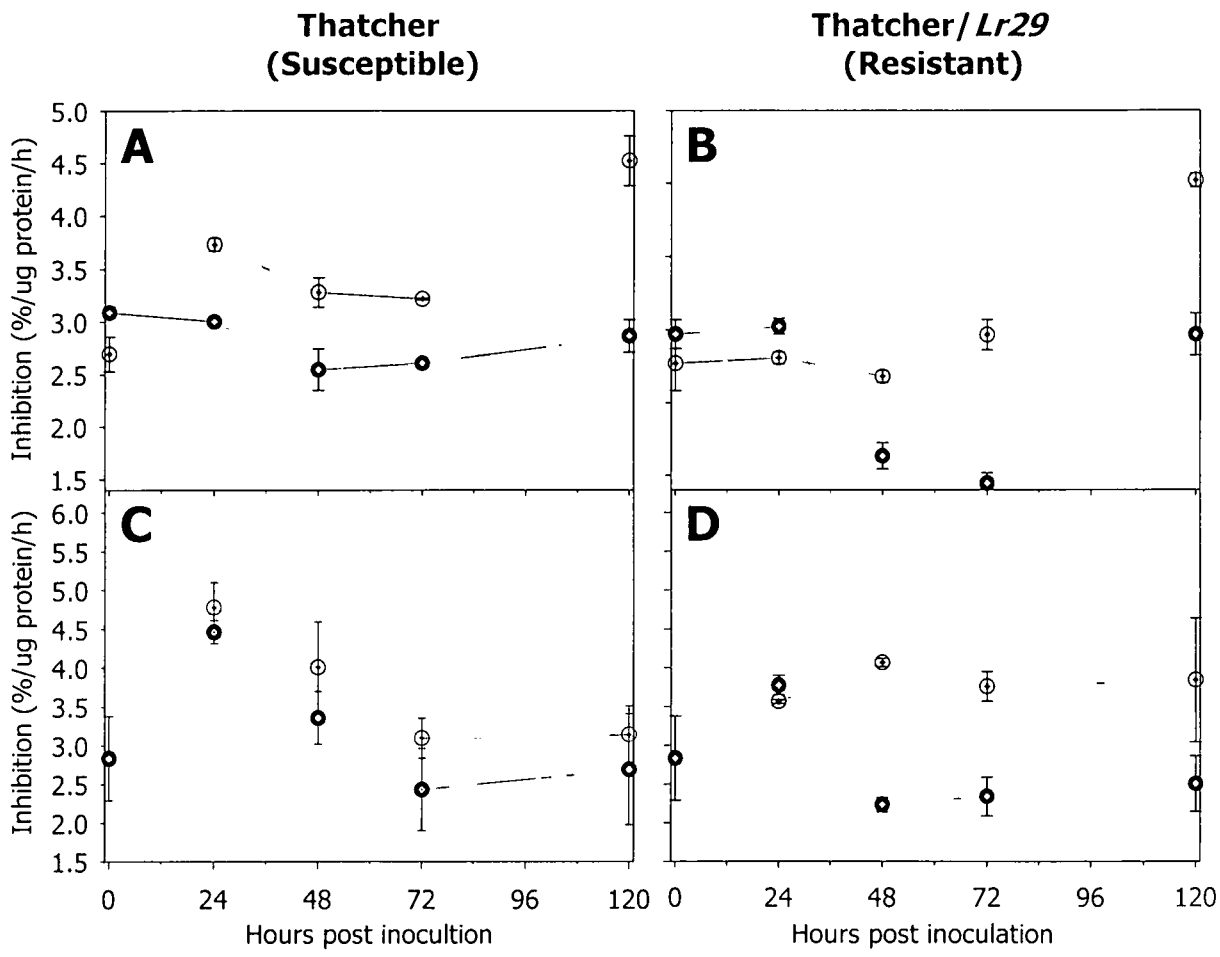


Fig. 4.1. Inhibition of *C. sativus* endopolygalacturonase (expressed as a percentage EPG inhibition) by intercellular protein extracts of leaf rust infected (-●-) and uninfected (-○-) susceptible and resistant wheat cultivars over a period of 120 h. A) Susceptible Thatcher plants. **B)** Resistant Thatcher/*Lr29* plants. **C)** **Repeat**. Susceptible Thatcher plants. **D)** **Repeat**. Resistant Thatcher/*Lr29* plants. Error bars represent the standard deviation (n=3).

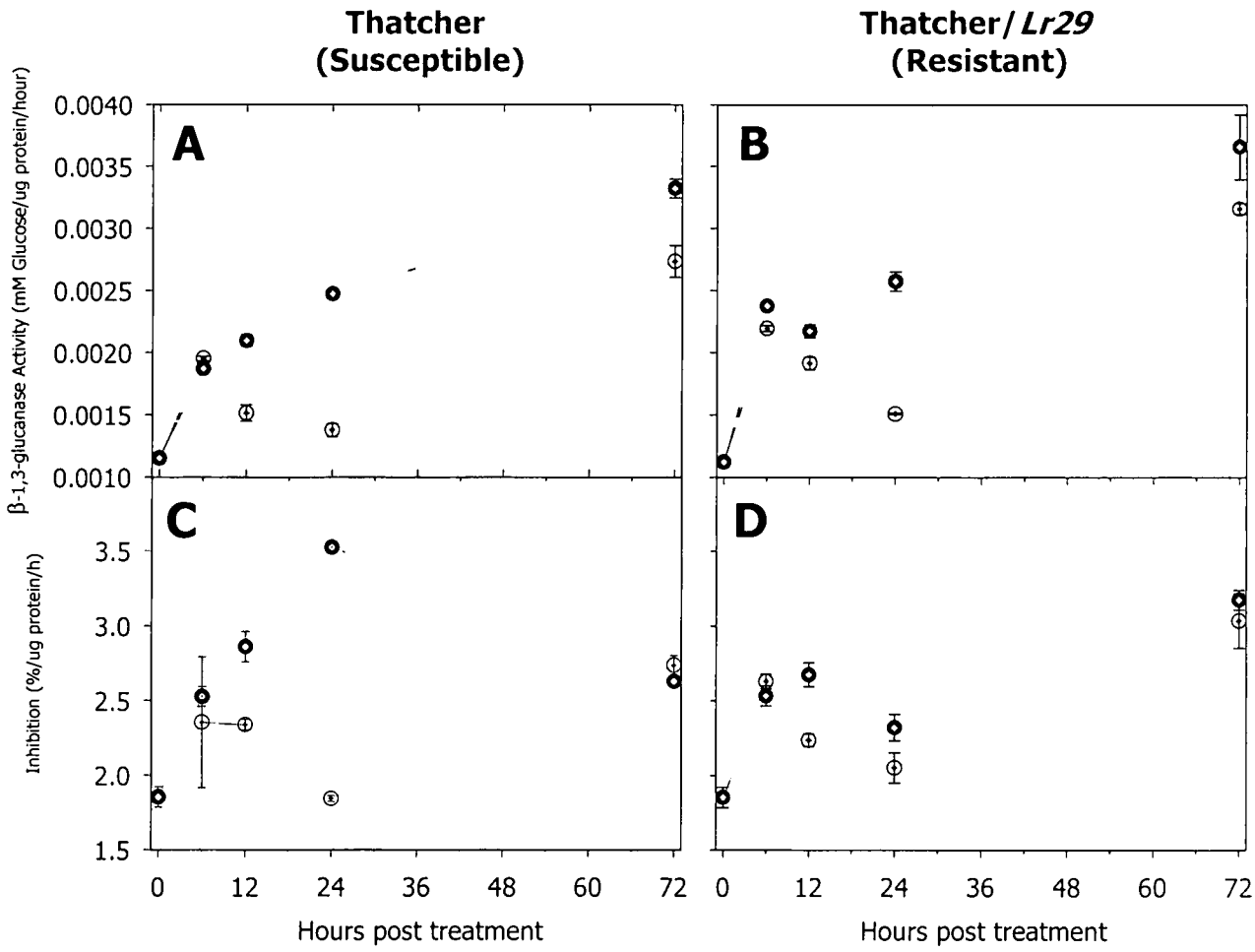


Fig. 4.2. Expression of β-1,3-glucanase activity (A, B) as an infection control, and inhibition of *C. sativus* endopolygalacturonase (expressed as a percentage EPG inhibition) (C, D) by intercellular protein extracts of 50 mM SA treated (-●-) and untreated (-○-) susceptible and resistant wheat cultivars over a period of 72 h. **A)** β-1,3-glucanase activity in susceptible Thatcher plants. **B)** β-1,3-glucanase activity in resistant Thatcher/*Lr29* plants. **C)** PGIP activity in susceptible Thatcher plants. **D)** PGIP activity in resistant Thatcher/*Lr29* plants. Error bars represent the standard deviation (n=3).

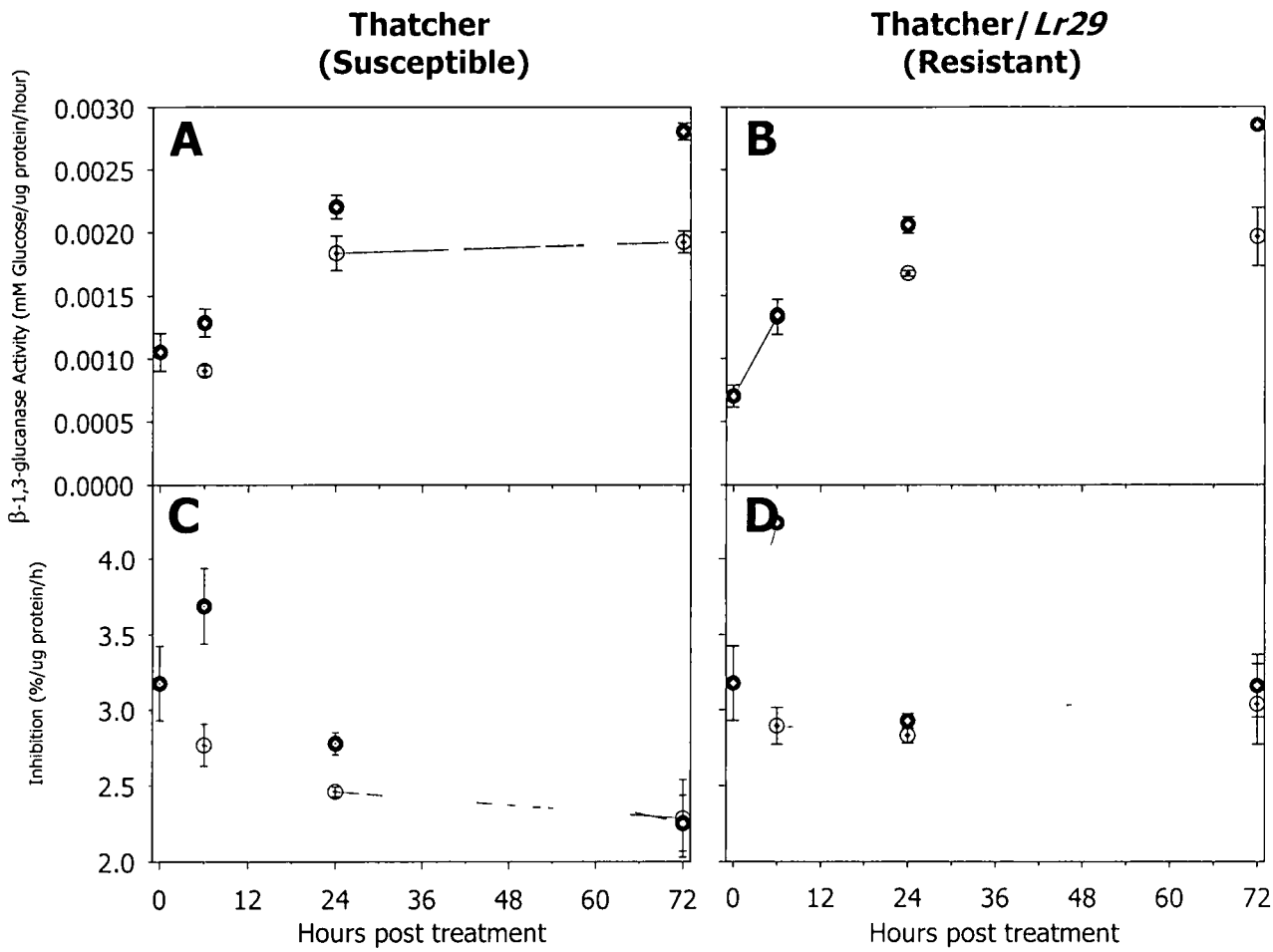
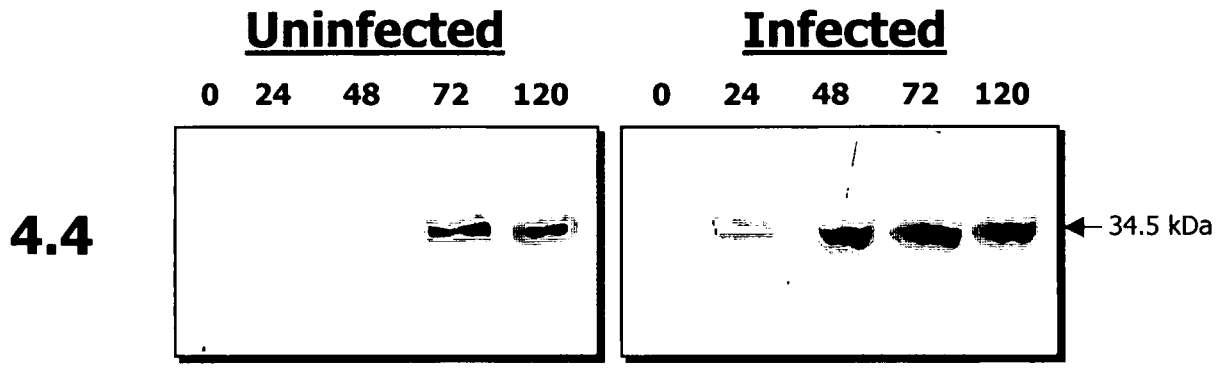
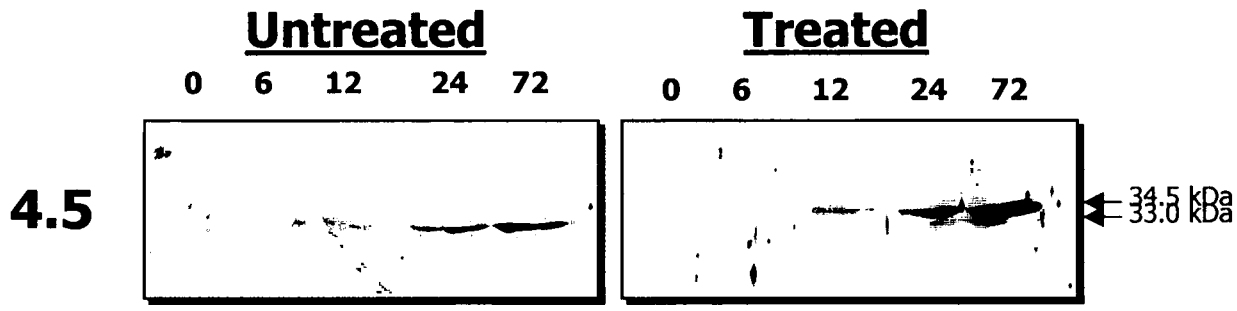
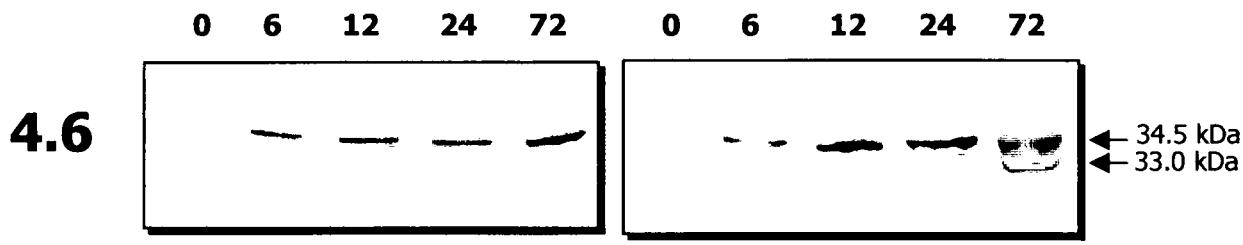


Fig. 4.3. Repeat. Expression of β-1,3-glucanase activity (A, B) as an infection control, and inhibition of *C. sativus* endopolygalacturonase (expressed as a percentage EPG inhibition) (C, D) by intercellular protein extracts of 10 mM SA treated (-●-) and untreated (-○-) susceptible and resistant wheat cultivars over a period of 72 h. A) β-1,3-glucanase activity in susceptible Thatcher plants. **B)** β-1,3-glucanase activity in resistant Thatcher/*Lr29* plants. **C)** PGIP activity in susceptible Thatcher plants. **D)** PGIP activity in resistant Thatcher/*Lr29* plants.







PGIP levels as detected by PGIP-II during immunoblotting of intercellular protein extracts over 72 h. Fig. 4.4 Uninfected and rust infected resistant Thatcher/*Lr29* wheat from 0 h.p.i. to 120 h.p.i. **Fig. 4.5** Untreated and SA treated susceptible Thatcher wheat from 0 h.p.t to 72 h.p.t. **Fig. 4.6** Untreated and SA treated resistant Thatcher/*Lr29* wheat from 0 h.p.t. to 72 h.p.t.

■ DISCUSSION:

It has been demonstrated (Chapter 3) that purified wheat PGIP effectively and specifically inhibits endopolygalacturonase from *C. sativus*. Therefore the inhibition profile of uninfected and leaf rust infected susceptible and resistant wheat, as previously conducted (Chapter 2), was repeated using *C. sativus* EPG rather than *A. niger* EPG. The aim here is to show that wheat PGIP responds to stimuli that would normally induce the plant's natural defense responses.

The PGIP-II antibody used in this investigation differed from the PGIP-I antibody previously used, in that this antibody, generated against a synthetic peptide corresponding to the 11 amino acids from residue 10-21 of *P. vulgaris* PGIP, is considerably more specific for PGIPs (Bergmann *et al.*, 1994). In the previous chapter it has been shown that this antibody specifically recognizes purified wheat PGIP, and is therefore considered more suitable than PGIP-I for immunoblotting of wheat extracts.

Following rust infection, the immunoblots probed with PGIP-II showed a clear induction of a ±34.5 kDa band, while the assay for the accompanying PGIP activity exhibited lower PGIP levels. In comparison, the uninfected controls exhibited a higher PGIP activity, as was previously recorded (Chapter 2). In contrast, treatment of the wheat plants with SA clearly showed the expected induction in PGIP activity, compared to the untreated control plants that showed no induction in PGIP activity levels. This increase in PGIP activity was confirmed through immunoblotting as an induction in the ±34.5 kDa with a subsequent induction of a ±33.0 kDa band. The almost constant increase in PGIP transcript level compared to the increase and drastic decrease in PGIP activity following SA treatment is noteworthy. Although difficult to prove with this investigation, this phenomenon is often encountered where post-translational control, (e.g. suppression) over enzyme activity plays an important role.

The induction in PGIP protein levels following rust infection and SA treatment clearly confirmed the findings of Bergmann *et al.* (1994) that PGIP and its mRNA levels in *P. vulgaris* respond to fungal infection and SA treatment. The subsequent induction in PGIP activity in the SA treated plants was anticipated, but the unexpected drop in PGIP activity following fungal infection was not. These findings, which confirm the results reported in Chapter 2, were attributed to several possibilities; two of these probable

explanations were now partially addressed by using different wheat lines and a more suitable EPG.

Susceptible and resistant wheat lines containing leaf rust resistance *Lr29*, conferring resistance to wheat seedlings, were used in this study. It was hypothesized in Chapter 2 that the plants carrying the resistance gene *Lr34* for adult plant resistance were not old enough at the time of harvest and might therefore not have produced the defense products required for rust resistance. By now using a resistance gene conferring rust resistance to seedlings, the correlation between hypersensitive resistance and the expression of PGIP activity in the two-week-old seedlings was investigated.

Since rust infected plants showed lower PGIP activities, the role of the *Lr29* gene could not be established. Although the induction in PGIP activity observed following SA treatment was confirmed by treating the plants with the lower SA concentration, it did not confirm higher wheat PGIP activity levels attributed to the resistance gene contained within the genome. Slightly higher PGIP transcript levels are however evident from the immunoblot in the resistant line (SA treated and untreated) compared to the susceptible line, which could be attributed to the resistance gene.

The EPG used is effective for inhibition assays, as was seen following SA treatment, but does not account for the activities observed following rust infection. It would appear that the factor responsible for the negative influence on assayable wheat PGIP remains a mystery. In Chapter 2 it was hypothesized that the lower PGIP activity levels recorded following rust infection could be attributed to either, a shortage in extractable free PGIP due to the complexation of wheat PGIP and rust EPG, to an extracted component responsible for the induction of EPG, or the suppression of PGIP activity.

Since the use of *C. sativus* EPG leads to a repeat of the trend in PGIP activity results obtained with *A. niger* EPG (a proven non-ligand), the possible involvement of a biochemical component (in either the plant or the fungus), which indiscriminately either induces the EPG used in the assay of PGIP activity, or suppresses wheat's PGIP have to be considered.

CHAPTER 5

IMMUNOGOLD LOCALIZATION OF PGIP IN WHEAT

For immunoblotting, proteins were extracted from the leaves with the pooled proteins giving valuable insight into the expression of PGIP in wheat leaves following various treatments. Immunoblotting, however, did not provide information on the subcellular location of PGIP. Determining the *in situ* location of PGIP in wheat could contribute to the understanding of the possible role of PGIP in the defense mechanism of monocots, as it pinpoints the exact location in the cell where wheat PGIP is active. Knowledge about its location in the cell will allow wheat PGIP expression on a subcellular level to be directly compared to the location of expression of other dicotyledonous PGIPs.

Immunogold labeling is a powerful tool for determining the exact location of a protein of interest at the subcellular level. In this technique, ultra thin sections of fixed and resin imbedded wheat leaves were probed with the specific PGIP-II antibody. In this manner, wheat PGIP was recognized in the cell and visualized with a gold conjugate antibody using a transmission electron microscope. This technique has been used extensively in the localization of various plant proteins during pathogen attack, e.g. callose formation in soybean following *Phytophthora sojae* infection (Enkerli *et al.*, 1997) and HRGPs in tobacco following tobacco mosaic virus infection (Benhamou *et al.*, 1990). However, very little work has been done on the immunogold localization of PGIP in plants. Bergmann *et al.* (1994) contributed greatly to this through localizing PGIP in *Phaseolus vulgaris* hypocotyls using PGIP-II.

The objective of this publication was to localize PGIP in wheat leaves using the same antibody, PGIP-II.

■ MATERIALS:

■ CHEMICALS

Gluteraldehyde and uranyl acetate were obtained from Merck[29], while LR-White was a London Resin Company[30] product. All other chemicals used were of analytical grade.

[29] Merck KgaA, Darmstadt, Germany
[30] London Resin Company Ltd, Berkshire, England

■ PLANT MATERIAL

Wheat (*Triticum aestivum* L.) plants resistant [Thatcher/*Lr29*] and susceptible [Thatcher] to *Puccinia triticina* were supplied by the Department of Plant Pathology, University of the Free State, Bloemfontein.

■ ANTIBODIES

Anti-PGIP antibody (PGIP-II) was supplied by Dr. CW Bergmann[31] (Bergmann *et al.*, 1994). As secondary antibody a 15 nm gold-labeled goat-anti-rabbit IgG (GAR) (BBI[32]) was used.

■ METHODS:

■ WHEAT GENOTYPES AND GROWING CONDITIONS

Thatcher and Thatcher/*Lr29* plants were grown in a sterilized soil:peatmoss mixture (1:1 v/v), in an environment free from leaf rust, at continuous 20-25°C. Cool-white fluorescent tubes, emitting 120 $\mu Em^{-2}s^{-1}$, provided a 14 hour (h) daylength.

■ INOCULATION WITH RUST

Seedlings were inoculated 14 days after planting by spraying the whole plant with freshly harvested spores of *Puccinia triticina* pathotype UVPrt9, suspended in distilled water with a drop of Tween-20. Control plants were sprayed with distilled water/Tween-20 only. Inoculated and control plants were allowed to dry for one hour before placement in the dark in a dew-simulation chamber at 18-20°C for 12 h. Plants were then transferred to the greenhouse.

■ TISSUE PREPARATION FOR ELECTRON MICROSCOPY

Replicate tissue pieces (2 of each) of ±1 cm in length were cut from wheat plants at 120 h.p.i. The samples were fixed in 3% (v/v) gluteraldehyde in 100 mM phosphate buffer pH 7.4 for 1 h at room temperature with constant agitation, washed in phosphate buffer (5x) followed by washes in distilled water (3x). The samples were subsequently dehydrated in an ethanol series (30-100% ethanol; 20% increments;

[31] Complex Carbohydrate Research Center, University of Georgia, USA
[32] British Biocell International Ltd, Cardiff, England

3x100%; 15 min. each), infiltrated with LR-White acrylic resin (1:1 LR-White:100% EtOH; 60 min.; 3x100% LR-White; 8 h), and cured for 40 h at 50°C.

Sections (60 nm thick) of selected material were cut using a LKB III ultramicrotome and collected on 3.05 mm 400 mesh nickel grids (Agar Scientific[33]).

■ IMMUNOCYTOCHEMISTRY

Grids bearing sections were floated with the section side down on drops of phosphate buffered saline (PBS) with 0.5% Tween-20 containing 1% (w/v) bovine serum albumine (BSA) (2x15 min.) to block unwanted active sites. Grids were subsequently incubated for 18 h at 4°C in PGIP-II (1:4) in PBST-BSA and washed in PBST-BSA (3x15 min.). This procedure was followed by incubation in the secondary antibody diluted (1:100) in PBST-BSA for 1 h at 4°C, washed in PBST-BSA (3x15 min.) and ddH_2O (3x15 min.). Samples were finally stained with 6% uranyl acetate and lead citrate (Reynolds, 1963) prior to examination with a Philips CM100 transmission electron microscope at 60 kV.

Incubation with the primary antibody was omitted for control treatments to detect possible nonspecific binding of the secondary antibody to sections.

■ RESULTS:

To gain an overall impression of the distribution of PGIP in resistant and susceptible wheat lines in the presence and absence of rust, both lines were prepared for immunogold labeling. The fixed plant material exhibited good antigenicity, but the sections were fragile and disrupted easily during microscopy and the immunogold labeling procedure. Therefore, the results did not allow for a quantitative analysis comparing the amounts of label between different tissue sections from different wheat lines. Results are shown in Figs. 5.2 – 5.4, which illustrate typical examples from approximately eight grids per leaf segment, sampled from infected and uninfected wheat plants.

Following the standard 18 h incubation of ultra thin sections of infected leaves with PGIP-II, exceptionally high labeling was associated with haustoria inside the plant

[33] Agar Scientific Ltd, Essex, England

cells (Fig. 5.3B, C), while fungal hyphae in the intercellular spaces labeled with a lower frequency (Fig. 5.3D). PGIP expression also appeared more concentrated near fungal tissue. Sections showing the haustorium mother cell (Fig. 5.4A - D) displayed dispersed labeling around the periphery of the mother cell wall, with localized labeling in the plant cell wall at the point of contact between the host and pathogen (Fig. 5.4C, D).

A low level of non-specific labeling, as noted by Bergmann *et al.* (1994), was noted in the chloroplast of both infected and uninfected controls (results not shown). Increasing the incubation time to 48 h also increased labeling of the cell wall dramatically (Fig. 5.2D), with a corresponding increase of non-specific labeling in the chloroplast. Little or no labeling was found in other parts of plant or fungal tissue. Fig. 5.1 illustrates the relevant fungal structures.

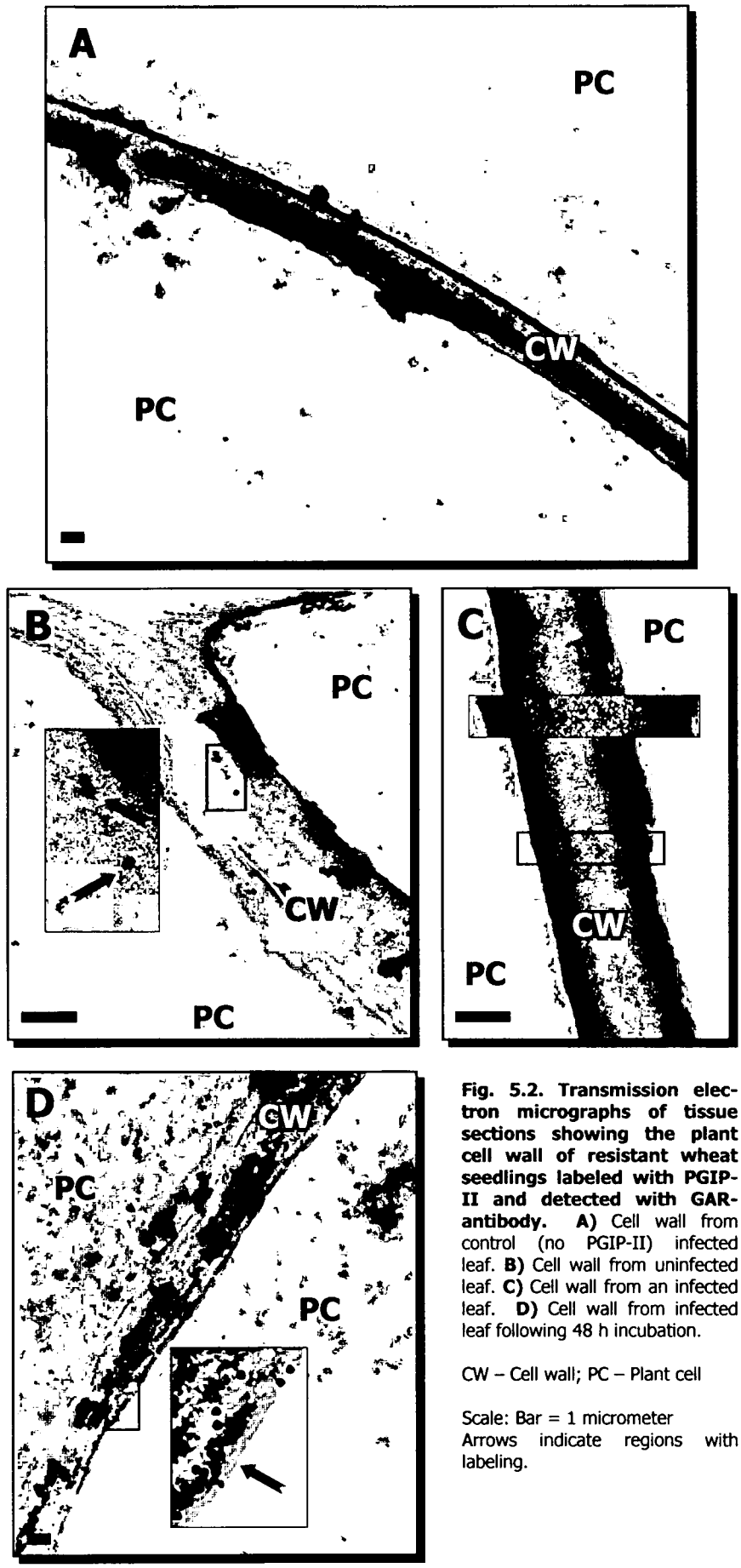


Fig. 5.2. Transmission electron micrographs of tissue sections showing the plant cell wall of resistant wheat seedlings labeled with PGIP-II and detected with GAR-antibody. **A)** Cell wall from control (no PGIP-II) infected leaf. **B)** Cell wall from uninfected leaf. **C)** Cell wall from an infected leaf. **D)** Cell wall from infected leaf following 48 h incubation.

CW – Cell wall; PC – Plant cell

Scale: Bar = 1 micrometer
Arrows indicate regions with labeling.

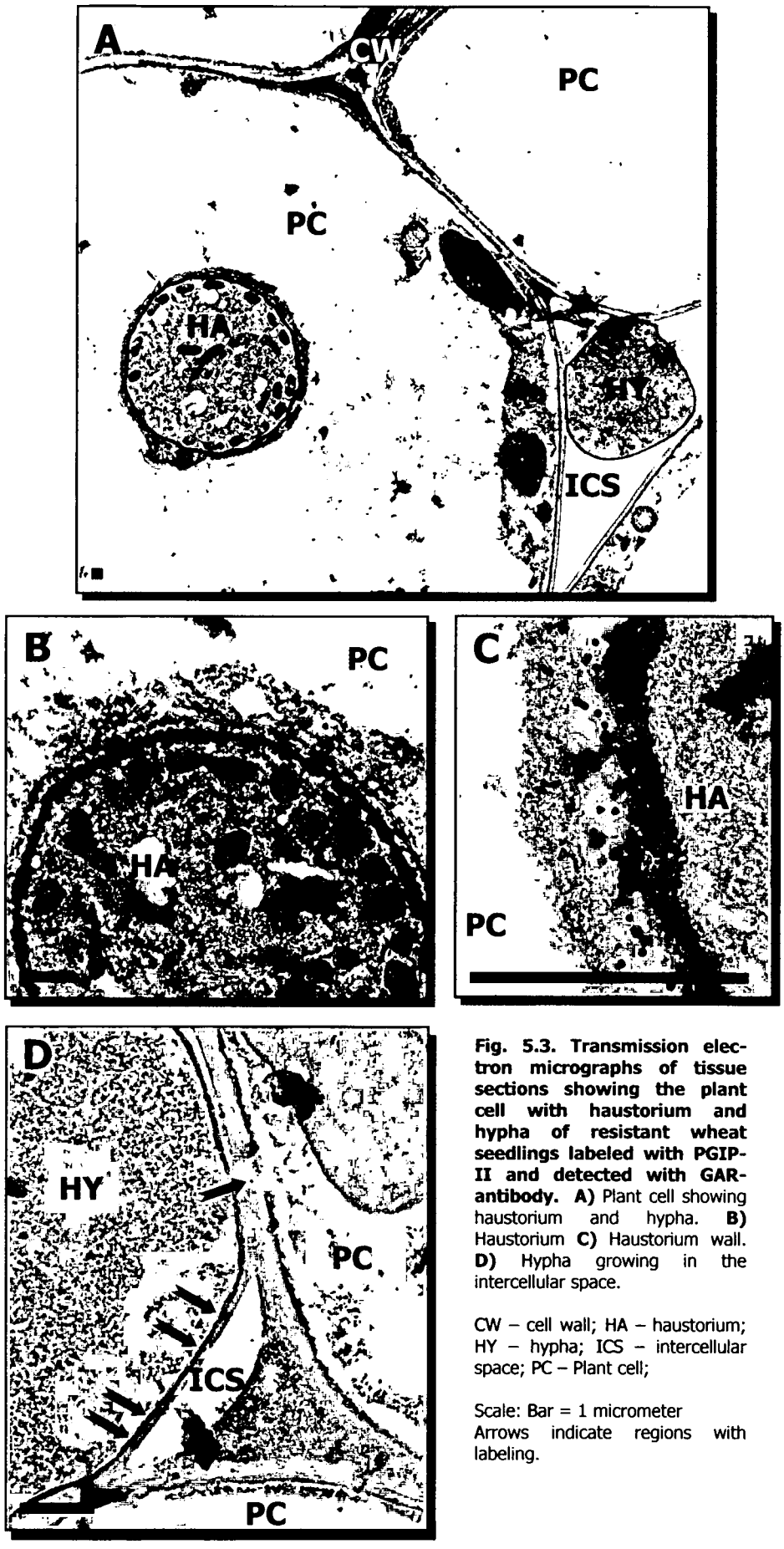


Fig. 5.3. Transmission electron micrographs of tissue sections showing the plant cell with haustorium and hypha of resistant wheat seedlings labeled with PGIP-II and detected with GAR-antibody. A) Plant cell showing haustorium and hypha. **B)** Haustorium **C)** Haustorium wall. **D)** Hypha growing in the intercellular space.

CW – cell wall; HA – haustorium; HY – hypha; ICS – intercellular space; PC – Plant cell;

Scale: Bar = 1 micrometer
Arrows indicate regions with labeling.

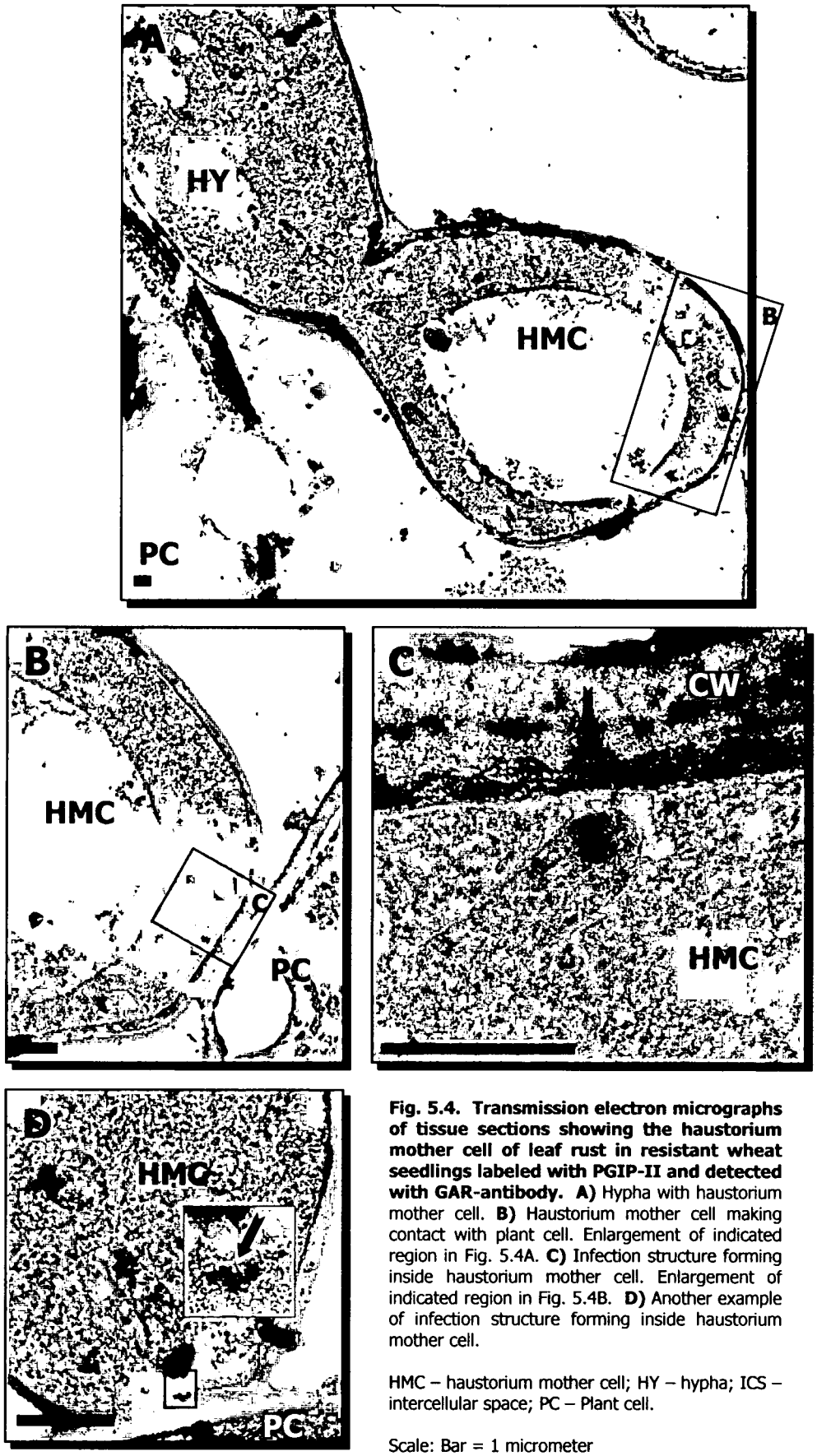


Fig. 5.4. Transmission electron micrographs of tissue sections showing the haustorium mother cell of leaf rust in resistant wheat seedlings labeled with PGIP-II and detected with GAR-antibody. A) Hypha with haustorium mother cell. **B)** Haustorium mother cell making contact with plant cell. Enlargement of indicated region in Fig. 5.4A. **C)** Infection structure forming inside haustorium mother cell. Enlargement of indicated region in Fig. 5.4B. **D)** Another example of infection structure forming inside haustorium mother cell.

HMC – haustorium mother cell; HY – hypha; ICS – intercellular space; PC – Plant cell.

Scale: Bar = 1 micrometer
Arrows indicate regions with labeling.

■ DISCUSSION:

Results from the present TEM study confirm the only other immunocytochemical study where PGIP was found at tissue level in uninfected and *Colletotrichum lindemuthianum*-infected *P. vulgaris* hypocotyls (Bergmann *et al.*, 1994). Locating PGIP in the cell walls of uninfected wheat plants confirms the findings of Cervone *et al.* (1989) that PGIP is present at low levels in the cell walls of healthy plants. This low expression of PGIP would account for the low levels of constitutively expressed PGIP recorded with the immunoblots in Chapter 4, which could act as an early biochemical barrier by protecting the epidermal cell walls from the fungal polygalacturonases (Bergmann *et al.*, 1994). Following infection, labeling of the haustorium was quite prominent with much less labeling of hyphae being observed in the intercellular spaces or near the cell walls, indicating that haustorium labeling is not due to a non-specific cross reaction between antibody and fungal tissue. This is in contrast to the findings of Bergmann *et al.* (1994) who makes no mention of labeling of fungal tissue inside infected cells, and who found a strong accumulation of gold particles on the cell walls. In agreement to the findings of Bergmann *et al.* (1994), gold labeling also decreased drastically with an increase in distance from fungal infection. This made it difficult to attribute the recorded PGIP localization to *Lr29* in the resistant lines, as it was impossible to determine on such a small scale whether lower labeling was the result of an incompatible reaction of the resistant plants or due to an increased distance from the point of infection.

Labeling of sections displaying the haustorium mother cell wall in contact with a host plant cell clearly showed the localization of PGIP at the point of contact between pathogen and host. In both examples shown in the micrographs, the localization of PGIP at this crucial point is unmistakable as the host cell combats the fungal hydrolysis of its cell wall by the haustorium mother cell. During the infection process of wheat, an infection peg develops from the rust haustorium mother cell at the point of contact where it penetrates the host cell wall and gives rise to the haustorium. Once inside the cell, the haustorium obtains nutrients from inside the cell as the fungus develops and prepares to colonize the plant. It is not clear if these micrographs represent this process. According to Rijkenberg[34] it is doubtful whether this is the actual point of penetration, as the localized cell wall thickening

[34] Personal Communication: Prof. FHJ Rijkenberg, University of Natal, Pietermaritzburg, South Africa

that often accompanies this process appears to be absent, and the presence of a vacuole is suggestive of a relatively old haustorium mother cell promising very little likelihood of host penetration. The cigar shaped object opposite the observed labeling could not be identified by either Rijkenberg or Mendgen[35].

The reason why wheat PGIP would attack the haustorium, as seen by the high level of localization around this structure, is unclear and requires further investigation. De Lorenzo *et al.* (2001) stated that the mature PGIP protein contains a signal peptide that is responsible for translocation of PGIP out of the ER to the cell wall, where it is in direct contact with the pathogen's EPG. This is true for all PGIP localized with the PGIP-II and GAR antibodies on the cell wall near the pathogen. It does not, however, explain how some PGIP can remain cytosolic and thus be able to inhibit any EPG released by the haustorium, nor is it clear why the fungus would produce EPG in the absence of polygalacturonic acid. A possible explanation for this might be found in the constitutive expression of EPG as it has been found that some fungi may able to express a constitutive and inducible EPG (Parenicová *et al.*, 2000; Wubben *et al.*, 2000).

Since a gene family, with between five and fifteen genes, often codes for PGIP (Frediani *et al.*, 1993), it could be speculated that not all isoforms are transported to the cell wall, and that some remain inside the cytosol as a possible defense mechanism. Whatever the case may be, these results confirm previous findings that wheat contains PGIP, which is present at very low levels in healthy cells, and is associated with fungal infection.

[35] Personal Communication: Prof. K Mendgen, Universitat Konstanz, Konstanz, Germany

CHAPTER 6

CLONING OF THE POLYGALACTURONASE-INHIBITING PROTEIN (PGIP) GENE FROM WHEAT THROUGH THE POLYMERASE CHAIN REACTION

Researchers often employ conserved DNA sequences, obtained from published sequence data to produce primers for polymerase chain reaction (PCR) amplification of equivalent sequences from other organisms. These conserved primers accommodate any change in sequence between proteins of different species through the incorporation of degenerate bases.

Degenerate primers, and primers patented to amplify pear *pgip*, were used to attempt the amplification and cloning of wheat *pgip*.

■ MATERIALS:

■ PLANT MATERIAL

The Department of Plant Pathology, University of the Free State, Bloemfontein, supplied seed of the wheat line Thatcher.

■ METHODS:

■ GENOMIC DNA ISOLATION

DNA was obtained by grinding mature wheat (*Triticum aestivum*, cv. Thatcher) leaves in a mortar and pestle to a fine powder in liquid nitrogen. Extraction buffer (500 mM NaCl, 100 mM Tris-HCl pH 8.0, 50m M EDTA, 1.25% (w/v) SDS and 37 mM Nabisulfite) heated to 65°C was added to the ground powder and the resulting mixture was incubated in polypropylene tubes at 65°C for 20 min. with intermittent inverting of the tube. The tubes were subsequently filled with chloroform:isoamyl alcohol (24:1) and the mixture vigorously mixed followed by centrifugation for 15 min. at 10000 rpm. Isopropanol (0.7 volumes) was added to the upper phase and the precipitated DNA strands were scooped out, transferred to 1.5 mL eppendorf tubes, washed with 70% ethanol, dried and dissolved in sterile water.

■ DEGENERATE PRIMER DESIGN

In order to design primers for amplification of *pgip* from wheat, the *pgip* DNA sequences from other plants, as listed below[36], had to be considered. Two regions, one upstream and one downstream, with a high level of homology between the DNA sequences were considered important and were subsequently used for the primer design.

The bases with uncertainties that remained after alignment were incorporated into the degenerate primers.

The final degenerate primers were as follows,

Region 1 (**UP**PGIP):

5′ **TG<u>Y</u> AA<u>Y</u> CC<u>V</u> <u>VAH</u> GA<u>Y</u> AA<u>R</u> <u>MA</u>** 3′

Region 2: (**DN**PGIP):

5′ **GCA <u>BYK</u> <u>R</u>TT <u>RTK</u> <u>RRM</u> ATA** 3′

Where:

R=A+G
Y=C+T
M=A+C
K=G+T
H=A+C+T
B=G+T+C
V=G+C+A

■ PCR PRIMERS

The two degenerate primers as described above were used to amplify an approx. 800 bp fragment.

Degenerate primers:

UPPGIP: 5′ TGY AAY CCV VAH GAY AAR MA 3′

DNPGIP: 5′ GCA BYK RTT RTK RRM ATA 3′

Other primers used for DNA amplification were those published and patented (US 5,569,830) by Stotz *et al.* (1993); patented for the isolation of nucleic acid sequences encoding plant polygalacturonase inhibitor proteins (PGIP):

STOTZUP: 5′ ACA TCT CTC AGG CTC TCA ACC 3′

STOTZDN: 5′ AAA TTG CTG GCC AAA TCT GCA G 3′

■ PCR REACTION

PCR was performed in 0.5 mL thin-walled tubes in a Hybaid thermal cycler. Standard PCR reactions were carried out in 50 μL volumes with the following final concentrations:

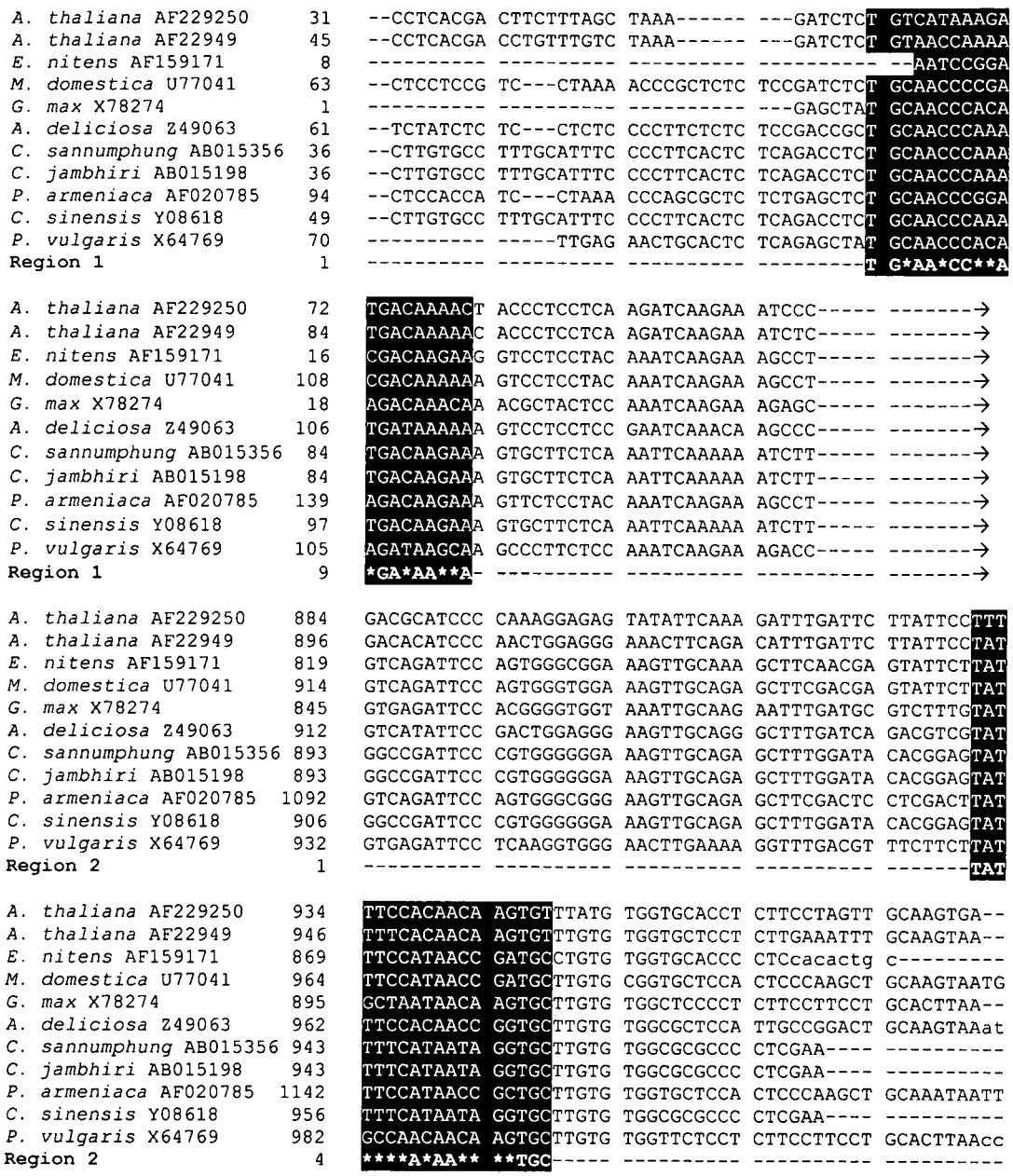


Fig. 6.1. Plant species (with Genbank accession numbers) and DNA sequences coding for regions at or near the beginning and end of their respective *pgips*. Regions of these sequences used for primer design are indicated in black with degenerate bases indicated with an asterisk (*).

STOTZUP

```
1    ACATCTCTCA GGCTCTCAAC CAAAACCAAA ACAATGGAAC TCAAGTTCTC CACCTTCCTC TCCCTAACCC
     ACATCTCTCA GGCTCTCAAC C
                                                    UPPGIP
71   TACTCTTCTC CTCCGTCCTA AACCCCGCTC TCTCCGATCT CTGCAACCCC GACGACAAAA AAGTCCTCCT
                                                  TGYAAYCCV VAHGAYAARM A
141  ACAAATCAAG AAAGCCTTCG GCGACCCCTA CGTCTTGGCC TCATGGAAAT CAGACACTGA CTGCTGCGAT
211  TGGTACTGCG TCACCTGTGA CTCCACCACA AACCGCATTA ACTCCCTCAC CATCTTTGCC GGCCAGGTGT
281  CAGGCCAAAT CCCCGCCCTA GTAGGAGACT TGCCATACCT TGAAACCCTT GAATTCCATA AGCAACCCAA
351  TCTCACTGGC CCAATCCAAC CCGCCATTGC CAAGCTCAAA GGACTCAAGT CTCTCAGGCT CAGCTGGACC
421  AACCTCTCAG GCTCTGTCCC TGACTTCCTC AGCCAACTCA AGAACCTCAC ATTCCTCGAC CTCTCCTTCA
491  ACAACCTCAC CGGTGCCATC CCCAGCTCGC TTTCTGAGCT CCCAAACCTC GGCGCTCTTC GTCTAGACCG
561  CAATAAGCTC ACAGGTCATA TTCCGATATC GTTTGGGCAG TTCATTGGCA ACGTTCCAGA CCTGTATCTC
631  TCCCACAACC AGCTTTCTGG TAACATTCCA ACCTCATTCG CTCAGATGGA CTTCACCAGC ATAGACTTAT
681  CACGGAACAA GCTCGAAGGT GACGCATCCG TGATATTTGG GCTGAACAAG ACAACCCAGA TTGTGGACCT
751  GTCCAGGAAC TTGCTGGAAT TTAATCTGTC AAAGGTGGAG TTTCCGACAA GCTTGACCTC GCTGGATATC
821  AACCACAATA AGATCTACGG GAGTATCCCA GTGGAGTTTA CGCAACTGAA TTTCCAGTTC CTGAACGTGA
                                                    DNPGIP
891  GCTACAACAG GCTGTGTGGT CAGATTCCTG TGGGTGGAAA GTTGCAGAGC TTCGACGAGT ATTCTTATTT
                                                  GCABYKR TTRTKRRMAT A
                                                                        STOTZDN
961  CCATAACCGA TGCTTGTGCG GTGCTCCACT CCCAAGCTGC AAGTAAAGGC CACAACTGCA GATTTGGCCA
                                                          CTGCA GATTTGGCCA
1031 GCAATTT
     GCAATTT
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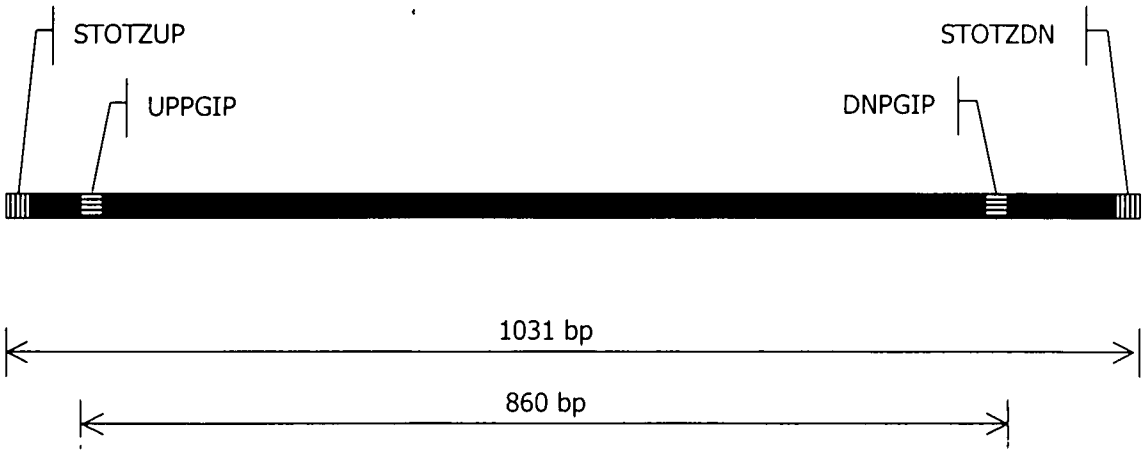

Fig. 6.2. The full DNA sequence as well as an illustration of the complete sequence of *pgip* from *P. communis*. Primers STOTZUP and STOTZDN amplifying the 1031 bp *pgip* gene are indicated, as well as the degenerate primers UPPGIP and DNPGIP responsible for the amplification of a potential 860 bp fragment of *P. communis pgip*. Both these sets of primers are to be used in cloning *pgip* from wheat.

10 mM Tris-HCl pH 9.0, 50 mM KCl and 0.1% Triton X-100, 2 mM $MgCl_2$, 250 μM each of dATP, dCTP, dGTP, dTTP (dNTP[37]), 1 μM of each primer and 1.25 U Promega Taq polymerase[38]. Fifteen nanograms of genomic DNA were added. PCR cycling conditions were optimized to be as follows: an initial denaturation at 94°C for 3 min., followed by 30 cycles of 94°C for 30 sec., annealing at 42°C for 1 min., and extension at 72°C for 2 min., and a final extension step of 72°C for 10 min.

The role of each primer in the banding pattern was investigated through amplification with only one primer at a time followed by separation on 0.8% agarose gel through electrophoresis in TAE (40 mM Tris acetate, 1 mM EDTA) buffer. Bands that resulted from both primers were investigated further by excision from the agarose gel, re-amplification, excision and purification through Genelute agarose spincolumns (Sigma)[39].

■ SOUTHERN BLOT ANALYSIS

Forty micrograms of total genomic wheat DNA were digested overnight at 37°C with 100 U HindIII restriction enzyme in a digestion buffer with the following final concentrations: 1 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.5 mM $MgCl_2$, 0.1 mM 2-mercaptoethanol.

The digested DNA was loaded into extended casting wells of a 1% agarose gel and separated in TAE separation buffer at 0.7 $V.cm^{-1}$.

The separated digested DNA was blotted onto positively charged Hybond N^+ (Amersham)[40] nylon membrane through an alkaline capillary transfer technique as described by Chomczynski (1992).

The amplified DNA fragments were, after separation on agarose, excised from the gel, and purified with Sigma's Genelute agarose spincolumn. One microgram aliquots of the purified fragments were subsequently nonradioactively labeled with digoxigenin (DIG) (Boehringer Mannheim) according to the manufacturer's specifications. The Southern blot was performed as described by Boehringer Mannheim with probe hybridization

[37] Boehringer-Mannheim, GmbH, Mannheim, Germany
[38] Promega, Madison, WI, USA
[39] Sigma Chemical Company, St. Louis, MO, USA

being facilitated in DIG Easy Hyb at 50°C, while detection of hybridized probes were done with CDP-Star™.

■ CLONING AND SEQUENCING

Purified fragments were ligated into the EcoRI site of the polylinker region of the pGEM-T-Easy Vector system II (Promega). Ligation was done at 4°C for 16 h and transformed to competent *Escherichia coli* (JM109) (Promega). Transformants were screened on LB-ampicillin plates using the blue/white phenotype (results not shown).

Recombinant plasmid clones were confirmed by restriction enzyme digestion with HindIII and agarose gel separation. Recombinant clones were sequenced using the BIG Dye terminator cycle sequencing kit with an ABI Prism model 377 sequencer (Perkin Elmer)[41]. T7 and SP6 primers were used for forward and reverse sequencing of the double-stranded plasmid template.

■ DATA ANALYSIS

DNA sequence data were translated to amino acids using DNA translate software[42]. The resulting DNA and amino acid sequences were used in BLAST[43] searches to detect homologous polypeptide and nucleotide sequences (Altschul *et al.*, 1997). Peptide and nucleotide sequence alignment was performed using the CLUSTALW (EBI) software[44].

■ RESULTS:

■ PCR

Using the designed degenerate primers, three major bands were amplified from the genomic wheat DNA (Fig. 6.3). These bands were analyzed through re-amplification with individual primers to investigate the role of each primer in the final amplification product. Of the three bands (Fig. 6.3) amplified by both primers, only one band (FDEGEN) was shown to be the product of both primers, while the other amplification products resulted from multiple recognition sites with one primer.

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[40] Amersham International plc, Amersham Place, Buckinghamshire, England
[41] Perkin Elmer Corp., Norwalk, CT, USA
[42] http://www.expasy.ch/tools/
[43] http://www.ncbi.nlm.nih.gov
[44] http://www2.ebi.uk

Following the same optimized protocol, numerous fragments were amplified from genomic wheat DNA using the patented Stotz-primers (Fig. 6.4). Three of these fragments were selected and excised from the gel for further investigation. These three fragments (F1, F2 and F3) were purified from the agarose gel and re-amplified (Fig. 6.5). Because it is present in the water control, fragment number 3 was identified as being DNA contamination from an unknown source. Fragments 1 and 2 were further analyzed by amplification with individual primers (Fig. 6.6).

Fragment 1 (F1) appeared to be the amplification product of only one primer. Fragment 2 (FSTOTZ) was unique to both primers, therefore it and FDEGEN were chosen for sequencing and Southern blot analysis (Fig. 6.6).

■ DNA SEQUENCING

The amplified bands, following excision and purification from agarose, were cloned and sequenced in both directions using the regions SP6 and T7 as upstream and downstream primers. The expected primers, as outlined above, were found at the terminal ends of all but one fragment (DNPGIP) (Fig. 6.8). DNA sequencing revealed FSTOTZ to be 658 bp, which is considerably shorter when compared to the expected 1031 bp sequence of *pgip* from *P. communis* from where these primers originated. Sequencing also revealed FDEGEN to be 731 bp in length, which is close to the length as predicted from their distances apart (800+ bp) on known *pgips*.

However, a considerable overlap of the two sequences was anticipated (Fig. 6.2) as the product (FDEGEN) of UPPGIP and DNPGIP were amplified from inside the amplification product (FSTOTZ) of STOTZUP and STOTZDN in *P. communis*, this was not the case in wheat.

Upon translation FSTOTZ revealed an open reading frame (ORF) of 194 amino acids out of a total of 221 amino acids (87%), while FDEGEN translated to a largest ORF of 132 amino acids out of a possible 243 amino acids (53%) (Fig. 6.8).

SOUTHERN BLOT ANALYSIS

Non-radioactively labeled amplified fragments (FDEGEN and FSTOTZ) were used as DNA probes against total genomic wheat DNA and revealed a number of bands with both probes. Probe FSTOTZ hybridized several times with HindIII digested DNA to reveal at least 6 bands (Fig. 6.7), ranging from 3.3 kb to 1.1 kb in size. FDEGEN exposed two major bands at 2.3 kb and 1.5 kb as well as a continuous smear in the background (Fig. 6.7). These results confirmed the wheat origin of the amplified fragments and pointed to the copy number of each fragment in the wheat genome.

SEQUENCE DATA ANALYSIS

A DNA sequence similarity search (BLAST) against the entire plant DNA database revealed a high level of similarity in two different regions, for the fragments FSTOTZ and FDEGEN, on the same 211 000 bp fragment. This 211 kb fragment (accession # AF 326781) is a recent addition to the database and originates from chromosome $1A^{m}S$ of *Triticum monococcum*, diploid wheat, in the genomic region orthologous to the *Lr10* leaf rust resistance locus from *Triticum aestivum* (Stein *et al.*, 2000). FDEGEN is 81% identical over the entire 731 bp (with a score of 611 and an expected (E) value of 1e-173) to a region of this fragment that contains an unclassified element (Fig. 6.9). FSTOTZ is 90% identical over the entire 658 bp (with a score of 797 and an E-value of 0.0) to this fragment containing a copia-like retrotransposon (Fig. 6.9). Alignment of database *pgip*'s as well as PGIP sequences to the *T. monococcum* fragment revealed a low level of similarity, excluding the possibility of this fragment containing a "*pgip*-like" sequence.

The predicted translation of these amplified fragments, searched against all six frames of the 211 kb *T. monococcum* revealed a high level of similarity between the translated sequences of FSTOTZ as well as FDEGEN and the *T. monococcum* sequence.

Direct alignments (CLUSTALW) between *pgip* sequences obtained from the database (Genbank), and the amplified sequences also revealed a low level of homology (Table 6.1). DNA sequence homology between *pgip* from *P. trifoliata* and FSTOTZ exhibited the highest identity (48%), while *pgip* from *C. iyo* had the highest identity with FDEGEN (47%) (Tabel 6.1). This was confirmed by aligning the translated FDEGEN and FSTOTZ sequences with PGIP sequences obtained from the database (Table 6.2). Here, the

highest identity existed between translated FSTOTZ and PGIP from *A. thaliana* (AAF69828) (20%) with translated FDEGEN having the highest identity with PGIP from *A. deliciosa* (20%) (Tabel 6.2).

The relevance of a low level of identical base pair and amino acids should also be noted. A comparison of DNA sequences between *pgip* from *E. saligna* (AF159170) and *pgip* from *G. max* (AW432387) revealed only a 47% base pair homology, with the amino acid sequences of PGIP from *E. saligna* (AAF22252) and PGIP from *G. max* (AAD45503) being 45% identical. Although identical in function, structurally the PGIP's from these two species are two of the most diverse.

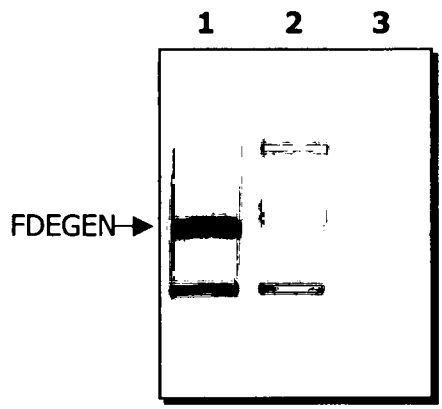

Fig. 6.3. Amplification of genomic wheat DNA with degenerate primers. Lane 1) Amplification products with upstream (UPPGIP) and downstream (DNPGIP) primers. **Lane 2)** Amplification products with only UPPGIP. **Lane 3)** Amplification products with only DNPGIP. Band of interest (FDEGEN) is indicated.

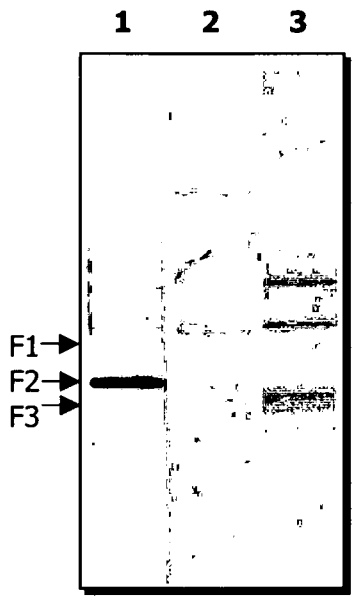

Fig. 6.4. Amplification of genomic wheat DNA with patented Stotz-primers. Lane 1) Amplification products with upstream (STOTZUP) and downstream (STOTZDN) primers. **Lane 2)** Amplification products with only STOTZUP. **Lane 3)** Amplification products with only STOTZDN. Bands of interest (F1, F2 and F3) are indicated.

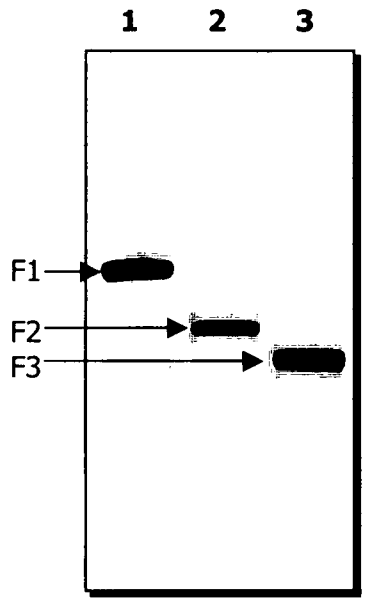

Fig. 6.5. Re-amplification of selected bands with Stotz-primers. Lane 1) First band of interest (F1). **Lane 2)** Second band of interest (F2). **Lane 3)** Third band of interest (F3).

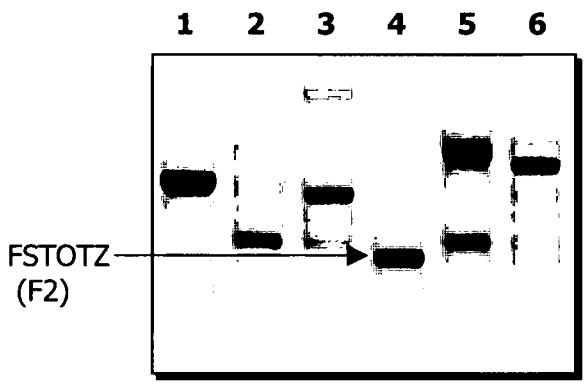

Fig. 6.6. Amplification of selected fragments with Stotz primers individually. Lane 1) Amplification products of F1 with upstream (STOTZUP) and downstream (STOTZDN) primers. **Lane 2)** Amplification products of F1 with only STOTZUP. **Lane 3)** Amplification products of F1 with only STOTZDN. **Lane 4)** Amplification products of F2 with upstream (STOTZUP) and downstream (STOTZDN) primers. **Lane 5)** Amplification products of F2 with only STOTZUP. **Lane 6)** Amplification products of F2 with only STOTZDN. Band of interest (F2 – FSTOTZ) is indicated.

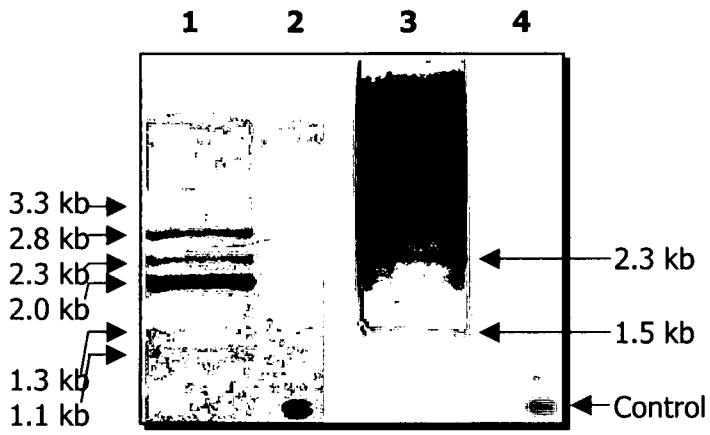

Fig. 6.7. Southern blot of 40 μg genomic DNA probed with amplification products. Lane 1) HindIII digested genomic wheat DNA probed with FSTOTZ. **Lane 2)** FSTOTZ as positive control. **Lane 3)** HindIII digested genomic wheat DNA probed with FDEGEN. **Lane 4)** FDEGEN as positive control.

DNA

FSTOTZ (658 bp) (primer homology is indicated in black):

```
1   ACATCTCTCA GGCTCTCAAC CACTCATGAA AAGCTTCCCT ATGATTATCT TGAAAGGAAG
61  CAAGAGCTTG AGAAATTGAG AGGGGCTCAT GAAGATCTTC AAAAAGAGAA TGAGTCACTT
121 CGCGCTCAAC AGACCAGTCC CGCTCAGGAA GGATTTGAAC CACCATGTCT AAAATGCCTT
181 GAGCGTGATA ACGCTACTTC TGTCGCTGAA TGTTCTACTG CTGCTACTGT TGCAATATCT
241 TCAACTATTG ATGTGGTAAC TAACCCCTCT GCTGAGGATA CCACTAGTAT TGCTGATGAA
301 AATGCTAGGT TGAAGACATT GCTTGAAACA GGGATGTACA AAAGTCTCAA AGGGCATCAG
361 ACACTGTGTG ATGTCCTCAA AAAGCAGATT CTGAACCGAA ACCCTAGGAA AGAGGGTGTA
421 GGGTTCGAGA GGAAAATAAA TGTTGATGGA TCCTACTGGA AGCCTGAGCA GTACCCCAAA
481 ACCACATGGG TTGCTGCAAA GGGACCTTCA GTGGATCCAT CCACCTTATC TGGTTTCACT
541 TGTGCTAACC CTATTATCAT TGATGAATCC TTTGATGCAA ACTAAGTTGT TTAAGAATCA
601 GAATGGTGAA GTGTTTGCCA GGTATATTGG TACTATCTGC AGATTTGGCC AGCAATTT
```

FDEGEN (731 bp) (primer homology is indicated is indicated in black):

```
1   TGCAATCCCG ACGACAAGCA GAAGTTTGGT TGCGGCAGCG CTCAAGAGTT CGTTGGCTGC
61  GCAGATGATC ATGCCAGGAT ATTCTCATAA CTATGCACTT TTCTATCAAT TGCTCGACAG
121 TAATTTGTTT ACCCGCCATA ATAATTATGC TATCTTGAGA GAAGCCACTA GTGAAACCTA
181 TGTCCCCCGG GTCTATTCTC TATCATATAA GTTTCCAATC TACTTTATTT TGCAATCTTT
241 ACTTTGCAAT CTATATCATA AAAATACCAA AAATATTTAT CTTTTCATAT TATCTCTATC
301 AGATCTCACT TTCGCAAGTG GCCGTGAAGG GATTGTCAAC CCCTTCATTG CGTTGGTTGC
361 GAGGTTCTTG TTTGTTTCTG TAGGCGCGTG GGACTTGTGA GGAGCCTCCT ACTAGATTGA
421 TACCTTGGTT CTCAAAACTG AGGGAAATAC TTACGCTACT TTGCTACATC ACCCTTTCCT
481 CTTCGAGGGA AAAACCAACG CATGCTCAAG AGGTAGCAAG AAGGATTTCT GGTGCCGTTG
541 CTGGGGAGGT CTTCACTCAA GTAAAGACAT AACAAGTACC CATCACAAAC TCATCTCCCT
601 CGCATTACAT TATTTGCCAT TTGCCTCTCG TTTTCCTCTC CCCCACTTCA CCTTTGCCGT
661 TTAATTCGCC CTCTCTTTTC CGTTTGCCTC TTTTTTGCTT GTCTCTTGTG TGCTTGTCTT
721 GCGGGTTACA A
```

Translated amino acid sequence data

FSTOTZ (193 aa) (largest open reading frame is indicated in black):

```
1   TSLRLSTTHE KLPYDYLERK QELEKLRGAH EDLQKENESL RAQQTSPAQE GFEPPCLKCL
61  ERDNATSVAE CSTAATVAIS STIDVVTNPS AEDTTSIADE NARLKTLLET GMYKSLKGHQ
121 TLCDVLKKQI LNRNPRKEGV GFERKINVDG SYWKPEQYPK TTWVAAKGPS VDPSTLSGFT
181 CANPIIIDES FDAN-VVS-E SEWS-SVCQV YWYYLQIWPA I
```

FDEGEN (132 aa) (largest open reading frame is indicated in black):

```
1   AIPTTSRSLV AAALKSSLAA QMIMPGYSHN YALFYQLLDS NLFTRHNNYA ILREATSETY
61  VPRVYSLSYK FPIYFILQSL LCNLYHKNTK NIYLFILSLS DLTFASGREG IVNPFIALVA
121 RFLFVSVGAW DL-GASY-ID TLVLKTEGNT YATLLHHPFL FEGKTNACSR GSKKDFWCRC
181 WGGLHSSKDI TSTHHKLISL ALHYLPFASR FPLPHFTFAV -FALSFPFAS FLLVSCVLVL
241 RVT
```

Fig. 6.8. DNA and amino acid sequences (translated) of FSTOTZ and FEGEN.

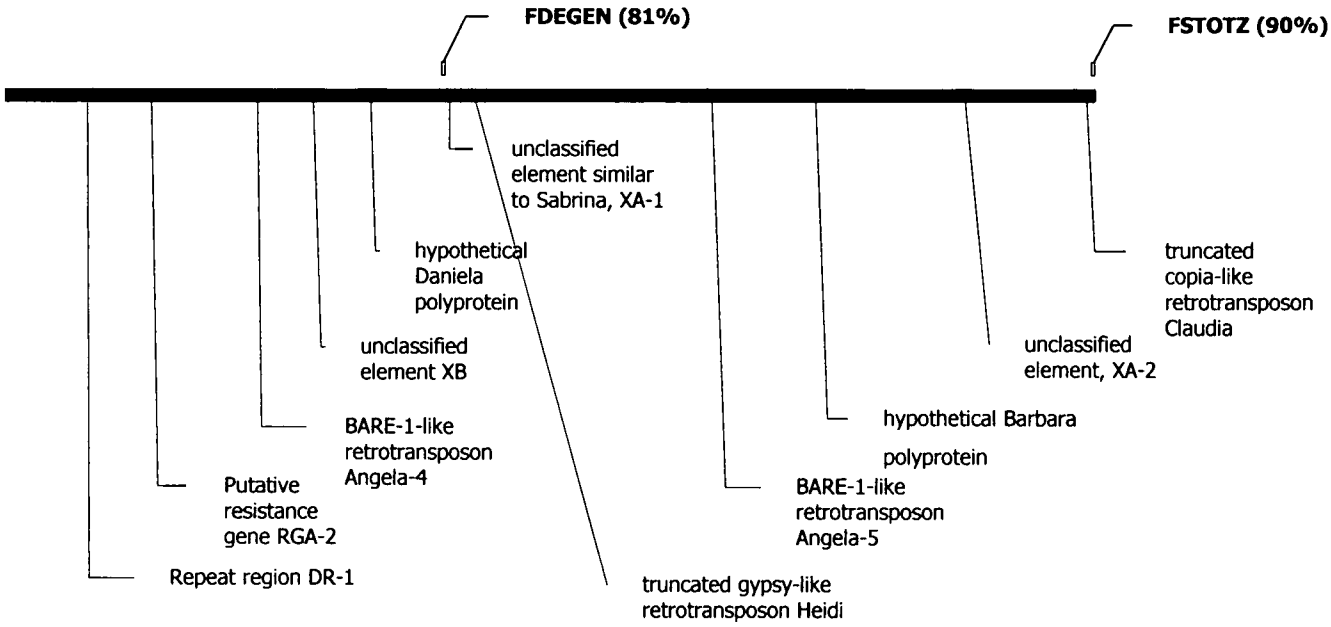

Fig. 6.9. The 211 kb *T. monococcum* fragment with identified regions that exhibit a high level of similarity with the amplified fragments. Percentage similarities are indicated in parentheses following the fragment name. Identified regions on this 211 kb fragment are labeled and indicated in blue.

Table 6.1
Percentages of identical nucleotides between *pgip* DNA sequences from Genbank, and FSTOTZ as well as FDEGEN.

Between FSTOTZ and ...		**Between FDEGEN and ...**	
P. trifoliata AB020528	48%	*C. iyo* AB016206	47%
C. iyo AB016206	47%	*C. jambhiri* AB015198	47%
C. jambhiri AB015198	47%	*C. sannumphung* AB015356	47%
C. sannumphung AB015356	47%	*C. sinensis* Y08618	47%
C. sinensis Y08618	47%	*C. unshiu* AB016204	47%
F. margarita AB020529	47%	*F. margarita* AB020529	47%
G. max X78274	47%	*P. trifoliata* AB020528	47%
P. communis L09264	47%	*A. deliciosa* Z49063	46%
P. sinensis AF196921	47%	*A. thaliana* AF229250	46%
P. vulgaris X78417	47%	*E. grandis* AF159167	46%
S. chinensis AF196953	47%	*P. vulgaris* X78417	46%
S. densiflora AF196950	47%	*P. vulgaris* X64769	45%
Unknown Organism I28276	47%	*V. vinifera* AF305093	45%
A. thaliana AF229249	46%	*A. thaliana* AF229249	44%
M. domestica U77041	46%	*P. mahaleb* AF263465	44%
P. pyrifolia AB021791	46%	*R. californica* AF196935	44%
R. californica AF196935	46%	*Unknown Organism* I28276	44%
C. unshiu AB016204	45%	*A. thaliana* AY035121	43%
E. grandis AF159167	45%	*G. Max* AF130974	43%
G. max AF130974	45%	*P. communis* L09264	43%
P. armeniaca AF020785	45%	*P. emarginata* AF196926	43%
P. emarginata AF196926	45%	*P. pyrifolia* AB021791	43%
A. deliciosa Z49063	44%	*S. chinensis* AF196953	43%
A. thaliana AF229250	44%	*S. densiflora* AF196950	43%
A. thaliana AY035121	44%	*V. californica* AF196962	43%
M. crystallinum BE035885	44%	*G. max* X78274	42%
P. mahaleb AF263465	44%	*M. crystallinum* BE035885	42%
R. scandens AF196946	44%	*M. domestica* U77041	42%
V. californica AF196962	44%	*O. sativa* BE039831	42%
P. fortuneana AF196932	43%	*P. armeniaca* AF020785	42%
S. sorbifolia AF196947	43%	*P. sinensis* AF196921	41%
O. sativa BE039831	42%	*R. scandens* AF196946	41%
V. vinifera AF305093	42%	*S. sorbifolia* AF196947	41%
P. tridentata AF196928	41%	*P. fortuneana* AF196932	40%
P. vulgaris X64769	41%	**FSTOTZ**	**39%**
FDEGEN	**39%**	*P. tridentata* AF196928	39%
G. max AI938045	35%	*G. max* AI938045	34%
G. max AI938190	32%	*G. max* AI938190	26%
G. max AW432387	29%	*G. max* AW432387	25%
G. max AW307262	21%	*G. max* AW307262	19%

Table 6.2
Percentages of identical amino acids between PGIP protein sequences from Genbank, and FSTOTZ as well as FDEGEN.

Between FSTOTZ and ...		Between FDEGEN and ...	
A. thaliana AAF69828	20%	*A. deliciosa* CAA88846	20%
A. thaliana AAK59626	20%	*L. esculentum* S47965	20%
G. max S60713	19%	*P. vulgaris* CAA46016	19%
C. sannumphung AB015356	17%	*P. vulgaris kb* S23764	19%
C. sinensis CAA69910	17%	*P. vulgaris* P35334	19%
C. sinensis T10263	17%	*P. vulgaris* X64769	19%
C. unshiu AB016204.1	17%	*A. thaliana* AAF69828	18%
A. thaliana AAF69827	16%	*A. thaliana* AAK59626	18%
G. max CAA55081	16%	*E. grandis* AF159167	18%
L. esculentum S47965	16%	*V. vinifera* AAK14075	18%
P. armeniaca AAB80732	16%	*A. thaliana* AAF69827	17%
P. trifoliatus AAK43429	16%	*C. iyo* AB016206.1	17%
Pgip 1 AAF69827	16%	*C. sannumphung* AB015356	17%
Pgip 1 BAB11144	16%	*C. sinensis* CAA69910	17%
R. scandens AAK43451	16%	*C. sinensis* T10263	17%
A. deliciosa CAA88846	15%	*C. unshiu* AB016204.1	17%
S. densiflora AAK43459	15%	*F. margarita* BAA34814	17%
V. vinifera AAK14075	15%	*G. max* CAA55081	17%
G. max AAD45503	14%	*P. trifoliata* BAA34813	17%
P. emarginata AAK43436	14%	*Pgip 1* AAF69827	17%
P. fortuneana AAK43442	14%	*Pgip 1* BAB11144	17%
P. tridentata AAK43438	14%	*C. jambhiri* AB015198	16%
P. trifoliata BAA34813	14%	*P. armeniaca* AAB80732	16%
P. vulgaris CAA46016	14%	*P. communis* AAA33865	16%
P. vulgaris kb S23764	14%	*P. communis pear* JQ2262	16%
P. vulgaris P35334	14%	*P. communis* Q05091	16%
P. vulgaris X64769	14%	*G. max* AAD45503	15%
E. grandis AF159167	13%	*G. max* S60713	15%
M. domestica U77041	13%	*M. domestica* U77041	15%
P. communis AAA33865	13%	*P. mahaleb* AAF79181	15%
P. communis pear JQ2262	13%	*P. dulcis* AAK43435	13%
P. communis Q05091	13%	*P. fortuneana* AAK43442	13%
P. dulcis AAK43435	13%	*P. trifoliatus* AAK43429	13%
P. mahaleb AAF79181	13%	*R. californica* AAK43444	13%
P. pyrifolia BAA96450	13%	*V. californica* AAK43466	13%
V. californica AAK43466	13%	*P. pyrifolia* BAA96450	12%
F. margarita BAA34814	11%	*P. tridentata* AAK43438	12%
C. iyo AB016206.1	10%	*R. scandens* AAK43451	12%
C. jambhiri AB015198	10%	*S. sorbifolia* AAK43456	12%
FDEGEN	**10%**	*P. emarginata* AAK43436	11%
R. californica AAK43444	10%	*S. densiflora* AAK43459	11%
S. chinensis AAK43462	10%	**FSTOTZ**	**10%**
S. sorbifolia AAK43456	10%	*S. chinensis* AAK43462	10%

DISCUSSION:

The use of PCR and degenerate primers for the amplification and subsequent cloning of *pgip* genes, is well published (for a review see De Lorenzo *et al.*, 2001). Using this technology to gain more information on these genes in wheat, two fragments were revealed that were further investigated for possible *pgip* characteristics.

As no information on PGIP in wheat exists, it was assumed that wheat *pgip* would structurally be similar to *pgip* from other dicotyledonous plants. This assumption lead to the design of degenerate primers around conserved regions of 12 *pgip* sequences. As a backup, two additional primers were used. Originally patented to isolate PGIP from pear, they were considered as a fair alternative to the degenerate primers. Two fragments, one from each primer, were amplified and designated FSTOTZ (amplified by the patented primers) and FDEGEN (amplified by the degenerate primers). Both were shown to be around 700 bp and, through the Southern blot indicated to exist as multiple copies in the wheat genome. Existing as multiple copies in the genome is quite common for *pgip* genes, which can often exist as large gene families (Stotz *et al.*, 1993, 1994; Ramanathan *et al.*, 1997). Their small ORFs, and the absence of a methionine signaling the start of the peptide, obtained from the translation of these sequences, showed that these sequences are most probably only fragments of a putative functional protein.

To identify the sequences and confirm their possible relatedness to PGIP, the fragments were examined through database similarity (BLAST) searches. The DNA fragments were very much identical with regions on a 211 kb *T. monococcum* (diploid wheat) sequence. FDEGEN mapped with 81% homology to this sequence on an unclassified region carrying a miscellaneous feature, with FSTOTZ showing 90% homology to a region containing a truncated copia-like retrotransposon. This similarity to a retrotransposon is interesting as it has been suggested that transposons may contribute to the plasticity of the *pgip* locus (De Lorenzo *et al.*, 2001), and sequences homologous to transposable elements have been found to flank regions of *pgip* from *P.vulgaris* (Pereira *et al.*, 1986; Dong *et al.*, 1999).

Direct CLUSTALW alignments of FSTOTZ and FDEGEN DNA and amino acid sequences with Genbank accessed sequences showed the percentage of base

similarity between these fragments and PGIP sequences to be insignificantly low. In general, two DNA sequences should be at least 60% identical to be considered related. These alignments revealed the highest base pair identity to be 48% between FSTOTZ and *pgip* from *P. trifoliata* and 47% between FDEGEN and *pgip* from C.*iyo* on DNA level, while on amino acid level FSTOTZ and FDEGEN were 20% identical with PGIP from *A. thaliana* (AAF69828) and 20% alike with PGIP from *A. deliciosa*, respectively. In contrast, however, the homology between *pgip* DNA sequences from *E. saligna* (AF159170) and *G. max* (AW432387), which are two of the most genetically diverse *pgip* sequences, is only 47%, demonstrating the possible significance of homology levels below 60%. It is only when analyzing the PGIP amino acid sequences, that the similarity between these two PGIP-diverse species (*E. saligna* AAF22252; *G. max* AAD45503) reveals 45% of the sequence to be identical, compared to 20% between the amplified fragments and PGIP. This level of amino acid homology between the fragments and dicotyledonous PGIP sequences is too low for the fragments to be considered significant.

Further evidence exists which disqualifies these findings as a possible wheat-*pgip*. The multiple banding patterns observed through the Southern blot analysis, the small ORF's and the DNA sequence dissimilarity to published data could all be indicative of non-coding repetitive sequences. Furthermore, since the primer design would suggest that the two fragments share some identity, their resulting unrelatedness might also be seen as evidence of a non-*pgip*. In support of this is the fact that the two fragments were shown to be homologous to a region in diploid wheat, however, the regions of similarity on this sequence do not contain *pgip* genes or sequences remotely similar to known *pgip* sequences.

It could be argued that the current lack of information surrounding PGIP from monocotyledonous plants does not allow for a more accurate analysis, and therefore a small possibility will always exist that the amplified fragments might well be related to monocotyledonous PGIP. However, judging from the evidence as provided here, the amplified sequences (FDEGEN and FSTOTZ) are unrelated to *pgip* from dicotyledonous plants.

GENERAL DISCUSSION

The purification of PGIP was the central element of this investigation into its presence in wheat. This study revealed the molecular weight of wheat PGIP to be in the order of 36.0 kDa, placing it among PGIPs purified from numerous fruits (see Chapter 1), and confirmed the expectation that wheat PGIP would, like many other PGIPs, be highly specific for the endopolygalacturonase (EPG) it inhibits.

Evidence of a possible inhibitor with a mass in the order of 36 kDa was presented during early investigations in this study. At that time a band of approximately 37.0 kDa was induced on the autoradiograph when newly synthesized proteins were being examined. Probing of the same samples with the PGIP-I antibody, raised against purified bean PGIP, revealed a band of approximately the same size. During a study of apoplastic proteins over a period of three weeks following infections, a band of the same size was recognized which also showed induction. The existence following fungal infection, of a protein in the intercellular space, a region where PGIP expression has been found before (Salvi *et al.*, 1990) was considered significant. The fact that the protein was recognized by an anti-PGIP antibody, strengthened the evidence but, still did not provide conclusive proof of the presence of PGIP, due to the polyclonal nature of the antibody. The PGIP-I antibody is a polyclonal antibody generated against a purified, glycosylated bean PGIP, and shows numerous non-specific reactions, attributed to antibodies recognizing glycosyl side chains on other proteins, which have the same structure as the carbohydrate moieties of the PGIP (Bergmann *et al.*, 1994). This made it impossible to positively conclude that the ±36.0 kDa band seen was indeed wheat PGIP.

Low levels of PGIP were expected, due to the low levels of pectin in the cell walls of monocotyledonous plants compared to the walls of dicotyledonous plants (Bacic *et al.*, 1988; Jarvis *et al.*, 1988). This was confirmed by the extremely small amounts of wheat PGIP recovered during purification. As relatively large amounts (20 μg – 200 μg) (Dunbar & Schwoebel, 1990) of purified protein are required for the successful generation of polyclonal antibodies, the small amounts of purified wheat PGIP recovered did not allow for the generation of antibodies specific to wheat PGIP. This forced the investigation to work with an effective alternative, in the form of PGIP-II. This antibody, raised against a peptide corresponding to residues 10-21 of bean PGIP, was shown to be considerably more specific for PGIP and less prone to non-specific recognition than PGIP-I. Final confirmation of the presence of PGIP

thus came through the purification of a ±36.0 kDa band, as determined by MALDI-TOF-MS, which was linked to endopolygalacturonase inhibition, and was also recognized by the more specific PGIP-II antibody.

Once it was proven that PGIP-II recognizes purified wheat PGIP, the expression study was repeated by either treating wheat plants with salicylic acid (SA), a known inducer of plant defenses (Fritig *et al.*, 1998; Pieterse and Van Loon, 1999), and by infecting wheat seedlings with leaf rust. The results showed that wheat recognizes the chemical inducer, at both concentrations tested, and also reacts to fungal infection. Recognition results in the induction of a cross reacting band at approximately 34.5 kDa as well as a 33.0 kDa band in the SA treated plants, hinting at the possible presence and expression of a family of multiple *pgip* genes in wheat. The observation of an induction in PGIP levels was in line with previous results from both infected and SA treated bean hypocotyls, where an increase in *pgip* transcript and protein levels was recorded (Bergmann *et al.*, 1994; De Lorenzo *et al.*, 2001).

An essential part of the purification of PGIP from wheat was the demonstration of the specificity of wheat PGIP for an endopolygalacturonase from the fungal pathogen, *Cochliobolus sativus*.

The pathogen *C. sativus* is the causal agent of common root rot and occurs wherever cereals are grown. It is an aggressive saprophyte in soil and colonizes and sporulates profusely on infested host debris (Murray *et al.*, 1998).

The lack of inhibition of numerous EPGs observed upon screening a variety of EPGs with the wheat PGIP demonstrated the complex recognition mechanism involved in the PGIP-EPG interaction, as discussed in Chapter 1. More importantly, it raises critical questions about the assay results from the preliminary expression study (Chapter 2). These results were based on the inhibition of *A. niger* EPG by wheat extracts containing a presumed inhibitor. The same *A. niger* EPG was later demonstrated to not be inhibited by the purified wheat PGIP. These results uniformly demonstrated, across all repeats spanning the three weeks investigated, that the infected lines exhibited either a lower PGIP activity, or possibly an increase in EPG activity, compared to the uninfected wheat lines. Initially these findings were considered insignificant except for the confirmation that the plant did recognize and

respond to the invader, even though in a negative manner. After the successful purification of PGIP from wheat by using the EPG from *C. sativus*, the importance of repeating the expression study with the *C. sativus* EPG became clear.

The use of *C. sativus* EPG to measure the change in PGIP activity following salicylic acid treatment of the wheat plants now gave results as expected, with an increase in PGIP activity, in comparison to the untreated control plants, evident from about six hours post treatment. Together with the increase in β-1,3-glucanase activity, this observed PGIP induction confirmed the uptake and reaction to the defense elicitor, and more importantly, confirmed that *C. sativus* EPG was ideal for use with wheat PGIP.

Following the success in assaying for PGIP activity following SA treatment, plants were infected with leaf rust, to examine the reaction to fungal infection through the expression of PGIP. Once again, PGIP expression dropped to below that of the uninfected control plants following infection. This decrease in PGIP expression now reproduced previous results (Chapter 2) using the EPG from *A. niger*. This ruled out the possibility of an unsuitable EPG being responsible for the decrease. The evidence now suggests factors related to the fungal infection as being responsible, as the decrease was not observed following SA treatment.

The assay for PGIP activity is based on correlating a decrease in EPG activity to an increase in PGIP. A decrease in observed PGIP activity therefore requires either a decrease in the amount of PGIP or an increase in EPG activity or concentration. This can be achieved in a limited number of ways:

Firstly by adding EPG, other than that used for the assay, to the assay reaction. The increase in EPG concentration will be observed as a decrease in PGIP activity. However, since no other EPG was added to the assay reaction, and as the 'reducing sugar control' in the activity equation accounts for any EPG that may be associated with the pathogen in the leaf, the possibility of an increase in EPG concentration can be discounted.

Secondly, through a decrease in the amount of inhibitor in the extract. A decrease can be achieved in three ways: 1) less PGIP can be transcribed, which the

immunoblots have shown not to be the case, 2) less PGIP is free to be assayed because it is complexed with its EPG ligand, or 3) the suppression of PGIP. The decrease in PGIP activity observed during the assay with the *A. niger* EPG discounts the second possibility as wheat PGIP does not interact with *A. niger* EPG. This leaves only the possible suppression of PGIP responsible for a decrease in PGIP activity (see below).

Finally, an increase in the activity of the EPG in the assay reaction will appear as a decrease in PGIP activity. After eliminating the possibilities above, the PGIP results hint at a possible indiscriminate induction of the EPG used (either from *C. sativus* or *A. niger*) in the assay. This proposed activation factor is speculative and of unknown structure or biochemical mechanism. However, this induction in EPG activity appears to be along the line of recent findings by Bergmann *et al.* (2001). They found evidence that for certain PGIP-EPG interactions, under certain pH conditions, the EPG was activated rather than inhibited. In fact, bean PGIP was able to increase the activity of the *A. niger* EPG isoform A 30 fold at pH 5.0.

Such an induction in activity of a pathogen's EPG could potentially have a suicidal effect on an infected wheat plant, if its own PGIP, alone or in combination with another factor, effectively activates the pathogen's EPG to speed up the colonization and subsequent destruction of the host. On the other hand, the observed EPG activation could be an isolated case between wheat PGIP and the EPGs from *C. sativus* and *A. niger* used in the assays, while the interaction between wheat PGIP and *P. triticina* (leaf rust) EPG is totally different. Although rust pathogens of cereals are obligate parasites growing only on their biological hosts, Fasters *et al.* (1993) were able to grow large amounts of stem rust in liquid culture. Until such time that this method can be optimized for the production of leaf rust EPG isoforms with sufficient activity that can effectively be harvested in large enough quantities for the assay of wheat PGIP, the effectiveness of wheat PGIP during fungal infection *in vivo* remains questionable.

In the quest for finding an explanation for the observed assay results, evidence exists that argues the release of suppressors during plant-pathogen interactions (Moerschbacher *et al.*, 1999).

This investigation did not examine the function of pectic fragments produced by fungal enzymes in wheat beyond their roles as elicitors as discussed in Chapter 2. However, the findings of Moerschbacher and co-workers (1999) give some valuable insight into the role of these fragments in wheat, and offer yet another possible explanation for the observed behaviour of PGIP expression in wheat. By examining the effect of wheat cell wall fragments on phenylalanine-lyase (PAL) and peroxidases (POD) activity, both enzymes with a role in disease resistance (Fritig *et al.*, 1998), it has been shown that the fragments, consisting mainly out of dimeric and trimeric polygalacturonic acid, were able to actively suppress these two enzymes during wheat's defense reactions (Moerschbacher *et al.*, 1999). These findings would therefore suggest that the products of the cell wall degrading enzymes responsible for hydrolyzing PGA in pectin (e.g. EPG), also act to suppress the ability of wheat's natural defense reactions. This would have the same detrimental effect on the plant, as discussed above, with products from its structure responsible for its own demise. Unfortunately, Moerschbacher and co-workers do not explain the exact mechanism of suppression, and it is therefore not known whether this suppression is relevant to all defense proteins or only those with enzymatic activity like PAL and POD.

PGIP-II, proven to be specific for the recognition of wheat PGIP, was subsequently used to probe ultra-thin sections of leaf segments, both uninfected and infected with leaf rust in a qualitative immunogold labeling study. Polygalacturonase-inhibiting protein has been identified as a cell wall localized protein. It was therefore expected to find high levels of labeling in the cell wall and perhaps the intercellular spaces, as found by Bergmann *et al.* (1994). Indeed, labeling was found in the cell wall of infected wheat leaves, especially close to infection sites and near developing fungal structures, as was recorded in infected bean plants by Bergmann *et al.* (1994). However, in cases where plant cells were penetrated and haustoriums formed in the cytoplasm, high levels of labeling were observed on the periphery of the haustorium. This observation is quite unique, as PGIPs are known to be exported to the cell wall due to the signal peptide in the mature protein's structure, and no PGIP has ever been reported to be active in the cytosol. No pre-immune serum was available to act as a control to verify whether identified cross-reactions may be the result of non-specific rabbit antibodies. Therefore, it can be argued that the observed haustorium labeling is the direct result of non-specific cross-reactions between fungal tissue and

rabbit antibodies. However, a significantly lower level of labeling was observed between hyphae outside the cell compared to the haustorium inside the cell, a clear indication that the observed cross-reaction was the result of a specific recognition with cytosolic fungal tissue.

Although the substrate for EPG, polygalacturonic acid, is located in the cell wall, and therefore absent from the cytoplasm the exact reason for a continued expression of EPG by the haustorium is not clear. It could be attributed to constitutively expressed EPG since it is known that fungi may express both constitutive and inducible EPGs (Parenicová *et al.*, 2000; Wubben *et al.*, 2000).

Favaron (2001) recently showed that PGIP from leek, a monocot, consists of not less that 20 isoforms with varying levels of inhibition, confirming the assumption that monocotyledonous PGIP, like PGIP from dicotyledonous plants, could exist as a gene family. With this in mind it can be speculated that some of these isoforms are not exported to the cell wall as dictated by its molecular make-up (De Lorenzo *et al.*, 2001), but that some remains inside the cytosol as part of the plant's defense mechanism. This assumption lends some support to the previously proposed concept of a decrease in assayable PGIP being extracted during vacuum infiltration, resulting in a decrease in assayable PGIP as observed in the assays. It can be speculated that not all of these PGIP isoforms are targeted for the cell wall, but that some remain behind in the cytosol. An induction in *pgip* transcription following fungal attack could selectively induce the cytosolic isoforms, or induce a PGIP isoform with less inhibitory activity in the cell wall, or perhaps redirect cell wall bound PGIP to the cytosol, all of which would result in less active PGIP being extracted. It could also be speculated that some of these PGIP isoforms has the ability to induce EPG to a higher activity, as mentioned above. These assumptions do not attempt to explain the recorded assay results, but provide a possible explanation of how PGIP ends up in the cytosol of pathogen infected cells, and explains how a decrease in PGIP activity can result.

Seen on its own, the cytosolic labeling serves to shift the focus of a possible explanation for the previously discussed observed decrease in PGIP activity. Although this evidence supports the possibility that less PGIP is being extracted, the assay data seem to support both the theory of an induction in EPG, as found by

Bergmann *et al.* (2001), and the suppression of disease resistance (possibly PGIP activity) as found by Moerschbacher *et al.* (1999).

It is surprising to find that PGIP from monocotyledonous plants differs markedly from PGIPs expressed in dicotyledons. This could explain the results obtained from the attempts to clone wheat PGIP by way of PCR. Based on the assumption that monocotyledonous PGIP and dicotyledonous PGIP are very similar, degenerate primers were designed and used together with patented PGIP primers. According to the DNA and translated amino acid sequences of the two fragments amplified (FSTOTZ and FDEGEN), they appear not to be related to PGIP from dicotyledonous plants in any way. As mentioned in Chapter 6, PGIPs from dicotyledonous plants often exhibit a high level of divergence. With this in mind, the observed low level of homology between PGIPs and these fragments could support the theory of PGIPs from monocots having unique properties.

As pointed out before, a polyclonal antibody like PGIP-I and PGIP-II (or any other) is only as specific as the antigenic determinant present during the antibody generation process. It is generally accepted that it is impossible to establish the exact determinant used by the animal's immune system in the generation process. Therefore, some level of uncertainty will always surround evidence produced with any polyclonal antibody and subsequently needs further evidence to comfirm the relevance of results. In this investigation such evidence was produced in the form of purified wheat fractions with the ability to inhibit *C. sativus* EPG activity (which is the minimum requirement of a PGIP), while simultaneously being recognized by PGIP-II during blotting as well as, being localized to a region of the plant cell where PGIP has been found. Three critically important pieces of evidence were provided to prove for the first time that PGIP is present in wheat.

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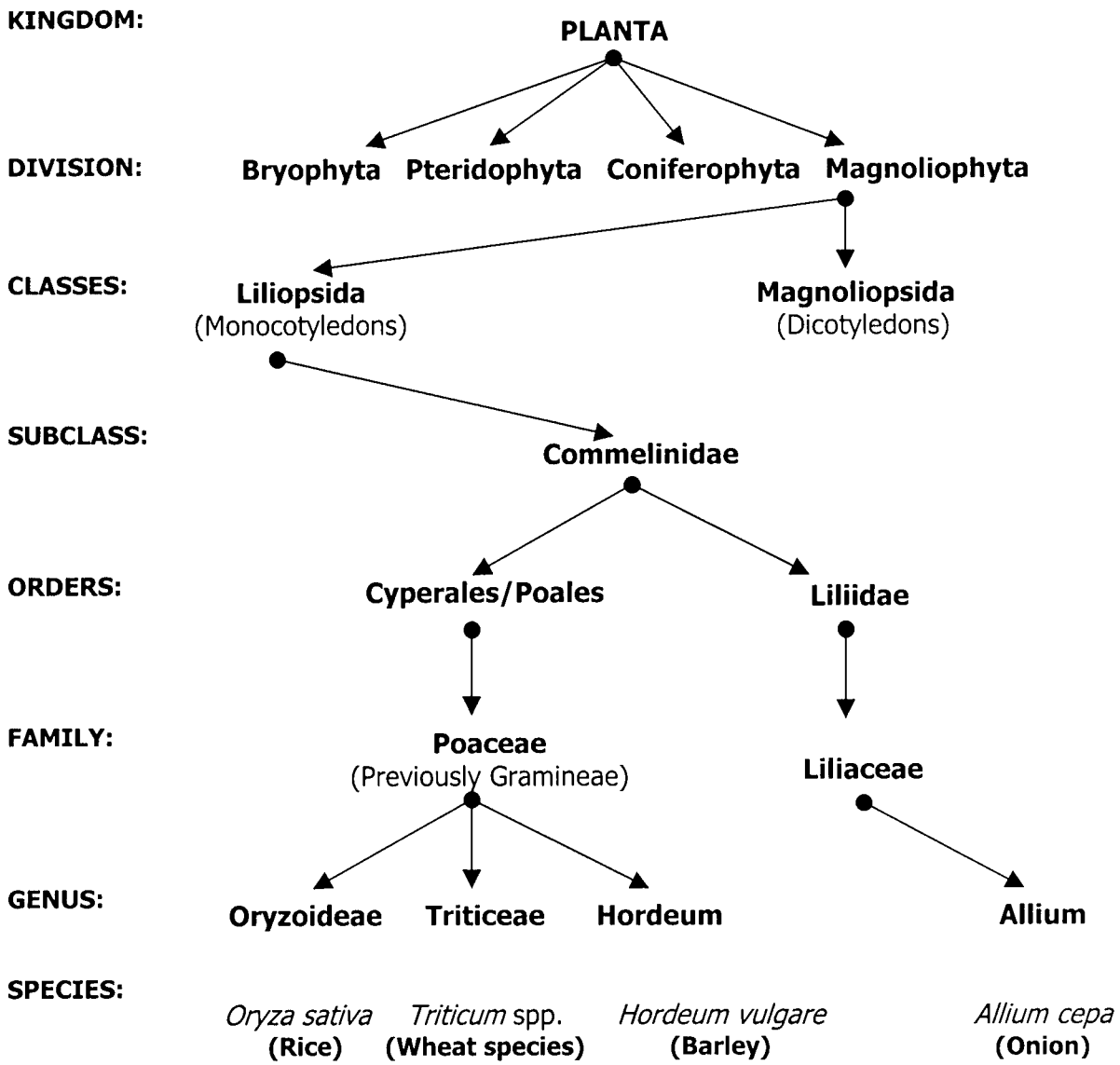
APPENDICES



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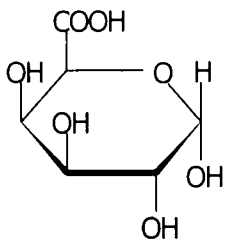

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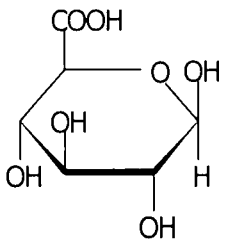

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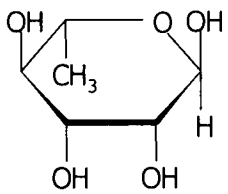

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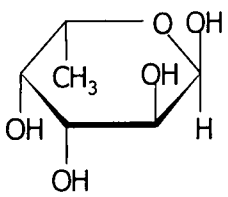

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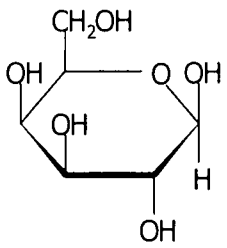

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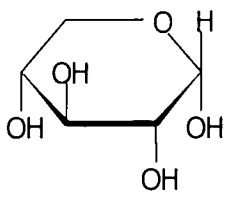

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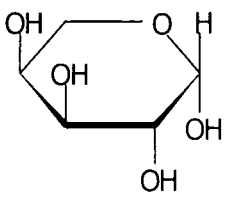

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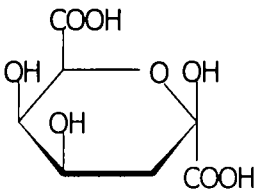

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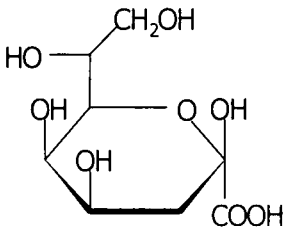

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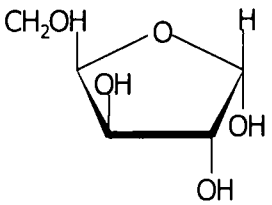

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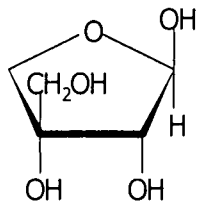
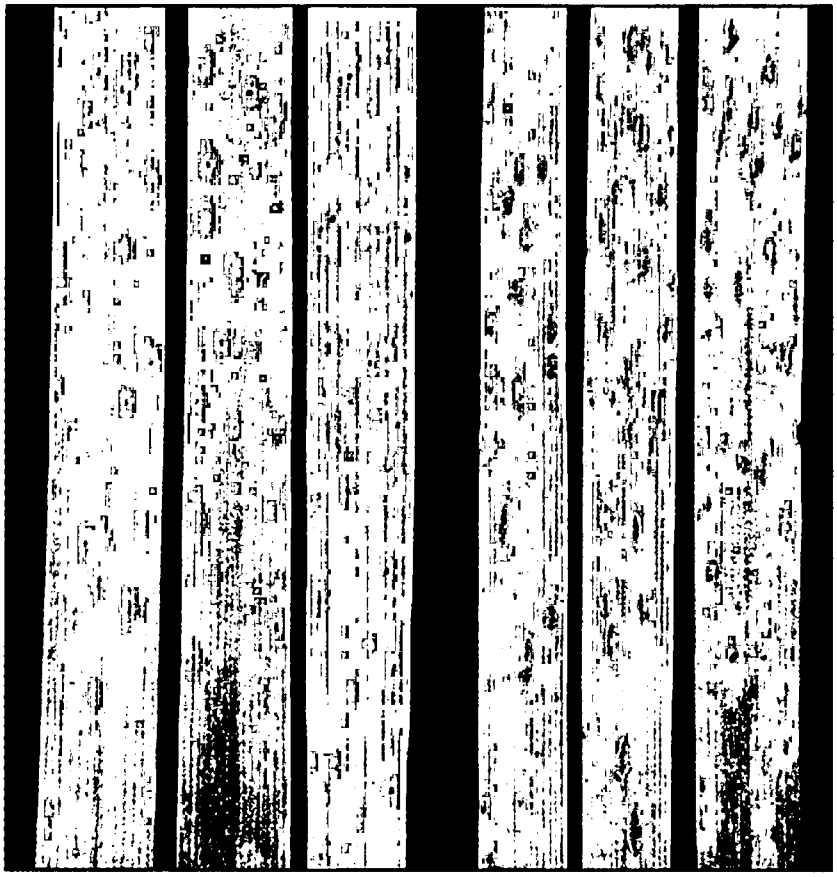


β-D-Api*f*

Resistant Thatcher/*Lr29*

Susceptible Thatcher



A. deliciosa	*Actinidia deliciosa*
A. thaliana	*Arabidopsis thaliana*
C. iyo	*Citrus iyo*
C. jambhiri	*Citrus jambhiri*
C. sannumphung	*Citrus sannumphung*
C. sinensis	*Citrus sinensis*
C. unshiu	*Citrus unshiu*
E. camaldulensis	*Eucalyptus camaldulensis*
E. grandis	*Eucalyptus grandis*
E. nitens	*Eucalyptus nitens*
E. saligna	*Eucalyptus saligna*
E. urophylla	*Eucalyptus urophylla*
F. margarita	*Fortunella margarita*
G. max	*Glycine max*
L. esculentum	*Lycopersicon esculentum*
M. crystallinum	*Mesembryanthemum crystallinum*
M. domestica	*Malus domestica*
O. sativa	*Oryza sativa*
P. armeniaca	*Prunus armeniaca*
P. communis	*Pyrus communis*
P. dulcis	*Prunus dulcis*
P. emarginata	*Prunus emarginata*
P. fortuneana	*Pyracantha fortuneana*
P. mahaleb	*Prunus mahaleb*
P. pyrifolia	*Pyrus pyrifolia*
P. sinensis	*Prinsepia sinensis*
P. tridentata	*Purshia tridentata*
P. trifoliata	*Poncirus trifoliata*
P. trifoliatus	*Porteranthus trifoliatus*
P. vulgaris	*Phaseolus vulgaris*
R. californica	*Rhamnus californica*
R. scandens	*Rhodothypos scandens*
S. chinensis	*Stephanandra chinensis*
S. densiflora	*Spiraea densiflora*
S. sorbifolia	*Sorbaria sorbifolia*
V. californica	*Vauquelinia californica*
V. vinifera	*Vitis vinifera*

SUMMARIES

Hierdie studie het die moontlike rol van poligalakturonase-inhiberings proteïen (PGIP) teenwoordig in koring (*Triticum aestivum*), wat aktief optree as deel van die plant se verdedigingsreaksie tydens blaarroes (*Puccinia triticina*) -infeksie, ondersoek.

Hierdie proteïen en sy rol in die verdedigingsarsenaal in diktotiel plante is goed bekend. PGIP beskerm die plant deur die hidrolitiese ensiem, endopoligalakturonase (EPG), wat deur die patogeen vrygestel word om die selwand te vernietig, te inhibeer. Die rol van hierdie proteïen in monokotiele en veral die graangewasse is nog nooit bestudeer nie.

'n Voorlopige immunologiese ondersoek met 'n poliklonale teenliggaampie wat opgewek is teen gesuiwerde boontjie PGIP (PGIP-I) het aangetoon dat 'n moontlike PGIP van ±37.0 kDa geïnduseer word met blaarroesinfeksie. Die gepaardgaande aktiwiteit soos gemeet aan die inhibisie van *Aspergillus niger* EPG het 'n afname in PGIP aangetoon. Met behulp van ioon-uitruilings en molekulêre siftings-chromatografie is die teenwoordigheid van PGIP in koring finaal bevestig. 'n Proteïen van ±36.0 kDa is gesuiwer wat spesifiek is vir *Cochliobolus sativus* EPG, maar nie vir *A. niger* nie. Hierdie proteïen is ook herken met 'n meer spesifieke anti-PGIP poliklonale teenliggaampie (PGIP-II) tydens 'n immunologiese studie.

Hierdie EPG is vervolgens gebruik om die uitdrukking van koring PGIP te bestudeer na onderskeidelik salisielsuurbehandeling en swaminfeksie. Salisielsuurbehandeling het 'n induksie in beide PGIP aktiwiteit en op proteïenvlak aangetoon, terwyl swaminfeksie 'n afname in PGIP aktiwiteit te weeg gebring het met 'n toename op proteïenvlak soos waargeneem met PGIP-II. Deur middel van immunogoudmerking is vasgestel dat PGIP uitdrukking in die koringselle beperk is tot die selwand en die haustorium van die patogeen in die plant sitosol.

Pogings om die koring *pgip* geen te kloneer deur middel van die polimerase kettingreaksie en nie-spesifieke DNA inleiers was onsuksesvol. Fragmente wat sodoende geamplifiseer is het geen betekenisvolle ooreenkomste met PGIP van dikotiel plante getoon nie.

Hierdie studie het aangetoon dat koring oor 'n 36.0 kDa PGIP beskik wat tydens salisielsuur en swaminfeksie geïnduseer word, maar 'n swamverwante faktor induseer EPG aktiwiteit of onderdruk PGIP aktiwiteit wat 'n afname in laasgenoemde veroorsaak. So 'n afname in PGIP aktiwiteit kan lei tot die swam se ontwikkeling en die gevolglike verlaging in plantproduktiwiteit.

The presence and possible role of polygalacturonase-inhibiting protein (PGIP) in wheat (*Triticum aestivum*) as part of the plant's defense reaction following leaf rust (*Puccinia triticina*) infection were investigated.

Through its ability to inhibit fungal endopolygalacturonase (EPG) that breaks down the plant cell wall during colonization, this protein is known to play an important role in the natural defense arsenal of dicotyledonous plants. The presence of PGIP in monocotyledonous cereals has never before been conclusively proved.

A preliminary investigation using a polyclonal antibody raised against a purified bean PGIP (PGIP-I) revealed the induction of a possible PGIP of ±37.0 kDa following fungal infection, while an inhibition assay of EPG from *Aspergillus niger* showed a decrease in PGIP activity. Through ion-exchange and size exclusion chromatography the presence of wheat PGIP was subsequently confirmed by the purification of a ±36.0 kDa inhibitor, which proved specific for the EPG of *Cochliobolus sativus* and not *A. niger*. Using a more specific anti-PGIP antibody (PGIP-II) the presence of this protein in wheat was also confirmed through immunoblotting.

The expression of PGIP in wheat following salicylic acid (SA) treatment and fungal infection in terms of *C. sativus* EPG inhibition was recorded. While SA treatment showed an induction of PGIP at protein and activity levels, fungal infection repeated the reduction in PGIP activity as previously observed. Using PGIP-II in immunogold localization the expression of PGIP in wheat leaves was confined to the plant cell wall and the periphery of the haustorium in the cytosol.

Attempts to clone the wheat *pgip* gene through the polymerase chain reaction (PCR) using degenerate primers were inconclusive, as fragments amplified did not exhibit significant similarity to PGIP from dicotyledonous plants.

These results therefore indicate that wheat expresses a ±36.0 kDa PGIP in reaction to fungal and SA treatment, but fungus-related factors originating from either the plant or the fungus apparently induce the EPG activity to higher levels, or suppress the PGIP activity to lower levels, both recordable as a decrease in PGIP activity and having the potential to enhance plant disease.