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The role of pectins in the regulation of plant defence responses against pathogens

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1. INTRODUCTION

The increase of the world population in the twentieth century requires an increase of crop productivity and therefore a better control of pests and epidemics. In order to control plant diseases many pesticides and fertilizers have been used which cause environmental pollution and human/animal health problems. The knowledge of plant defence mechanisms and the selection of plant varieties that are resistant to disease may provide a convenient, inexpensive, and environmentally mild alternative to the use of chemicals.

Plants are naturally resistant to most pathogens and the ability of a pathogen to cause disease in a host plant is usually the exception and not the rule. This is because plants have an innate ability to recognize potential invading pathogens and to mount successful defences. In a converse manner, successful pathogens cause disease only because are able to evade recognition or suppress host defence mechanisms. There is a continuous progress in our understanding of genes and mechanisms involved in plant defence responses. This knowledge indicates that there is a molecular recognition between molecules secreted by pathogens or produced by pathogen attack (elicitors) and molecules produced by plants.

1.1 The plant cell wall is the first barrier against pathogens

The plant cell wall is a structure rich of polysaccharides, surrounding the external face of the plasma membrane. It is responsible for the shape of the cell, it plays a role as a reserve storage and in the intercellular transport control but also has a defence function (Carpita and Gibeaut 1993). It is the first structure that comes into contact with the invading organisms and many of the recognition events of the plant pathogen interactions occur at the cell wall level. Pathogenic and saprophytic fungi, that extensively invade the plant tissue either intracellularly or extracellularly, cause the degradation of plant cell wall (Cooper 1984).

The plant cell wall is composed of cellulose fibrils which form an insoluble and inelastic crystalline material, interconnected with high molecular weight hemicellulose (typically xyloglucan or arabinoxylan) molecules. These are embedded in a matrix of pectin, a term to indicate the galacturonic acid-rich fraction of the cell wall (Fig 1.1). The most abundant component of pectin is rhamnogalacturonan I (RGI), which consists of a backbone of alternating rhamnose and galacturonic acid with various side groups,

principally galactans and arabinans. The three major components are homogalacturonan (HG), xylogalacturonan (XGA) and rhamnogalacturonan II (RGII).

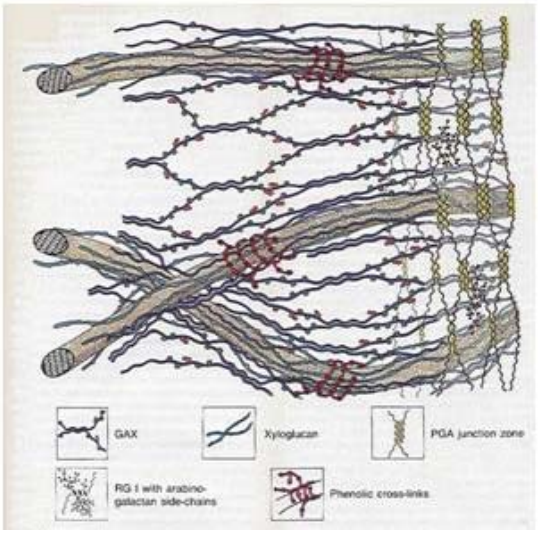

Fig 1.1 A schematic representation of the plant cell wall.

Homogalacturonans are linear 1,4-linked α-D-galactopyranosyluronic acid chains, in which some of the residues carry methyl or acetyl groups (Ridley et al. 2001). The carboxylic acid moieties of pectins confer them the capability of binding calcium and other divalent cations by lateral association of two different chains in a structure known as "egg-box" (Grant et al. 1973) (fig 1.2). This characteristic structure is responsible for gel formation in the presence of calcium and gives to pectins a specific defence function in the cell wall. Many pathogens release enzymes such as polygalacturonases and pectate lyases that degrade cell wall polysaccharides; some of their degradation products elicit defensive responses by plants (see later).

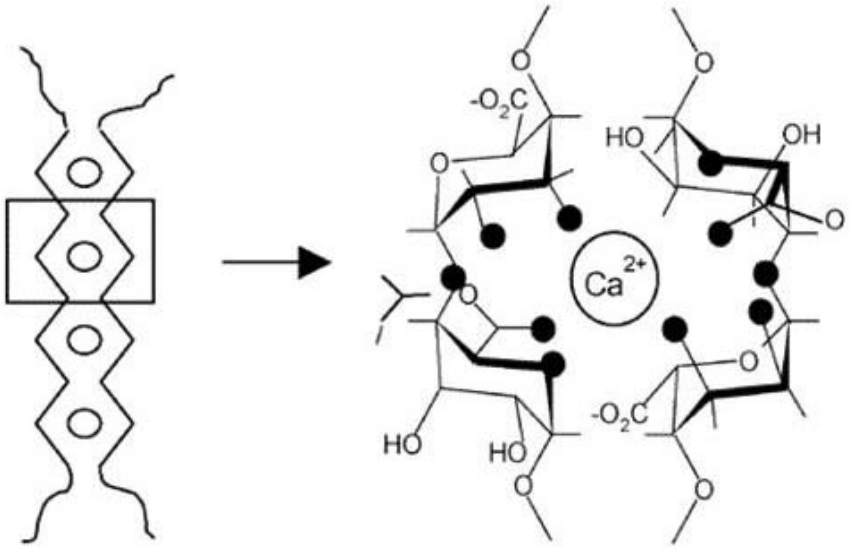

Fig 1.2. Egg-box model.

1.2 The plant defence mechanisms

The ability of plants to defend themselves from fungi, bacteria, viruses and nematodes does not rely on circulating antibodies but on a number of defence responses that are activated at the site of infection. This activation sometimes follows a recognition event mediated by plant receptors which perceive specific molecules, called elicitors, derived from the pathogen or from the plant following pathogen infection. Recognition of elicitors leads to the activation of different defence-related pathways (Montesano et al. 2003; Nimchuk et al. 2003; Nurnberger et al. 2004). In wild type plant populations, most plants are healthy most of the time and only a small proportion of pathogen infections are likely to result in disease (Staskawicz et al. 2001). In some cases plant defence mechanisms are constitutive (pre-infection), while, in other cases, they are induced once the pathogen attack has occurred (post-infection).

A plant is defined susceptible to a potentially pathogenic microorganism, when infection is followed by symptoms of disease (compatible interaction), while in the opposite case is defined as resistant (non compatible interaction) (Clarke and Knox 1978). Resistance is due to a combination of physical and chemical barriers, which are either preformed or induced after the infection. Some antimicrobial compounds are constitutively present, but accumulation of molecules called phytoalexins is induced upon pathogen

attack. In addition to phytoalexin accumulation, synthesis of enzymes against pathogens (glucanases, chitinases), plant cell wall fortification (crosslinking, lignification), production of proteinase inhibitor and polygalacturonase inhibiting proteins (PGIP) are defence mechanisms induced by the infection. Within minutes of pathogen attack, plant defences are locally activated and may also lead to a sort of programmed cell death called hypersensitive response (HR). Within hours, defence responses are elaborated in tissues far from the invasion site and, sometimes, even in neighbouring plants. Timely perception of the pathogen by the plant is central to the activation of defence responses.

The pathogen often locally diffuses effectors or elicitors that are perceived by the host. These fall into two broad categories. The avirulence gene products (Avr) act as elicitors that are specific and unique for a particular pathogen and have been developed as a microbial adaptation to the unique environment within the host. The resistance depends on the presence of a specific product of avirulence (Avr) genes in the pathogen and a specific gene of a resistance (R) gene in the plant host (gene-for-gene theory) (Keen 1990).

The second category of effectors are non-specific elicitors that activate a broader and more basal surveillance. This involves sensitive perception systems for numerous microbe-derived molecules or plant-derived molecules during the infection, which mediate activation of plant defence responses in a non-cultivar-specific manner (Boller 1995). Several of these non-specific elicitors are pathogen-associated molecular patterns (PAMPs) and are derived from essential components of the pathogen cell wall (chitin, glucan) or other structures like the bacterial protein flagellin. They are constitutively present in the pathogen and are essential or "vital factors" for the functioning of the microorganism. As a result, they are conserved within a class of microbes. Other non-specific elicitors like oligogalacturonides are produced by the enzymatic action of microorganisms during pathogenesis.

1.3 Fungal *endo*polygalacturonases are pathogenicity factor which may release elicitors from the cell wall

Because the plant cell wall is the first physical barrier against infection, microorganisms or erbivores need to breech this structure to gain access to the plant tissues. During the early stages of infection pathogens secrete cell wall degrading enzymes (CWDEs) in order to degrade the cell wall polymers. These enzymes are normally inducible and are secreted sequentially (Cervone et al. 1986). Due to the fact that pectin is

a major component of the plant cell wall and is more external than other cell wall components, pectinolytic enzymes (pectinases) are the first microbial enzymes to be secreted followed by hemicellulases and cellulases (Jones et al. 1972; Mankarios and Friend 1980). Among pectinases polygalacturonases (PGs) are the very first secreted enzymes and their action is a prerequisite for the accessibility of substrate to other degrading enzymes (Bateman and Basham 1976; Hahn et al. 1989).

Fungal PGs with an *endo* mode of action catalyse the fragmentation and solubilization of homogalacturonan and are important pathogenicity factors (De Lorenzo et al. 2001). On the other hand PGs also may elicit a number of defence responses such as the accumulation of phytoalexins (Lee and West 1981) (Favaron et al. 1992), proteinase inhibitor I (Walker-Simmons and Ryan 1984), lignin formation (Robertsen 1987), peroxidases and β-1-3-glucanases (Lafitte et al. 1993). PGs are not *per se* elicitors but they are involved in the production of oligogalacturonides (OGs) (Bruce and West 1982), the true elicitors of defence responses (Bruce and West 1989).

1.4 Polygalacturonase inhibiting protein (PGIP), a defence protein which determines the formation of oligogalacturonides

Plants have developed different systems to defend themselves from pathogens. When phytopatogenic fungi secrete PG to breech the cell wall during the early stages of infection, in the wall is present a leucine rich repeat protein (LRR) which inhibits PG activity. This protein, called polygalacturonase inhibiting protein (PGIP), interacts with PG, slows down its pectin degrading activity and promotes the formation of active elicitors (OGs) (Dixon and Lamb 1990). The occurrence of PGIP has been reported in a variety of dicotyledonous plants and in the pectin–rich monocotyledonous plants such as onion and leek (De Lorenzo et al. 2001). It has been recently reported that PGIP plays a direct role in plant defence and that transgenic *Arabidopsis* plant overexpressing PGIPs exhibit enhanced resistance to *Botytis cinerea* (Ferrari et al. 2003a). Like many other defence proteins, PGIPs are encoded by gene families and in Arabidopsis two tandemly duplicated genes (AtPGIP1 and AtPGIP2) are upregulated coordinately in response to *Botrytis cinerea* infection, through separate signal transduction pathways (Ferrari et al. 2003b).

In many plants PGIP is constitutively present and ready to interact with PGs to form active elicitors. A model of PGIP as a component of the cell surface signalling system that leads to the formation of elicitor-active oligogalacturonides is presented in figure 1.5. In

this scheme it is shown that PGIP not only inhibits PGs activity by slowing down pathogen penetration, but also avoids fast homogalacturonan depolymerization allowing the formation of OGs with a degree of polymerization between 10 and 15; these oligogalacturonides belong to the class of non-specific elicitors and induce defence responses in many plant species and genotypes. The oligogalacturonides are perceived by plants as indication of the presence of the pathogen and activate defence responses through a signal transduction pathway whose components are mostly still unknown (Coté and Hahn 1994). Among the early signalling events involved in the OG signal transduction pathway the activation of ion fluxes and membrane depolarization with H^+ influx and K^+ efflux have been reported. OGs also influence both Ca^{2+} influx and Ca^{2+} efflux and a PM Ca^{2+}-ATPase is involved in the oxidative burst induced in plant cells by OG (Romani et al. 2004). The activation of GTP binding proteins (Ridley et al. 2001), the stimulation of phospholypase A_2 and C (Legendre et al. 1993b), the induction of protein phosphorylation and phosphatase activity and the activation of MAPK activity (Droillard et al. 2000) are additional effects played by OGs.

A response to OG that is clearly involved in plant defence is the production of active oxygen species (H_2O_2, and O_2). This response occurs within a few minutes after the addition of OGs to suspension-cultured soybean (Legendre et al. 1993a), tobacco (Binet et al. 1998; Rout-Mayer et al. 1997), and tomato (Stennis et al. 1998) cells. Reactive oxygen species are thought to have direct (through cytotoxicity) and indirect (through signalling) roles in the plant cell death required for the hypersensitive response (HR).

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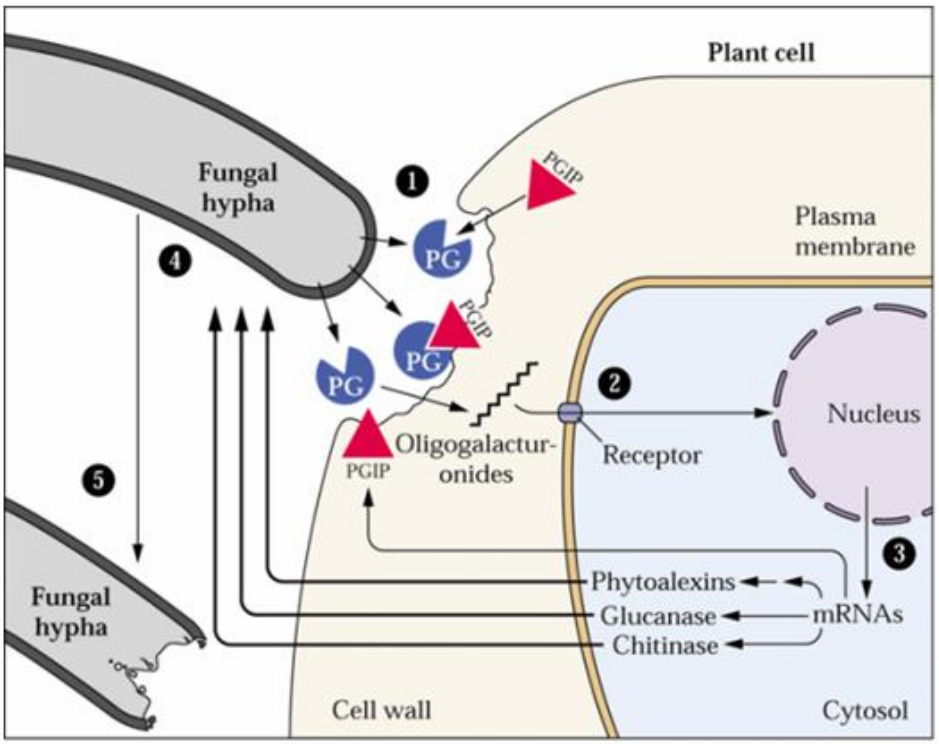

Fig. 1.5. Model of PGIP as a component of the cell surface signalling system that leads to the formation of elicitor-active oligogalacturonides.

The transcriptional activation of genes involved in the biosynthesis of antimicrobial compounds, the accumulation of phytoalexins (Davis et al. 1986), the activation of defence genes such as phenylalanine-ammonia lyase (PAL) and chalcone synthase (CHS) are among the late responses to OGs (De Lorenzo et al. 1987). OG also induce resistance to the necrotrophic fungus *Botrytis cinerea* infection independently of jasmonic acid (JA), salycilic acid (SA) and ethylene (ET) up-regulating genes encoding enzymes required for the biosynthesis of the phytoalexin camalexin (Ferrari et al. in preparation). Synthesis and accumulation of fungal cell wall degrading enzymes such as chitinases (Broekaert and Peumans 1988) and β-1,4-glucanases (Davis and Hahlbrock 1987), activation of the synthesis of lignin (Robertsen 1986) up-regulation of PGIP gene (Ferrari et al. 2003b) and a block of the increase of cytosolic calcium in response to OG is also reported (Navazio et al. 2002).

Oligogalacturonides are not only involved in the defence but also in plant growth and development; they inhibit auxin-induced pea stem elongation (Branca et al. 1988), induce formation of flowers (Marfà et al. 1991), inhibit the expression of late auxin-responsive genes (Mauro et al. 2002) and rizogenesis (Bellincampi et al. 1993) and activate stomatic mytosis (Altamura et al. 1998).

The structure of the isoform 2 of *Phaseolus vulgaris* PGIP (*Pv*PGIP2) has recently been solved (Di Matteo et al. 2003) showing that the central LRR domain consists of a set of 10 tandemly repeating units, each derived from modification of a 24-amino acids leucine-rich peptide. The LRR element matches the consensus GxIPxxLxxLxxLxxLxLxxNxLx and has regularly spaced Leu residues and conserved Gly, Pro, and Asn in the 1st, 4th and 21st position (De Lorenzo et al. 1994). A long parallel β-sheet (B1) occupies the concave inner side of the structure. The β-sheet B1 corresponds to the predicted β-sheet where the residues determining the affinity and specificity of PGIP2 are known to reside (Leckie et al. 1999). An additional extended parallel β-sheet (B2) characterizes the fold of PGIP2 (fig 1.3).

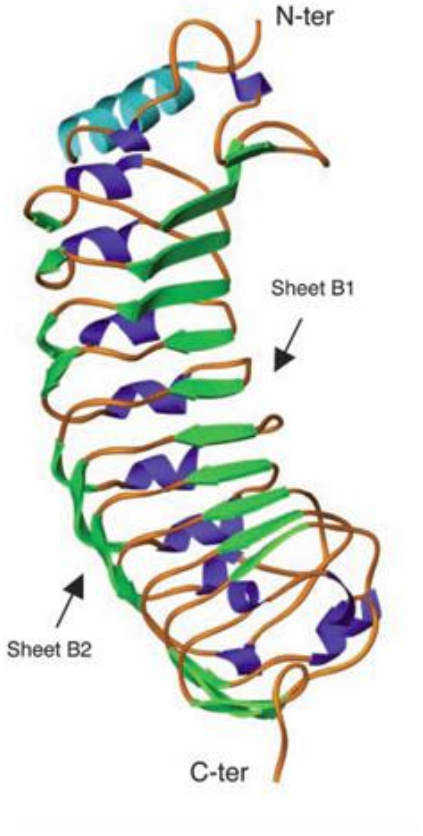

Fig 1.3. The 3-D structure of *Pv*PGIP2.

The LRR is a versatile structural motif found in many proteins of diverse origin (microbia, animals and plants) and responsible for both protein-protein interactions and protein-non protein interactions (Kinoshita et al. 2005). The leucine residues of the LRRs form a hydrophobic core while the amino acids flanking the leucines are exposed to the solvent and interact with the ligands (De Lorenzo et al. 2001). In plants, LRR proteins play a crucial role in development and in resistance. The majority of resistance gene products (R) are LRR proteins such as the products of the resistance *Cf* genes in tomato, that confer resistance to the fungus *Cladosporum fulvus* (Jones and Jones 1997), and *Xa*21 of rice, that confers resistance to the fungus *Xhantomonas oryzae* (Wang et al. 1996) (fig. 1.4), indicating that plants have selected this secondary structure organization for their immunity function and pathogen perception (Jones and Jones 1997).

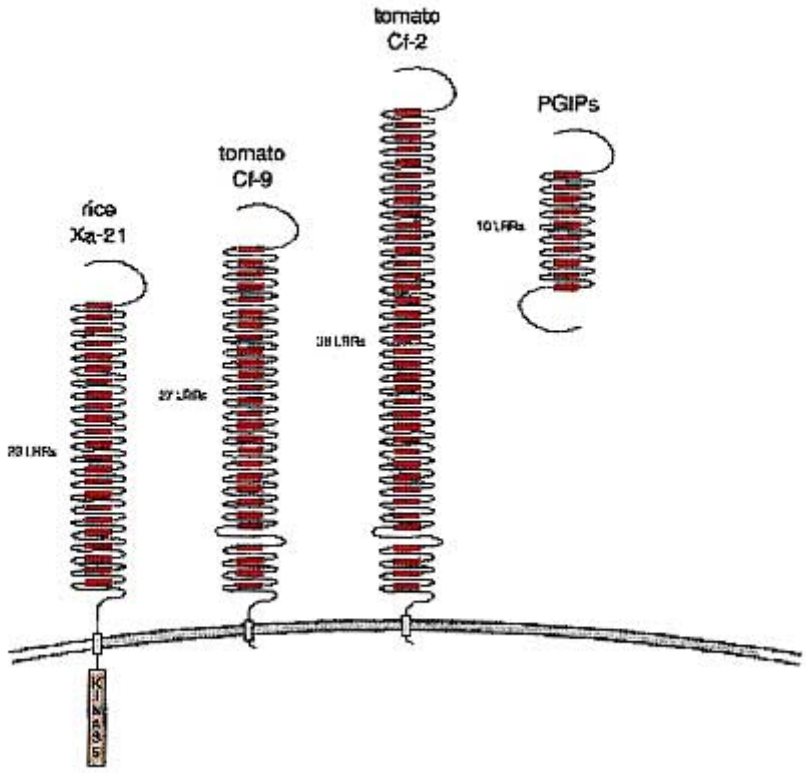

Fig. 1.4. Schematic representation of some resistance gene products in plants displaying an extracytoplasmic LRR domain. PGIPs have not be classified as R products but share with them many similarities.

Plants have evolved different PGIPs with specific recognition capabilities against the many PGs produced by fungi (De Lorenzo et al. 2001). Consistent with their role in defence, PGIPs are constitutively expressed at low levels and their expression is induced in response to several stress stimuli. Interestingly, oligogalacturonides are capable of inducing PGIP expression, suggesting the existence of a feed-forward mechanism for the accumulation of oligogalacturonides (De Lorenzo et al. 1994).

1.5 Proteomics

The word proteome means the pool of PROTEin products of the genOME and proteomics is the systematic analysis of protein profiles of a tissue, organ or organism in a particular state (Wasinger et al. 1995). The importance of proteomics derive from the awareness that the information given by the sequencing of a genome are not enough to unravel how the proteins inside an organism operate individually or synergistically to fulfill biological functions. So, while we know the complete genome sequences of different organisms still do not understand how the simplest living organisms actually work. While

the genome is virtually static and can be well-defined for an organism, the proteome continually changes in response to external and internal events.

On one hand the knowledge of the genome sequence and the chromosome location of all genes provides an anatomical description, on the other hand functional genomics, through the analysis of mRNA levels, provides information about expression pattern and function of gene products. Nevertheless, the lack of direct correspondence between mRNA levels and those of proteins has often been reported (Anderson and Seilhamer 1997). Moreover protein functionality can depend on post-translational modifications (e.g. N or C terminal truncation, phosphorylation, glycosylation). Protein turnover and cell compartment localization and cannot be determined from DNA sequence and transcripome analysis alone. Proteomics complements and extends genomics and is a new field with a major impact in the post-genome era. It is based on technologies for the differential display and mass screening of proteins and their post-translational modifications in whole organisms, as well as in their tissues or organelles. The novelty is that it is now possible to conduct parallel studies on many proteins and obtain a real time connection between genomic and proteomic data.

The achievement of the sequencing of the *Arabidopsis thaliana* genome in the year 2000 opened the possibility to use proteomic approach to study plants. It is now possible to determine profiles of protein expression in different tissues of Arabidopsis and under different conditions and to identify post-translational modifications of proteins in response to different stimuli.

Proteomic analysis involves the identification of proteins in complex mixtures. Two steps are usually required: 1) separation and visualization of a big number of proteins at the same time, 2) identification of proteins of interest by mass spectrometry. The sample preparation and/or prefractionation is important and is usually followed by two dimensional gel electrophoresis, which first separate proteins according to their isoelectric points (pI) and then according to their molecular mass. The first dimension is obtained running proteins on a strip of polyacrylamide gel with a preformed pH gradient, which is then placed on the top of the gel for the second dimension in SDS-PAGE.

Separation and visualization of more than 1000 proteins on the same gel sometimes allows the identification of only the most abundant ones. To study low abundant proteins it is necessary to simplify the complexity of the mixture by considering, for example, specific cellular compartments (cell wall, vacuole, nucleus, Golgi, ER, etc). Electrophoresis is

usually paired with mass spectrometry (MS) that embraces a range of technologies differentiated by the type of instrumentation constructed for obtaining the mass-to-charge ratio (m/z) of the analyzed biomolecule. One of the most used MS approach for the identification of proteins is the peptide mass fingerprint (PMS) using an high mass accuracy Matrix-assisted laser desorption/ionization (MALDI).
Usually proteins of interest are cut from the gel, digested with trypsin and then peptides obtained are analysed by MS. The mass to charge ratio values obtained from the analysis of tryptic peptide mixtrures are searched for matching against theoretically predicted digests of proteins in databases.

1.6 AIM OF THE THESIS

Oligogalacturonides (OGs) are well known elicitors of plant defence responses (Ridley et al. 2001). OGs act via a signal transduction pathway that functions independently of the known pathways involving salicylic acid (SA), jasmonate (JA), and ethylene (Et) as secondary messenger molecules (Ferrari et al. 2003b). The signalling pathway regulated by OGs is still to be characterized. Global transcript profiling has been carried out in the laboratory using the model plant *Arabidopsis thaliana* (Ferrari, Dewdney et al, unpublished). One of the objectives of my work was to contribute to the dissection of the OG-regulated signal transduction pathway and responses in Arabidopsis and complement the microarray data with information at the proteome level.

OGs are formed in the cell wall during pathogenesis by the action of polygalacturonase (PG) secreated by the pathogen to breech the cell wall. PG rapidly hydrolizes cell wall pectins producing short and inactive pectic fragments (OGs). The interaction of PG with PGIP slows down its pectin degrading activity producing a transient accumulation of oligogalacturonides with a degree of polymerization between 10 and 15 that are active elicitors. PGIP has been shown to interact with pectins. A second objective of my work was to clarify the structural basis of the interaction of the PGIP with cell wall pectin, the source of OGs. In the 3-D structure of PGIP a motif of four clustered arginines and lysine (R183, R206, K230, R252) was identified as a putative pectin binding site; by using site directed mutagenesis and matrix polysaccharide/polyacrylamide affinity chromatography**,** PGIP-pectin interaction was studied.

2. MATERIALS AND METHODS

2.1 Arabidopsis seeds sterilization:

Arabidopsis thaliana (ecotype Columbia) seeds were weighted directly in 2 ml Eppendorf, washed for 1 min with isopropanol and then washed three times with MilliQ water. 3 volumes of MilliQ water and 1 volume of sodium hypochlorite (Carlo Erba) were added, immediately mixed by vortex and left under shaking for 18 min in order to sterilize seeds. Sodium hypochlorite solution was removed and seeds were washed 8-10 times with sterile MilliQ water under laminar flow. Water was removed and seeds were left for 3 days at +4 °C in the dark for the stratification.

2.2 Arabidopsis seedlings growth conditions and OG treatment:

Arabidopsis seedlings were grown for 10 days in 12 well plates under controlled temperature and light conditions. The temperature in the growth room was 21°C, the photoperiod was 16h light and 8h dark.

18-20 seedlings per well were grown in 1 ml of sterile medium (Murashige and Skoog (MS) -medium pH 5.7 and 0.5% sucrose) and after 8 days the medium was completely removed and replaced with a fresh one in order to avoid stressed seedlings.

2 days after medium exchange seedlings were treated (under laminar flow) with 100 μg/ml oligogalacturonides (OGs) adding 20 μl of 5 mg/ml OGs stock sterile solution to the medium; sterile water was added to the control.

2.3 Extraction of apoplastic proteins of *Arabidopsis thaliana*:

In order to obtain intercellular washing (ICW) fluids a vacuum infiltration of 10 days-old seedlings was performed using 25 mM Tris/HCl pH 7.4 + 50 mM EDTA + 150 mM $MgCl_2$ extraction buffer.

Seedlings were weighted and carefully placed in 10 ml syringe filled with extraction buffer (2.5g per syringe). The syringe was then placed in a 250 ml vacuum flask and the vacuum was applied for 3 min and then was released quickly by disconnecting the flask from the vacuum pump.

The syringe was placed in a 50 ml Falcon and centrifuged at 1500 x g for 3 min to recover the ICW fluids. Proteins were then precipitated o/n at –20°C with 80% acetone and 10% trichloroacetic acid (TCA).

Proteins were recovered by centrifugation and the pellet was washed three times with cold 80% acetone and then air dried.

Proteins were then solubilized with a small volume (50-100µl) of isoelectric focusing buffer containing ASB-14 as detergent (ASB-14 Lysis buffer = 10mM Tris pH 8.0, 5mM magnesium acetate, 7M urea, 2M thiourea, 2% ASB-14) for 1 h under shaking. A second extraction could help to solubilize more proteins.

In order to eliminate salts and concentrate proteins the Clean-up kit (Amersham biosciences) was used following the manufacturer's instructions. Finally proteins were solubilized in 40µl of isoelectric focusing buffer containing ASB-14 as detergent.

2.4 Protein quantification:

Proteins were then quantified using 2D Quant kit (Amersham Biosciences) following the manufacture's intructions. This procedure is compatible with the isoelectric focusing buffer containing urea and thiourea .

2.5 Protein labelling for DIGE:

2.5.1 Overview of the technique:

DIGE (Amersham Biosciences) is a 2D gel based technique that allows a sensitive and quantitative comparison of protein changing abundance using fluorescent dyes (Cy 2, Cy 3, Cy5). Three different dyes can be used to label three samples that will be run in a single gel.

To compare two samples, one sample is labelled with Cy3 whilst the second sample is labelled with Cy5. After labelling, the samples are combined and the signals compared. Reciprocal labelling is completed to rule out abnormalities in labelling e.g. Cy3 control versus Cy5 OG treated and Cy5 OG treated versus Cy3 control CyDyes label the lysine residues on proteins, each dye adding 0.5KDa in MW to the bound protein.

Prior to labelling need to determine the concentration of each sample to allow equal labelling of protein essential for comparison of signals.

The optimal conditions for DIGE are a minimal labelling with 1 dye molecule binding 1 in every five proteins. To this purpose 50µg of total protein are mixed with 1µl of 0.2mM dye (200pmoles) in a volume of 10µl at pH 8.

For DIGE labelling of 2 samples, each sample was labelled under identical conditions in terms of amount of protein labelled, amount of dye added and labelling reaction volume.

2.5.2 Methodology

Samples were spinned at 13,000rpm / 3mins and use only supernatant for labelling.
In separate 1.5ml Eppendorf tubes for sample #1 and sample #2, the volumes required for 50µg of protein were transferred and then normalised using ASB-14 Lysis buffer.
Dyes were vortexed and briefly spinned. 1µl of 0.2mM working Cy3 stock was added to sample #1 and 1µl of 0.2mM working Cy5 stock was added to sample #2.
Tubes were vortexed and the contents of each tube were briefly spinned down.
The labelling reaction was incubated for 30mins / 4^{o}C / dark.
To block unreacted linkers 1µl of 1mM lysine was added to each tube (10nmoles = 50x excess).
Tubes were vortexed and the contents of each tube were briefly spinned down.
The quenching reaction was incubated for 10mins / 4^{o}C / dark.
The contents of each tube were pooled into a single tube.

2.6 Two-dimensional gel (2D gel)

2.6.1 Overview of the technique

2D gels is a high resolution technique used to separate, visualize and compare proteins in complex mixture. To obtain a 2D gel an isoelctric focusing is used as first dimension in order to separate proteins according to their isoelectric point (pI) and an SDS-PAGE is used as second dimension in order to separate proteins according to their molecular weight. The first dimension is the critical step and is carried out on supported polyacrylamide gel stips with immobilized pH gradient. Different length and pH gradient strips are available. Because they are in dried form they need to be rehydrate before first dimension.
The second dimension is an SDS-PAGE in which the strip containing proteins after first dimension is layered on the top of the gel.

2.6.2 First dimension

The first dimension can be used to separate both labelled and unlabelled proteins. In both cases an equal volume of the appropriate 2x sample buffer (2% ASB14, 7 M urea, 2 M thiourea, 20 mg/ml DTT and 2% IPG buffer/Pharmalytes fresh added) was added to each sample, vortexed and spinned briefly.

The mix was incubated for 15min. A volume of the appropriate rehydration buffer that is sufficient to rehydrate the length of IPG strip being used was added.

Strip length	Rehydration volume
7cm	125µl
13cm	250µl
18cm	340µl

Sample was spinned at 13,000 rpm / 2 mins and only the supernatant was loaded onto the strip. This removes any protein that has precipitated.

The IPG coffins to be used were soaked with ASB-14 buffer for 30 min. After soaking IPG coffins were rinsed with water and then dried thoroughly.

The IPG strips to be used were thawed out 5 mins before use.

Each sample was loaded into different IPG coffins. Backing stripe was removed from IPG strip and IPG strip was layed into each coffin, gel side down.

The IPG strip was overlayed with Drystrip cover fluid (Amersham Biosciences) and the lid was placed on top of the coffin ensuring that no air bubbles are formed within the chamber upon placement of the lid onto the coffin.

The excess oil was wiped off. The coffins were placed onto the IPGphor with the pointed end electrode sitting on the anodic plate (+) and the blunt end electrode sitting on the cathodic plate (-).

IPG strips of all lengths have to be rehydrated prior to use for at least 10 hours at room temperature. As sample can be included in the rehydration volume, sample loading is therefore achieved automatically during IPG strip rehydration.

Rehydration sample loading is enhanced by rehydrating for 10hours / 20^{o}C / 20V. *(10 hours at 20V = 200Vh).*

The rehydration time is independent of strip length, there are specific focusing times for each IPG strip length.

7cm IPG pH 3-10 or 4-7 strip	Run parameters
1	200Vh (10.00h @ 20V)
2	250Vh (0:30h @ 500V)
3	500Vh (0:30h @ 1000V)
4	8000Vh (1:00h @ 8000V)

13cm IPG 3-10 or 4-7 strips	Run parameters
1	200Vh (10.00h @ 20V)
2	500Vh (1:00h @ 500V)
3	1000Vh (1:00h @ 1000V)
4	40000Vh (5.00h @ 8000V)

2.6.3 Preparation of IEF strips for the second dimension

The IPG strips were removed from the IPGphor coffin and the excess oil was washed away from the plastic back-side of the strip.

The IPG strip was transferred to a sterile petri dish with the plastic back-side of the strip facing the inside edge of the petri dish.

In order to reduce disulfide bonds before the second dimension, a minimum of 10ml of equilibration buffer (100 mM Tris pH 6.8, 30% glycerol, 8 M urea, 1% SDS, 0.2 mg/100 ml bromophenol blue, 5 mg/ml DTT) was added to each dish and incubated for no more than 15 mins / room temperature on a rotator.

To alkylate proteins after reduction the last step was repeated using 25 mg/ml iodoacetamide (IAA) instead of DTT in the same equilibration buffer.

The excess of equilibration fluid was removed from the strip using SDS running buffer or MilliQ water and the strip was loaded onto the surface of the second dimension.

2.5.7 Adding IPG strip to the second dimension

1% agarose in running buffer containing a few milligrams of bromophenol blue was prepared and kept molten at 55^{o}C prior to use.

The top of the gel was washed adding SDS running buffer for three times to remove loose acrylamide.

The IPG strip from the petri dish into equilibriation buffer was then loaded onto the gel with the plastic side to the back and the pointed end of the strip (anode) to the left.

The running buffer was drained off from the top of the gel and a layer of molten agarose that covers the surface of the entire length of the strip was pipetted.

The gel were run using the following parameters: 20 mA./ gel for 15 mins and 40 mA / gel until the blue dye front runs off the bottom of the gel.

2.7 Gel staining

2.7.1 Coomassie brilliant blue staining

All the steps were performed on a shaking table, in a closed box to avoid keratin contamination.

A solution was prepared containing 2% (v/v) ortho-phosphoric acid and 10% (w/v) ammonium sulphate in MilliQ water; add an appropriate volume of a 5% Coomassie brilliant Blue G-250 (CBB) to obtain a final concentration of 1.6%, and immediately before the use methanol was added slowly and under stirring until 20% is reached.

100 ml of this final solution were used to stain small gels (7 cm strips), while use 500 ml to stain big gels (13 cm or 18 cm strips).Gels are placed in this solution for 6h to over night (o/n). The gels were destained with MilliQ water (several changes) until the background is clear.

2.7.2 Silver staining for mass spectrometry

All the steps were performed on a shaking table, in a closed box to avoid keratin contamination.

The gel was fixed with a solution containing methanol : water : acetic acid = 45 : 5: 50 for 30 min, rinsed with water for 60 min to remove the excess of acid, sensitized using freshly made solution of 0.02% sodium thiosulfate for 5 min, rinsed again twice with MilliQ water for 1 min . The gel was incubated with cold 0.1% $AgNO_3$ for 40 min, rapidly rinsed twice with MilliQ water and developed using 0.04% formalin and 2% Na_2CO_3. The reaction was quenched when sufficient staining is obtained with 1% acetic acid solution.

2.7.3 Sypro ruby staining

All the steps were performed on a shaking table, in a closed box to avoid keratin contamination.

The gel were fixed with 10% methanol, 7% acetic acid for 30 min; then the fix solution was removed and the gel was stained o/n with an appropriate volume of Sypro ruby gel staining solution, a volume 10 times the volume of the gel was used. The gel was washed three time for 30 min with the fix solution to remove excess of staining solution and to avoid spotted gels due to Sypro ruby precipitates.

The gel was acquired using Typhoon 9200 using an appropriate filter.

2.8 OG-induced protein changing analysis

Gels obtained from control seedlings were compared with those obtained from seedlings treated with OG in order to obtain information about proteins induced or repressed by the treatment. These information were achieved using a commercial software for image analysis.

To analyse visible stained gels and Sypro ruby stained gels was used Image Master 2D platinum from Amersham Biosciences, while to analyse DIGE gels was used DeCyDer software from Amersham Biosciences. This software can be used to perform two types of analysis: 1) DIA analysis that allows direct comparison of two samples within the same gel and does not provide results with a statistical significance; 2) BVA analysis that allows multiple gels comparison using an internal standard and provides statistical results using 1 way ANOVA or T-test.

In this thesis only results with T-test score or ANOVA score <0.001 were considered as significant results.

2.9 Spot excision and protein digestion

In order to identify proteins induced or repressed by OGs, spots that significantly change after the treatment were excised from gel with a plastic tip and placed in a Millipore ZipPlate plate for trypsin digestion.

Protein digestion was carried out using In-Gel Digest$_{zp}$ Kit (Millipore), following manufacturer's instructions. Briefly each gel piece, placed in a well of the multiwell, was washed once with 25 mM ammonium bicarbonate, 5% acetonitrile, twice with 25 mM ammonium bicarbonate, 50% acetonitrile and once with 100% acetonitrile. Proteins were

digested using trypsin according to the manufacturer recommendation for 3 h at 37°C. Digestion was stopped adding 100% acetonitrile and extraction / wash buffer (0.2% trifluoroacetic acid (TFA)) for 30 min. Elution of peptides from the ZipPlate plate was performed directly on a MALDI target using 1.3µl of 0.2% TFA, 50% acetonitrile and 3mg/ml α-cyano-4-hydroxycynnamic acid.

2.10 MALDI-ToF and MALDI-ToF-toF analysis

Peptides eluted from ZipPlate plate were mixed with the matrix (α-cyano-4-hydroxycynnamic acid), air dried and analysed with MALDI-ToF (Voyager DE-STR Applied Biosystems, MA) or MALDI-ToF-ToF (4700 Applied Biosystems, MA).
The spectrometers were operated in positive ion mode with reflector; the following parameters were used for the analysis: delay time of 180 ns, acceleration voltage of 20kV, % grid voltage 68. Each final spectrum was obtained from the average of 1000 single shots spectra. All MALDI spectra were internally calibrated using trypsin autoproteolysis products.

2.11 Plant material

Lycopersicon esculentum cv. Moneymaker and *35S::Pvpgip1* transformed tomato plants were grown in growth chamber at 25°C under a 16/8 hr photoperiod for one month and subsequently transferred in greenhouse.

2.12 Intercellular washing fluid (IWF) isolation from tomato

IWFs were collected from tomato stems by centrifugation. 2-cm long stem sections were excised from stems isolated from three-months old 35S::Pvpgip1 plants. Segments were stacked upright on a 105 µm nylon mesh in the bottom of a 20-ml plastic syringe. The packed sections were washed with 20 mM Na acetate buffer pH 5.0 for 5 min. Afterwards they were vacuum-infiltrated for 5 min with 20 mM Na acetate buffer pH 5.0 in the presence or in the absence of 0.3 M NaCl. IWF was recovered by centrifuging the vacuum-infiltrated stems at 500 g for 5 min at 4°C. The amount of IWF obtained from 1 g of tissue (fresh weight) was 0.2-0.3 ml. The degree of cytosolic contamination of IWF was determined by measuring the G6PD activity. Total extracts were obtained by homogenizing fresh stem tissue with cold 20 mM Na acetate buffer pH 5.0 containing 1M NaCl (1ml per g of tissue) in a Waring blender at 4°C. The homogenate was than shaked

for 1 hour, centrifuged at 10,000 x g for 20 min at 4°C and the supernatant was collected. Protein concentration was determined by using BioRad (Biorad, Milan, Italy) reagents and bovine serum albumin as standard. SDS-PAGE was performed.

For immunoblotting experiments, the proteins were electrophoretically transferred to nitrocellulose after SDS-PAGE and a polyclonal rabbit antibody raised against the N-terminal peptide (17 amino acids) of mature PGIP1 was used. The antigen-antibody complex was detected with ECL kit (Amersham, Rome, Italy) following manufacturing user's guide.

2.13 Molecular biology materials and strains:

The following materials and reagents were obtained commercially as indicated: *P. pastoris* wild-type strains X-33, *E.Coli* TOP10F', *P.pastoris* expression vector pGAPZαA and Zeocin (Invitrogen); Quick-Change Site-Directed Mutagenesis Kit (Stratagene); VivaFlow 200 (Sartorius), EcoRI (Promega), XbaI (Promega), AvrII (Roche), QiuaPrep spin plasmid miniprep kit (Quiagen); agarose (Invitrogen), polygalacturonic acid from citrus (Sigma);

2.14 Construction of expression vector encoding PvPGIP-2 gene:

The wild-type PvPGIP2 gene was cloned in pGAPZαA between EcoRI and XbaI sites to generate a construct with the PGIP2 native signal sequence replaced by the yeast α-factor signal sequence. The construct was amplified by transforming *E.Coli* TOP10F' competent cells. Transformants were selected on LSLB plates containing 25 μg/ml Zeocin and confirmed by direct PCR amplification to have the gene of interest using two primers, one on the gene and one on the pGAPZαA. One PCR positive colony was picked with a sterile tip and used to inoculate 3 ml of LSLB (1% tryptone, 0.5% yeast extract, 0.5% NaCl pH 7.4) liquid medium containing 25 μg/ml Zeocin and the culture was grown o/n at 37 °C at 250 rpm. The plasmid was extracted from the cells using a plasmid mini prep kit. It was quantified by agarose gel, linearized with *AvrII* and concentrated by isopropanol precipitation to obtain 3 μg of the plasmid in a total volume of 5 μl. Before *P. pastoris* transformation the construct was analysed by digestion with *EcoRI* and *XbaI* restriction enzyme followed by agarose gel to see the presence of the gene in the plasmid.

2.15 Pv-PGIP2 site-directed mutagenesis:

Site-directed mutagensis was performed to modify the arginines and lysine predicted to be critical for the binding of the PGIP to pectins. Four mutants were designed: a double mutant in which Arg (183) and Lys (230) were replaced by Gln (QRQR), two triple mutants, one with Arg (183), Lys (230) and Arg (252) replaced by Gln (QRQQ) and one with Arg (183), Lys (230) and Arg (206) replaced by Gln (QQQR) and one quadruple mutant with Arg (183), Lys (230) Arg (252) and Arg (206) replaced by Gln (QQQQ).
Mutagenesis of PvPGIP-2 gene were made through PCR-based site-directed mutagenesis using Quick-Change Site-Directed Mutagenesis Kit. PCR was carried out directly on PGIP-2/pGAPZαA construct. Internal overlap primers were designed that hybridize at the site of the desired mutation and contain the relevant mismatched bases. The primers used were:
5'-GCGTTCGTTGACTTGTCTCAAAACATGCTGGAGGGTGAT-3'
and 5'-GTCACCCTCCAGCATGTTTTGAGACAAGTCAACGAACGC-3' for R206Q,
5'-TTGAACGGGTTGGATCTGCAGAACAACCGTATCTATGGG-3' and
5'-CCCATAGATACGGTTGTTCTGCAGATCCAACCCGTTCAA-3' for R252Q,
5'-ACGTCGATGACCATCTCCGAGAACCGCCTCACCGGGAAG-3' and
5'-CTTCCCGGTGAGGCGGTTCTGGGAGATGGTCATCGACGT-3' for R183Q,
5'-CAGAAGATACATCTGGCGCAGAACTCTCTTGCCTTTGAT-3' and
5'-ATCAAAGGCAAGAGAGTTCTGCGCCAGATGTATCTTCTG-3' for K230Q.
The mutations were confirmed by sequencing of the gene (GeneLab ENEA-CASACCIA Roma).

2.16 Transformation of *P. pastoris* and selections of transformants:

The construct was used to transform wild-type *P. pastoris* X-33 competent cells. The transformants were selected on YPDS (1% yeast extract, 2% peptone, 2% dextrose and 1M sorbitol) plates containing 100 μg/ml Zeocin. Ten or more transformant colonies were picked with a sterile tip and used to inoculate fresh YPDS plates containing 100 μg/ml Zeocin and 3 ml of liquid "BMMY modified" medium (0.4% yeast extract, 0.6% tryptone 50 mM phosphate buffer pH 6, 1.34% YNB, $4x10^{-5}$ % biotin, 2% glucose) containing 100 μg/ml Zeocin. After 5 days of incubation at 28°C at 300 rpm the cultures were centrifuged and the supernatants were used to perform an agar diffusion-assay (Cup-plate) to choose the higher level expression clones. The colony that expressed the highest level of protein was used for the production of PGIP or mutants proteins. The selected colony on YPDS

plate was first picked with a sterile tip to 1 ml of liquid "BMMY modified" containing 100 μg/ml Zeocin and after 3 days of incubation at 28°C at 300 rpm the culture was used to inoculate 100 ml of fresh BMGY medium containing 100 μg/ml Zeocin. When the new culture reached an OD_{600}= 20-30 it was used to inoculate 2l of fresh BMGY (1% yeast extract, 2% peptone, 1% glycerol, 100 mM potassium phosphate (pH 6)) medium and was incubated at 28°C at 300 rpm for 5 days. At the end of this time the culture was centrifuged and the supernatant was concentrated by VivaFlow 200 and dialyzed against 20 mM Na acetate pH 4.7.

2.17 Agar diffusion-assay

Agar diffusion-assay (cup-plate) was performed according to Taylor and Secor (Taylor and Secor 1988). 15 μl (or 40 μl) of culture medium containing PGIP and PG from *Colletotrichum acutatum* was added to 0.5 cm wells on plates containing 100 mM sodium acetate, pH 4.7, 0.5% polygalacturonic acid, and 0.8% agarose. Plates were incubated for 12 h at 30°C, and the halo caused by enzyme activity was visualized after 5 min of treatment with 6 N HCl. The smaller halo corresponds to the higher amount of PGIP.

2.18 Purification of PGIP-2 wild-type and mutants:

After dialysis wild-type or mutated PGIP-2 expressed in *P.pastoris* were mixed with a suspension of diethylaminoethyl (DEAE) cellulose (DE52, Whatman, Kent, U.K.) pre-equilibrated with 20 mM Na acetate pH 4.7. The non-absorbed proteins were loaded on a cation exchange SP-Sepharose (Amersham Biosciences) column pre-equilibrated in 20 mM Na acetate pH 4.7. Adsorbed proteins were eluted with a linear gradient of 0-0.2 M NaCl in 20 mM Na acetate pH 4.7 in 10 min followed by 5 min of 0.2 M NaCl in 20 mM Na acetate pH 4.7. Flow-rate was 1 ml/min and fractions of 1 ml were collected. One peak of activity was detected. Fractions containing PGIP-2 wild-type or mutants, detected by SDS-PAGE analysis, were pooled, dialyzed against 10 mM Na acetate pH 4.7 and purified by isoelectrofocusing in a IEF Rotofor system (Bio-Rad) in a pH 3-10 gradient. Fractions containing PGIP were collected and dialyzed against 20 mM Na acetate pH 4.7 and finally concentrated to 1 mg/ml by using VIVASPIN-2 concentrator with a cut-off of 5 kDa.

2.19 Preparation and purification of PGIP and PG

PvPGIP1 was purified from transgenic tomato plants and PvPGIP2 was purified from PVX–infected tissues of *Nicotiana benthamiana* plants. PvPGIPs, PG from *Fusarium moniliforme* and PGII from *Aspergillus niger* I used for this study were already available in the laboratory.

2.20 Isolation of cell wall fractions

Different cell wall fractions used in this study were already available in the laboratory and they were isolated using the following protocol.

Cell walls were isolated from tomato stems of three-months-old plants grown in greenhouse.

Alcohol Insoluble Solids (AIS) were prepared by adding ethanol to tomato stems until the final concentration of 70%. The mixture was heated at 70°C for 30 min, blended and centrifuged at 7000xg for 15 min. The pellet was suspended in 70% ethanol, stirred overnight and centrifuged at 7000xg for 20 min. Washing was repeated twice with 70% ethanol and twice with 95% ethanol. AIS was subsequently washed with 80% acetone and finally suspended in acetone and air dried in a fume cupboard.

Buffer soluble solids (BSS) were obtained by extracting 2g of AIS with 50 mM Na acetate buffer pH 5.2 at 70°C for one hour with occasional stirring. Supernatant was separated by centrifugation (7000 x g, 20 min) and the pellet was suspended in 50 mM Na acetate buffer pH 5.2. Extraction of BSS was repeated twice. Supernatants were concentrated by rotary evaporation, dialysed against distilled water and dried by lyophilization.

To remove proteins the pellets obtained after BSS separation were suspended in sodium dodecyl sulphate (1.5 %) in 50 mM sodium metabisulphite. The mixture was stirred at room temperature for three hours, centrifuged (7000 x g, 20 min) and the supernatant decanted. The pellet was re-extracted twice with SDS-metabisulphite.

Chelating agent soluble solids (ChASS) was extracted from the de-proteinated pellets by using 50 mM Na acetate buffer containing 50 mM of CDTA and ammonium oxalate**.** Extraction was carried out by stirring at room temperature for 6 hours and was repeated three times. All supernatants were combined and dialysed extensively against 100 mM ammonium acetate pH 5.2 and concentrated by rotary evaporation. The pellet was washed overnight with distilled water to remove CDTA and centrifuged (7000 x g, 20 min). The "Residue" pellet was extensively washed with distilled water and lyophilised.

2.21 Matrix polysaccharide/polyacrylamide affinity chromatography

Affinity chromatography gels were prepared according to Penel and Greppin (Penel and Greppin 1996) with some modifications. 800 μg of cell wall fractions, PGA or pectins with different DM were mixed with 1 ml of 12% polyacrylamide solution in the presence or absence of 10 mM $CaCl_2$, 10 μl APS and 12.5 μl Temed were added and the polymerization was allowed overnight. Lime pectins with different patterns (blockwise and non-blockwise) and defined DM were prepared by enzymatic treatment of highly methyl-esterified lime pectin (E81, GRINDSTED^{TM} Pectin URS 1200) as described previously (Willats et al. 2001). Briefly, one series was produced by blockwise de-esterification of E81 with plant PME isolated from orange peel (P-series), while another series was produced by non-blockwise de-esterification of E81 with a fungal PME from *Aspergillus niger* (F-series). After polymerization the gels were fragmented in small particles and packed into chromatography columns (50 x 5 mm) that were equilibrated with 20 mM Na acetate buffer pH 5.0 or with 20 mM Hepes pH 7.0 in the presence or absence of $CaCl_2$. Columns prepared without $CaCl_2$ were washed with buffer containing 1 mM EGTA. Each column was equilibrated with 20 mM Na acetate buffer pH 5.0 or 20 mM Hepes pH 7.0 in the presence or in the absence $CaCl_2$, loaded with 10 μg PGIP2 (alone or preincubated for 1 h with 1 mg/ml of oligogalacturonides (10<DP<15)) and extensively washed with the buffer used for column equilibration. We have excluded that pectins entrapped in the polyacrylamide columns were eluted by high salt concentration by performing, several times, uronic acid assay of the eluate. The bound protein was eluted with PBS containing 1 M NaCl and 1mM EGTA. To determine the amount of PGIP eluted or retained by the columns all the collected fractions were analyzed by SDS-PAGE and assayed for PGIP inhibitory activity and densitometry.

2.22 Effect of PGII from *A.niger* and PG from *F. moniliforme* on the binding of PvPGIP2 and PvPGIP1 to pectins

PvPGIP2 (15 μg) and PvPGIP1 (15 μg) were separately loaded onto two Ca^{2+}-PGA/polyacrylamide columns equilibrated in 20 mM Na acetate buffer containing 100 μM $CaCl_2$. The columns were washed with 20 mM Na acetate buffer containing 100 μM $CaCl_2$. Subsequently, AnPG (15 μg) or FmPG (15 μg) dissolved in the same buffer was loaded onto each column. Flow trough, the fraction collected from the column after

loading PG, was recovered; the columns were washed again with the buffer and the bound proteins were eluted with PBS containing 0.3 M NaCl. To determine the amount of PGIP eluted or retained by the columns all the collected fractions were analyzed by SDS-PAGE and western blot and a densitometric analysis was done.

2.23 Glucose 6-P dehydrogenase activity (G6PD) activity

The presence of intracellular contamination in intercellular washing fluids (IWFs) was assessed by measuring glucose 6-P dehydrogenase activity (G6PD).

G6PD assay is based on the reduction of $NADP^+$ into NADPH by G6PD during oxidation of glucose 6-P into glucono lacton. When G6PD is present follows an increase of absorbance at 340 nm due to the production of NADPH.

Solutions containing 0.2mM $NADP^+$ (20 µl), 1mM glucose 6-P (20 µl), 1m M $MgCl_2$ (20µl) and samples to be analysed (up to 60 µl) were added to Gly-NaOH 100 mM pH 8 buffer to reach a final volume of 1 ml. The reaction was followed for 5 minutes by reading the variation of absorbance at 340 nm. An extinction coefficient (ε_m) of $6.22*10^3$ $M^{-1}cm^{-1}$ was used to convert absorbance values at 340 nm to nmoles of NADPH produced.

2.24 H_2O_2 assay

The presence of H_2O_2 in Arabidopsis growth medium can be estimated using a xylenol orange assay; this is a spectrophotometric assay based on the detection of ferric ion (Fe^{3+}) produced from the redox reaction between Fe^{2+} ion and H_2O_2. Concentration of Fe^{2+} is proportional to the concentration of H_2O_2 initially present in the sample. In the presence of xylenol orange H_2O_2 concentration follows Lambert-Beer's law and it can be estimated measuring absorbance at 560 nm.

A solution was prepared mixing 10 ml of 250 mM H_2SO_4 + 1mM xylenol orange, 10 ml of 1 M sorbitol, 10 ml of $(NH_4)_2Fe(SO_4)_2$ and 20 ml of MilliQ water; 500 µl of this solution were placed in a Eppendorf tubes, 500 µl of H_2O_2 solution were added (different dilution for each tube) for the standard curve or 500 µl of the sample and mixed immediately, the mix was incubated for 45 min and then the absorbance at 560 nm was read.

2.25. Lipid rafts (detergent resistant membranes DRMs) protein extraction

To extract DRMs I decide to use mixed organelle membranes prepared as follows: Arabidopsis callus tissue was resuspended in two volumes of cold STE homogenisation

buffer (12% w/v sucrose, 1mM EDTA, 100mM Tris-HCl pH 8.0). Three pusles of 15 s at 7 K rpm with a polytron (Kinematica, Switzerland) were used to homogenise the tissue at 4°C. The homogenate was centrifuged 2 times for ten minutes at 1600 x g to remove cell debris. The membrane suspension was then pelleted onto a 1.8 M sucrose cushion at 140 x g for 35 min. The membrane fraction was harvester and diluted five-fold in cold TNE (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA pH 7.5). After centrifugation at 100000 x g for 90 min, membrane pellets were resuspended in cold TNE and homogenized in a 1 ml Dounce glass homogeniser. Membrane preparations were frozen in liquid nitrogen and stored at -80°C.

Detergent resistant membranes (DRMs) were prepared by low-temperature detergent extraction adapting the protocol described in Fielder et al. (1993). Membranes were resuspended in cold TNE containing 4:1 (detergent to protein) excess of triton X-100. Triton concentration were routinely 2.0%. Extractions were carried out on ice shaking at 100 rpm for 35 min. Extracts were adjusted to 1.8 M sucrose/TNE by addition of three volumes of cold 2.4 M sucrose/TNE. Extracts were overlaid with sucrose step gradients 1.6/1.4/0.14 M and centrifuged at 240000 x g for 18 h at 4°C. DRMs were visible as white bands with a flocculent appearance at the 0.15/1.4 M and above 1.4/1.6 M interface. Membranes fractions (0.5 ml above and 0.5 ml below the centre of the band) were collected to harvest the DRMs. Membranes were diluted in five volumes of cold TNE and pelleted at 145000 x g over night. Pellets were resuspended in 50 µl of cold TNE, pooled together and centrifuged 25 min at 45000 rpm at 4°C and then stored at -80°C.

Proteins were extracted adding 20-50 µl of ASB-14 lysis buffer and shaking for 1 h at room temperature.

3. RESULTS

3.1 Proteomic analysis of responses induced by oligogalacturonides

OGs with degree of polymerization 10-15 are elicitors of plant defence responses. Microarray experiments show a dramatic change in gene expression profiles occurring in Arabidopsis seedlings treated with these elicitors. After 1 h and 6 h of treatment primary metabolism is "switched off" while secondary metabolism is "switched on" and many genes involved in protein trafficking and secretion are induced (Ferrari et al. unpublished data).

To obtain information about changes induced by OGs at the protein level, a proteomic analysis was undertaken. Proteomics uses 2D gel electrophoresis for the separation and visualization of a complex mixture of proteins and MALDI-ToF mass spectrometry for protein identification. More than 1000 proteins can be separated and visualized in the same gel. However, there is main problem when total proteins extracts are analysed: the most abundant proteins (mainly photosynthetic proteins) hide the less abundant ones. In order to overcome this problem, it is necessary to decrease the complexity of the mixture to be analysed. This can be achieved, for example, by limiting the study to specific cellular compartment (cell wall, vacuole, nucleus, Golgi, ER, etc).

Because the cell wall is the first structure that comes into contact with pathogens and OG are formed and probably perceived in this compartment, I chose to analyze the change of apoplastic proteins in response to OGs.

3.1.1 Optimization of the extraction of apoplastic protein from Arabidopsis seedlings

Extraction of apoplastic proteins from plants is a difficult task because many of them are embedded in the cell wall matrix and interact with different strength with the different components of this structure. On the other hand, extraction of proteins from the cell wall with high ionic strenght buffers may result in damage of the plasma membrane, and therefore contamination by intracellular proteins. Most of the studies on apoplastic proteins have been carried out using cell cultures because it is much easier to recover apoplastic proteins from the liquid medium than from intercellular spaces (Chivasa et al. 2002a; Kwon et al. 2005a). In the only proteomic study carried out using Arabidopsis, proteins

were recovered from the liquid medium where seedlings had been grown under shaken conditions (Charmont et al. 2005). A similar approach can not be used to study OG-induced responses because shaking itself induces stress responses and masks the action of OGs. On the other hand, attempts to recover apoplastic proteins from Arabidopsis seedlings grown without shaking were unsatisfactory because the amount of proteins recovered from the medium was very low (not shown).

In order to obtain suitable amounts of apoplastic proteins, Arabidopsis seedlings were therefore vacuum infiltrated with different buffers and subsequently centrifuged to recover intercellular washing fluids (IWFs). Different extraction buffers were tested in order to minimize intracellular contamination of the IWFs, which was determined by assaying glucose 6-P dehydrogenase (G6PD), an abundant cytosolic enzyme and a good marker of contamination (Table 3. I).

	Buffer	**G6PD units** [a]
A	25 mM Tris/HCl pH 7.4 + 50 mM EDTA	0.0016
B	**25 mM Tris/HCl pH 7.4 + 50 mM EDTA + 150 mM $MgCl_2$**	**0.0014**
C	50 mM Tris/HCl pH 7.4 + 50 mM EDTA	0.94
D	50 mM Tris/HCl pH 7.4 + 50 mM EDTA + 150 mM $MgCl_2$	0.64
E	50 mM NaOAc pH 6.5	0
F	50 mM NaOAc pH 6.5 + 50 mM EDTA	0.48
G	50 mM NaOAc pH 6.5+ 150 mM $MgCl_2$	0.64
H	50 mM NaOAc pH 6.5 + 50 mM EDTA + 150 mM $MgCl_2$	1.1

Tab. 3.I G6PD activity in intercellular washing fluids (IWFs) obtained from Arabidopsis seedlings using different buffers. The buffer chosen for further analyses is indicated in bold.

[a] 1 unit is the amount of enzyme that produces 1µmol of NADPH during 1 minute in a volume of 1 ml. The assay was carried out using 100 µl of IWFs.

For proteomic analysis, proteins of the IWF, were first separated according to their isoelectric points (pI) and then according to their molecular weight (Mw) by 2D gel electrophoresis and stained. Spots corresponding to different proteins were cut from the gel, digested with trypsin, a sequence specific protease that cleaves C-terminal of arginines and lysines, and the mixture of tryptic peptides was analyzed by MALDI-ToF mass spectrometry. The mass to charge ratio (m/z) values obtained from the analysis were searched for matching against theoretically predicted digests of Arabidopsis proteins in the database for the identification. After identification, the presence of a predicted signal sequence for translocation into the ER was searched using the program SignalP (www.expasy.org/tools/#proteome). The presence of proteins lacking this predicted signal sequence was used as an additional indicator of contamination by intracellular proteins.

Proteomic analysis was performed for IWFs obtained with four different buffers. One showed no contamination (buffer E) while two had a very low contamination level (Buffers A and B). The one showing high contamination (buffer H) was used as a negative control.

The sample obtained with the buffer E (50 mM NaOAc pH 6.5; G6PDH activity = 0) had a very low protein content and showed no visible protein spots on the gel. IWFs obtained with buffer H (50 mM NaOAc pH 6.5 + 50 mM EDTA + 150 mM $MgCl_2$; G6DH activity = 1.1) showed a large number of proteins on the gel; however, the most abundant of them had a putative intracellular localization (At1g13440 - glyceraldehyde 3-phosphate dehydrogenase; At2g05710 - aconitate hydratase; At3g60750 - transketolase). IWFs obtained with buffer A (25 mM Tris/HCl pH 7.4 + 50 mM EDTA; G6DH activity = 0.0016) and buffer B (25 mM Tris/HCl pH 7.4 + 50 mM EDTA + 150 mM $MgCl_2$; G6PDH = 0.0014) displayed a good pattern of proteins and a comparable low contamination level; however buffer B showed more proteins probably because of its high ionic strength. The most abundant proteins obtained with both buffers were identified by MS and all proteins exhibited a signal peptide for secretion in their primary amino acid sequence, consistent with their apoplastic localization (table 3.II). Buffer B was therefore chosen for further experiments.

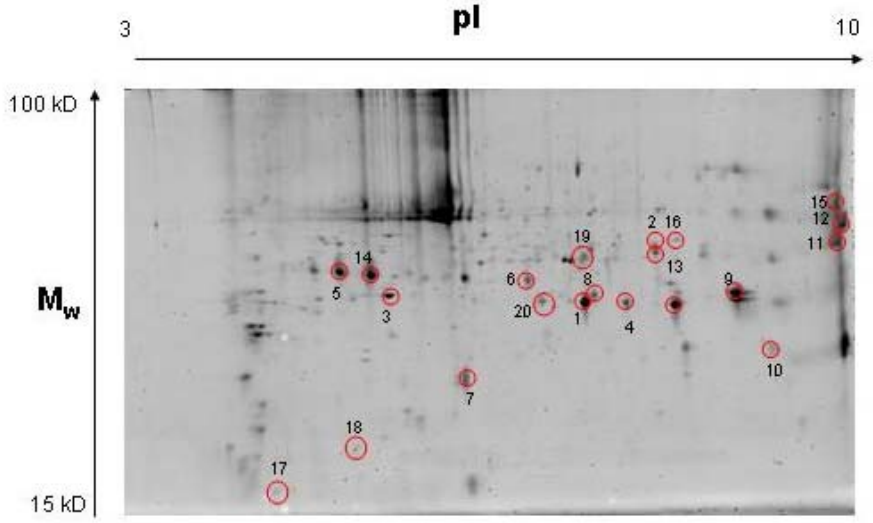

Fig. 3.1. 2D gel obtained from Arabidopsis seedlings infiltrated with buffer B (25 mM Tris/HCl pH 7.4 + 50 mM EDTA + 150 mM $MgCl_2$). Proteins chosen for identification are indicated by red circles.

Spot number	Protein name and accession number
1	At3g16530 Lectin-like protein
2	At1g54010 Myrosinase-associated protein
3	At3g16420 Jacalin lectin family Jasmonate inducible protein
4	At4g30270 (MERI-5B) Endo-xyloglucan transferase
5	At1g78830 Curculin-like protein
6	At1g78820 Curculin-like protein
7	At5g20630 Germin-like protein GER-3
8	At4g37800 Xyloglucan endo 1,4 glucanase
9	At3g15356 Legume lectin family protein
10	At5g38940 (At5g38930) Germin-like protein
11	At1g03220 Extracellular dermal glycoprotein
12	At4g19410 Putative pectinacetylesterase
13	At1g54000 Putative myrosinase-associated protein
14	At1g78830 Curculin-like protein
15	At3g54400 Aspartyl protease
16	At3g32980 Peroxidase 32 precursor
17	At1g08830 Superoxide dismutase
18	At4g39260 Glycin rich protein
19	At1g54000 Myrosinase-associated protein
20	At5g44120 Seed storage protein

Tab. 3.II. Proteins from 2D gel identified using MALDI –ToF MS are reported with name and accession number. The presence of a putative signal peptide is present in all proteins reported. Proteins were obtained using buffer B (25 mM Tris/HCl pH 7.4 + 50 mM EDTA + 150 mM $MgCl_2$). Numbers refer to the spots in Fig, 3.1.

3.1.2 Arabidopsis seedlings respond to OGs by accumulating H_2O_2 and PGIP

Among the defence responses activated in Arabidopsis by OGs, production of reactive oxygen species (ROS) such as H_2O_2 (unpublished data) and induction of AtPGIP1 (Ferrari et al. 2003b) have been characterized in our laboratory. ROS produced in the early stages of infection have a double effect: on the one hand, they are toxic for the pathogen and slow down the infection; on the other hand they are responsible for the cell wall crosslinking and reinforce this structure against pathogen penetration. Accumulation of ROS and induction of AtPGIP were therefore monitored in the Arabidopsis seedlings to assess the effectiveness of OG treatment.

Response of Arabidopsis seedling to OGs with a degree of polymerization between 10 and 15 was assessed by measuring accumulation of extracellular H_2O_2 at different times of incubation in the presence of 100 µg/ml of OGs using the xylenol orange assay (Fig. 3.2). A typical transient accumulation of H_2O_2 occurred in response to the OGs, with maximum levels at 3 h of treatment. H_2O_2 decreased at 6 h and returned to the basal level after 24 h. The slight accumulation of H_2O_2 that accumulated in the control plants was probably due to the minimal shaking occurring during the experiments.

Incubation of *Arabidopsis* seedlings with OGs is also known to cause a transient increase of AtPGIP1 transcripts, detectable as early as 90 min after treatment and sustained for at least 6 h; to the increased level of AtPGIP1 transcripts corresponds an increased amount of the protein (Ferrari et al. 2003b). Total protein extracts were therefore prepared from the OG-treated and control seedling and tested for PGIP activity. Accumulation of PGIP was detected after 24 h of incubation in the presence of OGs (fig. 3.2).

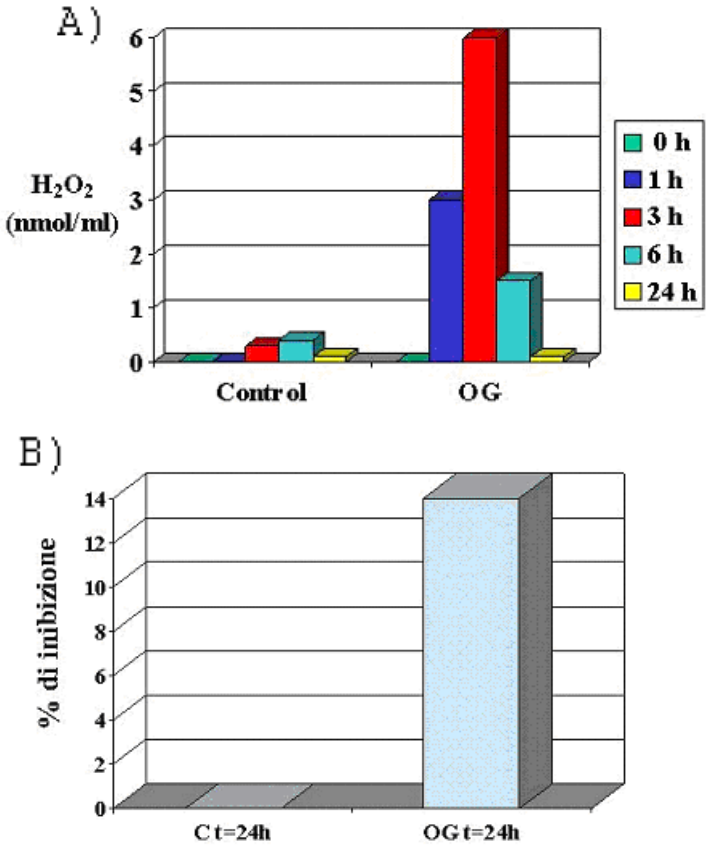

Fig 3.2. Accumulation of H_2O_2 and AtPGIP1 in response to OGs.

A) Hydrogen peroxide accumulation in the growth medium of Arabidopsis seedlings incubated for different times in the presence or in the absence of OGs seedlings.

B) Accumulation of PGIP activity at 24 h in response to OGs. Activity is reported as % of inhibition of PG activity. One agarose plate unit of enzyme was used in this experiment.

3.1.3 Preliminary proteomic analysis of apoplastic proteins affected by OG treatment

I chose to analyze seedlings treated with OGs for 6 h, because this is an intermediate time between early responses (such as H_2O_2 accumulation) and late responses (such as PGIP accumulation). At 6h main changes in protein expression and secretion are expected to occur. IWFs were prepared from treated and control seedlings. Each sample was subjected to 2D gel electrophoresis and, after staining, each protein pattern was acquired using a densitometer. Protein patterns obtained from control seedlings and seedlings treated for 6 h with OGs were compared using a software for image analysis (PDQuest,

BioRad). Three independent experiments were performed. Only few apoplastic proteins (in bold in Table 3.III) were found to vary consistently in all three experiments; other proteins varied in one or two experiments only.

For these proteins a comparison with transcriptomic analysis was done in order to establish if the induction/repression by OGs correlates to the increase/decrease of the corresponding transcripts determined by cDNA microarray experiments.

Spot n.	Accession number and protein name	Experiments [a]	Fold change [b] (average)	Microarray analysis [c]	
				OG	
				1 h	3 h
1	**At3g16530 Lectin-like**	3/3	+4.1	+11	+9.8
2	**At1g54010 Myrosinase-associated protein**	3/3	+2.8	-1.2	-1.1
3	**At3g16420 Jacalin lectin family Jasmonate inducible protein**	3/3	+2.5	+1.0	-1.1
4	**At 4g30270 (MERI-5B) Endo-xyloglucan transferase**	3/3	+2.2	-1.0	-1.4
5	At1g78830 Curculin-like	1/3	+2.6	+2	+1.5
6	At1g78820 Curculin-like	1/3	+3.5	+1.9	+2.5
7	At5g20630 Germin-like protein GER-3	1/3	+4.3	-1.3	-1.7
8	At4g37800 Xyloglucan endo-1,4-glucanase	1/3	+3.5	-1.5	-1.6

Tab 3.III. Proteins induced by OG treatment in Arabidopsis seedlings identified using MALDI –ToF MS are reported with name and accession number.

[a] The number of experiments in which each protein was found to vary; proteins in bold varied in all the three experiments, other proteins varied only in one or two experiments. Numbers in the first column refer to protein spots as shown in Fig, 3.1.

[b] Fold change for each protein; plus (+) indicates induction while minus (-) indicates repression after OG treatment.

[c] Variations in the transcript levels of the corresponding genes after treatment with OGs for 1 and 3 hours, as determined by global transcript profiling (Ferrari et al. unpublished data).

3.1.4 DIGE analysis of apoplastic proteins that change after OG treatment

A major problem of the proteomic approach described in 3.1.3 derives from gel-to gel variation which in most of cases provides results with little or no statistical consistency. The problem of gel-to-gel variation can be avoided by using differential in-gel electrophoresis (DIGE) analysis. This analysis, that employs dyes to label different protein populations run in the same gel, eliminates inter-gel variation and facilitates quantitative evaluation of differentially expressed proteins.

DIGE analysis using the DIA (DIfferential Analysis) mode of the software was used to study variation of the apoplastic proteome in response to OGs. This mode allows a direct comparison between two samples labelled with different dyes, loaded in equal amounts and run in the same gel. Differences in proteins abundance were evaluated by recording the intensity of fluorescence for each protein spot using a Typhoon 9200 (Amersham Biosciences). Because the intrinsic fluorescence of each dye is different, to avoid false positive results, control and OG-treated samples were run twice labelled with reciprocal dye (gel 1: control labelled with Cy3 and OG labelled with Cy5; *vice versa* for gel 2) as shown in the scheme reported in figure 3.3.

For each experiment, among the differential proteins found, only those common between the two gels run with reciprocal dyes were considered as proteins of interest**.**

The analysis was performed using samples obtained from three independent experiments, therefore running six gels (two for each experiment) according to the scheme in figure 3.3. Proteins that were differentially expressed (induced or repressed) in all three experiments are shown in figure 3.4.

Only few spots of interest had an intensity that allowed the identification by MALDI-ToF MS (Table 3.IV).

To further minimize the problem of gel-to-gel variation the analysis was also carried out using the BVA mode (Biological Variation Analysis), that includes the use of an internal standard consisting of a pool of all the samples analyzed, labelled with Cy2. The internal standard is run in each gel together with the Cy3 and Cy5 labelled samples. The results of a BVA analysis is a value indicating protein variation level paired with a statistical score (p) obtained from ANOVA or T-test analysis which supports the result. Proteins identified using the BVA mode are also reported in the table 3.IV.

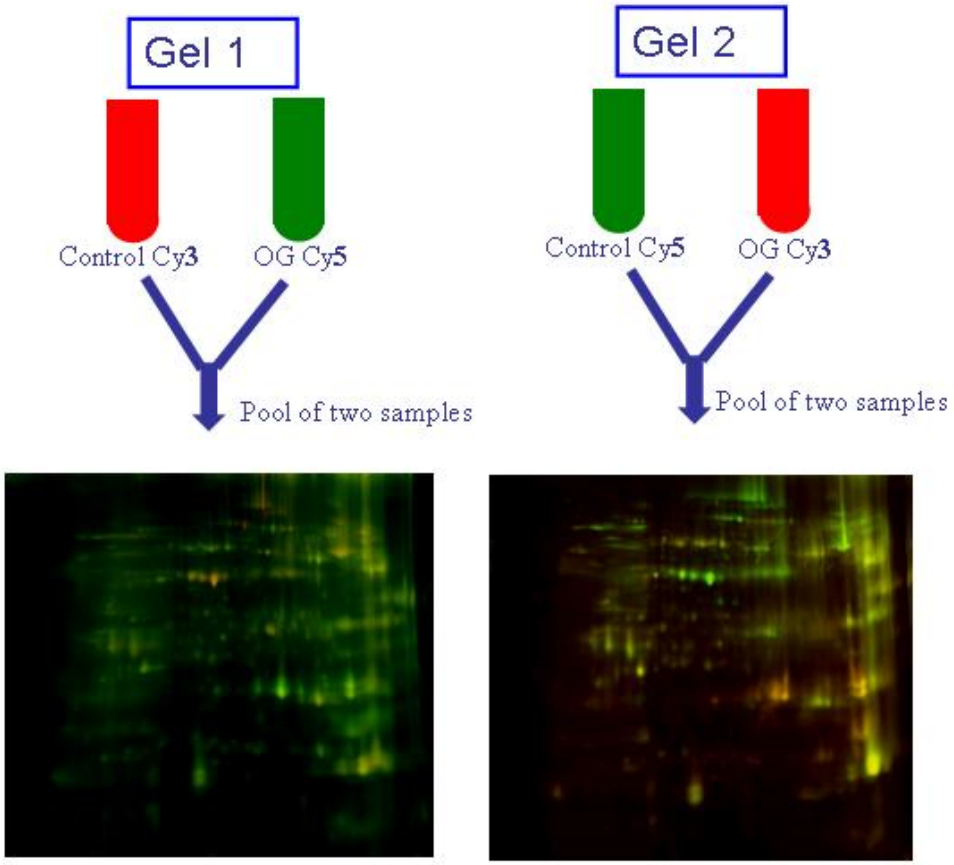

Fig. 3.3. Scheme of DIA analysis for one experiment: two samples are labelled with different dyes (Cy3 and Cy5), then pooled and run in the same gel. For gel number 2, conditions are the same but reciprocal labelling is done to avoid false positives. The output image shows mixed colours for proteins expressed at the same level and one colour preferentially for proteins induced or repressed by treatment.

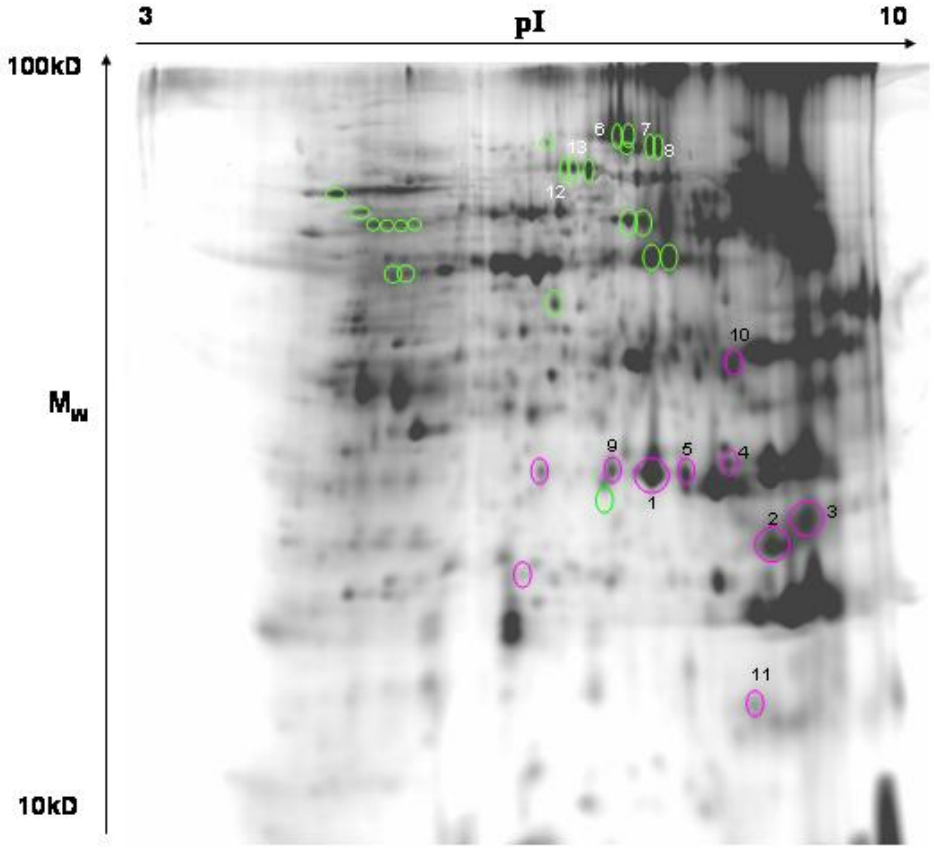

Fig 3.4. Gel obtained with IWFs. Proteins that were induced more than 1.5 fold OG are highlighted in pink and those repressed more than 1.5 fold are highlighted in green. Proteins with numbers were identified by MALDI-ToF MS and are reported in table 3.IV.

Spot n.	Accession number and protein name [a]	Mode of analysis	Fold change [b]	Microarray analysis [c]	
				OG	
				1 h	3 h
1	At3g16530 lectin like	DIA/BVA	+2.6	+11	+9.8
2	At3g22060 receptor protein kinase related	DIA	+1.7	+6.0	+5.0
3	At1g78830 curculin like	DIA	+1.9	+2.0	+1.5
4	At3g16530 lectin like	DIA	+1.9	+11	+9.8
5	At4g30270 xyloglucan endo 1,4 beta glucanase	DIA	+1.8	0	-1.4
6	At1g68560 alpha-xylosidase precursor	DIA/BVA	-4.1	-1.5	-1.4
7	At1g68560 alpha-xylosidase precursor	DIA/BVA	-3.4	-1.5	-1.4
8	At1g68560 alpha-xylosidase precursor	DIA/BVA	-3.5	-1.5	-1.4
9	At3g16530 lectin like	DIA	+2.4	+11	+9.8
10	At5g06860 polygalacturonase inhibiting protein 1 (PGIP1)	DIA/BVA	+1.5	+3.6	+10
11	At1g52400 alpha-galactosidase	DIA/BVA	+3.6	-1.0	-1.0
12	At1g68560 alpha-xylosidase precursor	DIA/BVA	-1.4	-1.5	-1.4
13	At1g68560 alpha-xylosidase precursor	DIA/BVA	-1.4	-1.5	-1.4

Tab.3.IV. Proteins induced or repressed by OG treatment in Arabidopsis seedlings identified using MALDI –ToF MS are reported with name and accession number; numbers in the first column refer to the spots in Fig, 3.4.

[a] Some proteins are reported more than one time in this table because they were present as different spots in the gel.

[b] Fold change for each protein; plus (+) indicates induction while minus (-) indicates repression after OG treatment.

[c] Variations in the transcript levels of the corresponding genes after treatment with OGs for 1 and 3 hours, as determined by global transcript profiling (Ferrari et al. unpublished data).

3.1.5 DIGE analysis of lipid rafts proteins that change after OG treatment

Lipid rafts are membrane microdomains rich in sphingolipids and cholesterol and are part of the machinery ensuring correct intracellular trafficking of proteins and lipids. They are able to exclude or recruit specific protein and play a role in sorting and vesicle formation, as well as in vesicle movement, cytoskeletal connections, vesicle docking and fusion (Ikonen 2001).

Protein changes induced by 6 h of OG treatment in these structures were analysed by DIA analysis.

Total membrane lipid rafts were prepared as described in the paragraph 2.25 of the section “Materials and Methods”; after protein extraction and quantification protein labelling was performed. Differential proteins (induced and repressed) obtained from the DIA analysis are shown in figure 3.5. proteins identified by MALDI-ToF mass spectrometry are shown in table 3.V.

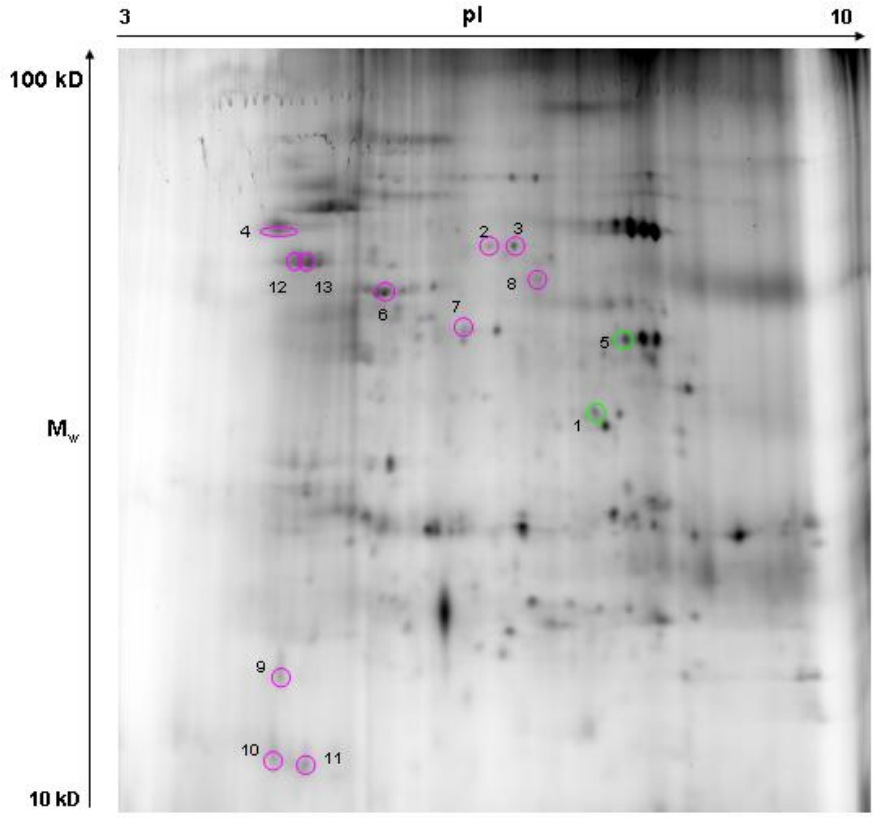

Fig. 3.5. 2D gel of lipid raft proteins. Proteins induced more than 1.5 fold OG were highlighted in pink and those repressed more than 1.5 fold were highlighted in green. Proteins with number were identified by MALDI-ToF MS and are reported in the table 3.V

Spot n.	Accession number and protein name [a]	Fold change [b]	Localization	Microarray analysis [c]	
				OG	
				1 h	3 h
1	At3g55110 ABC transporter – like	-9.4	membrane	-1.4	-1.0
2	At3g02090 putative mitochondrial processing peptidase	+2.3	soluble	+1.0	+1.1
3	At3g02090 putative mitochondrial processing peptidase	+2.8	soluble	+1.0	+1.1
4	At5g61790 calnexin - like protein	+2.8	membrane	-1.0	+2.6
5	At1g66270 beta-glucosidase	-1.6	plasma membrane	+1.0	+1.0
6	At1g51980 metalloendopeptidase	+1.5	soluble	-	-
7	Not analysed	+1.6	-	-	-
8	At2g17120 receptor-like GPI-anchored protein	+1.8	plasma membrane	+3.3	+2.7
9	At2g43160 putative clathrin binding protein	+1.7	soluble	+1.3	+1.0
10	Not analysed	+1.6	-	-	-
11	Not analysed	+1.9	-	-	-
12	At1g76030 vacuolar-type H+-ATPase subunit B1	+1.6	Vacuolar/soluble	+1.0	-1.1
13	At4g38510 vacuolar-type H+-ATPase subunit B2	+1.7	Vacuolar/soluble	-	-

Tab. 3.V. Proteins induced or repressed by OG treatment in Arabidopsis seedlings identified using MALDI –ToF MS are reported with name and accession number; numbers in the first column refer to the spots in Fig, 3.5.

[a] Some proteins are reported more than one time in this table because they were present as different spots in the gel.

[b] Fold change for each protein; plus (+) indicates induction while minus (-) indicates repression after OG treatment.

[c] Variations in the transcript levels of the corresponding genes after treatment with OGs for 1 and 3 hours, as determined by global transcript profiling (Ferrari et al. unpublished data).

3.2 Study of PGIP pectin binding site

3.2.1 PGIP interacts with cell walls both *in planta* and *in vitro*

PGIP is extracted from cell wall only with high ionic strength buffers. Stem segments of transformed tomato plants over-expressing the isoform 1 of PGIP from *Phaseolus vulgaris* (PvPGIP1) (Desiderio et al. 1997) were vacuum-infiltrated with 50 mM Na acetate buffer pH 5.0 in the presence or in the absence of 0.3 M NaCl to obtain intercellular washing fluids (IWFs) according to the procedure described in section 2.12 of "Material and Methods". Contamination of IWFs by cytoplasmic components was ruled out by measuring glucose-6-phosphate dehydrogenase (G6PD) activity which accounted for less than 1% of total extractable activity. Immunoblotting analysis showed that PvPGIP1 is present in IWFs obtained with high ionic strength buffer and is absent in IWFs

obtained with buffer alone (Fig. 3.6) thus demonstrating that PvPGIP1 is strongly ionically bound to the cell wall.

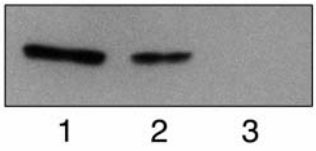

Fig. 3.6. Western blot analysis of PvPGIP1 extracted from *35S::Pvpgip1* tomato stems.
Lane 1, purified PvPGIP1 (10 ng); lane 2, IWF of tomato stems obtained with 50 mM Na acetate buffer containing 0.3M NaCl (4 µg of proteins); lane 3, IWF obtained with 50 mM Na acetate buffer (7 µg of proteins).

To single out which component of the wall binds PvPGIP1, tomato walls were fractionated according to the scheme shown in Figure 3.7. The wall material, i.e. the alcohol insoluble solid (AIS), was suspended in acetate buffer and subsequently fractionated into a pellet and a buffer soluble solid (BSS) fraction. After deproteination the pellet was fractionated into the chelating agent soluble solid (ChASS) and "Residue". The monosaccharide composition and degree of methyl-esterification (DM) of each fraction is reported in Table 3.VI. Each fraction is characterized by a distinctive distribution of sugars and contains various amounts of uronic acids which are potentially involved in binding the positively charged PGIP (pI ~ 9).

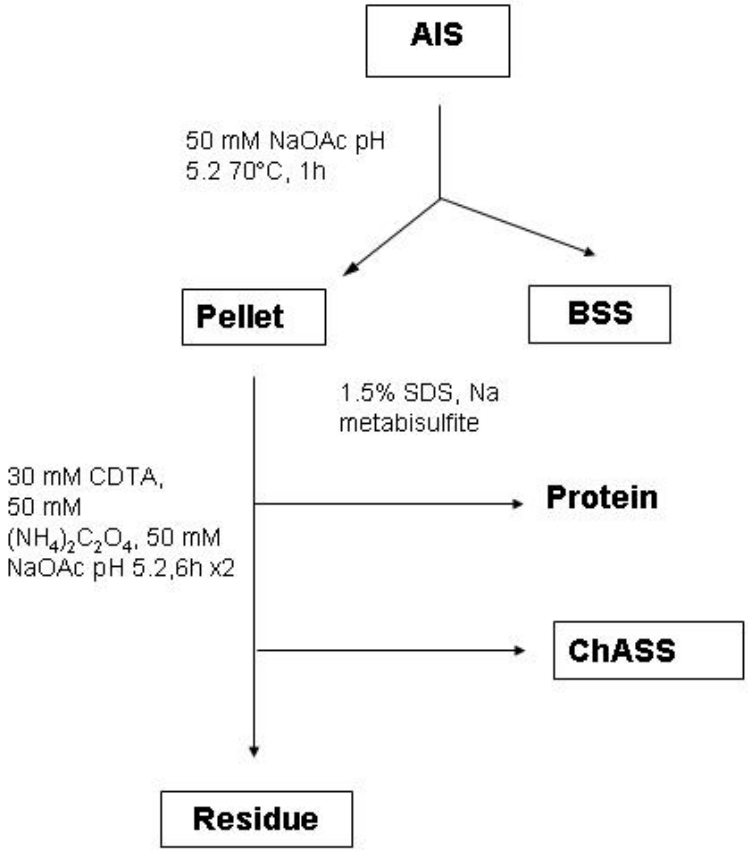

Fig. 3.7. Scheme of the polysaccharide extraction from tomato cell wall. Alchool Insoluble Solid (AIS), Buffer Soluble Solid (BSS), Chelating Agent Soluble Solid (ChASS).

Cell wall fraction	Rha	Ara	Xyl	Man	Gal	Glc	UrAc	DM %
AIS	6.0	10.2	15.3	2.2	16.8	24.1	25.4	89.7
BSS	5.7	14.6	1.9	3.7	14.5	46.0	13.5	89.3
ChASS	5.8	6.8	14.7	7.2	7.5	8.3	49.7	49.8
Residue	3.0	6.4	15.6	5.6	6.8	56.2	6.3	50.0

Tab. 3.VI. Sugar composition (% of moles) and degree of methyl-esterification (DM) of the cell wall fractions from tomato stems.

By using an affinity chromatography approach, the different wall fractions were tested for their ability to bind PvPGIP1. Columns containing polyacrylamide gel entrapping AIS, BSS, ChASS and "Residue" in the presence of $CaCl_2$, were prepared according to Penel

and Greppin's procedure (Penel and Greppin 1996). Homogeneous PvPGIP1 was loaded onto each column and the non-bound and bound protein was recovered by washing the column with 20 mM Na acetate buffer (pH 5.0) containing 100 μM $CaCl_2$ and then eluting the bound PvPGIP1 with PBS containing 0.3 M NaCl. The percentage of bound PGIP with respect to the amount of loaded PGIP (100%) was determined by inhibition assay against PGII from *Aspergillus niger*. Control columns containing only polyacrylamide did not retain PvPGIP1. PvPGIP1 displays different propensity towards various wall fractions; the level of bound PvPGIP1 was maximal (63%) on columns containing the uronic acid-rich fraction ChASS and minimal (7%) on the fraction "Residue" which contains a low amount of uronic acids (Table 3.VII). A similar interaction with pectin enriched components of the cell wall was observed for the isoform 2 of PGIP from *Phaseolus vulgaris* (PvPGIP2) (not shown), which differs from PvPGIP1 by only eight aminoacids in the LRR motif (Leckie et al. 1999).

Column containing	**Bound PGIP (%)**
AIS	58 (± 4)
BSS	38 (± 3
ChASS	63 (± 6)
Residue	7 (± 2)
Control	n.d.

Tab. 3.VII. Amount of PvPGIP1 bound to polyacrylamide gel columns containing different cell wall fractions. The Ca^{2+}-wall fraction/polyacrylamide columns equilibrated with 20 mM Na acetate buffer pH 5.0 containing 100 μM $CaCl_2$ were loaded with PvPGIP1, extensively washed with the equilibration buffer and eluted with PBS containing 0.3 M NaCl. The control was performed by loading PvPGIP1 onto a polyacrylamide gel columns; n.d stands for not detectable. The percentage of bound PGIP with respect to the amount of loaded PGIP (100%) was determined by inhibition assays against PGII from *A.niger*. The percentage reported is the average (±SD) of three different experiments.

3.2.2 The 3-D structure of PGIP reveals a binding site for pectins

The 3-D structure of PvPGIP2 (Di Matteo et al. 2003) (Fig. 3.8 A) revealed a positively

charged patch located between the two β-sheets B1 and B2, directly below the negative pocket putatively involved in PG binding (Fig. 3.8 B). The positive patch consists of a cluster of regularly spaced Arg and Lys residues (R183, R206, K230, R252) which protrude into the solvent creating a regular distribution of charges (Fig. 3.8A). We investigated whether the patch of Arg and Lys acts as a pectin binding site. The arginines and the lysine were substituted with the polar amino acid glutamine to generate variant proteins. Two single mutants in which R183 and K230 were mutated into glutamine (QRKR and RRQR) were obtained. Also, the double mutant QRQR having both R183 and K230 mutated into Q, the triple mutants QRQQ and QQQR with the mutations R183Q, K230Q and R252Q or R183Q, R206Q and K230Q respectively, and the quadruple mutant QQQQ with the mutations R183Q, R206Q, K230Q and R252Q were constructed. Both the wild-type PvPGIP2 (RRKR) and mutants were individually expressed in *Pichia pastoris*, purified and their capability to interact with polygalacturonic acid (PGA) was analyzed at pH 5.0 and 7.0 either in the presence or in the absence of calcium. The wild-type PGIP showed maximum binding capacity in the presence of 100 μM Ca^{2+} at pH 5.0 (Figure 3.9 A) while in the absence of calcium and in the presence of EGTA the binding was reduced by approx. 50% (Table 3.VIII). Binding of PvPGIP2 to PGA was not observed at pH 7.0. Alginate (ALG) and polyacrylamide alone (not shown) were also used as controls, both in the presence or absence of calcium at pH 5.0 and pH 7.0. The binding of the inhibitor to ALG and polyacrylamide was not observed in all the conditions used (Table 3.VIII).

PvPGIP2 also binds oligogalacturonides (OGs) (Figure 3.9 B) because if the inhibitor is pre-incubated with OGs before affinity column, it does not bind to PGA and is totally recovered in the flow through (Figure 3.9). In contrast with the wild-type, the variants with single mutation exhibited reduced capacity of binding PGA in the same conditions; remarkably, the decrease of affinity towards PGA was more evident in the presence of Ca^{2+}. Moreover the variants with double, triple and quadruple mutations showed not binding to PGA (Table 3.VIII).

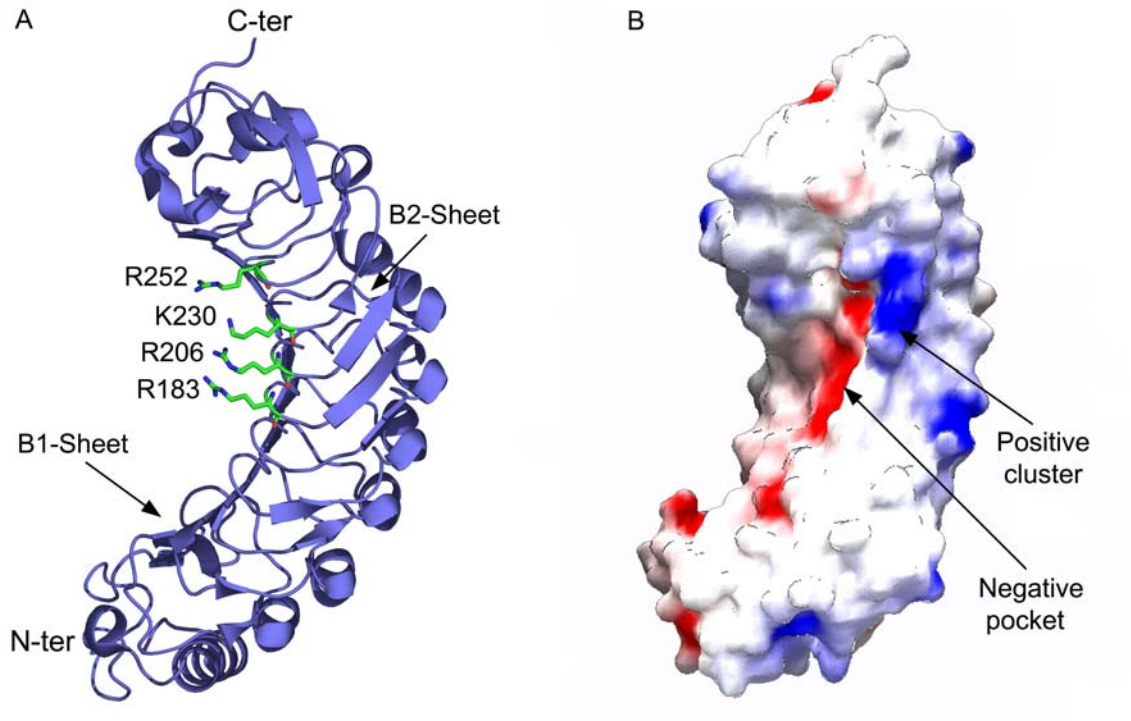

Fig. 3.8. **A,** View of the pectin binding motif composed by four positively charged residues (R183, R206, K230, R252). protruding into the solvent and located between sheet B1 and sheet B2. The residues are shown in ball and stick representation. **B,** GRASP electrostatic potential surface of PvPGIP2 calculated at pH 4.6. Regions of negative and positive potential are shown in red and blue, respectively. A wide negative pocket, putatively involved in binding the polygalacturonase, is located in the middle of the inner concave surface of the protein (Di Matteo et al. 2003). A positively charged cluster likely involved in pectin binding is located directly below the negative pocket.

	Bound PGIP (%)	
	PGA	
	EGTA	Ca^{2+}
RRKR (w.t.)	47	100
QRKR	43	32
RR**Q**R	7	17
QR**Q**R	0	0
QR**QQ**	0	0
QQQR	0	0
QQQQ	0	0

Tab. 3.VIII. Amount of PvPGIP2s bound to polyacrylamide gel columns containing either PGA or ALG at pH 5.0. The percentage of bound PvPGIP2 with respect to the amount of loaded PvPGIP2 (15 μg; 100%) was determined by SDS-PAGE. Wild type (w.t.) and mutated PGIP variants are indicated in the first column. Mutated residues are in bold.

Fig. 3.9. A) SDS-PAGE obtained with fractions from PGA/polyacrylamide affinity column with PvPGIP2; B) SDS-PAGE obtained with fractions from PGA/polyacrylamide affinity column with PvPGIP2 pre-incubated with OGs for 1h.
FT stands for flow through.

All PvPGIP2 variants showed inhibitory activity against PGII of *Aspergillus niger* (*An*PGII).

The proximity of the pectin binding site to the region known to interact with

polygalacturonase (Leckie et al. 1999) suggested that, upon binding the enzyme, PvPGIP2 may be removed from the pectic matrix. Consequently, *An*PG and PG of *Fusarium moniliforme* (*Fm*PG) were tested for their ability to compete for the interaction of PvPGIP1 and PvPGIP2 with pectin. Columns of polyacrylamide entrapping pectin were separately loaded at pH 5.0 with the two inhibitors and, after washing, were subsequently loaded with buffer containing *An*PGII or *Fm*PG. PvPGIP1 interacts with *An*PGII and is unable to interact with *Fm*PG, while PvPGIP2 interacts with both fungal PGs, though with different affinity (K_D= 0.96 nM for the interaction with *An*PG and 47.7 nM for the interaction with *Fm*PG) (Leckie et al. 1999). Figure 3.10 A and 3.11 A show that in the absence of PGs (buffer alone) the inhibitor was not observed in flow through and was totally recovered in the bound fraction. Both enzymes were able, with different efficiency, to displace the bound PvPGIP2 from the column (Fig. 3.10 B, C, D), while only *An*PGII displaced PvPGIP1 (Fig. 3.11 B, C) consistently with the different specificity of the inhibitors.

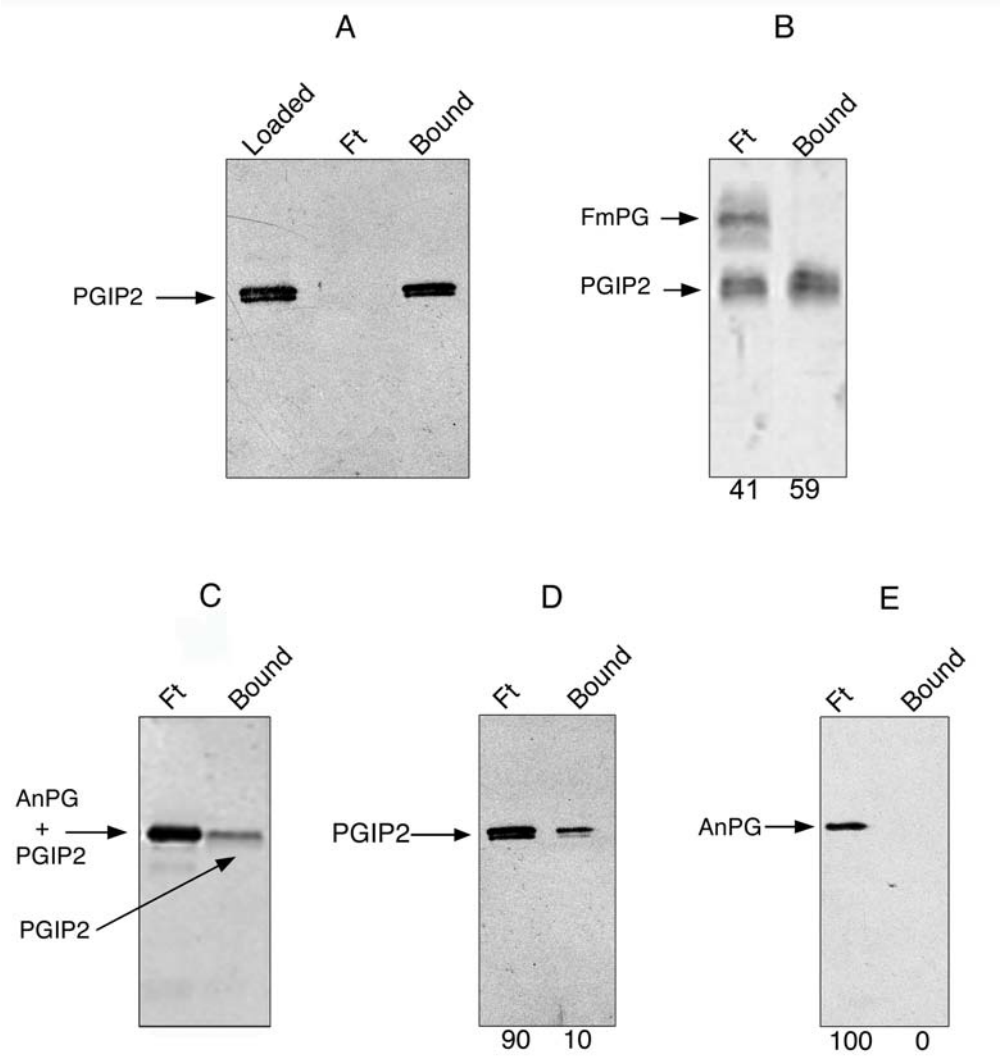

Fig. 3.10. Effect of *An*PGII or *Fm*PG on the binding of *Pv*PGIP2 to Ca^{2+}-PGA/ polyacrylamide columns.

A) Silver stained SDS-PAGE showing that columns washed with washing buffer alone do not release PGIP2.

B) Silver stained SDS-PAGE showing the effect of the *Fm*PG on the binding of *Pv*PGIP2 to Ca^{2+}-PGA/ polyacrylamide column; numbers at the bottom of the figure indicate the relative amount of PGIP determined by densitometer.

C) Silver stained SDS-PAGE showing the effect of *An*PGII on the binding of *Pv*PGIP2 to Ca^{2+}-PGA/ polyacrylamide column.

D) Western blot obtained from the gel showed in C using an antibody against *Pv*PGIP2; numbers at the bottom of the figure indicate the relative amount of PGIP as determined by densitometer.

E) Western blot obtained from the gel showed in C using an antibody against *An*PGII;

Flow trough (FT) is referred to the fraction collected from the column after loading PG and "Bound" is the fraction collected after elution with PBS containing 0.3 M NaCl.

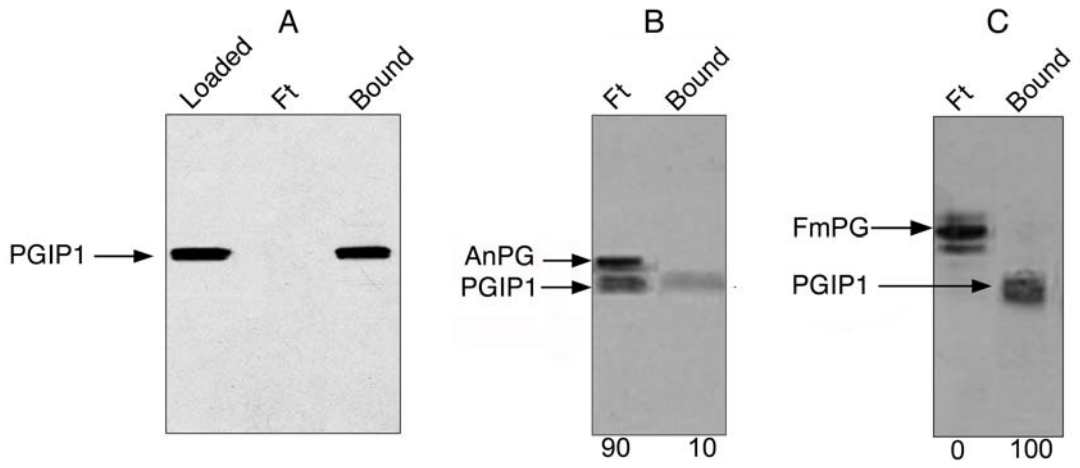

Fig. 3.11. Effect of *An*PGII or *Fm*PG on the binding of *Pv*PGIP1 to Ca^{2+}-PGA/ polyacrylamide columns.

A) Silver stained SDS-PAGE showing that columns washed with washing buffer alone do not contain PGIP1.

B) Silver stained SDS-PAGE showing the effect of *An*PGII on the binding of *Pv*PGIP1 to Ca^{2+}-PGA/ polyacrylamide column.

C) Silver stained SDS-PAGE the effect of *Fm*PG on the binding of *Pv*PGIP1 to Ca^{2+}-PGA/ polyacrylamide column.

Flow trough (FT) is referred to the fraction collected from the column after loading PG and "Bound" is the fraction collected after elution with PBS containing 0.3 M NaCl.

3.2.3 The Pattern of Methyl-Esterification Influences the Interaction of PGIP with Pectin

PvPGIP1 was used for binding experiments either with partially de-esterified pectins, obtained from sugar beet pectin by blockwise de-esterification using a plant pectin methylesterase (PME), with a DM of 41% (P41), and non-blockwise pectins, obtained by de-esterification using a fungal PME, with a DM of 43% (F43). When PvPGIP1 was loaded on P41 column in the presence of 100 μM Ca^{2+} at pH 5.0 the interaction was strong and comparable to that observed with PGA, indicating that the presence of blockwise de-esterified stretches is sufficient to confer to pectin the capability to interact with PGIP. On the other hand when PvPGIP1 was loaded on F43 column at pH 5.0 in the presence of calcium only 15 % of the inhibitor was retained (Fig. 3.12).

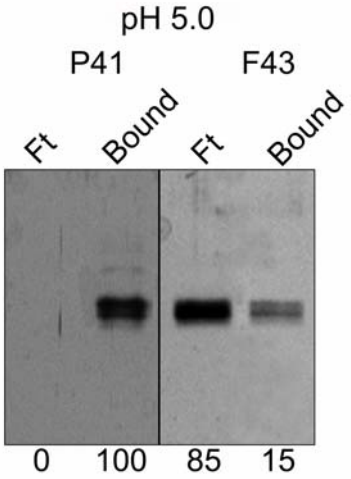

Fig. 3.12. SDS-PAGE analysis of fractions obtained from affinity chromatography of *Pv*PGIP1 loaded onto polyacrylamide columns polymerised in the presence of 100 μM $CaCl_2$ at pH 5.0 entrapping blockwise de-esterified pectin with a methyl-esterification degree of 41% (Ca^{2+}-P41/polyacrylamide) and non blockwise de-esterified pectin with a methyl-esterification degree of 43% (Ca^{2+}-F43/polyacrylamide columns). The columns equilibrated with 20 mM acetate buffer pH 5.0 containing 100 μM $CaCl_2$ were loaded with *Pv*PGIP1, extensively washed with the equilibration buffer and eluted with PBS containing 0.3 M NaCl. Bound and not bound (FT) fractions were analysed by SDS-PAGE. Numbers at the bottom of the figure indicate the relative amounts of bound and not bound *Pv*PGIP1, as determined by densitometer and inhibitory activity against PGII from *A.niger*. Uronic acid assay was carried out in the eluate to exclude that pectins entrapped in the gel were not released. Bound and not bound (FT) fractions were analysed by SDS-PAGE. Numbers at the bottom of the figure indicate the relative amounts of bound and not bound PvPGIP1, as determined by densitometer and inhibitory activity against PGII from *A.niger*. Same relative amounts of PGIP were revealed with the two different procedures.

4. DISCUSSION

4.1 Proteomic analysis of responses induced by oligogalacturonides

In the first part of my thesis I reported the study of OG-activated defence responses in 10-days old *Arabidopsis thaliana* seedlings using a proteomic approach. In particular I focused my research on the study of apoplastic proteins that vary after OG treatment. The cell wall not only is the source of OGs but also is the first place that comes into contact with pathogens during the infection. Both seedlings and cell cultures are suitable as systems to study OG responses. In this research I used seedlings rather than cultured plant cells for several reasons. First cultured cells are not representative of the whole plant, being composed by undifferentiated cells; secondly, one of the aim of my work was provide information at the proteome level to complement data obtained in the laboratory by global transcript profiling carried out using Arabidopsis seedlings.

Many different papers regarding proteomic studies of cell wall proteins have been published (Charmont et al. 2005; Chivasa et al. 2002b; Ndimba et al. 2003; Oh et al. 2005) but most of them have been carried out using Arabidopsis cell cultures because it is much easier to recover apoplastic proteins from the liquid medium than from intercellular spaces (Borderies et al. 2003; Chivasa et al. 2002b; Kwon et al. 2005b; Oh et al. 2005). In the only proteomic study carried out using Arabidopsis seedlings, proteins were recovered from the liquid medium where seedlings had been grown under shaking conditions (Charmont et al. 2005). Because a protocol to recover apoplastic proteins from intercellular spaces of Arabidopsis seedlings is not available, the first step of my study was the development of a good protocol to obtain proteins of intercellular washing fluids (IWFs) from Arabidopsis seedlings avoiding intracellular contaminations. As reported in the section "results" to this purpose I tested different buffers to vacuum infiltrate seedlings and extract proteins; I tested the effect of the pH, EDTA presence and ionic strength on proteins extraction. Without EDTA and salts the extracts had very low protein content and showed no visible protein spots on the gel demonstrating that even loosely bound proteins need at least chelating agents or ionic strength to be extracted. Buffers containing both chelating agents (EDTA) and high ionic strength ($MgCl_2$) gave the same contamination level of the buffer without $MgCl_2$ but higher protein content in the extracts. This result suggests that the ionic strength of the buffer is important for the extraction of apoplastic proteins that can be ionically bound to the cell wall, and does not necessarily affect plasma

membrane integrity. EDTA is also important because by chelating calcium cell wall is weakened, thus allowing an easier recovery of proteins embedded in the matrix.

The extracts obtained using the buffer containing both EDTA and $MgCl_2$ showed some proteins that have not been previusly identified in other proteomic studies of apoplastic proteins, such as At4g30270 endo-xyloglucan transferase, At1g78820 curculin-like protein, At4g37800 xyloglucan endo 1,4 glucanase, At5g38940 germin-like protein, At1g03220 extracellular dermal glycoprotein, At3g32980 peroxidase 32 precursor, At1g08830 superoxide dismutase, At4g39260 glycin rich protein and At5g44120 seed storage protein. This suggests that this new protocol can complement other protocols available for the recovery of cell wall proteins.

Two different approaches both based on 2D gels were used to perform differential analysis on apoplastic proteins upon OG treatment; one was the "classic" approach in which two samples are loaded on to two different gels. The gels are compared to obtain information about differential proteins. The main problem of this approach is that, even if the gels are run in the same conditions, they are slightly different making the comparison difficult. The second approach utilized the DIGE (DIfference in Gel Electrophoresis) methodology that limits this problem because two samples are labelled with two fluorophores and loaded onto the same gel.

The results obtained with the two approaches showed several overlaps, as expected, but also some differences. This is probably due to the fact that DIGE allows an easier comparison between control and treated because these two samples are run in the same gel allowing an automatic matching between spots in the control and the corresponding spots in the treated sample.

Only proteins found to vary using DIGE analysis are considered in this discussion.

The isoform 1 of polygalacturonase inhibiting protein (PGIP1 At5g06860) was induced by OG treatment, in agreement with the reported induction of the gene *AtPGIP1* (Ferrari et al. 2003b). PGIP is involved in the defence responses by interacting with polygalacturonases secreted from fungi during infection. The inhibitor is capable to slowing down the degrading activity of PG causing a transient accumulation of active OGs (De Lorenzo and Ferrari 2002). It has been recently reported that PGIP plays a direct role in plant defence and that transgenic *Arabidopsis* plants over-expressing PGIP exhibit enhanced resistance to *Botytis cinerea* (Ferrari et al. 2003a). Like many other defence proteins, PGIPs are encoded by gene families and in Arabidopsis two tandemly duplicated

genes (*AtPGIP1* and *AtPGIP2*) are present. Both genes are up-regulated coordinately in response to *Botrytis cinerea* infection, but only *PGIP1* is induced in response to OGs (Ferrari et al. 2003b). The identification of AtPGIP1 as an induced protein confirms that the differential analysis and the identification system worked properly.

Two proteins induced by OG after 6 h of treatment are lectin-like proteins (At3g16530, At1g78830). Plant lectins are proteins possessing at least one non-catalytic domain that reversibly binds specific mono-or oligosaccharides. Most plant lectins are directed to bind foreign polysaccharide and are believed to play a role in pathogen recognition and take a part in the defence responses (Sharon and Lis 2004). Recent progress in glycobiology has revealed that cell surface oligosaccharides play an essential role in recognition events. Lectins may function as antibodies to protect plants against harmful soil bacteria, control seed germination, or be involved in the transport and storage of sugars. Lectins protect plants against phytopathogenic microorganisms and insects as well as against predatory animals. They are also involved in the association between leguminous plants and their symbiotic nitrogen-fixing bacteria (Rudiger and Gabius 2001). The suggestion that lectins may be involved in the protection of plants against pathogenic microorganisms was originally based on the observation that some lectins inhibited the sporulation and growth of fungi such as *Trichoderma viride*, *Penicilium notatum*, and *Aspergillus niger* (Barkai-Golan et al. 1978). Potato lectin was subsequently shown to act in a similar manner on *Botrytis cinerea* (Sharon and Lis 2004) a fungal phytopathogen that secretes PG and produces OGs in different plants. A possible explanation is that lectins can bind to fungal cell wall polysaccharides interfering with its growth.

The biosynthesis of most plant lectins proceeds via the secretory pathway (Bowles and Pappin 1988; Vitale and Chrispeels 1992) and many lectins are found in the inner periphery of the cells in loose association with the cell wall (Etzler et al. 1984). Under this light the observation that lectins are induced by OG treatment in the apoplast is not surprising. Both lectins that I found to increase are induced at both the transcript level and the protein level as shown in table 3.IV.

OGs are produced by the action of PGs (secreted for example by *Botrytis cinerea*) on the plant cell wall pectins during the early stages of fungal infection so it is likely that a significant portion of the defence responses induced by *Botrytis cinerea* may be mediated by the accumulation of OGs in the apoplast. Therefore many responses induced by both Botrytis and OGs are likely to be directed against the fungus. The lectins At3g16530 and

At1g78830 could be involved either in the recognition of complex polysaccharides present in the fungal cell wall during the infection or in the inhibition of the sporulation and growth of fungus (Sharon and Lis 2004).

Lectin-like proteins induced by OGs are both soluble extracellular proteins never characterized before. At3g16530 protein shows a sequence similarities with legume lectin with only one β-domain; At1g78830 has sequence similarities with bulb-type mannose specific binding involved in the mannose recognition. Functional studies are needed to assess their biological role and to establish if these proteins are able to bind polysaccharides.

A protein I found to be repressed by OG treatment is an α–xylosidase (At1g68560) a polysaccharide modifying enzyme able to specifically release the unsubstituted side chain xylosyl residue attached to the glucosyl backbone situated nearest to the non-reducing end of the molecule. It has been reported that this gene encodes for an apoplastic α–xylosidase active against xyloglucan oligosaccharides (Sampedro et al. 2001); higher levels of transcripts of the gene corresponding to this protein were found in younger rosettes leaves of Arabidopsis indicating developmental regulation of its expression (Sampedro et al. 2001). Cell wall xyloglucan are complex branched polysaccharides and the sequential action of the different enzymes are necessary to completely degrade xyloglucan oligosaccharides to their monomers. The action of α-xylosidase on xyloglucans is very important because removing xylosyl residue attached to the backbone gives access to other degrading enzymes (Sanchez et al. 2003). The repression of the expression of this protein caused by OGs is probably due to the fact that plants need to slow down cell wall degradation during pathogen infection limiting the presence of polysaccharides modifying enzymes that leads to an easier degradation. Notably, this protein is present as multiple spots in the gel (same molecular weight but different isoelectric point) due to protein phosphorylation (Kwon et al. 2005b); only some of this spots seems to be repressed by OGs while others do not change after the treatment. These observations raise the possibility that proteins differently modified exhibit different enzymatic activity on xyloglucan and probably only some of them need to be repressed. They may also suggest the repression of the activity of specific kinases.

Instead, the amount of a xyloglucan *endo*-1,4 β-glucanase (At4g30270) was found to increase in the apoplast after OG treatment. Xyloglucan *endo*-1,4 β-glucanases are *endo*-

1,4 β-glucan hydrolyzing enzymes found in both prokaryotes and eukaryotes (Nicol et al. 1998). In plants these enzymes are thought to be involved in the hydrolysis of the cell wall xyloglucan. A different xyloglucan *endo*-1,4 β-glucanase was found to be induced 3-fold by chitosan and *Fusarium moniliforme* treatment in Arabidopsis cells cultures suggesting that this proteins are involved in pathogen response but their role is still unclear (Ndimba et al. 2003). It also important to note that the increase of this proteins after OG treatment does not correspond to an increase of transcript of the corresponding gene as shown in the table 3.IV. Changes in protein trafficking and secretion into the apoplast may be responsible for this apparent inconsistency. Interestingly microarray data show that many genes involved in protein trafficking and secretion are induced by OG treatment (Ferrari et al unpublished data).

A receptor protein kinase related (At3g22060) was found to vary in the apoplast in response to OGs both at the protein level and at the transcript level. This protein has not been previously studied and it comprises two DUF26 domains with unknown function. The DUF26 domain contains four conserved cysteines that probably form two disulphide bridges, and is usually associated to kinase domains. However the At3g22060 protein does not present a kinase domain in the sequence. The function of this protein is thus unknown and other studies need to be done to assess its biological function and relevance.

Finally I also carried out a preliminary analysis of lipid rafts proteins that change in response to OGs. Lipid rafts are specialized microdomains within the plasma membrane and intracellular membranes that possess a liquid-ordered structure resulting from enrichment in specific lipid components such as sterols and sphingolipids. They are involved in many different cell processes such as the regulation of signal transduction and trafficking of macromolecules (Schlegel et al. 1998; Simons and Toomre 2000) and are part of the protein sorting machinery operating as platforms for the inclusion of sorting receptors as well as cargo molecules to the forming vesicle. Rafts are believed to function as signalling platforms in which interactions between receptor proteins, adapter molecules, and effectors are tightly regulated to modulate signal generation. They are also thought to serve as sites of signal amplification and diversification and can be also used by several pathogens as gates into the cells.

The results shown in this thesis show that OG responses involve lipid rafts structures. A receptor-like GPI-anchored protein (At2g17120) was found to be increased in lipid rafts in response to OG after 6 h of treatment. Glycosylphosphatidylinositol-anchored proteins

(GPI-APs) are a functionally and structurally diverse family of post-translationally modified membrane proteins in which glycosylphosphatidylinositol (GPI) moiety is attached at the C terminus of the protein. They are found mostly in the outer leaflet of the plasma membrane in a variety of eukaryotic cells; anchoring to the lipid bilayer confers the GPI anchored proteins (GPI-APs) a number of physicochemical properties that are shared with intrinsic plasma membrane proteins. It has been recently demonstrated that many GPI anchored proteins are localized in plasma membrane lipid rafts in Arabidopsis (Borner et al. 2003; Elortza et al. 2003).

Although the general role of GPI-APs remains unclear, they have attracted attention because they act as enzymes and receptors in cell adhesion, differentiation, and host-pathogen interactions and may also be involved in intracellular sorting and transmembrane signalling processes (Horejsi et al. 1999; Muniz and Riezman 2000).

The lipidic part of the anchor has been shown to act as a signaling molecule, e.g. mammalian protein kinase C is activated by diacylglycerol (Nishizuka 1995).

The protein At2g17120 contains two lysine motif (LysM) domains. The LysM domain is about 40 residues long and is found in a variety of enzymes involved in bacterial cell wall degradation. The presence of LysM domains is generally associated with binding of glycans. It has also been reported that LysM domain receptors are involved in the direct recognition of rhizobial Nod factors (lipochitin oligosaccharides) (Spaink 2004). This protein is a good candidate as an oligogalacturonide receptor.

4.2 Study of PGIP pectin binding site

Pectin partially consists of polygalacturonic acid bearing evenly distributed negative charges, which may interact with proteins exposing positive charges in favourable orientation. The crystal structure of PvPGIP2 revealed a cluster of regularly spaced Arg and Lys residues (R183, R206, K230, R252) protruding into the solvent and creating a regular distribution of positive charges (Fig. 3.8 A). A similar arrangement of positive charges defining a pectin binding site has been previously predicted by homology modelling for the zucchini peroxidase APRX (Carpin et al. 2001). Site directed mutagenesis of these residues in PvPGIP2 demonstrates that this array of positive charges defines a specific pectin binding site close to the LRR motif of the inhibitor. Indeed, the lack of binding of PGIP to alginate strength the vision that a specific interaction arises from the exact spatial fitting of arginines and lysine and the negatively charged motif of

homogalacturonan, suggesting this arrangement in homogalacturonan as a specific docking site for different protein ligands (Carpin et al. 2001). Interestingly docking sites for numerous protein ligands and receptors involved in different biological processes have been identified in anionic polymers of the animal extracellular matrix, such as heparin (Esko and Selleck 2002).

Inhibitors mutated in the Arg-Lys cluster shows altered or lost affinity for PGA. Furthermore, these mutants retain quite the same level of interaction with PG and the specificity of PGIP-PG interaction correlates with the capability of PG to displace the PGIP binding to pectin. This indicates that PGIP contacts PG and pectin by engaging the same region but with distinct binding epitopes. The observation that PGIP binds ligands such as fungal PGs and pectins, with no obvious structural similarities suggests that it is a versatile protein capable of establishing diverse interactions. Noteworthy, a specific region of decorin, an animal LRR protein which presents intriguing similarities, with PGIP, has been involved in binding either to collagen type I and to the transforming growth factor-β (Santra et al. 2002). Leucine-rich repeat sequences are also involved in steroid binding, as demonstrated for the brassinosteroid receptor BR1 (Kinoshita et al. 2005). Possibly some LRR repeats can adopt atypical folds that are suitable to form binding domains also for non-protein molecules.

Our results suggest that stretches of un-methylated pectin are the anchorage place of the protein in the wall in physiological conditions: un-esterified pectins are able to bind PGIP at acidic pH, close to the physiological apoplastic enviroment, and Ca^{2+} can modulate the binding. In fact Ca^{2+} is required for optimal binding to PGA, indicating that the distribution of negative charges of homogalacturonan in the egg-box conformation best matches the regular arrangement of positive charges on PGIP. This optimal matching is strongly affected by the mutation of any of the residues on PGIP.

The interaction of PGIP2 with PGA in the absence of Ca^{2+} is reduced by approximately 50%; the mutation of K230, that disrupts the positive cluster, affects the binding more than the mutation of R183, in which the charge density created by the stretch of three positive charges is sufficient to maintain the binding almost at the same level. Interaction at pH 7 is not observed either in the absence or in the presence of Ca^{2+}. The lack of interaction at neutral pH cannot be ascribed to different protonation of positive residues (arginines and lysine) of PGIP or carboxylic groups of polygalacturonic acid. It is possible instead that a conformational transition of the PGA structure occurs increasing the pH, thus creating a

charge distribution not suitable for PGIP binding (Cesaro et al. 1982). Indeed, we have observed by circular dichroism analysis that PGA at pH 7.0 in the presence of 100 μM calcium is subjected to a conformational change with respect the form present at pH 5.0 (data not shown). A change of the apoplastic pH to values higher than 5 occurs in response to pathogen infection (Bolwell et al. 2002), and this may cause the release of PGIP from pectin to initiate the defence response.

Remarkably, PGIP does not bind exclusively Ca^{2+}-pectate as observed for several peroxidases, but also homogalacturonan chains without egg-box conformation, indicating that the protein can be anchored to different domains of the cell wall and not only to egg-box rich domains such as that observed in middle lamella and cell corners. PGIP strongly interacts with pectin having a blockwise pattern of de-esterification but not with pectin with a random pattern of methylation. This reflects the way by which PGIP is localized in the cell wall and has the physiological significance of protecting the substrate of fungal polygalacturonase. Moreover, PGIP is strategically located in the wall to favour the release of elicitor-active OGs which may further strengthen the plant defence against invading fungi.

A role of PGIP in the accumulation of OGs with DP between 10 and 15 has been shown (Cervone et al. 1989). Our evidence that PGIP binds both PGA and OGs suggests that PGIP may act not only by slowing down the PG activity, but may protect stretches of homogalacturonan of a definite size, favouring the release of elicitor active fragments.

In the presence of a fungal PG, PGIP may be removed from pectin and acts as enzyme inhibitor. The inhibition of PGs is thought to be an important event of plant defence against fungi (Mattei et al. 2001; Federici et al. 2001; Ferrari et al. 2003b; Manfredini et al. 2005). Fungal PGs are the first cell wall degrading enzymes (CWDEs) produced when fungi are grown on cell wall material and are a prerequisite for wall degradation by other CWDEs. The inhibition of PGs by PGIP is also thought to cause the accumulation in the plant apoplast of oligogalacturonides (OGs), which act as elicitors of a wide range of defence responses (De Lorenzo et al. 2001). Our evidence that PGIP specifically interacts with homogalacturonan indicates that the protein could be strategically positioned to efficiently protect pectin from fungal PGs and strengthens the vision that PGIP is involved in the release of biologically active OGs. It remains to be investigated how the relative amounts of PG and PGIP and/or the relative strengths of the interactions may influence the release of OGs and the plant defence.

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