THE STUDY OF POLYGALACTURONASE AND POLYGALACTURONASE INHIBITING PROTEIN USING SURFACE PLASMON RESONANCE / BIOMOLECULAR INTERACTION ANALYSIS – MASS SPECTROMETRY

by

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(Under the Direction of Dr. Ron Orlando)

ABSTRACT

Polygalacturonase Inhibiting proteins (PGIPs) are one of a plant's first lines of defense against fungal attack. PGIPs are found in the plant cell wall and work to both inhibit the destruction caused by phytopathogenic fungal enzymes know as polygalacturonases (PGs), and to regulate the activity of these enzymes. Once secreted by the attacking fungi, PGs break through the plant's polysaccharide rich cell wall allowing for penetration of the plant tissue by the fungi. PGIPs help defend the plant by binding to the PGs therefore regulating their activity, and permitting for the induction of defense elicitors. Through the use of surface plasmon resonance - biomolecular interaction analysis and mass spectrometry it is possible to study the specific interactions of PGIPs with PGs and identify PGIPs contained in a particular plant.

INDEX WORDS: Polygalacturonase, Polygalacturonase Inhibiting Proteins, Surface Plasmon Resonance / Biomolecular Interaction Analysis, Mass Spectrometry

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BS, The University of Georgia, 1998

A Thesis Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

MASTER OF SCIENCE

ATHENS, GEORGIA

2003

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ACKNOWLEDGEMENTS

I would like to thank my major professor, Dr. Ron Orlando, for his guidance and support and Dr. Jonathan Amster and Dr. Carl Bergmann for serving on my committee. I would like to especially thank Carl for his diligent effort in helping me throughout my project. I would like to thank Kumar Koli for his instruction and knowledge of the Biacore and mass spectrometry instruments. I would also like to thank Erica Fletcher for her help with the biacore and wish her luck in the experiments to come. Lastly I would like to thank my husband Bricker Daughtry, and my parents Gerald and Carol Cummings for all of their kindness, patience and support.

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

Introduction

The investigation of protein-protein interactions has made great advances in the last few years due to the combination of surface plasmon resonance – biomolecular interaction analysis (SPR/BIA) with mass spectrometry (MS). SPR-BIA provides a rapid, sensitive, and nondestructive means for analyzing the real time interactions between biomolecules. With its ability to detect the presence of binding partners, calculate such things as kinetic rate constants, concentrations, and binding affinities, and allow for further analysis of recovered biomolecules with MS, SPR-BIA can be an excellent tool for studying the interactions between fungal polygalacturonases (PG) and plant polygalacturonase inhibiting proteins (PGIPs).

Polygalacturonase inhibiting proteins (PGIPs) are one of the plant's first lines of defense against fungal attack. Found in the plant cell wall, PGIPs work to both inhibit the destruction caused by phytopathogenic fungal enzymes know as polygalacturonases (PGs), and to regulate the activity of these enzymes. Once secreted by the attacking fungi, PGs break through the plant's polysaccharide rich cell wall and allow for penetration of the plant tissue by the fungi. PGIPs help defend the plant by binding to the PG, thereby regulating their activity, and permitting for the induction of defense elicitors. The presence of multiple isoforms of PGs found in fungi has been matched by the production of multiple isoforms of PGIPS in plants. This has served to provide the plant

with a means to resist fungi. The selectivity of the different isoforms of PGIPs for specific isoforms of PGs has been under investigation for the last decade.

The research presented in this thesis lays down the groundwork for studying interactions between the different isoforms of both PGs and PGIPs using SPR/BIA-MS. The capabilities provided for by the use of SPR/BIA-MS will allow us to study such things as the effects of point mutations on the interactions of PGIPs with PGs, the differences between constitutively expressed PGs versus induced PGs, effects of pH on binding interactions, as well as allow us to isolate and identify PGIPs from a complex mixture. The wealth of information that stands to be gained from these studies will hopefully lead to the engineering of pathogen resistant plants.

The cell wall

A cell wall is a characteristic unique to plants, which provides strength and shape to the cell, rigidity to the whole plant, and a layer of protection against attack by pathogens and predators. The cell wall consists of three layers composed of cross-linked macromolecules such as celluloses, pectic polysaccharides, and hemicelluloses. The first layer, known as the middle lamella, is derived from the cell plate and can be found at the most exterior part of the cell wall – the layer that lies in the middle of two adjoining cells. Once the middle lamella is fully developed the next layer, the primary cell wall, is deposited by the daughter cells and continues to form as long as the cell is growing in surface area. The third layer, the secondary cell wall, is found only in specialized cells and is deposited at the onset of differentiation[1,2]. With each layer the strength of the cell wall increases and the cell continues to take on more of its role in the life of the plant.

Each layer of the cell wall consists of two networks, the microfibrillar network which allots tensile strength to the cell wall, and the matrix network that enables the cell wall to resist compression. The microfibrillar network is composed of extremely long, thin structures with circular or oval cross-sections known as microfibrils. Each of these microfibrils are made up of thirty to one hundred cellulose molecules, contain a high degree of crystallinity and are relatively homogeneous in chemical composition. The matrix, on the other hand, is a non-crystalline mixture composed primarily of two types of polysaccharides, the hemicelluloses and pectins, along with several structural proteins[1,3,4,5]. It is the interconnection of these two networks that provides the cell with its strength and stability.

The complex cross-linking of these two networks forms the skeleton of the cell and ultimately provides the cell wall with strength and stability. In each layer microfibrils lie approximately 20 – 40 nm from one another in an overlapping parallel array, meaning that their reducing ends all lie at the same end of the microfibrils[3]. These mibrofibrils are connected to one another by the long hemicelluloses, xyloglucan or arabinoxylan, of the matrix[6]. The hemicellulose molecules attach to the microfibrils by hydrogen bonds, forming a tight cellulose-hemicellulose network. The orientation of the microfibrils within each layer of the cell wall further enhances the strength of the wall. The strength of the wall is greatest in the direction parallel to the microfibrils and weakest in the direction perpendicular to them [3]. Therefore, by changing the orientation of the microfibrils in different layers the cell wall is able to increase its strength and complexity. The other major component that lies within the interlacing of the cellulose – hemicellulose network is the matrix pectic polymers. The structural complexity of these

polysaccharides present pathogens with a large array of differently linked glycosyl residues and non-carbohydrate substituents to overcome in order to penetrate the plant cell wall [7,8]. The most common pectic polysaccharides found in the cell wall are homogalacturonan, rhamnogalacturonan I (RG-I), and rhamnogalacturonan II (RG-II) [6]. The pectic polymers are more or less structurally independent of the cellulose-hemicellulose network, however functionally they are dependent on the cellulose-hemicellose network[9,10]. The primary roles of these pectins are thought to be control of the wall's mechanical properties, and cementing adjacent cells together. The structural connectivity of these pectin polymers is difficult to ascertain because their extraction and purification often results in the recovery of molecular fragments, which are not representative of the whole molecules [3]. Some studies suggest pectins are held in place in the wall by their interactions with divalent cations, especially calcium. In cases where the pectins are heavily methylated or when low calcium levels are present in the cell wall they are then held together by hydrophobic or hydrogen bonds[11]. Other studies suggest that pectins are covalently cross-linked [12,13]. The interlacing of the microfibrils and the matrix gives the cell its structure and strength, however the cell wall is unable to take on its full function as a rigid barrier without the presence of structural proteins.

The two main structural proteins that help hold the layers together, increase the strength of the cellulose-hemicellulose bonds, and provide overall rigidity to the cell wall, are extensin and lignin. The three layers of the cell wall (primary, secondary and middle lamella) are held together with hydroxyproline-rich glycoproteins, called extensins. These proteins are believed to be non-covalently bound to the other molecules found in the cell wall, however their exact mode of binding is not known for sure. Extensins are

elongated molecules that lie perpendicular to the cellulose microfibrils, holding the layers together and increasing the rigidity of the cell wall [14]. Lignin, a three dimensional polymer built from monomers called monolignols, forms parallel to the hemicellulose molecules and replaces the water found in these regions [3,15]. By replacing the water between the hemicellulose molecules, lignin forces the hydrophilic gel to become hydrophobic which in turn causes the strength of the hydrogen bonds that connect the cellulose and hemicelluslose to increase. Both of these structural proteins in combination with the microfibrilar and matrix phases provide the cell wall with strength, shape, and rigidity. (Figure 1)

Plants response to pathogenic attack

The complexity of the cell wall and the ability of the plant to defend itself against pathogens severely limits the number of successful pathogenic attacks. In order for a pathogen to invade a plant, it must be able to penetrate the cell wall either through enzymatic digestion of the cell wall or through breaks found in the cell wall. Since the pores found in the cell wall are so small that viruses can not even pass through them, the latter of these two mechanisms is rare[3]. Therefore even in a passive state, the cell wall is able to act as an extremely effective physical barrier, and serves as a first line of defense. However upon attack the plant elicits a series of active responses that further enable the plant to defend itself. The wall's active lines of defense include such things as a hypersensitive response, callose deposition, accumulation of pathogenesis-related proteins, and the synthesis of phytoalexins[15]. The first of these defenses, hypersensitive response (HR), occurs at the site of infection. When a pathogen attacks a cell wall, the cells closest to the point of attack undergo lignification and rapid, localized, cell death.

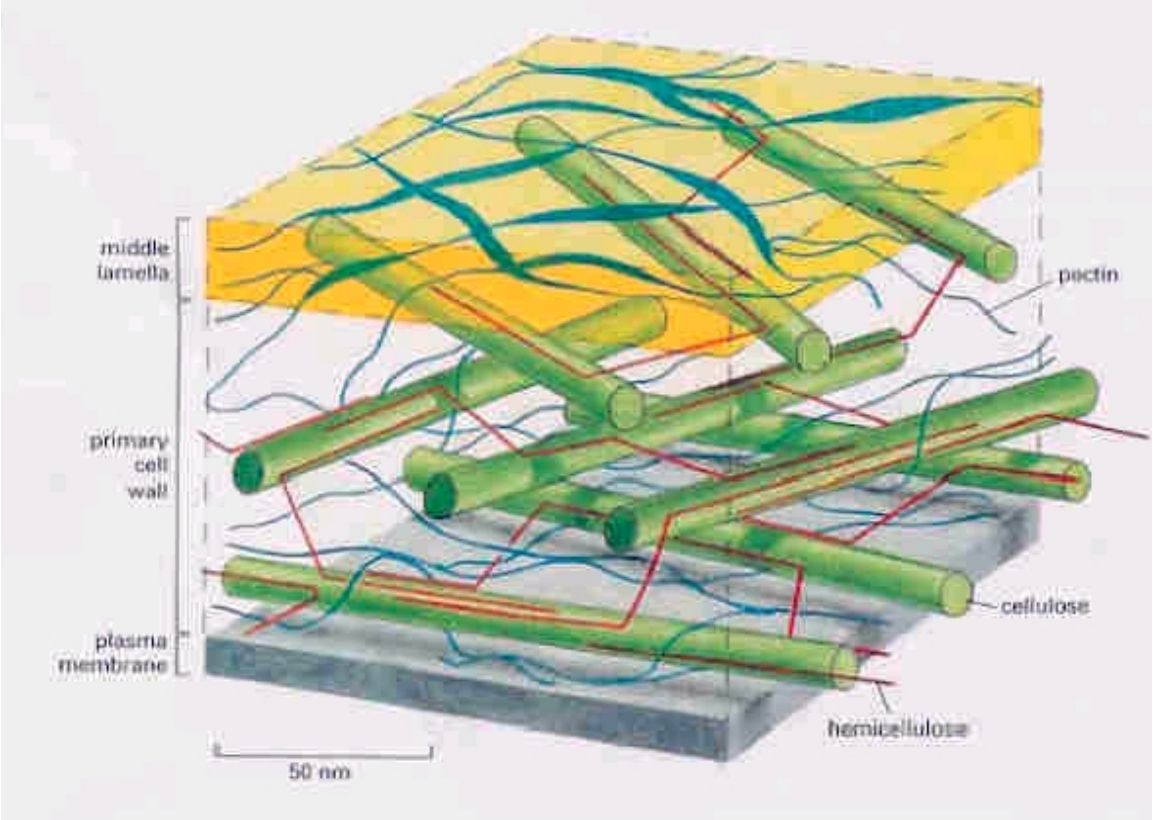

Figure 1: Illustration of the cell wall[5].

The increase in lignin within the cell wall aids in increasing its strength, making it impermeable to attacking pathogens[16]. Lignification combined with the necrosis of cells at the point of infection allows the plant to deprive the pathogen of water and nutrients. The HR is often accompanied by several other reactions that serve to prevent the further spread of an attacking pathogen.

Another common line of defense against the initial penetration of a pathogen is the deposition of callose, β1,3-glucan, inside the surface of the cell wall. The β1,3-linkage of callose produces a helical conformation that allows it to form either a gel or microfibrils. It is believed that when a pathogen disturbs the plasma membrane it causes an influx of calcium to enter the cell thus triggering the deposition of callose gel to the inside surface of the cell wall. This new layer of callose, known as a papilla, provides the cell with an additional barrier for the pathogen to pass through in order to reach the protoplast[17]. Hypersensitive response, increase in lignin, and the deposition of callose all act to confine the pathogen to its initial point of penetration.

The last two types of defenses against pathogenic attack, the accumulation of pathogenesis-related proteins, and the accumulation of phytoalexins, both actively work to counteract the presence of the attacking pathogen. Pathogenesis-related proteins are proteins coded for by the host plant, but are not induced until the presence of a pathogen is detected[18]. These proteins are therefore not defined because of their antipathogenic activities, but because of their increased presence during pathenogenic attack[19]. However, several of these proteins are known for their specific roles in preventing the invasion of pathogens. An example of a pathogenesis-related protein is Polygalacturonase inhibiting protein. It's goal is to limit the destruction to the cell wall

caused by certain pectin degrading enzymes and to regulate the activity of these enzymes. Phytoalexins, on the other hand, are non-specific phenolic compounds that destroy both the pathogen and the surrounding plant tissue upon their release[20-22]. All of these defense systems, hypersensitive response, callose deposition, accumulation of pathogenesis-related proteins, and the synthesis of phytoalexins help protect the plant from the wide array of pathogens, however, the plant must first be able to detect the presence of a pathogen before these defenses can be initiated.

Compounds that induce a defense response in plants are known as elicitors. Elicitors can be found in both the host, where they are known as endogenous elicitors, and the pathogen, where they are termed exogenous elicitors. Elicitors include a variety of compounds such as oligosaccharides, glycoproteins, peptides and lipids [21,23]. Some common elicitors from fungal cell walls include chitosan, which induce the formation of cell wall barriers such as papillae, and arachidonic acid, which causes the release of phytoalexins [24-26]. Several types of pectic fragments produced by the hydrolysis of the host cell wall serve as endogenous elicitors. One examples of this would be rhamnogalacturonan I, which induces proteinase inhibitors in sycamore [27,28]. Other well known elicitors produced by the action of polygalacturonases are oligogalacturonic acids with degrees of polymerization between 10 and 15. These elicitors are responsible for the induction of lignification, the synthesis of proteinase inhibitors, and the induction of phytoalexins[23, 27-32].

Polygalacturonases

Given that the cell wall is the plant's first line of defense against pathogens, a pathogen must be able to break through the cell wall in order to successfully invade the

plant. The primary means of accomplishing this is through the use of cell wall degrading enzymes. Polygalacturonases, the most abundant form of pectic enzymes, are the first cell wall degrading enzymes produced by fungi when grown on isolated cell walls[33,34]. This is consistant with observations that fungi initially attack at the middle lamella. The middle lamella contains the highest concentrations of pectic polymers, therefore, the fungus uses these enzymes as a pretreatment so that other cell wall degrading enzymes, such as cellulase, and hemicellulase, are able to successfully attack their substrate. The oligosaccharides that are produced by these enzymes serve as a carbon source for the pathogen, but they can also serve as early elicitors which give the plant time to defend itself before the pathogen is able to fully establish itself in the cell wall[35]. The complex structure of the cell wall polysaccharides, along with the plant's ability to defend itself, is often enough to provide the plant with a successful resistance to pathogens.

The diversity and complexity of plant cell walls has forced pathogens to evolve over time in order to ensure a successful invasion. The concentration of pectic polysaccharides, and the types of pectin available play an important role in how well a pathogen is able to invade its host. The difference in the amount of pectic polysaccharides found in germinaceous monocots versus dicots provides an example of how pathogens have adapted in order to overcome their host. The cell walls of germinaceous monocots contain less than ten percent of the amount of pectic polysaccharides than dicots. Therefore, a pathogen that feeds off of dicots must produce more pectin degrading enzymes then pathogens that use monocots as a host [36-38]. Along with being able to adapt to its host, a pathogen must also be able to overcome obstacles within its host. The actions of pectin degrading enzymes need to be altered due to the

complex structures of pectin polysaccharides. Furthermore, the products from the degradation of pectin polysaccharides by the action of endo-PGs can be greatly altered due to the distribution of methyl groups along individual pectin chains [39,40]. The diversity and complexity of the pectin polysaccharides found in plant cell walls can significantly alter the ability of the pathogen to penetrate the cell wall.

In order to combat the diversity presented by cell wall polysaccharides several isoforms of PGs may exist within a single fungus. Multiple isoforms have been isolated from numerous fungal species including the plant pathogen *Fusarium oxysporum*, molds such as *Trichoderma koningil*, *Aspergillus japonicus* and *Aspergillus niger*[41] the white-rot fungus *Phanerochaete chrysosporium*[42] and the brown-rot fungus *Postia placenta*[43]. The differences between isoforms affect their degrees of stability, specific activity, optimum pH levels, substrate preference, and pattern of hydrolysis[44,45,46,47]. The most recognizable and highly studied difference amongst PGs is their pattern of hydrolysis. PGs hydrolyse pectin polymers in either an endo or exo fashion. Endo-PGs generally randomly cleave internal regions of the homogalacturonan polysaccharide releasing oligogalactronic acid, whereas exo-PGs only cleave the non-reducing terminal end of a homogalacturonan polysaccharide releasing galacturonic acids[39,48]. A third class, combines the aspect of both the previous mechanisms, and hydrolyses PGs in both an endo and exo fashion. The variety of isoforms that exist, either within a single pathogen, or amongst different pathogens, may be the result of the wide range of hosts available for pathogens, as well as the complexity of the pectin network.

Due to the complexity of the pectin network a successful pathogenic attack is often not accomplished solely by the action of a single polygalacturonase isoform. A

pathogen can contain several isoforms of PGs and it is the combination of these isoforms that determines the success of the pathogen. A number of studies using either targeted gene deletions or recombinant clones that over express a PG gene have been performed to demonstrate the involvement of multiple PGs required for a successful pathogenic attack[10]. One such study was done using *Botrytis cinerea*, a fungus that causes gray mold rot or *Botrytis blight*. *B. cinerea* affects most fruits and vegetables as well as a large number of shrubs, trees, flowers and weeds. When one of the genes that codes for endopolygalacturonases was mutated a significant decrease in (but not the total elimination of) secondary infection was observed[49]. The concentration of polygalacturonases found within a fungus not only determines its ability to break through the complex pectin network of the cell wall, but it may also determine its ability to avoid resistance from the plant by rapidly digesting elicitor-active forms of oligogalacturonides.

When a pectic degrading enzyme digests the plant cell wall, oligogalacturonides with varying degrees of polymerization are released. Oligogalacturonides that exhibit degrees of polymerization (dp) in the range of ten to fifteen act as elicitors to the plant, signaling the presence of a pathogen, and thus provoking a defense response[50-52]. A determination of the activity of elicitor-active compounds may be performed by measuring the levels of phytoalexins, small lipophilic antimicrobial compound having static or toxic effects on fungal and bacterial growth[28]. Oligogalacturonides with a dp of less than 10 or more than 15 exhibited little or no induction of phytoalexins[53-58]. Defense responses observed due to these oligogalacturonides differ from plant to plant but include maceration of plant tissues, nercrosis of plant cells, lignification, accumulation of phytoalexins, and accumulation of protease inhibitors[30,31,56-59]. The oligogalacturonides

are only bioactive in the range of ten to fifteen dp, therefore further hydrolysis by pectin enzymes can quickly convert active elicitors into inactive ones[7,8].

Polygalacturonase inhibiting proteins

In order to control the direct damage to the cell wall caused by PGs, and to reduce the rate at which PGs diminish active galacturonic acid elicitors into inactive products, the plant produces a class of proteins known as polygalcturonase inhibiting proteins .[48] PGIPs have been found in a variety of dicotyledonous plants, such as peas, green peppers, tomatoes, cucumbers, apples, pears, oranges, and alfalfa, and some pectin-rich monocotyledonous plants such as onion and leek [44, 60-69]. In the absence of these proteins, PGs are able to hydrolyze elicitor-active oligogalacturonides to elicitor-inactive oligogalacturonides within fifteen minutes. However, in the presence of excess PGIP it can take up to forty-eight hours to degrade elicitor-active oligogalacturonides to elicitor-inactive oligogalacturonides[35]. This extra time allotted as a result of the interactions of PGIPs with PGs increases the lifetime of elicitor active oligogalacturonides and allows for their accumulation in plant tissues.

The ability of PGIPs to inhibit specific PGs appears to lie within a characteristic Leucine-rich repeat (LRR) motif found in its amino acid sequence. This motif affiliates PGIPs with a class of resistance genes where specific protein-protein interactions are required [70]. The leucines within this xxLxLxx motif create a hydrophobic core, therefore forming a solvent-exposed β-sheet/β-turn structure important for ligand binding[71,72]. Through the use of site-directed mutagenesis, Leckie et al. were able to demonstrate the importance of the amino acids that lie within the solvent-exposed β-sheet/β-turn structure to the specificity of interactions. Two PGIPs isolated from *Phaseolus vulgaris* were

used in their study. PGIP-1, which inhibits PG from *Aspergillus niger*, only varies by eight amino acids from PGIP-2, which inhibits PGs from both *A. niger* and *Fusarium moniliforme*. Five of these eight amino acids lie within the β-sheet/β-turn structure listed above. By replacing some of the variant amino acids of PGIP-2 with the corresponding amino acids of PGIP-1 a decrease in affinity for PG from F.moniliforme was oberved. In contrast, by replacing some of the variant amino acids of PGIP-1 with those of PGIP-2 an increase in activity was observed. The recognition capabilities of these proteins toward pathogenic PGs enable them to regulate the activity of the PGs, allowing for the accumulation of active elicitors, which ultimately provides the plant with the ability to resist fungal attack.

Although effective in their ability to regulate a wide range of PGs, PGIPs are typically only effective against fungal PGs, and not against other PGs of either microbial or plant origin[73]. The specificity of PGIPs for PGs requires the plant to contain a variety of isoforms of PGIPs to combat the number of isoforms of PGs secreted by fungi[44,71]. It is proposed that the combination and concentration of isoforms of PGIPs present within a plant and their specific interaction with the PG isoforms of the attaking fungi are one of the determinants as to whether the plant can successfully defend itself against fungi[13].

The study of the interactions of PGIPs with PGs plays an important part in the understanding of how plants are able to resist certain pathogens. The knowledge gained from these studies is of great importance to the agricultural industries; because it can lead to the engineering of pathogen resistant plants. Currently the use of chemical fungicides is the only means agriculturalists have in defending their plants against fungal pathogens. This type of solution is not only expensive but it is rapidly gaining opposition from the

"green" community. Therefore the understanding of how plants are able to resist pathogenic attack is rapidly gaining importance in the hopes of one day being able to genetically manipulate plants in order to give them enhanced resistance to pathogens.

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CHAPTER 2

SURFACE PLASMON RESONANCE/BIOMOLECULAR INTERACTION ANALYSIS-MASS SPECTROMETRY

Surface plasmon resonance/biomolecular interaction analysis

The investigation of protein-protein interactions has made great advances in the last few years due to the combination of surface plasmon resonance - biomolecular interaction analysis (SPR-BIA) with mass spectrometry (MS). SPR-BIA provides a rapid, sensitive, nondestructive means for analyzing the real time interactions between biomolecules[1-5]. SPR-BIA allows for the separation of proteins from a complex biological mixture with the added advantage of real time monitoring of the biomolecular interactions, which also allows for the determination of binding rates between molecules as small as 180 Da up to molecules as large as 150 kDa[6-9]. After binding interactions have been studied, and binding partners have been isolated, the power of SPR-BIA can be further enhanced by employing the characterization techniques of tandem mass spectrometry.

The basis behind SPR-BIA lies within its unique chip based optical biosensor that detects changes in the refractive index from a sensor surface. The sensor surface used in this technique consists of a glass slide, or prism, coated on one side with a thin film of gold. Once this metal film is placed at an optical interface where the incoming light source is totally internally reflected, any changes in the refractive index on the surface of the metal film is observed as a decrease in the reflected light intensity at a specific

angle[10]. Therefore, when one molecule is covalently bound to the sensor surface the interactions between it and any molecule found in a solution can be studied using SPR.

The research presented in this thesis uses the the Biacore 3000, a SPR/BIA instrument. Some of the advantages made available with the Biacore 3000 systems include a wide variety of sensor chips, a precise sample handling system, and an ability to recover bound material. The variety of sensor chips available increase the potential to study biomolecular interaction due to the different matrices attached to them. The matrix, which is covalently bound to the glass slide through a linker layer, not only serves as a surface where immobilization may take place but it also increases the binding capacity of the surface, therefore increasing the sensitivity of the instrument. Further, the matrix provides an environment, which limits non-specific binding to the surface, and provides a hydrophilic atmosphere suitable for most biological interactions[121]. The most widely used chip, and the one used in our studies, is the CM5 chip. The CM5 chip has a carboxymethyl dextran matrix, which allows for a wide range of immobilization techniques, such as surface thiol, ligand thiol, amine, avidin-biotin, and aldehyde coupling. The immobilization capacity on the surface of a CM5 chip is 50ng/mm^2 compared to the 1-5 ng/mm^2 immobilization capacity of an untreated gold surface. Carboxymethyl dextran is also advantageous because it is a linear dextran, which greatly increases the surface area, it is chemically stable in most biomolecular interaction buffers, and it does not deteriorate with short periods of exposure to extreme pH[11]. Other types of sensor chips available include SA (streptavidin) which captures biotinylated DNA, proteins, and lipids; NTA (nitrilotriacetic acid) which binds His-tagged ligands to

chelated nickel; HPA (hydrophobic absorption) which anchors membrane-bound ligands onto a hydrophobic surface; and a variety of pioneer chips used to solve problems with non-specific interactions experienced amongst any of the existing chips. Reproducible results with low nonspecific binding may be obtained as long as the correct sensor chip is chosen for immobilization. The success of biomolecular interaction analysis is therefore dependent on how well a molecule is immobilized to the sensor surface.

Immobilization of a biomolecule is done by first activating the chip surface with a suitable agent or sequence of agents, which depends on the immobilization technique chosen, followed by a washing step which removes any excess activation agent, injection of the biomolecule, and deactivation of any remaining active groups by injection with a suitable agent[12]. The most common and generally applicable form of coupling chemistry is Amine coupling, also used in our studies. In Amine coupling N-hydroxysuccinimide esters are introduced to the surface matrix by modifying the carboxyl groups through an injection of N-hydroxysuccinimide (NHS) and N-ethyl-N-(dimethylaminopropyl)-carbodiimide (EDC). The resulting esters provide an activated carboxyl for the covalent binding of amine groups and other nucleophilic groups to the matrix[12]. After immobilization has taken place any active sites that remain are deactivated using ethanolamine. Choosing the right sensor chip for immobilization may be important in how well the biomolecule is able to interact with its specific binding partners. If the correct sensor chip is selected immobilization of the biomolecule should not change its conformation in any way, therefore it should behave much as it would in solution, with the bulk of the biomolecule freely accessible for interactions. (Figure 2)

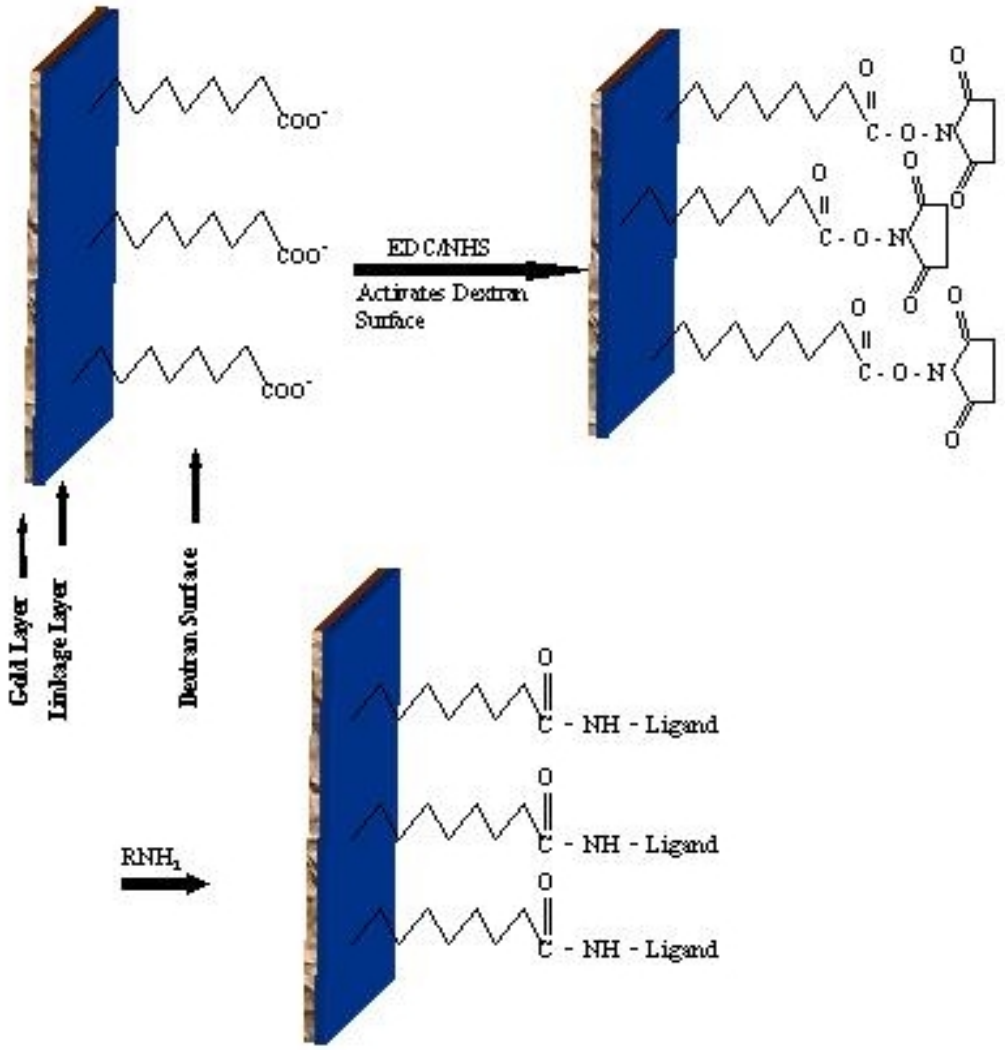

Figure 2: Amine coupling to a CM5 sensor chip.

Once a biomolecule has been successfully bound to the sensor surface, accurate binding interactions with any complimentary biomolecules are obtained as a result of the precision of the microfluidic flow system[9]. The microfluidic system is made up of an integrated micro fluidics cartridge (IFC), a hard polymer plate consisting of a series of precision-cast channels, and the sensor surface. The sensor surface forms one wall of the flow cells, while the IFC forms the remaining parts of the microfluidic system. A series of valves in the microfluidic flow system allows the user to switch between buffer and sample with a limited amount of dispersion between the two. The precision of the microfluidic flow system allows the sample to be passed over the sensor surface as a defined liquid segment in a continuous, pulse-free and controlled flow, which maintains a constant analyte concentration at the sensor surface[10]. With the ligand properly immobilized onto the sensor surface and the sample accurately delivered to the sensor surface the optical sensing technique of SPR is able to detect subfemtomole amounts of protein binding to the ligand[13].

In SPR, light is focused through the use of high output light emitting diodes onto the sensor surface, where a particular wavevector of it couples with the plasmon mode of the metal film, and the rest is reflected off the metal film to an optical photodetector. Any changes in the composition of the sensor surface results in a change in the effective refractive index of the metal film. This in turn affects the wavevector at which light couples into the surface plasmon mode of the metal. Therefore by measuring the plasmon coupling angle, and reflected intensity, any changes in the refractive index of the sample can be monitored[14]. In the case of most proteins, a change in concentration on the sensor surface of approximately 1pg/mm^2 is equivalent to a change of .001° in the angle

of the intensity minimum. This amount of change is recorded by the Biacore 3000 as one resonance unit (RU)[8]. All binding interactions that take place on the sensor surface can be viewed in real time as a plot of RUs versus time, known as a sensogram[11]. Based on the information acquired during a binding interaction analysis the specificity, concentration, kinetic rate constants, and binding affinities can be determined. After biomolecular interactions have been studied the interacting molecule can then be recovered from the surface and further analyzed using MS. (Figure 3)

Isolated proteins from the sensor surface can be recovered through the use of the microrecovery procedure. During the microrecovery procedure a series of small liquid volumes separated by air-bubbles pass over the sensor surface. One of the liquid volumes contains a recovery buffer, a reagent chosen by the user that is suitable for dissociating the binding pairs, into which the bound material will diffuse. The other liquid volumes consist of a wash solution segment and another segment of recovery buffer[16]. The procedure begins with the wash solution being injected over the sensor surface. This is done to ensure that all running buffer is removed from the sensor surface so that only the sample and recovery buffer will be recovered. Next a plug of recovery buffer is injected onto the sensor surface and the flow is stopped for a user specified amount of time in order to allow for the diffusion of the sample into the recovery buffer. Once the bound material has moved into the recovery buffer the flow is reversed and deposited into a collection vial. A second segment of recovery buffer is placed after the first one in order to prevent contamination of the recovered sample with running buffer. The microrecovery procedure can be run several times in order to obtain enough sample material for further analysis. Advantages of the microrecovery procedure are: low

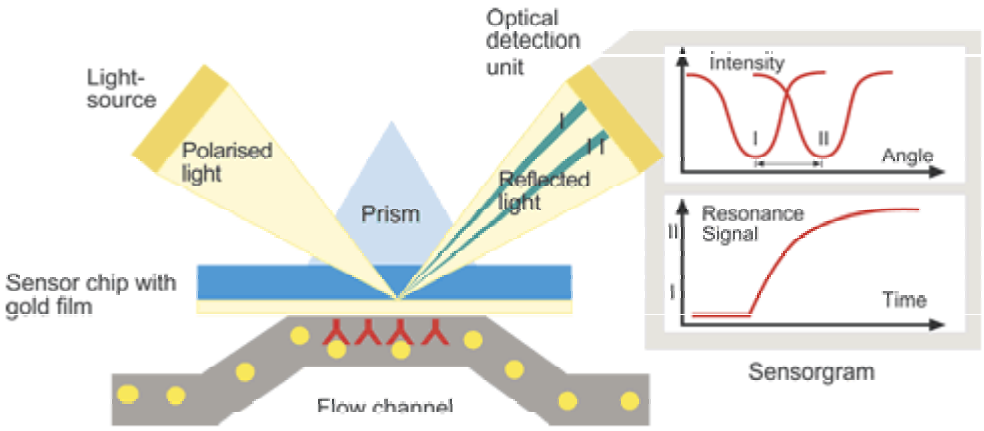

Figure 3: SPR technology[15]

sample volumes, ability to select reagents suitable for mass spectrometry and the ability to reuse the sensor surface for future analysis[17]. All the characteristics of a surface plasmon resonance – biomolecular interaction analysis instrument allow for the accurate investigation of binding events, and the non-destructive elution of the analyte from the sensor surface allows for the identification of the bound material using mass spectrometry.

Mass spectrometry

Mass spectrometry, a technique that allows for the identification of a biomolecule based on its molecular mass, has become the most sensitive technique used for the structural characterization of biomolecules[18]. The most commonly used MS technique for structural identification of unknown organic molecules is Electrospray-tandem MS. With this method the sample is introduced as a beam of ions into the mass spectrometer where a precursor ion is then mass-selected and induced to fragment giving structurally significant product ions[19]. The combination of electrospray ionization with the quadrupole mass filter and the time-of-flight mass analyzer provides a highly sensitive technique ideal for identifying unknown proteins.

The process of electrospray ionozation creates multiply charged ions that are ideal for the study of biomolecules. In ES ionization a sample that has been dissolved in a solvent is pumped through a thin metal capillary with a sharp pointed open end, which is held at a high potential of approximately 4V. The capillary typically has an inner diameter of 0.1 mm and is located about 1 to 3 cm from a large planar counter electrode that contains an orifice leading to the mass analyzer[20]. As the sample inside the capillary becomes charged it pushes out of the capillary tip in a form known as a Taylor cone. The

newly formed charged droplets then push away from one another forming a fine spray known as a plume. This plume then moves across the electric field where it passes through a jet of N_2, which evaporates the neutral solvent molecules from the charged droplets. As the droplets continue to travel down a pressure and potential gradient towards an orifice in the counter electrode they become more and more desolvated, which ultimately increases the charge concentration to a point where Coulombic repulsion overcomes the droplet's surface tension and the droplets explode. This process continues until individually charged analyte ions are formed[21,22]. The multiply charged ions are then introduced into the high vacuum mass spectrometer through a pinhole aperature[23,24]. The advantage of using ES over other ionization techniques is it provides a means for the direct analysis of polar and thermally labile biomolecules without a need for prior derivitization, and can be easily attacked to an HPLC[18]. (Figure 4)

The next step in our ES-QTOF setup is the quadrupole analyzer. Once the multiply charged ions pass through the pinhole aperture they then proceed through four parallel rods with fixed DC and alternating RF voltages applied to them. The electric field produced by these voltages causes ions of a particular m/z to be focused on the detector while all other ions are deflected into the rods. Varying the strengths and frequencies of the electric fields allows for different ions to be detected, producing the mass spectrum. The advantages provided by the quadrupole are its simple structure and fast scanning ability[20, 25]. (Figure 5)

After the ions have been mass selected by the quadrupole they can then be broken down and further analyzed with the use of a second mass analyzer. After leaving the quadrupole mass analyzer the ions are focused onto a collision cell where they

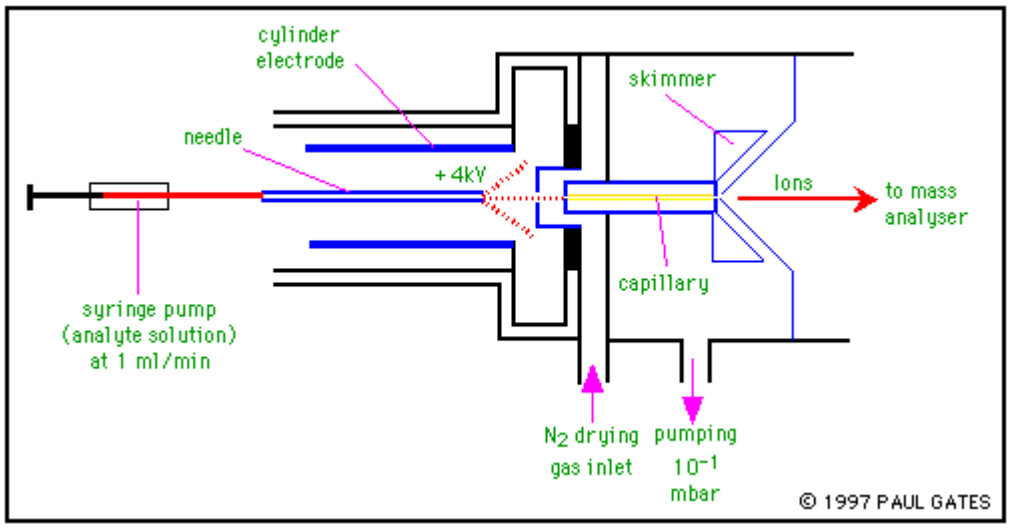

Figure 4: Illustration of Electrospray ionization[26].

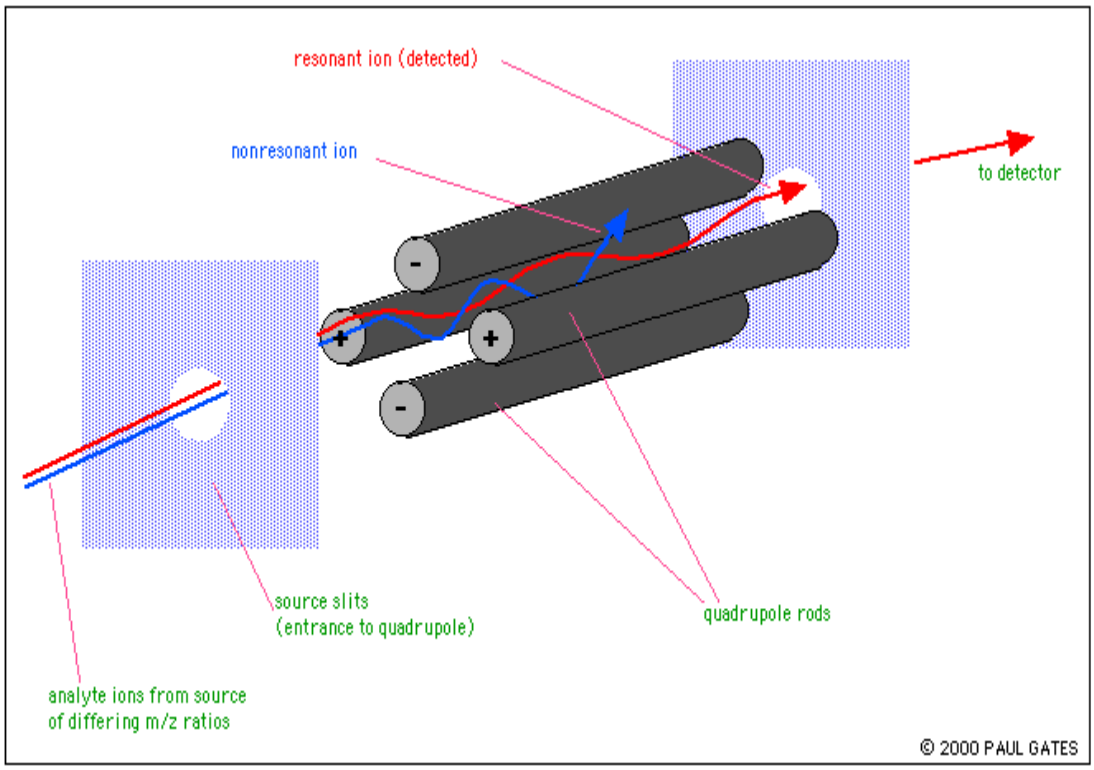

Figure 5: Illustration of quadrupole[27].

collide with inert gas. The collisions between the ions and the inert gas molecules cause the translational energy of the precursor ions to be converted into internal energy, therefore, causing the ion to decompose. Once the ions have fragmented they are introduced into the TOF analyzer. The simplest type of charged-particle analysis is the TOF analysis[25]. Ions are simply accelerated down a flight tube due to a voltage that is applied at the entrance of the TOF analyzer. Since all of the ions receive the same acceleration voltage their velocity down the flight tube is dependent upon their mass[20, 25]. ES/Q-TOF has gained great recognition for the identification of unknown biomolecules due to its high sensitivity, simplicity, and rapid data acquisition rate. (Figure 6)

The use of SPR-BIA in conjunction with MS provides a highly sensitive technique that allows for the rapid analysis of interacting biomolecules. With its ability to detect the presence of binding partners, calculate such things as kinetic rate constants, concentrations, and binding affinities, and allow for further analysis of recovered biomolecules with MS, SPR-BIA is rapidly gaining popularity in the proteomics communitiy[28]. The wealth of information that can be gained from the combination of these two systems opens the doors to a better understanding of the molecular basis of the interactions seen between plants and pathogens, with the possibility of someday engineering pathogen resistant plants.

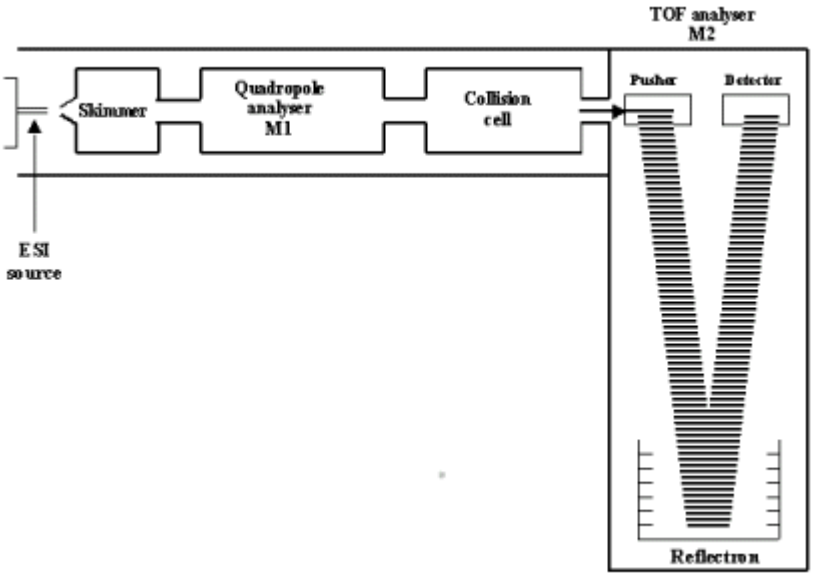

Figure 6: Illustration of MS/MS[29]

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CHAPTER 3

RESEARCH

Introduction

The interactions between the secreted fungal polygalacturonases and the plant regulatory polygalacturonase inhibiting proteins have proven to be complex. For example, as discussed earlier, the mutation of only a few amino acids within the solvent exposed β-sheet / β-turn region of the leucine rich repeats (LRR) can markedly alter the specificity of the inhibitory activities of PGIPs toward certain PGs[1]. This suggests that point mutations are the major driving force for the adaptation of the PGIP families.(Stotz et al) The differences recently observed in the interactions of constitutively expressed PGs versus induced PGs with PGIPs demonstrates an important yet complex aspect of PG/PGIP interactions. Interactions with PGIPs usually lead to inhibition for induced PGs, however the presence of PGIPs has lead to both activation and inhibition for constitutively expressed PGs[2]. Through the use of surface plasmon resonance/biomolecular interaction analysis-mass spectrometry (SPR/BIA-MS) the hypervariable regions of PGIPs that appear to be crucial to specificity of interactions as well as any differences between constitutively expressed and induced PGs that may attribute to the observed responses, can be studied.

A major focus of study for understanding how polygalacturonase inhibition is controlled in plant species is the LRR motif found in PGIPs. This LRR motif closely relates PGIPs to several plant and mammalian resistance (R) proteins, which are involved

in specific protein-protein interactions[3-6]. The structural characterization of these LRR proteins was first studied in 1993 through the crystallographic studies of porcine ribonuclease inhibitor (RI)[7]. In these studies it was shown that the leucine residues found in the LRR motif formed a hydrophobic core producing a β-sheet/β-turn structure, and exposing the amino acid side chains to the surrounding solvent [8]. (Figure 7) Later it was determined that the binding sites responsible for the specific recognition of porcine RI for RNAse lay within this solvent exposed β-sheet/β-turn region of the LRR motif [7,9]. These studies led to a better understanding of how specific interactions, driven by the products of resistance genes, are accomplished.

Modeling studies performed on PGIPs show similar characteristics of the LRR motif to those found in porcine ribonuclease inhibitor where the β-sheet/β-turn structures form a solvent exposed surface[1]. Also, like porcine RI, the contact sites responsible for specific interactions are believed to lie within the solvent exposed β-sheet/βturn region of the LRR motif. A mature PGIP contains 10.5 LRR, which ultimately provides the different genes that code for PGIPs with altering recognition patterns and allows for the evolution of new recognition properties with just a few amino acid substitutions[10]. Several plant species, such as raspberry, tomato, pear, apple, Arabidopsis, bean and soybean have been found to contain more than one PGIP gene[11-15]. The presence of multiple PGIP genes presents the plant with a composite of different inhibitory abilities, therefore improving its resistance to a variety of fungal species.

P. vulgaris has been found to contain at least 5 PGIP genes. Two members of the pgip gene family, pgip-1 and pgip-2, differ from one another by only 26 nucleotides with eleven of them being nonsynonymous substitutions that occur within or very close to the

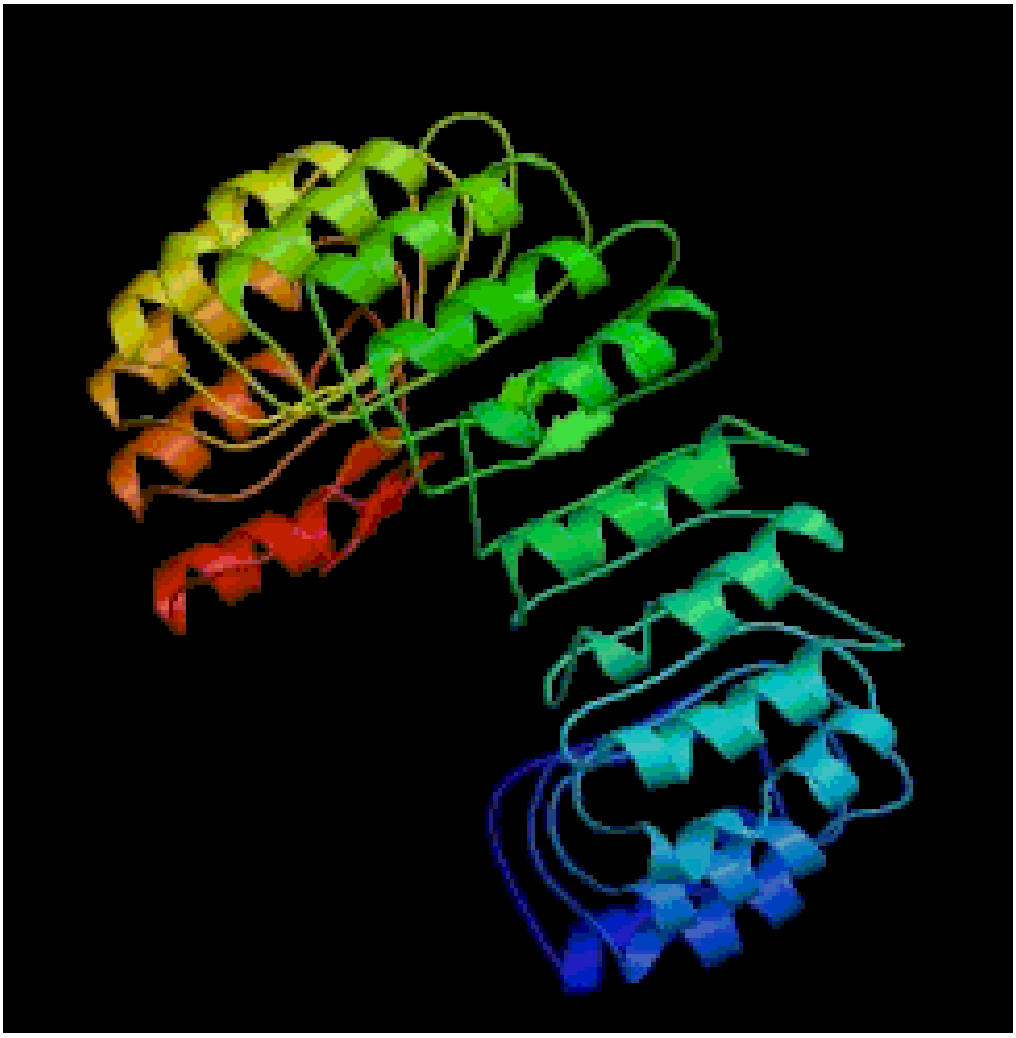

Figure 7: Structure of Porcine Ribonuclease Inhibitor

β-sheet/βturn region formed by the LRR[1]. Two other members of the pgip gene family pgip-3 and pgip-4 share a 93.4% identity with one another, but only share a 79% identity with pgip-1 and pgip-2. The PGIP gene products characterized by these genes are distinguishable not only by their sequences but also by their ability to inhibit different PGs.

The differences found between the PGIPs expressed by pgip genes provide the plant with its total inhibitory activity. One example of this comes from a study performed by Desiderio *et al*. where they compared the inhibitory abilities of *P. vulgaris* PGIP-1 overexpressed in transgenic tomato plants to that of PGIPs isolated from *P. vulgaris*[16]. In this study they were able to demonstrate that bulk bean PGIP is able to completely inhibit PGs from *F.moniliforme* and *B. cinerea*, where PGIP-1 was unable to inhibit PGs from *F. moniliforme* and only slightly able to inhibit PGs from *B. cinerea*. They then took this information and compared it to the result of inhibition tests performed on an isolated PGIP obtained from a *P. vulgaris* PGIP mixture, which showed similar inhibition characteristics to the PGIP produced from the pgip-1 gene, and deduced that *P. vulgaris* contains multiple PGIP forms which ultimately lead to its total capabilities to resist a wide range of fungi.

On the opposite end of the pathogen-plant interaction is the ability of the fungi's polygalacturonases to avoid inhibition by the plant, therefore opening the way to a successful attack. The presence of several isoforms of PGs in a single fungus is believed to have evolved in order to overcome the complex pectin network of the cell wall. It is also possible that they evolved in order to avoid inhibition by plant PGIPs. However, constitutively expressed PGs have demonstrated a different response to PGIPs than what

is believed to be the normal response observed by induced PGs. For induced PGs an interaction with PGIPs has always been shown to slow down the hydrolytic actions of the PG. In constitutively expressed PGs, it has been reported that at pH 5.0 (the pH of the plant before enzymatic digestion occurs) the hydrolytic actions of these PGs actually increase in the presence of PGIPs[2]. The reason for these differences are unknown, however further studies by SPR/BIA could lead to a better understanding of the roles of constitutively expressed PGs versus those of induced PGs.

The research presented in this paper lays the groundwork for studying the binding interactions of PGs with PGIPs using the Biacore 3000 SPR/BIA instrument. The principal advantages of this technique over the traditional techniques used in studying PG/PGIP interactions is its ability to study interaction in real time in a non destructive, label free environment. This capability allows us to study the effects of point mutations on the interactions of PGIP with PGs, the kinetic rate constants and binding affinities of PGIPs to PGs, and the differences in binding interactions seen between constitutively expressed PGs and induced PGs. Another advantage presented by the Biacore is its ability to separate PGIPs from a complex mixture in a more rapid and cleaner means than the traditional separation technique of affinity chromatography. Following the elution of bound materials from the sensor surface further analysis using MS could lead to the possible identification of new isoforms of PGIPs. The wide range of potential experiments that can be performed using this technique will hopefully allow us to gain a better understanding of the selection pressures of PGs and PGIPs and how variations in both PGs and PGIPs affect the plant's ability to resist fungi.

Methods and materials

Polygalacturonase B and Polygalacturonase II

These PGs were kind gifts from the laboratory of Jaap Visser, Wageningen University. They were both obtained as previously reported[17,18].

Recombinant and native PGIPs

Recombinant PGIP-1 and PGIP-2 were kind gifts of Dr. Felice Cervone (University of Rome, "La Sapienza"). Native *P. vulgaris* cv. Pinto PGIPs were separated into two separate pools, corresponding in activity to PGIP-1 and -2, by affinity chromatography on an *F. moniliforme* EPG affinity column, as reported in Desiderio *et al*[16]. Native PGIP-1 was further purified on an *A. niger* PG I affinity column. All native bean PGIPs were purified by ion exchange on a Hi-Trap S cartridge as a final step [19].

Pear PGIP

Purified as per Stotz et. al.[20]

Immobilization

Standard Biacore 3000 protocol was used for the amine coupling of PGs to a CM5 sensor chip. The running buffer used in the immobilization processes was a sterile filtered and degassed solution of 0.01 M HEPES pH 7.4, 0.15 M NaCl, 3 mM EDTA, and 0.005% Surfactant P20. A mixture of .1M N-hydroxysuccinimide (NHS) and .4N-ethyl-N'-(dimethylaminoproppyl)carbodiimide (EDC) was injected for 7 min. over the sensor chip in order to activate the carboxyl groups of the matrix. Next a solution containing 30µl of PGB in 300 µl of 10mM Sodium Acetate buffer at pH 5.0 was injected over the sensor surface for 1 hour at a flow rate of 5µl per minute. The last step of immobilization

consisted of a 7 minute injection of 1M ethanolamine hydrochloride pH 8.5 over the sensor chip in order to block any remaining active sites.

Reducing sugar

Generation of reducing sugar during EPG-catalyzed hydrolysis of homogalacturonan was determined by the PAHBAH (p-hydroxy benzoic acid hydrazide) colorimetric assay[21], as previously reported [22]. For PGIP-inhibition assays, a 10% excess of PGIP was added, to ensure maximum inhibition of PG activity. Experiments were performed at least in duplicate.

Tandem MS

The recovered sample was introduced into the Q-TOF2 (Micromass) mass spectrometer using a Waters Capillary LC. The original Capillary LC solvent delivery configuration was modified to minimize the dead volume between mixer and nanocapillary column. The mobile phases used for gradient elution consisted of water (A) with 0.1% formic acid and acetonitrile (B) with 0.1% formic acid. The gradient conditions used to elute the peptides were 10% B to 70%B in 70 minutes with a flow rate of 0.180 µl per minute. The Q-TOF2 was operated in a data-dependant scan mode. The survey MS spectra were acquired from 450 – 1700 and the switch criteria for MS to MS/MS mode were the ion count and charge state. The Q-TOF was set to ignore singly charged ions and acquire MS/MS data for up to 3 co-eluting peaks. The collision energy was varied depending on the peptide mass and charge state.

Results

In these experiments we were able to prepare a sensor chip capable of performing multiple analyses of PG/PGIP binding interactions. Requirements for such a chip

included an RU greater than 1000 for the immobilized PG, a blank surface to test for non-specific binding to the sensor chip surface, and a recovery solution capable of completely dissociating any PG/PGIP interactions. Due to the relatively low pIs found in most PGs several steps had to be taken to accomplish an immobilization RU of greater than 1000. First a binding solution of 10mM NaAc at pH 4.0 demonstrated the highest amount of RUs for immobilization of PGB to a CM5 sensor chip. Doubling the concentration of the NHS/EDC mixture from the standard protocol also allowed for optimum amounts of PGs to be bound to our sensor surface. This permitted for the activation of more carboxyl groups than the standard protocol allowed, providing a greater density of active sites for immobilization. Next, if concentrations are determined, through electrostatic interactions, to be near saturation levels an injection time of one hour is sufficient to maximize immobilization of PGs to our sensor surface. Times greater than one hour did not lead to any further increase in binding; therefore we can deduce saturation of our sensor chip is reached at one hour. A final step of doubling the injections of ethanolamine hydrochloride was taken to ensure the sensor chip was optimized for studying binding interactions. We found that with only one injection we were not able to block all the active sites, leaving behind areas where our analyte could bind to the surface and give false binding responses. Following these steps enabled us to obtain consistent immobilizations with over 1000RUs of PGs bound to our CM5 sensor chip.

Following preparation of our PGB surface, we prepared a blank surface that allowed us to test for any non-specific binding to our sensor chip. The blank surface was activated with the same concentrations of NHS/EDC used in the PG immobilization, and immediately deactivated using two injections of ethanolamine hydrochloride. We then

used this blank surface to test for non-specific binding to the sensor surface in all of our binding studies. A buffer of 50mM NaAc, .15M NaCl, and 25mM MES pH 5.0 was used for both the running buffer and for sample dilutions. Binding to the blank flow cell was observed in situations where less than 1000 RUs were bound to the sensor surface or buffer solutions with lower salt concentrations were used. In binding studies where more than 1000 RUs of PG was immobilized to the sensor surface and the above running buffer was used no binding was observed in the blank. (Figure 8)

The CM5 sensor chip used in our binding studies consisted of a blank flow cell, followed by a flow cell with PGB immobilized onto it, and then a flow cell with PGII immobilized onto it. The binding observed on our PGB surfaces demonstrated no specific selection for binding with differing PGIPs, where binding studies performed on our PGII surface showed binding of PGIP-2, and no binding of pear PGIP. (Table 1) The binding interactions for PGII are consistent with the inhibition results from reducing sugar assays; however inhibitions results from reducing sugar assays show no inhibition of PGB from pear PGIP.

Following binding studies a microrecovery procedure was performed that removed any bound material from the sensor chip so that further studies requiring tandem MS could be performed. The microrecovery procedure consisted of a 5 μL injection of native PGIP solution, followed by a 5 μL injection of 0.01 M HEPES pH 7.4, 0.15 M NaCl recovery buffer. All binding of PGIP to the PG surface could be removed with 0.01 M HEPES pH 7.4, 0.15 M NaCl. Once the native PGIP dissociated from the PG into the recovery buffer the flow was then reversed and the recovered native PGIP was collectedin a vial. The amount of sample recovered from one microrecovery was not

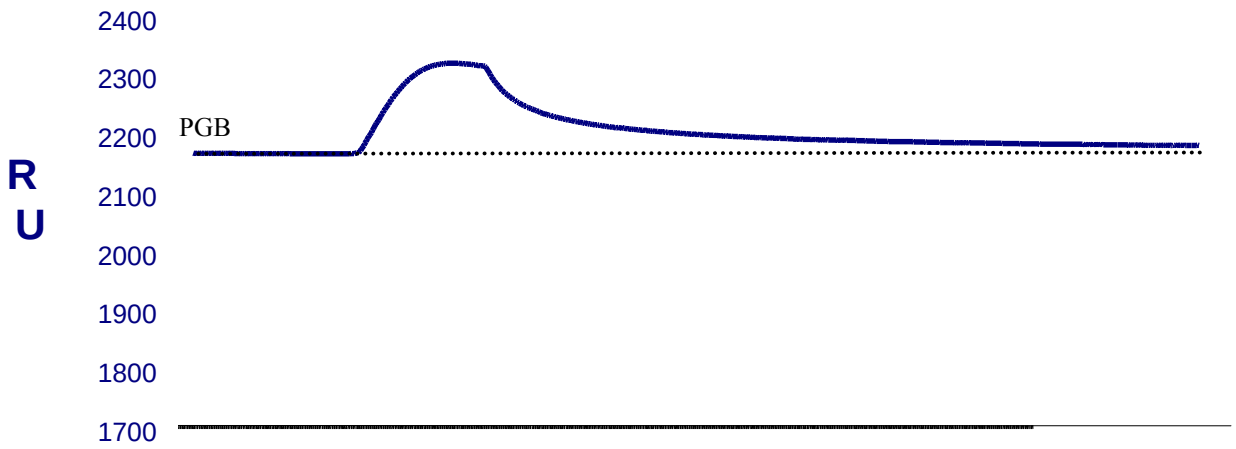

Figure 8: Binding interactions of native PGIP to PGB ~ and native PGIP to a blank flow cell. ***** PGIP only binds to surface with bound PGB.

Table 1: Observed binding interaction of PGB and PGII to Pear PGIP and PGIP-2.

	PGB	PGII
Pear PGIP	Binding	No Binding
PGIP-2	Binding	Binding

sufficient for further studies; therefore, the microrecovery procedure was performed in 10 repeating loops, and recovered sample was collected into the same vial. Once the native PGIP had been recovered, the presence of native PGIP was verified using tandem MS.

Prior to injecting the recovered PGIP into the Q-TOF2 (Micromass) mass spectrometer, the recovery buffer was removed from the sample using a 10,000 molecular weight cut off centrifugal filter. The recovered sample was then added to 10 µl of .1M NH_4HCO_3 and 10µl of 1mg/ml modified porcine sequencing grade trypsin and placed in a 37°C oven for 36 hours. After digestion, the sample was concentrated and purified using a Millipore® $ZipTip_{C18}$. The recovered, digested sample was injected into the Q-TOF2 (Micromass) mass spectrometer and several LRR sequences were detected. (Figure 9-11)

Discussion

The research presented in this paper lays down the groundwork for studying PG/PGIP interactions using SPR/BIA-MS. Past studies using SPR/BIA have demonstrated the ability of this technique for studying interactions between PGIPs immobilized onto a sensor surface with the PGs present in a passing solution, and its capability for studying the effects of point mutations on inhibition[23]. However in order to increase our ability to study variations in PGIPs, and differences in constitutively expressed PGs versus induced PGs, without the cost of using large numbers of sensor chips, we designed a means to gain optimal binding results with immobilized PGs on our sensor surface.

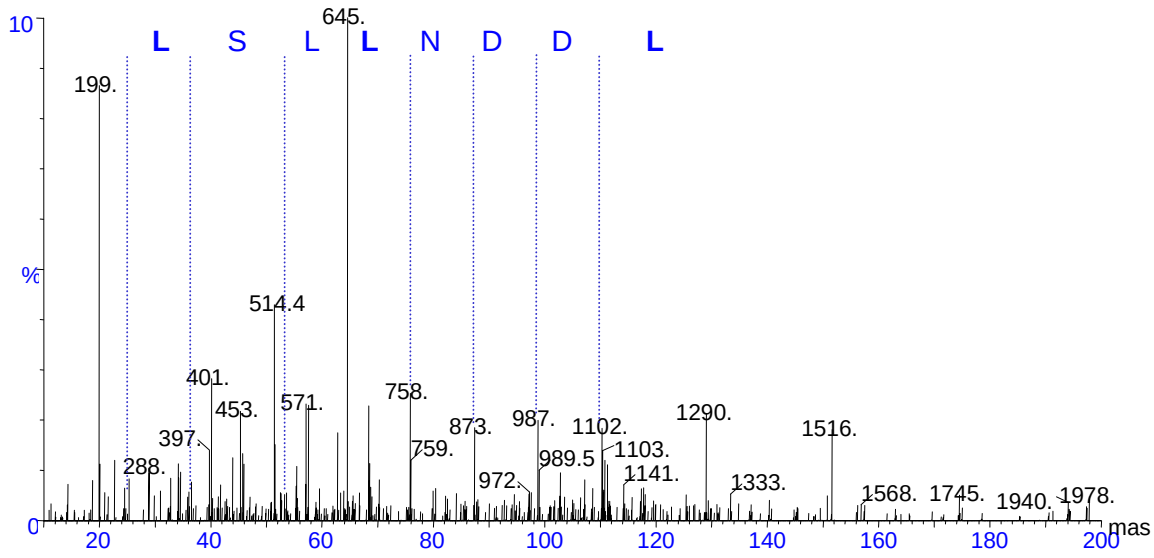

Figure 9: Example of a peptide sequence obtained from MS/MS data. Only the Y ions are labeled. The Leucine Rich Repeat motif is indicative of a PGIP.

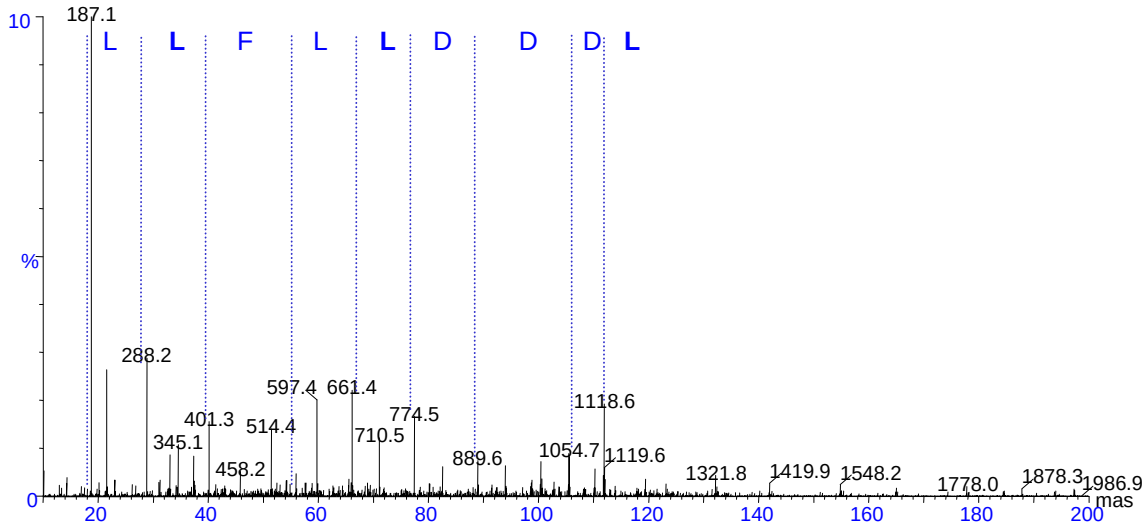

Figure 10: Example 2 of a peptide sequence obtained from MS/MS data. Only the Y ions are labeled. The Leucine Rich Repeat motif is indicative of a PGIP.

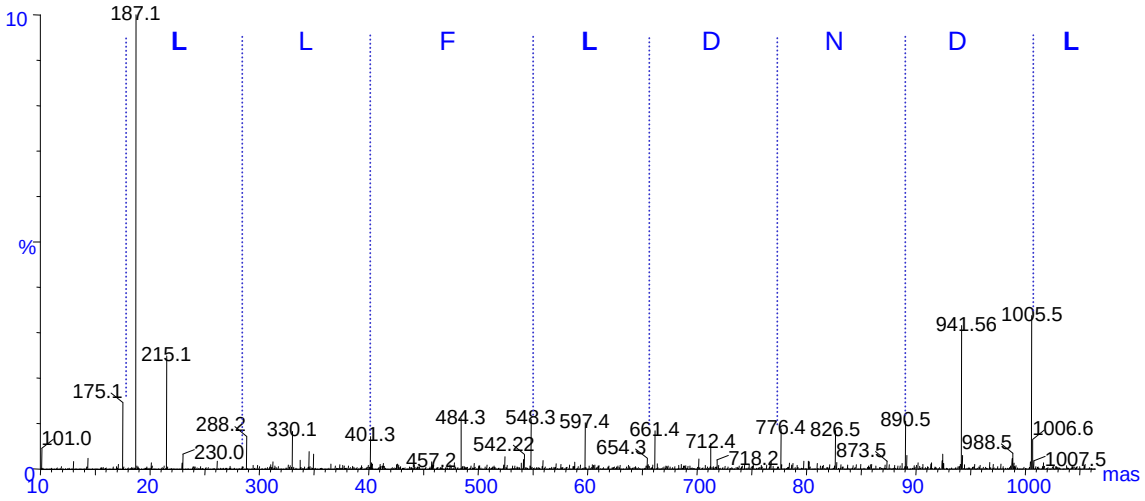

Figure 11: Example 3 of a peptide sequence obtained from MS/MS data. Only the Y ions are labeled. The Leucine Rich Repeat motif is indicative of a PGIP.

In order to demonstrate the capabilities of our binding surfaces we tested the binding interactions of PGIPs with two different PGs immobilized onto our sensor surface. The first PG, PGB, was immobilized onto one flow cell on our sensor surface, and PGII was immobilized onto a second flow cell on our sensor surface. With the assumption that inhibition of PGs occurs when PGIPs bind to them we concluded from reducing sugar assays that we should see binding between PGB and native PGIP-2, but not between PGB and pear PGIP. We should also see binding between PGII and native PGIP-2, but not between PGB and pear PGIP. However, the data we obtained from the Biacore only followed the expected binding patterns for PGII, and not those for PGB. Upon further investigation of our selected PGs we decided that these interactions should not be looked at as a demonstration that our surfaces are not capable of showing specific interactions, but as a possible result of our assumption that binding leads to inhibition. As demonstrated in the study discussed in the introduction constitutively expressed PGs, such as PGB, have demonstrated both an activation and inhibition when in the presence of PGIP. Therefore, our deduction that pear PGIP should not bind to PGB because it does not inhibit it may be wrong. Further studies using SPR/BIA can help us gain information on the kinetic rate constants and binding affinities of these two different types of PGs, which may lead to a better understanding of their roles in pathogenic attack.

Another aspect of binding PG to our sensor surface that enhances the capabilities of the Biacore is its ability to recover bound materials from its sensor surface. Due to the similarities in the molecular weight and amino acid sequence found in PGIP isoforms it is difficult to separate them by size exclusion, ion exchange or ConA columns. The most

popular technique used for separating individual PGIP isoforms from a complex mixture is affinity chromatography. The new technique of SPR/BIA replaces the packed columns of beads found in affinity chromatography columns with a thin-layer surface, and replaces post-column detectors with a single surface-integrated detector. These advances allow for cleaner separations, more rapid analysis, label free detection, and a smaller sample requirement for creating a specific separation surface. In our results we demonstrated the ability of the Biacore to bind recombinant PGIP to a PG surface, dissociate the bond formed between them, and recover the bound material in a collecting vial. We then analyzed the recovered sample using tandem MS and were able to verify the presence of recombinant PGIP.

The groundwork laid out in this research has provided us with a highly sophisticated means of studying the complex interactions of PGs and PGIPs. With the added advantages of being able to study kinetic rate constants and binding affinities in a nondestructive label free environment, SPR/BIA-MS leaps ahead of the conventional techniques used for studying PG/PGIP interactions. Now, instead of just being able to see if point mutations have any effect on binding interactions between PGs and PGIP we should be able to determine how much of an effect they have. We should also be able to study the correlations between binding and inhibition, as well as be able to isolate and recover PGIPs from a complex mixture in a search for new isoforms. The potential of this technique will hopefully lead to a better understanding of PG/PGIP interactions with the possibility of someday engineering pathogen resistant plants.

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CHAPTER 4

CONCLUSION

The complexity in the interactions between polygalacturonases (PGs) and polygalacturonase inhibiting proteins (PGIPs) have forced scientists to continuously look for new techniques that will help determine the presence and strength of binding interactions between different isoforms of PGs and PGIPs. New studies of these types of interactions are made possible with the use of surface plasmon resonance/biomolecular interaction analysis (SPR/BIA). SPR/BIA allows for the determination of the presence of binding partners and calculates the kinetic rate constants. An added advantage of SPR/BIA is that it is a nondestructive technique that does not require the use of labels. Therefore, after all analysis has been performed the bound material can be further characterized with the use of mass spectrometry.

The different isoforms of both PGs and PGIPs have demonstrated a range of different activities when in the presence of one another. For example, minor mutations in the leucine rich repeat motif found in PGIPs has been shown to alter its specificity for different PGs[1]. Further, the activity of some constitutive PGs has shown to increase in the presence of PGIPs at pH of 5.0, but when the pH drops below 4.5 they become inhibited by the PGIPs[2]. The differences in whether a PGIP binds to a PG or whether it activates or inhibits that PG can be studied using SPR/BIA.

The research presented in this thesis lays the groundwork for studying the interactions of different isoforms of PGs and PGIPs using SPR/BIA. An immobilization

procedure for PGs to a CM5 sensor chip was determined, the amount of PG that needs to be immobilized and the buffers that needs to be used in order to limit nonspecific binding to the sensor chip have been determined. A procedure for the microrecovery and further analysis by mass spectrometry of the bound PGIPs has also been characterized. These findings will allow us to study binding affinities, kinetic rate constants, and isolate PGIPS from a complex mixture, that will hopefully lead to the engineering of fungal resistant plants.

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