DISSERTATION

STUDY OF BIOACTIVE PROTEINS IN THE ROOTS AND ROOT EXUDATES OF MODEL PLANTS

Submitted by

Clelia De-la-Peña

Department of Horticulture and Landscape Architecture

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY CLELIA DE LA PEÑA ENTITLED STUDY OF BIOACTIVE PROTEINS IN THE ROOTS AND ROOT EXUDATES OF MODEL PLANTS BE ACCEPTED AS FULFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

Committee on Graduate Work

Cecil Stushnoff, Ph.D.

Mark Paschke, Ph.D.

Daniel Manter, Ph.D.

Jorge M. Vivanco, Ph.D. (Advisor)

Stephen J. Wallner, Ph.D. (Department Head)

ABSTRACT OF DISSERTATION

STUDY OF BIOACTIVE PROTEINS IN THE ROOTS AND ROOT EXUDATES OF MODEL PLANTS

The plant root system serves many roles, including anchorage and uptake of nutrients and water. The ability of roots to release a wide range of organic and inorganic compounds into the rhizosphere to communicate with roots of other plants and other organisms has been the focus of recent studies. Among the compounds released into the rhizosphere, proteins comprise an important amount of energy secreted by roots but have not been studied in detail.

In the present study, I conducted a proteomic and enzymatic analysis of *Arabidopsis thaliana* root exudation across a developmental gradient to track the changes that occur in the root-secreted proteins at different plant developmental stages. Further, I found that the secretion of proteins (including pathogenesis-related [PR] proteins, myrosinases, and enzymes related to protein refolding) was qualitatively and quantitatively related to the growth stage of the plant. For instance, the intensity and activity of PR proteins such as chitinases were higher at peak flowering times than at any other time during Arabidopsis development.

I also studied the root secretion of proteins by two model plants (*Medicago sativa* and *A. thaliana*) during their interaction with the symbiont of one of these species (*Sinorhizobium meliloti)* and with an opportunistic pathogen of *A. thaliana* (*Pseudomonas syringae* pv. tomato DC3000). I found that the early interactions between *M. sativa* and *S. meliloti* induced exudation of enzymes such as acid chitinases, thaumatin proteins, PR10 and PR1 proteins. However, these proteins were not induced when *M. sativa* was inoculated with *P. syringae* DC3000. In addition, I found that *P. syringae* DC3000 could differentially induce the secretion of proteins related to defense in *A. thaliana*, whereas *S. meliloti* did not provoke the same response.

The final study of my dissertation focused on the activity of ribosome-inactivating proteins (RIPs, EC 3.2.2.22) in *Arabidopsis thaliana*. Based on amino acid sequencing, it was determined that the purified RIP had homology to the mature form of a pectin methylesterase (PME, *At1g11580*); this purified protein showed PME activity. Further the *A. thaliana* full-length and mature PME forms were cloned into the expression vector PQE30 and both constructs were expressed in *Escherichia coli*.

Clelia De la Peña

Department of Horticulture and Landscape Architecture

Colorado State University

Fort Collins, CO 80526

Fall 2007

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CHAPTER 1

Understanding the role of bioactive root-exuded proteins in nature

1.1 INTRODUCTION

Roots have many functions in nature (Marschner, 1995), but an important function is the capacity to release a wide range of organic and inorganic compounds into the rhizosphere (Rovira, 1969; Hinsinger, 1998; Bertin et al., 2003; Bais et al., 2004; Uren, 2007). The rhizosphere is defined as the soil closely surrounding the plant roots (Kang and Mills, 2004) and it is influenced by the flux of mineral nutriments into the plant roots and accumulation of plant root exudates in the surrounding soil. In addition, the rhizosphere is a place where soil, plants, and microorganisms interact, and it is also an environment in which roots and microorganisms can mutually benefit by utilizing each other's exuded organic materials (Harrison, 2005; Shaw et al., 2006) or, in contrast, in which some organisms are inhibited by the secretions of others (Macias et al., 2003). Moreover, the exudation of root compounds into the rhizosphere is influenced by the microbial population, the availability of nutrients, and plant competition with other plants (Reynolds et al., 2003).

Recognition of the importance of root exudates has increased as new technologies have been developed and new ecological problems have arisen. Root exudation plays important roles in biological, biochemical and ecological processes (Rovira, 1969; Vancura and Hanzlíková, 1972; Currier and Strobel, 1976; Bertin et al., 2003; Nóbrega et al., 2005; Liao et al., 2006; Bais et al., 2006). Organic rhizodeposition consists of border cells, mucilage, carbon-containing compounds, and proteins that are released from root cells into the soil (Rovira, 1969; Vancura and Hanzlíková, 1972; Uren, 2007). These compounds play a role as intermediaries in negative and positive interactions with other organisms (Bais et al., 2006). For instance, plants can inhibit the

growth of other plants by secreting allelopathic metabolites from roots (Macias et al., 2003; Belz and Hurle, 2005). Plants under attack can also release root exudates as a defense response (Guerrieri et al., 2002), or can even secrete flavonoids to induce *nod* genes in bacteria for nitrogen fixation processes (Scheres et al., 1990; Perret et al., 2000; Suominen et al., 2003). In addition, secondary metabolites from root exudates are involved in mycorrhizal associations (Tamasloukht et al., 2003), plant growth-promoting bacteria (Steenhoudt and Vanderleyden, 2000), quorum sensing (Teplitski et al., 2000; von Bodman et al., 2003), and rhizoremediation (Kuiper et al., 2004). On the other hand, while there has been exhaustive investigation of the secondary metabolites secreted as root exudates, the role of most root-exuded proteins in the rhizosphere is largely unknown.

In annual plant species, 30-60% of the photosynthetically fixed carbon is translocated to the roots, and around 70% of that carbon can be released into the rhizosphere (Rovira, 1969). Proteins can also be secreted by roots but relatively little is known about their identity or function, or about the processes related to protein exudation from roots.

Proteins are usually transported by vesicles to different destinations in the cell. While passing through the Golgi apparatus, proteins are separated from other proteins destined for the vacuolar compartment, and are transported to the plasmalemma by transfer vesicles (Chrispeels, 1976; Chrispeels, 1991). Some of these proteins containing signal peptides are transported into the cell wall where they finally are secreted out of the cell (Battey and Blackbourn, 1993; Darrah, 1993; Neumann et al., 2003; Tesfaye et al., 2005; Qin et al., 2006).

The nature of most enzymes in soil, particularly due to their size and charge characteristics, is such that they have very low mobility in soils. Therefore, if a secreted enzyme is to have any effect, it must operate close to the point of secretion, and its substrate must be able to diffuse to the enzyme (Uren, 2007). Proteins involved in root exudation have been the subject of increasing interest (Gramss and Rudeschko, 1998; Walz et al., 2004; Nóbrega et al., 2005; Wen et al., 2007). For instance, extracellular enzymes are released from roots to mineralize N, P, and S (Rovira, 1969; Vancura and Hanzlíková, 1972; Wasaki, 1997). In addition, secretory acid phosphatase is produced in response to P-deficiency by epidermal cells in the main roots of white lupine and in the cell walls and intercellular spaces of lateral roots (Wasaki, 1997).

Enzymes in the rhizosphere originate from various sources (Tarafdar and Marschner, 1994), mostly microbial (Nannipieri et al., 2001), and are found at much higher levels in the rhizosphere than in bulk soil (Rouatt et al., 1960; Rouatt and Katznelson, 1960). Moreover, extracellular enzymes released from soil microorganisms initiate the degradation of high-molecular-weight substrates in plants, such as cellulose, chitin, lignin, etc. However, as a counter attack many of the different compounds in root exudates have both antibacterial and antifungal properties, although the compounds typically work synergistically to achieve general antimicrobial results (Bais et al., 2004). Further, some antimicrobial proteins such as Ribosome Inactivating Proteins (RIPs) have been shown to be secreted by roots (Park et al., 2002).

This dissertation was focus on understanding the participation of proteins, particularly those found in root exudates, in defense and plant-microbe interactions and their regulation at different stages of development. Using two model plants, *Arabidopsis*

thaliana and *Medicago sativa*, I initiated this dissertation with the study of bioactive proteins secreted into the rhizosphere in order to understand the biological function and role of these proteins. The use of model plants with an already-sequenced genome provide the tools for the identification of genes involved in plant defense responses allowing the elucidation of plant-related disease control in future studies. I also presented the results of my study of the regulation of protein secretion at different developmental stages using *A. thaliana* and different defense- and flowering-related mutants. Finally, in this dissertation, I discussed the dual activity found in an RIP purified from *A. thaliana*.

1.2 Participation of root-exuded proteins in development

Recent evidence has established that many plant genes expressed naturally at specific times of vegetative and reproductive growth can also be activated at other times elsewhere in the plant in response to adverse environmental stimuli (Shao et al., 2007). Sometimes, it is the same gene that responds to these developmental and environmental signals; at other times, closely related genes within a multigene family are expressed. There are some proteins strongly regulated by defense that also are associated with development (Delp and Palva, 1999). For instance, interest in understanding the contribution of β-1,3-glucanases to plant defense as well as to normal development processes in plants has been growing since the characterization of two genes encoding for β-1,3-glucanases, which were found to be expressed during development, specifically at flowering time (Delp and Palva, 1999). There is also increasing evidence that other defense-related proteins, besides being induced upon pathogen attack, have a

function during plant development (Samac and Shah, 1991; Leung, 1992; De Jong et al., 1992; Goldman et al., 1992; Gu et al., 1992). Moreover, it has been discovered that phosphatase activity increases with plant age (Uren, 2007). Expression data supports possible non-defense functions of β-1,3-glucanases including hormonal induction and tissue-specific expression of class I glucanase genes in tobacco with the highest expression levels in roots (Castresana et al., 1990). Proteins such as chitinases and glucanases have been highly expressed in roots during de novo flower formation in tobacco (Meeks-Wagner et al., 1989). The physiological significance of the association between the secretion of these proteins in the root exudates and flower production is currently unclear. Furthermore, it has been shown in *Celosia cristata* leaves that the level of two antiviral proteins that act against a systemic host-virus is higher at the pre-flowering stage than at the post-flowering stage (Balasubrahmanyam et al., 2000).

1.3 Defense Proteins (RIPs)

It has been proposed that Ribosome-Inactivating Proteins (RIPs), one type of plant defense proteins, are compartmentalized into the apoplast (Park et al., 2004). Upon infection, RIPs directly target the pathogens either in cytosolic or extracellular regions, inhibiting the growth of the microbes (Park et al., 2004). RIPs are well documented to be secreted via the endoplasmic reticulum (Chow et al., 1990; Roberts and Lord, 2004; Stirpe and Batelli, 2006), but it has also been discovered that RIPs can be secreted in ways that do not involve secretory signal sequences (Walz et al., 2004). RIPs have been considered to be cytotoxic defense-related proteins for their *N*-glycosidase activity, which disrupts protein synthesis by removing a specific adenine residue from the highly

conserved α-sarcin/ricin (S/R) loop in the large rRNA (Endo et al., 1987; Endo and Tsurugi, 1988). Such cytotoxic enzymes have been found in the exudates of *Phytolacca americana* (PAP) hairy roots (Park et al., 2002). PAP has been considered a defense-related protein because it depurinates ribosomes from a wide variety of organisms. Its expression in transgenic plants leads to resistance to pathogen infection (Zoubenko et al., 1997; Wang et al., 1998). Furthermore, it has been hypothesized that the penetration of PAP into fungal cells is facilitated by PR proteins such as chitinases, β-1,3-glucanases, and proteases (Park et al., 2002).

1.4 Proteins involved in plant-microbe interactions.

How the rhizosphere microbial population is influenced by the amount and composition of plant root exudates has been studied for several decades (Rouatt et al., 1960). Root exudates directly or indirectly influence the microbial composition and species richness at the soil-plant interface, stimulating different interactions. One of the most studied plant-directed root-microbe interactions is that of the secretion and accumulation of antimicrobial compounds in and around the roots (Bais et al., 2004; Bais et al., 2005); also, of course, many microbes act as root pathogens. There can also be positive aspects to the plant-microbe relationship. The microbes take advantage of nutrients provided by the plant. In return, microbes may help the plant; for instance, by making nutrients available or by producing plant growth-promoting compounds. In general, the microbes that inhabit the rhizosphere serve as an intermediary between the plant, which requires soluble inorganic nutrients, and the soil, which contains the

necessary nutrients but mostly in complex and inaccessible forms. Rhizosphere microorganisms thus provide a critical link between plant and soil environments.

Microorganisms can recognize specific signal molecules in the exudates of their host plants, and this recognition leads to the induction of their virulence- or symbiosis-specific gene systems. Furthermore, plants have developed systems for monitoring the presence of microorganisms, or of microbial molecules (Morgan et al., 2005; Barea et al., 2005; Watt et al., 2006). This detection of microorganisms induces developmental pathways in the plant that culminate in a symbiotic or defense response (Baron and Zambryski, 1995). Many of the phenolics and flavonoids that are released by plant roots have very specific roles in the formation of symbiotic relationships in the rhizosphere (Currier and Strobel, 1976; Peters and Long, 1988; Maxwell et al., 1989; Fraysse et al., 2003). In the case of proteins, the function is the same (Mauch et al., 1988; Fritig et al., 1998; Minic et al., 1998; Xie et al., 1999; Minic et al., 2000). Root-exuded plant proteins act as signal molecules to microbes in the rhizosphere (Morgan et al., 2005; Bais et al., 2006; Wen et al., 2007). Recognition of microorganisms by the plant cell depends on the generation of elicitors by the pathogen (Benhamou, 1996; Parker, 2003). Protein-protein interactions play a role as signaling intermediates in a reaction cascade of recognition (Fritig et al., 1998; Janeway and Medzhitov, 2002). Recently a root-microbe interaction has been discovered in which different antimicrobial compounds are produced and accumulate around roots (Bais et al., 2004). The number of tools that plants have to defend themselves against pathogen invasion is enormous, such as the synthesis of antimicrobial cell wall-degrading chitinases and glucanases, the ability to reinforce the cell wall with lignin and callose (Dixon et al., 1994), and the release of

antimicrobial metabolites named phytoalexins into the rhizosphere (Dakora, 2003). Because microorganisms in the rhizosphere are so dependent on root exudates, plants largely control the interactions, microbes, and processes that exist in the rhizosphere (Kourtev et al., 2003).

1.5 CONCLUSIONS

Undoubtedly there are numerous ways root-exuded proteins are directly or indirectly involved in different cellular mechanisms such as development, defense, and the complicated interactions that occur in the rhizosphere. The use of model plants combined with biochemical approaches, such as proteomics, could help us to expand our knowledge of the function of proteins in the rhizosphere. High degrees of activity by both the plant roots and the microbial community are often difficult to conclusively characterize. However, identifying the proteins involved in symbiosis and pathogenesis will likely establish their biological function in the rhizosphere. Furthermore, the use of mutants in the development of new agricultural technologies will enable faster genetic modification technologies for employment in the future.

CHAPTER 2

Protein exudation by Arabidopsis roots is constitutive and is correlated to the developmental stage of the plant

ABSTRACT

In the present study, I conducted a proteomic and enzymatic analysis of *Arabidopsis thaliana* root-secreted proteins at different plant developmental stages. I found that the secretion of proteins (including pathogenesis-related [PR] proteins, myrosinases, and enzymes related to protein refolding, among others) was qualitatively and quantitatively related to the growth stage of the plant. For instance, the intensity and activity of PR proteins such as chitinases was higher at peak flowering times than at any other time during Arabidopsis development, and accordingly I used several mutant and transgenic plants to examine this relationship. I found that a defense-impaired transgenic line, *NahG*, and a defense-impaired mutant, *npr1-1*, secreted fewer PR proteins (chitinase, thaumatin protein and glucanase) while a mutant resistant to Pseudomonas, *cpr5-2*, had enhanced accumulation of PR proteins that were found to inhibit the growth of both pathogenic *Pseudomonas syringae* pv. *lycopersici* (DC3000) and non-pathogenic *Pseudomonas syringae* pv. *phaseolicola* (Psph 3121) strains, while the proteins from the wild type only inhibited the growth of Psph 3121. By analyzing the root exudates of three flowering-related mutants (*fca-1*, *stm-4* and *co-1*), I found an increase in PR protein secretion at flowering time, suggesting that these protein root secretions seemed to be regulated by developmental changes.

2.1 INTRODUCTION

The plant root system serves many roles, including anchorage and uptake of nutrients and water. The ability of roots to communicate with roots of other plants and other organisms has been the focus of increasing attention (Walker et al., 2003; Bais et al., 2004; Weir et al., 2004; Macias et al., 2005). Root secretions of secondary metabolites and volatile organic compounds (VOCs) have been shown to play offensive, defensive and symbiotic roles (Bais et al., 2004; Kappers et al., 2005; Cramer et al., 2005). Recent studies using *Arabidopsis thaliana* have shown that phytochemicals present in the root exudates may serve as the first line of defense for plants battling pathogen attack (Bais et al., 2005). Likewise, the symbioses between *Rhizobium* species and various members of the legume family have been extensively studied and involve the initial secretion of specific flavonoids by roots (Bernett et al., 2004). Recently, a multitrophic interaction has been uncovered in which VOCs released by *Medicago truncatula* were shown to attract soil nematodes which could in turn bring Rhizobia, used as food by the nematodes, to the roots and thus facilitate the symbiotic process (Horiuchi et al., 2005).

These examples of bioactive root secretions all involve carbon-containing compounds present in the root exudates. Although numerous reports show changes in the protein profile of leaves and roots in response to wounding (Li and McClure, 1990), insect attack (Van der Westhuizen and Pretorius, 1996), fungal or oomycete infection (Olivieri et al., 1998; Hiilovaara-Teijo et al., 1999; Pearce et al., 2001; Mithöfer et al., 2002; Rep et al., 2002; Hugot et al., 2004), or the defense-related hormone jasmonic acid (Grunwald et al., 2003), fewer reports have focused on protein secretion by the

roots (Gramss and Rudeschko, 1998; Yu et al., 2003; Charmont et al., 2005; Nóbrega et al., 2005; Basu et al., 2006; Wen et al., 2007).

Two-dimensional gel electrophoresis (2-DE) has proven to be a useful tool for identification of proteins in root exudates of both Arabidopsis and pea (Esaka et al., 1990; Charmont et al., 2005; Basu et al., 2006; Wen et al., 2007). Although Basu et al. (2006), by using proteomics, reported several extracytosolic proteins released from Arabidopsis roots, we still do not understand the temporal accumulation or regulation of secreted proteins or whether they are predisposed to be secreted in response to plant developmental changes. Such baseline information is clearly critical to a thorough understanding of how these root exudate proteins react to developmental stimuli.

In order to understand the nature of proteins found in the root exudates of the model plant Arabidopsis throughout its developmental process, I systematically profiled protein differences in the root exudates of the wild type (wt) and six different mutants/genotypes involved in defense and flowering processes at different stages of development. The data were correlated with plant phenotype and defense-related functional ability. This work provides a biological description through time of changes in the secretion of the 18 most variable proteins in the exudates of unstressed Arabidopsis wt and mutants, describing the dynamics of the protein secretion and providing evidence that secretion profiles change dramatically throughout the course of a normal plant's lifespan during a 49-day time course. My data also presents evidence that the secreted proteins, separated from other root-exuded metabolites, may account for root defense responses and may be associated with flowering time.

2.2 MATERIALS AND METHODS

2.2.1 Plant material and growth conditions

A. thaliana ecotype Col-0 (wt) seeds were purchased from Lehle Seeds (Round Rock, TX). The transgenic line *NahG*, which lacks the ability to accumulate SA through the constitutive expression of a bacterial salicylic acid hydroxylase gene (Delaney et al., 1994; Friedrich et al., 1995), and the mutant line *npr1-1,* which does not express the positive regulator NPR1 involved in SAR (Systemic Acquired Resistance) (Cao et al., 1994; Cao et al., 1997), were kind gifts from Dr. Xinnian Dong (Duke University, NC.). The mutant *cpr5-2*, which accumulates large amounts of salicylic acid (Bowling et al., 1994); the mutant *co-1*, which flowers later (after day 28) than the wt (after day 21 but before day 28) under long-day conditions, possibly due to a mutation on the *CO* gene impairing a putative transcription factor required to promote flowering under long-day conditions (Putterill et al., 1995); the mutant *fca-1*, which shows late flowering due to a mutation that disrupts the vernalization response (Chandler et al., 1996), and the mutant *stm-4*, defective in the gene SHOOT MERISTEMLESS (STM) and thus impaired in the initiation and maintenance of the shoot apical meristem (Cole et al., 2006), were obtained from the Arabidopsis Biological Resource Center (Ohio State University, Columbus). The seeds were surface-sterilized with 3% (v/v) sodium hypochlorite for two minutes followed by three washes with sterile distilled water. The seeds were germinated on solidified MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) sucrose in a growth chamber at 25°C and 16/8 h day/night cycle at 55 μmol $m^{-2}s^{-1}$. Replicates containing ten seven-day-old plants were transferred under a laminar flow hood into each Magenta box containing 30 ml of liquid MS medium supplemented

with 3% (w/v) sucrose and placed on a shaker set at 80 rpm, 24 ± 2°C under photoperiod of 16/8 h at 80 μmol $m^{-2}s^{-1}$. Aseptic protocols were used and no evidence of contamination was observed throughout the experiment. The fresh weight (FW) of the ten plants in each Magenta box was measured every seven days, and the ratio of micrograms of exuded protein to gram of FW for each genotype was determined. For each genotype, five Magenta boxes containing ten plants each were used, and each experiment was repeated three times (n=15).

2.2.2 Time course collection of root exudates

A. thaliana Col-0 (wt), *npr1-1*, *NahG, cpr5-2, fca-1, stm-4,* and *co-1* plantlets were grown in Magenta boxes as described earlier. At seven-day intervals, the total root exudates of each box's ten plants were collected and centrifuged at 8,000 g for 15 min at 4°C to remove the root sheathing. The supernatant was filtered through a 0.2 μm syringe filter and the filtration was concentrated to one milliliter by passing it through Amicon Ultra Centrifugal Filter Devices (MWCO of 5000 D, Millipore) to remove the salts. The protein concentration of the samples were determined as described by Bradford (1976) using a protein assay kit (Bio-Rad) and bovine serum albumin (BSA) was used as a standard. Exudate proteins were stored at -80°C until use.

2.2.3 Electrophoresis

To evaluate the proteins in the root exudates of all the mutants/genotypes at every time point, I used sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), following the method of Laemmli (1970) using a Bio-Rad Mini–protean

II slab gel unit. PAGE gels of 4 and 12% (1 mm thickness) were used as stacking and separating gels, respectively. Seven micrograms of each plant genotype's root-exuded proteins from each time point were diluted in Laemmli 5x buffer and heated at 95°C for five min before loading. Gels were electrophoresed at 100 V for 90 min at room temperature, according to the manufacturer's specifications. Protein Molecular Mass Markers (6 – 200 kD; Invitrogen) were used as a source of molecular mass standards. The protein bands were visualized by silver staining (Blum et al., 1987).

2.2.4 Two-Dimensional Electrophoresis (2-DE) separation

Separation and quantification of secreted protein exudates sampled throughout the development of the wt was performed by two-dimensional SDS-PAGE electrophoresis (2-DE) (O'Farrell, 1975). Proteins were analyzed in the exudates for all time points in the wt by 2-DE. Fifty micrograms of each mutant/genotype total root protein exudate for each time point was analyzed by 2-DE using the protocol described by Lei et al. (2005). Briefly, exudate proteins were precipitated by centrifugation with 12.5% (w/v) TCA plus 1% (v/v) 2-mercaptoethanol after an incubation at -20°C for 45 min. The protein pellet was washed with a cold solution of 80% (v/v) acetone and 20% water containing 0.05% (v/v) 2-mercaptoethanol three times to remove residual TCA and highly soluble phenolics (Watson et al., 2003; Watson and Sumner, 2007), air dried, and resuspended in 2-DE solubilization buffer consisting of 9 M urea, 3% (w/v) CHAPS, 2% (v/v) Triton X-100, 20 mM DTT and 0.5% ampholytes. Immobilized pH gradient (IPG) strips (ImmobilineTM Dry Strips, 24 cm, pH 3–10 non-linear, Amersham Biosciences) were rehydrated at 20°C with 50 μg of protein in 450 μl 2-DE

solubilization buffer for 12 h. Isoelectric focusing (IEF) of proteins was performed using the following stepped gradient: 500 volts for one h, 1000 volts for one h, and 8000 volts until a total of 50,000 V-h had been achieved. After focusing, the IPG gel strips were incubated for 10 min in equilibration buffer (6 M urea, 50 mM Tris-HCl pH 8.8, 30% glycerol, 2% SDS, and traces of bromophenol blue) containing 2% (w/v) DTT followed by 10 min incubation with equilibration buffer containing 2.5% (w/v) iodoacetamide. After equilibration, the IPG strips were sealed on the top of resolving SDS polyacrilamide gel (12.5%, 1 mm thick) with 1% (w/v) agarose and electrophoresed at 110 mA overnight at 10°C. Separated proteins were visualized using silver staining (Blum et al., 1987) and resultant gels digitally imaged with a Bio-Rad FluorS equipped with a 12-bit camera. Protein spot detection, quantification and comparative analyses were performed using Phoretix 2D Expression software (v 2005, Nonlinear Dymanics, Durham, NC) from three different replicate gels. Total spot volume was calculated and each spot was assigned a normalized spot volume as a portion of this total value prior to analysis by ANOVA. Each individual protein spot was then matched with the identical protein spot from each replicate gel.

2.2.5 In-gel trypsin digestion

Qualitative identification of 18 proteins was performed using in-gel digestion followed by high performance liquid chromatography tandem mass spectrometry (HPLC/MS/MS) and database searching. Briefly, silver-stained protein spots were manually excised from the gels and transferred to 96-well polypropylene plates. The gel plugs were de-stained (Sumner et al., 2002) and dehydrated with 25 µl of acetonitrile

(ACN) for 15 min at room temperature. The gel spots were dried under vacuum and rehydrated in 20 µl of sequencing-grade modified bovine trypsin (10 ng/µl in 25 mM ammonium bicarbonate, Roche Diagnostics). After rehydration for 30 min on ice, excess trypsin solution was removed, and 15 µl of 25 mM ammonium bicarbonate was added to each well to prevent dehydration during incubation. Proteolysis was allowed to continue overnight at 37°C and stopped by adding 15 µl of 10% (v/v) formic acid. The supernatant was recovered and the spots were extracted twice more with 25 µl of a 1:1 [v/v] solution of ACN and 25 mM ammonium bicarbonate and once more with 25 µl of ACN. All peptide extract fractions were pooled, concentrated to dryness and resuspended in a 95% water/5% acetonitrile solution containing a final concentration of 0.1% formic acid.

2.2.6 HPLC/MS/MS

Separations of the protein digests were achieved using a nanoscale HPLC (LC Packings, San Francisco, CA) consisting of an autosampler (Famos), a precolumn switching device (Switchos), and an HPLC pump (Ultimate). Samples (5 µL) were loaded onto a C18 precolumn (0.3-mm inner diameter x 5.0 mm, 100 Å, PepMap C18, LC Packings) for desalting and concentrating at a flow rate of 50 µl/min using mobile phase A (5% ACN and 95% water containing 0.1% formic acid). Peptides were then eluted from the precolumn and separated on a nano-analytical C18 column (75 µm i.d. x 15cm, 100Å, PepMap C18, LC Packings) at a flow rate of 200 nl/min. Peptides were eluted with a linear gradient of 5% to 40% mobile phase B (95% ACN and 5% water containing 0.08% formic acid) over 40 min. The separated peptides were analyzed using

tandem mass spectrometry and an ABI QSTAR Pulsar I hydrid Q-TOF mass spectrometer (Applied Biosystems) equipped with a nanoelectrospray ionization source (Protana) similar to that described previously (Lei et al., 2005).

2.2.7 Database queries and protein identification

Protein identifications were determined through queries of the acquired mass spectral data against the NCBInr database which was downloaded from the NCBI ftp site (ftp://ftp.ncbi.nih.gov/blast/db/FASTA/) on 4-22-2003. Although, it contains a total of 1415660 sequences (455667871 residues), only the 38475 *Arabidopsis thaliana* annotated sequences were searched using a taxonomic filter in MASCOT Daemon (version 1.8.0, Matrix Science Ltd., London, UK) search engine (Perkins et al., 1999; Creasy and Cottrell, 2002) and raw tandem mass spectral data (.wiff). No peak list was generated with the instrument software or any other third party software and used for the database search. The search used a mass tolerance of 100 ppm, and allowed for up to one trypsin mis-cleavage and variable amino acid modifications consisting of methionine oxidation and cysteine carbamidomethylation. Protein identifications with single-peptide match were manually validated through for the assignment of fragment ions in the MS/MS spectra to avoid false positive identification.

2.2.8 Enzymatic assays

Enzymatic assays of the total protein exudates were performed to correlate protein identification results with the biological activity of PR proteins present in the root exudates. Chitinase activity was assayed as described by Gómez-Ramírez et al.

(2004), using chitinase azure as a substrate. β-1,3-Glucanase activity was assayed according to Abeles and Forrence (1970), using laminarin from *Laminaria digitata* as substrate. Protease activity was assessed to evaluate the disappearance of several proteins on certain days of wt exudation as well as mutant/genotype development as related to degradation due to proteolysis. Root exudate protease activity was measured with a zymogram gel. A 12% SDS-PAGE gel containing 0.05% (w/v) gelatin was used and electrophoresis was performed according to the manufacturer's instructions. Following electrophoresis, the gel was incubated at room temperature for 30 min in 100 mL of 2.5% (v/v) Triton X-100 with gentle agitation and incubated overnight at 37°C in a solution of 50 mM Tris-HCl, pH 7.4 containing 200 mM NaCl, 5mM $CaCl_2$, 1 mM cysteine and 0.02% (v/v) Triton X-100. The gel was then stained with 0.5% (w/v) Coomassie brilliant blue R-250 and destained slightly to reveal zones of proteolysis. The activity was visualized as a white band on a blue background.

2.2.9 Antimicrobial assays

The antimicrobial activity of total root protein exudates, isolated as explained above, was tested against *Pseudomonas syringae* pv. *phaseolicola* (Psph 3121) and *P. syringae* pv. *lycopersici* (DC3000) using the broth microdilution antimicrobial susceptibility test as described in published methods of the National Committee for Clinical Laboratory Standards. Briefly, bacteria grown overnight from serial dilutions with twenty micrograms of protein from root exudates were plated on LB agar plates with appropriate antibiotics to determine viable cell concentration as colony-forming

units (cfu) per milliliter in order to investigate the antibacterial activity at every time point and for each Arabidopsis type.

2.2.10 Protein sequence analyses

The proteins identified in the root exudates were first clustered into gene families according to the "*Arabidopsis* Gene Family Information," available at "The Arabidopsis Information Resource (TAIR)" website (http://www.arabdopsis.org). A second approach was used to confirm the protein family identity by searching all proteins against InterPro with InterProScan tool (Zdobnov and Apweiler, 2001) at the European Bioinformatics Institute (EBI) (http://www.ebi.ac.uk/InterProScan/). All protein sequences used in this study were obtained from the TAIR website and from the "UniProt Knowledgebase" tool for querying by cross-reference available at the Swiss Institute for Bioinformatics (SIB).

2.2.11 Prediction of subcellular localization of the exuded proteins

I used four different softwares to predict the subcellular localization of all proteins used in this study. The Proteome Analyst Specialized Subcellular Localization Server (PA-SUB) (Lu et al., 2004), WoLF PSORT (Nakai and Horton, 1999), LocTREE (Nair and Rost, 2005), TargetP (Emanuelsson et al., 2000) and Multiloc (Hoglund et al., 2006). Both TargetP (http://www.cbs.dtu.dk/ services /TargetP/) and Multiloc (http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc/) use the presence/absence of N-terminal targeting sequences to predict the multiple cellular locations of the proteins such as chloroplast, cytoplasm, ER, extracellular space,

lysosomes mitochondria, Golgi apparatus, peroxisomes, plasma membrane, and vacuoles. In addition, tissue-specific expression and development-stage-specific expression patterns of the genes for the 18 identified proteins were downloaded from publicly available microarray data using the GENEVESTIGATOR software (Zimmermann et al., 2004) (https://www.genevestigator.ethz.ch/).

2.3 RESULTS

2.3.1 Plant development influences Arabidopsis root protein secretion.

The results from my temporal experiment designed to correlate plant development, biomass and proteins found in the root exudates of Arabidopsis wt (see Materials and Methods) indicated that the ratio of micrograms of total secreted protein to gram of fresh weight (FW) of *A. thaliana* wt (Fig. 2.1) decreased slightly during the second week of development and then rose steadily until plant death at approximately day 49 (7^{th} week). Using 2-DE, approximately 90 proteins were resolved from day 7 until day 49 in wt root exudate proteins. I focused on the 18 proteins which varied most dramatically across the temporal course for more detailed examination (Fig. 2.2). All 18 proteins were excised from the gel, digested with trypsin, and analyzed by nano-HPLC coupled to an ABI hybrid quadrupole time-of-flight mass spectrometer (ABI QSTAR Pulsar i) (Lei et al., 2005).

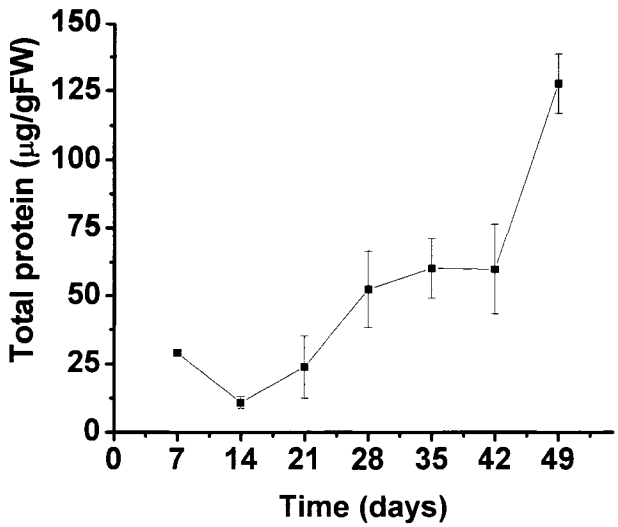

Figure 2.1. Time course for protein secreted in root exudates of *Arabidopsis thaliana* wt. Total protein as the ratio of micrograms of exuded protein to gram of fresh weight for *A. thaliana* wt was evaluated every seven days. Plants were grown in 30 ml of liquid MS medium; the exudates were collected and evaluated every seven days. The values are means ± SE, n=15.

To eliminate possible redundancy, I examined the database search results to determine whether the same peptide set identified several different proteins. I used the gel of each sample to rule out the possibility that any one protein was appearing in the database under multiple accession numbers or that protein isoforms were sharing the same peptide set. In this study I did not find any redundant proteins identified by the same set of peptides. Therefore, I was confident that the data is not redundant. Once the 18 most dynamic proteins were confidently identified (Table 2.1), I was then able to chart the emergence, change, and disappearance of these proteins from the root exudates throughout the lifetime of the wt plant. These changes were further visualized using the differential software, which made it possible to trace each protein's rise and fall as a normalized spot volume (Fig. 2.2B; Materials and Methods).

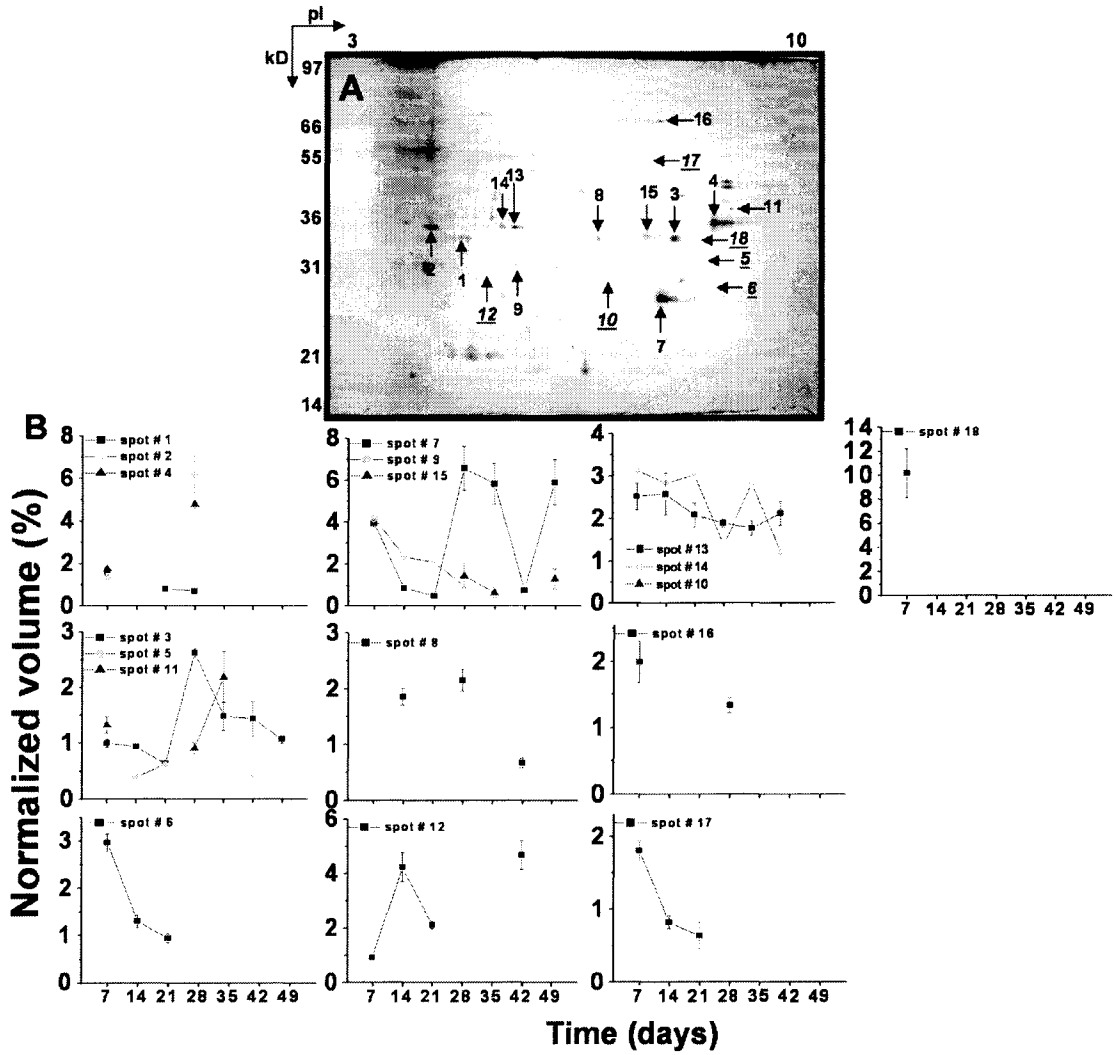

Figure 2.2. Two-Dimensional Electrophoresis (2-DE) of total *Arabidopsis thaliana* Col-0 (wt) root-secreted proteins in a temporal course. Fifty micrograms of protein were isoelectrically focused and separated by SDS-PAGE gel. **A,** Representative 2-DE gel from root-secreted proteins at day 28 is shown. The molecular masses (kD) of protein standards are indicated to the left of the gel and the isoelectric point (pI) is indicated at the top of the gel. The arrows with numbers represent identified proteins which are listed in Table 2.1. The underline numbers represent the proteins that are not present in the representative gel, but are present in other samples. **B,** Histograms of normalized volume values of each spot throughout the whole 49-day temporal course. Three replicate gels were analyzed at each time point. Each graph is a subset of proteins grouped together by class/isoforms. 1,3, beta glucosidases (spots 1, 2, and 4), chitinases (spots 3, 5, and 11), secretory protein (spot 6), isomerases (spots 7, 9, and 15), lectin (spot 8), thaumatin protein (spot 12), jasmonate inducible protein (spots 10, 13 and 14), subtilisin (spot 16), myrosinase (spot 17), peroxidase (spot 18).

Up to 75% of the exuded proteins identified in wt were related to defense (Table 2.1) and were exuded in the greatest quantity at days 7 and day 28 (after flowering) (Fig. 2.2B). The intensity of most of both PR proteins, chitinases and glucanases (spots # 1-5), decreased or disappeared after day 28, and other proteins such as plant basic secretory protein, lectin, and thaumatin like-protein (spot 6, 8 and 12 respectively) disappeared just before the onset of flowering and appeared again after flowering (day 28).

I compared the gene expression of the most variable 18 proteins with their secretion pattern in the root exudates by using Gene Chronologer and Gene Atlas tools of GENEVESTIGATOR database (Table 2.2). For instance, the genes corresponding to the protein spots 1, 3, 4, 5, 6, 8, 10, 11, 13, 14 and 17 were highly expressed in roots and genes of the protein spots 2, 7, 9, 12, 15, 16 and 18 were highly expressed in leaves. The genes highly expressed in roots and leaves correspond mostly to proteins related to defense and refolding respectively (Tables 2.1 and 2.2). On the other hand, examining the expression pattern of these genes at different developmental stages of Arabidopsis, I found that seventy-seven percent of the 18 genes showed higher expression before flowering than at any other developmental stages of the plant. Genes related to defense, like chitinases (At3g12500, At2g43590 and At1g56680), showed higher expression (five-fold more) before flowering than after flowering (Table 2.2). Similarly, the genes encoding jasmonate inducible proteins (At1g52050, At1g52060 and At1g52070) showed at least 20 times more expression before flowering than after flowering. Only one gene (At5g67360), encoding a subtilisin–like protein, showed a higher expression in

leaves compared to roots (Table 2.2). It was found to be highly expressed at the senescent stage.

2.3.2 Proteome analysis of the root exudates of defense-related mutants.

Based on the changes in accumulation of defense-related proteins (spots # 1-5, 8, 11, 12, and 17) in the root exudates of the wt at days 21 and 28 (Fig. 2.2B; Table 2.1), root secretions of the defense-impaired mutants/genotypes *npr1-1* and *NahG* and the defense-enhanced mutant *cpr5-2* were analyzed by 2-DE and the normalized volumes of the 18 proteins are shown in Figure 2.4. An initial assessment indicated that all defense-related mutant plants had the same pattern of ratio of micrograms of total exuded protein to gram of FW (Fig. 2.3) as the wt, with a rapid increase of protein secretion starting at day 21 (before flowering starts) and stopping by day 35 (after flowering). Thus, further analyses were performed on protein root exudates of the mutants and wt at days 21 and 28 (Fig. 2.4), days at which I found the most dynamic changes in protein root secretion and accumulation of defense-related proteins in the wt correlated to the flowering stage of the plant.

Table 2.1. List of the proteins identified in the root exudates of Arabidopsis.
TargetP: http://www.cbs.dtu.dk/services/TargetP/. cTP: chloroplast transit peptide; mTP: mitochondrial targeting peptide; SP: secretory pathway signal peptide. MultiLoc: http://www-bs.informatik.uni-tuebingen.de/Services/MultiLoc. Database: NCBInr 042203 (1415660 sequences; 455667871 residues). Taxonomy: *Arabidopsis thaliana* (thale cress) (38475 sequences). The NCBI non-redundant protein database (NCBInr) was downloaded on 4-22-2003. It contains a total of 1415660 sequences (455667871 residues) and the 38475 sequences for taxonomy of *Arabidposis thaliana* against which the database was searched.

									TargetP			MultiLoc
Spot Number	Protein ID	Protein Accession	Gene Locus	Score	Peptides	Coverage %	MW (D)	*pI*	cTP	mTP	SP	Location (prob estimate)
1	Endo 1,3, beta glucosidase	NP_193361	At4g16260.1	98	2	9	33535	5.3	0	0.1	0.8	Secretory (0.98)
2	Beta 1,3 glucanase 2 (PR-2)	AAM63339	At3g57260.1	228	5	19	31376	4.8	0	0.03	1	Secretory (0.97)
3	Basic endochitinase	BAA82814.1	At3g12500.1	123	2	8	36174	5.3	0	0.1	0.8	Secretory (0.98)
4	Endo 1,3, beta glucosidase	NP_193361	At4g16260.1	134	2	9	33535	6.4	0	0.1	0.8	Secretory (0.98)
5	Chitinase	NP_181887	At2g43590.1	64	1	5	28334	8.4	0	0.02	1	Secretory (0.99)
6	Plant Basic Secretory Protein	NP_565369	At2g15220.1	155	3	13	25149	8.6	0	0.01	1	Secretory (0.99)
7	Peptidylprolyl isomerase ROC4	NP_191762	At3g62030.1	136	3	13	28190	8.8	0.9	0.08	0	Chloroplast (0.75)
8	Putative lectin	NP_001030710	At3g16420.1	60	2	10	32138	5.5	0.1	0.15	0.1	Other (0.98)
9	Peptidyl-prolyl cis-trans isomerase	NP_565069	At1g73655.1	118	2	13	25721	6.8	0.8	0.27	0	Chloroplast (0.98)
10	Jasmonate inducible protein	NP_175617	At1g52050.1	74	1	4	32309	5.8	0.1	0.03	0.9	Secretory (0.98)
11	Chitinase	NP_176061	At1g56680.1	76	3	10	31183	8.7	0	0.01	1	Secretory (0.98)
12	Thaumatin-like protein (PR-5)	NP_177641	At1g75040.1	78	1	5	25236	4.8	0.1	0.01	1	Secretory (0.98)
13	Jasmonate inducible protein	NP_175618	At1g52060.1	119	2	9	31776	5.4	0.1	0.11	0.1	Other (0.8)
14	Jasmonate inducible protein	NP_175619	At1g52070.1	76	1	5	32676	5.5	0.1	0.04	0.9	Secretory (0.98)
15	Putative triosephosphate isomerase	NP_179713	At2g21170.1	92	2	13	33325	7.7	0	0.03	1	Secretory (0.97)
16	Subtilisin-like protein	NP_569048	At5g67360.1	51	1	1	78164	5.8	0.1	0.02	1	Chloroplast (0.54)
17	Myrosinase-associated protein	NP_564647	At1g54010.1	129	3	12	43116	8.8	0	0.04	1	Secretory (0.99)
18	Peroxidase, putative	AAM64354	At5g64120.1	166	3	13	34868	8.6	0	0.18	0.6	Secretory (0.99)

Table 2.2. Tissue-specific expression and development-stage-specific expression pattern of 18 identified proteins corresponding to genes examined by using Gene Chronologer and Gene Atlas tools of Genevestigator database.

Gene id	Tissue specific expression		Developmental stage specific expression								
	Roots	Rosettes	1.0-5.9	6.0-13.9	14.0-17.9	18.0-20.9	21.0-24.9	25.0-28.9	29.0-35.9	36.0-44.9	45.0-50.0
At4g16260	7687 (302)	1521 (188)	443 (122)	4395 (182)	4154 (246)	3228 (555)	879 (797)	2449 (508)	1260 (163)	397 (146)	85 (15)
At3g57260	108 (12)	4816 (291)	37 (6)	399 (46)	1512 (292)	5445 (616)	700 (187)	6457 (581)	4386 (360)	3429 (1130)	80 (11)
At3g12500	7653 (355)	609 (89)	1555 (603)	2131 (83)	4285 (284)	2225 (414)	199 (63)	559 (164)	581 (84)	225 (60)	98 (16)
At4g16260	7687 (302)	1521 (188)	443 (122)	4395 (182)	4154 (246)	3228 (555)	879 (797)	2449 (508)	1260 (163)	397 (146)	85 (15)
At2g43590	8418 (379)	1221 (111)	2406 (328)	2983 (163)	5135 (313)	1738 (389)	1042 (334)	1265 (159)	931 (63)	138 (18)	680 (242)
At2g15220	2382 (138)	141 (14)	114 (30)	478 (24)	1313 (95)	547 (145)	63 (16)	155 (24)	101 (10)	58 (21)	81 (11)
At3g62030	2108 (92)	27165 (424)	16682 (2550)	19490 (582)	17444 (825)	27024(1573)	27671(3243)	20830(571)	24065 (674)	13463(1285)	6858 (771)
At3g16420	27009 (433)	3597 (185)	24111 (4111)	21585 (424)	17587 (582)	7999 (1128)	2185 (521)	1972 (245)	1756 (256)	156 (43)	114 (27)
At1g73655	694 (19)	6522 (118)	1741 (302)	3031 (86)	3517 (169)	6356 (384)	4695 (695)	5908 (213)	6142 (198)	3484 (205)	1289 (139)
At1g52050	2700 (148)	133 (6)	653 (137)	794 (73)	1279 (85)	355 (64)	96 (11)	140 (5)	156 (16)	85 (6)	81 (8)
At1g56680	999 (41)	39 (2)	336 (110)	217 (14)	526 (33)	120 (21)	72 (12)	50 (6)	75 (7)	99 (18)	41 (7)
At1g75040	37 (4)	4680 (256)	30 (4)	231 (42)	1705 (276)	5446 (665)	2031 (351)	4548 (410)	4940 (381)	4747 (1267)	350 (133)
At1g52060	4339 (309)	115 (10)	697 (174)	1501 (164)	1839 (135)	616 (127)	100 (11)	104 (6)	163 (28)	75 (5)	124 (14)
At1g52070	4891 (266)	39 (8)	576 (155)	1237 (127)	2315 (160)	449 (94)	34 (6)	37 (7)	56 (16)	25 (5)	53 (8)
At2g21170	5269 (87)	14313 (164)	8726 (901)	13144 (251)	10677 (308)	14694 (577)	13645(1114)	11955(238)	13584 (248)	9414 (435)	4054 (512)
At5g67360	2882 (67)	4556 (76)	7867 (1048)	5256 (93)	3646 (77)	3661 (107)	5400 (219)	5184 (174)	4680 (106)	7359 (970)	8427 (1243)
At1g54010	8029 (204)	1061 (46)	10811 (1939)	7290 (158)	4748 (203)	1844 (234)	1645 (308)	1068 (107)	734 (70)	235 (24)	351 (46)
At5g64120	3055 (247)	3394 (288)	2866 (684)	4216 (215)	2655 (185)	2368 (409)	436 (200)	8135 (786)	760 (87)	702 (79)	167 (42)

This table was generated based on information extracted from the GENEVESTIGATOR web site from public gene profiling databases. All data were generated using the 22K Affymetrix ATH1 Arabidopsis Genome array and wild type Col-0. Numbers in the table denote the expression values. The parenthesis represents the standard error.

It was found that the mutant *cpr5-2* had the highest accumulation of PR protein chitinase (spots # 3, 5, and 11; Fig. 2.4) in the root exudates at day 21 (before flowering) while in both *NahG* and *npr1-1* it was totally absent. Also, the roots of the mutant *cpr5-2* secreted the highest amount of plant basic secretory protein (spot 6) on days 21 and 28. Interestingly, myrosinase (spot 17), an enzyme involved in the hydrolysis of glucosinolates (defense-related secondary metabolites widely present in Arabidopsis), was only found in this mutant at day 28 (Fig. 2.4; and in the wt at days 7, 14 and 21; Fig. 2.4B). Lectin protein (spot 8) was absent in the wt and in very low quantities in *NahG* and *npr1-1* in comparison with the mutant *cpr5-2* at day 21 while at day 28 it was absent only in *npr1-1*. Proteins related to refolding such as the isomerases (spots 7, 9) were observed highly accumulated in *cpr5-2* at day 21, and only one of these proteins (spot 9) was not present in the defense-impaired mutants *NahG* and *npr1-1* at day 28. Meanwhile, jasmonate inducible proteins (spots 10, 13 and 14) and endochitinase (spot 3) were present in all mutants on both days (Fig. 2.4). Peroxidase (spot 18) was absent in the root exudates of all plants at days 21 and 28.

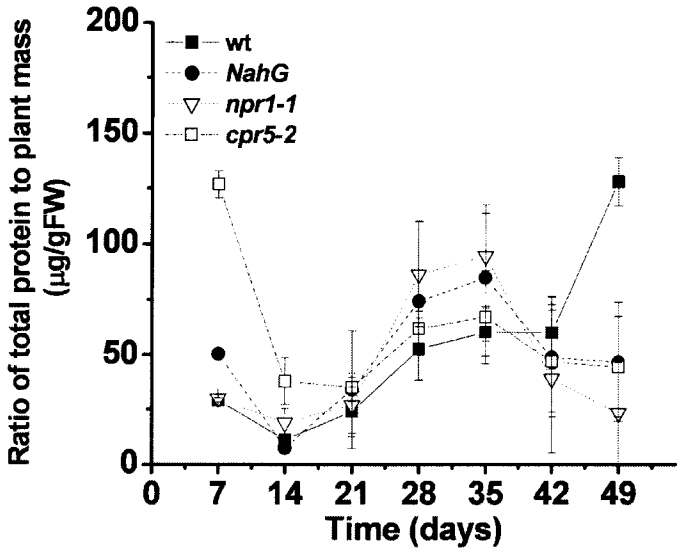

Figure 2.3. Time course for protein secreted in root exudates of *Arabidopsis thaliana* wt, *NahG*, *npr1-1* and *cpr5-2*. Total protein as the ratio of micrograms of exuded protein to gram of fresh weight for *A. thaliana* was evaluated every seven days. Plants were grown in 30 ml of liquid MS medium; the exudates were collected and evaluated every seven days. The values are means ± SE, n=15.

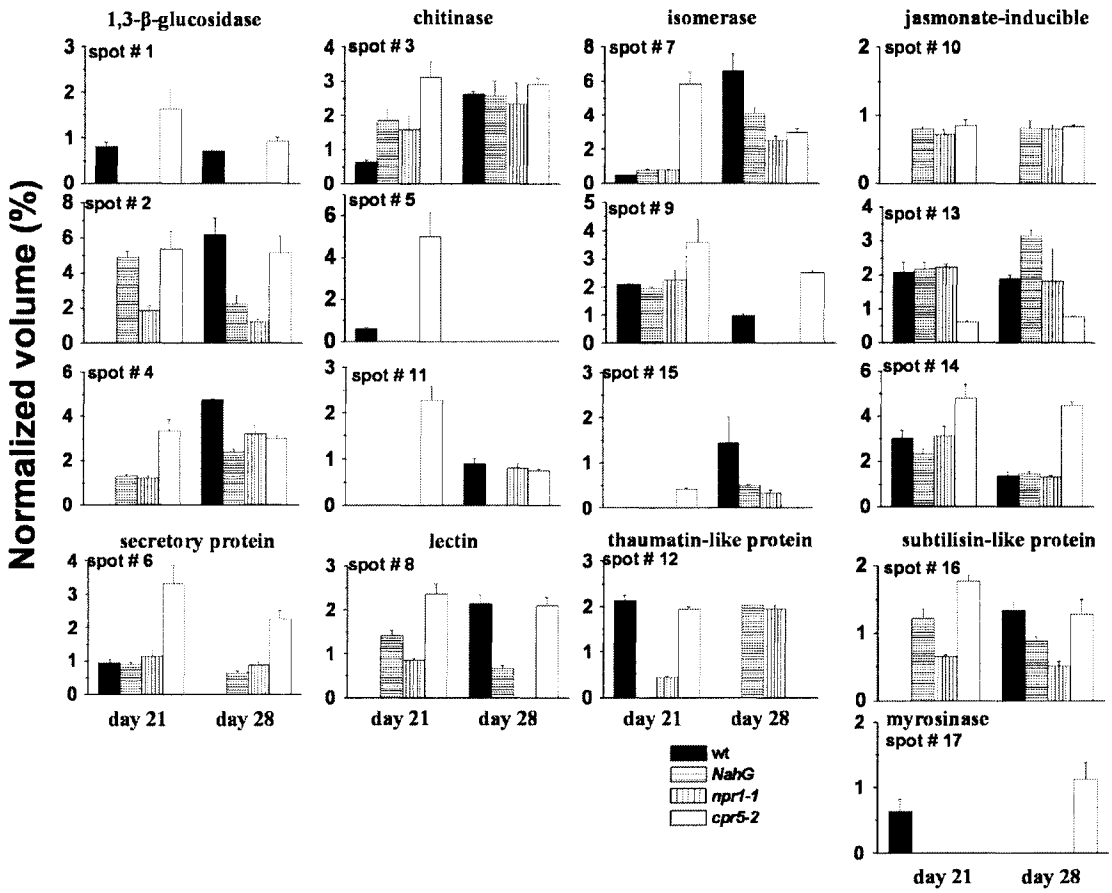

Figure 2.4. Comparative histogram of normalized volume values from each of the 18 spots from 2-Dimensional Electrophoresis (2-DE) of *Arabidopsis thaliana* wt and the defense-related mutants. The major differences between the root secreted proteins of *A. thaliana* ecotype Col-0 (wt), impaired-defense mutant *npr1-1*, impaired-defense transgenic *NahG*, and the defense-related mutant *cpr5-2* at days 21 and 28 are shown. Fifty micrograms of protein for every genotype were analyzed by 2-DE. The spot numbers represent the identified proteins which are listed in Table 2.1. 1,3, beta glucosidases (spots 1, 2, and 4), chitinases (spots 3, 5, and 11), secretory protein (spot 6), isomerases (spots 7, 9, and 15), lectin (spot 8), thaumatin protein (spot 12), jasmonate inducible protein (spots 10, 13 and 14), subtilisin (spot 16), myrosinase (spot 17). Peroxidase (spot 18) is not present in the root exudates of any plant at day 21 or day 28. The error bars illustrate the SE values of three repetitions (n=3).

2.3.3 Bioactivity of proteins found in the root exudates.

To determine whether the PR proteins found in the root exudates of Arabidopsis were still functional once secreted into the rhizosphere, enzymatic assays were performed to determine β-1,3-glucanase, chitinase and protease activity (Fig. 2.5) as

described in Materials and Methods. For the wt as well as *NahG*, day 7 had the highest glucanase activity (Fig. 2.5A). For the defense-impaired mutant *npr1-1*, day 28 and 42 had the highest β-1,3-glucanase activity, with 112 mg of glucose produced per mg of protein. The resistant mutant *cpr5-2* showed its highest ß-1,3-glucanse activity, with 506 mg of glucose produced per mg of protein, at day 14 (Fig. 2.5A). Another PR protein that was evaluated is chitinase (Fig. 2.5B). The chitinase activity was evaluated for *npr1-1*, *NahG*, *cpr5-2* and the wt during the time course of 49 days using chitinase azure as the substrate. The mutant *cpr5-2* showed the highest chitinase activity at days 21 (before flowering was observed) and 28 (after flowering was observed). The high activity in this mutant at day 21 is correlated with the proteome results where three chitinases identified (spots 3, 5 and 11; Fig. 2.4) show the highest accumulation at this time point. However, this was not the case for day 28, which suggests the accumulation of chitinases that were not identifiable by proteomic means, but were clearly detected by enzymatic assays. In the case of the transgenic *NahG,* it showed on average 200 units of chitinase activity throughout the whole temporal course (Fig. 2.5B) with only a small increase at days 21 and 28. The chitinase activity of *npr1-1* as well as the wt reached maximum activity in the early days of development (Fig. 2.5B). It was also examined the protease activity in the root exudates throughout plant development in the wt and the mutants (Fig. 2.5C). Protease activity was found at days 42 and 49 for the wt, while such activity in the mutants *NahG* and *npr1-1* was observed at four different days: 14, 21, 28 and 35 for *NahG*, with the highest activity at day 21 and 28, 35, 42 and 49 for *npr1-1*. The mutant *cpr5-2* showed protease activity only at day 42.

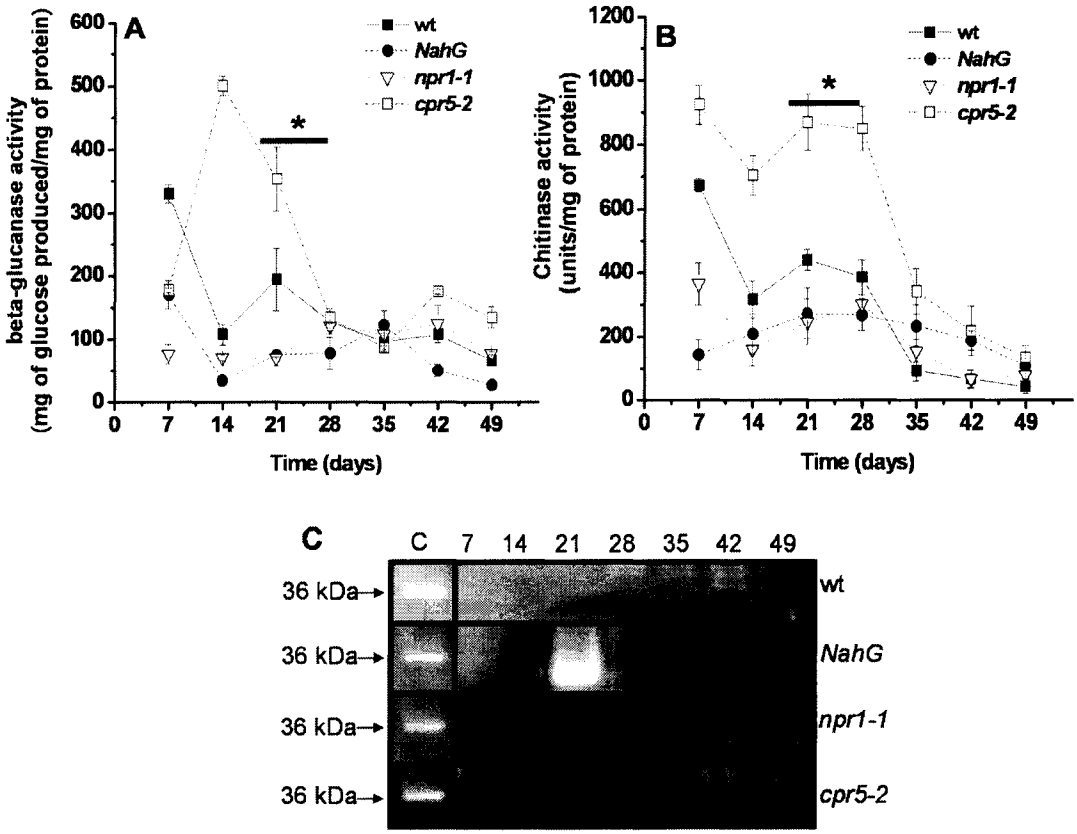

Figure 2.5. Enzymatic activity of β-1,3-glucanases, chitinases and proteases in *Arabidopsis thaliana* Col-0 (wt) and defense-related mutants. **A,** β-1,3-glucanases activity was quantified every seven days in *A. thaliana* Col-0 (wt), the impaired-defense mutant *npr1-1*, the impaired-defense transgenic *NahG*, and the defense-related mutant *cpr5-2* throughout the whole temporal study. The bar with the star represents the days when flowering was observed. **B,** Enzymatic activity of chitinases. Chitinolytic activity in root exudates of *A. thaliana* Col-0 (wt), impaired-defense mutant *npr1-1*, impaired-defense transgenic *NahG*, and the defense-related mutant *cpr5-2* was quantified using chitinase azure as a substrate. The bar with the star represents the days when flowering was observed. The error bars indicate the SE values. The results represent experiments repeated three times with five replicates each (n=15). **C,** Protease activities. Twenty-five μg of protein exudate from *A. thaliana* ecotype Col-0 (wt), impaired-defense mutant *npr1-1*, impaired-defense transgenic *NahG*, and the defense-related mutant *cpr5-2* were loaded in each lane. The arrow indicates protease activity. Papain from Papaya Latex (10 μg) was used for a control.

2.3.4 Protein root exudates with antimicrobial activity.

In order to analyze the antimicrobial activity of root-exuded proteins from the wt and the mutants *NahG*, *npr1-1* and *cpr5-2*, I used *Pseudomonas syringae pv. lycopersici* (DC3000), a compatible pathogen to Arabidopsis, and *Pseudomonas syringae* pv. *phaseolicola* (Psph 3121), considered a non-pathogen to Arabidopsis (Fig. 2.6). The secreted proteins from 21- and 28-day-old *Pseudomonas*-resistant mutant *cpr5-2* roots inhibited the growth of the non-pathogenic *Pseudomonas* strain Psph 3121, as well as that of the pathogenic strain DC3000. In contrast, the root exudates of the wt (day 21) only inhibited the growth of the non-pathogen Psph 3121, and the defense-impaired mutants *npr1-1* and *NahG* were not able to inhibit either bacteria. Further, *NahG* secretions were found to induce the growth of both Psph 3121 and DC3000. It should be noted that this assay was performed with secreted proteins alone as explained in the Materials and Methods section, and thus no other cellular components (i.e., carbohydrates, secondary metabolites, etc.) are likely to be responsible for the microbial inhibition.

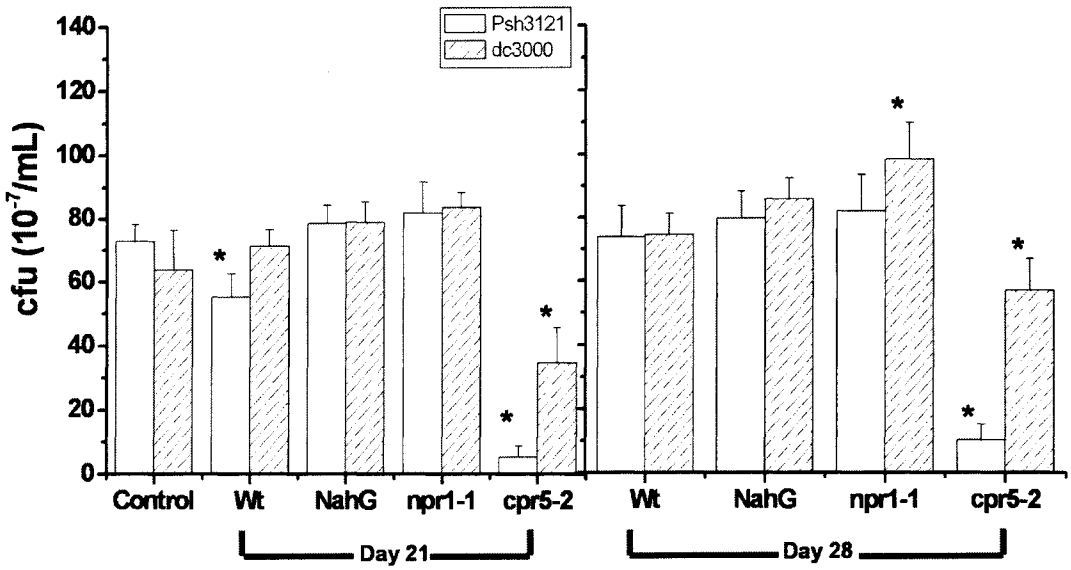

Figure 2.6. Antibacterial assay from protein root exudates of *Arabidopsis thaliana* Col-0 (wt) and the defense-related mutants. Antibacterial activity assays of protein root exudates from the wt, impaired-defense mutant *npr1-1*, impaired-defense transgenic *NahG*, and the defense-related mutant *cpr5-2* at days 21 and 28. Twenty μg of protein exudates from every genotype were tested against *P. syringae* pv. *lycopersici* (DC3000) and *Pseudomonas syringae* pv. *phaseolicola* (Psph 3121). The asterisk indicates the values that are statistically significant at $p \leq 0.05$ in a *t test*, n=10. The error bars illustrate the SE values. The presented values are from two independent experiments with five replicates each (n=10).

2.3.5 Proteome analysis of the root exudates of flowering-impaired mutants.

Based on the large change in the secretion of defense-related proteins seen at flowering in the wt as well as in the mutant *cpr5-2* (Figs. 2.1, 2.2 and 2.3), flowering-related mutants were studied to better understand secretion of proteins to the rhizosphere relative to developmental changes such as flowering. The proteins secreted by roots were examined over the lifespan of three mutants related to flowering, *fca-1*, *stm-4*, and *co-1* (Fig. 2.7), to determine whether specific protein patterns could be triggered by flowering processes. An initial assessment of total protein secretion by gram of FW of plant indicated that the protein secretion during development of the flowering mutants

was different than the wt's and the defense-related mutants *NahG*, *npr1-1* and *cpr5-2* (Fig. 2.3). The late-flowering mutant *co-1*, for example, had a delayed exponential phase of total protein root secretion, at day 35. The *fca-1* and the *stm-4* mutants had more total proteins secreted by the root than the wt at the exponential phase. The *fca-1* mutant flowered around day 28, *stm-4* did not flower at all, and *co-1* flowered around day 35 while the wt flowered between days 21 and 28 (data not shown).

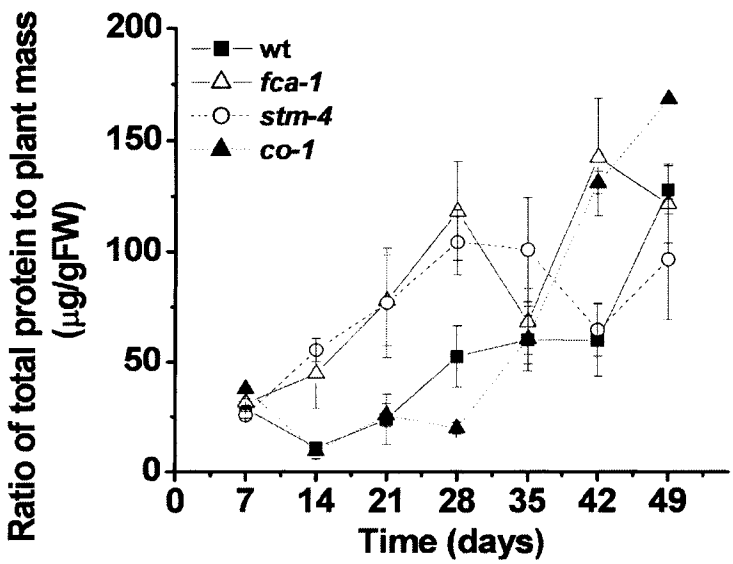

Figure 2.7. Time course for protein secreted in root exudates of *Arabidopsis thaliana* wt, *fca-1*, *stm-4* and *co-1*. Total protein as the ratio of micrograms of exuded protein to gram of fresh weight for *A. thaliana* was evaluated every seven days. Plants were grown in 30 ml of liquid MS medium; the exudates were collected and evaluated every seven days. The values are means ± SE, n=15.

Using comparative 2-DE analysis of the proteins exuded by the roots of three flowering-related mutants and the wt on days 21 and 28 (Fig. 2.8), I analyzed the normalized volumes of each of the 18 identified proteins and several significant differences were observed. For example, an endo 1,3-beta glucosidase (spot 1; Fig. 2.8) is not exuded at day 21 by any of the flowering mutants, but it is secreted by the

flowering mutants *fca-1* and *stm-4* at day 28 at the same quantity as wt at day 21. The proteins that were present in both days in the wt as well as the three mutants were basic endochitinase, peptidyl-prolyl isomerase ROC4, and two jasmonate inducible proteins (spots 3, 7, 13 and 14, respectively; Fig. 2.8). On the other hand, the myrosinase (spot 17) was the only protein present in the root exudates of the wt at day 21 and the jasmonate inducible protein (spot 10) was present only in the mutant *co-1* at day 21 and 28. Another important difference between the proteins secreted by the mutants and the wt is that endo 1,3-beta glucosidase, peptidylprolyl isomerase ROC4, lectin, triosephosphate isomerase, and subtilisin-like protein (spots 4, 7, 8, 15 and 16, respectively) were more secreted in the wt than in the mutants at day 28. Peroxidase (spot 18) was the only protein absent in the root exudates of all plants at days 21 and 28.

In order to analyze the enzymatic activity of glucanases, chitinases and proteases in the root exudates of the flowering mutants in comparison with the wt (Fig. 2.9), I analyzed first the glucanase activity which was found to be highest at day 7 in the wt and day 49 in *fca-1*, with an additional peak on day 14 for *stm-4* and *fca-1*. The delayed-flowering mutant *co-1*, in contrast to the other delayed flowering mutant *fca-1*, had its lowest activity on day 14 (Fig. 2.9A). I also compared the chitinase activity of the flowering mutants with the wt (Fig. 2.9B). The two delayed-flowering mutants (*fca-1 and co-1)* both had very low or undetectable chitinase activity in contrast to the high level of chitinase activity in the wt and *stm-4* until day 21. The proteolytic activity of the late flowering-related mutants (*fca-1 and co-1)* was found more often than that of the wt and the *stm-4* (non-flowering mutant) (Fig. 2.9C).

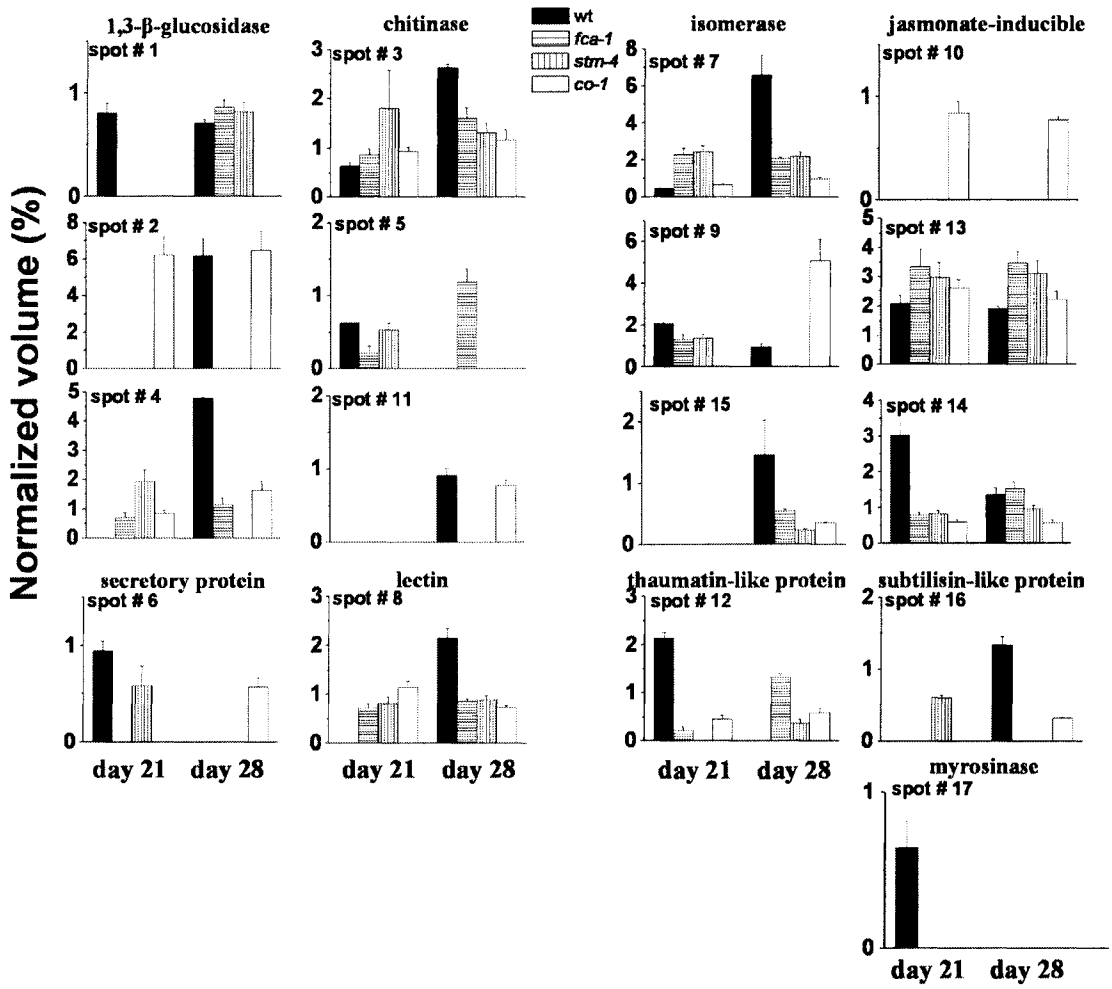

Figure 2.8. Comparative histogram of normalized volume values from each of 18 spots from 2-Dimensional Electrophoresis (2-DE) of *Arabidopsis thaliana* wt and the flowering-related mutants. The major differences between the root secreted proteins of *A. thaliana* ecotype Col-0 (wt) and three flowering-related mutants *fca-1, stm-4* and *co-1* at days 21 and 28 are shown. Fifty micrograms of protein for every genotype were analyzed by 2-DE. The spot numbers represent the identified proteins listed at Table 2.1. Endo 1,3, beta glucosidases (spots 1, 2, and 4), chitinases (spots 3, 5, and 11), secretory protein (spot 6), isomerases (spots 7, 9, and 15), lectin (spot 8), thaumatin protein (spot 12), jasmonate inducible protein (spots 10, 13 and 14), subtilisin (spot 16), myrosinase (spot 17). Peroxidase (spot 18) is not present in the root exudates of any plant at days 21 and 28. The error bars indicate the SE values from the normalized values of three different gels (n=3).

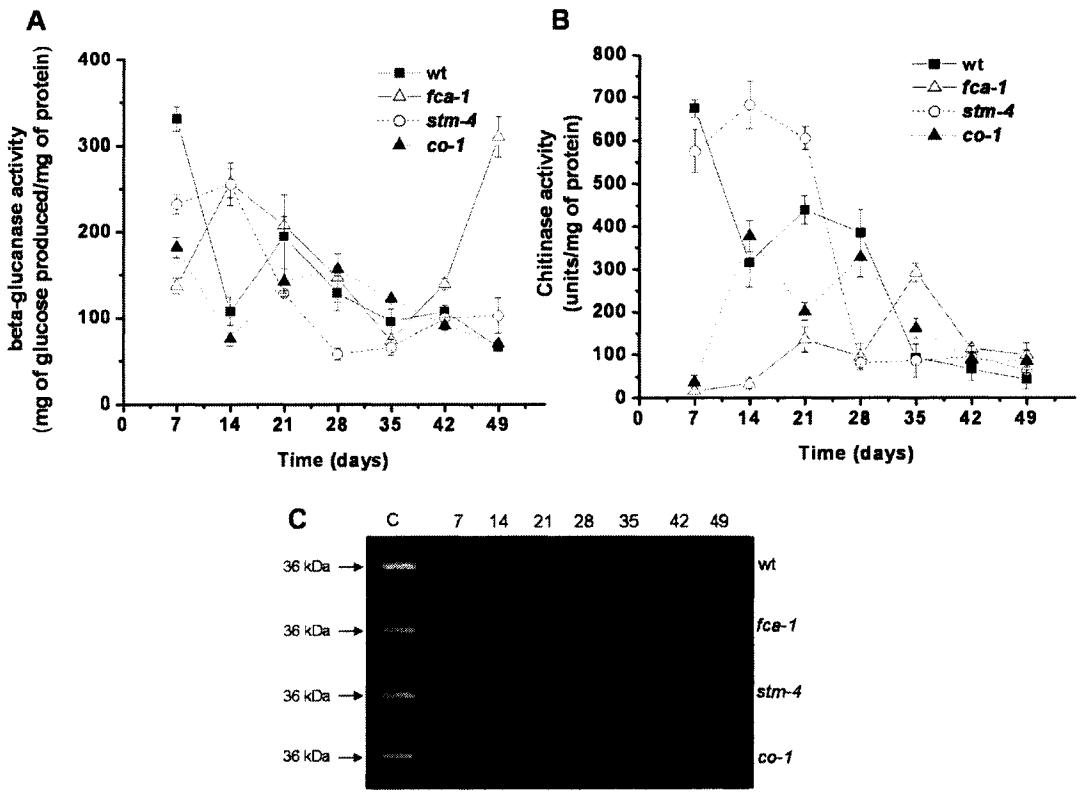

Figure 2.9. Enzymatic activity of β-1,3-glucanases, chitinases and proteases in *Arabidopsis thaliana* Col-0 (wt) and flowering-related mutants. **A,** β-1,3-glucanases activity was quantified every seven days in *A. thaliana* Col-0 (wt), the delayed-flowering mutant *fca-1*, the impaired-flowering mutant *stm-4*, and the delayed-flowering mutant *co-1* throughout the whole temporal study. The bar with the star represents the days when flowering was observed. **B,** Enzymatic activity of chitinases. Chitinolytic activity in root exudates of *A. thaliana* Col-0 (wt), the delayed-flowering mutant *fca-1*, the impaired-flowering mutant *stm-4*, and the delayed-flowering mutant *co-1* was quantified using chitinase azure as a substrate. The bar with the star represents the days when flowering was observed. The error bars indicate the SE values. The results represent experiments repeated three times with five replicates each (n=15). **C,** Protease activities. Twenty-five μg of protein exudate from *A. thaliana* ecotype Col-0 (wt), the delayed-flowering mutant *fca-1*, the impaired-flowering mutant *stm-4*, and the delayed-flowering mutant *co-1* were loaded in each lane. The arrow indicates protease activity. Papain from Papaya Latex (10 μg) was used for a control.

2.4 DISCUSSION

In the present study, I found that protein exudation is constitutive for all genotypes studied in this work (Figs. 2.1, 2.3 and 2.7), despite the fact that root exudation represents a significant cost to the plant (Shepherd and Davies, 1993). I found that particular root-exuded proteins were secreted at specific time points; the timing of various proteins' secretion seemed to be influenced by genotype as well as by plant developmental stage (Figs. 2.2, 2.4 and 2.8). The results presented here were based on careful methodology in which more than 70% of identifications include multiple peptides and protein coverage greater than 9%. All identified proteins have a MASCOT score of twice the $p<0.05$ significance level.

Root protein secretion has been studied by other groups (Borisjuk et al., 1999; Charmont et al., 2005; Basu et al., 2006; Wen et al., 2007), but the possible functions of this process are poorly understood. For instance, Arabidopsis roots secrete proteins into culture media (Charmont et al., 2005; Basu et al., 2006), but so far it is unknown what possible role(s) the secretion of such energy-consuming compounds may have in the rhizosphere. Basu et al. (2006), using different protein extraction methods and 18-day-old plants, identified fifty-two secreted proteins, some of which are defense-related, from Arabidopsis root exudates, but did not address the possible roles for these proteins in the exudates. Likewise, Charmont et al. (2005) reported several proteins secreted from the roots of 14-day-old Arabidopsis etiolated seedlings, but did not address the potential effect of etiolation on root secretion. Some of the identified defense-related proteins could be related to severe stress on plastids and cell membranes due to the etiolation process (Kleffmann et al., 2004; Solymosi et al., 2007). In my study, I set out

to investigate the possible biological role of the proteins that changed the most dramatically in the root exudates at different developmental stages.

The data in my work suggest a few possible roles for root exudate proteins found in the wt and different mutants. To my knowledge, this is the first report describing how protein secretion in the absence of stress (besides the *in vitro* culturing process) can change dramatically from one time point to the next (Fig. 2.2) and between the wt and a range of mutants (Figs. 2.4 and 2.7). In this study, important defense-related proteins were identified in the root exudates of plants growing under aseptic and non-stressed conditions (Table 2.1). These proteins were shown to change dramatically at different time points (Fig. 2.2B), suggesting that these proteins may provide the plant with a baseline level of defense against soil-borne pathogens (Greenberg and Ausubel, 1993; Kus et al., 2002) or that they may serve other functions in addition to defense, such as developmental changes (Whalen, 2005). I found a strong relationship between the defense-related proteins identified in the exudates at different developmental time points and their corresponding genes' tissue-specific expression and development-stage-specific expression pattern by using GENEVESTIGATOR database (Table 2.1 and Table 2.2).

Because I identified several proteins related to defense in the root exudates of Arabidopsis (Table 2.1), I examined the effect of protein root exudates on Arabidopsis host and non-host pathogens to determine whether the identified defense-related proteins have an effect on pathogens once they have been released to the rhizosphere (Fig. 2.6). I found that the wt as well as *cpr5-2* root exudate proteins inhibited a non-pathogen, Psph 3121, but only exudates of the mutant resistant to Pseudomonas *cpr5-2* inhibited the

growth of the pathogen DC3000. The root exudate proteins of *npr1-1* and those of the transgenic *NahG* did not show any antimicrobial activity against both the non-pathogenic Psph 3121 and the pathogenic DC3000. This lack of antimicrobial activity in *npr1-1* and *NahG* mutants may be related to the fact that both mutants fail to induce systemic acquired resistance (SAR) (Stinzi et al., 1993; Cao et al., 1994; Friedrich et al., 1995; Wang et al., 2005) and, as a consequence, PR proteins are not secreted (Wang et al., 2005). However, the presence of defense proteins with both chitinase and glucanase activities in these susceptible genotypes' root exudates indicates that an additional protein(s) is required for effective pathogen defense. According to Ludwig and Boller (1990), fungi are temporarily inhibited by chitinases and glucanases, but they can adapt to these proteins very quickly. A similar adaptation by bacteria could explain my observation of more growth of Psph 3121 in the presence of the exudates of *NahG* (Fig. 2.6). It is conceivable that Psph 3121 hydrolyzed the protein exudates of the mutants, suggesting that this pathogen is able to obtain amino acids from the proteins of *npr-1* and *NahG*.

The significance of certain proteins to plant defense was established decades ago (Lotan et al., 1989; Neale et al., 1990; Stinzi et al., 1993; Graham and Sticklen, 1994). These proteins have included proteases (Estelle, 2001; Valueva and Mosolov, 2004; Xia et al., 2004), glucanases, chitinases and other defense-related proteins (Bowles, 1990; Bol and Linthorst, 1990), several of which have also been demonstrated to possess dual activity (Wasternack and Hause, 2002; Sharma et al., 2004; Matarasso et al., 2005; Roopashree et al., 2006). For instance, MJ0109, a protein from *Methanococus jannaschii*, has both inositol monophosphatase and fructose-1,6-bisphosphatase

activities (Stec et al., 2000). Furthermore, it has been shown recently that a plant cystein protease has dual function, acting enzymatically in the cytoplasm and as a transcription factor in the nucleus to induce the ethylene pathway expression (Matarasso et al., 2005). Thus, it is not surprising that proteins related to defense could be related to other functions in the rhizosphere such as markers for developmental changes as hypothesized in this study. In the present study, I found a lectin in the root exudates that has been reported to be involved in defense against insects (Peumans and Van Damme, 1995) and to agglutinate and immobilize bacteria as a defense response (Chrispeels and Raikhel, 1991). My results showed that lectins (spot 8) tend to accumulate more in the root exudates before flowering in the resistant mutant *cpr5-2* than in the wt and the impaired defense-related mutants (Fig. 2.4), suggesting a possible protective function for the plant during flowering.

Finally, I looked at the possible function of several defense-related proteins as developmental markers. A potential developmental role for chitinases and glucanases was supported by enzymatic analysis (Figs. 2.5A and 2.5B) in which there was strong relationship between high levels of chitinase activity and flowering time in both wt and defense-related mutants. This relationship with chitinase activity and flowering time was also found in flowering-related mutants at day 35 for *co-1* and day 28 for *fca-1* (Fig. 2.9B), days at which those plants start flowering. It has been hypothesized that PR proteins such as chitinases (Neale et al., 1990; Leung, 1992) and β-1,3-glucanases (Lotan et al., 1989) might have a function in the sexual reproduction of higher plants. Similarly, it has been shown that the appearance of chitinase activity in roots is related to the development of tobacco (Neale et al., 1990). Chitinase activity in the roots of *A.*

thaliana has also been shown to be preceded by an increase in mRNA levels that are organ-age-dependent (Samac et al., 1990). Therefore, I speculate that because those proteins have signal peptides in order to be secreted into the apoplast, chitinases and glucanases are possibly secreted into the rhizosphere due to their high levels in roots' gene expression (Table 2.2). The abundance of chitinases that I observed during flowering is supported by other studies that suggest the involvement of chitinases in flowering (Lotan et al., 1989; Neale et al., 1990; Leung, 1992). Neale et al. (1990) suggest the possibility that a number of hydrolytic enzymes, including defense-related proteins, are directly involved in the vegetative-to-floral transition and the subsequent sequential development of floral organs. The physiological significance of the association between the secretion of these proteins in the root exudates and flower production is unclear at the moment, but there are studies in tobacco that indicate that leaves can have increased resistance to pathogens in the developmental transition from vegetative to flowering phase (Hugot et al., 1999). Furthermore, it has been shown in *Celosia cristata* leaves that the level of two antiviral proteins against a systemic host-virus is higher at the pre-flowering stage than at the post-flowering stage (Balasubrahmanyam et al., 2000). The results of these and other studies reinforce my suggestion that the secretion of defense-related proteins is not simply related to the constitutive defense process of Arabidopsis roots or a waste of energy, but to developmental changes in plants.

In summary, my present findings suggest that protein secretion by Arabidopsis roots is directly related to constitutive plant defense and developmental changes during flowering, although further molecular approaches are needed to determine the

mechanisms involved in protein secretion by roots. Thus, it is possible to infer that specific proteins may be predictably secreted at specific time points in a plant's development, a factor that is critical to take into account when examining root exudates to assess a plant's response to either biotic or abiotic stresses. Root-exuded proteins clearly changed throughout a plant's life cycle based on plant developmental changes. Further studies are necessary to determine how biotic and abiotic stresses interact with internal plant signals to produce certain changes in root exudates at different stages of development.

CHAPTER 3

Roots modify protein exudation in response to different soil microbes

ABSTRACT

Biotic interactions in the rhizosphere are biologically important and although many of those interactions are well studied, the role of root protein secretion in these interactions is not well understood. Using proteomic analysis, I studied protein root secretion by two model plants (*Medicago sativa* and *Arabidopsis thaliana*) during interaction with the symbiont of one of these species (*Sinorhizobium meliloti)* or an opportunistic pathogen of *A. thaliana* (*Pseudomonas syringae* pv. tomato DC3000). I found that the early interactions between *M. sativa* and *S. meliloti* induced exudation of enzymes such as acid chitinases, thaumatin proteins, PR10 and PR1 proteins. However, these proteins were not induced when *M. sativa* was inoculated with *P. syringae* DC3000. In addition, I found that *P. syringae* DC3000 could differentially induce the secretion of proteins related to defense in *A. thaliana*, whereas, *S. meliloti* did not provoke the same response. My results showed that plants can induce the secretion of specific proteins when they were in the presence of a pathogen vs. in the presence of a non-pathogen, and possibly that these responses may be manipulated by the microbes.

3.1 INTRODUCTION

Plants are exposed to multiple biotic interactions under natural conditions (Wardle, 2006), and in the rhizosphere roots tend to interact with soil microbes using highly sophisticated mechanisms (Butler et al., 2003; Singh et al., 2004; Morgan et al., 2005; Barea et al., 2005; Bais et al., 2006). The initial recognition between roots and microorganisms depends on a wide range of signals coming from both plants and microbes. On the root side, the main signals come in the form of root exudates, a

complex mixture of chemicals including carbohydrates, phenolics, flavonoids, and proteins (Bais et al., 2004). The composition of the phytochemicals present in the root exudates tends to vary depending of the nature of the interaction and is likely controlled by a network of root transporters and specific genes (Taylor, 1998; Neumann and Martinoia, 2002; Yazaki, 2006; Loyola-Vargas et al., 2007). On the microbial side, bacteria release quorum-sensing signals to regulate their population density and these signals have been shown to induce changes in the accumulation of root proteins (Mathesius et al., 2003). Relatively less is known about signals released by fungi in the soil (Hause et al., 2007) although it is well known that host root exudates play a key role in the stimulation of hyphal growth (Harrison, 2005), and a recent study has identified strigolactone 5-deoxy-strigol released by roots of *Lotus japonicus* as the compound involved in establishing the initial interaction with mycorrhizae (Akiyama et al., 2005).

Roots and soil bacteria are engaged in highly specific chemical communication leading to biologically significant interactions (Hammond-Kosack and Parker, 2003; Bais et al., 2006; Kiely et al., 2006). The symbiotic association between the Rhizobiaceae family (including the genera *Azorhizobium*, *Bradyrhizobium* and *Sinorhizobium*) and leguminous plants constitute the best known example of this chemical exchange of information. It has long been known that root flavonoids and betaines released by the plant root are perceived by the bacterial NoD family of transcriptional activators in Rhizobia (Halverson and Stacey, 1984; Egelhoff and Long, 1985; Hartwig et al., 1989; Hartwig et al., 1991; Hungria et al., 1991; Long, 1996) and lead to the release of nod factors that cause the root hairs to curl, providing a haven for the bacterial colonizers. However, new information has shown that not only the nod genes are the responsible for

early nodulation events. Recently, it has been shown that the photosynthetic Bradyrhizobium does not have nod genes in its genome and that purine derivatives are required to initiate the nodule formation (Giraud et al., 2007).

The studies of signals between rhizobia and legumes have been restricted to secondary metabolites released from the roots or carbohydrates secreted from the bacteria (Currier and Strobel, 1976; Peters et al., 1986; Maxwell et al., 1989; Hartwig et al., 1991; Fraysse et al., 2003). However, other compounds, particularly macromolecules such as proteins, may also be involved in this process. For instance, in the symbiosis between the squid *Euprymna scolopes* and the luminous bacterium *Vibrio fischeri*, the bacteria enhance the production of an enzyme in the symbiotic organ of the squid, functionally similar to the mammalian myeloperoxisase that plays an important role in the initial interaction required for the squid's luminescence (Weis et al., 1996). This enzyme plays an important role in the interaction between the squid and the bacteria, controlling the symbiont specificity and bacteria growth; in mammals, this protein participates in a complex antimicrobial response. Further, a member of the mindin-F-sapondin family of secreted extracellular matrix proteins in bacteria accounts for a unique pattern-recognition molecule in the extracellular matrix of the bacteria that is essential for the initiation of the immune response in mice (He et al., 2004). In the case of plants, proteins are beginning to be recognized as key players in recognition of and resistance to bacterial pathogens (Chinchilla et al., 2007). For instance, the most abundant bacterial protein elongation factor Tu elicits innate immunity in *A. thaliana* which contributes to resistance against bacterial pathogens (Kunze et al., 2004). Given the lack of information about the participation of protein signals in the interaction

between plants roots and soil microbes, in this study I provided a detailed proteomic analysis and comparison of proteins secreted by the roots of two model plants *Arabidopsis thaliana* and *Medicago sativa* during their initial interaction with *Pseudomonas syringae* pv. tomato DC3000 or *Sinorhizobium meliloti* strain Rm1021.

3.2 MATERIALS AND METHODS

3.2.1 Plant material and growth conditions.

A. thaliana ecotype Col-0 (wt) seeds were purchased from Lehle Seeds (Round Rock, TX) and *M. sativa* seeds were obtained from a local store (Fort Collins, CO). The seeds were surface sterilized with 3% (v/v) sodium hypochlorite for two minutes for *A. thaliana* and twenty minutes for *M. sativa* followed by three washes with sterile distilled water. The seeds of both plants were germinated on solidified MS (Murashige and Skoog, 1962) medium supplemented with 3% (w/v) sucrose in a growth chamber at 25 $\pm$ 2 °C and 16/8 h day/night photoperiod. One seven-day-old plant was transferred into one Magenta box containing 15 ml of liquid MS medium supplemented with 3% (w/v) sucrose and placed on a shaker set at 70 rpm, 24 $\pm$ 2°C under photoperiod of 16/8 h. Sixty Magentas containing one plant each were used for each replicate of the different experiments as described below.

3.2.2 Time course collection of root exudates.

A. thaliana Col-0 (wt) and *M. sativa* plantlets were grown in Magenta boxes as described earlier. At twenty one-days, the plants were washed three times with sterile distilled water and transferred to a new Magenta box containing 10 ml of fresh MS

media. At intervals of 6, 12 and 24 hours, the exudates of plants in sixty separate Magenta boxes were collected, pooled and centrifuged at 8,000 g for 15 min at 4°C to remove the root sheathing. The supernatant was filtered through a 0.2 μm syringe filter and the filtrated solution was concentrated to 500 μl by passing through Amicon Ultra Centrifugal Filter Devices (Millipore) Mr of 5000 Da. Root exuded proteins were stored at -80°C until use. For the collection of exudates from roots inoculated with microorganisms, I used *P. syringae* pv. tomato DC3000 (Moore et al., 1989), a pathogen of *A. thaliana* and an innocuous microbe to *M. sativa*, and *S. meliloti* Rm1021 (Meade et al., 1982), a N_2-fixing symbiont of *M. sativa*, which is neither beneficial nor pathogenic to *A. thaliana*. The bacteria *P. syringae* pv. tomato DC3000 and *S meliloti* strain Rm1021 were grown overnight in LB liquid media with 50 μg/ml of rifampicin and 100 μg/ml of streptomycin respectively, then the overnight cultures were mixed with MS liquid media separately to a final OD_{600} of 0.02. The proteins secreted after 6, 12, and 24 h from the bacteria in MS media were used as a control. The plants were washed three times with sterile distilled water and then were transferred to fresh MS media previously inoculated with the bacteria having a final OD_{600} of 0.02 as described above. The roots of each type of plant were inoculated separately with the bacteria and after 6, 12 and 24 hours the root exudates of sixty plants (grown separately) were collected and centrifuged at 8,000 g for 15 min at 4°C to remove root debris and the microorganisms. The supernatant was filtered through a nylon syringe filter of pore size 0.22 μm (Pall Life Sciences, East Hill, NY, USA, Cat. PN 4612 or Nalagene, Rochester, NY, USA, Cat. 195-2520) and the filtration was concentrated to 500 μl by passing through Amicon Ultra Centrifugal Filter Devices (Millipore) Mr of 5000 Da to separate the secondary

metabolites from the proteins and to remove the salts. Root-secreted proteins were stored at -80°C until use. For each type of plant and treatment three biological repetitions (n=3; 180 plants in total) were used, and each experiment was repeated three times. The same volume of MS media used for plants alone was used also for bacteria alone.

3.2.3 Two-Dimensional Electrophoresis (2-DE) Separation.

To identify the proteins secreted in the exudates of roots interacting with bacteria, I performed a complete two-dimensional gel resolution (2-DE) (O'Farrell, 1975). I analyzed the proteins in the root exudates of *A. thaliana* alone, *A. thaliana* inoculated with *P. syringae* DC3000, *A. thaliana* inoculated with *S. meliloti*, *M. sativa* alone, *M. sativa* inoculated with *P. syringae* DC3000, *M. sativa* inoculated with *S. meliloti*, *P. syringae* DC3000 alone and *S. meliloti* alone by 2-DE at 6, 12 and 24 h. One hundred and fifty micrograms of total exuded protein for each time point/treatment were run in 2-DE. I followed the protocol described by Lei et al. (2005). Briefly, the exudates were brought to a final concentration of 12.5% (w/v) TCA plus 1% 2-mercaptoethanol and incubated at -20 °C for 45 min. IPG strips (ImmobilineTM Dry Strips, 24 cm, pH 3–10 non-linear, Amersham Biosciences) were rehydrated for 12 h at 20°C with 150 µg of protein in 450 µL of 2-DE solubilization buffer consisting of 9 M urea, 3% (w/v) CHAPS, 2% (v/v) Triton X-100, 20 mM DTT and 0.5% ampholytes. IEF of proteins was performed using the following stepped gradient: 500 volts for one h, 1000 volts for one h, and 8000 volts until a total of 50,000 V-h had been achieved. After focusing, the IPG gel strips were incubated for 10 min in equilibration buffer (6 M urea, 50 mM Tris-

HCl pH 8.8, 30% glycerol, 2% SDS, and traces of bromophenol blue) containing 2% DTT followed by 10-min incubation with equilibration buffer containing 2.5% iodoacetamide. After equilibration, the IPG strips were placed on the top of resolving SDS polyacrylamide gel (12.5%, 1 mm thick) and run at 110 mA overnight at 10°C. Separated proteins were visualized using silver staining (Blum et al., 1987) and resultant gels were digitally imaged with a Bio-Rad FluorS equipped with a 12-bit camera. Protein spot detection, quantification, background subtraction and comparative analyses were performed using Phoretix 2D Expression software (v 2005, Nonlinear Dymanics, Durham, NC) from three different replicate gels. Total spot volume was calculated and each spot was assigned a normalized spot volume as a portion of this total value. Each individual protein spot was then matched with the identical protein spot from each replicate gel.

3.2.4 In-gel trypsin digestion.

In order to identify the proteins resolved in gel that changed in the interactions between plants and microbes, the proteins were separately digested with trypsin. Briefly, silver-visualized protein spots were manually excised from the gels. These gel plugs were transferred to polypropylene 96-well plates for further processing. I followed the protocol described by Sumner et al. (2002) for removal of silver stain. Then the gel spots were dehydrated with 25 µl of acetonitrile (ACN) for 15 min at room temperature. After ACN removal, the gel spots were dried under vacuum and rehydrated in 20 µl of sequencing-grade modified bovine trypsin (10 ng/µl in 25 mM ammonium biocarbonate, Roche Diagnostics). After rehydration for 30 min on ice, excess trypsin solution was

removed, and 15 µl of 25 mM ammonium bicarbonate was added to each well to prevent dehydration during incubation. Proteolysis was allowed to continue 13 hrs at 37°C and stopped by adding 15 µl of 10% formic acid. All peptide extract fractions were pooled, concentrated until dry and resuspended in a 50:50 (%v/v) water-acetonitrile solution containing a final concentration of 0.1% formic acid.

3.2.5 LC/MS/MS.

Separations of the protein digests were achieved using a nanoscale HPLC system (LC Packings, San Francisco, CA) consisting of an autosampler (Famos), a precolumn switching device (Switchos), and an HPLC pump system (Ultimate). Samples (5 µl) were loaded onto a C18 precolumn (0.3-mm inner diameter x 1.0 mm, 100 Å, PepMap C18, LC Packings) for desalting and concentrating at a flow rate of 50 µl/min using mobile phase A (5% ACN and 95% water containing 0.1% formic acid). The separated peptides were directly analyzed with an ABI QSTAR Pulsar I hydrid Q-TOF mass spectrometer (Applied Biosystems) equipped with a nanoelectrospray ionization source (Protana).

3.2.6 Database queries and protein identification.

In order to confirm protein identifications, the acquired mass spectral data were queried against the NCBI non-redundant protein database (NCBInr), downloaded on 4-22-2003, using the MASCOT (version 2.2, Matrix Science Ltd., London, UK) search engine (Perkins et al., 1999; Creasy and Cottrell, 2002), a mass tolerance of 100 ppm, and allowance for up to one trypsin mis-cleavage and variable amino acid modifications

consisting of methionine oxidation and cysteine carbamidomethylation. Only protein identifications with a molecular weight search (MOWSE) score greater than the generally accepted significant threshold (determined at 95% confidence level as calculated by MASCOT; $p < 0.05$) and at least two matched peptides are reported in this study. The NCBInr also provides the origin of the protein by which I could differentiate between the proteins that come from plant or from microorganisms.

3.3 RESULTS

3.3.1 Quantification of protein secretion and microbial growth.

I quantified the total proteins secreted by plants and microbes alone and during plant-microbe interactions (Fig. 3.1A). The accumulation of the proteins secreted by the plants and the microbes were very different when they were grown alone compared to the accumulation during plant-microbe interactions. In the case of *A. thaliana, M. sativa, S. meliloti* and *P. syringae* DC3000 grown alone, I observed that *S. meliloti* secretes more proteins than *P. syringae* DC3000 over a time course. In addition, I found that *M. sativa* secretes on average more protein than *A. thaliana* at 6 hours and *A. thaliana* secretes more than *M. sativa* at 12 h (Fig. 3.1A). The total secreted proteins during all interactions increased compared with that secreted when the plants or the microbes grew alone. When *A. thaliana* was inoculated with *S. meliloti* the increase of protein secretion was 49% higher after 24 h than when *A. thaliana* was inoculated with *P. syringae* DC3000 at the same time point (Fig. 3.1A). On the other hand, the protein exudation at 24 h during the interaction between *M. sativa* and *S. meliloti* increased by 41% in comparison with the interaction *M. sativa – P. syringae* DC3000 (Fig. 3.1A).

To ascertain that the increase in the secretion of proteins into the media was not due to an increase in the microbial growth during the interactions, I measured the optic density (O.D.) of *P. syringae* DC3000 and *S. meliloti* alone and in the presence of plants at 6, 12 and 24 h after inoculation. I observed that the two microbes alone grew similarly over time (Fig. 3.1B). However, in the case of *A. thaliana* inoculated with *P. syringae* DC3000 at 24 h, the growth of the bacteria was more than three times higher than *S. meliloti* grown with *A. thaliana*, yet I saw more protein secreted in the *A. thaliana* - *S. meliloti* interaction (Fig. 3.1A, 3.1B). The growth of *P. syringae* DC3000 with *M. sativa* was more than five-fold higher than the growth of *S. meliloti* with *M. sativa* at 12 and 24 h (Fig. 3.1B) but again I saw more protein secretion in the *S. meliloti* – *M. sativa* interaction. Therefore, it seems that the accumulation of protein in the interactions is not necessarily due to microbial growth.

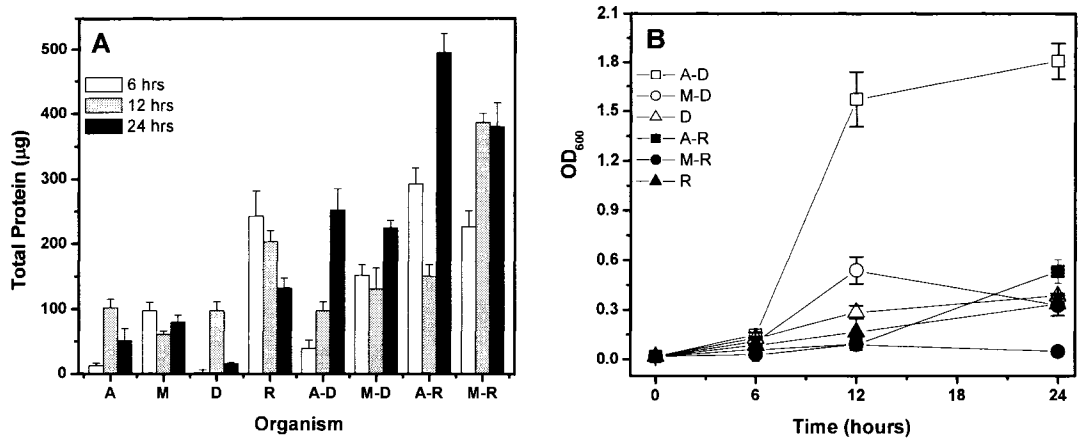

Figure 3.1. Time course of exudate protein accumulation in the plant-microbe interaction and bacterial growth. **A**, Total protein from exudates of *A. thaliana* (A), *M. sativa* (M), *Pseudomonas syringae* pv. tomato DC3000 (D), *Sinorhizobium meliloti* (R), *A. thaliana* inoculated with *P. syringae* DC3000 (A-D), *A. thaliana* inoculated with *S. meliloti* (A-R), *M. sativa* inoculated with *P. syringae* DC3000 (M-D), *M. sativa* inoculated with *S. meliloti* (M-R). The root exudates of plant alone or with bacteria cultivated in 10 ml of liquid MS medium were collected and evaluated at 6, 12 and 24 hours. The values are means ± SE, n=3. **B**, Analysis of growth of bacteria alone or in the presence of plants at 6, 12 and 24 hours quantified as OD at 600 nm. *A. thaliana* (A), *M. sativa* (M), *P. syringae* DC3000 (D), *S. meliloti* (R), *A. thaliana* inoculated with *P. syringae* DC3000 (A-D), *A. thaliana* inoculated with *S. meliloti* (A-R), *M. sativa* inoculated with *P. syringae* DC3000 (M-D), *M. sativa* inoculated with *S. meliloti* (M-R). The values are means ± SE, n=3.

3.3.2 Proteome analysis of the root-exuded proteins in the plant-microbe interactions.

Applying a proteomic approach to the secretions found in each interaction and to those of organisms alone, I found remarkable differences in the identity of proteins exuded by plants and microbes alone and those in the plant-microbe interactions (Tables 3.1, 3.2 and 3.3). In Figure 3.2, I showed the protein spots resolved by two-dimensional electrophoresis (2-DE) and how those proteins change in host and non-host interactions. Although I did the proteomic analysis for each interaction and controls at 6, 12, and 24 h, I only showed representative pictures at 6 hours for all interactions and for organisms

alone. Using the control gels from plants and bacteria alone, I could corroborate the origin of the identified proteins with the NCBInr database.

In the *M. sativa* – *S. meliloti* interaction (Fig. 3.2B), I observed that protein secretion above pI 5 clearly decreased in *M. sativa* inoculated with *S. meliloti* compared with *M. sativa* alone (Fig. 3.2A), and the secretion of proteins of high molecular weight disappeared compared with *S. meliloti* alone (Fig. 3.2D). When *M. sativa* was inoculated with *P. syringae* DC3000 (Fig. 3.2C) this led to the accumulation of proteins of low molecular weight and proteins around pI 5 mainly from bacterial origin (Table 3.1) compared with *M. sativa* alone (Fig. 3.2A) and *P. syringae* DC3000 alone (Fig. 3.2H). In the interaction between *A. thaliana* with *S. meliloti* the number of low molecular weight proteins increased after *S. meliloti* inoculation (Fig. 3.2F) compared with *A. thaliana* alone (Fig. 3.2E) and *S. meliloti* alone (Fig. 3.2D). On the other hand, in the interaction between *A. thaliana* and *P. syringae* DC3000 (Fig. 3.2G) an increase in nine plant proteins related to defense [three peroxidases (spots 4, 15 and 42), a glycosyl hydrolases family 17 (spot 5), a proteinase K (spot 14), an expressed protein (spot 18), a beta-1,3-glucanase class I precursor (spot 19), a basic chitinase (spot 37) and a glycosyl hydrolases family 18 (spot 40); Table 3.2] was observed compared with *A. thaliana* alone (Fig. 3.2E).

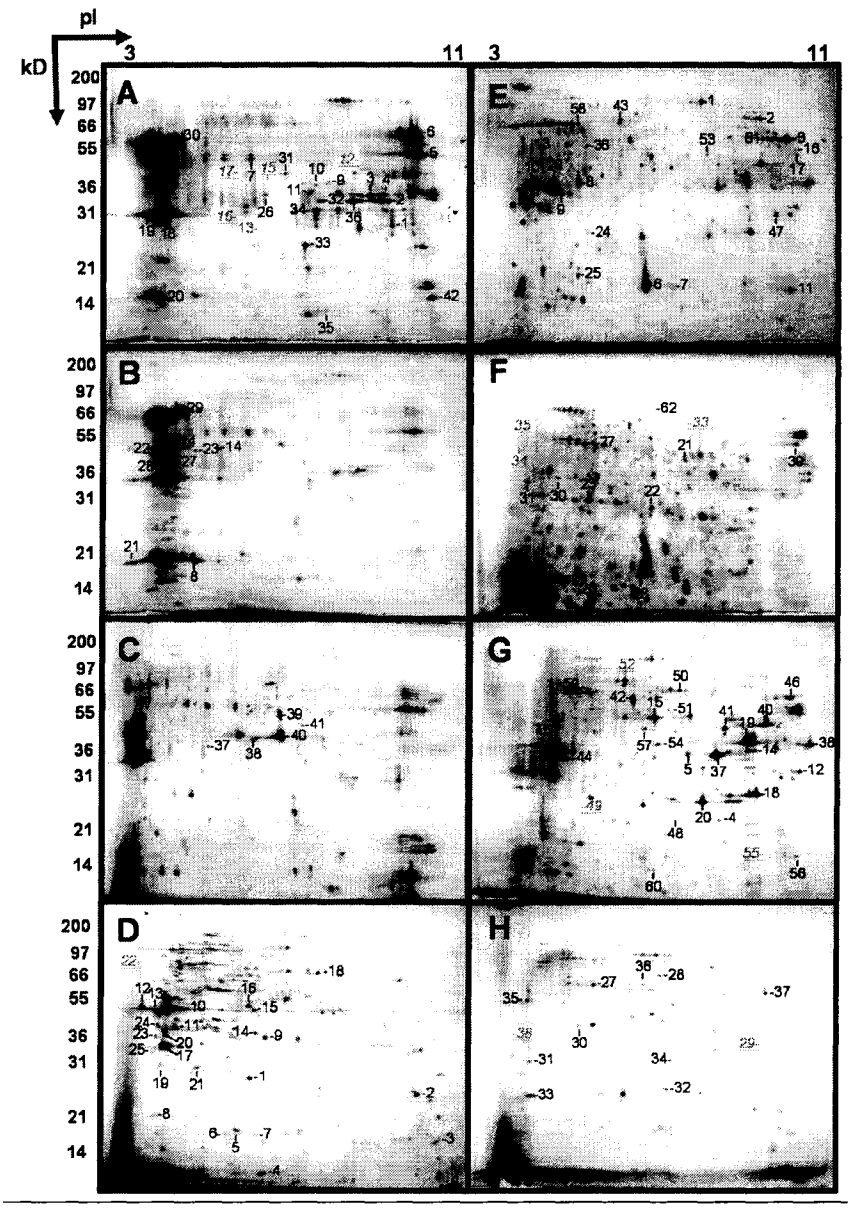

Figure 3.2. 2-DE of total secreted proteins in the interaction between plants and bacteria. Representative 2-DE gel showing root-secreted proteins at 6 hours after treatment of **A,** *M. sativa* alone; **B,** *M. sativa* inoculated with *S. meliloti*; **C,** *M. sativa* inoculated with *P. syringae* pv. tomato DC3000; **D,** *S. meliloti* alone; **E,** *A. thaliana* alone; **F,** *A. thaliana* inoculated with *S. meliloti*; **G,** *A. thaliana* inoculated with *P. syringae* DC3000; **H,** *P. syringae* pv. tomato DC3000 alone. One hundred and fifty micrograms of protein were isoelectrically focused and separated by SDS-PAGE gel. The molecular masses (kD) of protein standards are indicated to the left of the gel and the isoelectric point (pI) is indicated at the top of the gel. The arrows with numbers represent identified proteins which are listed in Table 3.1 (for A, B and C), Table 3.2 (for E, F and G) and Table 3.3 (for D and H). The underlined gray numbers represent the proteins that are not present in the representative gel, but are present in other samples.

Compatible interactions

I defined compatible interactions as those that led to the development of a symbiosis (*M. sativa-S. meliloti*) or a disease (*A. thaliana-P. syringae* DC3000). For a more detailed analysis of how the proteins changed over time, I analyzed the quantitative data collected at 6, 12, and 24 h, using the Phoretix 2D Expression software for each interaction and microorganism alone (Tables 3.1, 3.2 and 3.3; Materials and Methods). The interaction between *M. sativa* and *S. meliloti* caused an increase in the secretion of eleven proteins from plant origin (spots 7, 18, 19, 23, 24, 26, 28 and 31-34; Table 3.1) in two or more time points compared with the plant control. From those eleven proteins three were determined to be from the glycosidase hydrolase family 18 (spots 18, 19 and 28), one was a peptidase (spot 23), one a peroxidase precursor (spot 31) and one a thaumatin-like protein PR-5b (spot 33) were accumulated in the exudates more than two-fold in comparison with the plant alone. In addition, eight proteins from bacterial origin were increased in the *M. sativa* - *S. meliloti* interaction in two or more time points (spots 11, 22, 25, 29, 30, 37 and 38; Table 3.1). On the other hand, it was observed that seven proteins, all of plant origin except one [the putative phosphate-binding periplasmic protein (spot 27) that was identified as a bacterial protein], were decreased specifically in the *M. sativa* and *S. meliloti* interaction at two or more time points (spots 1, 2, 5, 20, 27, 35 and 42; Table 1). Of these seven proteins, a beta-1,3-glucanase (spot 2), a peroxidase (spot 5), a putative phosphate-binding periplasmic protein (spot 27) and a PR-1 pathogenesis related protein (spot 42) were secreted more than two-fold compared with the plant and microbe controls. Furthermore, seven proteins were undetectable in two or more time points in this compatible interaction

(spots 3, 4, 15, 16, 17, 39 and 41) from which a phosphate ABC transporter (spot 39) and a PotF1 protein (spot 41) were shown to be from bacterial origin.

In the interaction between *A. thaliana* and *P. syringae* DC3000 nine of the 62 identified proteins (spots 15, 17, 25, 33, 42, 43, 49, 51 and 55; Table 3.2) were induced at two time points. From these nine proteins, only two peroxidases (spots 15 and 42) and a polygalacturonase-like protein (spot 43) were secreted more than two-fold at 6h. In addition, sixteen proteins (spots 16, 22, 24, 26, 27, 41, 44, 45, 46, 48, 50, 54, 56, 57, 59 and 60; Table 3.2) were highly secreted at all time points (6, 12 and 24 h), from which a phosphate ABC transporter (spot 54), a hypothetical protein PSPTO_5175 (spot 56), a glucose ABC transporter protein (spot 57), a flagellar hook-associated protein FlgK (spot 59) and a secreted protein Hcp (spot 60) were of bacterial origin. On the other hand, twelve proteins of the 62 identified were decreased in secretion at only two time points (spots 4, 5, 14, 18, 19, 20, 37, 38, 40, 47, 53 and 61; Table 3.2); from these only one protein, of the glycosyl hydrolase family 18 (spot 40), was found to be secreted three-fold less than the plant control. Furthermore, nine proteins were decreased in secretion at all time points (spots 1, 2, 3, 8-12 and 58; Table 3.2); of these nine, four, a glycosyl hydrolase family 17 (spot 10), a pathogenesis-related PR-1-like protein (spot 11), a chitinase (spot 12) and a beta-amylase (spot 58) are considered to be defense-related proteins (Fritig et al., 1998). In addition, fifteen proteins (spots 7, 13, 21, 23, 28-32, 34-36, 39, 52 and 62; Table 3.2) were not detected in at least two time points. From those fifteen proteins, only five proteins [a putative phosphate-binding periplasmic protein (spot 31), a flagellin a protein (spot 34), a flagellin b protein (spot 35), a

translation elongation factor Tu (spot 36) and an outer membrane porin (spot 52)] are from bacterial origin.

Incompatible interactions

I defined incompatible interactions as those that did not lead to symbiosis (*A. thaliana* – *S. meliloti*) or disease (*M. sativa* – *P. syringae* DC3000). To determine whether the secreted proteins identified in the compatible interactions had a similar response in the incompatible interactions, I compared the quantitative data given as a normalized volume of 42 proteins in the interaction between *M. sativa* with *P. syringae* (Table 3.1) and 62 proteins in the interaction of *A. thaliana* with *S. meliloti* (Table 3.2).

In the incompatible interaction between *M. sativa* with *P. syringae*, I found that the response in protein exudation was different from the compatible reaction. In response to *P. syringae* DC3000 inoculation, *M. sativa* increased the secretion of eight proteins in at least two time points compared with the plant control (spots 3, 18, 19, 23, 24, 26, 28 and 31; Table 3.1) from which only one protein, an endo beta-1,3-glucanase (spot 3; Table 3.1) was not induced in the compatible interaction with *S. meliloti*. Furthermore, nine proteins [a translation elongation factor EF-Tu (spot 14), a putative phosphate-binding periplasmic protein (spot 27), an oligopeptide uptake ABC transporter periplasmic soluble-binding protein precursor (spot 29), a probable periplasmic dipeptide-binding protein (spot 30), a flagellin (spot 37), an outer membrane protein (spot 38), a phosphate ABC transporter, periplasmic phosphate-binding protein (spot 39), an amino acid ABC transporter, periplasmic amino acid-binding protein (spot 40) and a PotF1 protein (spot 41); Table 3.1] from *P. syringae* origin were induced in at least two time points. On the other hand, it was observed that in this incompatible

interaction secretion of eleven proteins (spots 2, 5, 6, 7, 20, 21, 32-35 and 42; Table 3.1) decreased in at least two time points. Three of those proteins [an unknown protein (spot 32), a thaumatin-like protein PR-5b (spot 33) and a thaumatin-like protein PR-5b precursor (spot 34)] were secreted by more than 1.5 fold in at least one time point in the compatible interaction with *S. meliloti*. Furthermore, I found that a polygalacturonase-inhibiting protein (spot 1), a proteinase K (spot 4) and a 50S ribosomal protein L9 (spot 8) were not detected at two time points; however, eight proteins (spots 9, 12, 13, 15, 16, 17, 22 and 25; Table 3.1) from the 42 identified were not detected at all time points in the *M. sativa* - *P. syringae* interaction.

I also studied the differences in protein secretion in the incompatible interaction between *A. thaliana* and *S. meliloti*. Significant differences were observed in this interaction in comparison to the compatible interaction between *A. thaliana* and *P. syringae*. For instance, seventeen proteins from plant origin (spots 6, 13, 16, 17, 21, 22, 24-28, 30, 33, 37, 39, 44 and 62; Table 3.2) were induced in at least two time points for this incompatible interaction, but six of those, a putative lectin (spot 6), an aspartyl protease (spot 13), a beta-fructofuranosidase (spot 21), a GSH-dependent dehydroascorbate reductase (spot 28), a xyloglucan endotransglycosylase (spot 30) and a neutral protease (spot 33), were not at all, or were only slightly secreted during the compatible interaction of *A. thaliana* and *P. syringae*. Furthermore, it was observed that fifteen proteins (spots 3, 4, 5, 8, 9, 10, 11, 15, 18, 19, 20, 29, 38, 42 and 58; Table 3.2) were decreased in secretion in at least two time points and from those seven proteins [two peroxidases (spots 4 and 42), a glycosyl hydrolases family 17 (spot 5), a peroxidase (spot 15), an expressed protein (spot 18), a beta-1,3-glucanase class I precursor (spot 19)

and an osmotin protein (spot 20)] were secreted in abundance at 6h in the compatible interaction. In addition, twenty proteins (spots 2, 7, 12, 14, 40, 41, 45, 46-57 and 61; Table 3.2) were not detected at two or more time points, but three proteins of the twenty [meprin and TRAF homology domain-containing protein (spot 41), glycosyl hydrolases family 17 (spot 45) and expressed protein (spot 46)] were secreted in abundance in *A. thaliana* with *P. syringae.*

Table 3.1. List of the proteins identified in the root exudates of *Medicago sativa*.

Spot No.	Protien Name/NCBInr	PAN[¶]	S[†]	P_{Num}[‡]	MW	pI	Medicago 6h			Medicago 12h			Medicago 24h		
							Control	*S.meliloti*	DC3000	Control	*S. meliloti*	DC3000	Control	*S. meliloti*	DC3000
1	Polygalacturonase-inhibiting protein	CAF04462.1	127	2	31373	9.05	0.431 (0.095)	3.174 (0.412)	n.d.	1.124 (0.102)	1.037 (0.373)	0.8 (0.91)	0.283 (0.012)	0.184 (0.066)	n.d.
2	Beta-1,3-glucanase	AAB24398	82	2	37423	5.98	0.674 (0.015)	0.116 (0.012)	0.04 (0.001)	0.216 (0.010)	0.512 (0.125)	0.555 (0.041)	0.34 (0.028)	0.158 (0.019)	0.251 (0.02)
3	hydrolase, family 17	ABD32327	310	5	40288	8.79	0.131 (0.016)	n.d.	0.04 (0.001)	0.02 (0.003)	n.d.	0.488 (0.011)	n.d.	n.d.	0.161 (0.008)
4	Proteinase K	1205229A	304	4	28467	7.79	0.48 (0.032)	n.d.	n.d.	0.278 (0.002)	n.d.	n.d.	0.212 (0.045)	0.034 (0.002)	0.323 (0.019)
5	Peroxidase	CAA62227	475	8	38252	5.8	2.741 (0.152)	2.055 (0.15)	0.646 (0.033)	3.49 (0.38)	1.708 (0.192)	2.671 (0.176)	2.318 (0.38)	0.269 (0.021)	1.946 (0.13)
6	Aspartic, catalytic	ABE77449	283	6	46519	8.54	2.818 (0.364)	1.622 (0.105)	0.479 (0.05)	0.623 (0.078)	0.859 (0.093)	0.723 (0.06)	0.917 (0.1)	n.d.	0.427 (0.39)
7	Peroxidase pxdC precursor	AAB41811	245	4	38176	5.76	0.39 (0.066)	0.33 (0.019)	0.242 (0.022)	0.597 (0.030)	0.625 (0.043)	0.372 (0.022)	0.117 (0.018)	0.489 (0.013)	1.008 (0.23)
8	50S ribosomal protein L9	CAL22124	110	2	15852	6.3	n.d.	n.d.	0.344 (0.031)	1.02 (0.222)	0.432 (0.038)	n.d.	n.d.	0.329 (0.030)	n.d.
9	Glyceraldehyde 3-phosphate dehydrogenase A	NP_405702	488	9	35933	6.19	0.794 (0.095)	n.d.	n.d.	n.d.	0.075 (0.003)	n.d.	0.993 (0.062)	0.135 (0.026)	n.d.
10	Outer membrane protein II	AAA24807	260	4	25538	4.88	0.736 (0.098)	n.d.	0.011 (0.009)	n.d.	1.131 (0.199)	0.092 (0.007)	1.571 (0.199)	0.045 (0.003)	n.d.
11	Major outer membrane protein	AAK68978	115	2	15693	4.81	0.114 (0.025)	0.412 (0.034)	n.d.	0.055 (0.007)	0.071 (0.003)	0.071 (0.008)	0.317 (0.038)	0.065 (0.003)	0.222 (0.044)
12	Outer membrane protein a precursor	P04845	121	2	38402	8.27	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.623 (0.125)	n.d.
13	3-hydroxybutyrate dehydrogenase	ZP_00070199	85	1	27393	5.14	n.d.	n.d.	n.d.	1.43 (0.117)	1.12 (0.166)	n.d.	n.d.	1.375 (0.126)	n.d.
14	Translation elongation factor EF-Tu	S13560	422	7	43224	5.3	n.d.	n.d.	0.231 (0.037)	n.d.	n.d.	0.365 (0.051)	n.d.	2.117 (0.25)	0.877 (0.24)
15	Enolase	NP_406838	380	6	45440	5.18	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.945 (0.074)	n.d.	n.d.
16	6-phosphogluconate dehydrogenase	NP_405127	301	5	51541	5.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.001 (0.281)	n.d.	n.d.
17	Phosphoglycerate kinase	NP_457468	100	3	41107	5.09	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.582 (0.21)	n.d.	n.d.
18	Hydrolase, family 18	ABE87096	205	3	31418	4.88	2.705 (0.259)	5.078 (0.611)	4.720 (0.051)	5.508 (0.921)	0.529 (0.003)	3.485 (0.33)	1.503 (0.062)	7.435 (1.026)	2.093 (0.28)
19	Glycoside hydrolase, family 18	ABE87095.1	139	2	27628	4.59	2.811 (0.319)	5.922 (0.707)	3.114 (0.449)	5.010 (0.827)	2.103 (0.235)	3.135 (0.401)	1.522 (0.172)	5.194 (0.974)	2.942 (0.39)
20	PR10-1 protein	CAA69931	350	7	16647	4.59	4.938 (0.952)	9.28 (0.512)	0.035 (0.03)	5.212 (0.287)	7.458 (1.13)	5.087 (0.3)	2.865 (0.368)	1.6 (0.051)	1.409 (0.112)
21	Disease resistance response protein Pi49 (PR10)	P14710	100	2	16737	4.94	1.71 (0.059)	1.128 (0.11)	1.05 (0.082)	1.624 (0.333)	1.6 (0.103)	1.032 (0.475)	n.d.	1.175 (0.026)	4.18 (0.47)

Table 3.1. (Cont.) List of the proteins identified in the root exudates of *Medicago sativa*.

Spot No.	Protien Name/NCBInr	PAN[¶]	S[†]	P_{Num}[‡]	MW	pI	Medicago 6h			Medicago 12h			Medicago 24h		
							Control	*S.meliloti*	DC3000	Control	*S. meliloti*	DC3000	Control	*S. meliloti*	DC3000
22	Outer membrane lipoprotein transmembrane	NP_386994	58	1	27536	4.65	n.d.	1.484 (0.264)	n.d.	n.d.	4.686 (0.510)	n.d.	n.d.	0.718 (0.040)	n.d.
23	Peptidase C1A, papain	ABD32628	93	1	52187	5.93	0.011 (0.009)	0.133 (0.01)	0.03 (0.003)	0.343 (0.021)	0.548 (0.03)	0.655 (0.13)	0.081 (0.038)	0.392 (0.053)	2.681 (0.92)
24	Conserved hypothetical protein	NP_387319	145	2	32464	4.89	n.d.	0.16 (0.022)	0.032 (0.005)	n.d.	0.172 (0.020)	0.18 (0.026)	n.d.	1.031 (0.266)	0.098 (0.01)
25	Probable general l-amino acid-binding periplasmic ABC transporter protein	NP_385584	339	5	36833	5	n.d.	1.202 (0.098)	n.d.	n.d.	2.948 (0.314)	n.d.	n.d.	2.684 (0.184)	n.d.
26	Chitinase	CAA71402	64	1	34901	6.77	0.065 (0.01)	0.118 (0.031)	0.063 (0.003)	0.048 (0.011)	0.067 (0.021)	0.164 (0.036)	0.069 (0.001)	n.d.	1.08 (0.32)
27	Putative phosphate-binding periplasmic protein	NP_384615	371	6	36493	4.77	n.d.	2.637 (0.43)	0.216 (0.02)	n.d.	1.302 (0.057)	n.d.	n.d.	1.576 (0.25)	0.101 (0.015)
28	Glycoside hydrolase, family 18	ABE87095	112	2	63517	6.29	0.312 (0.09)	1.529 (0.1)	1.591 (0.104)	n.d.	0.028 (0.001)	0.339 (0.03)	0.074 (0.004)	0.19 (0.036)	4.643 (0.28)
29	Oligopeptide uptake ABC transporter periplasmic solute-binding protein precursor	NP_437401	295	5	58968	4.97	n.d.	2.605 (0.059)	0.385 (0.026)	n.d.	1.882 (0.098)	1.192 (0.4)	n.d.	1.194 (0.051)	n.d.
30	Probable periplasmic dipeptide-binding protein	NP_384837	103	2	59026	4.93	n.d.	1.294 (0.218)	0.189 (0.022)	n.d.	0.728 (0.031)	0.237 (0.025)	n.d.	0.988 (0.037)	n.d.
31	Peroxidase 1C precursor	CAA62227	475	8	38252	5.8	0.13 (0.025)	0.423 (0.03)	0.294 (0.031)	0.118 (0.012)	0.381 (0.097)	0.431 (0.02)	0.005 (0.005)	0.027 (0.002)	0.708 (0.037)
32	Unknown protein	AAM91539	112	2	32411	6.23	0.28 (0.032)	0.319 (0.017)	0.498 (0.081)	0.451 (0.025)	0.215 (0.012)	0.117 (0.012)	0.6 (0.08)	0.736 (0.18)	0.496 (0.023)
33	Thaumatin-like protein PR-5b	CAA09228.1	94	2	16204	9.1	0.058 (0.002)	0.131 (0.031)	0.022 (0.001)	0.226 (0.017)	3.294 (0.3)	0.142 (0.02)	1.035 (0.031)	0.027 (0.006)	0.043 (0.008)
34	Thaumatin-like protein PR-5b precursor	CAA09228.1	214	3	34604	7.51	0.773 (0.037)	0.422 (0.022)	0.023 (0.004)	1.488 (0.199)	2.742 (0.188)	1.161 (0.140)	0.965 (0.175)	1.012 (0.192)	1.147 (0.114)
35	Wound-induced protein	Q02243	311	4	11116	7.81	0.078 (0.023)	0.073 (0.039)	0.019 (0.011)	0.646 (0.023)	0.298 (0.022)	0.108 (0.047)	0.324 (0.023)	0.189 (0.007)	0.056 (0.03)
36	Chitinase	AAB41324	526	8	34900	7.79	1.331 (0.2)	0.979 (0.076)	0.35 (0.001)	0.665 (0.037)	0.679 (0.024)	0.992 (0.02)	n.d.	n.d.	n.d.
37	Flagellin	NP_791772	260	4	29158	5.25	n.d.	0.138 (0.011)	0.159 (0.03)	n.d.	0.446 (0.03)	1.475 (0.113)	n.d.	2.073 (0.191)	0.593 (0.04)
38	Outer membrane protein	NP_791545	259	4	45545	5.75	n.d.	0.465 (0.021)	0.325 (0.017)	n.d.	0.317 (0.025)	0.655 (0.042)	n.d.	0.366 (0.049)	1.272 (0.08)

Table 3.1. (Cont.) List of the proteins identified in the root exudates of *Medicago sativa*.

Spot No.	Protien Name/NCBInr	PAN¶	S†	P_{Num}‡	MW	pI	Medicago 6h			Medicago 12h			Medicago 24h		
							Control	*S.meliloti*	DC3000	Control	*S. meliloti*	DC3000	Control	*S. meliloti*	DC3000
39	Phosphate ABC transporter, periplasmic phosphate-binding protein	NP_793052	784	14	36657	8.31	n.d.	n.d.	3.709 (0.895)	n.d.	n.d.	0.709 (0.123)	n.d.	0.15 (0.014)	3.275 (0.4)
40	Amino acid ABC transporter, periplasmic amino acid-binding protein	NP_791085	259	5	36675	7.52	n.d.	0.036 (0.02)	0.257 (0.026)	n.d.	0.025 (0.012)	0.145 (0.012)	n.d.	0.32 (0.023)	1.332 (0.094)
41	PotF1	AAK15487	86	2	40054	7.62	n.d.	n.d.	0.079 (0.013)	n.d.	n.d.	0.086 (0.004)	n.d.	n.d.	0.479 (0.055)
42	PR-1 pathogenesis related protein	CAA69931	99	6	14216	9.21	0.651 (0.078)	0.31 (0.01)	0.034 (0.01)	1.018 (0.137)	0.394 (0.143)	0.393 (0.181)	0.194 (0.022)	1.448 (0.16)	n.d.

¶: protein accession number; †: Mascot MOWSE score; ‡: number of peptides matched to the protein; MW: molecular weight; *p*I: isoelectric point.

The numbers represent the normalized volume of three different gels. n.d. means that a particular protein was not detected. The number in parenthesis represents the standard error at $p<0.05$

Table 3.2. List of the proteins identified in the root exudates of *Arabidopsis thaliana.*

Spot No.	Protien Name/NCBInr	PAN[¶]	S[†]	P_{Num}[‡]	MW	pI	Arabidopsis 6h			Arabidopsis 12h			Arabidopsis 24h		
							Control	*S.meliloti*	DC3000	Control	*S.meliloti*	DC3000	Control	*S.meliloti*	DC3000
1	Subtilisin-like proteinase nodule-specific	CAA59963	705	11	78164	5.81	0.894 (0.152)	n.d.	0.048 (0.002)	1.135 (0.2)	0.037 (0.005)	0.297 (0.017)	0.51 (0.031)	0.57 (0.021)	0.25 (0.013)
2	Pectin methylesterase	AAM97070	691	11	60003	8.49	0.861 (0.089)	n.d.	0.193 (0.005)	0.967 (0.070)	n.d.	0.07 (0.003)	0.167 (0.013)	0.21 (0.034)	0.02 (0.02)
3	Chloroplast nucleoid DNA-binding protein-related	NP_563851	469	9	47631	7.48	2.213 (0.352)	0.085 (0.067)	0.444 (0.024)	1.576 (0.414)	0.25 (0.031)	0.504 (0.017)	0.931 (0.036)	0.031 (0.016)	0.149 (0.07)
4	Peroxidase	NP_201217	96	2	15966	6.74	0.77 (0.052)	0.623 (0.031)	2.612 (0.2)	1.694 (0.112)	1.633 (0.125)	0.991 (0.01)	0.868 (0.054)	0.796 (0.021)	0.522 (0.03)
5	Glycosyl hydrolase family 17	NP_193361	140	4	33565	5.3	1.115 (0.152)	1.28 (0.231)	3.263 (0.2)	1.511 (0.112)	0.387 (0.025)	0.688 (0.012)	1.383 (0.154)	0.433 (0.021)	1.127 (0.213)
6	Putative lectin	AAM65935	403	6	32138	5.46	4.63 (0.52)	3.494 (0.231)	n.d.	1.516 (0.112)	4.187 (0.523)	0.397 (0.012)	n.d.	1.447 (0.021)	0.408 (0.013)
7	RuBisCO small chain B2 precursor	CAA32701	80	2	20303	7.59	0.316 (0.123)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	Jasmonate inducible protein	At1g52070.1	51	2	32672	5.46	1.913 (0.134)	0.066 (0.007)	0.025 (0.03)	1.278 (0.098)	0.021 (0.03)	0.32 (0.013)	4.456 (0.513)	0.593 (0.037)	2.544 (0.08)
9	Methylglyoxalase	Q39366	93	2	31626	5.19	1.286 (0.089)	0.059 (0.022)	0.316 (0.297)	0.792 (0.018)	0.179 (0.05)	0.529 (0.047)	0.747 (0.025)	0.251 (0.03)	0.333 (0.05)
10	Glycosyl hydrolase family 17	NP_191285	694	11	37316	4.85	1.417 (0.177)	0.172 (0.023)	1.113 (0.141)	1.638 (0.131)	0.188 (0.019)	0.479 (0.021)	2.595 (0.216)	0.898 (0.062)	0.277 (0.01)
11	Pathogenesis-related PR-1-like protein	NP_179068	200	3	17666	9.08	0.576 (0.03)	0.058 (0.002)	0.175 (0.008)	1.874 (0.095)	0.01 (0.003)	0.551 (0.065)	1.686 (0.103)	n.d.	0.358 (0.022)
12	Chitinase	NP_181887	191	3	28334	8.43	0.545 (0.011)	n.d.	0.074 (0.02)	0.935 (0.1)	n.d.	0.383 (0.043)	0.856 (0.035)	n.d.	0.164 (0.024)
13	Aspartyl protease family protein	NP_188478	177	3	53201	5.27	0.762 (0.32)	0.823 (0.131)	0.292 (0.12)	n.d.	0.025 (0.015)	n.d.	1.504 (0.219)	0.062 (0.011)	n.d.
14	Proteinase K	1205229A	168	2	28647	7.79	0.426 (0.032)	n.d.	0.933 (0.12)	0.961 (0.41)	n.d.	0.595 (0.061)	0.734 (0.019)	n.d.	0.247 (0.03)
15	Peroxidase	AAA32842	219	4	36184	5.95	0.611 (0.032)	0.43 (0.01)	1.632 (0.12)	1.131 (0.241)	0.405 (0.023)	1.451 (0.161)	0.634 (0.019)	0.4 (0.049)	0.5 (0.08)
16	Peroxidase	CAA50677	532	10	38808	7.57	0.242 (0.032)	1.582 (0.21)	1.142 (0.032)	0.041 (0.011)	3.54 (0.23)	0.565 (0.161)	0.06 (0.09)	0.275 (0.019)	0.337 (0.013)
17	Peroxidase C1C precursor	P15233	226	5	36525	6.21	0.121 (0.04)	1.133 (0.08)	0.042 (0.03)	n.d.	0.148 (0.011)	0.089 (0.032)	n.d.	0.028 (0.002)	0.194 (0.042)
18	Expressed protein	NP_565369	740	11	25149	8.56	1.285 (0.321)	0.246 (0.053)	2.063 (0.199)	0.94 (0.034)	0.617 (0.166)	0.43 (0.033)	0.809 (0.104)	0.282 (0.028)	0.649 (0.102)
19	Beta-1,3-glucanase class I precursor	AAL36038	950	16	37688	6.43	0.235 (0.019)	0.08 (0.02)	1.694 (0.25)	0.937 (0.039)	1.055 (0.01)	0.525 (0.034)	0.354 (0.022)	0.209 (0.017)	0.187 (0.01)
20	Osmotin	CAA61411	255	4	26585	6.67	0.219 (0.1)	1.212 (0.072)	2.385 (0.911)	1.06 (0.073)	0.684 (0.033)	0.784 (0.047)	0.954 (0.128)	0.216 (0.018)	0.895 (0.099)
21	Beta-fructofuranosidase	CAA61624	235	6	62924	5.41	n.d.	0.238 (0.052)	n.d.	n.d.	0.105 (0.048)	0.467 (0.036)	0.214 (0.017)	n.d.	n.d.

Table 3.2. (Cont.) List of the proteins identified in the root exudates of *Arabidopsis thaliana.*

Spot No.	Protien Name/NCBInr	PAN[¶]	S[†]	P_{Num}[‡]	MW	pI	Arabidopsis 6h			Arabidopsis 12h			Arabidopsis 24h		
							Control	*S.meliloti*	DC3000	Control	*S.meliloti*	DC3000	Control	*S.meliloti*	DC3000
22	Superoxide dismutase (Fe)	CAB36752	96	1	25364	6.13	n.d.	0.506 (0.035)	0.241 (0.014)	0.09 (0.01)	0.454 (0.033)	0.243 (0.051)	n.d.	0.997 (0.17)	0.195 (0.032)
23	Dehydroascorbate reductase	NP_173387	656	14	23626	5.56	n.d.	1.315 (0.242)	n.d.	n.d.	0.804 (0.103)	n.d.	n.d.	0.247 (0.23)	n.d.
24	Putative trypsin inhibitor	NP_565061	209	4	23778	6.74	0.094 (0.023)	0.419 (0.021)	0.398 (0.032)	0.197 (0.03)	0.341 (0.102)	0.25 (0.048)	0.092 (0.009)	0.125 (0.016)	0.297 (0.011)
25	Beta-amylase	CAB10300	276	7	56126	5.2	0.518 (0.035)	1.384 (0.108)	0.678 (0.076)	n.d.	0.865 (0.025)	0.193 (0.011)	n.d.	0.09 (0.023)	n.d.
26	Thioredoxin; protein	S58118	100	3	13101	5.06	0.482 (0.13)	0.622 (0.092)	0.601 (0.1)	0.029 (0.005)	0.268 (0.12)	1.186 (0.359)	0.799 (0.4)	0.689 (0.023)	1.086 (0.299)
27	Beta-amylase	BAA07842	134	4	55993	5.2	n.d.	0.696 (0.04)	0.067 (0.008)	n.d.	0.415 (0.051)	0.51 (0.083)	n.d.	0.738 (0.095)	0.56 (0.03)
28	GSH-dependent dehydroascorbate reductase 1	NP_173386	81	2	17051	5.58	n.d.	0.666 (0.127)	n.d.	n.d.	0.374 (0.048)	n.d.	n.d.	0.107 (0.024)	0.319 (0.021)
29	Phytocyanin	NP_182006	109	2	20341	5.83	0.881 (0.041)	0.788 (0.08)	n.d.	n.d.	0.841 (0.127)	n.d.	2.731 (0.348)	1.023 (0.072)	n.d.
30	Xyloglucan endotransglycosylase (XTR-6)	NP_194311	152	3	32043	5.05	n.d.	0.13 (0.08)	n.d.	n.d.	0.125 (0.033)	n.d.	0.58 (0.071)	0.157 (0.04)	n.d.
31	Putative phosphate-binding periplasmic protein	NP_384615	286	5	36493	4.77	n.d.	0.132 (0.022)	n.d.	n.d.	0.725 (0.132)	n.d.	n.d.	0.076 (0.02)	n.d.
32	Thioredoxin m2	AAF15949	180	4	20360	9.35	n.d.	2.617 (0.314)	n.d.	n.d.	0.951 (0.166)	n.d.	n.d.	1.911 (0.152)	n.d.
33	Neutral protease	AAK00602	321	6	61069	5.81	n.d.	n.d.	0.006 (0.002)	n.d.	0.201 (0.055)	0.108 (0.007)	n.d.	0.363 (0.071)	n.d.
34	Flagellin a protein [sinorhizobium meliloti]	NP_384775	696	10	40693	4.19	n.d.	0.24 (0.011)	n.d.	n.d.	0.427 (0.3)	n.d.	n.d.	1.882 (0.084)	n.d.
35	Flagellin b protein [sinorhizobium meliloti]	NP_384776	685	12	40717	4.18	n.d.	0.241 (0.03)	n.d.	n.d.	0.756 (0.047)	n.d.	n.d.	0.545 (0.077)	n.d.
36	Translation elongation factor Tu	NP_742606	130	2	43468	5.22	n.d.	0.031 (0.014)	0.067 (0.009)	n.d.	0.077 (0.01)	n.d.	n.d.	0.155 (0.007)	n.d.
37	Basic chitinase	1710349A	410	6	36091	8.05	0.108 (0.02)	0.248 (0.028)	3.243 (0.355)	0.889 (0.091)	1.239 (0.23)	0.863 (0.11)	1.609 (0.085)	0.525 (0.041)	0.556 (0.028)
38	Peroxidase	NP_201217	794	12	34868	8.6	1.419 (0.36)	0.503 (0.291)	1.294 (0.087)	3.539 (0.116)	0.231 (0.031)	3.311 (0.096)	1.09 (0.131)	0.031 (0.048)	2.207 (0.234)
39	Peroxidase	CAA50677	162	4	38927	8.08	n.d.	0.541 (0.073)	n.d.	n.d.	0.889 (0.044)	n.d.	n.d.	n.d.	n.d.
40	Glycosyl hydrolase family 18	NP_193716	749	12	41102	8.91	1.441 (0.11)	n.d.	3.18 (0.17)	0.948 (0.162)	0.03 (0.005)	0.238 (0.053)	0.958 (0.127)	n.d.	0.258 (0.011)
41	Meprin and TRAF homology domain-containing protein	NP_566660	254	4	43422	6.17	n.d.	n.d.	0.58 (0.186)	n.d.	n.d.	0.237 (0.055)	n.d.	n.d.	0.215 (0.094)

Table 3.2. (Cont.) List of the proteins identified in the root exudates of *Arabidopsis thaliana.*

Spot No.	Protien Name/NCBInr	PAN[¶]	S[†]	P_{Num}[‡]	MW	pI	Arabidopsis 6h			Arabidopsis 12h			Arabidopsis 24h		
							Control	*S.meliloti*	DC3000	Control	*S.meliloti*	DC3000	Control	*S.meliloti*	DC3000
42	Peroxidase	AAA32842	289	6	38148	5.95	0.226 (0.058)	0.043 (0.011)	2.556 (0.406)	1.23 (0.076)	0.158 (0.023)	0.921 (0.015)	n.d.	0.086 (0.014)	0.617 (0.122)
43	Polygalacturonase-like protein	BAA95779	323	6	48866	5.38	0.14 (0.08)	n.d.	0.816 (0.175)	n.d.	0.238 (0.084)	0.965 (0.218)	0.18 (0.022)	0.089 (0.042)	0.011 (0.023)
44	Glyoxalase I, putative (lactoylglutathione lyase)	NP_172648	418	8	31908	5.19	n.d.	n.d.	0.877 (0.134)	n.d.	0.299 (0.073)	0.082 (0.012)	n.d.	0.029 (0.006)	0.033 (0.012)
45	Glycosyl hydrolase family 17	NP_191285	116	6	37316	4.85	n.d.	n.d.	0.397 (0.062)	0.051 (0.02)	n.d.	0.299 (0.017)	n.d.	0.007 (0.002)	0.201 (0.033)
46	Expressed protein	NP_563851	249	5	47631	7.48	n.d.	n.d.	0.828 (0.075)	0.883 (0.073)	n.d.	1.42 (0.375)	n.d.	n.d.	0.129 (0.029)
47	Germin, putative	NP_173332	79	1	23169	8.91	0.653 (0.079)	n.d.	0.619 (0.112)	0.37 (0.044)	1.035 (0.478)	0.774 (0.063)	0.195 (0.071)	n.d.	0.065 (0.018)
48	YceI-like family protein	NP_794803	115	3	20621	6.59	n.d.	n.d.	0.382 (0.105)	n.d.	n.d.	0.519 (0.049)	n.d.	n.d.	0.547 (0.118)
49	Superoxide dismutase, Fe	NP_794118	124	3	21352	5.27	n.d.	n.d.	n.d.	n.d.	n.d.	0.342 (0.021)	n.d.	n.d.	0.727 (0.092)
50	Polygalacturonase	NP_793726	953	18	55422	6.7	n.d.	n.d.	0.364 (0.027)	n.d.	n.d.	0.638 (0.017)	n.d.	n.d.	0.561 (0.055)
51	Serine protease, MucD	NP_793982	93	2	50341	6.5	n.d.	n.d.	0.507 (0.022)	n.d.	n.d.	0.244 (0.019)	n.d.	n.d.	n.d.
52	Outer membrane porin, OprD family	NP_795049	790	12	48440	6.06	n.d.	n.d.	n.d.	n.d.	n.d.	0.255 (0.113)	n.d.	n.d.	n.d.
53	Myrosinase-associated protein	NP_175802	124	4	43166	6.65	0.073 (0.017)	n.d.	0.034 (0.027)	0.386 (0.114)	n.d.	0.298 (0.017)	0.277 (0.375)	0.328 (0.113)	0.402 (0.19)
54	Phosphate ABC transporter	NP_793052	849	17	36657	8.31	n.d.	0.385 (0.017)	0.556 (0.083)	n.d.	n.d.	1.717 (0.521)	n.d.	n.d.	0.753 (0.196)
55	Flagellin	NP_791772	644	10	29158	5.25	n.d.	n.d.	n.d.	n.d.	n.d.	0.263 (0.031)	n.d.	n.d.	0.141 (0.064)
56	Hypothetical protein PSPTO_5175	NP_794906	159	3	18463	9	n.d.	n.d.	0.059 (0.004)	n.d.	n.d.	0.073 (0.006)	n.d.	n.d.	0.254 (0.084)
57	Glucose ABC transporter	NP_791121	318	6	45652	6.17	n.d.	n.d.	0.683 (0.046)	n.d.	0.132 (0.028)	0.658 (0.054)	n.d.	n.d.	0.5 (0.12)
58	Glycosyl hydrolase family 14 (beta-amylase)	NP_567460	1055	17	56027	5.2	0.668 (0.103)	0.498 (0.119)	0.166 (0.052)	1.088 (0.188)	n.d.	0.241 (0.037)	0.563 (0.041)	0.248 (0.088)	0.143 (0.025)
59	Flagellar hook-associated protein FlgK	NP_791767	1415	18	69934	4.98	n.d.	0.238 (0.104)	0.41 (0.087)	n.d.	n.d.	0.048 (0.01)	n.d.	0.206 (0.191)	0.359 (0.041)
60	Secreted protein Hcp	NP_795162	459	8	19075	5.53	n.d.	0.719 (0.097)	0.771 (0.033)	n.d.	0.307 (0.123)	1.182 (0.198)	n.d.	n.d.	1.184 (0.175)
61	Translation elongation factor eEF-1 alpha chain	BAE98750	89	2	49457	9.19	2.782 (0.599)	n.d.	0.776 (0.035)	1.116 (0.167)	n.d.	0.662 (0.105)	0.61 (0.039)	0.434 (0.052)	2.252 (0.417)

Table 3.2. (Cont.) List of the proteins identified in the root exudates of *Arabidopsis thaliana.*

Spot No.	Protien Name/NCBInr	PAN¶	S†	P_{Num}‡	MW	pI	Arabidopsis 6h			Arabidopsis 12h			Arabidopsis 24h		
							Control	*S.meliloti*	DC3000	Control	*S.meliloti*	DC3000	Control	*S.meliloti*	DC3000
62	Putative lectin	At3g16460.1	86	2	72430	5.31	n.d.	0.762 (0.085)	n.d.	n.d.	0.939 (0.127)	n.d.	n.d.	n.d.	n.d.

¶: protein accession number; †: Mascot MOWSE score; ‡: number of peptides matched to the protein; MW: molecular weight; *p*I: isoelectric point.

The numbers represent the normalized volume of three different gels. n.d. means that a particular protein was not detected. The number in parenthesis represents the standard error at $p<0.05$

Table 3.3. List of the proteins identified secreted in *Pseudomonas syringae* pv. tomato DC3000 and *Sinorhizobium meliloti*

Spot No.	Protien Name/NCBInr	PAN[¶]	S[†]	P_{Num}[‡]	MW	pI	*S. meliloti* 6h			*S. meliloti* 12h			*S. meliloti* 24h		
							Control	*M. sativa*	*A. thaliana*	Control	*M. sativa*	*A. thaliana*	Control	*M. sativa*	*A. thaliana*
1	Superoxide dismutase	YP_350214	113	2	24490	5.81	0.867 (0.102)	n.d.	0.581 (0.082)	0.131 (0.017)	1.11 (0.021)	0.140 (0.033)	0.11 (0.03)	0.078 (0.011)	0.054 (0.066)
2	Putative signal peptide protein	NP_385748	286	5	22461	8.87	0.721 (0.102)	n.d.	n.d.	0.762 (0.092)	n.d.	n.d.	0.267 (0.061)	n.d.	n.d.
3	30 ribosomal protein S8	NP_742634	209	4	13966	9.63	0.363 (0.033)	n.d.	n.d.	0.773 (0.11)	0.214 (0.021)	0.197 (0.023)	n.d.	n.d.	n.d.
4	Cold acclimation protein CapB	AAK35071	89	2	7691	6.54	1.159 (0.677)	n.d.	0.866 (0.102)	0.254 (0.023)	n.d.	0.193 (0.062)	0.012 (0.01)	n.d.	n.d.
5	50S ribosomal protein L9	NP_794663	208	4	15461	5.39	1.161 (0.281)	0.229 (0.036)	0.404 (0.038)	0.032 (0.012)	0.314 (0.02)	0.133 (0.042)	0.008 (0.004)	n.d.	n.d.
6	Nucleoside diphosphate kinase	NP_791256	150	4	14942	5.41	0.12 (0.028)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
7	Ribosomal protein L9	YP_346270	117	2	15443	5.41	0.063 (0.025)	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
8	Protein-transport protein	Q93TF4	65	1	17741	4.49	0.189 (0.023)	0.113 (0.028)	0.255 (0.011)	0.308 (0.121)	0.551 (0.192)	0.3 (0.022)	0.42 (0.15)	0.483 (0.048)	0.399 (0.061)
9	Succinyl-CoA synthetase, alpha subunit	NP_746302	564	10	30092	5.89	0.898 (0.192)	n.d.	0.504 (0.048)	0.429 (0.077)	n.d.	0.683 (0.122)	1.013 (0.195)	0.833 (0.199)	1.513 (0.53)
10	Putative phosphate-binding periplasmic protein	NP_384615	1349	18	36493	4.77	4.025 (1.003)	5.85 (1.187)	0.132 (0.022)	4.757 (0.946)	6.48 (1.198)	0.725 (0.13)	11.344 (2.94)	18.77 (10.52)	0.072 (0.02)
11	Probable leu/ile/val-binding protein	NP_385029	84	2	37684	4.91	0.757 (0.176)	1.57 (0.521)	n.d.	0.61 (0.055)	1.03 (0.116)	n.d.	1.202 (0.328)	1.484 (0.294)	0.991 (0.111)
12	Flagellin A protein	NP_384775	881	13	40693	4.19	0.256 (0.052)	0.281 (0.023)	0.24 (0.011)	1.759 (0.222)	0.833 (0.067)	0.427 (0.03)	1.874 (0.292)	0.605 (0.08)	1.882 (0.084)
13	Flagellin B protein	NP_384776	779	11	40717	4.18	0.343 (0.043)	0.277 (0.075)	0.241 (0.03)	1.309 (0.21)	0.734 (0.045)	0.756 (0.047)	1.166 (0.119)	0.837 (0.077)	0.545 (0.077)
14	Hypothetical immunogenic protein	NP_436822	355	7	34841	6.01	0.505 (0.055)	n.d.	n.d.	n.d.	0.381 (0.032)	n.d.	0.04 (0.01)	n.d.	n.d.
15	Carbamate kinase	YP_350116	176	3	32730	5.81	0.787 (0.123)	1.391 (0.222)	0.321 (0.439)	n.d.	1.558 (0.129)	0.731 (0.055)	n.d.	n.d.	n.d.
16	Ketol-acid reductoisomerase	YP_262325	664	11	36213	5.48	0.814 (0.07)	n.d.	n.d.	0.705 (0.063)	n.d.	n.d.	0.87 (0.051)	n.d.	n.d.
17	Phosphate uptake ABC transporter periplasmic solute-binding protein precursor	NP_437382	880	15	32011	4.9	4.742 (1.002)	5.643 (0.943)	0.321 (0.231)	1.602 (0.278)	0.822 (0.077)	0.343 (0.042)	8.953 (1.255)	1.419 (0.298)	0.993 (0.117)
18	Signal peptide protein	NP_385656	98	2	34230	5.63	1.403 (0.104)	0.129 (0.044)	0.489 (0.063)	0.225 (0.015)	n.d.	0.310 (0.044)	0.379 (0.021)	n.d.	0.123 (0.032)
19	Hypothetical protein SMc02156	NP_384596	817	12	28535	4.8	0.382 (0.053)	0.512 (0.031)	0.616 (0.101)	n.d.	0.220 (0.033)	0.108 (0.01)	n.d.	0.316 (0.044)	0.47 (0.062)
20	Conserved hypothetical exported protein	NP_437971	300	6	32271	4.84	2.335 (0.875)	1.103 (0.4)	0.511 (0.068)	0.567 (0.066)	0.919 (0.11)	0.614 (0.078)	0.909 (0.05)	0.471 (0.043)	0.505 (0.107)

Table 3.3. (Cont.) List of the secreted proteins identified in *Pseudomonas syringae* pv. tomato DC3000 and *Sinorhizobium meliloti*

Spot No.	Protien Name/NCBInr	PAN[¶]	S[†]	P_{Num}[‡]	MW	pI	*S. meliloti* 6h			*S. meliloti* 12h			*S. meliloti* 24h		
							Control	*M. sativa*	*A. thaliana*	Control	*M. sativa*	*A. thaliana*	Control	*M. sativa*	*A. thaliana*
21	Putative amino-acid binding periplasmic protein	NP_385976	182	3	28011	4.85	0.859 (0.094)	0.017 (0.011)	0.111 (0.023)	0.099 (0.01)	n.d.	n.d.	n.d.	n.d.	n.d.
22	Endo-1,3-1,4-beta-glycanase, C-terminal secretion signal protein	NP_437595	96	3	50255	4.13	n.d.	n.d.	n.d.	0.516 (0.012)	n.d.	n.d.	0.311 (0.104)	n.d.	n.d.
23	Putative glycine betaine-binding ABC transporter protein	NP_386505	264	5	33994	4.62	0.22 (0.023)	0.433 (0.18)	n.d.	0.128 (0.027)	0.314 (0.028)	0.07 (0.01)	0.892 (0.105)	0.661 (0.11)	1.193 (0.199)
24	Putative outer membrane lipoprotein transmembrane	NP_386994	304	5	27536	4.65	1.215 (0.118)	1.484 (0.264)	n.d.	1.199 (0.194)	4.686 (0.518)	n.d.	1.175 (0.225)	0.718 (0.04)	0.325 (0.118)
25	Putative amino acid-binding periplasmic (signal peptide) ABC transporter protein	NP_387353	108	3	29241	4.65	0.226 (0.022)	n.d.	n.d.	0.141 (0.018)	n.d.	n.d.	0.727 (0.055)	n.d.	n.d.
Spot No.	**Protien Name/NCBInr**	**PAN[¶]**	**S[†]**	**P_{Num}[‡]**	**MW**	**pI**	***P. syringae* 6h**			***P. syringae* 12h**			***P. syringae* 24h**		
							Control	*M. sativa*	*A. thaliana*	Control	*M. sativa*	*A. thaliana*	Control	*M. sativa*	*A. thaliana*
26	Outer membrane protein	NP_791545	556	9	45545	5.75	2.364 (0.535)	0.325 (0.051)	0.555 (0.101)	1.789 (0.282)	0.655 (0.112)	1.298 (0.299)	2.262 (0.519)	1.272 (0.192)	3.985 (0.727)
27	Phosphate ABC transporter, periplasmic phosphate-binding protein	NP_793052	586	12	36657	8.31	1.187 (0.288)	3.709 (0.555)	1.331 (0.123)	2.894 (0.585)	0.709 (0.123)	0.023 (0.022)	0.631 (0.065)	0.15 (0.014)	0.888 (0.1)
28	Polygalacturonase	NP_793726	460	9	55422	6.7	0.591 (0.119)	0.033 (0.02)	0.364 (0.027)	0.661 (0.052)	0.051 (0.019)	0.638 (0.017)	0.58 (0.066)	0.617 (0.088)	0.561 (0.05)
29	Chitinase	AAB41324	385	8	34900	5.25	n.d.	0.129 (0.01)	0.471 (0.034)	4.059 (1.023)	0.451 (0.10)	0.238 (0.022)	n.d.	0.021 (0.01)	0.837 (0.065)
30	Flagellin	NP_791772	743	11	29158	5.25	0.972 (0.191)	0.159 (0.003)	n.d.	5.52 (1.002)	1.475 (0.113)	0.263 (0.031)	0.427 (0.023)	0.593 (0.04)	0.141 (0.006)
31	Outer membrane protein OmpW	NP_790526	489	10	24429	5.1	2.886 (0.339)	n.d.	n.d.	0.86 (0.119)	n.d.	0.123 (0.031)	11.574 (5.521)	0.178 (0.023)	0.041 (0.016)
32	P21 protein	P25096	116	2	21449	4.84	0.848 (0.099)	0.933 (0.128)	1.41 (0.144)	4.822 (1.022)	0.978 (0.281)	3.33 (0.22)	1.248 (0.311)	0.02 (0.01)	0.952 (0.192)
33	Endochitinase A precursor (CHN-A)	P08252	63	1	35134	8.4	5.951 (1.00)	1.66 (0.206)	6.67 (1.23)	0.667 (0.098)	0.188 (0.033)	0.793 (0.102)	12.741 (5.26)	0.129 (0.012)	3.20 (0.929)
34	Thiol:disulfide interchange protein DsbA	NP_790191	179	3	23325	6.43	1.145 (0.262)	0.306 (0.051)	1.833 (0.222)	1.499 (0.342)	0.456 (0.039)	1.849 (0.10)	n.d.	n.d.	n.d.
35	Outer membrane porin OprF	NP_792118	203	4	36499	4.77	2.796 (0.626)	n.d.	2.03 (0.2)	4.183 (0.377)	n.d.	2.53 (0.32)	1.972 (0.2)	n.d.	3.54 (1.043)

Table 3.3. (Cont.) List of the secreted proteins identified in *Pseudomonas syringae* pv. tomato DC3000 and *Sinorhizobium meliloti*

Spot No.	Protien Name/NCBInr	PAN¶	S†	P_{Num}‡	MW	pI	*P. syringae* 6h			*P. syringae* 12h			*P. syringae* 24h		
							Control	*M. sativa*	*A. thaliana*	Control	*M. sativa*	*A. thaliana*	Control	*M. sativa*	*A. thaliana*
36	Membrane protein involved in aromatic hydrocarbon degradation	YP_236739	72	2	45736	5.57	0.396 (0.042)	n.d.	n.d.	0.405 (0.1)	n.d.	0.209 (0.031)	0.32 (0.004)	0.373 (0.19)	n.d.
37	Translocator protein TolB precursor	YP_234504	486	10	47666	8.75	1.315 (0.318)	n.d.	0.376 (0.1)	0.817 (0.077)	0.124 (0.008)	0.405 (0.036)	0.402 (0.2)	0.103 (0.009)	n.d.
38	Putative phosphate-binding periplasmic protein	NP_384615	88	3	36493	4.77	n.d.	0.216 (0.02)	n.d.	1.306 (0.18)	n.d.	n.d.	n.d.	0.101 (0.015)	n.d.

¶: protein accession number; †: Mascot MOWSE score; ‡: number of peptides matched to the protein; MW: molecular weight; *p*I: isoelectric point. The numbers represent the normalized volume of three different gels. n.d. means that a particular protein was not detected. The number in parenthesis represents the standard error at $p<0.05$

3.4 DISCUSSION

Plants and microbes engage in several forms of interaction, including those leading to disease, symbiosis or innocuous associations. For the most part these interactions have been studied to identify signals that may affect the outcome (Bohlool and Schmidt, 1974b; Whalen et al., 1991; Esnailt et al., 1993; Keith et al., 2003; Ampe et al., 2003; Harrison, 2005; Takeda et al., 2007). I approached this study in a different way: I sought to eavesdrop on the initial communication between two plants (roots) and two soil-borne bacteria, treating the release of proteins by both types of organisms as the actual "words" of the communication. I reasoned that depending on the root or bacterial partner, the roots and the bacteria were going to respond differently. Accordingly, I used a proteomics approach to identify those specific proteins. In this study I analyzed the protein composition of the exudates related to the interactions between the roots of two model plants (*A. thaliana* and *M. sativa*) in the presence or absence of *P. syringae* or *S. meliloti* (Table 3.1 and 3.2).

Proteins secreted in the root exudates have been the subject of increasing interest for their different enzymatic activities in soil (Gramss and Rudeschko, 1998; Walz et al., 2004; Nóbrega et al., 2005; Wen et al., 2007). However, the role that root-secreted proteins may play in establishment of recognition between compatible and incompatible interactions among plants and microbes is a novel area of investigation. Here, I showed that protein secretion by roots increases depending on which microorganism is interacting with the plant (Fig. 3.1). This increase is totally independent of the amount of bacteria that is in contact with the roots (Fig. 3.2). This discovery, combined with previous studies indicating that plants have developed systems for monitoring the

presence of microorganisms or microbial molecules (Morgan et al., 2005; Barea et al., 2005; Watt et al., 2006), led to the hypothesis that plants can also respond to different bacteria by changing the amount of protein secreted into the media (Fig 3.1). Using 2-DE gels, I monitored the specific changes in protein secretion in compatible and incompatible interactions (Fig 3.2). The results of this work support the idea that detection of different microorganisms by a plant induces different responses that culminate in either a symbiotic or defense response (Baron and Zambryski, 1995). Based on my data I also conclude that the presence of a plant induces the differential secretion of proteins by a given bacteria.

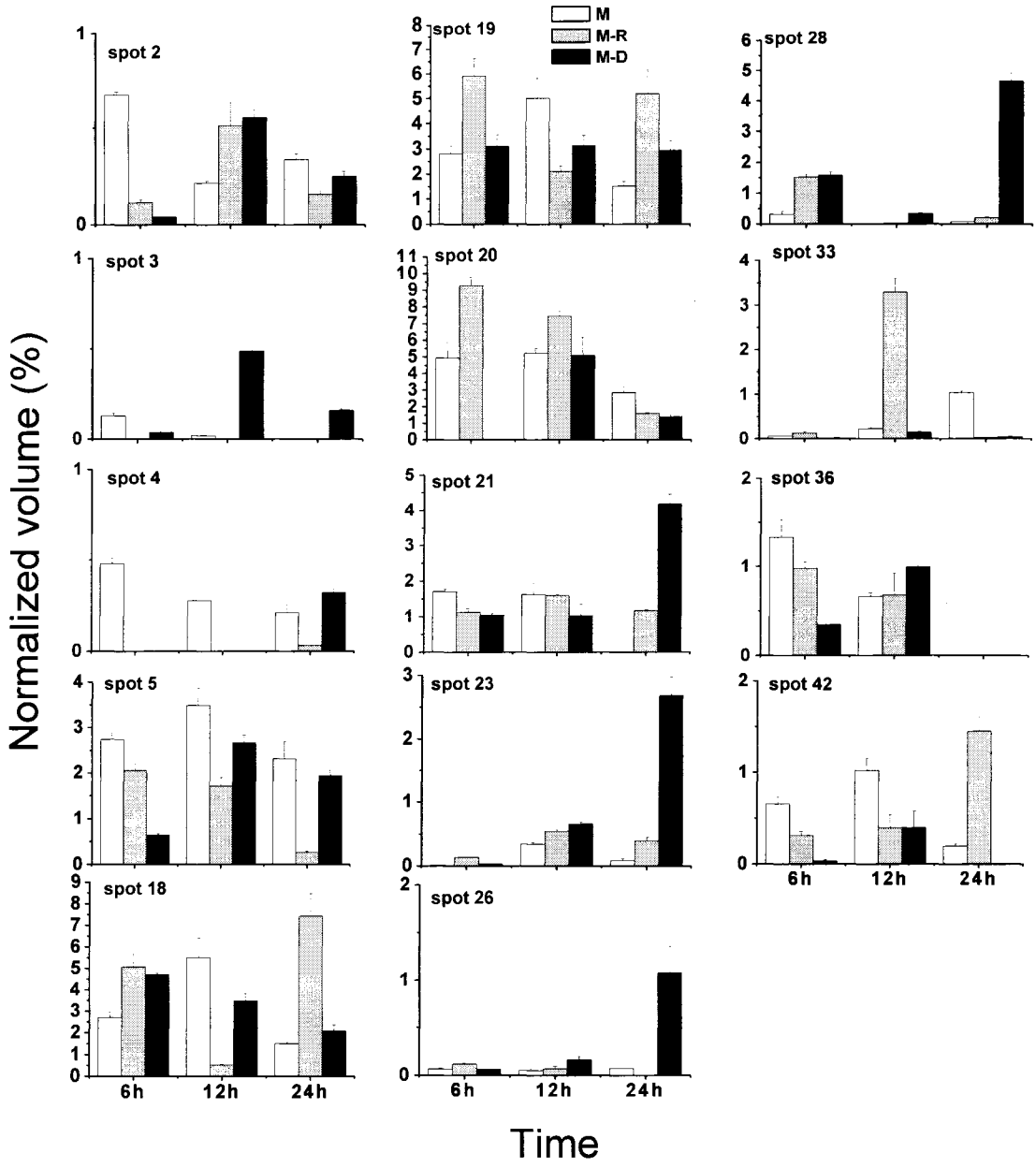

Figure 3.3. Comparative histogram of normalized volume values of the secreted proteins from *Medicago sativa* in the presence and absence of bacteria. The major differences between the secreted protein of *M. sativa* (M), *M. sativa* inoculated with *S. meliloti* (M-R), and *M. sativa* inoculated with *P. syringae* DC3000 (M-D) at 6, 12 and 24 hours are shown. The normalized volume differences were analyzed by Phoretix 2D Expression software. One hundred and fifty micrograms of protein for each experiment were analyzed by 2-DE. The spot numbers represent the identified proteins which are listed in Table 3.1; these are glucan endo-1,3 β-glucosidase (spots 2 and 3), proteinase K (spot 4), peroxidase (spot 5), chitinases (spots 18, 19, 26, 28 and 36), PR10 protein (spots 20 and 21), peptidase (spot 23), thaumatin protein (spot 33), PR protein 1 (spot 42). The error bars illustrate the SE values of three repetitions (n=3).

In this study forty-two secreted proteins were identified in the interaction between *M. sativa* with *S. meliloti* or *P. syringae* (Table 3.1). Five of them, which were identified as plant PR proteins [two glycoside hydrolases (spots 18 and 19), a PR10-1 protein (spot 20), a thaumatin-like protein PR-5b (spot 33) and a chitinase (spot 36)], were secreted in greater volume at six hours by *M. sativa* inoculated with *S. meliloti* but not when it was inoculated with *P. syringae* (Fig. 3.3; Table 3.1) in a non nitrogen-limiting condition. The fact that *M. sativa* responded faster by secreting proteins in the presence of *S. meliloti* but not in the presence of *P. syringae* suggests that an efficient signaling process similar to that operating during pathogenic interaction takes place during the early interaction with *S. meliloti* (Turrini et al., 2004; Harrison, 2005; Shaw et al., 2006). My results also strongly suggest that the secretion of a PR10-1 (spot 20) in the interaction between *M. sativa* and *S. meliloti* may indicate that this protein is a specific marker for compatible recognition from the roots at the early stages of the interaction between the two organisms (Fig. 3.3; Table 3.1), even if nodulation was not achieved under my experimental conditions (data not shown) of N abundance. PR10-1 has been considered to belong to the group of secreted and extracellular proteins (van Loon et al., 2006) and expressed exclusively during nodulation (Gamas et al., 1998). Moreover, it has been proposed that because PR10 is one of the pathogen-induced proteins, this protein can prevent pathogenic infection in the external media (Gamas et al., 1998). Interestingly, the genes that codified for some PR proteins are also induced in *M. truncatula-S. meliloti* interaction (El Yahyaoui et al., 2004).

In the incompatible interaction between *M. sativa* and *P. syringae*, two chitinases (spot 26 and 28) were secreted 15-fold in comparison with the plant control at 24 h (Fig. 3.3).

Although it is known that the expression of different chitinase classes are due to compatible or incompatible infection (Samac et al., 1990; Kasprzewska, 2003) and the type of the PR proteins can be induced by either salicylic acid, jasmonic acid or ethylene (Samac et al., 1990; Thomma et al., 1998; Kasprzewska, 2003), I argue that because the chitinases secreted in the presence of *S. meliloti* are acidic and the chitinases secreted in the presence of *P. syringae* DC3000 are basic (Table 3.1), *S. meliloti* can stimulate indirectly the salicylic acid accumulation in the plant while *P. syringae* induces ethylene or jasmonic acid accumulation. These results strongly suggest that the accumulation of salicylic acid or jasmonic acid in the plant due to the type of chitinase could provide the first recognition for a compatible or incompatible interaction. In addition, certain chitinase isoenzymes are specifically induced only in the early stage of soybean nodulation (Xie et al., 1999); therefore, it is not surprising to find specific proteins secreted in response to different microorganisms. In fact, chitinases of legumes have received particular attention because certain rhizobial Nod factors are substrates for chitinases (Perret et al., 2000). Thus, it has been proposed that cleavage of nod factors is necessary to limit the amount of active Nod factors after their perception by the host plant (Staehelin et al., 1995; Goormachtig et al., 1998).

I found that a peroxidase (spot 5; Fig. 3.3; Table 3.1) was secreted in the root exudates of *M. sativa*, but its secretion was decreased starting at 12 h when *S. meliloti* was present. This decline in plant peroxidase secretion after bacterial recognition may be the signal for nodulation and this hypothesis is corroborated by the data from Den Herder et al. (2007) who found that the expression of a gene Srprx1 in *Sesbania rostrata* roots, which codes for a class III plant peroxidase, depends on *Azorhizobium caulinodans*

nodulation factors. Moreover, Den Harder et al. (2007) have shown that this peroxidase gene is not induced after wounding or pathogen attack, indicating its participation as a symbiont-specific protein. This differential induction may indicate that when *M. sativa* is not experiencing nitrogen limiting conditions, it does not need to start the nodulation signaling process with *S. meliloti* and the plant stops the secretion of peroxidases. This clear message could be sent by inhibiting the secretion of peroxidases or other proteins into the media in the presence of *S. meliloti,* but not in that of *P. syringae.*

While secondary metabolites from root exudates have been shown to be involved in mycorrhizal associations (Tamasloukht et al., 2003), plant growth-promoting bacteria (Steenhoudt and Vanderleyden, 2000) and quorum sensing (Teplitski et al., 2000; von Bodman et al., 2003), the role of plant-secreted proteins involved in the communication establishing bacterial recognition has not been a topic of discussion. In this study, the compatible interaction between *A. thaliana* and *P. syringae* was also studied using proteomics (Fig. 3.2G; Table 3.2). The participation of endogenous root proteins in the interaction with pathogenic and nonpathogenic microorganisms has been well studied (Cook et al., 1995; Bueno et al., 2001; Fedorova et al., 2002; Kim et al., 2003; Prayitno et al., 2006; Den Herder et al., 2007; Oldroyd, 2007; Takeda et al., 2007). I report in this study that plants also secreted proteins into the rhizosphere as part of the process of differentiating between pathogen and nonpathogen. *Xylella fastidiosa* secretes a wide variety of proteins that are related to bacterial survival and pathogenesis (Bustamante Smolka et al., 2003). However, such extracellular proteins were not identified and the specific mechanism by which root exudates alter the production of extracellular proteins in the presence of other microorganisms remains unknown. I identified sixty-two

proteins in *A. thaliana* with *P. syringae* (Table 3.2) from which only proteins related to defense, such as peroxidases (spots 4, 15 and 42), glycosyl hydrolase family 17 (spot 5), proteinase K (spot 14), basic chitinase (spot 37) and glycosyl hydrolase family 18 (spot 40), were highly secreted soon after initial contact with *P. syringae* (by six hours), but not in the incompatible interaction with *S. meliloti* (Fig. 3.4; Table 3.2). However, after 12 h the secretion of these proteins was significantly reduced compared with the plant control (Fig. 3.4; Table 3.2), strongly suggesting that *A. thaliana* can modify root protein exudation in response to a compatible or incompatible interaction with microorganisms (Thilmony et al., 2006). Other important proteins that have been studied for their role in plant-microbe recognition have been lectins (Bohlool and Schmidt, 1974a; Rodriguez-Navarro et al., 2000). In this study, two lectins (spot 6 and 62; Fig. 3.4) were found to be secreted in greater quantity in the presence of *S. meliloti* than in the presence of *P. syringae*, suggesting that *S. meliloti* could be recognized by the plant while *P. syringae* could not. This result also indicates that because *P. syringae* is a pathogenic bacterium for Arabidopsis, it could suppress the plant defense response and freely infect the plant as has been suggested by other authors (Thilmony et al., 2006). Although it has been suggested that the proteins released along roots during cell separation could play a role in innate immunity in plants (Wen et al., 2007), this hypothesis has not been proven. Here, I demonstrated that *A. thaliana* can selectively secrete defense proteins at an early stage in the contact with *P. syringae.*

In this study, I also found evidence that bacteria are also able to change the proteins they secrete depending on the identity of the plant partner, a fact which argues for a two-way protein-based communication between the organisms (Table 3.3). As different

extracellular proteins can be found in different microorganisms (Antelmann et al., 2006; Boekhorst et al., 2006; Gilois et al., 2007), their role as communication molecules with plants remains unknown. It has been found that the addition of the leguminous flavonoids genistein and naringenin to the bacteria *Sinorhizobium fredii* induced and enhanced the secretion of bacterial proteins into culture (Lin et al., 1999). I found that two proteins related to recognition of *P. syringae* and *S. meliloti*, the elongation factors and flagellin (spot 34, 35, 36, 55 and 61; Table 3.2) which are known to function as key signals for the initiation of the defense response in bean (Zipfel et al., 2004; Chinchilla et al., 2007), were secreted in the interaction between *A. thaliana* and the two microorganisms studied and the elongation factor 4 (spot 61; Table 3.2) was only found to be secreted in the interaction between *A. thaliana* and *P. syringae* DC3000. On the other hand, one important bacterial protein for symbiosis is superoxide dismutase (Santos et al., 2000); this protein is specifically induced during the early process of infection (Ampe et al., 2003), and I found it to be secreted in large quantities by *S. meliloti* in only in the interaction with *M. sativa* and only at six hours (Table 3.3). This result suggests that there is microbe recognition only for *M. sativa*, but maybe because the interaction took place in the presence of adequate nitrogen, the secretion of this protein was decreased after 12 h (Table 3.3). Furthermore, I found that even the genes encoding the transport system, which constitute the largest class of the genome of *S. meliloti* (Galibert et al., 2001) and *P. syringae* (Buell et al., 2003), are decreased in secretion in the interaction with either *M. sativa* or *A. thaliana* compared with the bacterial control (Table 3.3).

Because microorganisms in the rhizosphere are so abundant it has been suggested that roots and these organisms engage in complex forms of communication (Kourtev et al., 2003) such as the one I described here. Therefore, I propose that protein root exudates play an important role in the initial crosstalk as two organisms establish compatible or incompatible interactions. This study opens up new research areas concerning protein-based plant-microbe communication and provides important information regarding the manipulation of gene expression of specific proteins with the purpose of modifying plant behavior related to compatible or incompatible interactions.

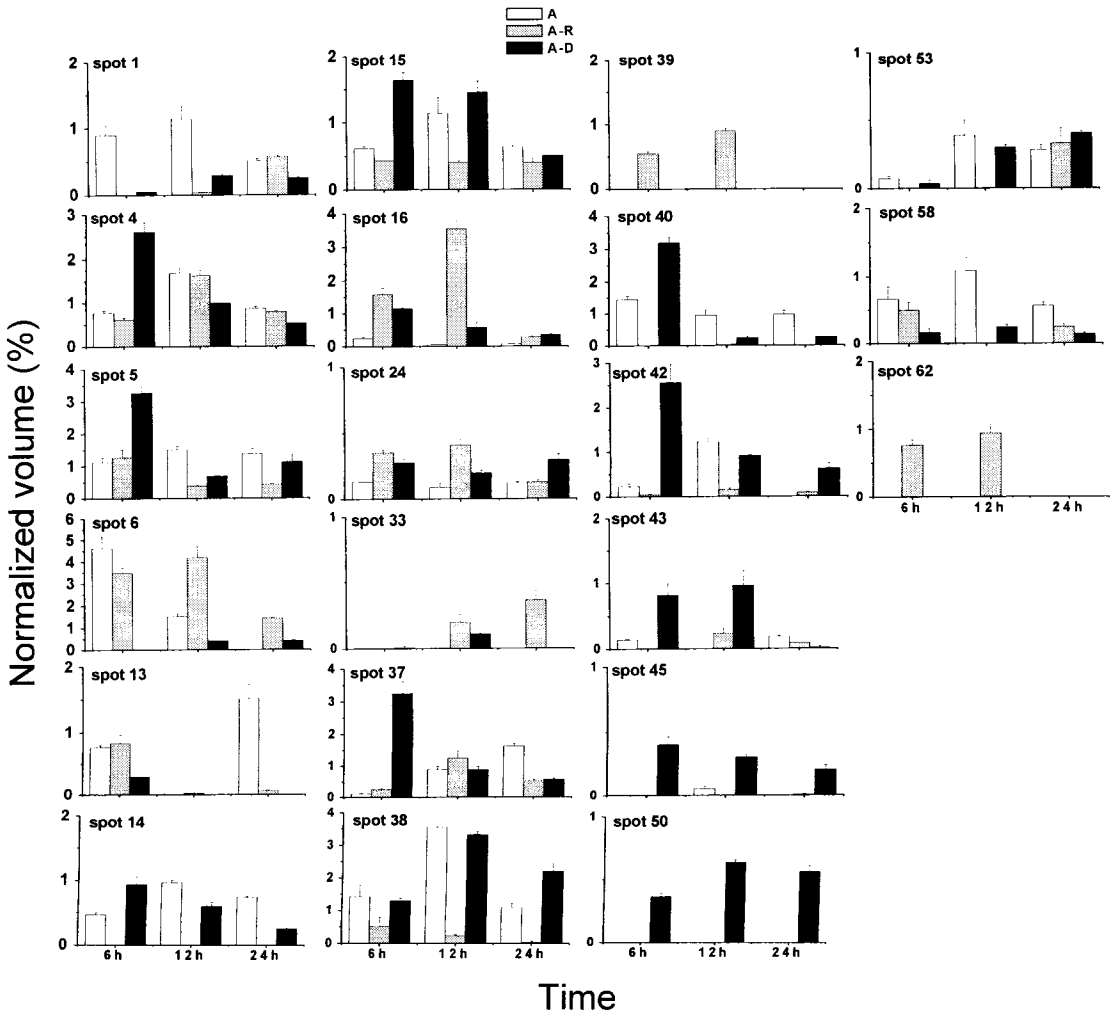

Figure 3.4. Comparative histogram of normalized volume values of the secreted proteins from *Arabidopsis thaliana* in the presence and absence of bacteria. The major differences in the secreted proteins of *A. thaliana* ecotype Col-0 (A), *A. thaliana* inoculated with *S. meliloti* (A-R), and *A. thaliana* inoculated with *P. syringae* DC3000 (A-D) at 6, 12 and 24 hours are shown. The normalized volume differences were analyzed by Phoretix 2D Expression software. One hundred and fifty micrograms of protein for each experiment were analyzed by 2-DE. The spot numbers represent the identified proteins which are listed in Table 3.2. Subtilisin-like protein (spot 1), peroxidase (spots 4, 15, 16, 38, 39 and 42), glucan endo-1,3 β-glucosidase (spots 5 and 45), lectin (spots 6 and 62), aspartyl protease (spot 13), proteinase K (spot 14), trypsin inhibitor (spot 24), neutral protease (spot 33), chitinase (spots 37 and 40), serine protease (spot 51). The error bars illustrate the SE values of three repetitions (n=3).

CHAPTER 4

Novel role for pectin methylesterase in Arabidopsis: a new function showing Ribosome-Inactivating Protein (RIP) activity

ABSTRACT

Ribosome-inactivating proteins (RIPs, EC 3.2.2.22) are plant enzymes that can inhibit the translation process by removing single adenine residues of the large rRNA. These enzymes are known to function in defense against pathogens, but their biological role is unknown; partly due to the absence of work on RIPs in a model plant. In this study, I purified a protein showing RIP activity from *Arabidopsis thaliana* by employing chromatography separations coupled with an enzymatic activity. Based on N-terminal and internal amino acid sequencing, the RIP purified was identified as a mature form of pectin methylesterase (PME, *At1g11580*). The purified native protein showed both PME and RIP activity. PME catalyzes pectin deesterification releasing acid pectin and methanol which cause cell wall changes. I expressed the full-length and mature form of cDNA clones into an expression vector and transformed it in *Escherichia coli* for protein expression. The recombinant PME proteins (full-length and mature) expressed in *E. coli* did not show either PME or RIP activity, suggesting that post-translational modifications are important for these enzymatic activities. This study demonstrates a new function for an old enzyme identified in a model plant and discusses the possible role of protein's conformational changes corresponding to its dual enzymatic activity.

4.1 INTRODUCTION

Ribosome-inactivating proteins (RIPs, EC 3.2.2.22) are plant enzymes that can inhibit the translation process by removing single adenine residues of the large rRNA (A_{4324} from rat liver rRNA) from the universally conserved sarcin/ricin loop (Endo et al., 1987; Endo and Tsurugi, 1988; Nielsen and Boston, 2001; Barbieri et al., 2006). These

enzymes have been classified into three distinct types (Stirpe and Barbieri, 1986). Type 1 RIPs consist of a single polypeptide chain, type 2 RIPs contain a catalytically active A-chain and a lectin B-chain connected by a disulphide bond, and type 3 RIPs have a single polypeptide chain of unknown function (Reinbothe et al., 1994) synthesized as inactive forms that require proteolytic processing to form an active RIP protein. Furthermore, RIPs have been considered of great interest for their role as endogenous defense proteins, and their catalytic activity has been useful to genetically engineer broad-spectrum pathogen resistance (Park et al., 2004).

Because RIPs may potentially inactivate ribosomes in the cells in which they are synthesized, these enzymes are targeted to vacuoles (Yoshinari et al., 1997), protein bodies (Goto et al., 2003) or cell walls (Bonness et al., 1994) as a sequestering mechanism. Based on their cellular localization and enzymatic activity, RIPs have been suggested to act as "suicidal agents" (Bonness et al., 1994) released from cell walls or vacuoles once the plant cell is damaged by pathogen attack or mechanical injury (Park et al., 2004). In addition to their *N*-glycosidase activity, some RIPs have DNAse, DNA glycosylase, and apurinic pyrimidinic lyase activities (Roncuzzi and Gasperi-Campani, 1996; Nicolas et al., 1998; Nicolas et al., 2000). Sharma et al. (2004) reported that iron-superoxide dismutase (Fe-SOD) from tobacco has dual function, showing RIP activity in addition to Fe-SOD activity.

Despite this biochemical and biological information, the role of these enzymes in plants is still a matter of debate. A critical reason why the biological role of RIPs *in planta* has been poorly elucidated is the lack of information about this group of enzymes in any model plant system. There are reports suggesting that *Arabidopsis thaliana* does

not appear in the group of plants that have RIPs (Hartley and Lord, 2004; Motto and Lupotto, 2004) while others claim that RIPs are ubiquitous among plants (Girbés and Ferreras, 1998). Generally, these studies in Arabidopsis were performed by comparing the sequence motifs of known RIPs from other plant species with the genome sequence information available in Arabidopsis with no apparent success (The Arabidopsis Genome Initiative, 2000; Motto and Lupotto, 2004). In the present study, I took a different approach and searched for an Arabidopsis RIP by biochemical fractionation coupled with an examination of enzymatic activity.

4.2 MATERIAL AND METHODS

4.2.1. Plant Material

Seeds of *Arabidopsis thaliana* wild type (Col-0) were obtained from Lehle Seeds (Round Rock, TX, USA). The seeds were surface-sterilized with 3% (v/v) sodium hypochlorite for two minutes followed by three washes with sterile distilled water. The seeds were germinated on solidified MS medium (Murashige and Skoog, 1962) supplemented with 3% (w/v) sucrose in a growth chamber at 25 $\pm$ 2°C and 16/8 h day/night photoperiod. Seven-day-old plants were transferred into flasks containing 250 ml of liquid MS medium supplemented with 3% (w/v) sucrose and placed on a shaker set at 80 rpm, 24 $\pm$ 2°C under a photoperiod of 16/8 h.

4.2.2. Protein extraction from Arabidopsis plants and chromatography separation

Five hundred and sixty-seven grams of 25-day-old *A. thaliana* plants cultivated as described above were ground in liquid nitrogen, homogenized in three volumes of extraction buffer (25 mM NaH_2PO_4, pH 7.0, with 250 mM NaCl, 10 mM EDTA, 5 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 1.5% [w/v] polyvinylpolypyrrolidone), and then centrifuged for 30 min at 10,000 g. The supernatant was dialyzed against 25 mM NaH_2PO_4 (pH 7.0), until it was free from salts. The dialyzed supernatant was filtered through a membrane filter of pore size 0.45 μm (Pall Life Sciences, East Hill, NY, USA). All extraction procedures were conducted at 4°C.

The dialyzed and filtered supernatant from *A. thaliana* was initially fractionated using a diethylaminoethyl sephadex (DEAE) column (22 x 4 cm) attached to an FPLC (Bio-Rad Laboratories, Hercules, CA, USA). Equilibration and loading of the crude extract (total protein; TP) was carried out using 25 mM sodium phosphate (pH 7.0) at flow rate of 1 ml min^{-1}. The flow-through protein (basic protein fraction; TB) was collected until no protein was found in the eluant. The basic protein fraction was concentrated (~150 ml) and desalted using 5-kD cutoff ultrafiltration membranes (Millipore, Bedford, MA, USA). The desalted proteins were further resolved by a cation exchange chromatography carboxymethyl cellulose (CM) column (4.6 x 130 mm) attached to an FPLC (Bio-Rad Laboratories, Hercules, CA, USA). Equilibration, loading, and washing were carried out in 25 mM sodium phosphate (pH 7.0) at a flow rate of 1 ml min^{-1}. The basic protein was eluted with a linear gradient of NaCl (0 – 1 M) and collected in fractions of 10 ml. The individual fractions were collected, concentrated, and desalted using 5-kD ultrafiltration devices (Millipore, Bedford, MA,

USA). Each fraction was assayed for RIP activity (see below), and only the fractions showing the activity were pooled and concentrated to 250 µl (CM fraction) using 5-kD ultrafiltration devices. After concentrating, the active fraction (250 µl) was loaded on the gel filtration column Sephadex SH-100 (90 x 1.5 cm) previously equilibrated with 25 mM sodium phosphate (pH 7.0) containing 250 mM NaCl and further fractionated. The proteins were eluted with an isocratic gradient of 25 mM sodium phosphate (pH 7.0) and 250 mM NaCl at a flow rate of 0.2 ml min^{-1} and each fraction (2 ml) was monitored at 280 nm. Each fraction was analyzed for RIP activity and only the active fractions were pooled (SH-100 fraction).

4.2.3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To visualize the proteins of every purification step, I used sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), following the method of Laemmli (Laemmli, 1970) using a Bio-Rad Mini–protean II slab gel unit (Bio-Rad Laboratories, Los Angeles, CA, USA). PAGE gels of 4 and 12% (1 mm thickness) were used as stacking and separating gels, respectively. Ten micrograms of each partially purified fraction and 2.5 µg of the purified protein (SH-100) were diluted in Laemmli 5x buffer and heated at 95°C for five min before loading on the gel. Gels were electrophoresed at 100 V for 90 min at room temperature, according to the manufacturer's specifications. Protein Mr markers (6 – 200 kD; Invitrogen) were used as a source of molecular mass standards. The protein bands and the purity of the purified protein were visualized by Coommassie brilliant blue (Bio-Rad Laboratories, Los Angeles, CA, USA). The protein concentration at all of the steps of purification was

determined according to the method of Bradford (1976) using a Bio-Rad protein assay kit.

4.2.4. NH_2- terminal amino acid and internal amino acid sequence analysis

The purified protein from *A. thaliana* showing RIP activity was sent to the Macromolecular Resources Facility, Colorado State University (Fort Collins, Colorado, USA) for N-terminal and internal amino acid sequencing analyses. Briefly, the purified protein band excised from the SDS-PAGE gel was digested by trypsin, and the generated fragments were analyzed by using MALDI-TOF/TOF mass spectrometry. Seven peptides generated from the in-gel enzymatic digestion of the protein were searched using a taxonomic filter in MASCOT Daemon (version 1.8.0, Matrix Science Ltd., London, UK) search engine (Perkins et al., 1999; Creasy and Cottrell, 2002) and raw tandem mass spectral data (.wiff).

4.2.5. Assay for N-glycosidase (RIP) activity

The depurination assay was conducted according to Tumer et al. (1997). In brief, ribosomes from yeast (*Saccharomyces cerevisiae*) were resuspended in RIP buffer (167 mm KCl, 100 mM $MgCl_2$, and 100 mM Tris-HCl, pH 7.2) and incubated with the different chromatographic fractions at 30°C for 30 min in a total volume of 100 µl. The ribosomes incubated in the absence of RIP was used as negative control and the ribosomes incubated with an RIP from *Mirabilis expansa* (ME1 (Vivanco et al., 1999)) was used as positive control. After incubation, the RIPs were removed from the mixture by phenol-chloroform extraction, and the rRNA was divided in half. One-half of the

extracted rRNA was incubated on ice for 30 min with 1 M aniline acetate (pH 4.5) and precipitated with ethanol. Both aniline treated and untreated rRNAs were subjected to electrophoresis in a 4.5% (w/v) polyacrylamide gel containing 7 M urea and stained with ethidium bromide.

4.2.6. Pectin methylesterase activity

PME activity was assayed as described by Downie et al. (1998) with the modifications of Bourgault and Bewley (2002). Briefly, 0.1g of pectin esterified 90% was dissolved in 100 ml of McIlvaine's buffer at pH 6.5 with 2% of agarose and gelatin (final concentration 500 μg/ml). The solution was heated to dissolve the agarose and then cooled to 60°C before applying it into the plates. Fifty-five nanograms of protein from every purification step (total protein, total basic protein, pooled active fraction from CM, and purified protein from SH-100) were used. Moreover, fifty-five nanograms of native pectin methylesterase (PMEN), 250 ng of recombinant pectin methylesterase mature protein (PMEM) and 250 ng of recombinant pectin methylesterase full-length protein (PMEF) were analyzed for PME activity. The gel was incubated at 30°C for 16 hours and then rinsed briefly with water and stained with 0.05% (w/v) ruthenium red. For the pH optimum assay, the McIlvaine's buffer was adjusted to a pH from 2, 3, 4.5, 5, 6, 7, or 9. I also tested other RIPs reported in the literature for PME activity. Fifty-five nanograms of PMEN (RIP from *A. thaliana*; purified in this study), and 250 ng of the purified RIPs ME1 (RIP from *M. expansa* roots; (Vivanco et al., 1999)), TRIP (RIP from *Nicotiana tabacum*; (Sharma et al., 2004)) and Ricin (RIP from *Ricinus communis* seeds; (Endo and Tsurugi, 1988)) were loaded into wells in 0.1% (w/v) of 90% esterified

pectin in McIlvaine's buffer. Pectinesterase from orange peel (Sigma, St. Louis, MO, USA) was used as a positive control and water as a negative control. After being stained and washed, the diameters of the stained zones were measured.

4.2.7. Gene cloning

Total RNA was isolated from whole plants of Arabidopsis with a Qiagen Plant RNA kit (Qiagen, Valencia, CA, USA) according to the manufacturer's directions and quantified by Nanodrop (Nanodrop technologies). Synthesis of cDNA was carried out using Superscript Reverse Transcriptase III (Invitrogen, Carlsbad, CA, USA) by using 2 μg of DNAse-treated total RNA as recommended by the manufacturer. The RT-PCR was carried out using gene-specific primers of PME: sense oligomer: 5'-AGACCAACATGTCTAATTCAAACC-'3; antisense oligomer: 5'–TCAAAGCCACTCTTGGAAAGTAAC-'3. The PCR was conducted with a jump start of 94°C for three min and then 25 cycles with a regime of 94°C for 30 s, 58°C for 30 s and 72°C for three min and then final extension of 15 min at 72°C. The PCR product was purified and cloned into a TA vector using a TOPO TA cloning kit (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's directions. The positive clones were selected and verified by restriction digestion and the product was further verified by DNA sequencing. The sequence was verified by comparison with the PME sequence available in the Arabidopsis genome database at "The Arabidopsis Information Resource (TAIR)" website (http://www.arabdopsis.org). After cloning and sequencing of PME, the full-length coding region and mature protein region was amplified using gene-specific primers with restriction digestion sites using the TA plasmid DNA

(spanning full-length PME cDNA) as a template for expression work. The primers were designed for full-length PME from sequences located at 1-22 (sense oligomer 5'-GCATGCATGTCTAATTCAAACC-'3, with a restriction site of *Sph1*) and 1650 to 1674 (antisense oligomer 5'-CCCGGGTCAAAGCCACTCTTGG-'3, with a restriction site *Xma1*). The primers were designed for mature region PME from sequences located at 948 to 975 (sense oligomer 5'-GCATGCGTGACCGCGAACGTTGTGGTG-'3 with a restriction site *Sph1*) and 1650 to 1674 (antisense oligomer 5'-CCCGGGTCAAAGCCACTCTTGG-'3, with a restriction site *Xma1*).

4.2.8. Expression and purification of *A. thaliana* PME in *E. coli*

The expression and purification of PME full-length and mature regions in *E. coli* was carried out with a QIAexpressionist kit (Qiagen, Valencia, CA, USA). The PCR fragments of the PMEF and PMEM were digested with *Sph1* and *Xma1* for 24 h and ligated into pQE-30 vectors supplied by the manufacturer (Qiagen, Valencia, CA, USA). A single colony of *E. coli* strain M15, transformed with the pQE-30 plasmid spanning PMEF and PMEM region cDNA, was cultured into 15 ml LB medium containing ampicilin and kanamycin (final concentration 100 μg/ml and 25 μg/ml, respectively) and incubated in the shaker at 260 rpm and 37°C overnight. The culture was then inoculated into 1.5 l of fresh LB medium containing the same antibiotics until it reached an OD_{600} value of 0.5 – 0.6 under the same conditions as described above. The temperature was reduced to 30°C and the culture induced with isopropyl-β-D-thiogalactoside (IPTG, Sigma, St. Louis, MO, USA) to a final concentration of 1 mM. The culture was incubated eight hours at 260 rpm at 30°C. The induced cells from 1.5 l were collected by

centrifugation at 7000 g for 15 min at 4°C and resuspended in 37.5 ml lysis buffer (50 mM Tris-HCl pH 8.0, 20 mM imidazole, 1 M NaCl, 5mM β-mercaptoethanol, 1 mM PMSF). The cells were sonicated with a microtip at 250 μA on ice in short pulses of 10 s each. Then the extract was centrifuged at 10000 g for 30 min at 4°C and the supernatant was saved. The recombinant PMEs were purified by using Ni-NTA His*Bind Resin (Novagen, San Diego, CA, USA) affinity chromatography according to the manufacturer's directions. Five micrograms of each recombinant pure protein were used for polyacrylamide gel electrophoresis as described above.

4.3 RESULTS

4.3.1. Purification of an RIP protein from *A. thaliana*

A protein showing RIP activity was purified to homogeneity from 25-day-old *A. thaliana* plants using anion exchange, cation exchange and gel filtration chromatography. The initial separation of the plant tissue extract throughout the DEAE anion exchange column resulted in recovery of a fraction with RIP activity in the flow-through, indicating the basic nature of the protein. The total basic protein showing RIP activity was passed through a CM column and the elution of bound protein in the column was performed with a linear gradient of NaCl (0 – 1 M) resulting in the recovery of a protein showing depurination activity in fractions 41 to 51 (Fig. 4.1A). After the protein fractions with RIP activity were recovered from the CM cationic column, they were pooled and concentrated to 250 μl and passed through gel filtration column where the RIP activity was located in fractions 37 to 40 (Fig. 4.1B), indicating an approximate protein size of 35 kDa under native conditions. The estimated yield of the pure protein

was 0.005 % of total starting material (Table 4.1). To rule out any contamination as the cause of two activities, the protein from this last purification step was examined on SDS-PAGE where a single protein band of around 35 kDa was observed without any contaminating protein (Fig. 4.2A). Furthermore, different concentrations of the purified protein (200 ng – 2.4 μg) were examined on SDS-PAGE was that further stained with silver (data not shown) to confirm the protein homogeneity and ruling out any other protein contamination.

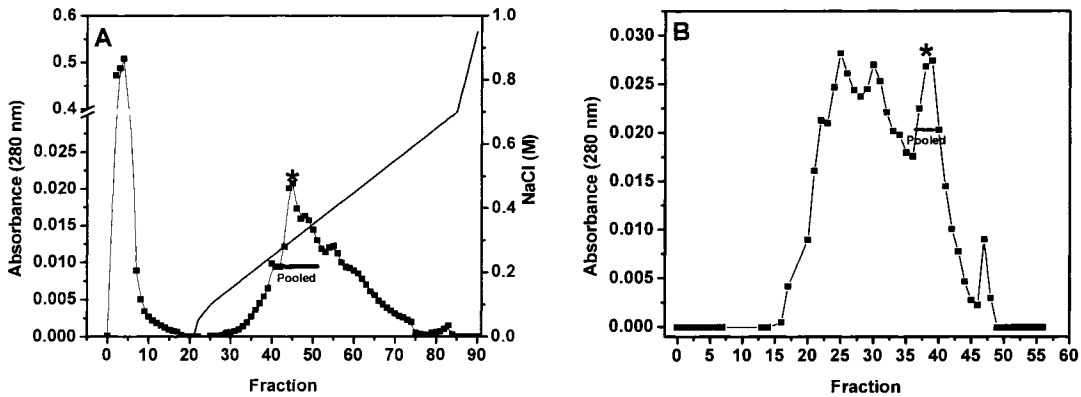

Figure 4.1. Purification of *Arabidopsis thaliana* Ribosome Inactivating Protein (RIP). **A,** The total soluble basic protein was eluted with a linear gradient of NaCl (0 – 1000 mM) on a cation exchange column. **B,** The basic protein fractions 41 to 51 showing RIP activity obtained from the cation exchange column, were pooled, concentrated, dialyzed and fractionated on a gel filtration column. The star represents the pooled fractions that represent only the majority of the RIP activity.

The RIP activity test was performed to confirm the presence of RIP activity in the chromatography fractions using yeast ribosomes as a substrate incubated with aniline and analyzed by electrophoresis, as shown in Fig. 4.2B and explained in detail in Materials and Methods. The release of a 367-bp "Endo fragment" (Endo et al., 1987) from the large rRNA upon treatment with acidic aniline indicates RIP activity. The approximate titer for *A. thaliana* RNA *N*-glycosidase activity was further examined by

incubating yeast ribosomes with concentrations of the pure protein ranging from 20 ng to 1 μg (Fig. 4.2C). As shown in the PAGE gel, the characteristic Endo fragment was visible starting at 40 ng of protein.

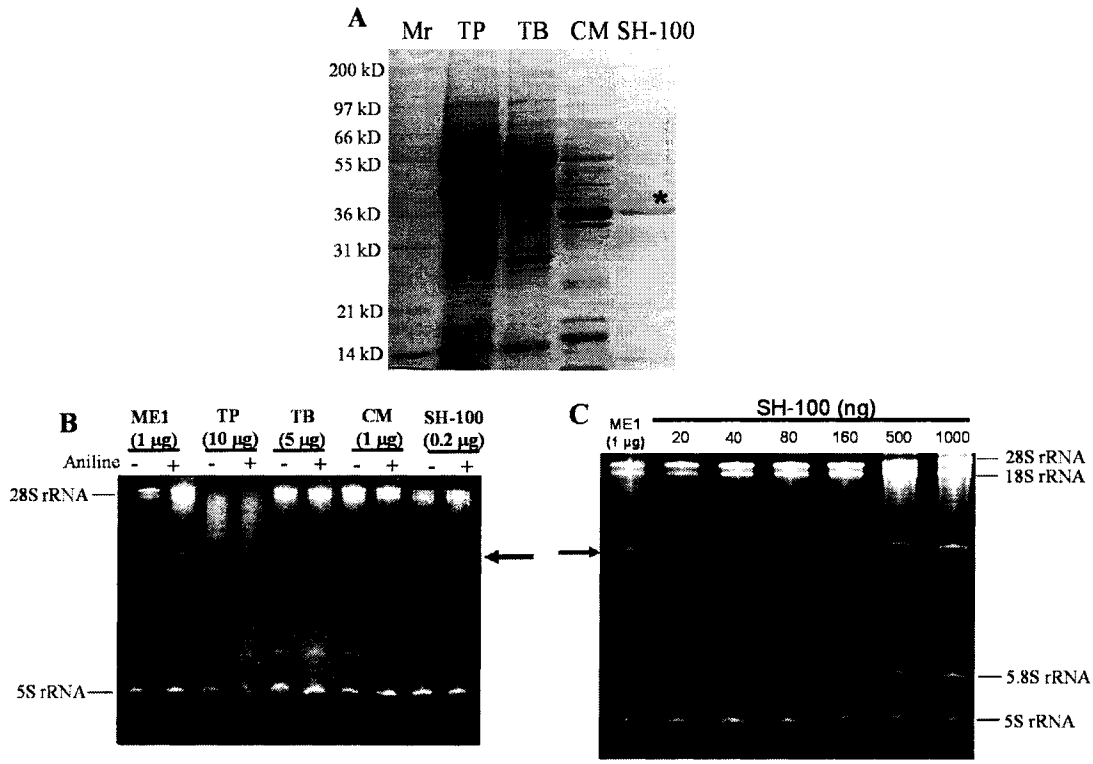

Figure 4.2. Association of Ribosome Inactivating Protein (RIP) activity with protein purification. **A,** Analysis by SDS-PAGE of protein fractions collected during purification steps. Molecular weight marker (Mr), total protein (TP), total basic protein (TB), cation exchange chromatography fraction with RIP activity (CM), and gel filtration fractions with RIP activity (SH-100) were resolved on 12% (w/v) SDS-PAGE. Ten micrograms of each partially purified fraction and 2.5 μg of the purified protein (SH-100) were loaded on the gel. The protein bands were visualized by Coommassie brilliant blue stain after electrophoresis. The * indicates the purified single protein band. **B,** Different protein fractions were examined for RIP activity using yeast ribosomes as a substrate as described in Materials and methods. The presence and absence of aniline is denoted. ME1 (RIP from *M. expansa* roots; (Vivanco et al., 1999)) was used as a positive control for RIP activity. The arrow mark indicates the release of an endo fragment due to aniline treatment. **C,** Concentration-dependent activity of purified RIP protein from the SH-100 fraction. Yeast ribosomes were incubated with different concentrations of the SH-100 gel filtration purified fraction. The rRNA was extracted, treated with aniline and separated on 4.5% (w/v) polyacrylamide gel containing 7 M urea and visualized by staining with ethidium bromide. The arrow indicates the release of endo fragment due to aniline treatment.

Table 4.1
Purification of Ribosome Inactivating Protein from *Arabidopsis thaliana*

Purification Steps	Amount of protein (mg)	Protein recovery (%)
Crude extract	1380.728	100
DEAE	200.418	14.51
CM	10.035	0.726
Shephacryl SH-100	0.071	0.005

4.3.2. Identification of RIP protein by N-terminal and internal sequencing

The purified native protein showing RIP activity from *A. thaliana* was subjected to trypsin digestion and peptide sequencing as described in Materials and Methods. Surprisingly, the seven peptide amino acid sequences revealed by trypsin digestion (Fig. 4.3) showed significant homology to pectin methylesterase (*At1g11580*) in the Arabidopsis genome database. The molecular mass of the native RIP protein purified in this study was 35 kD, but the full-length pectin methyl esterase (PMEF) amino acid sequence was predicted to be 62 kD, which suggests that the protein I purified was cleaved at some point. In order to determine the cleavage site of the RIP protein that I purified, I performed N-terminal sequencing using automated analysis by Edman degradation. The N-terminal sequencing revealed that the native RIP protein is only the mature protein (PMEM) without the N-terminal leader sequence which starts at valine (V-243) (Fig. 4.3).

Furthermore, the sequence of the full-length cDNA shows the characteristic N-terminal leader sequence, pectin methylesterase inhibitor (PMEI 1) domain, pectin esterase domain and pectin esterase signature sequences 1 and 2 (Fig. 4.3).

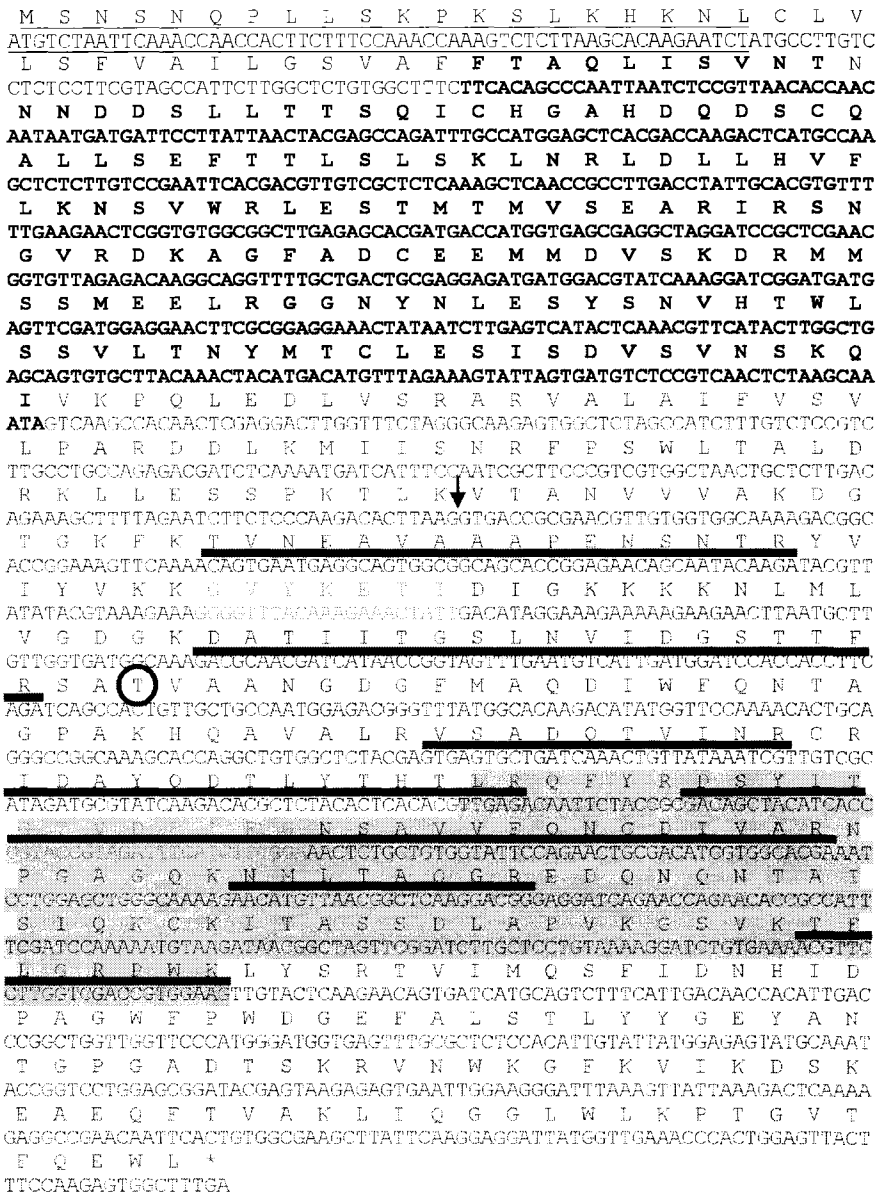

Figure 4.3. The nucleotide and deduced amino acid sequence of the *Arabidopsis thaliana* Ribosome Inactivating Protein (RIP) protein identified as pectin methylesterase (At1g11580) by N-terminal and internal amino acid sequencing analyses. The peptide sequences identified by trypsin digestion are denoted in bold underline. The predicted signal peptide sequence is underlined. The pectin methylesterase inhibitor (PMEI 1) domain (Pfam04043) is indicated in bold. The esterase-like domain (Pfam01095) is shaded. The pectin methylesterase signature sequences 1 and 2 are denoted in red. The arrow indicates the cleavage site of mature protein from the N-terminal leader sequence. The circle indicates the potential glycosylation site. The * indicates the stop codon. SignalP (Bendtsen et al., 2004) and SOSUIsignal (Gomi et al., 2000) programs were used to detect putative signal peptide and transmembrane domains respectively. The Pfam motifs were determined using the website URL http://www.sanger.ac.uk/Software/Pfam/.

After it was identified that the purified RIP protein is a PME, I evaluated the PME activity for all the purification steps (Fig. 4.4). The gel diffusion assay shows that the PME activity is increased based on the purity of the protein except for the basic fraction. The SH-100 fraction showed more than two-fold increase in PME activity compared with the total protein.

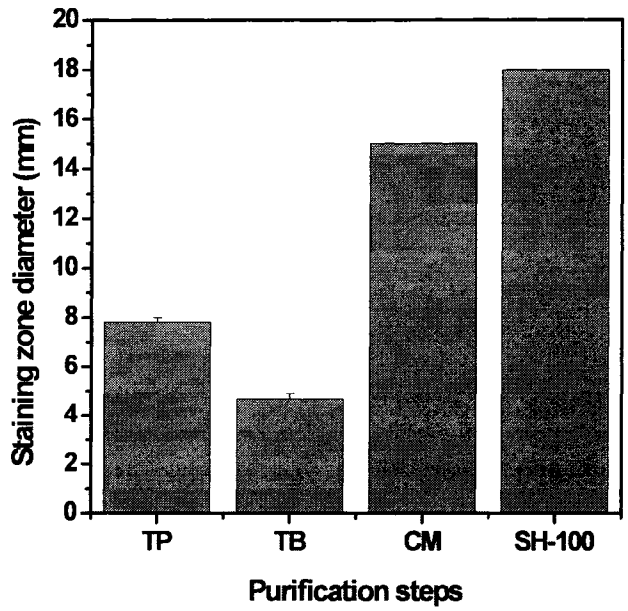

Figure 4.4. Pectin Methyl Esterase (PME) activity of different protein fractions derived from purification. A gel plate assay was used for determination of PME activity. Fifty-five nanograms of total protein (TP), total basic protein (TB), cation exchange chromatography fraction with RIP activity (CM), and gel filtration fractions with RIP activity (SH-100) were loaded into wells in 0.1% (w/v) of 90% esterified pectin in McIlvaine's buffer. After being stained and washed, the diameters of the stained zones were measured. The error bars indicate the SE values of three repetitions (n=3).

4.3.3. PME activity examination in other RIPs

I compared the active domain amino acid sequence of PME showed RIP activity, identified in this study with other RIPs (type I and type II) by using ClustalW (Thompson et al., 1994) (Fig. 4.5). Comparison of the sequences revealed four amino

acid residues (one phenylalanine, one tyrosine and two leucines) conserved in all RIPs and other nine residues (one leucine, one asparagine, two serines, one phenylalanine, one tyrosine, one valine, one glutamic acid and one isoleucine) conserved in at least two different RIPs and in the PME. Therefore, I performed the PME assay for other RIPs, at different pHs, in order to know whether these RIPs also has PME activity (Fig. 4.6). Based on gel diffusion assay plates at pHs from 2 to 9, the type 1 RIPs ME1 and TRIP both demonstrated PME activity, showed the highest activity at pHs 4.5 and 5 respectively, while ricin, a type 2 RIP, showed no activity. The PMEN purified in this study showed a good correlation in its activity by increasing in pH from 3 to 7 but no activity was found at pH 9.

4.3.4. Cloning of the RIP gene and enzymatic activity assays with the recombinant PMEs

To further reveal the activity of the native protein with RIP activity purified from *A. thaliana*, I cloned the full-length and mature regions of the cDNA clone by employing an RT-PCR approach using gene-specific primers designed according to the cDNA sequence available in the Arabidopsis genome database (TAIR). The full-length cDNA of PME was 1674 bp and the mature region was 945 bp, encoding proteins of 62 kDa and 34.6 kDa respectively (Fig. 4.7A). The molecular masses (Mr) of the pectin methylesterase full-length region (PMEF) and the pectin methylesterase mature region (PMEM) of the protein expressed by *E. coli* were the same as the estimated Mr derived from the cDNA clone. The Mr of the PMEM protein expressed by *E. coli* was slightly larger than the native purified RIP protein because of the His-tag (Fig. 4.7A). The *E.*

coli expressed PMEF and the PMEM proteins were purified under native conditions by His-tag (Ni-NTA) affinity chromatography and further used for enzymatic assays (Figs. 4.7B and 4.7C).

The depurination assay was performed using yeast ribosomes as a substrate to evaluate the presence of RIP activity in the native protein, the recombinant PMEF and the recombinant PMEM (Fig. 4.7B). Aniline treatment released an endo fragment from the large rRNA only in the presence of native PME purified from *A. thaliana*, whereas aniline treatment of rRNA in the presence of PMEF as well as PMEM failed to generate the endo fragment (Fig. 4.7B). I also performed the RIP assay in the presence of orange peel PME and the endo fragment was not released (data not shown). In addition to the RIP activity assay, I examined the PME activity of the native and recombinant proteins. The results show that only the native protein and the control (PME from orange peel) had PME activity (Fig. 4.7C). None of the recombinant proteins showed PME activity.

Figure 4.5. Comparison of the pectin methyl esterase (PME) amino acid sequence (At1g11580) with other Ribosome Inactivating Protein (RIP) sequences. Alignment was performed using Clustal W (Thompson et al., 1994). The * indicates the conserved residues among the RIPs with PME. The ‡ indicates that at least one residue is different among the RIPs and PME. Names of RIPs are indicated on the left. Ricin (RIP from *Ricinus communis* seeds; (Endo and Tsurugi, 1988)), Trichosanthin (RIP from *Trichosanthes kirilowii* root tubers; (Zhang and Liu, 1992)), ME1 (RIP from *Mirabilis expansa* roots; (Vivanco et al., 1999)), Mirabilis (RIP from *Mirabilis jalapa* root tubers; (Wong et al., 1992)), PAP (RIP from *Phytolacca americana* leaves; (Irvin et al., 1980)) and Saporin (RIP from *Saponaria officinalis* seeds; (Maras et al., 1990)).

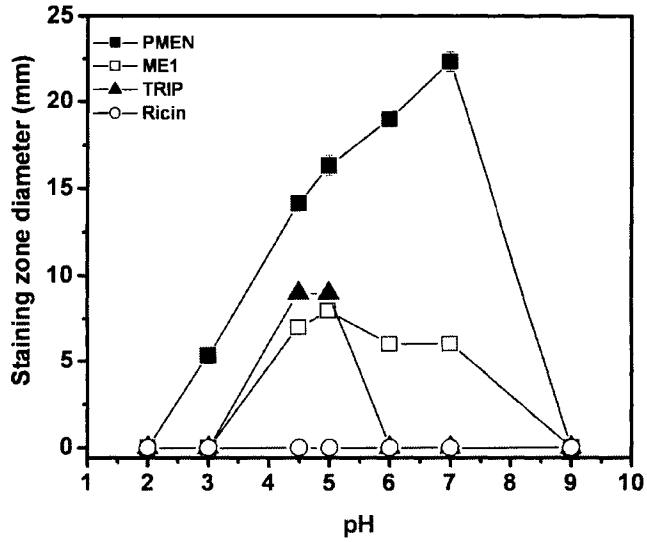

Figure 4.6. Pectin methylesterase activity assay with different Ribosome Inactivating Proteins (RIPs). A gel plate assay was used for determination of the optimum pH from 2 to 9. Fifty-five nanograms of PMEN (RIP from *Arabidopsis thaliana*; purified in this study), 250 ng of the purified RIPs ME1 (RIP from *Mirabilis expansa* roots; (Vivanco et al., 1999)), TRIP (RIP from *Nicotiana tabacum*; (Sharma et al., 2004)) and Ricin (RIP from *Ricinus communis* seeds; (Endo and Tsurugi, 1988)) were loaded into wells in 0.1% (w/v) of 90% esterified pectin in McIlvaine's buffer at different pHs. After being stained and washed, the diameters of the stained zones were measured. The error bars illustrate the SE values of three repetitions (n=3). Five nkat of PME from orange peel (Sigma) was used as a control.

In order to compare the role of pH in PME activity, I performed the assay at pHs from 2 to 9 in gel diffusion plates. None of the PMEs, including the control, showed activity at pH 2 or 9. Both the control and the native PME protein showed the highest activity at pH 7. However, none of the recombinant PMEs showed activity at any pH (Fig. 4.8). The native PME showed the same pattern of activity as the control from orange peel and the increase in the staining zone was correlated with the increase in pH.

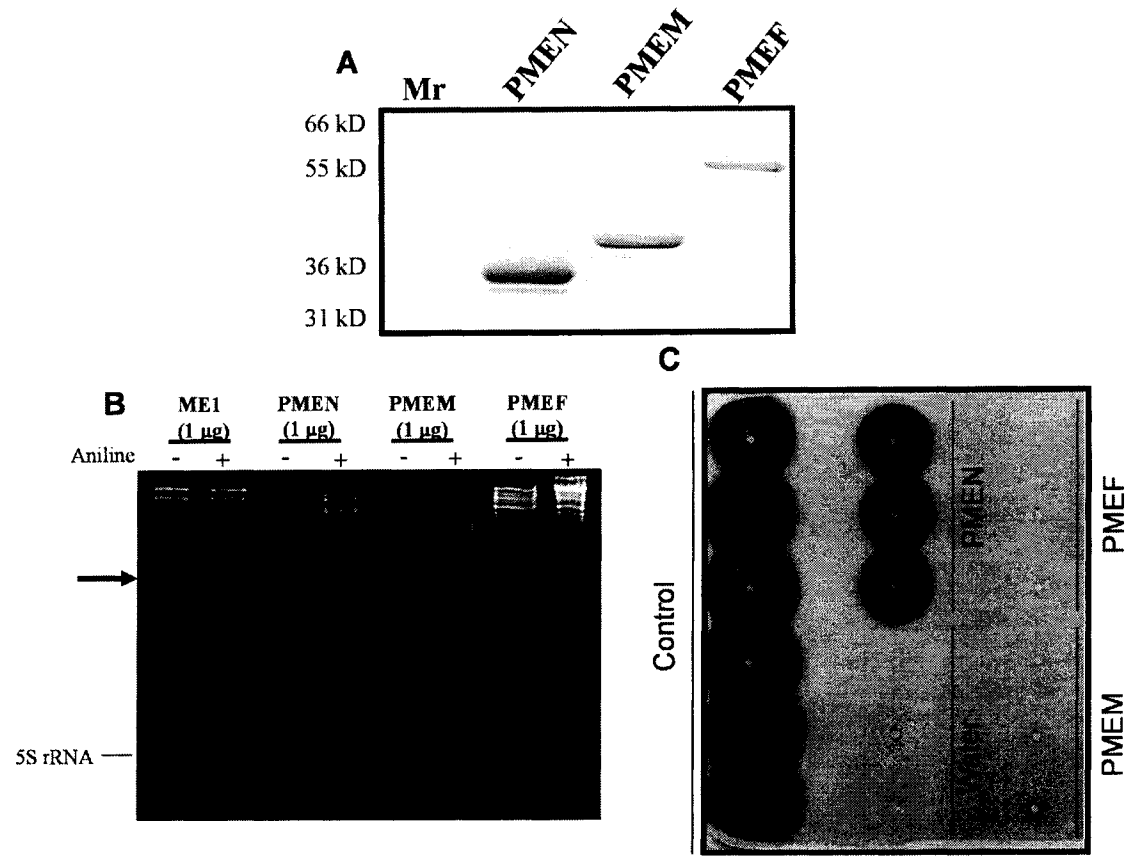

Figure 4.7. Association of Pectin Methyl Esterase (PME) and Ribosome Inactivating Protein (RIP) activity with recombinant protein purification. **A,** Analyses by SDS-PAGE of the native protein and the recombinants. Native pectin methylesterase (PMEN), recombinant pectin methylesterase mature protein (PMEM) and recombinant pectin methylesterase full-length protein (PMEF). The molecular mass (Mr) is indicated on the left of the gel. Five micrograms of PMEN, PMEN and PMEF were loaded on the gel. The gel was stained with Coommassie brilliant blue R250 stain. **B,** Enzymatic N-glycosidase activity of PMEN, PMEM and PMEF *in vitro*. Intact ribosomes isolated from *S. cerevisae* were used as a substrate for RIP activity as described in Materials and methods. The arrow indicates the 367-nucleotide endo fragment 28s rRNA cleavage. The presence and absence of aniline treatment is denoted. **C,** Pectin methylesterase activity assay with the native protein and the recombinants. Fifty-five nanograms of PMEN, and 250 ng of PMEM and PMEF were loaded into wells in 0.1% (w/v) of 90% esterified pectin. Hydrolysis proceeded at 30°C for 16 h, after which the gels were stained and washed with water. Standards of 9, 28, 47, 66, 84 and 94 nkat of PME from orange peel (Sigma) were used as positive controls and 15 µl of water were used as a negative control.

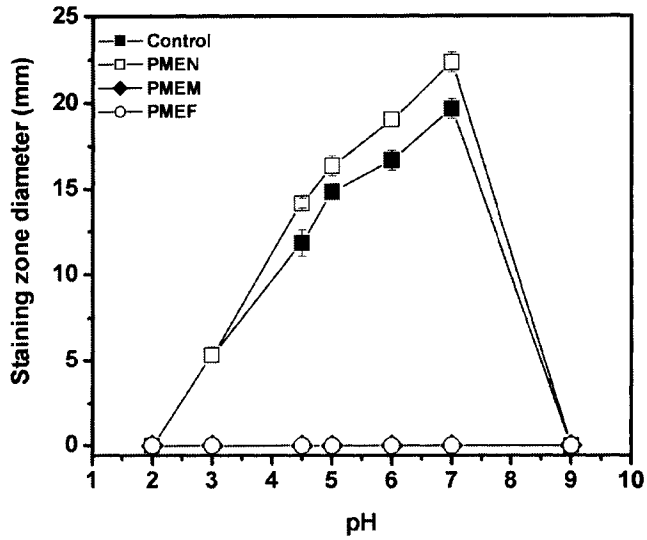

Figure 4.8. Pectin methylesterase activity assay at different pHs. A, pH optimum determination for native pectin methylesterase (PMEN), recombinant pectin methylesterase mature protein (PMEM) and recombinant pectin methylesterase full-length protein (PMEF) in McIlvaine's buffer at pH values from 2 to 9. Fifty-five nanograms of PMEN, and 250 ng of the recombinants PMEM and PMEF were loaded into wells in 0.1% (w/v) of 90% esterified pectin. Five nkat of PME from orange peel (Sigma) was used as a control. The error bars illustrate the SE values of three repetitions (n=3).

4.4 DISCUSSION

A. thaliana is one of the most studied plants and is considered to be a model system for plant biologists. Although the genome of Arabidopsis has been completely sequenced, most of the biochemical and physiological functions of its genes have not been discovered. In the present study, I purified to homogeneity an active RIP from *A. thaliana* (Fig. 2A) and identified it as a mature form of PME (At1g11580; Fig. 4.3) lacking the N-terminal extension (PRO-region) (Markovic and Janecek, 2004; Pelloux et al., 2007), which is thought to act as an inhibitor of PME activity (Giovane et al., 2004; Pelloux et al., 2007). The full length PME is targeted to the endomembrane system,

leading to the export of PMEs in mature form, without the leading polypeptide, to the cell wall (McFeeters and Armstrong, 1984; Micheli, 2001; Dorokhov et al., 2006b; Pelloux et al., 2007). In the cell wall, mature PMEs participate in cell wall expansion and elongation (Moustacas et al., 1991; Nari et al., 1991), and in several defense responses induced by biotic or abiotic stresses (Pelloux et al., 2007).

Protein maturation involving proteolytic removal of a peptide segment has also been previously reported in RIPs (Walsh et al., 1991; Hey et al., 1995; Krawetz and Boston, 2000; Rudhe et al., 2002; Kuroyanagi et al., 2002; Bass et al., 2004). In some cases, a precursor is processed into a two-chain holoenzyme (Bass et al., 2004). The processing of maize pro-RIP1 (b-32) occurs primarily during seed germination (Bass et al., 1992) and appears to be involved in a secretion process which does not involve vacuoles or protein bodies (Roberts and Lord, 2004). In the case of type 3 RIPs, they are synthesized as inactive forms that require proteolytic processing of internal regions to form active proteins (Kwon et al., 2000). In my case, I cannot conclude that the RIP I purified in *A. thaliana* shares the characteristics of a type 3 RIP processing because the full-length protein expressed in *E. coli* was not active (Fig. 4.7B).

The fact that recombinant PMEF and PMEM expressed in *E. coli* was inactive (Figs. 4.7B and 4.7C) suggests that posttranslational modifications are necessary for RIP and PME activity, such as protein glycosylation (Peng et al., 2005). In plants, it has been shown that biological functions of glycoproteins are highly influenced by *N*-linked glycans (Lerouge et al., 1998), a process that occurs in the secretory pathway, which could explain why the recombinant proteins do not show RIP or PME activity (Figs. 4.7B and 4.7C). It is known that PMEs need to be glycosylated in order to be functional

(Peng et al., 2005) and even that the existence of different activities varies according to the degree of glycosylation (Van Rijssel et al., 1993). In the case of RIPs, it has been found that glycosylation is important for the toxicity of the enzyme (Li et al., 1998). A detailed analysis of the mature PME shows a potential glycosylation site in the amino acid threonine 326 (T326; Fig. 4.3) that is very conserved among pectin methylesterases (Ding et al., 2002). Chen et al. (Chen et al., 1998) confirmed that a modification in a single amino acid is essential for the catalytic activity of the enzyme. Thus, changing or modifying a single amino acid residue essential for the activity would produce most of the times the complete catalytic inactivation. This suggests that the lack of post-translational modification (glycosylation) in the recombinant PMEs may account for it did not show either PME or RIP activity.

Although few known proteins are reported to have dual enzymatic activity, more proteins are being added to this list (Jeffery, 2003). Jeffery (1999) has defined this characteristic of one protein with multiple functions as "moonlighting protein". Moreover, the function of these proteins can vary based on many factors, such as cellular localization, oligomeric state, cellular concentration of a substrate, and binding site availability, among others (Jeffery, 1999). For instance, MazG protein from *Thermotoga maritime* has both nucleoside triphosphate pyrophosphohydrolase and pyrophosphatase activities (Zhang et al., 2003), and both enzymatic activities are unrelated. MJ0109, a protein from *Methanococus jannaschii*, has been discovered to also have two enzymatic activities: inositol monophosphatase and fructose-1,6-bisphosphatase (Stec et al., 2000). Jeffery (1999) suggests that such unrelated activities could have arisen through an ancestral active site that conferred an organism with the

ability to synthesize fewer proteins and, in consequence, replicate less DNA, saving energy for growth and reproduction. In this study, the enzymatic activities found (RIP and PME) of the purified protein are completely different. RIPs remove the adenine residue of the rRNA due to its *N*-glycosidase activity whereas PME catalyzes pectin deesterification releasing acid pectins and methanol, and these activities occur at different pH values (Figs. 4.7B and 4.7C; see Materials and Methods). It is well-known that upon changes in pH, conformational changes occur in enzymes such as PME (Nari et al., 1984). Recently, it has been reported that stress can stimulate conformational changes of proteins within cells (Johnson et al., 2007). I showed that the two enzymatic activities remain constant (Figs. 4.2B and 4.4). PME activity appeared in the crude extract, but RIP did not. However, there was a decrease in PME activity in the TB fraction which coincided with the rise in RIP activity. This suggests that the PME inhibitor is having an effect on the PME activity (Giovane et al., 2004) in the TB fraction. By the final purification step (SH-100), when I found protein homogeneity presumably without the PME inhibitor, there was high PME and RIP activity which gives my confidence in my conclusion that the two activities arise from the same protein. Moreover, I found that four amino acids, one phenylalanine (F), one tyrosine (Y) and two leucines (L), are conserved between RIPs and the PMEN (Fig. 4.5). This suggests an important role for those amino acids in the RIP activity. These amino acid residues are more than enough to make the difference between an active and an inactive enzyme (Chen et al., 1998).

Another common feature between the RIPs and PMEs, besides conserved amino acids, is their function in defense response. It was reported that the PME (At1g11580),

which I purified and demonstrated to have RIP activity in this study, is highly expressed in the presence of biotic stress but not in the presence of abiotic stresses as observed in other PMEs (Pelloux et al., 2007). The highest gene expression for this particular gene was observed in the presence of *Agrobacterium tumefaciens,* strongly suggesting its participation in defense response (Pelloux et al., 2007). Since the discovery of the possible participation of PMEs in cell-to-cell movement of TMV (Dorokhov et al., 1999; Chen et al., 2000), a novel activity of tobacco PMEs against some viruses as RNA silencing has been reported (Dorokhov et al., 2006a). The sequencing of the *A. thaliana* genome has led to the identification of 66 open reading frames annotated as PMEs (Pelloux et al., 2007); therefore, it is not surprising to find new functions for them. However, at this point I cannot conclude which activity (RIP or PME) is more prominent in nature.

In plants, PMEs have been linked to cell wall changes, thus it will be of great interest to define the molecular mechanism(s) or factor(s) by which a PME could be caused to perform RIP activity. Based on its dual function, it could be speculated that the RIP activity of this PME in *A. thaliana* might be related to temporal defense or a stress response. As a result of pH changes in the cell wall, PME could be subjected to conformational changes that expose the active site and confer specific binding capability to the rRNA substrate, in order to show RIP activity. It is of high biological relevance to know at what conditions *in planta* this protein functions as a PME or as an RIP; such information could stimulate the efforts to engineer plants for increased disease resistance.

CHAPTER 5

Conclusions

The present dissertation focused on the study of bioactive proteins secreted into the rhizosphere in two model plants, *Arabidopsis thaliana* and *Medicago sativa*, in order to lay the foundation for understanding the biological function and role of these proteins. Among the bioactive proteins found in the root exudates, several were defense related. The present study intends to further my understanding of the biological significance of defense-related proteins in the developmental changes of the plant and in plant-microorganism interactions. Furthermore, the study attempts to understand the discovery of a new function in a Ribosome-Inactivating Protein (RIP) in Arabidopsis. The results of this dissertation could potentially lead to new applications in agriculture research.

Based on the results of my investigation, I propose that roots secrete the proteins needed to communicate with the surrounding biota in an extraordinarily precise way. Moreover, the plant uses sophisticated mechanisms to perceive signals from developmental processes or from different microorganisms in order to translate that signal into the exudation of a precise amount and type of protein into the soil. The findings of this study provide new insights into the developmental and defense mechanisms in which protein root secretion is involved.

The major findings in this dissertation are as follows:

1. The protein profile of the root exudates of Arabidopsis throughout its lifespan were studied by employing proteomic and bioinformatics tools. The proteins secreted by Arabidopsis Col-0 (wt) were compared with different mutants related to defense and flowering time. The results showed that proteins secreted in the root exudates were

higher when the plant is ready to bloom. This protein secretion varied between Arabidopsis wt and other Arabidopsis mutants, a fact of critical biological significance.

2. The identity of the proteins related to development was found using MS/MS data. The secretion of chitinases and glucanases were strongly influenced by flowering time. In addition, I showed that the proteins secreted in the root exudates of Arabidopsis may account for root defense responses. Furthermore, bioassays were used to confirm the inhibitory effect of root-exuded proteins in pathogenic and non-pathogenic (to Arabidopsis) strains of *Pseudomonas syringae*. Proteins secreted by the *cpr5-2* mutant had a strong effect on the growth of *P. syringae* DC3000 and Psph3121.

3. Also reported was the identification of proteins from roots that were differentially secreted when *Medicago sativa* was alone and when it was inoculated with *Rhizobium meliloti*. Moreover, this root protein secretion was dramatically different when *M sativa* was in the presence of *Pseudomonas syringae* DC3000. Based on the proteomic results and normalized volume quantification, it was determined that the secreted enzymes acid chitinase and thaumatin proteins were the most induced in the interaction between *M. sativa-R. meliloti,* but not in that between *M. sativa-P. syringae* DC3000.

4. A comparison was made of the proteins secreted in the interaction between *A. thaliana* and *P. syringae* DC3000, well-known as an opportunistic pathogen of Arabidopsis. In addition, the comparison was made between the interaction of Arabidopsis with *R. meliloti* and that of Arabidopsis with *P. syringae* to differentiate between the proteins

secreted by Arabidopsis in the presence of a pathogen and in the presence of a non-pathogen.

5. In addition, my study showed that plants could recognize the microorganism with which it was interacting by secreting specific proteins into the culture media. Furthermore, this study pioneers a new method by which the exuded protein could be used to engineer the plants for disease resistance. The identification of an acid chitinase or a peroxidase from the model plant *Arabidopsis* and Medicago makes easier in future studies to mutate the factors by which pathogens cause disease *in planta.*

6. I identified, purified, cloned, and expressed a novel RIP from *A. thaliana* by employing a systematic biochemical approach. Based on the N-terminus region and internal amino acid sequencing analyses, I determined that a protein showing RIP activity was a pectin methylesterase (PME). This enzyme had a molecular weight of 62 kDa with an isoelectric point of 8.4. The mature form of this protein, however, was 35 kDa and its isoelectric point 9.24.

7. The identification of an RIP protein from the model plant *Arabidopsis* makes easier in future studies to identify the factors which cause PME to show RIP activity *in planta.* In addition, this study opens a new way by which the PME genes, considered to be a gene family in *Arabidopsis*, could be used to engineer the plants for disease resistance.

8. Comparing the catalytic activity of PME with other RIPs, it was found that the RIPs type I showed the highest PME activity at pH 4.5.

Taken together, these observations indicate that bioactive proteins are not just wasted energy secreted into the rhizosphere, but are an extraordinarily sophisticated means by which plants communicate with soil organisms. Therefore, I conclude here that because proteins can catalyze reactions outside of the cell, the secretion of these proteins are highly important in the metabolizing of compounds secreted by other organisms. Furthermore, plants can secrete defense proteins in order to avoid pathogen attack. Moreover, I conclude that because flowering is the most important developmental stage of the plant for offspring evolution, plants over-secrete a series of defense proteins to avoid being infected by soil pathogens during this critical time.

Transcriptional changes do not reflect the complete cellular regulatory processes, since post-transcriptional processes, altering the amount of active protein, such as synthesis, degradation, processing, and modification of proteins, are not taken into account. Thus, complementary approaches such as proteome-based expression profiling are needed to obtain a full picture of the regulatory elements in developmental processes and plant-pathogen interactions.

Root-exuded proteins represent a vast loss of energy to the plant; however, I found that this secretion provides great potential rewards as well. From this sophisticated secretion mechanism, root-exuded proteins related to defense represent an important portion; however, the function of many of the proteins secreted into the rhizosphere has yet to be discovered.

CHAPTER 6

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