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Molecular Characterization of Plant Defense Responses to *Erwinia carotovora*

Marcos Montesano

Division of Genetics, Department of Biosciences
Faculty of Science
University of Helsinki

Academic Dissertation

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Supervisor: Professor Tapio Palva
Department of Biosciences, Division of genetics
University of Helsinki, Finland

Reviewers: Professor Teemu Teeri
Department of Applied Biology
University of Helsinki, Finland

Professor Martin Romantschuk
Department of Ecological and Environmental Sciences
University of Helsinki, Finland

Opponent: Professor Thorsten Nürnberger
Department of Stress and Developmental Biology
Institute of Plant Biochemistry
Halle/Salle, Germany

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CONTENTS

LIST OF ORIGINAL ARTICLES

This thesis is based on the following original articles, which will be referred to in the text by their Roman numerals.

I. Vidal S, Eriksson ARB, Montesano M, Denecke J, Palva ET. (1998) Cell wall-degrading enzymes from *Erwinia carotovora* cooperate in the salicylic acid-independent induction of a plant defense response. Mol. Plant-Microbe Interact. 11:23-32

II. Montesano M, Kõiv V, Mäe A, Palva ET. (2001) Novel receptor-like protein kinases induced by *Erwinia carotovora* and short oligogalacturonides. Mol. Plant Pathol. 2:339-346

III. Montesano M, Hyytiäinen H, Wettstein R, Palva ET. (2002) A novel potato defense-related alcohol:$NADP^+$ oxidoreductase induced in response to *Erwinia carotovora*. Plant Mol. Biol. (in press)

IV. Montesano M, Scheller HV, Wettstein R, Palva ET. Downregulation of photosystem I by *Erwinia carotovora* derived elicitors correlates with H_2O_2 accumulation in chloroplasts of potato. (manuscript)

V. Mäe A, Montesano M, Kõiv V, Palva ET. (2001) Transgenic plants producing the bacterial pheromone *N*-acyl-homoserine lactone exhibit enhanced resistance to the bacterial phytopathogen *Erwinia carotovora*. Mol. Plant-Microbe Interact. 14:1035-1042

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ABBREVIATIONS

avr	avirulence gene
bp	base pair
cDNA	complementary DNA
CF	cell-free culture filtrates containing the PCWDEs secreted by *E.c. carotovora*
DNA	deoxyribonucleic acid
GUS	glucoronidase
HPL	hydroperoxide lyase
HR	hypersensitive response
INA	2,6-dichloroisonicotinic acid
ISR	induced systemic resistance
JA	jasmonic acid
LOX	lipoxygenase
LRR	leucine rich repeats
MeJA	methyl jasmonate
mRNA	messenger RNA
NADP(H)	nicotine adenine dinucleotide phosphate (reduced form)
OGAs	oligogalacturonides
OHL	*N*-oxoacyl-homoserine lactone
PAL	phenylalanine ammonia-lyase
PCR	polymerase chain reaction
PCWDEs	plant cell wall-degrading enzymes
PR	pathogenesis-related
PSI	photosystem I
R	resistance gene
RLKs	receptor-like protein kinases
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcriptase- polymerase chain reaction
SA	salicylic acid
SAR	systemic acquired resistance
TMV	tobacco mosaic virus

ABSTRACT

The present thesis is concerned with plant defense responses to the bacterial phytopathogen *Erwinia carotovora* subsp. *carotovora*, an etiological agent of bacterial soft-rot that affect a wide range of plants including several economically important crops. The aim of this work was to identify and characterize molecular components of plant defense responses induced by *E.c. carotovora* in potato and tobacco, and engineer plants with enhanced resistance to this pathogen.

The production and secretion of plant cell wall-degrading enzymes (PCWDEs) is central to the virulence of *E.c. carotovora*, and these PCWDEs were used as elicitors of potato and tobacco defense responses. The role of individual enzymes as well as combinations of different PCWDEs as elicitors of plant defenses was characterized in tobacco. This characterization indicated that the PCWDEs synergistically induce plant defense responses and systemic resistance. Furthermore, this PCWDE-induced systemic resistance is salicylic acid-independent.

Identification of potato genes responsive to PCWDEs from *E.c. carotovora* led to the isolation of several defense related genes including:
i) Four genes encoding novel receptor-like protein kinases (*prk*-1-4). These genes most likely constitute a family encoding related receptors. Characterization of these genes indicated that they are not only responsive to PCWDEs but also to short oligogalacturonides and wounding, suggesting that these receptors may be involved in perception of the damage produced by *E.c. carotovora*.
ii) A gene encoding a novel alcohol:$NADP^+$ oxidoreductase (*drd-1*). Biochemical characterization of DRD-1 indicated that this enzyme exhibits a preference for several aromatic and aliphatic aldehydes. The defense-related induction of DRD-1, the kinetics of the enzyme and the chemical structure of DRD-1 substrates and products suggest that this enzyme may play a role in plant defense signaling.
iii) *psaD*, a nuclear gene encoding the PSI-D subunit of photosystem I (PSI). Functional characterization of PSI complex indicated that the electron transport activity of PSI is altered in plants treated with PCWDEs from *E.c. carotovora*. This alteration of PSI is preceded by the downregulation of *psaD* transcripts induced by PCWDEs and correlates with an accumulation of hydrogen peroxide in chloroplasts of potato plants, suggesting that the downregulation of *psaD* leads to accumulation of reactive oxygen species in chloroplasts.

In order to generate plants resistant to *E.c. carotovora*, we produced transgenic tobacco overproducing N-oxoacyl-homoserine lactone (OHL), a signal that is essential for the quorum sensing that controls the production of PCWDEs in these bacteria. Plants overproducing OHL exhibited enhanced resistance to *E.c. carotovora* suggesting that plant-engineering strategies targeting the modulation of microbial signaling systems may be useful for agricultural applications.

INTRODUCTION

Plants are primary producers and they are able to convert the energy of sunlight into chemical energy such as carbohydrates, proteins, and lipids. These characteristics are extremely important because, directly or indirectly, they provide most of the energy necessary for other organisms to live. Archaeological evidence indicates that around 10,000 years ago humans started to practice agriculture, and since then, and probably before, plant diseases started to be a human concern. Diseased plants may be toxic for humans and animals, and they reduce food quality and production. A good example of how a plant disease can affect human life is given by the potato late blight epidemic in Europe in 1845-6 that importantly affected the Irish people. At the present, approximately 25% of crops are lost worldwide due to general diseases and insects (Agrios, 1997). Tens of thousands of diseases affect cultivated plants. On average, each kind of crop plant can be affected by hundred or more plant diseases (Agrios, 1997). Until now, the control of plant diseases has been dependent on pesticide application. The application of these toxic compounds is harmful for the environment. The ecological and economical cost that the application of these compounds has in short and long-term periods are difficult to estimate. Therefore, other environmentally less harmful approaches for controlling plant diseases and improving food quality and production are needed. At this moment, conventional breeding and genetic engineering for disease resistance seem to be good strategies for approaching these problems. As a part of developing better strategies for controlling plant diseases, a good understanding of the plant-pathogen interactions at the molecular level is needed.

Plants have been exposed to adverse environmental conditions, including biotic stresses, since their origins. Biotic stresses include fungi, bacteria, and viruses, which use different types of molecular mechanisms as virulence strategies to infect plants and eventually cause diseases. Because of these stresses, plants induce defense responses to protect themselves from the infecting microbes. Therefore, plant defense and microbe virulence strategies have a long history of constant coevolution.

Plant defense to pathogens results from a complex combination of structural plant characteristics and induced biochemical reactions. The structural characteristics, including the cuticle and the cell wall, constitute a preformed plant defense that acts as a physical barrier to prevent the entrance and spreading of pathogens throughout the plant. In addition to this constitutive defense, plants may perceive directly or indirectly the presence of a pathogen and subsequently induce plant defense responses. These inducible plant defense responses include the synthesis of signals such as salicylic acid, ethylene and jasmonates that regulate gene expression, and the production of defense molecules such as reactive oxygen species, phenylpropanoids, phytoalexins and pathogenesis-related proteins. All these inducible biochemical reactions tend to create protective physiological conditions to limit pathogen growth and invasion in the host tissues. The final result of the host-pathogen interaction, plant disease (compatibility), or plant resistance (incompatibility), depends on the combination of a number of different variables. These variables include the genetic characteristics and physiological state of both the plant and the pathogen, and several environmental conditions including light, temperature, and humidity, among others.

Perception of the pathogen

To infect a plant, pathogens must be able to evade or overcome the constitutive plant defense formed by physical barriers such as for example the cuticle and the cell wall. Different pathogens use different strategies to face this constitutive plant defense and gain entrance into plant tissues. Some of the pathogens are specific for one plant variety while others affect a wide range of plants. Irrespective of the type of pathogen, plants might be able to perceive its presence and trigger a defense response. Plants achieve the perception of a pathogen through molecules of a diverse nature called elicitors, which originate from the pathogen or from the plant, and are able to trigger plant defense responses. In some cases, the recognition of the pathogen by the plant is cultivar specific, as in the gene-for-gene type of interactions, while in other cases the plant nonspecifically recognizes the presence of the pathogen by general elicitors.

Gene-for-gene interactions

The gene-for-gene type of plant-pathogen interactions involve the specific plant recognition of a protein encoded by a dominant avirulence gene (*avr*) from the pathogen, via a complementary interacting protein encoded by a dominant plant resistance gene (*R*) (Flor, 1971; Keen, 1990; Staskawicz et al., 1995). When one of the *avr* or the complementary *R* genes is not expressed, plant recognition does not occur, the plant-pathogen interaction is compatible and disease may be the result. When both interacting members, Avr and the complementary R proteins, are present, recognition occurs resulting in an incompatible interaction where plant resistance is the result. The hypersensitive response (HR) is a typical plant defense response associated with resistance in these incompatible plant-pathogen interactions, and is characterized by the local death of plant cells around the site of infection, which is thought to restrain pathogen growth and spreading (Hammond-Kosack and Jones, 1996).

Several plant *R* genes and the corresponding *avr* genes from the pathogen have been identified from many plant-pathogen interactions. Based on the structural domains that different R protein sequences exhibit, they have been classified into different groups. Most of the R genes encode proteins containing leucine rich repeats (LRR) that are thought to mediate protein-protein interactions (Bent, 1996; Ellis et al., 2000; Takken and Joosten, 2000). In addition to the LRR regions, some R proteins contain nucleotide-binding sites (NBS) and they are classified as NBS-LRR. The NBS-LRR proteins are further subdivided according to the characteristics of their amino-terminal sequences, featuring either a coiled coil (CC) or a Toll-Interleukin receptor (TIR) homology domain (Bent 1996, Takken and Joosten, 2000). Furthermore, some R genes contain kinase domains and some of these, as for example Xa21, are transmembrane receptor kinases (Song et al., 1995).

In contrast to the R proteins, most of the Avr proteins show little or no homology to each other (Nimchuck 2001, Bonas, 2002). However, some regions such as myristoylation encoding sites are present in several Avr sequences and they may be required for the proper localization and function of the Avr protein (Nimchuk et al., 2000; Shan et al., 2000). There is evidence that Avr proteins play a role in pathogen virulence on susceptible hosts (Kjemtrup et al., 2000; White et al., 2000; Laugé and De Wit, 1998). For example, AvrPto enhances the

capacity of *P. syringae* pv. *tomato* to induce necrosis in tomato plants lacking the R gene Pto, an such a necrosis is correlated with an increase in bacterial growth (Chang et al., 2000).

The simplest model for gene-for-gene interaction is where R proteins are receptors and Avr proteins are ligands. However, in some cases there is evidence that R proteins are not the primary receptor for the corresponding Avr protein. For example, the Ry-mediated resistance response in potato requires the intact active site of the protease elicitor NIa from potato virus Y (Mestre et al., 2000). Therefore, in this case the R protein may mediate the recognition of the product released by the Avr protein. Based on recent biochemical data, different models for R-Avr interactions have been hypothesized (Bonas and Lahaye, 2002; Dangl and Jones, 2001).

General elicitors

In addition to the specific pathogen-derived Avr elicitors, other types of general elicitors are generated during plant-pathogen interactions. These elicitors are called general because they occur in a number of different plant-pathogen interactions. These general elicitors are molecules of a diverse nature including oligosaccharides, lipopolysacharides, glycopeptides and peptides, derived either from the plant or the pathogen. All these general elicitors are somehow perceived by the plant cells and trigger several plant defense responses (Ebel and Cossio, 1994; Benhamou, 1996; Dow et al., 2000).

Oligosaccharide elicitors were among the earliest elicitors characterized in detail (Darvill and Albersheim, 1984). These general elicitors are able to trigger several plant defense responses including oxidative burst (Apostol et al., 1989), induction of defense signal molecules such as jasmonic acid (JA) (Doares et al., 1995a) and ethylene, accumulation of phytoalexins, and induction of pathogenesis-related genes (Darvill 1992). Oligosaccharides, may originate from pathogen or plant structures. For example, hepta-β-glucoside, oligochitin, and oligochitosan derive from pathogens while oligogalacturonides derive from plants (Ebel, 1998). Plant oligogalacturonides (OGAs) may be released by the action of cell wall degrading enzymes derived from the invading pathogen, which may generate OGAs with different degrees of polymerization. The size range of OGAs that activates plant defense responses is narrow. For example, OGAs with a degree of polymerization between 10 and 15 are generally required to elicit most of the plant defense responses mentioned above (Darvill, 1992). However, OGAs with a degree of polymerization as short as 2 are also able to trigger induction of plant defense-related genes such as proteinase inhibitor and allene oxide synthase (Bishop et al, 1984; Norman et al., 1999). The perception of OGAs and other general elicitors most probably involve direct or indirect participation of membrane receptors (Hahn, 1996).

Receptors

Plant cell-surface receptors are key components that perceive extracellular stimuli from the environment. They are vital for an appropriate physiological role of the cells as functional units of the tissues and the whole organism. During the last decade, several types of receptors have been identified in plants. According to their structural characteristics, they have been classified into different categories including receptor-like protein kinases (RLKs), hisitidine

kinase receptors, and receptors with different numbers of transmembrane domains (Satterlee and Sussman, 1998; Walker, 1994; Grignon, 1999).

Of special interest are the RLKs, which represent almost 2% of the *Arabidopsis* genome (The *Arabidopsis* Genome Initiative, 2000). RLKs are characterized by an extracellular domain probably involved in signal perception, a transmembrane domain, and a cytoplasmic kinase domain, which may initiate a signal transduction cascade into the cell. All plant RLKs identified are serine-threonine kinases and based on the structural characteristics of the extracellular domain they have been divided into different categories (Fig. 1) (Shiu and Bleecker, 2001; Satterlee and Sussman 1998, Walker, 1994).

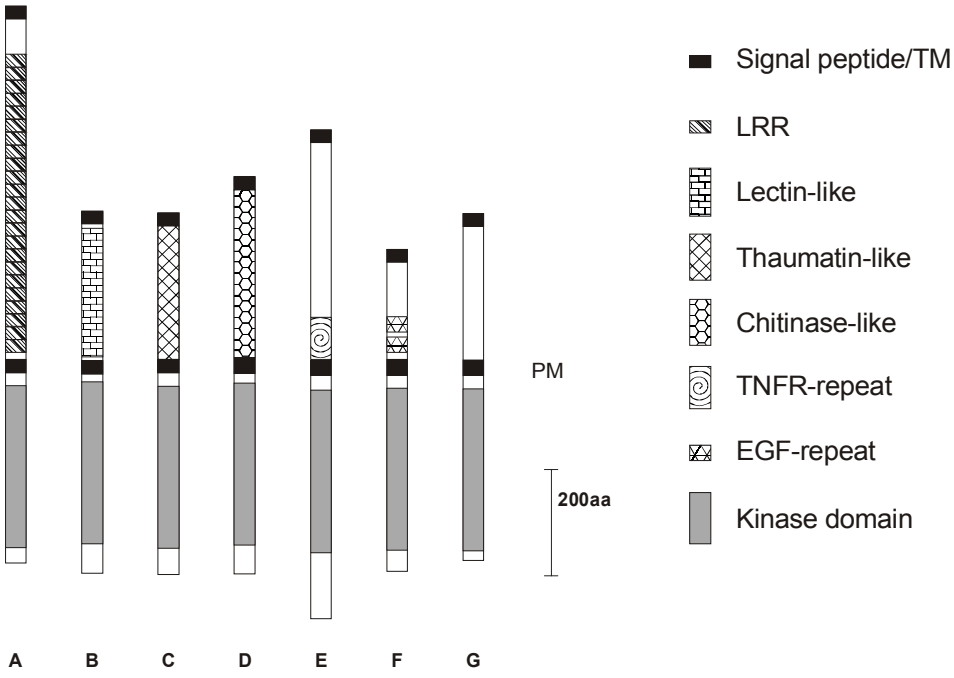

Figure 1. Schematic representation of receptor-like protein kinases (RLKs) identified in plants. These receptors are expected to be located at the plasma membrane (PM) and their extracellular domains exhibit similarity to diverse sequence motifs. A, Xa21 from rice contains leucine-rich repeats (LRR) (Song et al., 1995). B, *Ath*LecRK1 from *Arabidopsis* contains lectin-like motifs (Hervé et al., 1996). C, PR5K from *Arabidopsis* exhibits similarity to the PR protein thaumatin (Wang et al., 1996). D, CHRK1 from tobacco shows similarity to the PR protein chitinase (Kim et al., 2000). E, CRINKLY4 from maize display similarity to the ligand-binding domain of the mammalian tumor necrosis factor receptors (TNFR) (Becraft et al., 1996). F, WAK1 from *Arabidopsis* contains epidermal growth factor-like (EGF) repeats (He et al., 1999). G, LRK10 from wheat do not exhibit homology to a particular established sequence motif (Feuillet et al., 1997).

Several members of the RLK family contain a different number of leucine-rich repeats (LRR) in their extracellular domain. The LRR regions often participate in protein-protein interactions (Kobe and Deisenhofer, 1994) and hence, they may be involved in the binding of the protein ligands. For example, FLS2 an LRR-RLK from *Arabidopsis* (Gomez-Gomez and Boller, 2000), interacts with flagellin, a peptide component of the flagella of Gram-negative bacteria, and recently the MAP kinase signaling pathway and transcription factors activated by this receptor have been identified (Asai et al., 2002). CLAVATA1 represents another example of this class of RLK with a putative polypeptide ligand CLAVATA3 (Brand et al., 2000; Trotochaud et al., 2000). However, other regions of the extracellular domain of LRR-RLKs, which are not LRR, might mediate binding to the ligand. The LRR-RLK, BRI1, perceives the signal of the steroid hormone brassinolide and a 70 amino acid island region, interrupting LRR regions of the extracellular domain, is required for brassinolide binding to the receptor (Wang et al., 2001).

Another class of RLKs contains lectin-like motifs within their extracellular domain. By their similarity to lectin proteins, these lectin binding motifs of RLKs are thought to bind sugars. For example, the *Ath.*LecRK1 receptor (Hervé et al., 1996) from *Arabidopsis* and the SRK receptor from *Brassica oleracea* (Stein et al., 1991) contain a lectin-like motif (Shiu and Bleecker, 2001). A putative polypeptide ligand for SRK, *SCR*, has been identified (Schopfer et al., 1999); however, data about the extracellular domains interacting with the ligand remain elusive.

Other members of the RLK family exhibit similarity to different types of plant and animal proteins. For example, the extracellular domain of the WAK (wall associated kinase) receptors from *Arabidopsis* contain epidermal growth factor-like (EGF) repeats (He et al., 1999) and CRINKLY4 from maize exhibit similarity to the ligand binding domain of the mammalian tumor necrosis factor receptors (TNFR) (Becraft et al., 1996). Interestingly, other RLKs contain extracellular domains with similarity to pathogenesis-related (PR) proteins from plants. Examples of this class are PR5K from *Arabidopsis* with similarity to PR5 (thaumatin) (Wang et al., 1996) and CHRK1 from tobacco with similarity to chitinase (Kim et al., 2000). On the other hand, a number of RLKs do not exhibit homology to a particular established sequence motif.

Another classification for the different RLKs is through the location of cysteines in the extracellular domain. Cysteines may be involved in formation of disulfide bonds, which may determine the folding and final structure of polypeptides. In animals, the structural relationship of several families of growth factors has become evident through the analysis of their crystallized molecules and a common cystine knot structure has been described (McDonald and Hendrickson, 1993; Sun and Davies, 1995). In plants, different RLKs contain cysteine patterns in their extracellular domains (He et al., 1999; Kohorn et al., 1992; Walker, 1994), and recently a superfamily including a number of RLKs and other proteins with C-rich repeats has been described (Chen, 2001). For example, several RLKs classified into an S-domain class (with the extracellular domain similar to the *Brassicaceae* S-locus glycoproteins) contain an array of cysteine residues in combination with other conserved motifs (Walker, 1994).

The extensive diversity of plant RLKs and the large number of them present in the *Arabidopsis* genome suggest that RLKs may be involved in the perception of a wide range of stimuli including those occurring during plant-pathogen interactions. Indeed, some RLKs have been identified as R genes. For example, Xa21 (LRR-RLK) from *Oryza sativa* (rice) that

confers resistance to *Xanthomonas oryzae* pv. *oryzae* (Song et al., 1995) and LRK10 from *Triticum aestivum* (wheat) that confers resistance to wheat rust fungi (*Puccinia recondita*) (Feuillet et al., 1997). In addition, several other RLKs have been associated with plant defense responses to pathogens. In these cases, the association is usually based on the expression pattern that the RLKs exhibit in plants treated with pathogens, elicitors, or signal molecules related to plant defense responses. For example, RLK3 from *Arabidopsis* is induced by oxidative stress, salicylic acid and pathogen attack (Czernic et al., 1999) and SFR1 from *Brassica oleracea* is induced by wounding and bacterial infection (Pastuglia et al., 1997).

In addition to the RLKs, several high affinity binding sites for general elicitors including oligosaccharides, glycopeptides and peptides have been identified through biochemical approaches (Cosio et al., 1990; Cheong and Hahn, 1991; Ito et al., 1997; Basse et al., 1993; Nürnberger et al., 1994; Umemoto et al., 1997; Mithöfer et al., 2000). However, little functional data about these putative receptors are available. For example, similar 75 kDa proteins associated with the plasma membrane of *Glycine max* (soybean) and *Phaseolus vulgaris* (French bean) cells, bind β-glucan elicitors from *Phytophthora* species with high affinity (Umemoto et al., 1997; Mithöfer et al., 2000). However, none of these proteins exhibit any known functional domain that might be involved in signal transduction, suggesting that these β-glucan binding proteins may interact with other components to transduce the elicitor signal (Mithöfer et al., 2000).

Plant defense responses

Early events of plant defense

Following pathogen or elicitor recognition, a series of cytological changes and biochemical responses have been identified in plant cells. The cytological changes include papilla formation, increased cytoplasmic streaming and nuclear migration, which are associated with depolymerization of microtubules and microfilaments (Kombrink and Schmelzer, 2001). The biochemical responses include changes in the H^+, K^+, Cl^-, and Ca^{2+} fluxes across the plasma membrane, and the formation of reactive oxygen species (ROS) that occur within 2-5 minutes after elicitor treatment (Nürnberger, 1994; Dixon et al., 1994; Mehdy, 1994; Low and Merida, 1996). Some of these biochemical reactions have been associated with the transduction of signals that lead to defense responses. For example, Ca^{2+} fluxes have been associated with the induction of phytoalexin accumulation (Dixon et al., 1994). Protein phosphorylation/dephosphorylation is another early event that follows pathogen recognition and is involved in signal transduction cascades that trigger plant defense responses (Dixon et al., 1994). The identification of several MAP kinases and kinase receptors associated with defense responses highlight the relevance of the phosphorylation/dephosphorylation processes during plant defense signaling. Recently, all the components of the kinase-signaling cascade associated with the recognition of flagellin, a peptide of bacterial flagella, have been identified (Asai et al., 2002).

Local and systemic defense responses

Local defense responses to pathogens or elicitors involve the regulation of several genes, which contribute to generate protective physiological conditions against the invading pathogens. At the site of infection these responses include the generation of ROS (Wojstaszek, 1997), the synthesis of proteins involved in the production of signals such as salicylic acid (SA), jasmonates, and ethylene (Malamy et al., 1990; Enyedi et al., 1992; Creelman and Mullet, 1995) as well as enzymes related to the phenylpropanoid metabolism (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995) and the biosynthesis of phytoalexins (Smith, 1996; Hammerschmidt, 1999), and pathogenesis-related (PR) proteins (Linthorst, 1991). Eventually, cells at the site of infection could undergo programmed cell death that often becomes visible as a hypersensitive response (HR) (Greenberg et al., 1994; Dangl et al., 1996).

A local infection often leads to the induction of similar defense responses in uninfected systemic plant tissues that result in broad-spectrum disease resistance to subsequent infections (Kuć, 1982). Additionally, systemic resistance can also be induced by wounding (Schewizer et al., 1998) and non-pathogenic rhizobacteria (van Loon et al., 1998). Furthermore, the application of an extensive amount of natural and synthetic compounds including SA, jasmonates, ethylene, 2,6-dichloroisonicotinic acid (INA) induce similar defense responses leading to a subsequent plant resistance (Kuć, 2001; Oostendorp et al., 2001). The different systemic defense responses associated with pathogen infections include the induction of several PR genes, accumulation of phytoalexins, induction of ROS and micro-HR (Kuć, 2001; Alvarez, 1998). Interestingly, the systemic resistance to virulent pathogens generated by previous treatment of a plant with biotrophic bacteria (*Pseudomonas fluorescens*) is not associated with induction of PR genes in systemic tissues (Pieterse et al., 1996).

Different signal molecules originated at the local sites of infection are responsible for the systemic responses. The systemic responses induced depend on the pathogen and signal pathways triggered by this pathogen. For example, the systemic acquired resistance (SAR) has been associated with the signal molecule SA, which in turn is required for the induction of SAR genes associated with resistance (Mauch-Mani and Métraux, 1998). However, other systemic responses resulting in resistance are SA-independent (Penninckx et al., 1996). In addition, an extensive amount of emerging evidence suggest that the induction of plant disease resistance results from a complex signaling network involving cross-talk between different pathways (Feys and Parker, 2000)

The wound response

Wound stress and pathogen attack are closely related. Pathogens may gain entrance into plant tissues through wounds produced by mechanical stress and pest injury, e.g. wind, rain, feeding insects, etc. Some of the plant defense responses induced by pathogens are similar to those produced by wounding. For example, defense responses to wound damage include generation of ROS, and induction of signals such as ethylene, and JA and derivatives such as methyl jasmonate (MeJA) (Bowles, 1998). These similarities may reflect the fact that common elicitors such as plant oligogalacturonides are generated during tissue damage produced either by wound or pathogens. Recently, an overlap in pathogen-specific resistance and wound response gene expression profiles has recently been shown (Durrant et al., 2000).

The best characterized event during the wound response is the accumulation of proteinase inhibitor (PIN) in both local and systemic leaves. The induction of wound responses has been associated with several molecules including, oligogalacturonides (OGAs), systemin, abscisic acid, jasmonates, and electrical pulses (León et al., 2001). Oligogalacturonides have been shown to induce local and systemic expression of PIN and they are thought to be formed during leaf wounding. However, although oligogalacturonides may be responsible for the induction of PIN at the local level, it is unlikely that they could be the signal traveling from local to systemic leaves (Bowles, 1991). In contrast, there is evidence that systemin, a wound inducible 18-amino-acid oligopeptide inducing PIN locally and systemically, moves from local wounded leaves to systemic leaves (Ryan and Pearce, 1998). Electric pulses have also been suggested to be a mobile signal responsible for the systemic induction of wound responses (Wildom et al., 1992). However, it may be possible that both local and systemic wound responses result from a complex regulatory network that involves several components (León et al., 2001).

Reactive oxygen species

The production of reactive oxygen species (ROS) including hydrogen peroxide (H_2O_2), superoxide ($O_2^{-\cdot}$), hydroxyl radical ($^{\cdot}OH^-$) and singlet oxygen ($^1O_2$), normally occurs in the metabolism of plant cells. They are usually generated by the electron transport activities of chloroplasts and mitochondria, and by enzymes in other cell compartments and the apoplast, involved in reduction-oxidation processes of the plant cell (Mehdy, 1994; Foyer, 1994). The hydroxyl radical is believed to be the most reactive species among the ROS mentioned, especially for its ability to initiate radical chain reactions responsible for the irreversible modifications of macromolecules and damage to cell organelles. At the other end, hydrogen peroxide is a relatively stable ROS and it is able to diffuse across cell membranes and reach cell locations remote from the site of its generation (Wojtaszek, 1997). ROS exhibit a dual function in the normal metabolism of plant cells. On one hand, they are toxic compounds that damage plant cells; on the other hand, they may be beneficial as for example, in the lignin formation in cell walls (Foyer et al., 1994; Lewis and Yamamoto, 1990). To control the action of ROS, plant cells contain several enzymatic and nonenzymatic scavenging systems in all subcellular compartments and the apoplastic space. The enzymatic antioxidative systems include enzymes such as catalases, superoxide dismutases, peroxidases, and the enzymes involved in the glutathione-ascorbate cycle. The nonenzymatic antioxidative metabolites include ascorbate, glutathione, flavonoids, carotenoids, and α-tocopherol (Polle, 1997). In plant cells under normal physiological conditions these systems provide sufficient protection against ROS. However, the increased generation of ROS induced by external stimuli, may overcome these systems and produce oxidative stress (Foyer 1994).

In host-pathogen interactions, accumulation of ROS has been associated with local defense responses (Levine, 1994; Lamb and Dixon, 1997; Wojtaszek, 1997). In some type of plant-pathogen interactions two phases of oxidative burst have been observed. The first phase occurs within the first hour after elicitor treatment of the plant cells and the second phase starts approximately after 4 hours of treatment and continues for several hours (Lamb and Dixon, 1997). In addition, accumulation of ROS has also been reported in systemic tissues of plants responding to pathogen infection (Alvarez et al., 1998). The most studied sources of ROS formation during plant pathogen-interactions are the NADPH oxidase complex in the

plasma membrane and the generation of hydrogen peroxide by cell-wall peroxidases (Grant and Loake 2000).

The production of ROS during plant defense responses to pathogens is associated with both local and systemic HR (micro-HR) (Alvarez et al., 1998; Kombrink and Schmelzer, 2001). Furthermore, the oxidative burst has been proposed as a prime candidate for triggering HR (Greenberg, 1997; Lamb and Dixon, 1997; Kombrink and Schmelzer, 2001). Other plant defense responses associated with ROS include a direct antimicrobial activity, the cross-linking of plant cell-wall proteins, and the induction of defense related genes (Baker et al., 1997; Lamb and Dixon, 1997; Levine et al., 1994).

Defense signals

Salicylic acid

Salicylic acid (SA) is a phenolic compound that participates in several physiological processes of plant cells including defense responses to pathogens. The biosynthesis of SA is initially derived from the shikimate pathway. A number of studies carried out in several plants including tobacco, potato and *Arabidopsis* indicate that SA is synthesized from phenylalanine via cinnamic acid and benzoic acid, and that SA forms different conjugated products (Lee et al., 1995; Silverman et al., 1995; Mauch-Mani and Slusarenko, 1996; Shulaev et al., 1997; Coquoz et al., 1998) (Fig. 2). However, recent studies in *Arabidopsis* have shown that SA is synthesized from chorismate via isochorismate and that SA made by this pathway is required for local and systemic defense responses against pathogens (Wildermuth et al., 2001) (Fig. 2). These proposed cascades together with the question marks that they still have and the complexity of the phenylpropanoid pathway, probably reflect the fact that SA may be generated from alternative pathways.

Several pieces of data indicate that SA plays critical roles in plant defense responses. During plant defense responses to some pathogens an increase in SA levels has been observed in local and systemic tissues. For example, in tobacco plants treated with tobacco mosaic virus (TMV), SA increases 20 fold in locally treated leaves and 5-10 fold in systemic tissues (Malamy et al., 1990; Yalpani et al., 1991). The increase of SA acid during plant defense responses is correlated with the induction of PR proteins and resistance, and the exogenous application of SA or functional analogs of SA to plants induce the synthesis of PR proteins and resistance (White 1979; Ward et al., 1991). These lines of evidence suggest that SA plays a critical role during plant defense responses in local and systemic tissues.

SA was originally proposed to be the mobile signal for systemic acquired resistance (SAR) (Malamy et al., 1990; Métraux et al., 1990). Several studies supported this hypothesis. For example, transgenic plants expressing the *nahG* gene, encoding a salicylate hydroxylase that degrades SA to catechol, exhibit enhanced susceptibility to virulent pathogens and are unable to develop SAR (Gaffney et al., 1993; Delaney et al., 1994). In addition, SA has been shown to be transported to uninfected leaves in tobacco and cucumber (Shulaev et al., 1995; Mölders et al., 1996). However, grafting experiments using tobacco plants expressing nahG or reduced levels of phenylalanine ammonia-lyase (PAL), and wild-type plants, suggested that other primary systemic signals are involved in the onset of SAR (Vernooij et al., 1994; Pallas et al., 1996). Scions of chimeric tobacco plants exhibited SAR after TMV treatment of nahG

rootstock leaves (Vernooij et al., 1994). Similarly, wild-type scions of chimeric tobacco developed SAR after TMV inoculation of PAL-suppressed rootstock leaves (Pallas et al., 1996). Because, PAL-suppressed scions did not exhibit SAR after TMV inoculation of wild-type rootstock leaves, and SA only partially restored the SAR in PAL-suppressed plants, the authors suggested that, in addition to increased levels of SA the presence of other phenylpropanoid products may be important for SAR induction (Pallas et al., 1996).

There is not a clear picture about the mechanisms of action by which SA induce PR genes and resistance. However, several studies suggest that SA action during plant defense responses may involve the inhibition of catalase and ascorbate peroxidase, which link SA action with the action of ROS. In addition, other SA-binding proteins have been identified although the role of these proteins remains elusive. Furthermore, protein phosphorylation/dephosphorylation has also been shown to play a role in SA signal transduction. The identification of *Arabidopsis* mutants affected in SA-mediated responses is contributing in clarifying the mechanism of action of SA during plant defense responses (Dempsey et al., 1999; Cameron, 2000). For example, NPR1 encodes a protein with ankyrin repeats that function downstream SA in the SAR response and interacts with transcription factors that bind to promoter sequences required for SA-inducible PR gene expression (Cao et al., 1997; Zhang et al., 1999).

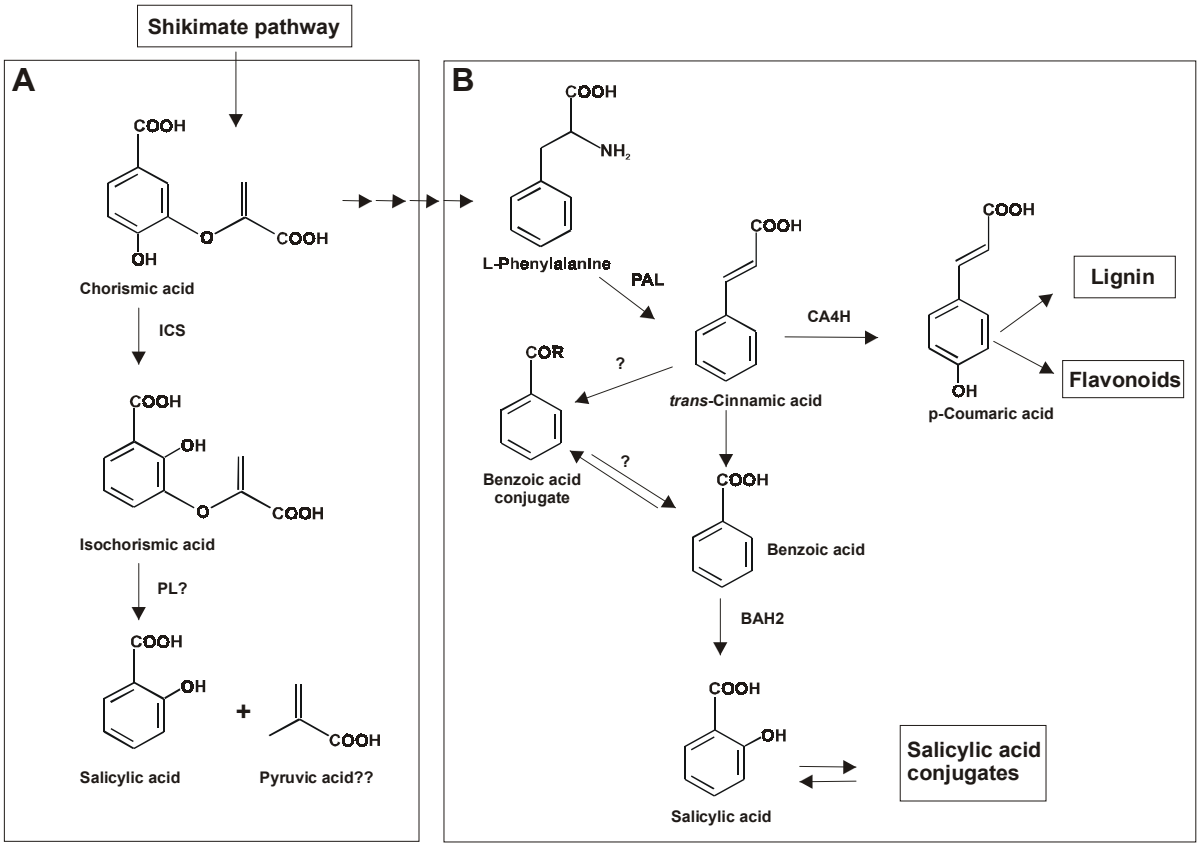

Figure 2. Proposed biosynthetic and metabolic pathways of salicylic acid in plants. In box A, the SA pathway occurring during *Arabidopsis* defense responses, recently proposed by Wildermuth et al. (2001). In box B, the traditional pathway proposed for SA biosynthesis, supported by several studies on different plant species including tobacco, potato and *Arabidopsis* (adapted from Lee et al., 1995). Abbreviations: ICS, isochorismate synthase; PL, pyruvate lyase; PAL, phenylalanine ammonia-lyase; CA4H, cinnamate 4-hydroxylase; BA2H, benzoic acid 2-hydroxylase.

Jamonates and C-6 volatiles

Jasmonic acid (JA) and other structural derivates including methyl jasmonate (MeJA), function as plant signals involved in plant defense to wounding and pathogens and developmental processes such as tuberization and pollen growth (Creelman and Mullet 1995; Sembdner and Parthier 1993). Jasmonates are synthesized from linolenic acid that is oxidized by lipoxygenase (LOX) to form 13-hydroperoxylinolenic acid. Jasmonic acid is produced from 13-hydroperoxylinolenic acid via a series of enzymatic reactions including allene oxide synthase (AOS), allene oxide cyclase (AOC) and other enzymes as indicated in Figure 3 (Creelman and Mullet 1997; Mueller 1997).

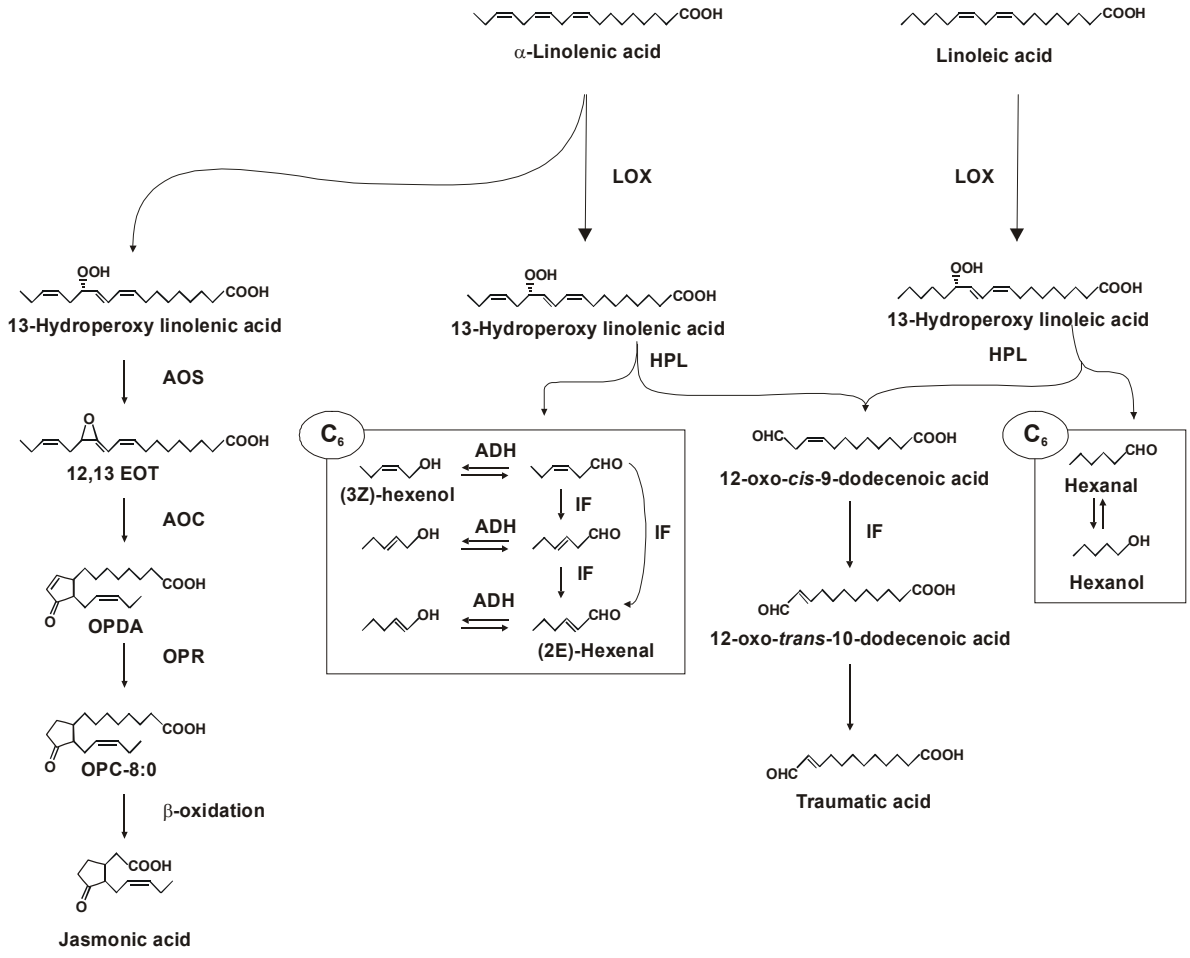

Figure 3. Biosynthesis of jasmonic acid and C_6-volatiles. Abbreviations: LOX, lipoxygenase; AOS, allene oxide synthase; AOC, allene oxide cyclase; OPR, 12-oxo-phytodienoic acid reductase; HPL, hydroperoxide lyase; ADH, alcohol dehydrogenase; IF, isomerization factor. (Sources: Hatanaka, 1993; Schaller, 2001).

Increased levels of jasmonates have been found in several plant species after wound and pathogen as well as elicitor treatment (Creelman and Mullet 1995). In addition, treatment of plants with JA or MeJA increases resistance to pathogens (Cohen et al., 1993). JA and MeJA regulate the expression of several genes including proteinase inhibitors, PR genes, and genes involved in phytoalexin biosynthesis (Farmer et al., 1992; Reinbothe et al., 1994; Wasternack and Parthier, 1997). Furthermore, octadecanoid precursors of JA and MeJA also activate the synthesis of proteinase inhibitors (Farmer and Ryan, 1992). On the other hand, photosynthesis related genes such as the small and large subunits of rubisco are downregulated by jasmonates (Creelman and Mullet 1997). The JA signal transduction pathway is mainly unknown. However, several *Arabidopsis* mutants have been identified, which exhibit reduced or increased sensitivity to jasmonates (Creelman and Mullet, 1997; Vijayan et al., 1998).

Another set of signal molecules, the C_6-volatiles, is related to the jasmonate pathway. C_6-volatiles are present in several plants and have been associated with defense responses upon tissue damage (Hatanaka et al., 1987; Turlings et al., 1995). C_6-volatiles derive from 13-hydroperoxy linolenic or linoleic acids, which are formed by LOX from linolenic or linoleic acids respectively (Fig. 3). The 13-hydroperoxides are cleaved by hydroperoxide lyase (HPL) to produce 12-oxo-dodecenoic acid and either *cis*-3-hexenal or hexanal, depending on whether the precursor was 13-hydroperoxy linolenic or linoleic acid, respectively. These C_6-volatile aldehydes are substrates for an alcohol dehydrogenase and an isomerization factor that produce other C_6-volatiles such as *trans*-3-hexenal, *trans*-2-hexenal, *cis*-3-hexenol, *trans*-3-hexenol, *trans*-2-hexenol and hexanol (Hatanaka, 1993).

These C_6-volatiles are rapidly released from damaged tissue and are responsible for the 'green odor' released from damaged leaves (Hatanaka et al., 1987, 1993; Turlings et al., 1995; Bate et al., 1998). The increased production of some of these volatiles including *cis*-3-hexenol and *trans*-2-hexenal has been shown during plant defense responses to pathogens (Croft et al., 1993). Some of the C_6-volatiles have been shown to induce expression of genes (Bate and Rothstein, 1998) and the production of phytoalexins (Zeringue, 1992). Furthermore, C_6-volatiles have been found to exhibit antimicrobial activity (Croft et al., 1993) and they reduce insect feeding rates (Hildebrand et al., 1993). Transgenic plants with reduced levels of HPL exhibited reduced levels of both hexanal and 3-hexenal and insects feeding from these plants exhibited a 2-fold increase in fecundity above those feeding on nontransformed plants (Vancanneyt et al., 2001).

Ethylene

Ethylene is a gaseous signal molecule that regulates numerous processes in plants including normal growth and development, and defense responses to biotic and abiotic stresses (Bleecker and Kende, 2000). Ethylene is synthesized from methionine via S-adenosyl-L-methionine (AdoMet) and 1-aminocyclopropane-1-carboxylic acid (ACC). The conversion of AdoMet to ACC is catalyzed by ACC synthase, and ACC oxidase catalyzes the conversion of ACC to ethylene. Ethylene exhibit the capacity to stimulate its own synthesis via a positive feed back loop, and for many biological responses it is effective at nanomolar concentrations (Kende, 1993; Bleecker and Kende, 2000). ACC synthase is the key enzyme responsible for the regulation of ethylene production and it is encoded by a multigene family. The expression of ACC genes is differentially regulated by diverse stimuli (Kende, 1993; Zarembinski and Theologis 1994).

Extensive data indicate that ethylene plays important roles in plant defense responses. The levels of ethylene have been shown to increase upon wounding, pathogen infection or treatment with elicitors of defense responses (Enyedi et al., 1992; O'Donnell et al., 1996). In addition, ethylene regulates the expression of several genes including those participating in plant defense responses to biotic stresses. For example, PR proteins such as glucanase and chitinase are induced by ethylene (Deikman, 1997).

Ethylene perception and signal transduction pathway have been characterized in *Arabidopsis* through the studies of several mutants (Fig. 4). Ethylene is perceived by histidine-kinase receptors. ETR1, ETR2, ERS1, ERS2 and EIN4 constitute a family of ethylene receptors, and mutations of these genes produce plants insensitive to applied ethylene (Johnson and Ecker, 1998; Bleecker and Kende, 2000). CTR1 is a protein kinase acting downstream of the ethylene receptors as a negative regulator of the ethylene response (Kieber et al., 1993). The *EIN2* gene encodes a 12-membrane-pass, metal-ion-transporter (Alonso et al., 1999) and is required for ethylene signaling. Although the role of this gene in the ethylene pathway remains elusive, genetic studies locate EIN2 between CTR1 and EIN3 (Bleecker and Kende, 2000). *EIN3* and the related *EIL1* and *EIL2* encode nuclear proteins (Chao et al., 1997) that regulate the expression of *ERF1* (Solano et al., 1998), a member of a large family of transcription factors referred to as ethylene-response-element-binding-proteins (EREBPs) (Ohme-Takagi and Shinshi, 1995).

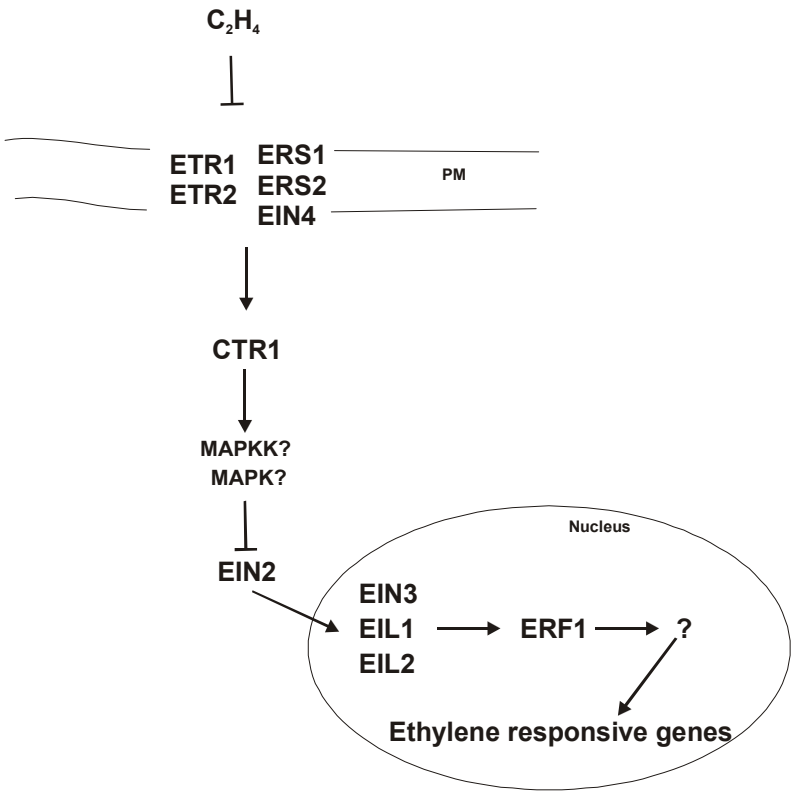

Figure 4. Ethylene perception and signal transduction pathway. For a description see the text. (Adapted from Bleecker and Kende, 2000)

Cross-talk between signal pathways

The signal transduction pathways triggered by signal molecules such as SA, JA and ethylene appear to be connected and form an intricate regulatory signaling network that modulates plant defense responses. Jasmonates and ethylene pathways act synergistically for the induction of some PR genes. For example, in tobacco plants ethylene and MeJA synergistically induce two PR genes, PR1b and osmotin (Xu et al, 1994), and in *Arabidopsis*, the expression of *PDF1.2*, a gene encoding an antifungal plant defensin, is concomitantly induced by ethylene and MeJA (Penninckx et al., 1998; Norman-Stterblad et al., 2000).

In contrast, SA and jasmonates have been shown to induce different set of PR genes (Thomma et al., 1998), and antagonism between SA and JA signal pathways has been shown during plant defense responses. For example, SA inhibits the synthesis of proteinase inhibitors induced by JA in tomato plants (Doares et al., 1995b). This antagonism is also reflected by the existence of different types of systemic resistance responses, e.g. SAR responses are SA-dependent whereas other induced systemic resistance (ISR) responses are JA/ethylene-dependent (Mauch-Mani and Métraux, 1998; Pieterse and van Loon, 1999). However, in rice, the SA-analogue INA synergistically stimulates the expression of genes induced by JA (Schweizer et al., 1997), and vice versa, JA enhances the regulation of the SA-induced pathway (Genoud and Métraux, 1999).

Recent studies in *Arabidopsis* with different mutant backgrounds indicate that SA-dependent and JA/ethylene-dependent pathways may require components that are active in both pathways. For example, NPR1, a protein with ankyrin repeats that interact with transcription factors (Cao et al., 1997; Zhang et al., 1999), is required for SAR and for ISR responses (Cao et al., 1994; Pieterse et al., 1998). In another example, fumonisin B1, a fungal toxin from *Fusarium moniliforme*, triggers program cell death in wild-type *Arabidopsis* protoplasts, and this response is light-dependent and requires SA-, JA-, and ethylene-dependent signaling pathways as well as other unidentified factors (Asai et al., 2000).

Pathogenesis-related proteins

PR proteins form an extensive and heterogeneous group of proteins. The single characteristic that links this group of proteins is that they are induced in pathological or related situations (Linthorst 1991, van Loon et al., 1994). However, some PR proteins are also developmentally regulated (Linthorst 1991). PR proteins are thought to play a major role in plant defense. Some PR proteins have been characterized and their biochemical functions are known. For example, PR-2 proteins are β1,3-glucanases and PR-3 proteins are chitinases (van Loon et al., 1994). Glucanases and chitinases may hydrolyze structural components of the pathogen (van Loon et al., 1994), and a synergistic effect of both enzymes has been observed on the inhibition of fungal growth (Mauch et al., 1988). Other PR proteins such as for example PR1 from tobacco have been shown to exhibit antifungal activity, however, its biochemical function remains elusive (van Loon and van Strien, 1999).

Phytoalexins

Phytoalexins constitute a large group of diverse compounds that are both synthesized and accumulated in plant cells undergoing defense responses against biotic stresses. The extensive variety of their structures makes it difficult to give a chemical definition for the group and usually phytoalexins are defined as low molecular weight compounds with antimicrobial activity (Smith, 1996; Hammeschmidt, 1999). More than 300 phytoalexins have been characterized including phytoalexins with structures like flavonoids and other phenylpropanoid derivatives, sesquiterpenes, polyketides, etc. (Smith, 1996; Hammeschmidt, 1999). The biosynthesis of phytoalexins may involve a single metabolic pathway or precursors from several different pathways (Kuć, 1995).

Phenylpropanoids

The phenylpropanoid pathway includes an intricate set of reactions producing an extensive and diverse amount of products that participate in several cellular processes including those induced by biotic stresses. Phenylpropanoids are derived from cinnamic acid, which is formed from phenylalanine by the action of phenylalanine amonia-lyase (PAL) (Dixon and Paiva, 1995; Hahlbrock and Scheel, 1989) (Fig. 5). A core set of reactions including the formation of cinnamic acid, its conversion into 4-coumaric acid, and the conversion of the latter into 4-coumaryl-CoA derivatives is common to the general phenylpropanoid metabolism (Hahlbrock and Scheel, 1989). Individual branches of the pathway may diverge at different points of these core reactions and lead to the formation of diverse phenylpropanoid derivatives including phytoalexins, anthocyanins, flavones, lignin, suberin, cell-wall bound phenolics and signal molecules such as SA (Hahlbrock and Scheel, 1989; Lee et al., 1995; Dixon and Paiva, 1995; Keller et al., 1996).

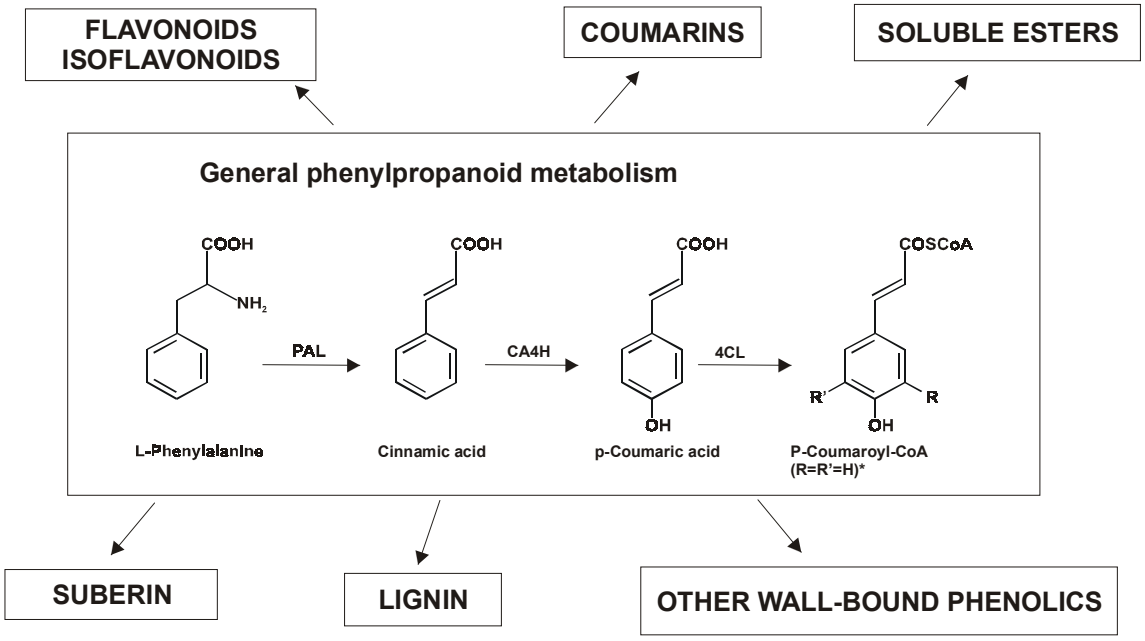

Figure 5. Diagram illustrating the major branch pathways derived from the core reactions common to the general phenylpropanoid metabolism. Abbreviations: PAL, phenylalanine ammonia-lyase; CA4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate:CoA ligase. (Adapted from Hahlbrock and Scheel, 1989)

Model of study

The phytopathogen *Erwinia carotovora* subsp. *carotovora*

Soft-rot Erwinias are facultative anaerobe, nonspore-forming, Gram-negative enterobacteria that cause disease in a wide range of plants including many economically important crops (Agrios, 1997; Pérombelon and Kelman, 1980). The extent of losses varies from country to country and is affected by the climate as well as the conditions of plant growth and storage (Pérombelon and Kelman, 1980). This group of bacteria is formed by several species and subspecies including *Erwinia carotovora* subsp. *carotovora*. Infections by *E.c. carotovora* can occur worldwide in plants growing on the field or in storage after harvesting. Dispersion of these bacteria occurs naturally by the rain, irrigation water, aerosols, insects, nematodes, earthworms and microfauna. Contaminated farm machinery and washing processes are also major means of *E.c. carotovora* dissemination. The bacteria can also spread from plant to plant especially in those plants which are vegetatively reproduced as for example potato (Pérombelon and Kelman, 1980).

Erwinia enter the plants through natural openings as stomata and lenticels or through wounds caused by feeding insects, herbivores, wind, rain, etc. Inside plant tissues, bacterial population multiply and secrete a large variety of extracellular enzymes that degrade the plant cell walls and the pectic substances of the middle lamella causing the maceration of the tissues (Pérombelon and Salmond 1995). The production of extracellular plant cell wall-degrading enzymes (PCWDEs) including several pectinases, cellulases, and proteases, is central to the virulence of *E.c. carotovora* (Kotoujansky, 1987). The relevance of PCWDEs for the virulence of *E.c. carotovora* has been demonstrated in several studies of mutants that are unable to produce these enzymes. For example, bacterial mutants affected in the production of the polygalacturonase PehA, exhibit reduced virulence (Saarilahti et al., 1992), and mutants affected in the expression of several PCWDEs are avirulent (Pirhonen et al., 1991, 1993). In addition, cell-free culture filtrates (CF) containing the PCWDEs secreted by *E.c. carotovora* reproduce the soft-rot maceration symptoms caused by the pathogen (Collmer and Keen, 1986; Norman-Setterblad et al., 2000).

In *E.c. carotovora* the production of PCWDEs is a very tightly regulated process that involve complex regulatory networks of sensor mechanisms that transduce environmental stimuli into gene expression. Induction of PCWDEs can be stimulated by different types of signals including plant-derived compounds, DNA-damaging agents and quorum sensing molecules (Barras et al., 1994). One of the central controls of PCWDEs production is through the quorum sensing, a signaling system that regulates various physiological responses in many Gram-negative bacteria. Quorum sensing is based on the extracellular accumulation of small diffusible signal molecules, usually different N-acylhomoserine lactones, that, over a threshold concentration correlated with the bacterial population density, induce the coordinated expression of specific target genes (Pierson et al., 1998; Whitehead et al., 2001). In *E.c. carotovora* the extracellular accumulation of the quorum sensing molecule, N-oxoacyl-homoserine lactone (OHL), is required for the coordinated activation of genes encoding PCWDEs (Pirhonen et al., 1993). The biosynthesis of OHL in *E.c. carotovora* is mediated by ExpI, and the inactivation of *expI* by mutagenesis results in downregulation of PCWDEs and an avirulent phenotype (Pirhonen et al., 1991, 1993).

Infection of plants with *E.c. carotovora* does not produce a visible HR in plants, and *avr* genes have not been found in these bacteria. However, some *Erwinia* species, including *E.c.*

carotovora strains SCC1 and *Ecc*71, harbor genes homologous to the hypersensitive reaction and pathogenesis (*hrp*) gene cluster (Rantakari et al., 2001; Cui et al., 1996). The *hrp* cluster encode components of the type III secretion system, which is present in several animal and plant pathogenic Gram-negative bacteria and is believed to transport bacterial effector proteins directly into the host cell (Galán and Collmer, 1999). In addition, *hrp* clusters may be associated with genes such as *avr* genes and genes encoding harpin-like molecules, which may be involved in elicitation of HR in plants (He, 1998). For example, the RsmA$^-$ mutant of *E.c. carotovora* strain *Ecc*71 overexpresses $hrpN_{Ecc}$ that encodes a harpin-like molecule that elicits HR in plants (Cui et al., 1996; Mukherjee et al., 1997). In the present work we have used the *E.c. carotovora* strain SSC 3193, and in this strain gene homology to harpin-like, *avr* or *hrp* genes have not been found (Saarilahti, personal communication).

The plant hosts

In the present work two plants from the *Solanaceae* family, *Solanum tuberosum* and *Nicotiana tabacum*, were used to study plant defense responses to *Erwinia carotovora* subsp. *carotovora.* Potato plants, including the most commonly cultivated species *Solanum tuberosum*, are the most important dicotyledonous plants used as a nutrition source for humans, and pathogens and pests damaging these plants generate extensive economical losses (Hooker, 1981; Agrios, 1997). *Solanum tuberosum* is a natural host of *E.c. carotovora* and soft-rot diseased plants may be found on the field and in storage. The control of soft-rot diseases in potato and other plants is difficult because the several sources of bacteria and the multiple modes of contamination mentioned above. Most of the methods for controlling bacterial soft-rots are almost exclusively based on sanitary practices. Chemical control is generally not recommended for the control of soft rots (Agrios, 1997; Pérombelon and Salmond, 1995).

Resistance to *E.c. carotovora* has been observed in *Solanum brevidens*, a wild nontuber-bearing potato species, which cannot be sexually crossed with *S. tuberosum*. However, tubers from somatic hybrids produced from *S. tuberosum* and *S. brevidens* exhibit enhanced resistance to *Erwinia* species (Austin et al., 1988).

A different approach in controlling soft-rot diseases is to generate transgenic plants resistant to *Erwinia* species. Transgenic potato plants expressing foreign genes such as PL3, a pectate lyase from *E.c. atroseptica*, or T4 lysozyme, a bacteriophage enzyme with lytic activity on bacterial cell walls, exhibited reduced susceptibility to *E.c. atroseptica* (Düring et al., 1993; Wegener et al., 1996). Similarly, transgenic potato plants expressing a fungal glucose oxidase that generates high levels of hydrogen peroxide, exhibited reduced growth of *E.c. carotovora* on tuber discs (Wu et al., 1995).

Tobacco plants have been extensively used as a host model in plant-pathogen interactions. An important amount of data about inducible defense responses to different pathogens including viruses, bacteria and fungi, has been accumulated. For example, several PR genes have been identified and characterized, and local and systemic defense responses against pathogens have been extensively studied in tobacco plants. In addition, tobacco plants are diploids and easily produce seeds in laboratory conditions; features that represent an advantage when screening, storing, and handling transgenic plants. These characteristics

together with the ability of *E.c. carotovora* to infect this plant, make tobacco plants a good complementary model to study plant defense responses to *E.c. carotovora*.

AIMS OF THE STUDY

The general aim of this thesis was to identify and characterize molecular components of plant defense responses induced by the phytopathogen *Erwinia carotovora* subsp. *carotovora*, and generate transgenic plants with enhanced resistance to this pathogen. Two plants of the *Solanaceae* family, *Solanum tuberosum* and *Nicotiana tabacum*, were used as hosts for *E.c. carotovora*, and studies of potato defense responses were especially emphasized. The specific aims of this study were:

- Characterization of tobacco defense responses induced by plant cell wall-degrading enzymes of *E.c. carotovora*.

- Identification and characterization of potato genes involved in plant defense responses triggered by the plant cell wall-degrading enzymes of *E.c. carotovora*.

- Biochemical analysis of the proteins encoded by the potato genes identified, and partial characterization of the physiological modifications they may produce.

- Generation and analysis of transgenic tobacco plants with enhanced resistance to *E.c. carotovora*.

MATERIALS AND METHODS

Biological material

The biological material used for the present work is precisely described in each specific study (I-V). Briefly, we have used *Solanum tuberosum* subsp. *tuberosum* and *Nicotiana tabacum* wild-type species. In addition, we also used transgenic tobacco plants. *Erwinia carotovora* subsp. *carotovora* and cell-free culture filtrates (CF) from these bacteria were used for pathogen and elicitor treatments, respectively. Additionally, different strains of *E. coli* and *Agrobacterium tumefaciens* containing different constructs have been used.

Methods

Technique:	used and described in paper:
Isolation of plant RNA	(I-V)
Isolation of plant DNA	(II, V)
Isolation of plant proteins	(III, IV)
Protein expression in *E. coli*	(III)
DRD-1 enzyme assays and kinetics	(III)
Thin layer chromatography	(III)
Differential Display	(III, IV)
Suppression subtractive hybridization	(II)
cDNA phage-library construction and screening	(III-IV)
RACE library construction and screening	(II)
PCRs	(II, V)
Vector construction	(III, V)
Southern blot analysis	(II, V)
Northern blot analysis	(I-V)
Western blot analysis	(IV)
Plant transformation	(V)
Quantitative GUS assays	(I, V)
Individual PCDWE preparations and enzyme assays	(I)
E.c. carotovora culture filtrates preparations	(I-IV)
Assays for bacterial virulence and bacterial growth in planta	(I, V)
Extraction and assay for *N*-oxoacyl-homoserine lactone	(V)
DNA sequencing and computer analysis	(II-V)
Isolation of thylakoid membranes	(III)
Analysis of the photosystem I electron transport	(III)
Detection of hydrogen peroxide in chloroplasts by transmission electron microscopy	(III)

RESULTS AND DISCUSSION

The results and the corresponding discussions of all five studies included in this thesis are adequately presented in each particular study. Therefore, only a summary of the results is presented and selected parts are discussed in this chapter.

Plant cell wall degrading enzymes from *Erwinia carotovora* synergistically induce SA-independent plant defense responses (I)

Previous studies have shown that PCWDEs from *E.c. carotovora*, including several pectinases, cellulases and proteases, are the main virulence factors of these bacteria. Previous studies have reported that cell-free culture filtrates from *E.c. carotovora* (CF) containing the PCWDEs elicit induction of defense gene expression in plants (Palva et al., 1994; Vidal et al., 1997). In this study we reported the role of individual PCWDEs from *E.c. carotovora* as elicitors of local and systemic defense gene expression and resistance in plants. In addition, we also demonstrated that a SA-independent pathway mediates this systemic resistance.

The role PCDWEs as elicitors of plant defense

In order to characterize the individual contribution of PCWDEs, several of these enzymes were individually produced in *E. coli* harboring the corresponding genes from *E.c. carotovora*, e.g. pectinases (PehA, PelA/D) (Saarilahti et al., 1990a; Heikinheimo et al., 1995) or cellulases (CelV1, CelS) (Saarilahti et al., 1990b; Mäe et al., 1995). The activity of individual enzymes in cell-free preparations from *E. coli* was equalized with the activity of the corresponding enzymes in CF before using these preparations for plant treatments. Tobacco plants treated with individual PCWDEs or combinations of them, and with CF, were analyzed for defense gene activation. This analysis was performed by both measuring glucoronidase (GUS) activity in transgenic tobacco, harboring a reporter gene (*uidA*) encoding β-glucoronidase under the control of a basic glucanase promoter (Castresana et al., 1990), and by measuring the accumulation of basic glucanase transcripts in tobacco control plants. The results show that mainly pectic enzymes (PehA and PelA/D) induce basic glucanase in locally inoculated leaves, although one cellulase (CelV1) also weakly induced this gene (I, Fig. 1). None of the individual enzymes could induce expression of the basic glucanase gene as high as the corresponding amount of CF. However, plants treated with a combination of all the individual enzymes (PehA+PelA/D+CelS+CelV1) showed an induction of the basic glucanase gene similar to that obtained with CF. Similarly, the combination of one pectic enzyme plus one cellulase resulted in enhanced induction of the basic glucanase gene as compared with the corresponding individual enzymes. However, such a synergism could not be observed when combinations of cellulases (CelV1+CelS) or pectinases (PehA+PelA/D) were used. When the induction of basic glucanase by CF and individual PCWDEs or a combination of them, was analyzed in systemic leaves, similar results were obtained (I, Fig. 2). Taken together, these results indicate that individual PCWDEs synergistically induce local and systemic defense gene expression in plants. One possible explanation of this synergistic effect of PCWDEs is that cellulases and pectinases may cooperate in the degradation of the plant cell wall, which may result in an increased release of plant cell-wall fragments active as plant defense elicitors. On the other hand, these enzymes

release different types of cell-wall fragments, which may be perceived by different plant receptors that in turn activate independent signal transduction pathways that cooperate in the induction of defense genes.

PCDWE-induced resistance is SA-independent

Previous studies indicated that pre-treatment of plants with CF from *E.c. carotovora* reduced bacterial maceration produced by this pathogen in a subsequent infection of the pre-treated tissue (Palva et al., 1993). Those studies and our results, indicating that PCWDEs elicited both local and systemic induction of the basic glucanase gene, prompted us to investigate whether the plant defense responses triggered by these enzymes may lead to a systemic resistance to *E.c. carotovora*. To carry out such a study, we treated axenic wild-type tobacco with CF or with PCWDEs (individual enzymes or combined) 24 hours prior to inoculation of *E.c. carotovora*, and scored the viable bacteria at different time points after inoculation. The results showed that the pre-treatment of plants with CF, or individual PCWDEs, or a combination of them, prior to *E.c. carotovora* inoculation, lead to enhanced resistance against this pathogen in systemic leaves (I, Fig. 3 and Table I). However, differences in the inhibition of bacterial growth could be observed between the different pre-treatments. Pre-treatments with CF or a combination of PCWDEs (PehA+Pel A/D+CelS+CelV1) resulted in the strongest inhibition of *E.c. carotovora* growth, while the pre-treatments with individual enzymes showed a less pronounced inhibition of bacterial growth. Furthermore, pre-treatment with pectic enzymes (PehA, PelA/D) produced a stronger bacterial growth inhibition than the cellulase tested (CelV1). These results correspond with the induction of defense gene expression previously observed in systemic leaves (I, Fig. 2), suggesting that PCWDEs from *E.c. carotovora* cooperate in the induction of the observed enhanced resistance. In order to determine if this enhanced resistance was specific to *E.c. carotovora* we repeated the pre-treatment of tobacco plants with CF prior to inoculation of *Xanthomonas campestris*, a bacterial pathogen responsible for black rot disease in several plant species (Williams, 1980) (I, Table II). The results showed that pre-treatment of plants with CF also inhibit *X. campestris* growth in systemic leaves, suggesting that the CF-induced systemic response enhances resistance to other bacterial pathogens.

These results suggested that the systemic resistance induced by CF might be indicative of a SAR-like response where SA plays a central role. In addition, previous studies showed that pre-treatment of plants with SA enhances plant resistance to *E.c. carotovora* (Palva et al., 1994). Therefore, the involvement of SA in the CF-induced resistance against *E.c. carotovora* was studied by following the growth of this pathogen in CF-pretreated transgenic NahG tobacco, which cannot accumulate SA (Gaffney et al., 1993) (I, Fig. 4A). The results show that CF leads to inhibition of bacterial growth in NahG tobacco in a similar way as it does in wild-type plants (I, Fig. 3). In order to exclude the possibility that NahG plants can still respond to SA, bacterial growth was evaluated in NahG plants treated with SA or with CF plus SA, and as a control we pre-treated wild type plants with SA. The results show that SA did not inhibit *E.c. carotovora* growth in NahG plants (I, Fig. 4B). In contrast, SA clearly inhibited bacterial growth in wild-type plants (I, Fig. 4D). Furthermore, SA plus CF inhibited bacterial growth in NahG plants (I, Fig. 4C) in a similar way as the treatment of these plants with only CF (I, Fig. 4A). Taken together, these results indicate that the CF-induced systemic resistance to *E.c. carotovora* is not mediated by SA and probably relies on a different signal. Molecules such as jasmonate and methyl jasmonate as well as ethylene have been shown to be

involved in plant-pathogen interactions (Creelman and Mullet 1995; Enyedi et al., 1992), therefore, these signals may be involved in the systemic resistance induced by CF.

Identification, isolation and characterization of potato target genes

The general aim of this work was to identify and characterize molecular components of plant defense responses induced by *E.c. carotovora*. As a strategy to reach this goal, we focused on the identification of potato genes differentially regulated after treatment with PCDWEs from *E.c. carotovora*. Studies II, III and IV of this thesis present data related to the genes identified.

Novel potato receptor-like protein kinases induced by *Erwinia carotovora* (II)

Isolation and sequence analysis of PRKs

A primary 206 bp cDNA fragment was isolated by the suppression subtractive hybridization technique (SSH) (Diatchenko, et al 1996) and RNA-gel blot hybridization experiments indicated that the corresponding gene was induced by CF treatment in potato leaves. Sequence information for this fragment was used to isolate the corresponding full-length cDNA by screening a CF-induced cDNA RACE-library. As result, 4 full-length cDNAs with different EcoRI restriction patterns were isolated. Sequence analysis of these cDNAs indicated that they encode receptor-like protein kinases. They contain a signal peptide (von Heijne, 1990) at their N-terminus followed by an extracellular domain of 255-280 amino acids containing 6-7 putative glycosylation sites, which is linked to a transmembrane spanning domain connecting to the cytoplasmic kinase domain (II, Fig. 1). The kinase domains contain all the characteristics of serine-threonine protein kinases including the 15 invariable amino acids with the correct organization (II, Fig. 2a) (Hanks et al., 1988; Hanks and Quinn, 1991). Based on the sequence information of these cDNAs we named them PRK-1, 2, 3, 4, for potato receptor-like protein kinase. The amino acid similarity between PRKs was 91-98%.

Several receptor-like protein kinases (RLK) have been identified in plants, and based on the structural characteristics of the extracellular domain they have been classified into different groups (Walker, 1994; Satterlee and Sussman 1998; Shiu and Bleecker, 2001). In order to investigate if PRKs exhibit characteristics of the already known RLKs, the extracellular domains of all the PRKs were aligned and compared with the extracellular domain of RLKs from databases and specific regions were analyzed. We did not find any already described motif for eukaryotic receptors including those described for plant RLKs. The highest amino acid sequence similarity of the extracellular domain was found with *Pv*PR20-1, an RLK of a new type from *Phaseoulus vulagaris* (Lange et al., 1999), and three different putative *Arabidopsis* RLKs. Alignment and comparison of the extracellular domain of these genes and the PRKs showed that: i) these domains exhibit 45-59% of amino acid similarity, ii) all the extracellular domains contain 6-9 putative glycosylation sites except for one *Arabidopsis* RLK that contain only four, iii) the relative position of four of the putative glycosylation sites were conserved and iv) all contain a conserved pattern of cysteine residues (II, Fig. 2c). In this conserved pattern of cysteine residues, two modules containing six cysteines each can be distinguished. The first module is located close to the N-terminus and contains a $C\text{-}X_{(49\text{-}53)}\text{-}C\text{-}X_{(8)}\text{-}C\text{-}X_{(2)}\text{-}C\text{-}X_{(11)}\text{-}C\text{-}X_{(12\text{-}14)}\text{-}C$ motif, which is followed by a 75-77

amino acid segment that links it to the second module containing a C-$X_{(8)}$-C-$X_{(2)}$-C-$X_{(10)}$-C-$X_{(0-1)}$-C-$X_{(12)}$-C motif that is followed by 40-42 amino acids connecting with the putative transmembrane domain. Interestingly, PRK-3 lacks a cysteine in the first module and PRK-4 lacks a cysteine in the second module. As mentioned in the introduction, several eukaryotic receptors exhibit conserved cysteine patterns including some already described in plant RLKs (He et al., 1999; Kohorn et al., 1992; Satterlee and Sussman, 1998; Walker, 1994); however, in the extracellular domain of those sequences we did not find the cysteine pattern described here. Interestingly, part of the bi-modular cysteine pattern we described (-C-$X_{(8)}$-C-$X_{(2)}$-C-) is also found in a recently described superfamily of plant proteins, which include plant RLKs and other proteins with cysteine-rich repeats (Chen, 2001). In conclusion, the structural similarities described and especially the conserved bi-modular cysteine pattern shared by the PRKs, PvPR20-1 from *Phaseoulus vulgaris* (Lange et al., 1999) and three different putative *Arabidopsis* RLKs, suggest that they represent a new class of plant RLKs.

PRK-3 may be generated by alternative splicing

The analysis of the extracellular domain of the PRKs also suggested that PRK-3 might have arisen as a result of alternative splicing. Alignment of the PRKs revealed that PRK-3 exhibited a gap of 25 amino acids in a region of the extracellular domain where the other PRKs contain a conserved cysteine and a putative glycosylation site (II, Fig. 1). Analysis of this region in *prk-3* and the other *prk* cDNAs revealed highly conserved sequences for splice sites (Brown and Simpson, 1998) in *prk-2* and *prk-4* (II, Fig. 2b). As a consequence of such putative alternative splicing, the 25 amino acids would be removed and, as an additional consequence, an amino acid substitution (serine instead of alanine) would occur in PRK-3. Southern blot analysis of the potato genome hybridized with a *prk* probe specific to the fragment covering the putative intron supported the notion that *prk-3* may result from alternative splicing (II, Fig. 3). Interestingly, a similar kind of splicing event, showing 25 amino acid insertion containing one or two cysteines plus one glycosylation site and one amino acid substitution at the splice junction, was suggested in the extracellular domain of TGF-β type II receptors from mouse and humans (Hirai and Fujita, 1996; Suzuki et al., 1994). The similarity of the splicing events between these mammalian TGF-β receptors and the PRKs, suggest that a similar mechanism may be involved in the processing of receptor-like serine-threonine kinases in different types of eukaryotic cells. On the other hand, it has recently been shown that alternative splicing occurred within the extracellular domain region of *inrpk1* transcripts, a gene from *Ipomea nil* encoding an LRR-RLK (Basset et al., 2000). Thus, it may be possible that modifications of the extracellular domain of RLKs created by alternative splicing may generate receptors with the same signaling function but with different affinity for a specific ligand. Additionally, these variations may create different isoforms of receptors that bind structurally related ligands. In the case of PRKs, PRK-3 lacks a putative glycosylation site and a cysteine residue in the first module and PRK-4 lacks a cysteine in the second module. Because cysteine residues are usually related to the formation of disulfide bonds, which may affect the three-dimensional structures of the proteins, it may both be possible that the different PRKs exhibit different binding affinities for the same ligand and that they bind structurally related ligands.

Expression of PRK transcripts

In order to study the role of *prk* genes in plant response to *E.c. carotovora*, we characterized their expression pattern in different plant tissues after CF treatment by RNA-gel blot hybridization and RT-PCR. Local induction of *prk* transcripts was observed in leaves and mini-tubers treated with CF. The transcripts were induced up to 24 hours after CF treatment exhibiting the highest accumulation within the first hour (II, Fig. 4). Furthermore, analysis by RT-PCR indicated that prk-1 and prk-4 transcripts were expressed in different tissues during the first hour of CF-treatment (II, Fig. 5). A low and delayed accumulation of *prk* transcripts was also observed in systemic leaves. The early accumulation of *prk* transcripts in different tissues responding to CF treatment suggest that PRKs may be involved in signal perception during potato defense responses to *E.c. carotovora*.

In animals, some receptors are up-regulated by their own ligands, for example the interleukin 2 and the epidermal growth factor receptors (Clark et al., 1985; Deeper et al., 1985). Thus, it may be possible that a similar situation could occur with PRKs. As mentioned in the introduction, the PCWDEs are the main virulence determinants of *E.c. carotovora* and cell wall fragments trigger plant defense responses (I). In addition, previous results from our group have shown that synthetic oligogalacturonides and CF similarly induce plant defense genes (Norman et al., 1999). Thus, in order to elucidate the nature of the inducer of PRKs, we characterized their expression pattern in leaves treated with di-oligogalacturonic acid (dimers) and tri-oligogalacturonic acid (trimers) (Fig. 5a and b). The results show that dimers, trimers and CF induce *prk* transcripts to similar levels during the first hour. However, clear differences between the expression patterns of CF and oligogalacturonide-treated plants were observed in the following hours. The accumulation of *prk* transcripts in CF-treated plants decreased during the second hour to a lower level that remained constant during the next hours. In contrast, the accumulation of *prk* transcripts in oligogalacturonide-treated plants increased during the second hour and decreased abruptly to control levels at four hours after treatment. In addition a low but detectable and reproducible induction of *prk* transcripts was observed in wounded plants. These results suggest that short oligogalacturonides released by CF or wound treatment are responsible for the induction of *prk* genes. The difference in the kinetics of *prk* accumulation between CF and oligogalacturonide-treated plants may be explained by the fact that the former is an enzymatic solution that continuously releases oligogalacturonides during several hours as maceration proceeds, while the latter is a solution with a fixed concentration of oligogalacturonide. Interestingly, the abrupt decrease of *prk* transcripts in oligogalacturonide-treated plants may reflect the involvement of a different type of signal that controls the temporal expression of *prk* transcripts. The characterization of *prk* expression suggest that PRKs are involved in potato defense responses to *E.c. carotovora* and show the relevance of short oligogalacturonides as elicitors of *prk* expression in this plant-pathogen interaction. The isolation of PRKs provides a new molecular tool to study the early stages of plant-*E.c. carotovora* interactions at the interface.

A novel potato defense-related alcohol dehydrogenase induced by *Erwinia carotovora* (III)

Identification, isolation and characterization of *drd-1*

The identification of *Solanum tuberosum* genes responsive to culture filtrates (CF) from *E.c. carotovora* by differential display (Liang and Pardee 1992, Liang et al. 1993) led to the isolation of a 550 cDNA fragment corresponding to a gene induced in potato leaves after CF treatments. Subsequently, this fragment was used as a probe for screening a potato CF-induced cDNA library. As a result, a full-length cDNA encoding a 359 amino acid polypeptide with high similarity to alcohol dehydrogenases was isolated. Based on the CF-induced mRNA expression and the sequence similarity of the protein we named the corresponding gene *drd-1*, for defense-related alcohol dehydrogenase. Computer analysis of DRD-1 sequence revealed specific patterns for zinc-containing alcohol dehydrogenases (Jörnvall et al., 1987; Sun and Plapp, 1992), and no clear targeting domains were found within the sequence, suggesting a cytoplasmic location of the protein (III, Fig. 1). Alignment of DRD-1 and comparison with similar protein sequences from databases showed 76-88% of amino acid similarity with *At*ELI3 from *Arabidospis thaliana* (Kiedrowski et al., 1992), *Ag*MTD from *Apium graveolens* (Williamson et al., 1995), *Le*RSE from *Lycopersicon esculentum* (Lauter, 1996), *Msa*CAD1 from *Medicago sativa* (Brill et al., 1999) and *Pt*SAD from *Populus tremuloides* (Li et al., 2001).

Characterization of the expression patterns of *drd-1* revealed that this gene is expressed locally and systemically in CF-treated potato leaves as well as in wound-treated leaves (III, Fig. 2). These results suggest that *drd-1* plays a role both locally and systemically in potato response to tissue damage whether produced by pathogens or wounding. Further characterization of *drd-1* expression in potato plants treated with SA, MeJA or ethylene indicated that all these defense signal molecules induced *drd-1* transcripts up to 24 hours after treatment; however, the expression patterns were clearly different (III, Fig. 3). SA showed a transiently strong induction of *drd-1* at 8-12 hours after treatment, while MeJA or ethylene-treated plants showed the highest level of expression at 24 hours. These results suggest that SA, MeJA and ethylene may mediate the induction of *drd-1* expression in potato plants responding to *E.c. carotovora*. On the other hand, as mentioned above, SA, MeJA and ethylene have been implicated in plant defense responses to different pathogens, e.g. virus, bacteria and fungi, and we have shown that *drd-1* expression is differentially induced by these defense signal molecules as well as by CF and wounding. Thus, it may be possible that *drd-1* participate in defense responses to damaged tissues irrespective of the source and type of such damages. If this is the case, then *drd-1* expression may be part of a general defensive physiological adaptation of the cells to diverse stresses.

Biochemical characterization of DRD-1

In order to determine the biochemical function of DRD-1, its cDNA was expressed in *E. coli*. The enzymatic assays revealed that DRD-1 is an alcohol:$NADP^+$ oxidoreductase with preference for various aromatic and aliphatic aldehydes as substrates (III, Fig. 4). The enzyme exhibited an absolute requirement for $NADP^+$/ NADPH as a cofactor that could not be substituted by NAD(H). The highest activity of DRD-1 was observed with 2-metoxy-

benzaldehyde as a substrate (Km 4.8 μM). However, the enzyme catalyzed the conversion of various other aldehydes with a similar high efficiency. These highly efficient substrates include: i) cinnamaldehyde and hydrocinnamaldehyde, ii) aliphatic aldehydes such as hexanal and octanal as well as iii) benzaldehyde derivatives such as 2-methoxy-benzaldehyde, 3-methoxy-benzaldehyde, salicylaldehyde, benzaldehyde and *o*-vanillin. In addition, DRD-1 exhibits significant activity with several other related benzaldehyde derivatives and aliphatic aldehydes including *trans*-2-hexenal, *trans*-2-nonenal, *trans*-2-*cis*-6-nonadienal, 4-methoxy-benzaldehyde and 3,4-dimethoxy-benzaldehyde. Low but detectable activity of DRD-1 was observed with syringaldehyde, 4-hydroxy-benzaldehyde, vanillin and isovanillin as substrates. In contrast, substrates such as coniferyl aldehyde and sinapaldehyde showed no detectable activity. Oxidation of the corresponding alcohols was also observed, although at least at 10 times slower rates. Analysis of the reaction products by thin layer chromatography revealed that, under the reaction conditions tested, DRD-1 enzyme carries out the conversion of aldehydes to alcohols (III, Fig. 5). The comparison of DRD-1 activities with all the different substrates and the chemical structure of them suggest that 4-methoxy and especially 4-hydroxyl groups in aromatic aldehydes reduce the efficiency of DRD-1 enzyme. This notion is supported by the kinetic studies of DRD-1 (III, Table II) and for example the low or not detectable activity of DRD-1 with p-hydroxy-cinnamaldehyde derivatives.

To determine DRD-1 enzyme activity in plants after treatment with CF we used plant crude extracts for DRD-1 enzymatic assays. A very high protease activity in CF-treated tissues did not allow such an assay. Instead, since SA and MeJA-treated plants exhibited the highest increase of *drd-1* transcripts, we decided to test DRD-1 activity in SA and MeJA-treated plants (III, Fig. 6). The activity of the enzyme was measured using 2-methoxy-benzaldehyde as a substrate (Km 4.8 μM). These results show a basal enzymatic activity with this substrate in control plants. After 12 hours of treatment with SA, leaf tissue exhibited almost a 4-fold increase in enzyme activity, while 24 hours MeJA-treated plants showed more than a 2-fold increase. These increases in DRD-1 activity correspond with the increase of *drd-1* transcripts observed in SA and MeJA treated plants, suggesting that DRD-1 activity might indeed play a role in plant defense response. However, although we did not measure the amount of *drd-1* transcripts, considering the increase of *drd-1* transcripts observed in SA or MeJA-treated plants, a higher activity of DRD-1 in plant proteins would be expected with these samples. This observation suggests that *drd-1* may probably be post-transcriptionally regulated. Although this post-transcriptional regulation remains to be confirmed, it suggests that a tight regulation of DRD-1 activity may be of high relevance for the physiological adaptation of cells undergoing a defense response.

As mentioned above, DRD-1 exhibited a high similarity (76-88%) with *At*ELI3 from *Arabidopis thaliana* (Kiedrowski et al., 1992), *Ag*MTD from *Apium graveolens* (Williamson et al., 1995), *Le*RSE from *Lycopersicon esculentum* (Lauter, 1996), *Msa*CAD1 from *Medicago sativa* (Brill et al., 1999) and *Pt*SAD from *Populus tremuloides* (Li et al., 2001). However, despite this high sequence similarity these proteins exhibit distinct functional properties. *Ag*MTD is a mannitol dehydrogenase (Williamson et al., 1995) and *Pt*SAD is a sinapyl alcohol dehydrogenase (Li et al., 2001). The product encoded by *LeRse*-1 gene is, by similarity, considered a cinnamyl alcohol dehydrogenase (CAD) (Lauter, 1996) but to our knowledge, there are no reports about the substrate specificity of the enzyme. In contrast, *Msa*CAD1, *At*ELI3 and DRD-1 appear to be functionally related. *At*ELI-3 has been shown to be pathogen induced (Kiedrowski et al., 1992) and *Msa*CAD1 was shown to be induced by wounding and salicylic acid treatments (Brill et al., 1999). In addition, another ELI3 protein, *Pc*ELI-3 from parsley has also been shown to be pathogen induced (Logemann et al., 1997).

All these enzymes catalyze the reduction of a broad spectrum of aldehyde substrates, however they exhibit differences in the kinetics and the affinity for different aldehydes. Thus, considering the sequence similarity, the enzyme properties and the participation in plant defense responses, DRD-1, *At*ELI-3, *Msa*CAD1 and *Pc*ELI-3 appear to form a group of related alcohol dehydrogenases although with different substrate specificity. These enzymes may play similar roles during plant defense. However, despite the known biochemical function, the biological role that these enzymes play during plant defense responses remains elusive.

The expression patterns of drd-1 transcripts observed with CF, wound, SA, MeJA and ethylene and the high activity of DRD-1 with several aromatic and aliphatic compounds suggest that this enzyme may carry out different roles in cells undergoing defense responses. The high activities with aromatic compounds suggest that this enzyme may be involved in phenylpropanoid metabolism, which has been shown to be activated by pathogen and wounding (Hahlbrock and Scheel, 1989; Dixon and Paiva, 1995). Phenolic compounds biosynthesized during plant defense responses may be associated with the cell wall. The structural similarity of some cell wall-bound phenolics synthesized in potato plants responding to fungal elicitors (Keller et al., 1996), with DRD-1 substrates suggest that DRD-1 activity may be related to the formation of cell wall-bound phenolics. On the other hand, SA is a compound derived from the phenylpropanoid pathway (Lee et al., 1995) and SA has been shown to be involved in plant signaling (Malamy and Klessig, 1992; Gaffney et al., 1993; Delaney et al., 1994). Thus, considering the close structural relationship of SA with the highly efficient DRD-1 substrates, such as benzaldehyde, methoxy-benzaldehydes and salicylaldehyde, it may be possible that DRD-1 plays a role in plant stress signaling (see Fig. 2 in the introduction and Fig. 4 in study III).

Interestingly, several aliphatic compounds that we have shown to be related to DRD-1 activity may also have a role in plant stress signaling. Some of these aliphatic aldehydes, substrates of DRD-1, and the corresponding alcohols, are C_6-volatiles released by tissue damage in several plants (Hatanaka et al., 1987; Turlings et al., 1995; Bate et al., 1998), and they can act as plant signals that affect gene expression (Bate and Rothstein, 1998). Furthermore, an alcohol dehydrogenase activity has been related to the interconversion of C_6-volatile alcohols and aldehydes (see Fig. 3 in the introduction and Fig. 4 in study III) (Hatanaka et al., 1987; Bate et al., 1998). Therefore, it is possible that DRD-1 is involved in modulation of these plant stress signals.

Another point to consider is that, as a consequence of DRD-1 activity on a broad spectrum of aldehyde substrates, NADP/NADPH homeostasis may be altered, which in turn may affect the reducing potential of plant cells undergoing defense responses. Generation of reactive oxygen species usually occurs during plant defense responses (Wojstaszek, 1997) and detoxification of these oxygen species may rely on NAD(P)/NAD(P)H homeostasis. In yeasts, four different NADPH oxidoreductase homologs from *Arabidopsis* have been shown to confer yeast tolerance to the thiol-oxidizing drug diamide (Babiychuck et al., 1995). Thus, it may be possible that DRD-1 activity is also related to the oxidative burst occurring in plant defense responses.

The overall contribution that the activity of DRD-1 plays in plant defense responses may be difficult to unravel because of the multiplicity of interrelated factors associated with such activity. On one hand, each of the products released by DRD-1 activity may participate in different pathways; on the other hand, the products released will depend on the substrate

availability for this enzyme. Subcellular localization of DRD-1 and cell integrity may play important roles for substrate availability, thus affecting the final output of DRD-1 activity.

Downregulation of photosystem I by *Erwinia carotovora* derived elicitors correlates with H_2O_2 accumulation in chloroplasts of potato plants (IV)

Isolation and characterization of *psaD*

In this study we report the isolation by differential display of a 300 bp cDNA fragment that was downregulated by CF treatment in potato leaves. Preliminary sequence analysis indicated that this fragment might correspond to *psaD*, a nuclear gene encoding the subunit II (PSI-D) of photosystem I (PSI). Subsequently, screening of a potato cDNA library using this fragment as a probe resulted in the isolation of a 780 bp full-length cDNA. The 624 bp open reading frame encodes a 207 amino acids polypeptide, and computer analysis revealed that this sequence contains a signal peptide domain characteristic of proteins targeted to the chloroplast (Emanuelsson et al., 2000). Comparison and alignment of potato PSI-D sequence with similar proteins showed extensive similarity (78-99%) to other PSI-D subunits from several plant species (IV, Fig. 1). When the signal peptide domain was excluded from such comparison, the sequence similarity of potato PSI-D with the other PSI-D subunits was 89-100%. This extensive similarity and the remarkable conservation of the primary structure of PSI subunits and the overall mechanism of PSI function in cyanobacteria, green algae, and plants (Chitnis et al., 1995), strongly suggest that the isolated gene correspond to *psaD*, which encodes the PSI-D subunit of PSI in potato plants.

PSI-D is located in the stromal side of PSI and plays multiple structural and physiological roles important for the correct function of PSI complex (Chitnis, 1996; Scheller et al., 1997). PSI-D interacts with PSI-C, -E, -H, -I and -L, and is important for the stabilization of PSI complex (Scheller et al., 2001). In particular, the interaction of PSI-D with PSI-C (a subunit containing two [4Fe-4S] clusters, F_A and F_B, the terminal electron transfer centers of PSI) is important for the right orientation of PSI-C in the core of PSI (Li et al., 1991; Naver et al., 1995). PSI-D is responsible for the docking of ferredoxin (Andersen et al., 1992; Merati and Zanetti, 1987; Zilber and Malkin, 1988; Pandini et al., 1999), which transfers the electrons donated by PSI to several reactions including the reduction of $NADP^+$ via ferredoxin:$NADP^+$ oxidoreductase (Knaff and Hirasawa, 1991), and the direct reduction of monodehydroascorbate radical to ascorbate (Miyake and Asada, 1994). The relevance of the role of PSI-D in the interaction with ferredoxin has been demonstrated in PSI-D-less mutants of *Synechocystis*, which are unable to reduce $NADP^+$ via ferredoxin (Xu et al., 1994).

Characterization of the expression patterns of *psaD* indicated that this gene is locally and systemically downregulated in leaves of CF-treated as well as in wound-treated plants (IV, Fig. 2). Further characterization of the *psaD* expression in plants treated with SA, MeJA and ethylene suggest that MeJA and ethylene might mediate the downregulation of *psaD* transcripts in CF-treated plants (IV, Fig. 3). Taken together these results suggest that the regulation of PSI may play a role in plant cells responding to tissue damage.

Functional characterization of photosystem I

As the first step to test whether the structure and function of PSI were affected by the downregulation of *psaD* transcripts, we analyzed PSI-D as well as several PSI subunits by western blot. Interestingly, despite the remarkable downregulation of *psaD* transcripts in CF-

treated leaves, the inmunoblot analysis revealed that PSI-D did not exhibit a visible change between CF-treated and untreated control plants (IV; Fig. 4). Similarly, analysis of several other subunits of the PSI complex such as PSI-A, -B, -C, -E, -F and -N, and PSI-related proteins such as ferredoxin:$NADP^+$ oxidoreductase (FNR) did not exhibit visible changes between CF-treated and untreated control plants (IV; Fig. 4). However, when the $NADP^+$ photoreduction activity of PSI was analyzed with these samples, we observed a 10-13% decrease in the PSI electron transport activity to $NADP^+$ after 12 and 24 hours of CF treatment. (IV; Fig. 5). Taken together, the results may suggest that the marked downregulation of *psaD* transcripts in CF-treated plants may slightly reduce the pool of PSI-D and this reduction may not be visible by western blot analysis but it is reflected by a decrease of the $NADP^+$ photoreduction activity of PSI. The critical roles that PSI-D subunit plays in the stability and physiology of the PSI complex, especially the docking of ferredoxin, suggest that a slight modification of this protein may affect PSI electron transport to ferredoxin. In agreement with this suggestion, recently it was shown that single-site mutations of the C-terminus of PSI-D from *Synechocystis*, showed PSI mutant complexes with reduced affinity for ferredoxin and decreased $NADP^+$ photoreduction activity (Lagoutte et al. 2001). Nevertheless, we cannot exclude that other factors different from a reduced level of PSI-D may be involved in the downregulation of the $NADP^+$ photoreduction activity of PSI observed in CF-treated plants.

Downregulation of photosystem I correlates with hydrogen peroxide accumulation

Previous studies focused on the normal physiology of PSI revealed that this complex is an important source of reactive oxygen species (ROS) (Asada et al., 1974), and the stromal and thylakoid-bound scavenging systems around PSI play an important role in detoxification of ROS (Asada, 1999). The superoxide photoproduced at the stromal side of PSI is converted by spontaneous disproportionation or by superoxide dismutase to hydrogen peroxide, which is scavenged by the stromal and the thylakoid-bound ascorbate peroxidases (Asada, 1999). These data together with those indicating that PSI-D play critical roles in the structure and function of PSI, prompted us to investigate whether the downregulation of the $NADP^+$ photoreduction activity of PSI observed in CF-treated plants correlates with an accumulation of hydrogen peroxide in chloroplasts. Hydrogen peroxide accumulation was histologically analyzed in untreated controls and CF-treated tissues by $CeCl_3$ staining and transmission electron microscopy (IV; Fig. 6). As a positive control for hydrogen peroxide accumulation in chloroplasts, plants were treated with methyl viologen, which binds to the stromal side of PSI and generates reactive oxygen species (Gillham and Dodge, 1987). The results show that in untreated control plants, where the PSI electron transport is not affected, there is no accumulation of hydrogen peroxide, suggesting that the normal photoproduction of hydrogen peroxide by thylakoid membranes is efficiently controlled by the scavenging systems of the chloroplasts. In addition, the results show that hydrogen peroxide accumulation was visible in the chloroplasts of CF-treated plants after 12 hours of treatment as well as in the positive controls treated with methyl viologen. These results indicate that the downregulation of PSI observed in CF-treated tissues correlates with the hydrogen peroxide accumulation observed, suggesting that the downregulation of PSI may cause an increase of reactive oxygen species in the chloroplasts of cells responding to *E.c. carotovora* elicitors.

A summary of the documented events normally occurring around PSI during the formation and scavenging of ROS are presented in Figure 7 of study IV (reviewed by Asada,

1999). Briefly, those data highlight the relevance of the electrons carried by PSI-reduced ferredoxin in the adequate recycling of the ascorbate pool and consequently, in the scavenging of hydrogen peroxide. Based on those data and the results presented in this report, we discuss the correlation observed between the downregulation of the PSI electron transport to $NADP^+$ and the accumulation of hydrogen peroxide in chloroplasts of CF-treated plants.

The downregulation of the $NADP^+$ photoreduction activity of PSI that we observed in CF-treated plants, is a direct measure of the PSI electron transport reducing ferredoxin, thus suggesting that in those chloroplasts less reduced ferredoxin may be available to subsequent reactions using this molecule. This may lead to the accumulation of hydrogen peroxide that we have observed in the chloroplast of CF-treated plants. Firstly, PSI exhibited a reduced capacity to transfer the electrons to ferredoxin, suggesting that an equal amount of light energy exciting the thylakoids of CF-treated and untreated control plants, may be excessive for the PSI complexes of CF-treated plants, resulting in a enhanced ROS formation. Secondly, a decrease in the pool of PSI-reduced ferredoxin would result in less reducing power available to reactions using this molecule, such as those implicated in the scavenging of hydrogen peroxide, thus enhancing ROS accumulation (IV; Fig. 7). This notion is supported by our results with methyl viologen, which induced accumulation of hydrogen peroxide in potato chloroplasts (IV; Fig. 6). Methyl viologen binds to the stromal side of PSI competing with the endogenous acceptor ferredoxin (Summers, 1980), and decreases $NADP^+$ photoreduction mediated by PSI (Albrecht et al., 1998). In addition, treatment of plants with methyl viologen induces accumulation of ROS in chloroplasts (Gillham and Dodge, 1987), which is histologically detected by $CeCl_3$ staining and transmission electron microscopy (Pellinen et al., 1999). Therefore, although we cannot exclude other sources, it may be possible that the downregulation of the $NADP^+$ photoreduction activity of PSI reflects a modification in the physiology of PSI that alters the normal electron transport, resulting in hydrogen peroxide accumulation in the chloroplasts of plants responding to *E.c. carotovora*.

Transgenic plants producing *N*-oxoacyl-homoserine lactone exhibit enhanced resistance to *Erwinia carotovora* (V)

As mentioned in the introduction, the production of the main virulence determinants of *E.c. carotovora*, the PCWDEs, is controlled through the quorum sensing system in these bacteria. This system is based on the production of small diffusible signals such as N-oxoacyl-homoserine lactone (OHL), the accumulation of which is required for the coordinated expression of PCWDEs in *E.c. carotovora* (Pirhonen et al., 1993). As a result of such a control mechanism, the massive production of PCWDEs starts when the OHL concentration has reached or exceeded certain threshold, ergo, the bacterial population has reached a certain density (quorum) (Salmond et al; 1995; Whitehead et al., 2001). On the other hand, we have also shown that PCWDEs trigger plant defense responses (I-IV). Thus, the control of the virulence through the quorum sensing in *E.c. carotovora* may facilitate the establishment of a successful infection, since the bacteria can multiply in plant tissues without triggering a plant defense response, and attack the host only when certain minimal bacterial population density has been achieved. Based on these assumptions we hypothesized that transgenic plants overexpressing OHL synthases, would in *E.c. carotovora* induce the premature production of PCWDEs already at low cell densities, which would trigger a premature induction of plant defense responses and affect the balance of this host-pathogen interaction in favor of the host. To test this hypothesis we generated transgenic tobacco expressing bacterial *expI*, a gene from *E.c. carotovora* responsible for the biosynthesis of OHL in this pathogen (Pirhonen et al., 1993).

Generation of transgenic plants producing N-oxoacyl-homoserine lactone

A vector containing the bacterial *expI* gene under control of the *Cauliflower mosaic virus* 35S promoter was used for tobacco transformation mediated by *Agrobacterium tumefaciens* (V, Fig. 1). As a control only the vector was used for plant transformation. Two lines of transgenic plants harboring *expI* and two lines only the vector (control) were used. The expression of the foreign *expI* gene in transgenic plants was measured by RNA-gel blot hybridization and RT-PCR (IV, Fig. 1). The production of biologically active OHL in transgenic plants was analyzed by a bioluminescence assay (V, Fig. 2). These results confirm that it is possible to produce OHL in plants by ectopic expression of the bacterial OHL synthase gene (*expI*).

To test if the amount of OHL produced by transgenic plants was enough to affect bacterial phenotype *in planta*, we first characterized whether the OHL-negative phenotype of the *expI*$^-$ mutant SCC3065 could be complemented in transgenic plants. This mutant is avirulent (Pirhonen et al., 1991, 1993), essentially because of the lack of OHL-dependent production of PCWDEs required for tissue maceration, and it is not able to multiply *in planta*. The infection of transgenic and control plants with *expI*$^-$ mutant showed a partial complementation only in OHL-producing plants. This complementation was partial because although we did not observed maceration in plants infected with *expI*$^-$ mutant, the bacterial could transiently recover the capacity to multiply in OHL-producing plants while this capacity was absent in control plants (IV, Fig. 3). These results indicated that the production of OHL by transgenic plants expressing bacterial *expI* gene was enough to affect bacterial behavior *in planta*.

Enhanced resistance to *Erwinia carotovora* in OHL-producing plants

To test our hypothesis, whether the production of OHL *in planta* would enhance plant resistance to *E.c. carotovora*, we compared the ability of these wild-type bacteria to cause plant disease in both vector-transformed control plants and OHL-producing transgenic plants (IV, Fig. 4). The ability of *E.c. carotovora* wild-type (SCC3193) to cause disease symptoms was reduced to about half in OHL-producing plants as compared with the vector-control plants. The OHL-producing lines were clearly more tolerant to the initiation of infection even after 48h incubation with *E.c. carotovora*. However, if the infection was established and tissue maceration started, we could not detect significant differences in bacterial growth between transgenic and control plants. Similarly, an increase of the inoculum size by a factor of four or more could overwhelm the enhanced tolerance to *E.c. carotovora* infection in OHL-producing tobacco. These results suggested that the OHL-production of transgenic plants expressing bacterial *expI* was enough to modulate the counteracting molecular events of plant-pathogen interaction in favor of the plant. However, this effect could be observed only to certain extent, since an increase in the number of *E.c. carotovora* cells infecting transgenic tobacco was enough to overwhelm plant resistance. This may reflect the fact that the amount of OHL produced by transgenic plants was not enough to affect bacterial behavior when the number of *E.c. carotovora* cells increased, e.g. induce premature production of PCWDEs in *E.c. carotovora*. On the other hand, it may also reflect that a higher number of bacteria is enough to overwhelm an earlier defense response triggered by the transgenic plants.

To test whether the enhanced resistance observed in transgenic tobacco is indeed due to a premature production of PCWDEs in *E.c. carotovora*, we first characterized the effect of added OHL on enzyme gene expression at low cell densities of bacteria in vitro (IV, Fig. 5). The results showed that the exogenous addition of OHL at low cell densities of *E.c. carotovora* induces expression of PCWDEs, which otherwise remains downregulated. Similar results had been previously observed by Flego (1994). In order to confirm that the production of OHL was the probable cause of the enhanced resistance observed in transgenic tobacco, we tested whether the exogenous application of OHL affects the infection process of *E.c. carotovora* wild-type in vector-transformed control plants (IV, Fig. 6 and Table I). The results showed that the wild-type bacteria without exogenous addition of OHL developed maceration symptoms in most of the plants while the addition of OHL clearly reduced the number of plants developing disease. Taken together, these results suggest that the production of OHL by transgenic tobacco may indeed induce a premature induction of PCWDEs by *E.c. carotovora* at low cell density, which favors plant defense during the early stages of the infection process. In conclusion, the results presented in this study demonstrated that it is possible to produce OHL in plants by ectopic expression of the bacterial OHL synthase gene (*expI*), and this OHL production in transgenic plants alters the early stages of the plant-pathogen interaction favoring the plant defense. These results are in accordance with and support our hypothesis (IV, Fig. 7).

Recently, Fray et al. (1999) reported similar results to ours using a different but related OHL synthase gene (*yenI*). Both of these studies describe a new strategy to improve plant disease resistance by producing bacterial signal molecules *in planta* that affect the coordinated signaling systems of the pathogen population. The same strategy can be extended to other plant-microbe interactions, including enhancement of beneficial interactions. Interestingly, Teplitski et al. (2000) have shown that pea plants naturally producing substances that mimic bacterial OHL affect population-density behaviors in associated

bacteria. Thus, it is conceivable that similar plant engineering strategies, targeting the modulation of microbial signaling systems, may have many applications in agriculture.

CONCLUDING REMARKS

The studies presented in this thesis revealed novel data about different aspects of the molecular events occurring during plant-*E.c. carotovora* interactions, and provide new starting points for further research. At the interface of the plant-*E.c. carotovora* interaction, the characterization of individual PCWDEs as elicitors of plant defense highlighted the synergism of pectinases and cellulases eliciting plant defenses. However, the role of other enzymes secreted by *E.c. carotovora* such as proteases as elicitors of plant defense responses remains to be analyzed; especially, to determine whether the action of such proteases counteract the synergism of the pectinases and cellulases as elicitors of plant defense responses. Additional characterization of molecules secreted by *E.c. carotovora* may contribute to the understanding of events occurring at the interface of these plant-pathogen interactions.

The PRKs are integrating components of plant defense responses to *E.c. carotovora*. These PRKs provide a novel starting point for analyzing both plant defense responses and molecular events occurring at the interface of the plant-*E.c. carotovora* interactions. Finding the ligand(s) interacting with PRKs is an important challenge that would significantly help to understand the function of these receptors and would indicate which molecules are important for the plant cell to perceive during the early stages of the potato-*E.c. carotovora* interaction. Furthermore, future studies with PRKs could also provide new insights into the downstream events triggered by the signal perceived by these receptors.

The data provided by the study of DRD-1 suggest that this enzyme may play a role in plant defense signaling. A priori, DRD-1 may be involved in multiple processes occurring during plant defense responses. Further studies characterizing the biological role of DRD-1 may reveal novel connections between different signaling and metabolic pathways. Subcellular localization of the enzyme and analysis of the biological role of the products released by DRD-1 activity would help to understand the role of this enzyme in defense. Furthermore, an analysis of the role that DRD-1 may play in NADP/NADPH homeostasis is also relevant.

The study of PSI indicated a correlation between the downregulation of *psaD* and PSI, and the accumulation of hydrogen peroxide in chloroplasts of cells responding to *E.c. carotovora*. This suggests that the accumulation of hydrogen peroxide in chloroplasts may be a consequence of the nuclear control of *psaD* triggered by PCWDEs. Little data is available about the regulation of molecular events participating in the electronic transport of chloroplasts that lead to ROS accumulation during plant-pathogen interactions. Therefore, future studies analyzing these events and the physiological role of such accumulation of hydrogen peroxide in chloroplasts of cells responding to PCWDEs from *E.c. carotovora* are needed. On the other hand, it is tempting to speculate that a tight regulation of the electron transport activity in chloroplasts is important, if not crucial, for regulating an appropriate physiological response of the cells responding to pathogen attack. Therefore, it may be possible that a signaling pathway between the nucleus and the chloroplast may coordinate such regulation. Thus, future studies exploring these topics would be useful in understanding plant defense responses to pathogens.

The generation and analysis of transgenic plants overproducing homoserine lactone supported the hypothesis stating that the production of OHL *in planta* would enhance plant resistance to *E.c. carotovora*. From such analysis we could infer that higher levels of OHL *in*

planta may enhance the resistance to *E.c. carotovora*. This could be achieved by targeting the *expI* gene to the chloroplast. On the other hand, although we did not observe any differential phenotype between OHL-producing and wild type tobacco, it may be interesting to know whether OHL production affects the physiology of plants; for example tuber formation and growth in potato plants. Nevertheless, the data presented in study V indicate that plant-engineering strategies targeted to modulate microbial signaling systems may be useful for agricultural applications.

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