Pathogen-Induced Defense Signaling and Signal Crosstalk in *Arabidopsis*

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Academic dissertation
To be presented for public criticism, with the permission of the Faculty of Biosciences of the University of Helsinki, in Auditorium 1041 of the Viikki Biocenter, Viikinkaari 5, Helsinki,
on October 6th, 2006, at 12 noon.

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ISSN 1795-7079
ISBN 952-10-3333-9 (paperback)
ISBN 952-10-3335-5 (PDF, online)

Edita Prima Oy
Helsinki 2006

Dedicated to the loving memory of my mother

Contents

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following publications, referred to in the text by their Roman numerals.

I Kariola, T., Palomäki, T.A., Brader, G., and Palva, E.T. (2003). *Erwinia carotovora* subsp. *carotovora* and *Erwinia*-derived elicitors HrpN and PehA trigger distinct but interacting defense responses and cell death in *Arabidopsis*. Mol Plant Microbe Interact 16, 179-187.

II Kariola, T., Brader, G., Li, J., and Palva E.T. (2005). Chlorophyllase 1, a damage control enzyme, affects the balance between defense pathways in plants. Plant Cell 17, 282-294.

III Kariola, T., Brader, G., Helenius, E., Li, J., Heino, P., and Palva, E.T. (2006). EARLY RESPONSIVE TO DEHYDRATION 15 – a negative regulator of ABA responses in *Arabidopsis*. Manuscript provisionally accepted (pending revision) to Plant Physiology.

ABBREVIATIONS

ABA	abscisic acid
APX	ascorbate peroxidase
BABA	β-aminobutyric acid
CAT	catalase
CC	coiled-coil
CDPK	calcium dependent protein kinase
CSN	COP9 signalosome
DAB	3, 3´-diaminobenzidine
EDS	enhanced disease susceptibility
ET	ethylene
IR	induced resistance
ISR	induced systemic resistance
JA	jasmonic acid
LPS	lipopolysaccharide
LRR	leucine-rich repeat
MAPK	mitogen-activated protein kinase
MeJA	methyl jasmonate
MeSA	methyl salicylate
NBS	nucleotide binding site
NDR	nonrace-specific disease resistance
NO	nitric oxide
NOS	NO synthase
OGA	oligogalacturonic acid
OPDA	12-oxo-phytodienoic acid
PAD	phytoalexin-deficient
PAMP	pathogen-associated molecular pattern
PCWDE	plant cell wall-degrading enzyme
PHY	phytochrome
PI	proteinase inhibitor
PR	pathogenesis-related
RES	reactive electrolyte species
RLK	receptor-like kinase
ROS	reactive oxygen species
SA	salicylic acid
SABP	SA binding protein
SID	SA induction-deficient
TIR	Toll and IL-1 receptor
TTSS	type III secretion system
WAK	wall-associated kinase

ABSTRACT

Erwinia carotovora subsp. *carotovora* is a bacterial phytopathogen that causes soft rot in various agronomically important crop plants. A genetically specified resistance to *E. carotovora* has not been defined, and plant resistance to this pathogen is established through nonspecific activation of basal defense responses. This, together with the broad host range, makes this pathogen a good model for studying the activation of plant defenses. Production and secretion of plant cell wall-degrading enzymes (PCWDE) are central to the virulence of *E. carotovora*. It also possesses the type III secretion system (TTSS) utilized by many Gram-negative bacteria to secrete virulence- promoting effector proteins to plant cells. This study elucidated the role of *E. carotovora* HrpN ($HrpN_{Ecc}$), an effector protein secreted through TTSS, and the contribution of this protein in the virulence of *E. carotovora*. Treatment of plants with $HrpN_{Ecc}$ was demonstrated to induce a hypersensitive response (HR) as well as resistance to *E. carotovora*. Resistance induced by $HrpN_{Ecc}$ required both salicylic acid (SA)- and jasmonate/ethylene (JA/ET)-dependent defense signaling in *Arabidopsis*. Simultaneous treatment of *Arabidopsis* with $HrpN_{Ecc}$ and PCWDE polygalacturonase PehA elicited accelerated and enhanced induction of defense genes but also increased production of superoxide and lesion formation. This demonstrates mutual amplification of defense signaling by these two virulence factors of *E. carotovora*.

Identification of genes that are rapidly induced in response to a pathogen can provide novel information about the early events occurring in the plant defense response. *CHLOROPHYLLASE 1 (AtCLH1)* and *EARLY RESPONSIVE TO DEHYDRATION 15 (ERD15)* are both rapidly triggered by *E. carotovora* in *Arabidopsis*. Characterization of *AtCLH1* encoding chlorophyll-degrading enzyme chlorophyllase indicated that it might have a role in chlorophyll degradation during plant tissue damage. Silencing of this gene resulted in increased accumulation of reactive oxygen species (ROS) in response to pathogen infection in a light-dependent manner. This led to enhanced SA-dependent defenses and resistance to *E. carotovora*. Moreover, crosstalk between different defense signaling pathways was observed; JA-dependent defenses and resistance to fungal pathogen *Alternaria brassicicola* were impaired, indicating antagonism between SA- and JA-dependent signaling.

Characterization of ERD15 suggested that it is a novel, negative regulator of abscisic acid (ABA) signaling in *Arabidopsis*. Overexpression of *ERD15* resulted in insensitivity to ABA and reduced tolerance of the plants to dehydration stress. However, simultaneously, the resistance of the plants to *E. carotovora* was enhanced. Silencing of *ERD15* improved freezing and drought tolerance of transgenic plants. This, together with the reducing effect of ABA on seed germination, indicated hypersensitivity to this phytohormone. ERD15 was hypothesized to act as a capacitor that controls the appropriate activation of ABA responses in *Arabidopsis*.

INTRODUCTION

Plants provide, directly or indirectly, all the food upon which humans and animals depend. Therefore, throughout history, diseases affecting plants have been feared as much as human diseases and war. Since the early days of agriculture, plant diseases have been responsible for crop losses, resulting in devastating times of hunger and famine. Thousands of years ago, these were seen as punishments meted out by gods for sins committed by people. Thus, the plant protection methods used in those days mostly comprised praying and sacrifices, which, understandably, were not very efficient. The Romans even created a special god, Robigus, to protect grains from rust (Agrios 2005). Eventually, crop protection developed from being merely a matter of faith to a more practical direction. Invention of the microscope in the late 17th century enabled scientists to discover many previously invisible microorganisms in diseased plant tissue. However, a further 100 years passed, before it was generally accepted that viruses, bacteria, fungi, and protozoa were not spontaneously occurring natural products of diseases, but actually the causative agents behind these diseases (Holub 2001; Agrios 2005).

The estimated total crop loss from diseases, insects, and weeds is about 36% of potential worldwide production, the proportion of diseases being 14% (Agrios 2005). However, added to this figure should be a further 6-10%, resulting from after-harvest damages, a problem especially encountered in developing tropical countries. Fighting plant diseases is therefore a major challenge when striving for successful agriculture. For millions of people who still cultivate their own food, plant diseases can make a significant difference between a comfortable life and a life agonized by hunger. The Irish famine in the 1840s that resulted from a potato (*Solanum tuberosum*) blight epidemic caused by the fungus *Phytophtora infestans* as well as part of the hunger in developing countries today are examples of the devastating power of plant diseases (Agrios 2005). In more developed countries, where food is plentiful, diseases manifest in economic losses to the farmers, followed by increases in consumer prices.

Diseases can affect plants in many ways, such as by reducing the quality and quantity of the crops. For example, infection by a pathogen can simply make the plants toxic to consumers. Ergot is a disease of cereals and grasses caused by *Claviceps purpurea* and related fungi. These pathogens develop a fruiting structure that replaces the seed of the plant and produces toxic secondary metabolites (Keller et al. 2005). The harvested grain is then contaminated and those who consume it may contract ergotism, a disease with symptoms ranging from blistering of the skin to hallucinations, insanity, and even death (Agrios 2005). Plant diseases can also limit the kinds of plants that grow in certain areas. This is exemplified by the fate of American chestnut (*Castanea dentata*); before the turn of the century, this tree, which provided people with timber and chestnuts, dominated the eastern half of the United States. The invasion of the fungus *Cryphonectria parasitica*, the causal agent of chestnut blight virtually eliminated this species of tree from North

America (Agrios 2005). Similarly, novel pests or pathogens or strains of pathogens could greatly reduce the area in which the major crop species wheat (*Triticum* sp.), rice (*Oryza sativa*), or maize (*Zea mays*) can be grown or eliminate these as vital crops, having considerable effects on the nutritional status of the world.

Over the last 100 years, the control of plant diseases has increasingly depended on the use of toxic chemicals. These chemicals are not only applied on plants and plant products but also to the soil. This leads to environmental pollution, eventually making the fields unsuitable for further cultivation. Also, traces of the chemicals may remain in the plant and make them harmful or even toxic for consumers. The use of pesticides also adds to the total costs of production; these chemicals as well as the machinery needed to spread them are expensive. Moreover, the human population continues to grow rapidly, resulting in an increasing demand for cultivating land. Formerly rare plants, such as maize, have become some of the most abundant species on earth. This has also influenced the disease-causing microbes; potential pathogens that had never before encountered these species now do so frequently, leading to an increased need for protection (Tillman 1999).

For the reasons presented above, much of the research today in the field of plant-pathogen interactions aims at finding both environmentally friendly and more efficient means of controlling plant diseases. Classical breeding for plant resistance has been important since the early days of the 20th century, but these methods, i.e. seed selection, backcrossing, etc., can be quite complicated and time-consuming (Stascawicz 2001; Agrios 2005). Recently, such promising approaches as genetic engineering, including RNA- and gene-silencing techniques, have started to emerge (Lindbo and Dougherty 2005). However, often only one dominant or semi-dominant gene, such as the resistance (*R*) gene, has been employed in breeding crop resistance (Stascawicz 2001). If the resistance is directed against specific pathogens or pathogen races (as it is with *R* genes), it is not necessarily enduring. The appearance and spread of a mutation in the pathogen population can adapt the pathogens to the presence of the *R* gene and break down the resistance (McDonald and Linde 2002).

The study of plant-pathogen interactions can provide tools for development of more durable approaches. For example, knowledge of the kinds of molecular responses various pathogens activate in the plant during the infection can have tremendous potential. Plant defense responses are a result of a complex network of signaling events that involves the interplay of kinases, hormones, and reactive oxygen species (ROS), leading to reprogramming of the plant transcriptome. These responses aim at the production of defensive compounds and, finally, resistance. Elucidation of the molecular components acting in these cascades provides useful tools for engineering more durable crops and resistance that is not so easily broken down. Modifications of signaling components can speed up defense activation upon pathogen attack, thus improving the chances of the plant to successfully respond to current and future encounters with the invaders. In addition to engineering disease-resistant plants, plant resistance can also be improved by the use of nontoxic chemical substances that elicit the activation of natural defense mechanisms, a process called “priming” (Conrath et al. 2002; Kohler et al. 2002). Knowledge about plant-pathogen interactions will hopefully lead to solutions for achieving broad-spectrum protection, long-lasting effects, and reduced chemical input in modern-day agriculture.

PLANT-PATHOGEN INTERACTION

The causative agents of plant diseases belong to the same groups as those causing disease in animals – pathogenic microorganisms such as fungi, viruses, bacteria, protozoa, and nematodes (Agrios 2005). To be a successful pathogen, a microorganism needs to interfere with one or more essential functions of the plant, thereby causing disease. Regardless of the type of pathogen, a prerequisite for pathogenicity of a microorganism is the ability to gain access to the plant interior. Pathogens force their way through plant surfaces by different means; some take advantage of natural openings, such as stomata or lenticels, or enter the plant through wounds, while others simply penetrate the leaf surfaces. Fungi, such as powdery mildew, for instance, can grow a fine hypha directly into plant epidermal cells. Oomycetes and nematodes usually use the penetration method, while bacteria utilize wounds and natural openings. In most fungal diseases, the fungus penetrates not only the cuticle but also the cell wall – the next obstacle for pathogens after reaching the intercellular spaces (apoplast). Some pathogens use chemical activities to overcome this barrier. For example, certain bacteria secrete cutin-degrading enzymes, cutinases, while others produce an arsenal of extracellular enzymes, including pectinases, cellulases, and polygalacturonases, that degrade the cell wall (Toth et al. 2003; Agrios 2005).

The virulence mechanisms the pathogen uses to reach its final goal – to take advantage of the plant as a source of nutrients – include secretion of toxins, growth regulators, and other substances that disturb the metabolism of plant cells or interfere with the plant defenses (Agrios 2005). The virulence strategy depends on how the pathogen intends to utilize the plant; biotrophs obtain nutrients from living plant tissue without killing the cells, whereas necrotrophs kill the cells and make use of their contents during invasion (Glazebrook 2005). Some pathogens, called hemi-biotrophs, fill the requirements of both biotrophs and necrotrophs, depending on the prevailing conditions they are in or the stages of their life cycles (Glazebrook 2005). Most pathogens continue multiplying indefinitely within the infected tissue, using it for nutrients until the plant is dead.

Indefinite multiplication would be true for every pathogen if plants were passive and defenseless organisms, which is not the case. Plants are subject to attack by a wide variety of microbial pathogens; nevertheless, the ability of a microorganism to cause disease in the plant is usually an exception rather than the rule. Due to plants´ fortress-like cell structure as well as their innate ability to recognize potential invading pathogens and activate effective defenses, plants are generally resistant to most pathogens (Heath 2000; Nürnberger et al. 2004). Thus, in order to be successful, a pathogen also needs to evade the plant surveillance system or suppress plant defenses. The ability to detect potential pathogens has been essential to the development of modern plants (Chisholm et al. 2006). Perception of the pathogen is achieved through receptors, a surveillance system capable of recognizing both conserved molecular patterns and specific effector proteins, and activation of the corresponding defenses (Montesano et al. 2003). Plants defend themselves against invaders in two ways: with structural barriers that inhibit the pathogen from gaining entrance and spreading throughout the plant and with biochemical reactions

taking place in plant tissues. These reactions produce toxic substances that inhibit the growth of the pathogen. The combinations of these two defense types vary between different plant-pathogen interactions (Glazebrook 2005). If the plant fails to recognize the pathogen or an elicitor, appropriate defenses might not be mounted and disease results. Alternatively, if the plant responds with a rapid and well-aimed activation of defenses, the attempted infection is halted.

PHYTOPATHOGENIC BACTERIA

Approximately 1600 bacterial species are known, and of these, roughly 100 cause diseases in plants (Agrios 2005). Bacteria are a significant group of phytopathogens and the diseases they cause are considerably difficult to control. This is due to the capability of bacteria to multiply at an astonishing rate and produce enormous numbers of cells in a short period of time. In addition, bacteria can alter their chemical environment significantly by secreting toxins that contribute to their pathogenicity. In contrast to many bacterial pathogens of animals that enter the cells of their hosts, phytopathogenic bacteria multiply in the apoplast of plant cells and remain extracellular (Staskawicz et al. 2001). Bacterial diseases affect all kinds of plants and occur in every place where it is reasonably moist and warm. The spread of bacteria is aided by, for example, water, insects, animals, and humans. Insects wound the plant organs and thus facilitate the entry of the pathogen into the plant. They also act as vectors for some bacteria; the corn flea beetle (*Chaetocnema pulicaria*) is the main vector of the bacterium *Erwinia stewartii*, which causes Stewart´s wilt on corn (Agrios 2005; Cook et al. 2005). Humans, on the other hand, help the long-distance spread of diseases by transporting infected plants to entirely new areas. Some of the most common plant pathogenic genera of bacteria include *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, *Xanthomonas*, and *Streptomyces* (Agrios 2005). Infected plants show a variety of symptoms, such as leaf spots and blights, soft rots, wilts, and cancers. Of these, members of the genera *Pseudomonas* and *Xanthomonas* are causative agents of almost all bacterial spots and blights of leaves, stems, and fruits, while *Agrobacterium* is the main cause of grown gall on many woody plants (Agrios 2005).

One example of a damaging bacterial plant disease is soft rot. Pathogens causing the most common and destructive soft rots are found in the genus *Erwinia* (Toth et al. 2003; Agrios 2005). This disease predominantly occurs in the fleshy tissues of vegetables and root crops. *Erwinia*s invade the plant through natural openings like stomata or take advantage of wounds in the plant tissue caused by, for example, feeding insects or mechanical damage. After invasion, these bacteria are able to reside in intercellular spaces of the plant until environmental conditions, such as temperature, oxygen availability, and water content, become appropriate for disease development (Toth et al. 2003). Soft-rot symptoms begin as a water-soaked lesion that enlarges further, culminating in slimy masses of bacteria and cellular debris oozing out of plant tissues (Perombelon and Kelman 1980; Toth et al. 2003). In their noninfective phase, soft-rot *Erwinia*s undergo endophytic,

epiphytic, and saprophytic lifestyles on plants and are also found in the soil and ground water (Perombelon and Kelman 1980; Toth et al. 2003; Agrios 2005).

PLANT DEFENSE

Even if plants live a sessile life, they are dynamic organisms that fight the pressure of pathogens with advanced defense strategies, including both preformed and inducible defense systems. Resistance of an entire plant species to all strains of a pathogen is called nonhost resistance, the most common type of resistance expressed by plants (Heath 2000; da Cunha et al. 2006). Simply put, this means that, for example, the pathogens of tomato (*Lycopersicon esculentum*) do not infect spruce (*Picea* sp.), and vice versa. Significant components of nonhost resistance are the preformed or constitutive defenses associated with plant structures and chemical compounds already present in the plant. These include structural barriers, such as the plant cell wall, as well as inhibitory compounds, e.g. phenolics and tannins (Heath 2000; Nürnberger et al. 2004; Agrios 2005).

Inducible defenses are triggered by the recognition of the pathogen. Basal defense, a constituent of both nonhost and host resistance, provides basal-level resistance (also called innate immunity or local induced resistance) that prevents infection by a wide range of microbes (Heath 2000; Thordal-Christensen 2003; Nürnberger et al. 2004; Oh and Collmer 2005). Some pathogens have acquired the ability to suppress basal defense responses and enhance their virulence by delivering specific effector proteins to the plant cells that interfere with plant defense. Gene-for-gene or race-cultivar-specific resistance occurs when specific members of a plant species, but not the species as a whole, have acquired resistance to a particular pathogen. This type of resistance is usually restricted to a particular pathogen species, being expressed against specific genotypes of that pathogen (Dangl and Jones 2001; Bonas and Lahaye 2002; Hammond-Kosack and Parker 2003; Chisholm et al. 2006).

PREFORMED DEFENSES

Noninducible, preformed structural defenses, such as a dense epidermal layer and wax and cuticle coverings on leaves, are the first line of plant defense to invading pathogens. Structures found on surfaces, such as spiky hairs called trichomes, can prevent feeding by insects or insect larvae. Hairs on leaves can also have a water repelling effect, hence making it more difficult especially for bacteria to establish contact with the plant. Rigid cell walls composed of fibrils of cellulose embedded in a matrix of several other kinds of polymers, such as pectin and lignin, also serve as an efficient barrier to the invasion of pathogens (Agrios 2005).

Chemical defenses include various antimicrobial peptides, proteins, and nonproteinaceous secondary metabolites present in plant cells that can prevent ingress of the invader (Heath 2000; Nürnberger et al. 2004). Toxic secondary metabolites stored in

specialized plant compartments can be activated or released upon tissue damage. Besides being directly harmful to the invader, they can operate by inactivating the extracellular enzymes secreted by the pathogen (Zhao et al. 2005). Secondary metabolites are often restricted in their distribution to particular plant families, genera, or species. For example, avenacin A-1 is a triterpenoid saponin found in the roots of oat plants. It is highly effective against the fungus *Gaeumannomyces graminis* var *tritici*, a major pathogen of wheat and barley roots, but not oats due to the presence of this secondary metabolite (Buchanan et al. 2000). The glucosinolate-myrosinase system is an example of a sophisticated chemical defense system characteristic of *Arabidopsis* and other *Brassicaceae* species (Halkier and Gershenzon 2006). Glucosinolates, preformed amino acid-derived secondary metabolites and myrosinase, an endogenous β-thioglucosidase, are stored in separate compartments in plant cells. Upon tissue disruption, such as wounding, myrosinase cleaves nontoxic glucosinolates, resulting in the release of such products as isothiocyanates, which can be harmful to a wide range of plant enemies, including mammals, insects, and bacteria (Halkier and Gershenzon 2006).

INDUCIBLE DEFENSES

Inducible plant defenses are triggered by the perception of a pathogen or pathogen-derived molecules called elicitors. The elicitors can be either general, common to a group of microbes, or specific to certain pathogen strains. In addition, pathogens can release polysaccharide oligomers from the plant surface, which can induce defenses (Montesano et al. 2003; Nürnberger et al. 2004; Chisholm et al. 2006). Perception of elicitors takes place in receptors located either at the cell surface or inside the cell (Dardick and Ronald 2006). According to current knowledge, recognition of general and specific elicitors triggers overlapping signaling responses in the plant (Espinosa and Alfano 2004; Kim et al. 2005). Interestingly, by comparing changes in plant mRNA profiles in response to avirulent and virulent *P. syringae*, Tao et al. (2003) demonstrated that the induction of defense genes was more rapid and enhanced in response to specific elicitors (i.e. the avirulent strain). This indicates a difference in the speed rather than the quality of response triggered by the two elicitor types (Espinosa and Alfano 2004; Kim et al. 2005).

Recognition of the elicitor induces several early responses (Figure 1): phosphorylation and dephosphorylation of plasma membrane proteins, increase of cytosolic Ca^{2+}, ion fluxes, and alkalization of the apoplast. Synthesis and deposition of callose in the form of papillae can be initiated rapidly at the site of pathogen invasion. Mitogen-activated protein kinases (MAPK) and NADPH oxidase are activated, and ROS is produced within minutes of contact with the elicitor (Zhao et al. 2005). Activation of transcription factors and early expression of defense genes also occurs. The activated kinase cascades and ROS further amplify the defense signal to downstream reactions (Dardick and Ronald 2006).

A series of alarm signals are triggered that are transmitted intracellularly and also to adjacent cells. These are sequentially followed by late defense gene activation and phytoalexin accumulation. Phytoalexins are toxic antimicrobial substances and can, for example, be flavonoids, alkaloids, and terpenoids produced in healthy cells in response to

signals from damaged cells adjacent to them (Hammerschmidt 1999; Zhao et al. 2005). Production of various defense-related proteins, such as pathogenesis-related (PR) proteins, which have antimicrobial activity and thus serve to contain the infection, is also activated (Wojstaszek 1997; Hammerschmidt 1999; Van Loon and Van Strien 1999). The formation of a hypersensitive response (HR), a rapid, localized cell death that restricts the growth of the pathogen, is more frequently associated with the recognition of a specific than a general elicitor (Greenberg 1997; Espinosa and Alfano 2004; Greenberg and Yao 2004) (Figure 1). The signals originating from the local infection site can then evolve into a systemic defense response involving distal, undamaged parts of the plant and conferring resistance to future pathogen infections.

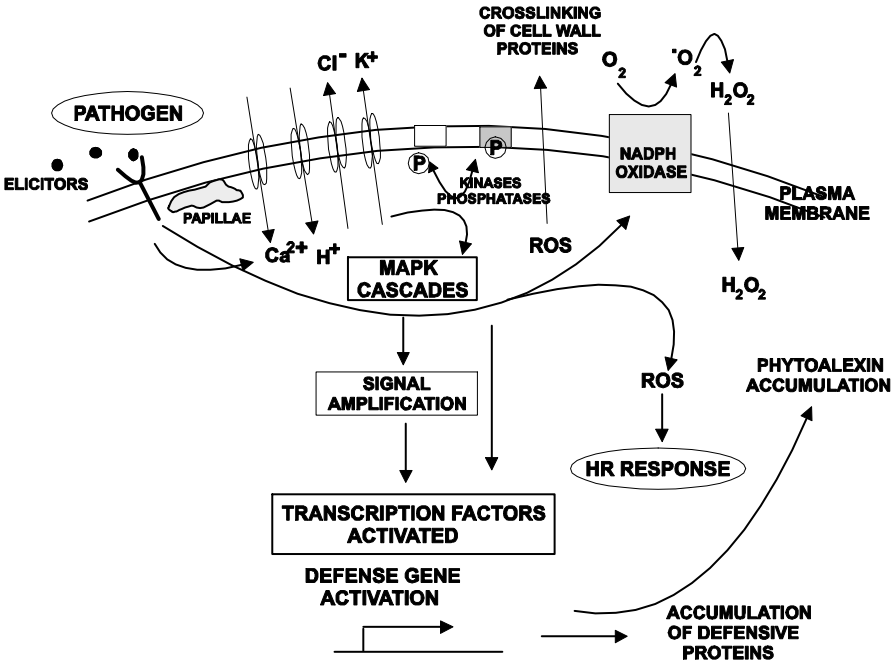

Figure 1. *Plant responses induced by the recognition of a pathogen (adapted from Buchanan et al. 2002).*

Elicitation of plant defense

General elicitors

Evolutionary ancient innate immunity, the ability to discriminate between self and nonself, is a quality of both animals and plants (Medzhitov and Janeway 2002; Parker 2003). It relies on the detection of pathogen-associated molecular patterns (PAMPs) characteristic of a whole class of potentially harmful microbial organisms (Heath 2000; Nürnberger and Brunner 2002; Nürnberger et al. 2004). Pathogen-derived molecules of diverse nature,

including lipopolysaccharides (LPS), flagellins, glucans, and chitins, serve as general elicitors that trigger basal defense responses independently of the genotype of the individual pathogen (Figure 2). For example, flagellin, the protein subunit of the bacterial surface structure flagellum, acts as a PAMP in both animals and plants (Felix et al. 1999; Gomez-Gomez and Boller 2002; Smith et al. 2003). General elicitors are usually molecules that are indispensable in the lifestyle of microbes and thus provide a fitness penalty for the pathogen if recognized by the plant surveillance system (Nürnberger and Brunner 2002; Nürnberger et al. 2004). Endogenous plant cell wall-derived structures released by the hydrolytic enzyme activities of invading microbes can also act as general elicitors (Benhamou 1996; Nürnberger et al. 2004) (Figure 2).

Race-specific elicitors

During evolution some pathogens have developed to overcome the PAMP-triggered basal resistance by acquiring the ability to deliver effector proteins into plant cells (Espinosa and Alfano 2004; Chisholm et al. 2006) (Figure 2). These effector proteins interfere with, manipulate, or suppress disease signaling, thereby enhancing pathogen growth and disease development (Oh and Collmer 2005; Chisholm et al. 2006; da Cunha et al. 2006; Truman et al. 2006). In response, during co-evolution plants have adapted to detect these specific pathogen-derived molecules. This cultivar-specific, gene-for-gene disease resistance system is determined by pathogen-encoded effector proteins and the corresponding plant-derived R proteins (Hammond-Kosack and Jones 1997; Bonas and Lahaye 2002).

Many Gram-negative bacterial pathogens possess the *hypersensitive response and pathogenicity* (*hrp*) gene cluster that encodes the type III secretion system (TTSS). TTSS is utilized by the bacteria for injection of the effector proteins into plant cells (Feys and Parker 2000; Lahaye and Bonas 2001; Alfano and Collmer 2004) (Figure 2). Chisholm et al. (2006) speculated that the effectors have developed to interfere with the components of PAMP-triggered defense or to promote the pathogenicity of the microorganism by affecting a variety of host proteins (Figure 2). Kim et al. (2005) demonstrated that *P. syringae* effector proteins AvrRpt2 and AvrRpm1 suppress PAMP-triggered defense responses in *Arabidopsis* by inhibiting flagellin-induced accumulation of callose. Moreover, another *P. syringae* effector, AvtPto, suppressed *Arabidopsis* genes encoding secreted cell wall and defense proteins (Hauck et al. 2003). Some avirulence factors act by suppressing HR response (Jamir et al. 2004), which is central in activating certain plant defense responses. Although many effector proteins have been cloned, the biochemical function of most remains unknown. AvrPtoB has been shown to have ubiquitin ligase activity in vivo (Janjusevic et al. 2005; Abramovitch et al. 2006). Deletion of key residues from this protein eliminated ubiquitin ligase activity and the capability of AvrPtoB to inhibit cell death. Thus, this effector was suggested to act by targeting proteins responsible for regulation of programmed cell death to degradation mimiking ubiquitin ligase of the host (Janjusevic et al. 2005; Chisholm et al. 2006).

However, if the effector protein meets a matching *R* gene in the plant, it becomes a specific elicitor and the plant defense system is activated by the R protein (Figure 2). Several *R* genes confer specific resistance to fungal, viral, or bacterial pathogens carrying

the matching effector gene (Staskawicz et al. 2001; Bonas and Lahaye 2002). Resistance is manifested by HR response, one of the most prominent features of gene-for-gene resistance, and inhibition of pathogen growth (Feys and Parker 2000; Bonas and Lahaye 2002). The oxidative bursts in the tissues undergoing HR response also appear to be important in propagating systemic defense signals (Oh and Collmer 2005; Truman et al. 2006).

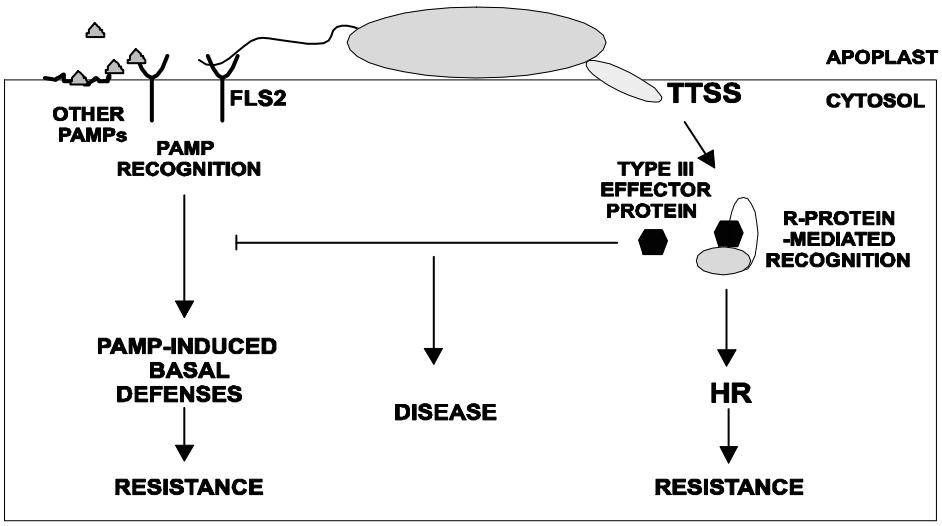

Figure 2. *General and specific elicitors of plant defense (modified from Abramovitch et al. 2004 and da Cunha et al. 2006).*

Elicitor perception

Receptors functioning in pathogen surveillance are located either at the plant cell surface or inside the cell, and they rapidly activate defense signaling pathways following infection (Dardick and Ronald 2006). Given the vast array of different elicitors, the identification of receptors is a major challenge. Several types of putative receptors have been identified in plants, including receptor-like kinases (RLKs), which form a large family of over 400 members in *Arabidopsis* (Johnson and Ingram 2005). RLKs are implicated in all aspects of plant biology, from early embryogenesis to disease resistance. They are composed of an extracellular domain, a single transmembrane-spanning region, and a cytoplasmic part containing a conserved kinase domain, as well as other more variable segments (Johnson and Ingram 2005). The expression patterns exhibited by certain RLKs in response to elicitor, pathogen, or signal molecule treatment have also been associated with pathogen responses (Montesano et al. 2003). The best-characterized example of a RLK interacting with a microbial elicitor is the PAMP receptor, the leucine-rich repeat (LRR)-containing FLAGELLIN SENSITIVE 2 (FLS2), which is essential for flagellin perception in *Arabidopsis* (Gómez-Gómez et al. 1999; Gómez-Gómez and Boller 2002; Robatzek et al. 2006). Other RLKs implicated in plant defense responses to pathogens include members

of the WALL-ASSOCIATED KINASE (WAK) family of RLKs (He et al. 1998). Interestingly, some RLKs have been identified as *R* gene products, e.g. Xa21 (an LRR-type of RLK) that confers resistance against strains of the bacterial pathogen *X. oryzae* pv. *oryzae* that carry AvrXa21 activity (Song et al. 1995).

The classical receptor-ligand model for gene-for-gene resistance suggests that effector proteins act as ligands to bind and activate a matching *R* gene-encoded receptor, which then results in resistance (Bonas and Lahaye 2002; Hammond-Kosack and Parker 2003). Despite the wide array of pathogens, isolation of *R* genes has revealed that most of them are structurally related. Depending on structure and function, *R* genes have been divided into five classes that encode both cytoplasmic and transmembrane proteins (Agrios 2005). Many R proteins contain a series of LRRs, a nucleotide-binding site (NBS), and an amino-terminal TIR (Toll and IL-1 receptor) or CC (coiled-coil) structure (Feys and Parker 2000; Ellis J. et al. 2002; Holt et al. 2003). For example, among the TIR-NBS-LRR class of cytoplasmic R proteins are RPP5 and RPS4, which confer resistance to oomycete *Peronospora parasitica* and bacterium *P. syringae*, respectively, in *Arabidopsis* (Gassmann et al. 1999; Nöel et al. 1999). RPM1 and RPS2 are of the CC-NBS-LRR-type and render plant a resistant to different *P. syringae* strains that express the corresponding effector genes (Holub 2001). In the elicitation of defense response, different *R* genes employ common downstream elements, such as NDR1 (NONRACE-SPECIFIC DISEASE RESISTANCE 1) and EDS1 (ENHANCED DISEASE SUSCEPTIBILITY 1) of *Arabidopsis* (Aarts et al. 1998).

However, experimental data that support the receptor–ligand model in gene-for-gene resistance are rare, most probably indicating a more complicated interaction requiring additional proteins. This has inspired the guard hypothesis (Dangl and Jones 2001; Bonas and Lahaye 2002; Hammond-Kosack and Parker 2003). This view suggests that the R protein does not interact directly with a pathogen effector but rather with another plant protein (the guardee). The attempt of the pathogen to modify the guardee activates the R protein, and plant resistance is triggered (Dangl and Jones 2001). *Arabidopsis* RIN4 is an example of a guarded protein. Two *P. syringae* effector proteins, AvrRpm1 and AvRpt2, manipulate RIN4, a regulator of PAMP signaling, and thus, interfere with the activation of basal defenses. The RIN4-associated perturbations are sensed by R proteins RPM1 and RPS2 and transduced into defense responses (Mackey et al. 2002; Kim et al. 2005).

Defense signaling

Recognition of a pathogen triggers diverse cellular events in plants (Figure 1). As discussed earlier, several immediate and local responses take place in cells, including changes in ion fluxes and alkalization of the cytoplasm (Wojstaszek 1997; Peck 2003). Many of these events are activated within minutes of pathogen perception. Kinase cascades involving MAPKs and CDPKs (calcium-dependent protein kinases) undergo rapid activation and amplify early responses (Peck 2003; Ludwig et al. 2005). Moreover, pathogen recognition and the early events trigger the production of the endogenous

signaling hormones salicylic acid (SA), jasmonic acid (JA), and ethylene (ET). They operate in two major defense pathways in plants: one dependent on SA and the other dependent on JA and ET, conferring resistance to different pathogens (Thomma et al. 1998). ROS and nitric oxide (NO) also contribute to the transmission of defense signals (Karpinski et al. 2003; Crawford and Guo 2005). In addition, reactive electrophilic species (RES), lipid oxidation products containing α, β-unsaturated carbonyl groups, accumulate during pathogen attack. Together with ROS and NO, these have been suggested to play important roles in the activation of defense genes (Farmer 2001; Alméras et al. 2003).

Upon appropriate stimulation, resistance can also be induced systemically in the noninfected tissues of the plant. Pathogens, soilborne microorganisms, various chemicals, and several forms of stress can enhance the tolerance of the plant to future pathogen attacks. The induced resistance (IR) phenomena are often associated with an enhanced capacity to mobilize cellular defense responses – i.e. the plants expressing IR are "primed" for potentiated induction of defense responses when they encounter a pathogen attack (Conrath et al. 2002; Van Hulten et al. 2006).

The classic form of IR is systemic acquired resistance (SAR) controlled by a signaling pathway that depends on endogenous accumulation of SA (Malamy et al. 1990; Métraux et al. 1990; Uknes et al. 1992; Durrant and Dong 2004). SAR is associated with the accumulation of defense compounds, such as PR proteins, in the uninfected parts of the plant, and it is mainly effective against biotrophic pathogens (Glazebrook 2005). Depending on the stimulus, also JA/ET-dependent IR can be triggered, and it has a different spectrum of effectiveness than SAR. For example, treatment of plants with elicitors of JA/ET signaling induces the systemic accumulation of defense-related proteins and enhances the resistance of the plant against attacks by necrotrophic pathogens (Vidal et al. 1998). In addition, certain strains of nonpathogenic rhizobacteria induce JA/ET-dependent IR, and this is referred to as induced systemic resistance (ISR) (Pieterse et al. 1998). ISR is effective against infection by different types of pathogens such as *P. syringae* pv *tomato* and fungal pathogens *Fusarium oxysporum* and *P. parasitica*. Interestingly, similar to SAR, rhizobacteria-induced ISR is dependent on the regulatory protein NPR1 (NONEXPRESSOR OF PR1) (Pieterse et al. 1998). Moreover, another type of systemic resistance is induced upon application of β-aminobutyric acid (BABA) (Jakab et al. 2001; Ton et al. 2005). BABA-induced resistance (BABA-IR) is effective against biotrophic and necrotrophic pathogens as well as abiotic stress. Depending on the stress, it involves either SA or ABA signaling (Ton and Mauch-Mani 2004; Ton et al. 2005). Rhizobacteria-triggered ISR and BABA-IR are not associated with direct activation of defense-related genes (Pieterse et al. 1998; Jakab et al. 2001), and therefore, they involve considerably lower fitness costs (i.e. reduction of growth or reproduction) than directly induced defense (Van Hulten et al. 2006).

Induction of systemic resistance seems to be a fairly common phenomenon in plants. Besides the types mentioned above, enhanced resistance to pathogens can also be induced by treatment with many natural compounds or chemicals as well as by wounding caused by insects or certain pathogens (Fought and Kuc 1996; Schilmiller and Howe 2005).

Role of reactive oxygen species in defense signaling

In plants, normal, unstressed photosynthetic and respiratory metabolism taking place in chloroplasts and mitochondria results in endogenous generation of such ROS as superoxide radical ($O_2^{-\cdot}$), hydroxyl radical (OH^-), and hydrogen peroxide (H_2O_2) (Grene 2002). ROS is also generated by cytoplasmic, membrane-bound, or exocellular enzymes involved in redox reactions (Foyer et al. 1994; Wojtaszek 1997). To avoid potential damage, plant cells contain several enzymatic and nonenzymatic antioxidant scavenging systems that take care of ROS detoxification. These include ascorbate peroxidases (APXs), superoxide dismutases (SODs) and catalases (CATs) as well as such antioxidants as ascorbic acid and glutathione (Noctor and Foyer 1998; Mittler 2002). Under unstressed conditions, the formation and scavenging of ROS are in balance. However, several forms of biotic and abiotic stress, such as pathogen invasion, excess light energy, dehydration, and low temperature, increase the generation of ROS. This can result in cellular damage, manifested in inactivation of enzymes or cell death, if the amount of ROS generated exceeds the capacity of the scavenging systems (Foyer et al. 1994; Bartosz 1997; Dat et al. 2000; Grene 2002).

Although potentially damaging, ROS has been shown to promote plant resistance to pathogens in several ways. During defense responses, ROS is produced by plasma membrane-bound NADPH oxidases and cell wall-bound peroxidases and amine oxidases in the apoplast (Mahalingam and Fedoroff 2003; Laloi et al. 2004) (Figure 3). One of the earliest pathogen-induced defense responses is the oxidative burst, a rapid and transient production of large amounts of ROS at the site of attempted invasion (Doke 1983; Wojtaszek 1997). A likely source for this apoplastic $O^{2-\cdot}$ generation is a NADPH oxidase homologous to that of activated mammalian phagocytes and neutrophils (gp91phox) (Keller et al. 1998; Overmyer et al. 2003; Laloi et al. 2004) (Figure 3). *AtRBOHD* and *AtRBOHF* genes encoding NADPH oxidase in *Arabidopsis* are required for full ROS generation during bacterial and fungal challenge (Torres et al. 2002). Hydrogen peroxide is also produced in vitro by some peroxidase isoforms at an alkaline pH. Since the apoplast is alkaline following pathogen recognition, peroxidases have been suggested to contribute to the oxidative burst (Bolwell et al. 1995; Wojtaszek 1997; Grene 2002). The accumulation of extracellular hydrogen peroxide induced by pathogen challenge has been proposed to crosslink the cell wall proteins, thus strengthening the wall (Neill et al. 2001). The oxidative burst can be directly harmful to invading pathogens but it also contributes to cell death: ROS generated via the oxidative burst play a central role in the development of host cell death during the HR reaction (Lamb and Dixon 1997; Grant and Loake 2000).

Importantly, ROS is thought to have potential for being a signal in plant defense responses (Mullineaux et al. 2000; Neill et al. 2001). Hydrogen peroxide is a relatively stable form of ROS and has the ability to diffuse across membranes and reach locations far from the site of its original generation (Wojtaszek 1997). Increased ROS generation enhances the accumulation of SA as well as the transcripts of *PR* genes (Chen et al. 1995; Van Camp et al. 1998; Maleck and Dietrich 1999). Furthermore, SA has been shown to have inhibitory effects on CAT and APX activities, which may lead to accumulation of hydrogen peroxide, free radicals, and other ROS (Chen et al. 1993; Durner and Klessig

1995). SA has also been suggested to potentiate the production of NADPH oxidase-dependent $O_2^{-\cdot}$ via a positive feedback loop (Van Camp et al. 1998).

Photo-produced hydrogen peroxide and other ROS in the cell also participate in controlling biotic and abiotic stress responses (Karpinski et al. 2003), and recently, mechanisms for plant defense against pathogens were linked to the light-sensing network. For example, induction of *PR1* by SA and its functional analogs was found to correlate strictly with the activity of the signaling pathway controlled by PHYA and PHYB photoreceptors (Genoud et al. 2002). Moreover, the growth of avirulent *P. syringae* pv. *tomato* was enhanced in *Arabidopsis phyA* and *phyB* mutants (Genoud et al. 2002).

Plant responses to pathogens seem to share common elements with responses to excess light (Karpinski et al. 2003). A rapid increase in ROS concentration, depletion of antioxidant pools, chlorosis and necrosis of leaves, local and systemic defense responses, and induction of defense gene expression are markers of both responses (Karpinski et al. 2003). However, while the ROS burst during pathogen infection is considered to originate mainly from cytoplasmic NADPH oxidase, during excess light stress ROS is produced in the chloroplast and peroxisome (Karpinski et al. 2003) (Figure 3). High light also induces the accumulation of SA, a central hormone in pathogen defense; Karpinski and coworkers (2003) demonstrated that high-light-acclimated plants had several-fold greater foliar SA than plants cultivated in low light.

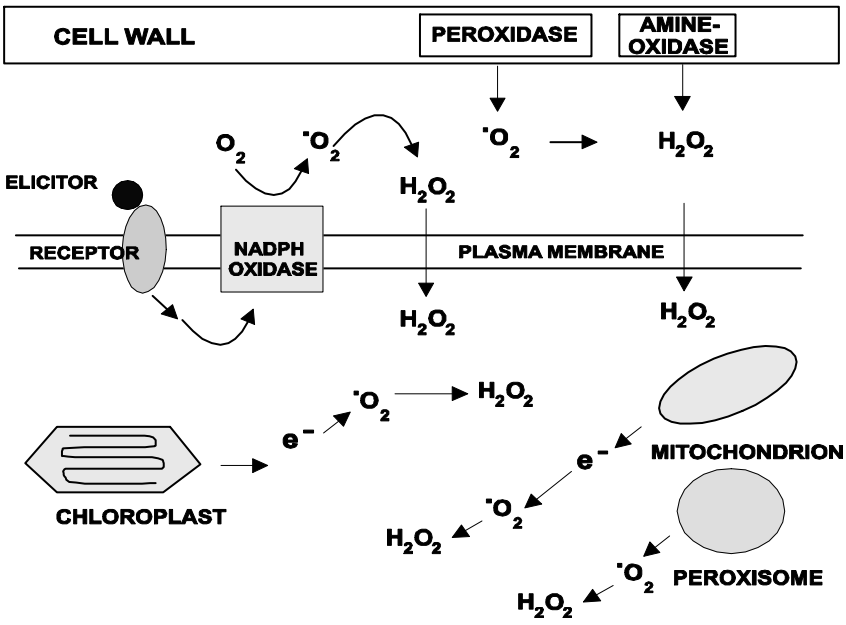

Figure 3. *Stress-triggered formation of reactive oxygen species (ROS) in the plant cell (adapted from Mahalingam and Fedoroff 2003).*

Role of nitric oxide in defense signaling

Nitric oxide (NO) was first identified as an important messenger in animal cells (Mayr and Hemmes 1997). However, it is becoming increasingly clear that it has diverse signaling functions in plants as well (Wendehenne et al. 2004; Mur et al. 2006). Besides developmental regulation and promotion of germination, NO is an important mediator in plant defense signaling (Wendehenne et al. 2004; Delledonne 2005). In animals, the NO burst is a hallmark of innate immunity response, and also in *Arabidopsis* recognition of bacterial LPS induces a rapid burst of NO (Zeidler et al. 2004). LPS from animal and plant pathogens were shown to induce NO synthase *AtNOS1* as well as activate several defense genes (Zeidler et al. 2004). Zeidler et al. (2004) also demonstrated the essential role of NO in basal resistance; *AtNOS1* mutants were more susceptible to virulent *P. syringae* pv. *tomato* than wild-type plants. Besides contributing to the local and systemic induction of defense genes, NO can also trigger cell death, and thus, it has been suggested to play an important role as an intercellular signal contributing to spread of HR (Romero-Puertas et al. 2004; Tada et al. 2004; Zeidler et al. 2004).

Salicylic acid-mediated defense signaling

The phytohormone salicylic acid (SA) has long been known to play a central role in plant defense signaling. SA levels increase in response to pathogen attack at the site of infection, and this is essential in resistance against various pathogens (Glazebrook 2005). Moreover, exogenous application of SA protects plants against pathogens and induces the expression of defense-related genes (Van Loon et al. 1997). SA is required also in the establishment of systemic acquired resistance (SAR). SAR is an induced state of resistance that is manifested throughout the plant in response to pathogen-triggered localized necrosis (Malamy et al. 1990; Métraux et al. 1990; Uknes et al. 1993; Durrant and Dong 2004). It can last from weeks to even months and is effective against a wide variety of normally virulent pathogens, including viruses, bacteria, fungi, and oomycetes (Thomma et al. 2001; Durrant and Dong 2004). The induction of SA signaling and SAR is associated with accumulation of such PR proteins as beta-1,3-glucanases, thaumatin-like proteins, chitinases, and PR1, which are thought to contribute to resistance (Van Loon 1997). Many of the PR proteins have antimicrobial activity in vitro, but their roles in the establishment of SAR are unclear. Nevertheless, they serve as molecular markers for the onset of the defense response (Van Loon 1997; Durrant and Dong 2004).

SA-mediated defense signaling and SAR are often induced by infection with avirulent pathogens that trigger gene-for-gene resistance and HR, but also in response to necrotizing cell death-causing pathogens (Glazebrook et al. 1997; Durrant and Dong 2004; Glazebrook 2005). However, while virulent pathogens do not usually trigger HR, they can induce SA signaling as part of the basal defense response to contain their growth (Glazebrook et al. 1997). SA-dependent defense responses are considered effective mainly against biotrophic pathogens that feed on living tissues, such as the oomycete *P. parasitica*, the fungus *Erysiphe orontii*, and the bacterium *P. syringae* (Glazebrook 2005). Accordingly, impaired SA production leads to increased susceptibility to various pathogens. For example, SA production is significantly reduced in *sid2* (*SA induction-*

deficient) plants, resulting in increased susceptibility to both virulent and avirulent forms of *P. syringae* and *P. parasitica* (Nawrath and Métraux 1999). *SID2* encodes isochorismate synthase (ICS1), and the drastic reduction in the accumulation of SA in the *sid2* mutant indicates that the majority of this hormone in *Arabidopsis* is produced via isochorismate (Wildermuth et al. 2001) rather than via the shikimate-phenylalanine pathway, as earlier presumed (Lee et al. 1995).

EDS1 and PHYTOALEXIN-DEFICIENT 4 (PAD4) are important activators of SA signaling (Aarts et al. 1998; Wiemer et al. 2005). These proteins are essential for basal resistance against virulent pathogens, but they are also needed in mediating cultivar-specific resistance activated by R proteins (Feys et al. 2001). For instance, *eds1* mutant has defects in basal defense to virulent isolates of *Erysiphe* sp. and *P. syringae*, and it is also impaired in specific resistance to certain strains of *P. parasitica* (Parker et al. 1996; Glazebrook et al. 1997). EDS1 and PAD4 interact in vivo and are induced by both pathogen infection and SA application, suggesting that they act upstream of SA production (Aarts et al. 1998; Feys et al. 2001). SA also contributes to the expression of both EDS1 and PAD4 as part of a positive feedback loop that seems to be important in defense signal amplification (Feys et al. 2001; Wiemer et al. 2005) (Figure 4). Several *R* gene products require the *NDR1* gene in establishing resistance after inoculation with certain avirulent pathogens (Century et al. 1995; Aarts et al. 1998). EDS5, a member of the multidrug and toxin extrusion (MATE) transporter family, is also required for pathogen-induced SA accumulation downstream of EDS1 and PAD4 (Nawrath et al. 2002). In addition, ROS forms an amplification loop with SA; it enhances the SA signal (Shirasu et al. 1997; Durrant and Dong 2004) and SA then inhibits hydrogen peroxide-scavenging enzymes CAT and APX, enhancing ROS accumulation (Durrant and Dong 2004) (Figure 4).

The first studies highlighting the importance of SA in defense signaling employed transgenic *Arabidopsis* plants expressing the bacterial SA-degrading enzyme salicylate hydroxylase (*NahG*), which converts SA to catechol (Gaffney et al. 1993; Delaney et al. 1994). *NahG* plants display enhanced susceptibility to several fungal, bacterial, oomycete and viral pathogens, interpreted to result from the lack of SA (Gaffney et al. 1993; Delaney et al. 1994). However, recent studies comparing *NahG* plants with SA-deficient mutants indicate that the observed disease susceptibility phenotype might partly arise from the SA degradation product catechol rather than the lack of SA itself (Heck et al. 2003; Van Wees and Glazebrook 2003). Treatment of *NahG* plants with catalase seems to reverse the susceptibility to *P. syringae* pv. *phaseolicola*. This suggests that the accumulation of catechol might trigger increased production of hydrogen peroxide, interfering with the true effects of the lack of SA (Van Wees and Glazebrook 2003).

Mutant screens aimed at finding components involved in SA signal transduction identified multiple alleles of a single gene: *NPR1/NIM1/SAI1* (*NONEXPRESSOR OF PR1*, Cao et al. 1994; *NON-INDUCIBLE IMMUNITY 1*, Delaney et al. 1995; *SA-INSENSITIVE 1*, Shah et al. 1997) (Figure 4). *NPR1* encodes an ankyrin repeat-containing protein that plays a central role in SA signal transduction. In mammalian systems, the NPR1 homolog IκB is involved in the repression of immune and inflammatory responses (Cao et al. 1997; Ryals et al. 1997). *npr1* mutant plants accumulate SA in response to pathogen challenge,

but are unable to induce SAR-marker genes. They also display enhanced susceptibility to virulent pathogens and are impaired in some *R* gene-mediated resistance responses (Cao et al. 1994; Delaney et al. 1995; Glazebrook et al. 1996). Overexpression of *NPR1* does not result in constitutive *PR* gene expression, but does enhance resistance to *P. parasitica*, *P. syringae*, and *E. cichoracearum* (Cao et al. 1998; Friedrich et al. 2001). This indicates that NPR1 needs to be activated for SAR induction even if it is expressed at high levels (Cao et al. 1998; Durrant and Dong 2004). Indeed, in an uninduced state, NPR1 resides in the cytosol as an oligomer. Accumulation of SA induces a redox change, reducing NPR1 to a monomeric, active form that is localized to the nucleus. There it activates the expression of *PR* genes through interaction with TGA transcription factors (Després et al. 2003; Mou et al. 2003; Spoel et al. 2003) (Figure 4).

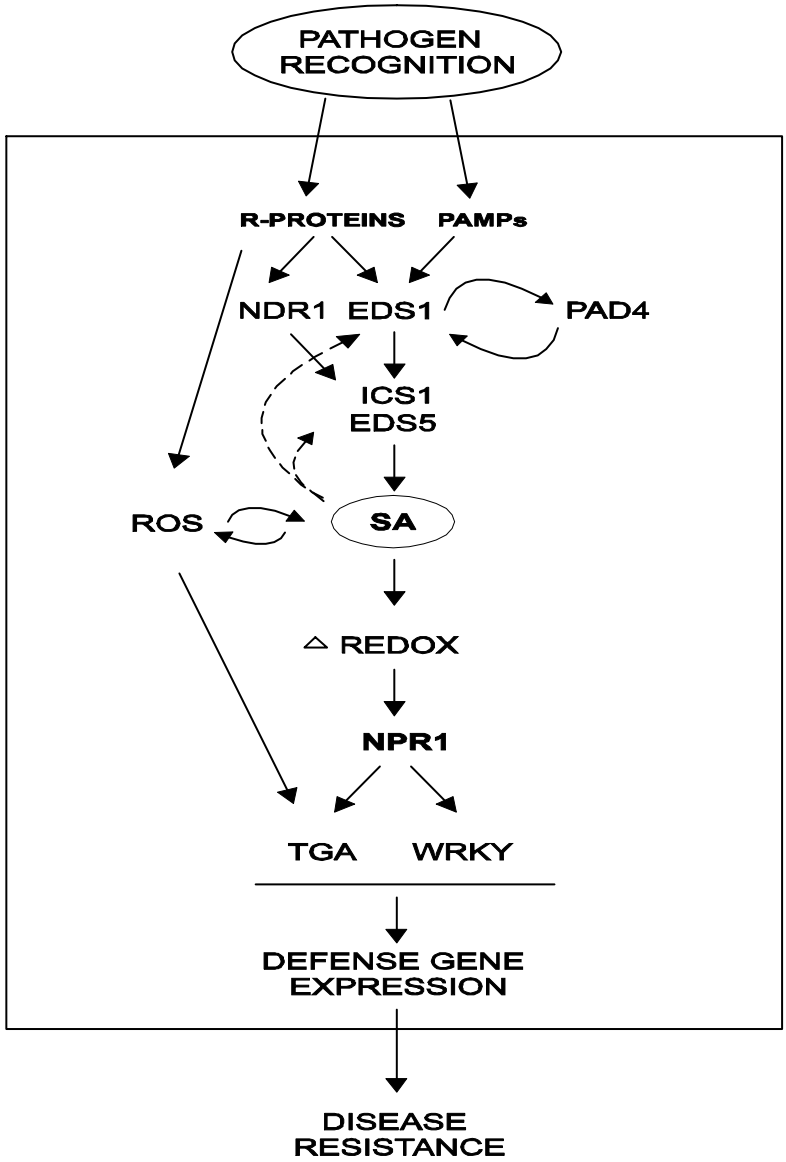

Figure 4. *Sequence of events from pathogen recognition to gene induction in defense signaling involving salicylic acid (modified from Durrant and Dong 2004).*

Characterization of various lesion mimic mutants from *Arabidopsis* highlights the role of cell death in the induction of SA-dependent defenses and SAR. Mutants such as *accelerated cell death* (*acd2)* and *lesions stimulating disease resistance* (*lsd1-7)* develop lesions due to light-induced (*acd2*) and spontaneous cell death (Weymann et al. 1995; Dietrich et al. 1997; Mach et al. 2001; Yao and Greenberg 2006). The common phenotype of these lesion mimic mutants includes an elevated level of SA, constitutive expression of *PR* genes, and enhanced resistance to virulent pathogens (Durrant and Dong 2004).

Nature of the systemic signal

SA has long been recognized as essential to the establishment of SAR; it accumulates in infected tissues in concert with the induction of *PR* genes and resistance (Malamy et al. 1990; Métraux et al. 1990; Uknes et al. 1993; Durrant and Dong 2004). SA was originally proposed as the putative signaling molecule mediating the induction of SAR based on the results obtained with cucumber (*Cucumis sativus*) (Métraux et al. 1990) and tobacco (*Nicotiana tabacum*) (Malamy et al. 1990; Malamy and Klessig 1992). Using *Arabidopsis* plants, Shulaev and coworkers (1995) showed that ^{18}O-labeled SA is transported from pathogen-inoculated leaves of tobacco to systemic, noninoculated leaves, indicating that SA itself is the signal. SA was also suggested to be converted to volatile methyl salicylate (MeSA), which could induce resistance not only in distal tissues of the infected plant but also in neighboring plants (Shulaev et al. 1997).

Evidence arguing against SA as the mobile signal also exists. When a scion from wild-type tobacco was grafted to a pathogen-inoculated rootstock of a plant expressing the SA hydroxylase *NahG* gene, and hence, unable to accumulate SA, the SAR signal was still transmitted to the wild-type plant (Vernooij et al. 1994). However, the authors showed that SA was needed in receiving the SAR signal since *NahG* scions grafted to wild-type rootstock were unable to establish SAR after the inoculation of the rootstock (Vernooij et al. 1994). Also, detachment of leaves from *P. syringae*-infected plants before SA levels rose did not block SAR development (Rasmussen et al. 1991). In addition, high SA concentrations have been detected in other plant species, such as potato and rice, under noninducing conditions (Coquoz et al. 1995; Silverman et al. 1995).

Recent work suggests that the mobile SAR signal may be a lipid-based molecule. *DIR1* encodes a putative apoplastic lipid transfer protein, and *dir1-1* (*defective in induced resistance 1-1*) plants exhibit wild-type local resistance to virulent *P. syringae*, but fail to develop SAR in systemic, uninoculated tissues (Maldonado et al. 2002). The phloem sap from *dir1* is deficient in the mobile signal, but the plants were able to establish SAR in response to sap of wild-type plants. This indicates that DIR1 might function in the transmission of the signal (Maldonado et al. 2002). Tobacco SA-BINDING PROTEIN 2 (SABP2) is also a lipase (Du and Klessig 1997; Kumar and Klessig 2003), and silencing of this gene diminished both local resistance and SAR (Kumar and Klessig 2003). In

addition, both EDS1 and PAD4 have homology to lipase-like proteins (Wiemer et al. 2005).

Jasmonic acid-mediated defense signaling

Certain oxygenated fatty acids, oxylipins, have key roles as regulators of different plant responses (Farmer et al. 2003). Interestingly, these lipid-derived molecules have biological activities that resemble some of the roles of well-known mediators in animals, most notably, prostaglandins, which are involved in inflammatory responses (Thoma et al. 2004). Jasmonates, especially phytohormone jasmonic acid (JA) and its methyl ester, methyl jasmonate (MeJA), regulate developmental processes, including embryogenesis, pollen and seed development, and root growth (Creelman and Mullet 1997; Farmer et al. 2003; Liechti et al. 2006). Moreover, JAs also mediate resistance to insects, microbial pathogens, and abiotic stress responses to wounding and ozone (Creelman and Mullet 1997; Reymond and Farmer 1998; Norman-Setterblad et al. 2000; Overmyer et al. 2000). However, while JA is a terminal product of the octadecanoid pathway, it is not the only one with biological activity. Recent studies suggest that a cyclopentenone precursor of JA, 12-oxo-phytodienoic acid (OPDA), can also induce defense gene expression (Farmer et al. 2003).

Arabidopsis mutants impaired in the synthesis (*fad3/7/8*) or perception (*coi1)* of JA exhibit enhanced susceptibility to a variety of pathogens, including the fungi *Alternaria brassicicola*, *Botrytis cinerea*, and *Pythium* sp., and the bacterium *E. carotovora* (Thomma et al. 1998, 2001; Norman-Setterblad et al. 2000). These pathogens have a common virulence strategy; they kill plant cells to obtain nutrients. Although JA responses are generally considered effective in defense against necrotrophic pathogens (Turner et al. 2002; Farmer et al. 2003), in some cases JA seems to contribute to plant resistance against biotrophs as well. For example, *Arabidopsis constitutive expression of vsp1* (*cev1*) mutant exhibits constitutive JA signaling and enhanced defenses against fungus *E. cichoracearum* and bacterium *P. syringae* pv. *maculicola* (Ellis C et al. 2002).

JA can be metabolized by a variety of routes, including methylation to MeJA and conjugation to amino acids (Liechti et al. 2006). A recent study demonstrates that JASMONIC ACID RESISTANT 1 (JAR1) is a JA-amino acid synthetase conjugating JA to isoleucine (Ile) (Staswick and Tiryaki 2004). *jar1* plants exhibit decreased sensitivity to exogenous JA, are susceptible to certain pathogens, and are unable to exhibit rhizobacteria-induced ISR (Pieterse et al. 1998; Staswick et al. 1998). They also have altered response to ozone (Overmyer et al. 2000). However, *jar1* plants are not male-sterile, suggesting that the activity of JAR1 is required for optimal JA signaling in some but not all responses in *Arabidopsis* (Staswick and Tiryaki 2004).

The perception and subsequent signal transduction of JA remain unclear. A receptor for JA has not yet been characterized (Liechti et al. 2006). However, a central element of the JA signaling pathway seems to be the COI1 (CORONATINE INSENSITIVE 1) protein (Feys et al. 1994; Xie et al. 1998). *coi1* mutants of *Arabidopsis* are male-sterile, fail to express JA-regulated genes, and are susceptible to pathogens (Thomma et al. 1998). COI1 is an F-box protein that forms an active SCF^{COI1} complex, which together with the

COP9 signalosome (CSN) plays an essential role in JA signaling (Devoto et al. 2002; Xu et al. 2002) (Figure 5). This machinery functions in vivo as an ubiquitin ligase complex that removes repression from JA-responsive defense genes. It is thought to target regulatory proteins, including transcriptional repressors, to ubiquitin-proteasome-mediated protein-degradation (Devoto et al. 2002; Xu et al. 2002; Feng et al. 2003). Feng et al. (2003) demonstrated that, like the *coi1* mutant, plants with reduced CSN function exhibit a JA-insensitive root elongation phenotype and an absence of specific JA-induced gene expression. Interestingly, the recently characterized auxin receptor TIR1 is an F-box protein that, like COI1, forms an ubiquitin protein ligase SCF^{TIR} complex (Dharmasiri et al. 2005). Thus, it is tempting to speculate that, similarly to TIR1, COI1 could act as a receptor for JA.

The production of JA eventually leads to the induction of many genes, including *VEGETATIVE STORAGE PROTEIN* (*VSP)* and *THIONIN 2.1* (*THI2.1*), used as markers for JA-dependent defense responses (Berger et al. 1995; Epple et al. 1995; Penninckx et al. 1998; Devoto and Turner 2003). Moreover, transcription of genes that regulate JA synthesis, e.g. *DAD1*, *LOX2*, *AOS*, and *OPR3*, is induced by JA (Devoto and Turner 2003). Some defense-related genes, such as *PLANT DEFENSIN 1.2* (*PDF1.2*), HEVEIN-LIKE PROTEIN (*HEL*), and *BASIC CHITINASE* (*CHIB*), are induced cooperatively by JA and ET in *Arabidopsis* (Penninckx et al. 1998; Norman-Setterblad et al. 2000) (Figure 5). Conserved MYC transcription factors are involved in JA signaling in both *Arabidopsis* and tomato (Boter et al. 2004; Lorenzo et al. 2004). *JASMONATE INSENSITIVE 1* (*JIN1*) encodes AtMYC2, a nuclear-localized basic helix-loop-helix-leucine zipper transcription factor whose expression is rapidly upregulated by JA in a COI1–dependent manner (Lorenzo et al. 2004) (Figure 5). AtMYC2 seems to differentially regulate the expression of two groups of JA-induced genes. Mutation in this locus prevents the activation of *VSP*, which is involved in JA-mediated plant responses to insects, herbivores, and mechanical damage. At the same time, the expression of JA-induced genes involved in pathogen defense is enhanced, and accordingly, *jin1/AtMYC2* mutant plants show enhanced resistance to the necrotrophic fungi *B. cinerea* and *Plectosphaerella cucumerina* (Lorenzo et al. 2004).

Jasmonic acid in systemic signaling

Plants have evolved to respond with sophisticated mechanisms to attack by herbivores and certain pathogens that rapidly destroy plant tissues. Wounding induces the expression of defensive foliar compounds that have toxic effects on the invader. In addition, plants under attack can also emit volatile substances that act indirectly by attracting predators of the herbivore (Schilmiller and Howe 2005; Wasternack et al. 2006). Importantly, signaling originating from the initial wound site induces systemic resistance in undamaged leaves located considerable distances away and protects the plant against a broad spectrum of future attackers (Howe 2004). Wound response has most been studied in tomato and other *Solanaceae* species, where it results in both local and systemic expression of defensive proteinase inhibitors (PIs) that act by blocking digestive proteases in the herbivore gut (Pearce et al. 1991). Many structurally different molecules play regulatory roles in wound

signaling and PI induction. These include cell wall-derived oligogalacturonides (OGAs), the oligopeptide systemin, and molecules with hormonal activity such as JA, ET, and ABA (León et al. 2001).

Gaps still exist in understanding the transmission of the systemic wound signal. The early events acting upstream of the octadecanoid pathway that couple tissue damage to the production of a primary wound signal are unknown. Nor is it clear how the wound response is transmitted from local to systemic tissues. JA with its volatile derivative MeJA and the oligopeptide systemin are considered central in mediating the long-distance signal (Bostock 2005; Ryan and Moura 2002; Schilmiller and Howe 2005). Recent studies suggest a central role for JAs; using different mutants of tomato, Li et al. (2002) demonstrated that mutations affecting either JA biosynthesis or JA signaling abolish the systemic expression of PI genes. Moreover, the requirement of JA biosynthesis at the site of wounding and the ability to perceive JA at remote tissues was shown in grafting experiments conducted with various tomato mutants (Li et al. 2002, 2005; Ryan and Moura 2002). Possible gene products involved in the transport of JA have not been characterized to date. Alternatively, JA could regulate the production of the actual signal (Li et al. 2005; Schilmiller and Howe 2005).

Wounding induces the production of systemin, which regulates the activation of over 20 defensive genes in response to herbivore and pathogen attack (Pearce et al. 1991; Ryan 2000). This 18-amino acid (aa) peptide is derived by proteolytic cleavage from a larger, 200-aa precursor protein called prosystemin (Ryan and Moura 2002). Systemin released from the primary wound site promotes *PI* gene expression and contributes to the long-distance defense response by activating and amplifying JA production in vascular tissues (Schilmiller and Howe 2005). Systemin binds to SR160, a cell-surface receptor homologous to brassinolide receptor BRI1 from *Arabidopsis* (Li and Chory 1997; Scheer et al. 2002). Interestingly, the existence and function of systemin or a related peptide have thus far been documented only in *Solanaceae* species (Ryan and Moura 2002).

Ethylene-mediated defense signaling

Ethylene (ET) is a gaseous plant hormone involved in various physiological processes, including seed germination, organ senescence, leaf abscission, fruit ripening, and morphological responses of organs (Bleecker and Kende 2000). ET also regulates plant responses to abiotic stresses, including those induced by flooding or drought, and to biotic stresses, such as pathogen attack (Penninckx et al. 1998; O'Donnell et al. 2003).

The production of ET is one of the earliest plant responses to pathogens. Diverse viral, bacterial, and fungal microbes trigger accumulation of ET, leading to induction of defense genes, such as basic *PR1*, basic *β-1,3-GLUCANASE*, and *CHIB*, which can also be induced by ET-independent pathways (Deikman 1997; Thomma et al. 1998). ET contributes to resistance in some interactions but can promote disease development in others (Thomma et al. 1998, 1999; Hoffman et al. 1999; Norman-Setterblad et al. 2000). *Arabidopsis ethylene-insensitive 2* (*ein2)* plants display enhanced susceptibility to *B. cinerea* and *E. carotovora* (Thomma et al. 1999; Norman-Setterblad et al. 2000). On the other hand, infection of *ein2* with virulent *P. syringae* and *X. campestris* resulted in

reduced disease symptoms (Bent et al. 1992). Insensitivity to ET has also been shown to reduce foliar disease development in tomato (Lund et al. 1998). The *never ripe* (*Nr*) tomato mutant impaired in ET perception displayed decreased disease symptoms in comparison with the wild-type after inoculations with *Pseudomonas, Xanthomonas,* and *Fusarium* pathogens (Lund et al. 1998). ET-insensitive tobacco was susceptible to the fungus *Pythium sylvaticum*, which normally is not pathogenic to this species (Knoester et al. 1998). The inability of ET response mutant *etr1* (*ethylene-resistant 1)* to develop pathogen resistance in response to nonpathogenic rhizobacteria demonstrated the requirement of ET in the establishment of ISR (Pieterse et al. 1998).

Characterization of ET-response mutants in *Arabidopsis* has identified components of the ET signal transduction pathway (Figure 5). One class of mutations, exemplified by *etr1*, led to the identification of ET receptors (Hua et al. 1998; Bleecker 1999). CTR1, which acts directly downstream of the ET receptors, is similar to the mitogen-activated protein kinase kinase kinases (MAPKKKs). This suggests that this signaling pathway might contain a MAP kinase cascade, but providing evidence supporting this possibility has proven tricky (Chang 2003; Ecker 2004). A role for MPK6 was thought to exist in ET signaling (Ouaked et al. 2003), altough recent evidence indicates that it instead functions as a key regulator of stress-responsive ET biosynthesis (Liu and Zhang 2004). EIN2 is a transmembrane protein required for ET signaling. While the role of this gene remains unclear, genetic studies locate it between CTR1 and EIN3 (Bleecker and Kende 2000). Some of the mutations affecting ET signal transduction have identified transcription factors such as the ERF1 protein. It is induced by the EIN3/EIL transcription factors, indicating that ethylene signaling involves a transcriptional cascade (Solano et al. 1998). ERF1 belongs to a family of ET response element binding factor (ERF) proteins (also known as ethylene response element binding proteins (EREBPs)) that are transcription factors unique to plants (Fujimoto et al. 2000). ERFs bind to a GCC box found in the promoters of several pathogenesis-related genes, including *β-1,3-GLUCANASE, CHIB*, and *PDF1.2* (Ohme-Takagi and Shinshi 1995; Solano et al. 1998; Wang et al. 2002) (Figure 5).

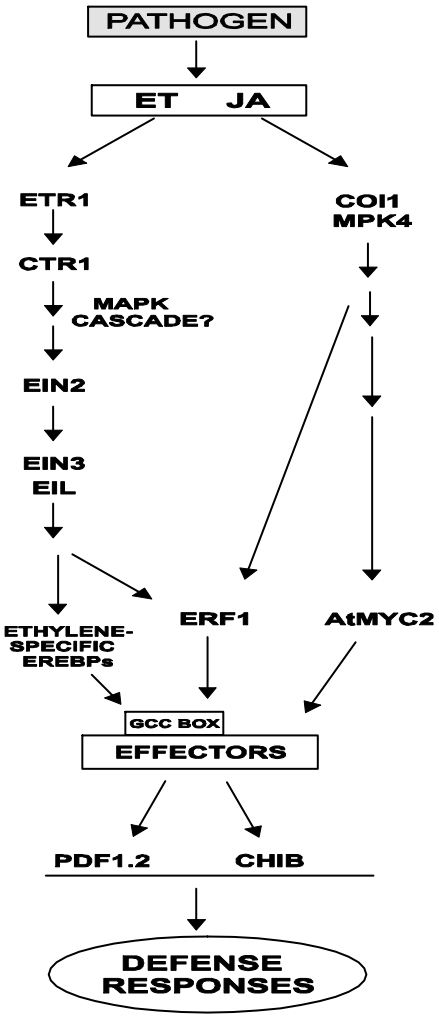

Figure 5. *JA and ET act together to activate production of defensive proteins such as* PDF1.2. *and* CHIB *(adapted from Glazebrook 2001 and Guo and Ecker 2004).*

Abscisic acid

The role of abscisic acid (ABA) in abiotic stress signaling has long been recognized, but, interestingly, the importance of this phytohormone in biotic stress responses is only beginning to be acknowledged (Mauch-Mani and Mauch 2005). Various studies elucidating ABA-mediated signal transduction have identified many of the cellular components that regulate or modulate ABA responses (Yamaguchi-Shinozaki and Shinozaki 2005, 2006). However, an unanticipated complexity has also been revealed (Finkelstein and Rock 2002; Himmelbach et al. 2003; Shinozaki et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2005, 2006). Transcriptome analyses have identified more than one thousand genes differentially regulated by ABA. Recently, the role of posttranscriptional regulation in ABA signal transduction has clearly emerged (Himmelbach et al. 2003; Kuhn and Schroeder 2003).

Role of ABA in abiotic stress responses

ABA is a carotenoid-derived hormone that has a wide range of essential functions in plant growth and development, including promotion of seed maturation and dormancy as well as inhibition of seed germination (Finkelstein and Gibson 2002). Several studies have shown that ABA is the key mediator in initiating adaptive responses to various abiotic stresses (Verslues and Zhu 2005). The increase of endogenous ABA in plants challenged by osmotic stresses, such as desiccation, acts as a signal to initiate defense responses, including limitation of transpirational water loss by the regulation of stomatal aperture (Zhu 2002). Moreover, ABA controls the expression of many genes leading to production of proteins that act as desiccation protectants (Shinozaki et al. 2003; Verslues and Zhu 2005). Endogenous ABA levels also rise in response to low temperature, which is needed for the establishment of plant freezing tolerance (Heino et al. 1990; Thomashow 1999). Furthermore, the decreased stress tolerance of mutants deficient in ABA synthesis or sensing has demonstrated the central role of ABA in abiotic stress responses (Finkelstein and Rock 2002).

Several components of the ABA signaling pathway have been identified, but the signaling network has proven very complex. Little is known of how the signal is transduced; however, one class of important components seems to be the type 2C protein phosphatases ABI1 and ABI2, which act as negative regulators of ABA responses (Leung et al. 1997; Gosti et al. 1999). Mutant lines *abi1-1* (*abscisic acid insensitive 1-1*) and *abi2-1*, which display considerable hormone resistance, carry dominant mutations and have constant phosphatase activity. These mutants demonstrate insensitivity to ABA in both germination and vegetative growth (Leung et al. 1997). Interestingly, loss of function for these genes results in only mild hypersensitivity to ABA (Gosti et al. 1999). Protein-protein analyses indicate that ABI1 and ABI2 interact with several cellular targets. Both have been shown to interact with PKS3, a Ser/Thr protein kinase and a global negative regulator of ABA responses (Guo et al. 2002). Other negative regulators of ABA responses include the farnesyl transferase ERA1 (Cutler et al. 1996) and inositol phosphatase FRY1 (Xiong et al. 2001).

Transcriptome analyses have revealed that ABA has a considerable impact on gene expression (Hoth et al. 2002). Promoters of many ABA-responsive, abiotic stress inducible genes, such as *RAB18* (Lång and Palva 1992) and *LTI78* (Nordin et al. 1993) contain an ABRE (ABA-responsive element) sequence that serves as a binding site for transcription factors AREBs (ABRE binding proteins) and ABFs (ABRE binding factors) (reviewed by Yamaguchi-Shinozaki and Shinozaki 2005, 2006) (Figure 6). ABA-dependent phosphorylation is required to activate these transcription factors and consequently the expression of ABRE-containing genes (Shinozaki et al. 2003; Yamaguchi-Shinozaki and Shinozaki 2005). Other cis-acting regulatory elements involved in ABA-dependent gene expression include MYB and MYC recognition sites found in, for example, the promoter of the drought-inducible *RD22* gene (Abe et al. 2003) (Figure 6). AtMYC2 and AtMYB2 transcription factors have been shown to activate the expression of *RD22* by binding the cis-acting elements in its promoter. Interestingly, AtMYC2 was also reported to function in JA-regulated defense responses in *Arabidopsis* (Abe et al. 2003), indicating that this transcription factor could mediate the crosstalk between ABA-

and JA-induced gene expression. Despite the central role of ABA in triggering abiotic stress responses, ABA-independent systems are also involved in the regulation of drought- and/or cold-inducible genes (Yamaguchi-Shinozaki and Shinozaki 2005) (Figure 6).

Many recent studies characterizing mutations behind altered ABA sensitivity suggest a link between posttranscriptional mRNA processing and ABA signal transduction as a means to influence transcript abundance (Himmelbach et al. 2003; Kuhn and Schroeder 2003). The *abh1* (*ABA-hypersensitive 1*) mutant is hypersensitive to ABA in seed germination and stomatal closure and has reduced wilting symptoms in response to drought stress (Hugovieux et al. 2001). *ABH1* encodes the large subunit of the *Arabidopsis* nuclear cap binding protein complex (CBC), which has been shown to participate in mRNA processing and nuclear export in human HeLa cells and yeast (Lewis and Izaurralde 1997; Ishigaki et al. 2001). Hugovieux and coworkers (2001) suggest a role for ABH1 in regulation of the strength of ABA response in *Arabidopsis*. *Arabidopsis* mutants *hyl1* (*hyponastic leaves 1*) and *sad1* (*supersensitive to aba and drought 1*) are both hypersensitive to ABA in germination and root growth. HYL1 is a double-stranded RNA binding protein shown to play a role in microRNA-mediated gene regulation (Lu and Fedoroff 2000; Han et al. 2004), and SAD1 is similar to multifunctional Sm-like snRNP (small nuclear ribonucleoprotein) proteins that participate in mRNA splicing, export, and degradation (Xiong et al. 2001).

However, a recently characterized receptor controlling ABA-dependent flowering, FCA (Razem et al. 2006), establishes the most direct link between ABA signaling and RNA processing. FCA is an RNA-binding protein that binds ABA with high affinity and together with mRNA processing factor FY negatively regulates the expression of flowering repressor FLC (Razem et al. 2006). Interestingly, ABA inhibition of flowering was not affected in *abi1-1* and *abi2-1* plants impaired in most stress-related ABA responses (Razem et al. 2006). Thus, FCA apparently does not operate through these pathways, indicating that additional receptors must exist. The diversity of ABA-mediated plant responses suggests that proteins completely unrelated to FCA might function as ABA receptors for responses other than flowering. This means that the hunt for a stress-associated ABA receptor continues.

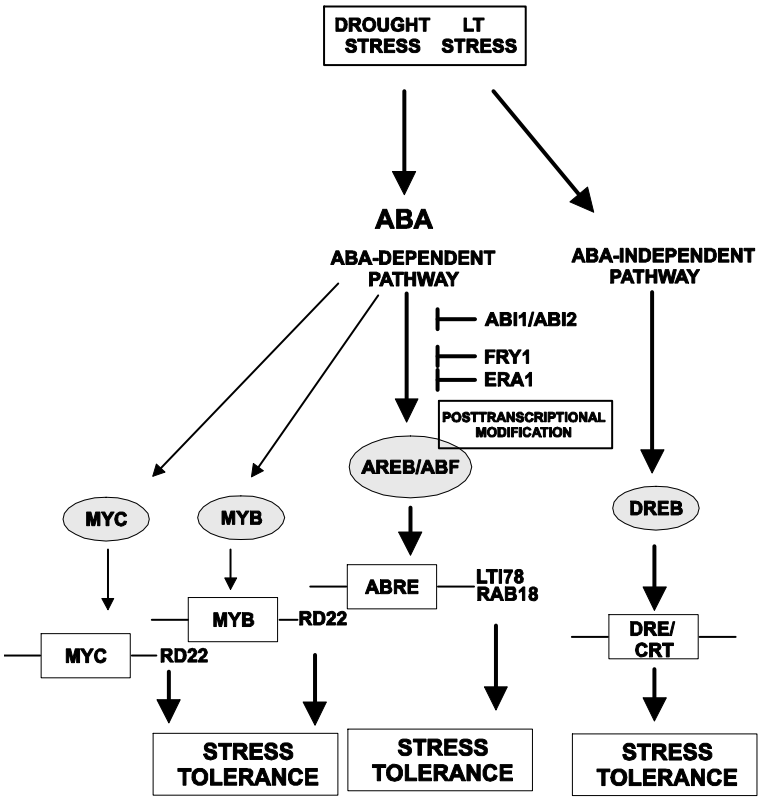

Figure 6. *Regulatory network of gene expression in response to drought and low temperature (LT) stress (modified from Yamaguchi-Shinozaki and Shinozaki 2005, 2006).*

Role of ABA in biotic stress responses

An endogenous increase of ABA has been observed in response to infection with viruses, bacteria, and fungi (Steadman and Sequeira 1970; Whenham et al. 1986; Kettner and Dörfling 1995), and increasing evidence suggests that ABA is significantly involved in the interactions between plants and pathogens (Audenaert et al. 2002; Anderson et al. 2004; Thaler and Bostock 2004; Ton and Mauch-Mani 2004). ABA seems to influence biotic stress responses by interfering with defense signaling regulated by SA, JA, and ET, but also through shared components of stress signaling (Mauch-Mani and Mauch 2005). However, recent research shows that ABA can also enhance plant resistance towards pathogens via its positive effect on callose deposition (Ton and Mauch-Mani 2004) (Figure 7).

Exogenous application of ABA prior to inoculation with the pathogen increases susceptibility of barley (*Hordeum* sp.), tomato, soybean (*Glycine max*), potato, and *Arabidopsis* (Edwards et al. 1983; Ward et al. 1989; Audenaert et al. 2002; Mohr and Cahill 2003). Accordingly, plant resistance against many pathogens is improved by ABA deficiency or by the use of the ABA biosynthesis inhibitor norflurazon (Kettner and Dörffling 1995; Audenaert et al. 2002; Mohr and Cahill 2003; Anderson et al. 2004).

Audenaert and colleagues (2002) demonstrated that ABA-deficient *sitiens* mutants of tomato are more resistant to the necrotrophic fungus *B. cinerea* than wild-type plants, and exogenous application of ABA restored the susceptibility. Also, treatment with SAR-inducing chemical BTH induced a stronger expression of the *PR1* gene in *sitiens* than in wild-type plants (Audenaert et al. 2002). Endogenous ABA accumulation induced by drought stress or ABA treatment prior to infection with avirulent *P. syringae* pv *tomato* resulted in necrosis and chlorosis in *Arabidopsis*, symptoms similar to susceptible infection. This suggests that ABA decreases SA-dependent defense responses in plants (Mohr and Cahill 2003). Furthermore, Mohr and Cahill (2003) demonstrated that inoculation of ABA-deficient mutant *aba1-1* with the virulent oomycete *Hyaloperonospora parasitica* resulted in HR-like necrotic spots and increased resistance.

Moreover, ABA interferes with JA-dependent defense signaling. Anderson et al. (2004) employed ABA-deficient *Arabidopsis* mutants and demonstrated that the lack of ABA positively affects resistance against the fungus *F. oxysporum* in *Arabidopsis*. The authors also showed that application of exogenous ABA suppressed both basal and JA/ET-induced expression defense genes. On the contrary, in ABA-deficient mutants, both basal and induced transcription from JA/ET-responsive defense genes was upregulated (Anderson et al. 2004). The application of MeJA or ET could not overcome the inhibitory effect of ABA, suggesting that abiotic stress signaling has the potential to override biotic signaling in situations where both stresses occur (Anderson et al. 2004). A point of convergence between JA and ABA signaling could be the transcription factor AtMYC2; it activates ABA-regulated gene expression and inhibits a subset of JA-regulated defense genes (Abe et al. 2003).

Interestingly, the speculated role of ABA in plant-pathogen interaction is controversial. While most studies report that ABA increases susceptibility, some observations suggest the opposite; in some cases, ABA is essential for pathogen resistance (Figure 7). BABA treatment has been shown to induce enhanced, or primed, defense to biotic and abiotic stresses (Zimmerli et al. 2000). A study by Ton and Mauch-Mani (2004) demonstrated that BABA priming of plant resistance is ABA-dependent. BABA treatment failed to induce resistance against *A. brassicicola* and *P. cucumerina* in ABA-deficient and ABA-insensitive *Arabidopsis* mutants (Ton and Mauch-Mani 2004). Callose (β-1,3-glucan), a major constituent of papillae located on the inner surface of epidermal cell walls in the apoplasm, and JA/ET defense signaling are important in resistance against these necrotrophic pathogens (Thomma et al. 1998). BABA treatment primes callose deposition and hence the formation of papillae at the site of infection (Ton and Mauch-Mani 2004; Flors et al. 2005). This is in accordance with Rezzonico and coworkers (1998) who demonstrated that ABA treatment downregulated the transcription of β-1,3-glucanase, a callose-degrading enzyme in tobacco. Interestingly, SA signaling might have the opposite effect on callose deposition: it induces the expression of *PR2*, a β-1,3-glucanase, which is also used as a marker of SAR induction (Van Loon and Van Strien 1999). A study by Nishimura et al. (2003) demonstrated that a mutation in callose synthase, which resulted in loss of the induced callose response, enhanced SA-dependent disease resistance. This led Ton and Mauch-Mani (2004) to speculate that the negative action ABA has on SA signaling could be mediated through callose accumulation. An element connecting ABA

and biotic stress responses might be the recently described poly (A)-specific endonuclease AtPARN from *Arabidopsis* (Nishimura et al. 2005). The regulation of gene expression by the mRNA-destabilizing activity of this protein is crucial for proper ABA and SA responses (Nishimura et al. 2005).

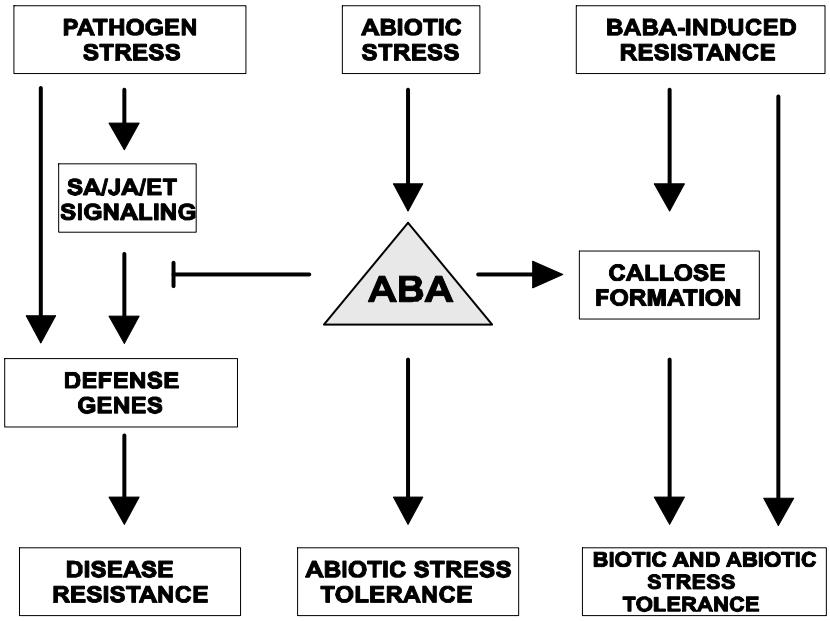

Figure 7. *The role of abscisic acid (ABA) in biotic stress responses (adapted from Mauch-Mani and Mauch 2005).*

CROSSTALK BETWEEN SIGNALING PATHWAYS

Plants respond to a variety of abiotic and biotic stimuli from the environment. Following perception of stress, several signal transduction pathways are switched on, resulting in physiological and molecular changes in the plant. When pathways operating in defense signaling are investigated, they are sometimes considered as independent units in order to simplify the interpretation. However, it would be naive to think that signal transduction is mediated through isolated, linear pathways. Defense pathways influence each other through a network of regulatory interactions, and thus, plant responses to various stress stimuli are a result of this complex interplay (Kunkel and Brooks 2002; Bostock 2005).

The term crosstalk is often used when discussing interactions in defense signaling. However, a good definition of what constitutes crosstalk does not exist, and differing opinions have been introduced concerning when it is appropriate to use this term to describe plant defense signaling (Taylor and McAinsh 2004; Mundy et al. 2006). Uncertainty results partly because not all the components operating in the defense pathways are known. Nevertheless, crosstalk is usually described as including a network of signal interactions in which functional outcomes can be positive, negative, or neutral

(Bostock 2005). In addition to different biotic stress signaling pathways, also biotic and abiotic pathways can “crosstalk”. This is exemplified by the effect of ABA on pathogen defense (Mauch-Mani and Mauch 2005), as discussed earlier. In conclusion, in most cases, ABA seems to have a negative effect on SA or JA signaling, impeding pathogen defense (Figures 7 and 8). However, on some occasions, ABA can have a positive impact on pathogen resistance; it enhances the accumulation of callose, and thus, increases resistance to certain necrotrophic pathogens (Ton and Mauch-Mani 2004).

Several studies have described crosstalk among SA, JA, and ET signaling pathways (Kunkel and Brooks 2003; Bostock 2005). SA and JA signaling interact on many levels, and in most cases, this relationship seems to be mutually antagonistic (Kunkel and Brooks 2002) (Figure 8). SA can inhibit the synthesis of JA and prevent the accumulation of PIs in response to JA, wounding, systemin, and oligosaccharides (Pena-Cortés et al. 1993; Doares et al. 1995). SA and its functional analogs have also been shown to prevent the expression of JA-dependent defense genes on several occasions (Pena-Cortés et al. 1993; Gupta et al. 2000; Kunkel and Brooks 2002). Moreover, Petersen et al. (2000) demonstrated that MAP KINASE 4 (MPK4) regulates negative crosstalk between JA and SA in the activation of defenses. Gene induction triggered by JA is blocked in *mpk4* mutants, indicating the importance of this gene for mediation of the JA signal (Figures 5 and 8). Simultaneously, this mutant constitutively expresses SA-regulated defense genes, probably as a result of the elevated SA levels. This suggests that a MAP kinase cascade involving MPK4 represses SA biosynthesis and promotes either JA perception or response (Petersen et al. 2000). A node of convergence between SA and JA signaling seems to be the plant-specific transcription factor WRKY70 (Li et al. 2004) (Figure 8). Plants overexpressing *WRKY70* showed decreased JA- but enhanced SA-dependent defense activation, hence improving resistance to *E. carotovora* and *P. syringae* (Li et al. 2004). This indicates that WRKY70 integrates defense signals, and thus, affects pathway activation (Li et al. 2004).

Some evidence also supports synergism between SA and JA defenses. Simultaneous activation of both SAR and rhizobacteria-triggered ISR resulted in an additive effect on induced protection against *P. syringae* (VanWees et al. 2000). Moreover, ROS has been shown to stimulate accumulation of SA and induction of SAR. At the same time, SA induces the production of ROS such as hydrogen peroxide and NO (Van Camp et al. 1998) (Figures 4 and 8). This synergism is thought to promote such defense responses as HR and killing of the pathogen.

Reported crosstalk between JA and ET signaling is mostly positive. Transcription factors AtMYC2/JIN1 and ERF1 are important regulators of these interactions in *Arabidopsis* (Lorenzo et al. 2004) (Figure 5). The expression of ERF1 (and its target genes) is synergistically activated by ET and JA, and ERF1 integrates these signals into the activation of plant defenses (Lorenzo et al. 2003) (Figures 5 and 8). ET seems to mediate the interaction between MAPK and CDPK, both of which are triggered by abiotic and biotic stress responses in *Arabidopsis*. Ludwig et al. (2005) demonstrated that CDPK signaling triggers high ET levels, leading to inhibition of stimulus-dependent MAPK activation. In rare cases, JA and ET have the opposite effects; in tobacco nicotine biosynthesis, a direct defense against some herbivores is stimulated by JA and inhibited by

ET (Shoji et al. 2000). The above-mentioned examples, especially the interaction between SA and JA signaling, demonstrate how plants can fine-tune their defense responses to different pathogens through crosstalk (Figure 8).

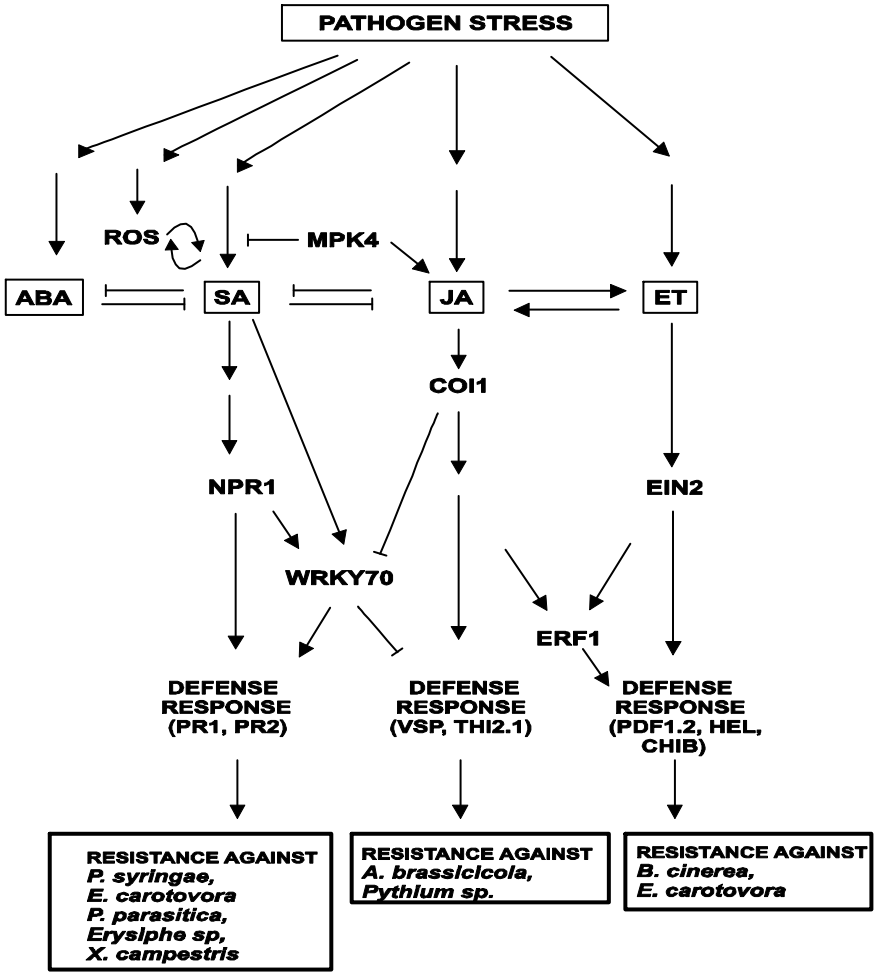

Figure 8. *Salicylic acid (SA), jasmonic acid (JA), and ethylene (ET) defense signaling pathways and signal crosstalk in Arabidopsis (modified from Kunkel and Brooks 2002 and Durrant and Dong 2004).*

MODEL ORGANISM *ERWINIA CAROTOVORA*

Soft-rot-causing *Erwinia*s (*E. chrysanthemi*, *E. carotovora* subsp. *atroseptica*, and *E. carotovora* subsp. *carotovora*) are Gram-negative phytopathogens that infect crop plants worldwide (Perombelon and Kelman 1980; Toth et al. 2003). *E. chrysanthemi* affects many tropical plants, whereas *E. carotovora* subsp. *atroseptica* causes blackleg of potato in cooler climate areas. *E. carotovora* subsp. *carotovora* (referred to as *E. carotovora* from here onwards) infects plants in both subtropical and temperate regions during the growing season as well as after harvest. The hosts include several economically important

crop species such as potato, carrot (*Daucus carota*), turnip (*Brassica rapa*), and celery (*Apium graveolens*) (Perombelon and Kelman 1980; Toth et al. 2003).

The main weapon of *E. carotovora* in causing disease is wide a variety of plant cell wall-degrading enzymes (PCWDE) including polygalacturonases, pectinases, cellulases, and proteases (Perombelon and Kelman 1980). These enzymes macerate plant cell walls and release nutrients for bacterial growth, hence the soft-rot phenotype of the infection. Besides extracellular enzymes, *E. carotovora* also secretes effector proteins to enhance its pathogenicity, and the *hrp* gene cluster encoding the TTSS has been characterized from this pathogen (Rantakari et al. 2001). One group of effector proteins secreted through TTSS are the harpins, acidic, glycine-rich, heat-stable proteins including HrpZ from *P. syringae* and HrpN from *E. chrysanthemi, E. amylovora*, and *E. carotovora* (Bauer et al. 1995; Cui et al. 1996; Rantakari et al. 2001). The role of harpins in virulence is controversial; the *hrpN* mutants of *E. amylovora* (Wei et al. 1992; Barny 1995) and *E. chrysanthemi* (Bauer et al. 1995) were less virulent than the wild-type form, whereas *E. carotovora* lacking *hrpN* remained fully pathogenic on tobacco plants (Cui et al. 1996). Harpins cause various effects on plants, including the HR, which is often associated with gene-for-gene interactions (Lahaye and Bonas 2001), despite no R-effector protein type of interaction involving harpin having been characterized thus far. The mechanism underlying induction of HR and plant defenses by harpin is unclear, but they seem to interfere with the ion balance of challenged cells by activating ion fluxes across the plasmalemma (Baker et al. 1993). Harpin from *P. syringae* binds lipid bilayers in vitro, resulting in the formation of ion-conducting pores, and induces formation of ROS (El-Maarouf et al. 2001; Lee et al. 2001; Samuel et al. 2005). Harpin can also induce resistance in plants; Jang et al. (2006) demonstrated that transgenic tobacco plants overproducing *E. amylovora* harpin are more resistant to the fungal pathogen *B. cinerea*.

A central factor influencing the success of the pathogen is the coordinate production of virulence determinants at the appropriate stage of the infection; not before the number of bacteria is sufficient for efficient attack, but before the plant becomes aware of the invasion and activates defenses. A sophisticated way to ensure correct timing is the use of a system called quorum sensing. *E. carotovora* secretes and detects small diffusible acylhomoserine lactone-based signal molecules called autoinducers (Pirhonen et al. 1993; Brader et al. 2005). The amount of secreted autoinducer increases as the bacterial population multiplies in the plant. It finally reaches a level sufficient to activate the production of PCWDEs, and hence, attack on the plant. Interestingly, autoinducers are unstable in alkaline pH, and one of the early counterattacks of the plant may take advantage of this by alkalinizing the site of bacterial invasion to a pH of >8.2 (Byers et al. 2002).

The virulence of *E. carotovora* is controlled by a complex regulatory network that can influence one or several virulence determinants. Many components that play a significant role in this complex system have been identified (Marits et al. 2002; Hyytiäinen et al. 2003). For example, inactivation of regulatory gene *expA* was shown to decrease the production of extracellular enzymes and result in an avirulent phenotype (Eriksson et al. 1998). Saarilahti et al. (1992) also demonstrated that mutation in polygalacturonase

(PehA) alone decreases virulence. Studies by Saarilahti et al. (1992) and Eriksson et al. (1998) highlight the central role of PCWDEs in the virulence of *E. carotovora*.

E. carotovora is a nonspecific pathogen, i.e. a race-cultivar-specific resistance to this pathogen has not been characterized. The broad host range, including the model plant *Arabidopsis*, makes this pathogen a good model for studying nonspecific plant-pathogen interactions. *E. carotovora* triggers basal plant defense responses presumably through the recognition of pathogen-delivered general elicitors, such as LPS, or OGAs released from plant cell walls by the action of PCWDEs. *E. carotovora* or *E. carotovora* elicitors trigger local and systemic induction of SA- and JA/ET-dependent defense genes in plants (Palva et al. 1993; Vidal et al. 1997, 1998; Norman-Setterblad et al. 2000). Interestingly, resistance against this pathogen can be generated by the induction of either JA/ET-mediated (Vidal et al. 1998; Norman-Setterblad et al. 2000) or SA-mediated (Palva et al. 1994; Li et al. 2004) defenses.

OPEN QUESTIONS

The study of defense mechanisms against pathogens is one of the faster moving fields within plant science. Interactions between plants and the different microorganisms attacking them have been elucidated, revealing some of the molecular components involved. Moreover, the pathogen-triggered plant defense signaling networks engaged in the induction of resistance are beginning to be pieced together. Plant defense responses against pathogens are regulated through pathways currently known to involve three central endogenous phytohormones: SA, JA, and ET. ABA has recently also been included in this team of hormones influencing the establishment of plant defense. Furthermore, ROS and NO add to the complexity of defense signal transduction.

Although progress and research producing novel data are impressive, many gaps in knowledge need to be filled before the big picture of plant defense signaling network can be drawn or the information applied to the development of durable crops. The defense pathways interact significantly with each other, and therefore, the characterization of potential convergence points where pathway crosstalk occurs is particularly important. Due to the abundance of the signals and the myriad of interactions therein, delineating the roles of these signals in integrated networks is challenging. Nevertheless, this information is critical for complete understanding of signaling events and outcomes in diverse stress situations.

What can the study of the interaction between the pathogen *E. carotovora* and plants contribute to the knowledge of the plant defense signaling network? Firstly, novel information would be yielded concerning the induction of basal defense responses in plants. Due to the nonspecific nature of the interaction between *E. carotovora* and plants, this knowledge can also be applied to interactions with other pathogens. Secondly, the resistance strategies developed to control *E. carotovora* are likely to be effective against a broad range of different plant pathogens. This can greatly benefit the development of efficient and durable applications for plant protection – the ultimate objective of plant-pathogen interaction studies.

AIMS OF THE STUDY

This work aimed at achieving novel information about the plant-pathogen interaction between the phytopathogen *E. carotovora* and the model plant *Arabidopsis*. This included characterization of both the plant defense triggered by pathogen elicitors and the plant genes involved in early responses to the pathogen.

Studies focused on the following topics:

- Characterization of plant defense responses triggered by the effector protein HrpN of *E. carotovora*, and the plant responses synergistically elicited by effector HrpN and PCWDE PehA

- Characterization of the specific roles of two *Arabidopsis* genes, *AtCLH1* and *ERD15*, involved in early plant defense responses triggered by *E. carotovora*

- Elucidation of how transgenic expression of these early pathogen responsive genes alters plant defense responses against *E. carotovora*

MATERIALS AND METHODS

BIOLOGICAL MATERIAL

The biological material used in this work is described in detail in Studies I-III. In brief, we used *Arabidopsis thaliana* and *Nicotiana tabacum* wild-type species and transgenic *Arabidopsis* plants. In addition, several different *Arabidopsis* mutants were employed. *Erwinia carotovora* subsp. *carotovora* strain SCC1 and *Alternaria brassicicola* strain CBSnr 567.77 were used as pathogen models. *Escherichia coli* strains JM109 and M15 (pREP4) were applied for protein expression.

Method	**Used and described in study**
Assay for bacterial virulence and growth in planta	I-III
Assay for freezing survival	III
Assay for drought tolerance	III
Chlorophyll extraction	II
Determination of bacterial growth in planta	I-III
Diaminobenzidine staining of H_2O_2	II, III
Electrolyte leakage assay for ABA-induced freezing tolerance	III
Elicitor treatments of plants	II, III
Extraction of chlorophyll	II
Generation and characterization of transgenic plants	II, III
High-light stress treatment	II
Infection of *Arabidopsis* with *A. brassicicola*	II
Infection of *Arabidopsis* with *E. carotovora*	I-III
Isolation of plant RNA	I-III
Isolation of plant proteins	II, III
Northern blot analysis	I-III
Phytohormone extraction and quantification	II, III
Plant growth conditions	I-III
Protein expression in *E. coli*	I
Purification of 6xHis-tagged proteins	I
Quantitation of fungal biomass by PCR	II
Seed germination assay for ABA sensitivity	III
Western blot analysis	I, III

RESULTS AND DISCUSSION

Erwinia carotovora and elicitors HrpN and PehA trigger distinct but interacting defense responses and cell death in *Arabidopsis* (I)

Induced defense responses in plants can be activated upon recognition of a pathogen, by a pathogen-derived elicitor molecule, or by elicitors released from the plant by the action of the pathogen (Montesano et al. 2003; Nürnberger et al. 2004). *E. carotovora* is a broad host range pathogen that uses an array of plant cell wall-degrading enzymes (PCWDE) as primary virulence factors (Pérombelon and Kelman 1980; Kotoujansky 1987). Earlier studies have demonstrated that PCWDEs release oligogalacturonide elicitors from plant cell walls (Palva et al. 1993) that elicit SA-independent defenses (Vidal et al. 1997, 1998). In addition, some *E. carotovora* strains have been shown to possess *hrp* genes that contribute to virulence (Rantakari et al. 2001). These include proteins required for type III secretion (TTSS) and delivery of effector proteins to plant cells (Rantakari et al. 2001). Study I characterizes plant defense responses triggered by effector protein HrpN of *E. carotovora* ($HrpN_{Ecc}$). Plant responses synergistically induced by $HrpN_{Ecc}$ and a PCWDE, the polygalacturonase PehA, were also investigated.

HrpN elicits lesion formation and induces resistance to *E. carotovora* in *Arabidopsis*

Harpins are acidic, glycine-rich, heat-stable effector proteins secreted through the TTSS. These proteins have been characterized from several phytopathogens, including HrpZ from *P. syringae* and HrpN from *E. carotovora* (Bauer et al. 1995; Cui et al. 1996; Rantakari et al. 2001). The impact of harpin on virulence is somewhat inconsistent: it is essential for pathogenicity in *E. amylovora* and *E. chrysanthemi* (Wei et al. 1992; Barny 1995), but dispensable in *E. carotovora* (Cui et al. 1996). Harpin has been demonstrated to cause various effects on plants, including the HR response. The role of HrpN in the virulence of *E. carotovora* is particularly difficult to assess due to the massive production of PCWDEs and maceration of plant tissue, which can mask any HR response. To overcome this problem, we treated tobacco and *Arabidopsis* plants with purified HrpN protein. The *hrpN* gene of *E. carotovora* strain SCC1 was isolated from a genomic lambda library, cloned to an expression vector, and the N-terminally His_6-tagged protein was purified. Infiltration of purified HrpN protein caused visible, HR-like symptoms in both tobacco and *Arabidopsis*, whereas no lesion formation was detected when plants were treated with controls, MES buffer, or acetylated bovine serum albumin (I, Fig. 1B). HrpN treatment was accompanied by increased ion leakage three to four days after infiltration (I, Fig. 1C), indicating cell death.

Earlier studies have shown that harpin treatment can induce resistance against *E. amylovora* in *Arabidopsis* (Dong et al. 1999) and *P. syringae* in cucumber (Strobel et al.

1996). This prompted us to examine whether *E. carotovora* HrpN could induce resistance in *Arabidopsis*. Leaves of wild-type *Arabidopsis* were pretreated with HrpN 24 h prior to inoculation with *E. carotovora*. After pathogen inoculation, a clear difference was observed in the maceration of the plants; HrpN-pretreated plants were significantly less macerated than MES-pretreated control plants (I, Fig. 2A). The bacterial numbers in inoculated plants also showed that after the initial multiplication the growth of *E. carotovora* was halted in HrpN-pretreated plants (I, Fig. 2B). This indicates that, similar to other harpins characterized, $HrpN_{Ecc}$ is a potent inducer of lesion formation as well as disease resistance in *Arabidopsis*.

Establishment of HrpN-triggered resistance requires both SA and JA signaling

Earlier studies have shown that resistance to *E. carotovora* can be generated by induction of either JA/ET-mediated (Vidal et al. 1998; Norman-Setterblad et al. 2000) or SA-mediated (Palva et al. 1994) defenses triggered by distinct *E. carotovora* elicitors (Palva et al. 1993; Norman-Setterblad et al. 2000). It was therefore of great interest to elucidate which defense pathways $HrpN_{Ecc}$ induced in *Arabidopsis*. Earlier work by Dong et al. (1999) and Strobel et al. (1996) demonstrates that *E. amylovora* and *P. syringae* harpins induce SAR in *Arabidopsis* and cucumber. To determine the contribution of $HrpN_{Ecc}$, we characterized the expression of two defense pathway specific marker genes: *PR1* responsive to SA and *PDF1.2* responsive to JA/ET after treatment with this protein. The expression of these genes in response to $HrpN_{Ecc}$ was compared with that induced by another *E. carotovora*-derived elicitor, the polygalacturonase PehA, a known inducer of JA/ET-dependent defenses (Vidal et al. 1998; Norman-Setterblad et al. 2000) and the pathogen itself. Inoculation of *Arabidopsis* with *E. carotovora* induced both *PR1* and *PDF1.2*, while PehA triggered only *PDF1.2* (I, Fig. 3A). Similar to harpins from *E. amylovora* and *P. syringae*, $HrpN_{Ecc}$ triggered accumulation of *PR1* transcripts (I, Fig. 3A). However, in addition to *PR1*, $HrpN_{Ecc}$ induced the expression of *PDF1.2*, which is in contrast to the earlier studies by Stroebel et al. (1996) and Dong et al. (1999) demonstrating that $HrpN_{Ecc}$ is capable of triggering both SA- and JA/ET-dependent defense signaling in *Arabidopsis*.

The involvement of both SA- and JA/ET-dependent pathways in the $HrpN_{Ecc}$-induced defense response was further elucidated by the use of *Arabidopsis* mutants and transgenic plants impaired in defense signaling. HrpN did not induce resistance in transgenic *NahG* plants unable to accumulate SA (I, Fig. 4). Nor was resistance induced in JA-insensitive *coi1* (Feys et al. 1994) or JA amino acid synthetase mutant *jar1* (Staswick and Tiryaki 2004) in response to HrpN treatment. The double mutant *NahG coi1*, where both SA- and JA-dependent defense pathways were affected, was most severely macerated, and the bacteria grew to the highest density (I, Fig. 4). These results demonstrate that HrpN-induced resistance requires functional SA-dependent as well as JA-dependent signal pathways in *Arabidopsis*.

Synergistic action of elicitors HrpN and PehA triggers enhanced defense gene expression and cell death

The enhanced expression of plant defense genes in response to inoculation with *E. carotovora* compared with either elicitor alone (I, Fig. 3A) prompted us to assess the effect of simultaneous treatment with HrpN and PehA on wild-type *Arabidopsis*. The combined effect of these two elicitors led to both faster and stronger induction of *PR1* and *PDF1.2* (I, Fig. 5A). Moreover, the systemic induction of these genes was clearly enhanced (I, Fig. 5B), indicating that HrpN and PehA synergistically trigger amplified plant defense response.

The role of ROS is central to the formation of the HR response, which is also induced by $HrpN_{Ecc}$ in *Arabidopsis* (I, Fig. 1B). The harpin of *P. syringae* has been suggested to amplify ROS production through the activation of AtMPK6 (Desikan et al. 2001). Moreover, simultaneous application of different elicitors has been shown to amplify the oxidative burst that follows (Chandra et al. 2000). Synergism of HrpN and PehA in inducing defense gene expression prompted us to test whether lesion formation and superoxide production were also enhanced. Indeed, simultaneous application of HrpN and PehA did result in a response that was clearly stronger than with either elicitor alone; the production of rapidly appearing HR-like lesions was accompanied by increased production of superoxide and enhanced ion leakage, indicating accelerated cell death (I, Fig. 6A-C).

How might *E. carotovora* benefit from triggering multiple responses in the host, including cell death, which can be an effective plant defense response limiting pathogen growth? Interestingly, responses, such as HR, that are effective against one pathogen can be ineffective against another (Kunkel and Brooks 2002; Glazebrook 2005). Enhanced ROS production can interfere with the plant metabolism, and the dying cells could provide nutrients for the growing bacterial population. Cell death responses of the plant are possibly also utilized by *E. carotovora* to spread infection. For example, in the case of the necrotrophic fungus *B. cinerea*, the HR response has been demonstrated to facilitate pathogenesis (Govrin and Levine 2000). Alternatively, perhaps *E. carotovora* does not aim at triggering cell death or benefit from it. Both elicitors might act as PAMPs and trigger plant defense, simultaneously being indispensable for the pathogenicity of *E. carotovora*. Saarilahti et al. (1992) have demonstrated that a mutation in the *pehA* gene reduces the virulence of this pathogen. Whether the same holds true for HrpN remains to be elucidated.

PLANT RESPONSES TO *E. CAROTOVORA* (II, III)

Early defense responses of the plant are crucial in determining the outcome of the plant-pathogen interaction, and thus, characterization of the molecular responses occurring early in the *E. carotovora-Arabidopsis* interaction was of great interest. To isolate the rapidly induced plant genes, *Arabidopsis* was treated with a preparation of *E. carotovora* elicitors. A cDNA library enriched for sequences preferentially expressed in elicitor-treated leaves

was established using a suppressive subtractive hybridization method (Brader et al. 2001). Approximately 200 differentially expressed genes were identified, and several of these were subjected to further analysis. Studies II and III elucidate the roles of two genes, *CHLOROPHYLLASE 1* (*AtCLH1*) (II) and *EARLY RESPONSIVE TO DEHYDRATION 15* (*ERD15*) (III), in plant stress responses.

Studies II and III demonstrated how transgenic modification of two early pathogen-induced genes can improve the basal, SA-dependent defense response and lead to enhanced resistance against *E. carotovora*. Both studies also implicated pathway crosstalk in modulating defense responses. Study II demonstrated antagonism between SA- and JA-dependent signaling pathways, whereas Study III revealed the occurrence of crosstalk between biotic (SA) and abiotic (ABA) defense pathways.

CHLOROPHYLLASE 1 AFFECTS INDUCTION OF DEFENSE PATHWAYS IN PLANTS (II)

RNAi silencing of *CHLOROPHYLLASE 1 (AtCLH1)* improves resistance to *E. carotovora* in *Arabidopsis*

Degradation of chlorophyll is initiated by the dissociation of chlorophyll and its phytol tail, resulting in the formation of chlorophyllide. This reaction is catalyzed by the first enzyme in the degradation pathway, chlorophyllase (Matile et al. 1999). *CHLOROPHYLLASE 1* (*AtCLH1*) is one of the two chlorophyllases characterized from *Arabidopsis*, and it is rapidly induced in response to wounding and methyl jasmonate (MeJA) (Benedetti et al. 1998; Tsuchiya et al. 1999; Benedetti and Arruda 2002). Interestingly, our studies showed a rapid induction of this gene also in response to the pathogen *E. carotovora*, while no induction was observed after treatment with SA (II, Fig. 1A). The expression pattern of this gene suggests that it could be a component of JA-dependent defense in *Arabidopsis*.

To elucidate the contribution of *AtCLH1* in plant defense, it was either overexpressed or silenced with RNAi in *Arabidopsis*. The effect of the transgenes on *AtCLH1* transcript accumulation was evaluated by Northern blot analysis using a gene-specific RNA probe for *AtCLH1*. Lines with enhanced and silenced expression of *AtCLH1* were chosen for further studies (II, Fig. 1B). In addition, the transgenic lines were evaluated by measuring chlorophyll breakdown in plants. Overexpression of *AtCLH1* resulted in increased accumulation of chlorophyllide, suggesting more efficient chlorophyll degradation in these plants, whereas RNAi silencing of *AtCLH1* led to a higher chlorophyll-chlorophyllide ratio in transgenic plants (II, Fig. 1D).

To elucidate the effect of altered *AtCLH1* expression on plant defense response, the transgenic plants were infected with *E. carotovora*. After 24 h, it was clear that silencing of this gene made the plants more resistant to the pathogen (II, Fig. 2A). No or only minor symptom development was observed in *AtCLH1* RNAi-silenced plants, while in the control and in *AtCLH1* overexpression plants the maceration had already spread

considerably (II, Fig. 2A), suggesting that *AtCLH1*, and thus, chlorophyll degradation might have a role in plant defense responses and that downregulation of this gene benefits the resistance of *Arabidopsis* against *E. carotovora*.

Resistance of *AtCLH1* RNAi-silenced plants is light-dependent and involves increased accumulation of ROS

Due to their capability to harvest light, chlorophylls and their porphyrin ring-containing derivatives are photoactive (Matile et al. 1999). If the resistance of *AtCLH1* RNAi plants resulted from impaired chlorophyll degradation and phototoxicity of the chlorophyll molecules, light conditions should make a difference in the outcome of the infection. To explore this, transgenic plants were inoculated with *E. carotovora* in low light conditions (~ 50 µmol min^{-2} sec^{-1} photons). The resistance of *AtCLH1* RNAi plants observed in normal light conditions was abolished, and maceration proceeded similarly to vector control and *AtCLH1* overexpression plants (II, Fig. 2C), indicating that the resistance arising from silencing of *AtCLH1* could indeed be due to the phototoxic effects of the chlorophylls.

Why would downregulated chlorophyll degradation have an impact on pathogen defense? *E. carotovora* infection causes damage to plant tissues, resulting in the release of chlorophyll molecules from the thylakoids. If not efficiently removed, these light-reactive molecules can increase ROS formation in the cells (Takamiya et al. 2000). To avoid such situations, chlorophyll degradation needs to be initiated effectively and evidently; decreased AtCLH1 activity can hamper this process. Since ROS has an impact on cellular metabolism and can also affect defense responses, it is not far-fetched that altered chlorophyllase activity could influence the plant resistance response to *E. carotovora*. To investigate this, we monitored ROS formation in response to *E. carotovora* inoculation in normal light conditions. Hydrogen peroxide accumulation was visualized by 3, 3´-diaminobenzidine (DAB) staining. Already 2 h after inoculation with the pathogen, the *AtCLH1* RNAi-silenced plants had increased amounts of hydrogen peroxide in comparison with vector control plants (II, Fig. 3A). This also correlated with enhanced induction of *GLUTATHIONE-S-TRANSFERASE I* (*GSTI*), a marker gene indicating the activation of antioxidant defenses needed for ROS detoxification in plant cells (Conklin and Last 1995) (II, Fig 3B).

The response of *AtCLH1* RNAi plants to pathogen stress in combination with the rapid inducibility of this gene in response to wounding and defense elicitors strongly indicate that AtCLH1 could act as a rapidly activated “emergency chlorophyllase” in initiating chlorophyll degradation after tissue damage. A role for AtCLH1 in tissue repair was also suggested by Benedetti et al. (2001). High light stress can also cause tissue damage, and indeed, it seems that AtCLH1 is essential in protection against this type of stress as well – an observation that further suggests an essential role for this chlorophyllase in situations where rapid chlorophyll degradation is needed. Similarly to the pathogen response of *AtCLH1* RNAi-silenced plants, high light stress resulted in increased accumulation of hydrogen peroxide and *GSTI* transcripts (II, Fig. 4A and B). Accordingly, previous studies show that transferring plants to high light conditions induces the degradation of the major

light-harvesting complex (LHCII) of photosystem II (PSII) (Yang et al. 1998). This probably leads to chlorophyll release from LHCII, creating a need for rapid degradation of these light-reactive molecules.

AtCLH1 RNAi plants do not develop lesions, which is unexpected when compared with, for example, *acd2* plants, which have a mutation in the *RED CHLOROPHYLL CATABOLITE REDUCTASE (RCCR)* gene and are compromised in chlorophyll degradation (Mach et al. 2001; Yao and Greenberg 2006). However, *AtCLH1* is only induced in response to tissue damage or injury, whereas *ACD2* is constitutively expressed and presumably participates in all chlorophyll degradation occurring in the plant cell (Mach et al. 2001). The absence of ACD2 results in constant accumulation of photoactive chlorophyll breakdown products, and hence, lesions (Yao and Greenberg 2006). Benedetti and Arruda (2002) also showed that the expression of the second *Arabidopsis* chlorophyllase gene, *AtCLH2*, did not vary in *AtCLH1* antisense and overexpression plants, and this could hypothetically mean that it can replace the missing AtCLH1 activity in case of injury. The free chlorophyll molecules would therefore eventually be degraded, albeit slowly, and ROS detoxified by the activated antioxidant defenses.

Silencing of *AtCLH1* affects the balance between SA- and JA-dependent defenses in *Arabidopsis*

What could be the mechanism through which altered chlorophyll metabolism affects plant defense responses? ROS can be directly harmful to invading pathogens, and it has also been implicated as a signal for activation of plant defense responses (Chen et al., 1995; Lamb and Dixon, 1997; Wojtaszek 1997; Bolwell 1999; Mullineaux et al., 2000). ROS can, for example, potentiate SA-dependent defense signaling (Durrant and Dong 2004). To elucidate the role of different defense pathways in the observed resistance of *AtCLH1* RNAi-silenced plants to *E. carotovora*, we characterized the expression of marker genes for these pathways. Moreover, to clarify the impact of light on pathway induction, expression analysis was done with plants infected in both normal and low light conditions. In the resistant *AtCLH1* RNAi-silenced plants, expression of SAR marker genes *PR1* and *PR2* was clearly enhanced compared with vector control or overexpression plants (II, Fig. 6A). Interestingly, this induction was abolished in low light conditions, where the expression of *PR1* and *PR2* was reduced to the level of vector control plants (II, Fig. 6B), underlining the importance of light and phototoxic chlorophyll molecules in the observed resistance.

The antagonism between SA- and JA-dependent defenses is well-characterized (Kunkel and Brooks 2002; Bostock 2005), and the enhanced SA defense prompted us to investigate whether the JA-dependent defense response of the transgenic plants was altered. Compared with vector control plants, the induction of *PDF1.2*, a marker gene for JA-dependent defense, was clearly suppressed (II, Fig. 6D), suggesting that the silencing of *AtCLH1* upregulates SA-dependent but downregulates JA-dependent defense in *Arabidopsis*. For further proof of the downregulation of JA-dependent defenses, we studied the response of *AtCLH1* RNAi-silenced plants to the necrotrophic fungus *A. brassicicola*. Resistance to this pathogen is dependent on JA defenses: *A. brassicicola* is

unable to infect wild-type *Arabidopsis*, but the *coi1* mutant is susceptible to it (Thomma et al. 1999, 1998). *AtCLH1* RNAi-silenced and vector control plants were infected with *A. brassicicola* under normal light conditions, and disease development was followed visually. After seven days, no disease symptoms were observed in the vector control plants. However, approximately 70% of the *A. brassicicola*-infected leaves of *AtCLH1* RNAi plants showed disease development (II, Fig. 7), indicating downregulation of JA-dependent defenses in these plants. This could be a result of enhanced SA-dependent defense that inhibits the activation of JA-dependent defenses.

Depending on the invading pathogen, plants employ different defense strategies. Fine-tuning the activation of defense genes and the balance between different signaling pathways is essential to achieve the best possible result in a battle against the invader. However, for a pathogen, ability to interfere with the defense response of the plant can be an asset when aiming at successful infection. How can *E. carotovora* benefit from the induction of chlorophyllase? This pathogen seems to take advantage of the activation of chlorophyll degradation (II, Fig 8); induction of *AtCLH1* decreases the accumulation of ROS in response to tissue damage at an early stage of infection. Increased ROS accumulation appears to enhance the basal SA-dependent defenses induced in response to *E. carotovora*, and without this effect, these defenses might not contain the infection and lead to resistance against this pathogen.

Activation of one defense signaling pathway can have an antagonistic effect on another (II, Fig. 8). For example, SA-dependent defenses are effective against *P. syringae* infection. However, this pathogen seems to exploit plant defense by producing the JA-mimicking toxin coronatine, which contributes to its virulence by inducing JA signaling and thereby repressing SA-dependent defense mechanisms (Bender et al. 1999; Greenberg 2005). Early induction of *AtCLH1*, and hence, JA-dependent signaling, could have an antagonistic effect on SA-dependent defenses effective against *E. carotovora*, thus giving an advantage to this pathogen in the plant-pathogen battle.

Interestingly, silencing of *AtCLH1* resulted in enhanced SA- but decreased JA/ET-defenses and resistance to *E. carotovora*. This is somewhat contradictory to the results of Study I, where HrpN-triggered resistance to *E. carotovora* required both SA- and JA/ET-dependent defense signaling. What makes it even more difficult to reach conclusions concerning plant resistance to *E. carotovora* is that earlier studies have shown that either JA/ET- or SA-dependent defenses alone triggered by different elicitors can be efficient against this pathogen (Palva et al. 1994; Norman-Setterblad et al. 2000; Li et al. 2004). Thus, resistance to the nonspecific pathogen *E. carotovora* can apparently be triggered through different defense pathways. Both the timing and degree of the active defense component might be critical to the outcome of the *E. carotovora*-plant interaction. One can hypothesize that in the case of silenced *AtCLH1* the modulation of one component of plant defense leads to induction of significantly enhanced SA responses that alone are efficient against *E. carotovora*. This response simultaneously antagonizes JA/ET signaling, which is not needed in addition to the strong SA response. HrpN-induced activation of these pathways could be different – less vigorous – and thus not lead to antagonism. The synergism between the activation of SA and JA/ET defenses then establishes the resistance to *E. carotovora*. The difference between study systems should,

however, be taken into account. The role of AtCLH1 was investigated in transgenic plants with an overexpression or RNAi-silencing construct of the gene, whereas the effect of HrpN was monitored from plants infiltrated with a preparation containing this protein. The activation of defense responses is likely to be affected differently depending on the study system used.

ERD15 IS A NEGATIVE REGULATOR OF ABA RESPONSES IN *ARABIDOPSIS* (III)

ERD15 is rapidly induced by both biotic and abiotic factors

EARLY RESPONSIVE TO DEHYDRATION 15 (*ERD15*), a gene originally identified as inducible by drought (Kiyosue et al. 1994), was rapidly induced after treatment with *E. carotovora* elicitors. Moreover, ABA, SA, *E. carotovora* infection, and wounding induced this gene rapidly, suggesting that it could be a component of both biotic and abiotic stress responses in *Arabidopsis* (III, Fig. 1A). Interestingly, while both SA- and JA-mediated defenses are effective against *E. carotovora* in *Arabidopsis*, SA but not MeJA induced the expression of *ERD15* (III, Fig. 1A). The rapid inducibility of *ERD15* indicates that it could mediate early steps in the activation of plant stress signaling.

Modulation of *ERD15* expression affects abiotic stress tolerance by influencing ABA sensitivity

The role of ERD15 in plant responses has been an enigma since it was first characterized (Kiyosue et al., 1994). Expression analysis of this gene (see above) did not shed any light on this issue, and thus, transgenic plants carrying overexpression and RNAi-silencing constructs of *ERD15* were created. The effect of the transgenes was verified by characterizing the expression levels in Northern blot analysis, and accordingly, three lines with increased and three lines with decreased expression of *ERD15* were chosen for further studies (III, Fig. 1B). Surprisingly, of approximately 50 different lines, very few with clearly decreased *ERD15* expression were found. The lines chosen were further evaluated with *ERD15*-specific antisera, demonstrating the effect of the transgenes also at the protein level (III, Fig. 1C).

The inducibility of *ERD15* by dehydration stress (Kiyuosue et al. 1994; III, Fig. 1D) prompted us to investigate the possible role of *ERD15* in abiotic stress responses. To this end, the transgenic *ERD15* plants were challenged with two related stresses, drought and freezing, both of which result in cellular dehydration (Thomashow 1999). The plants were drought-stressed for two weeks in low humidity conditions, and their survival was assessed visually. The difference between the transgenic lines was striking after two weeks; most of the plants overexpressing *ERD15* were dead, whereas the control plants remained alive, although they did not appear very healthy and showed a strong

accumulation of anthocyanin (III, Fig. 2A). However, the majority (83%) of the *ERD15* RNAi-silenced plants were still alive and, unlike the control plants, had maintained their green color (III, Fig. 2A, B). Applying freezing stress to the *ERD15* transgenic plants supported the observations from the drought experiment. Freezing stress brought about the greatest damage to the plants overexpressing *ERD15*, while the plants with the RNAi-silenced gene clearly survived better than the control plants (III, Fig. 2C and D).

The phytohormone ABA is central in plant responses to dehydration stress; it induces stomatal closure and production of desiccation protectants (Finkelstein and Gibson 2002; Zhu 2002; Zhu and Verslues 2005). The dramatic changes in the drought and freezing tolerance phenotypes of the transgenic *ERD15* plants suggested that this protein might be involved in ABA-mediated stress responses in *Arabidopsis*. The application of ABA can induce the development of freezing tolerance in higher plants such as wild potato (Chen and Gusta 1983) and *Arabidopsis* (Lång et al. 1989**;** Mäntylä et al. 1995). Interestingly, plants overexpressing *ERD15* were impaired in their capacity to increase freezing tolerance in response to ABA treatment. This was observed as increased electrolyte leakage compared with wild-type plants after the ABA-treated plants were exposed to freezing stress (III, Fig. 3A). This supports the argument that the changes in *ERD15* expression alter the sensitivity of the plants to ABA.

Expression of numerous plant genes involved in plant responses to abiotic environmental stresses, especially those involved in drought response, is regulated by ABA (Yamaguchi-Shinozaki and Shinozaki 2005). The expression of two ABA-responsive genes, *RAB18* (Lång and Palva 1992) and *LTI78* (Nordin et al. 1993), was elucidated in transgenic *ERD15* plants (III, Fig. 4A). In plants overexpressing *ERD15*, the drought-induced expression of these genes was reduced, indicating that ERD15 interferes with ABA signaling in *Arabidopsis* (III, Fig. 4A).

Studies with *Arabidopsis* mutant *abscisic acid-insensitive 1-1* (*abi1-1)* plants demonstrate that insensitivity to ABA can lead to an increase in the basal and stress-induced endogenous levels of ABA (Lång et al. 1989; Mäntylä et al. 1995). The same was observed in plants overexpressing *ERD15* after the extraction of phytohormones (Schmelz et al. 2004); both basal and drought stress-induced ABA concentrations were higher than those of the wild-type plants (III, Fig. 4B). This supports the hypothesis that *ERD15* overexpression makes plants insensitive to ABA and that the observed increase in the endogenous hormone concentration is due to feedback regulation. The question then arises: if overexpression of *ERD15* decreases the sensing of ABA in these plants, could silencing of this gene do the opposite, result in hypersensitivity? The ABA germination assays suggested exactly this; *ERD15* RNAi-silenced plants germinated poorly and only a very small number of them had green cotyledons (III, Fig. 3B and C). One can also hypothesize that the improved drought (III, Fig. 2A and B) and freezing (III, Fig. 2C and D) tolerance of *ERD15*-silenced plants could result from a more rapid response to the stress-induced ABA signal due to hypersensitivity of the plants. In conclusion, the drought-tolerance phenotype, inhibition of seed germination by ABA, and the gene-expression data of transgenic plants all indicate that ERD15 has an impact on the capability of *Arabidopsis* to sense ABA.

Insensitivity to ABA enhances resistance of *Arabidopsis* to *E. carotovora*

Interestingly, *ERD15* is also induced by *E. carotovora* and SA in *Arabidopsis* (III, Fig. 1A). Therefore, it was of interest to elucidate whether resistance to pathogens was altered in the transgenic *ERD15* plants. According to several recent studies, ABA is also involved in plant defense responses to pathogens (Audenaert et al. 2002; Mohr and Cahill 2003; Ton and Mauch-Mani 2004; Anderson et al. 2005; Mauch-Mani and Mauch 2005). ABA has been shown to have an antagonistic effect on SA (Audenaert et al. 2002; Mohr and Cahill 2003) and JA (Anderson et al. 2005) -dependent defense responses, but it seems to be essential in the BABA-induced callose accumulation needed to defend against certain necrotrophic pathogens (Ton and Mauch-Mani 2004). Since our results strongly indicated that modulation of *ERD15* expression in *Arabidopsis* decreased the sensitivity of the plants to ABA, we investigated whether this had an impact on the pathogen resistance of transgenic plants. Transgenic *ERD15* plants were inoculated with *E. carotovora*, and the disease symptoms were followed. The symptom development of plants overexpressing *ERD15* was clearly reduced compared with that of control plants or plants with RNAi-silenced *ERD15* (III, Fig. 6A). The enhanced resistance was also reflected in the low bacterial numbers in inoculated plants (III, Fig. 6B), indicating that overexpression of *ERD15*, besides reducing ABA sensitivity, also promotes resistance to *E. carotovora*.

Earlier studies (Palva et al. 1993, 1994; Vidal et al. 1997, 1998; Norman-Setterblad et al. 2000; Li et al. 2004; I, II) have shown that resistance to *E. carotovora* can be generated by either JA/ET- or SA-dependent mechanisms. The potential contribution of these pathways to the observed resistance in *ERD15* transgenics was elucidated by characterizing the induction of pathway-specific marker genes by Northern blot analysis. The expression pattern of these genes suggested that the resistance of *ERD15* overexpression plants was due to enhanced SA defense (III, Fig. 7B). This was seen after infection with *E. carotovora* but also after plant treatment with pathway elicitor SA. This treatment was done to eliminate possible difficulties in interpretation of gene expression in response to the pathogen due to variation in progress of the infection. In *ERD15* overexpression plants, the expression of *PR2*, a marker gene for SA signaling, was more rapidly induced in response to *E. carotovora* and enhanced after SA treatment (III, Fig. 7B). This suggests that insensitivity to ABA enhances the induction of SA-dependent defense responses. However, the induction of *PDF1.2*, a marker gene of the JA/ET pathway, was decreased in the more resistant *ERD15* overexpression plants (III, Fig. 7A). This might be due to the enhanced SA-dependent defense and the well-characterized antagonism between SA- and JA-dependent defense responses (Kunkel and Brooks 2002; Li et al. 2004; Bostock 2005).

The phenotype of *ERD15* overexpression plants indicating that ABA insensitivity could alter the resistance of *Arabidopsis* to *E. carotovora* by enhancing SA-dependent defense signaling prompted us to study the impact of ABA insensitivity on pathogen responses further. While earlier studies have characterized enhanced SA signaling only in ABA-deficient and not in ABA-insensitive mutants, the BABA-primed accumulation of

callose required in the resistance against certain necrotrophic fungi is impaired in both mutants (Ton and Mauch-Mani 2004), indicating that insensitivity can also affect defense responses. We employed the ABA-insensitive mutants *abi1-1* and *abi1-2* and determined whether the resistance of these plants was altered in response to *E. carotovora*. Interestingly, similar to *ERD15* overexpression plants, both of these mutants displayed improved resistance to this pathogen (III, Fig. 8A). This was accompanied by enhanced induction of *PR1*, a marker for SA-dependent defenses (III, Fig. 8C). These results demonstrate that not only ABA deficiency but also ABA insensitivity can have beneficial effects on resistance to certain pathogens, such as *E. carotovora*, by enhancing SA-dependent defense responses.

ERD15 is a negative regulator of ABA responses in *Arabidopsis*

Our results suggest that ERD15 is a novel, negative regulator of ABA responses in *Arabidopsis* (III, Fig. 9). Overexpression of *ERD15* results in insensitivity to ABA, whereas RNAi silencing leads to a hypersensitive seed germination phenotype in the presence of this phytohormone. The improved drought and freezing tolerance of the *ERD15*-silenced plants can also be explained by ABA hypersensitivity; the response is activated more rapidly, leading to enhanced tolerance. Moreover, in the case of *ERD15* overexpression, the insensitivity to ABA strengthens SA-dependent signaling (III, Fig. 9). This suggests that ERD15 might also be a factor influencing the selection of activation of either ABA- or SA-dependent stress responses. We hypothesized that ERD15 acts as a "capacitor" that prevents plants from responding too quickly after the onset of stress. It prevents stress signaling until it is certain that the stress prevails and the plant´s survival depends on defense activation. Simply put, the role of the capacitor is to ensure that the limited assets are invested in stress adaptation only when it is absolutely essential to do so. Activation of a large-scale defense response consumes plant resources that would otherwise be allocated to such targets as growth or reproduction. It is therefore beneficial for the plant to avoid these fitness costs before it is certain that the stress will prevail and adaptation is necessary. Heil (2002) introduced this concept for biotic stress.

The actual mechanism underlying the influence of ERD15 on ABA signaling can only be speculated without further biochemical experimentation. ERD15 has a PAM2 motif, indicating an interaction with the C-terminus of poly-A-binding proteins (PABP) (Albrecht and Lengauer 2004). Indeed, Wang and Grumet (2004) demonstrated the binding of ERD15 to PABP in a yeast two-hybrid screen. Thus, ERD15 may act by inhibiting translation of certain ABA-regulated transcripts in *Arabidopsis*. Accumulating evidence also suggests a central role for posttranscriptional regulation in ABA signaling (Hugovieux et al. 2001; Himmelbach et al. 2003; Kuhn and Schroder 2003). The recently characterized poly-A-specific ribonuclease AtPARN has been demonstrated to have poly (A) degradation activity in vitro (Chiba et al. 2004). This gene is induced by ABA and SA, and is presumed to be crucial for proper abiotic and biotic stress responses in *Arabidopsis* (Nishimura et al. 2005). ERD15, which also has an impact on both SA and ABA responses, could be involved in the same processes; it has been demonstrated to interact with PABPs that bind poly (A) tails of mRNAs, tails which AtPARN possibly degrades.

However, despite evidence of translational regulation in ABA signaling, a model putting these pieces together has thus far not been constructed. The role of ERD15 also remains elusive.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

The studies presented in this thesis provide novel data about the interaction between *Arabidopsis* and *E. carotovora*. Roles of harpins in the virulence of several plant pathogens, including *P. syringae* and *E. chrysanthemi*, have been investigated, but the role of *E. carotovora* HrpN as a virulence factor has remained uncharacterized until now. This is partly due to the massive maceration of plant tissue brought about by the action of PCWDEs, which potentially masks plant responses induced by effector proteins. Using purified HrpN, we showed that this protein elicits an HR response and triggers resistance to *E. carotovora* infection. The input of HrpN in *E. carotovora*-induced plant defense was further clarified; it induces both SA- and JA/ET-dependent defense signaling in *Arabidopsis*. Thus, HrpN might contribute to the capability of *E. carotovora* to trigger SA-dependent plant defense signaling. However, most probably other, currently unknown effector proteins exist that have an impact on elicitation of plant defense.

Also demonstrated was that the power and efficiency of *E. carotovora* resides not only in the massive production of PCWDEs but also in the parallel use of an effector protein. HrpN and the PCWDE PehA synergistically triggered enhanced cell death but also enhanced plant defense signaling. Future studies will reveal whether the enhanced cell death actually benefits the pathogenicity of *E. carotovora*; as a necrotroph, it does feed from dead plant tissue. Alternatively, the pathogen might not necessarily aim at producing cell death, with cell death instead resulting from the activation of plant defenses in response to pathogen elicitors. *E. carotovora* is nevertheless capable of overcoming this response and infecting the plant. The question of whether or not HrpN is indispensable as an effector for the pathogenicity of *E. carotovora* will be addressed in future studies characterizing the virulence of the *hrpN* mutant. Finally, this work emphasizes that classification of pathogens to certain categories according to their lifestyle does not necessarily mean that members of these groups trigger the same defense pathways. While induction of HR and SA signaling is usually considered to occur in response to biotrophic pathogens (Glazebrook 2005), the necrotroph *E. carotovora* is also capable of inducing this type of defense.

Studies II and III of this thesis characterized the plant defense-related roles of two genes, *AtCLH1* and *ERD15*, both rapidly induced in response to *E. carotovora*. Novel data demonstrating how the transgenic manipulation of these early stress-induced components can benefit (or weaken) plant stress tolerance were also uncovered. In addition, these studies demonstrated signal crosstalk between the defense pathways. First, silencing of

AtCLH1 suggested that the amount of ROS formed in plant cells increases after tissue damage if rapid chlorophyll degradation is impaired. This was shown to be light-dependent. ROS accumulation enhanced SA-dependent signaling and resistance of the plants to *E. carotovora*. Therefore, we speculated that AtCLH1 acts as a damage control enzyme that degrades free chlorophyll molecules in plant injury caused by, for example, pathogen attack.

Second, until now the function of ERD15 in plants was unknown, but our studies placed this gene in the signaling network mediating ABA responses in plants. We hypothesized, that ERD15 is a novel, negative regulator that affects the capability of plants to respond to a stress-related ABA signal. Transgenic modulation of *ERD15* expression influenced the ability of plants to tolerate abiotic as well as biotic stress. Improved pathogen resistance, presumably due to altered ABA sensing, was a further indication of the emerging theme that ABA influences biotic stress responses (reviewed by Mauch-Mani and Mauch 2005).

Third, we were able to demonstrate crosstalk between the plant defense signaling pathways and show that it influences the outcome of defense responses. Silencing of *AtCLH1* enhanced SA-dependent defenses and resistance to *E. carotovora*, but also had an antagonistic effect on JA/ET signaling and resistance to the fungus *A. brassicicola*. Moreover, the modulation of *ERD15* expression introduced crosstalk; it decreased ABA sensitivity of the plants, while SA-dependent defenses and resistance to *E. carotovora* were enhanced.

The long-term objective of the studies presented in this thesis is to enhance the development of stress-resistant crop plants. To this end, consideration of the strengths and weaknesses of AtCLH1 and ERD15 studies in the context of applying them to crop protection would be useful. However, certain facts should be borne in mind. Unlike in the laboratory, in the field plants are challenged with more than one stress at a time, with either combinations of different biotic stresses or situations where simultaneous biotic and abiotic stresses occur. Rendering the situation even more complex is the crosstalk between stress signaling pathways. Moreover, in the case of induced defenses, one must consider different fitness costs (Heil and Baldwin 2002). Studies characterizing the role of AtCLH1 and ERD15 in plant stress responses highlight different aspects that influence the total effectiveness of induced resistance. Often there is only a fine line between overall cost and benefit; improving one characteristic easily impairs another. Silenced AtCLH1 activity exemplifies a tradeoff between defenses; resistance against one group of pathogens might be detrimental if the plant is attacked by another group. For *AtCLH1*-silenced plants, the enhanced SA defense and resistance to *E. carotovora* also had a reverse side. Wild-type *Arabidopsis* cannot be infected by the fungus *A. brassicicola* since it has functional JA defenses to resist this pathogen. However, in plants with silenced *AtCLH1*, the resistance to *A. brassicicola* was compromised, indicating antagonistic crosstalk between SA- and JA-dependent defenses. Thus, in the situation where plants are under threat from different pathogens, improving only SA-dependent defenses would be insufficient. An interesting topic for future research would be determining whether the modulation of *AtCLH1* activity influences abiotic stress tolerance of transgenic plants.

Autotoxicity costs can be brought about by a continuously expressed resistance trait (Heil and Baldwin 2002); these are costs that establish a metabolic burden on the plant and have physiological effects that manifest in the plant morphology. For example, *Arabidopsis* mutants expressing constant SA (*cpr1*) or JA (*cev1*) defenses often have stunted growth phenotypes (Bowling et al. 1994; Ellis and Turner 2001), indicating that suppression of defenses in enemy-free conditions is beneficial. Continuous accumulation of ROS can be toxic to the plants and could result in physiological effects, including lesion formation. This has been observed in chlorophyll biosynthesis mutant *acd2* plants (Greenberg and Yao 2004). However, in *AtCLH1*-silenced plants, the increase in oxidative stress and the subsequent activation of defense genes only occur when needed – after pathogen invasion. Once the stress is over, ROS levels return to normal and the plant does not suffer from continuous metabolic imbalance. Thus, the phenotype of *AtCLH1*-silenced plants is normal, and they grow and produce seeds normally. In conclusion, while the manipulation of chlorophyll catabolism by silencing *AtCLH1* has certain costs for the plant (reduction in JA defenses), it may offer an elegant means of enhancing the tolerance of agronomically important plant species to pathogens, like *E. carotovora*, that can be controlled by SA-dependent responses.

Studies of ERD15 reveal crosstalk between abiotic and biotic stress signaling. *ERD15* overexpression resulted in diverse outcomes with regard to tolerance to drought and pathogen stress. SA-dependent defense signaling, and hence, resistance to *E. carotovora* was improved. However, at the same time, the plants paid a considerable price for this in the form of severely impaired tolerance to two abiotic stresses, drought and freezing, which arose from decreased ABA sensitivity. Silencing of *ERD15* clearly improved plant tolerance to these abiotic stresses, while the susceptibility of these plants to *E. carotovora* was only moderately increased. This raises the question of how moderate abiotic stresses, such as mild drought, commonly encountered in crops during the growing season influence the capability of plants to induce pathogen resistance, or vice versa.

Allocation costs can occur if large quantities of fitness-limiting resources are allocated to resistance (Heil and Baldwin 2002). Thus, induction of defenses is always a tradeoff with some alternative target for energy, such as growth and reproduction. Activating defense responses only when it is absolutely necessary for the survival of the plant is therefore critical. In this context, ERD15 is hypothesized to act as a capacitor that guards the use of plant resources; it delays the ABA signal, and thus, activation of defenses, until the stress has continued for a certain period of time and exceeds the negative regulation. Only then is the inhibition relieved and the stress signaling aimed at initiating adjustive responses induced. Further studies are needed to characterize the specific gene products “guarded” by ERD15 and to figure out how this is executed at a molecular level.

A topic for future research would be to determine whether pathogen resistance or drought tolerance of agronomically important species, such as potato could be altered by modulating the expression of *AtCLH1* or *ERD15*. Also awaiting elucidation is whether the effects observed on stress tolerance under laboratory conditions hold true in the field.

ACKNOWLEDGMENTS

This study was carried out at the Department of Biological and Environmental Sciences, Division of Genetics, University of Helsinki, in the Viikki Biocenter. Funding was provided by the Academy of Finland and the Viikki Graduate School of Biosciences.

I am grateful to my supervisors, Professor Tapio Palva and Docent Günter Brader. I thank Tapio for giving me the opportunity to do my Master´s work in his group and for then enabling me to continue with a PhD thesis. His guidance, ideas, and encouragement over these years have been invaluable. I am also indebted to Günter; without his guidance and his brilliant scientific mind finishing this thesis would have been much more difficult.

I am thankful to Professors Katri Kärkkäinen and Teemu Teeri for kindly reviewing this thesis. Carol Ann Pelli is acknowledged for editing the English language of the manuscript.

I thank all present and former members of Tapio Palva´s, Pekka Heino´s, Hannu Saarilahti´s, and Jaakko Kangasjärvi´s groups. My warmest thanks are due to my friends and colleagues Elina Helenius, Günter Brader, Solveig Sjöblom, Roosa Laitinen, Hanne Mikkonen, and Jorma Vahala with whom I have shared good moments during these years. I am also grateful to all plant-pathogen interaction people, especially Jing Li, Karen Sims-Huopaniemi, and Leila Miettinen, as well as the personnel on 5th floor; thank you for all the support and help over the years. I've enjoyed working with all of you! Pekka Heino and Hannu Saarilahti are also thanked for advising me about many practical matters related to this thesis as well as for giving sound scientific advice. I am grateful to Arja Ikävalko and Arja Välimäki for all generous help with all manner of things.

My friends Reetta Kariola, Heikki Vilen, Mari Kaunisto, Hanna Kivelä, and Tuula Siljander are warmly thanked for friendship and the good times that we have shared.

I am particularly indebted to all of my friends outside science, especially Petra and Juuso, Ninni and Heka, Kikka and Kari, Pasi, Timo and Minna, and Jarkko and Minna for their friendship. Thank you also for not asking too often that when will I finally finish my thesis☺.

My heartfelt thanks go to my father Reijo, my late mother Anna-Liisa, and my brother Risto and his family. Their endless support and faith in me as well as my father's continuous advice "Ota rauhallisesti" have helped me greatly particularly in moments of doubt.

Most of all, I thank my husband Kimmo, whose love, encouragement, and sense of humor are by far the most important things in my life.

Kotalahti, July 2006

Tarja Kariola

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