# Identification of Key *Arabidopsis* genes required for resistance against *Botrytis cinerea*

**Pedro Luiz Nurmberg**

**Doctor of Philosophy**

**Institute of Molecular Plant Science**

**School of Biological Science**

**The University of Edinburgh**

**September 2005**

# CONTENTS

# Declaration

I hereby declare that the work presented here is my own and has not been submitted in any form for any degree at this or other university.

I would also like to acknowledge Andrea Chini and Eleanor Gilroy for their contribution and help in the laborious, time-consuming mutant screen.

# Publications arising from this project

**Loake, G., and Nurmberg, P.** (2003). Role of Jasmonates in Growth, Development and Defence. (Ed. Murphy, D.M and Thomson, W.).

**Tani, H., Chen, X., Nurmberg, P., Grant, J.J., SantaMaria, M., Chini, A., Gilroy, E., Birch, P.R., and Loake, G.J.** (2004). Activation tagging in plants: a tool for gene discovery. Funct. Integr. Genomics **4,** 258-266.

**Nurmberg, P.L.; Yun, B.W., Hudson, A., Morris, P., Loake, G.** The dorsoventral factor *AS1* is an evolutionary conserved regulator of the plant immune response. (in preparation).

**Nurmberg, P.L., Yun, B.W., Loake, G.** *AS1* is involved in *R* gene-mediated resistance against *Pseudomonas syringae* DC3000. (in preparation).

**Sun, X.; Chini, A., Gilroy, E., Nurmberg, P.L., Loake, G.** *ADS1* encodes a MATE efflux protein involved in resistance against a wide range of plant pathogens. (in preparation)

# Acknowledgements

I would like to acknowledge in particular my supervisor Dr. Gary Loake for his advices and significant input into my PhD.

I also would like to thank Gabriela, my family and friends for being supportive and helpful along of these years.

In addition, I would like to say 'thanks' to all current and former members in Loake group, especially Andrea, Angela, Eleanor, Eun Jung, Hang, Hazel, Jeong-Gu, Joonseon, Lena, Lufei, Reza, Tanja, Xinli and Wook, for their contribution during the course of my PhD and also for making the lab such a nice and cheerful place.

Thanks as well to Dr. Peter Morris and Ben Mewburn for helping with the ethylene and SA/camalexin analysis, respectively.

My acknowledgements to CNPq (Brazil) and ORSAS (UK) for funding my PhD, and James Rennie Bequest and MolBio fund for the travel funds to attend conferences.

# Abstract

Despite the significant progress achieved in molecular biology in the last few years, our knowledge about the mechanisms of plant resistance against necrotrophic and non-host pathogens is still rudimentary. Here, we report the isolation and characterization of three *Arabidopsis* mutants selected for altered resistance against *B. cinerea*. Mutations in the *I*ncreased *B*otrytis *R*esistance (*IBR1*) gene resulted in significant resistance against *B. cinerea* and *A. brassicicola*, another necrotrophic fungus. Interestingly, *ibr1* plants also exhibited enhanced susceptibility to the host and non-host bacterial pathogens *P. syringae* pv. *tomato* and *P. fluorescens*, respectively. Conversely, resistance against *B. graminis* f. sp. *tritici* and *E. cichoracearum* was not affected, suggesting *IBR1* is required for resistance against some, but not all pathogens. By TAIL-PCR *IBR1* gene was found to be allelic to *asymetric leaves 1* (*as1*). *AS1* belongs to the *R2R3* subfamily of the MYB-domain and has been extensively studied because of its central function in leaf development. Interestingly, neither *as2* or *erecta* mutants or the conditional line *KN1* are affected in resistance against *B. cinerea* or *Pst*DC3000. These results suggest that *as1*-mediated disease resistance and susceptibility is independent from the *AS1*-dependent pathway regulating leaf development. Moreover, we show that *AS1* function in disease resistance & susceptibility is conserved in at least three evolutionary divergent plant species, suggesting *AS1* function in disease resistance is relatively ancient. The analysis of a substantial series of *as1* double mutants in *Arabidopsis* revealed that both the production and perception of jasmonate (JA) and ethylene (ET) are required for *as1*-mediated disease resistance against necrotrophic pathogens. Moreover, the expression of JA/ET-dependent defence genes was shown to be accelerated in *as1* plants in response to *B. cinerea* challenge. While *as1* resistance against necrotrophic pathogens seems to be associated with enhanced expression of JA/ET-dependent genes, susceptibility against the different strains of host and non-host bacterial pathogens occurred in the presence of normal *PR-1* expression and salicylic acid accumulation, suggesting susceptibility towards bacterial pathogens is not associated with defects in the activation of SA signalling pathway. In sum, our findings indicate that *AS1* is a negative regulator of resistance against necrotrophic fungi and a positive regulator of non-host resistance and basal protection against bacterial pathogens in both *Arabidopsis* and other plant species. In contrast to *ibr1*, the *ibr2* and *ebs1* (*Enhanced Botrytis susceptible 1*) mutants seem to specifically affect resistance against *B. cinerea*. The *ibr2* was mapped to a short interval on chromosome five. Despite *ibr2* cloning is not accomplished, we speculated that the mutation is in an alpha-tubulin gene because *ibr2* plant morphology resembles the phenotype of the previously characterized *lefty1* and *lefty2* mutants, which also encode alpha-tubulins. Our speculation is supported by the presence of two alpha-tubulins in the mapped region on chromosome 5, close to the predicted location of *ibr2*. The *Botrytis* susceptible line *ebs1* co-segregates with basta resistance, indicating that the mutated gene is tagged. However, the T-DNA inserted contains additional sequences on its left border that prevented successful cloning. The e*bs1* plants show impaired resistance against *B. cinerea,* but unaffected resistance against virulent and avirulent strains of *Pst*DC3000, suggetsing *EBS1* plays a more specific role in resistance against necrotrophic pathogens. Further characterization of *ibr2* and *ebs1* is required in order to elucidate their role in plant disease resistance.

# Abbreviations

| | |
|---|---|
| 4x*35S* | tetramer of *CaMV 35S* enhancer element |
| *Adr1* | *Acquired disease resistance 1* |
| *Ads1* | *Acquired disease susceptibility* |
| *as1* | *assymetric leaves 1* |
| *avr* | avirulence gene |
| BLAST | Basic Local Alignment Search Tool |
| BTH | benzothiadiazole |
| bZIP | Basic Leucine Zipper |
| CaMV | Cauliflower Mosaic Virus |
| CC | Coiled-Coil |
| CDPK | Calcium-Dependent Protein Kinases |
| CNL | CC-NBS-LRR |
| DEX | dexamethasone |
| DNA | deoxyribonucleic acid |
| *EBS* | *Enhanced Botrytis Susceptibility* |
| ET | Ethylene |
| GST | Glutathione S-Transferase |
| HR | Hypersensitive Response |
| HSP | Heat Shock Protein |
| *IBR* | *Increased Botrytis Resistance* |
| ISR | Induced Systemic Resistance |
| JA | Jasmonic Acid |
| KB | King's Broth media |
| LUC | luciferase |
| LRR | Leucine-Rich Repeat |
| MAPK | Mitogen Activated Protein Kinase |
| MEME | Multiple Expectation Maximisation for Motif Elicitation |
| Me-JA | Methyl Jasmonate |
| MS | Murashige and Skoog media |
| NBS | Nucleotide-Binding Site |
| PCR | Polymerase Chain Reaction |
| PDF | plant defensin |
| *Pst* Dc3000 | *Pseudomonas syringae* pv *tomato* DC3000 |
| PR protein | Pathogen Related protein |
| *R* gene | Resistance gene |
| ROI | Reactive Oxygen Intermediate |
| ROS | Reactive Oxygen Species |

| | |
|---|---|
| SA | Salicylic Acid |
| SAR | Systemic Acquired Resistance |
| SSC | Solution of Sodium Citrate |
| TAIL-PCR | Thermal Asymmetric Interlaced PCR |
| TAIR | The *Arabidopsis* Information Resource |
| TIR | Toll/Interleukin-1 Receptor |
| TNL | TIR-NBS-LRR |

# List of Figures

# List of Tables

# 1 Introduction

It is expected that human global population will rise up to 8 billion by 2025 and up to 10 billion by 2050, which means that in less than half a century the global population will almost double in size (Huang *et al.*, 2002a). Hence, despite the significant advances achieved in food production, we must continue attempting to develop novel plant varieties with new traits. For example, tolerance to biotic and abiotic stresses. We will then be able to produce sufficient food for all. Of course, however, we must take into account the problem of unequal distribution. In addition, we have to consider the demand for better quality of food, which is also an increasing requirement.

Since antiquity, a major hindrance for improving crop productivity has been disease susceptibility, which accounts for at least 12.7% of pre-and post-harvested global crop losses (Agrios, 1997). Considering that most arable land has already been incorporated into the productive system, the extra food required by the increasing population has to come from improvements made in areas like soil fertility and plant nutrition, crop protection and plant breeding. Plant disease can be controlled by chemical treatments, however, their application can be compromised by adverse environmental conditions and efficiency affected by resistant pathogen strains (McDowell and Woffenden, 2003). Moreover, it is an expensive measure, not easily accessed in developing countries and its inappropriate use results in significant environmental pollution, and intoxication of humans and animals. Therefore, plant breeders have been continually engaged in the development of new varieties genetically resistant to the adverse conditions, such as diseases.

Plant breeding techniques promoted an extraordinary increase in crop yield in the last century and will certainly remain as a major pivot for food production in future. The methodologies used have been updated and are efficient for a variety of species and different purposes, being of crucial importance for our society. Biotechnology associated to traditional plant breeding has a great potential for the future of agriculture. While, still rejected in most countries across Europe, use of genetically modified (GM) crops continues to grow in other continents for the ninth consecutive year. In 2004 there was a 20% increase in global area compared to 2003, totalling 81.0 million hectares (James, 2004). This expansion in area cultivated with GM crops is due, along with other factors, to an

extra 1.25 million new farmers, 90% of which are resource-poor farmers from developing countries.

This frank expansion of genetic engineered varieties reflects the substantial advantages in terms of productivity, reduced environmental pollution, cost cutting and health. For example, in China, use of *Bt* (*Bacillus thuringiensis)* transgenic cotton varieties carrying a microbial gene which conveys resistance to insects allowed up to 80% reduction in pesticide applications and a 25-30% cutting of cost of production (Huang *et al.*, 2002a; Huang *et al.*, 2002b). In 2004 there were 14 countries growing 50,000 hectares or more of transgenic crops from which nine were in the developing world and five from industrial countries (USA, Argentina, Canada, Brazil, China, Paraguay, India, South Africa, Uruguay, Australia, Romania, Mexico, Spain and Philipines) (James, 2004).

## 1.1 Plant-pathogens interactions

Continuous interaction between plants and pathogens favoured the evolution of complex resistance networks. The primary barrier that pathogens have to breach in order to cause disease is the plant surface, which shows a diversity of structure and defence compounds. The amount and composition of wax and cuticle that cover the epidermal cells, the structure and thickness of epidermal cell wall, the location, size and shape of stomata and lenticels are some of the factors that will interfere with resistance at the first stage. These pre-formed barriers are present independent of attempted pathogen ingress (Agrios, 1997). Wax, like thick layers of trichomes form a water repellent surface, preventing the formation of a film of water, which is needed for spore germination of many species of fungi and is also vital for many bacterial phytopathogens. Plants also synthesize, and exude a series of compounds which help preventing pathogen ingress.

In addition to pre-formed barriers, a plethora of inducible defence responses is activated following pathogen recognition (Glazebrook, 2001). One of the first steps in the plant defence response is the generation and accumulation of reactive oxygen species (ROS) at sites of infection (Grant and Loake, 2000). Nitric oxide (NO) also accumulates concomitantly with the oxidative burst (Delledonne *et al.*, 1998). Moreover, synchronized accumulation of ROS and NO is required for the development of the hypersensitive response (HR) (Delledonne *et al.*, 2001), a programmed cell death programme deployed by plants in order to stop pathogen spread (Dangl *et al.*, 1996; Jones and Dangl, 1996). Biotrophic pathogens require living host cells to survive while necrotrophic pathogens kill host cells to thrive on released nutrients. Therefore, the efficiency of HR as a defence mechanism will depend on the nature of the parasite, whether it is necrotrophic or biotrophic. In fact it has been shown that HR facilitates infection by the necrotrophic pathogen *Botrytis cinerea* on *Arabidopsis* plants (Govrin and Levine, 2000).

Pathogen recognition is normally associated with the expression of a given resistance (*R*) gene in the host plant together with its corresponding avirulent (*avr)* gene in the pathogen (Flor, 1971). This type of resistance, also called gene-for-gene resistance, is the most well understood genetic mechanism of defence in plants and will be discussed in more detail in

the next session. Subsequent to pathogen recognition, defence signalling molecules rapidly accumulate both locally and systemically. At least two independent defence signalling pathways have been reported, one of which is salicylic acid (SA) dependent and the other a jasmonic acid (JA) /ethylene (ET) dependent pathway (Thomma *et al.*, 1998; Ton *et al.*, 2002; Glazebrook *et al.*, 2003; Shah, 2003). Details of these signalling molecules and their roles in the defence response will be commented on item 1.2 of this introduction.

### 1.1.1 Gene-for-gene resistance and the guard cell hypothesis

In controlling plant diseases, one of the most successful means is the development of varieties genetically resistant to pathogen attack. Mankind has since the pre-historic period involuntary selected those lines genetically more resistant to diseases, pests and other biotic and abiotic adverse conditions. Nowadays, breeding for disease resistance is one of the most important goals of plant breeding programmes across the different continents. Specific or vertical resistance is easily manipulated in breeding programmes and therefore has been the most commonly used in crop species. The effectiveness of gene-for-gene resistance depends on the existence of matching pairs of R genes and *avr* genes in the host and parasite, respectively (Flor, 1971). If either *R* or *avr* gene is missing the plant host will be unable to recognise the invading pathogen resulting in disease, see Figure 1.1.1.

During the 1990s genes conveying resistance to bacteria, viruses, fungi, nematodes and even pest insects were cloned. However, despite the broad diversity of parasites with different life-styles, R genes cloned so far are classified into only five different classes (Figure 1.1.2) (Dangl and Jones, 2001; Jones, 2001). *Class 1*- NB-LRR are cytoplasmic proteins that are thought to function exclusively as R gene products. This class can be sub-divided into those gene possessing homology in their amino terminus to the Toll and Iterleukin-1 receptor (TIR) (the TIR-NB-LRR family) and those which do not, most of which carry a putative coiled coil (CC) at their terminus (the CC-NB-LRR family) (Jones, 2001). The LRR repeat domain is found in many proteins and is involved in protein-protein interactions, peptide-ligand binding and protein-carbohydrate interactions (Jones and Jones,

1996; Kobe and Kajava, 2001). It has been shown that the NB domain binds to ATP rather than any other nucleoside triphosphate (Tameling *et al.*, 2002).

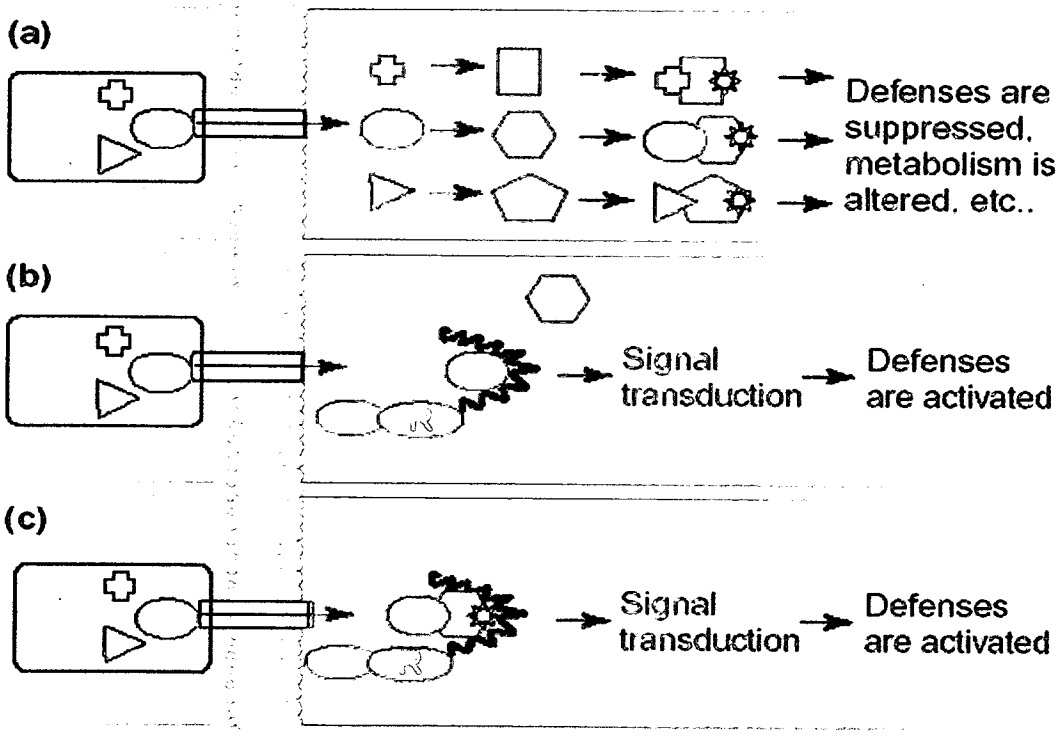


**Fig. 1.1.1 Interactions between pathogen Avr proteins and plant R proteins**. A hypothetical pathogen (grey) has attached to a plant cell and is expressing a suite of virulence proteins (red). These proteins are translocated into plant cells via Type III secretion (bacteria) (Nimchuk *et al.*, 2001) or other unknown mechanisms (fungi and oomycetes). Once inside, they target host proteins (green) that control defense responses, metabolism or other plant process that affect pathogen virulence (note that virulence proteins could also be targeted towards extracellular proteins). (a) In this panel, the plant cell does not express an R protein that is capable of recognizing any virulence protein. Thus, the plant cannot detect the pathogen efficiently and defenses are, at best, only weakly induced. Disease then results from the collective action of the virulence proteins. (b) This panel depicts the classic receptor–elicitor hypothesis, in which an R protein directly binds a virulence protein. This recognition event activates a complex signal transduction network, which in turn triggers defense responses. (c) This panel depicts the guard hypothesis, in which an R protein (guard) detects a modified host protein (guardee, red star), perhaps as a complex with the 'attacking' virulence protein. Source: **McDowell and Woffenden (2003).**

Members of TIR-NB-LRR gene family function through an *EDS1* dependent signalling pathway, while CC-NB-LRR are *NDR1*-dependent, with some exceptions (Aarts *et al.*, 1998). For example, *RPP8* does not require either *EDS1* or *NDR1* in isolate-specific resistance to *P. parasitica*, although resistance is weakly compromised by *eds1*. Similarly, the *EDS1*-dependent *RPP4* and *RPP5* mediated resistance is partially affected by *ndr1*

mutation. EDS1 (enhanced disease susceptibility) encodes a plant specific lipase-like protein (Falk *et al.*, 1999). NDR1 (non-race specific disease resistance) encodes a glycosylphosphatidylinositol (GPI) anchored protein (Holt *et al.*, 2003). Functions of *EDS1* and *NDR1* in disease resistance will be explored in more details together with non-host resistance SA-dependent responses later in this review.

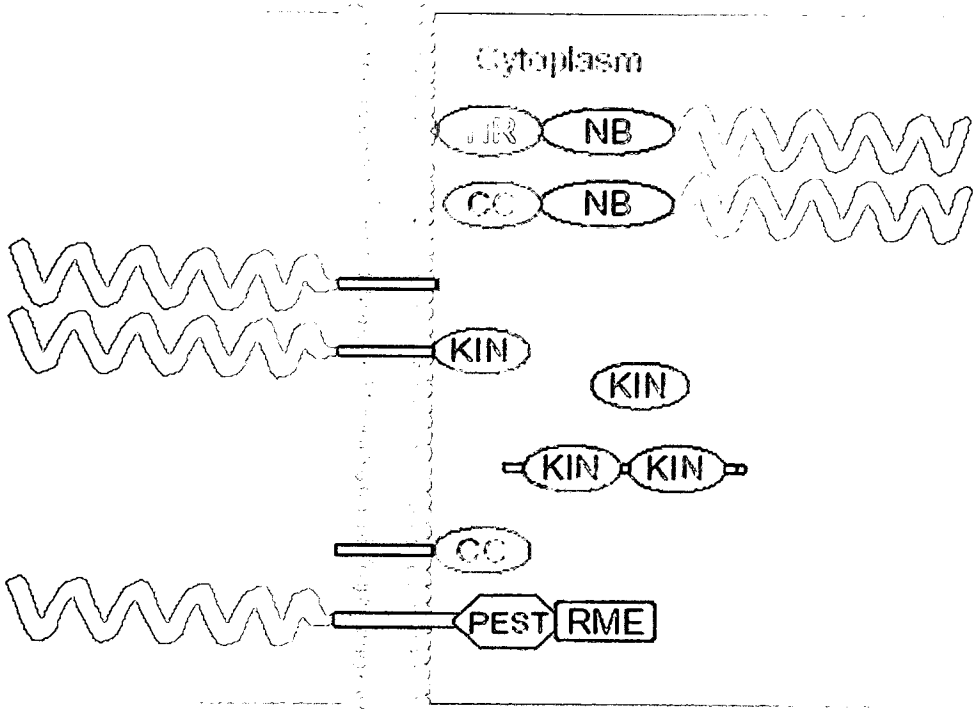


**Figure 1.1.2 Major families of R proteins**. The majority of R proteins contain tandem leucine-rich repeats (LRRs, depicted in blue), which have a major role in recognition specificity (Jones, 2001). Members of the most widely represented R protein family (NBLRR) also contain a nucleotide-binding site and a region of similarity to proteins that regulate programmed cell death in metazoans (Dangl and Jones, 2001; Kachroo *et al.*, 2001). NB-LRR proteins are likely localized in the cytoplasm, perhaps as peripheral membrane proteins. Some NBLRR proteins contain a TIR domain with homology to the metazoan superfamily of Toll-like innate immunity receptors. Other NB-LRR proteins contain a putative coiled-coil domain (CC) at the N-terminus. The eLRR protein family consists of extracytoplasmic leucine-rich repeats, anchored to a transmembrane domain. The LRR-kinase superfamily consists of an eLRR fused to a cystoplasmic serine-threonine kinase domain (KIN). One resistance protein (tomato Pto) is a Ser-Thr kinase, without LRRs, and another (maize Rpg1) contains two kinase domains. The Arabidopsis RPW8 protein contains a membrane anchor, fused to a putative coiled-coil domain (CC). The tomato Ve1 and Ve2 proteins contain putative extracellular LRRs, along with a PEST domain for protein degradation (found only in Ve2, and not Ve1), and short proteins motifs that might target the protein for receptormediated endocytosis (RME). Source: **(McDowell and Woffenden, 2003)**

The other four and less abundant classes of *R*-genes encode proteins with diverse structure and function, some of which have demonstrated functions in cellular and developmental processes unrelated to defence (Dangl and Jones, 2001)]. *Class 2*: Represented by the tomato *Pto* gene, possibly the most well studied *R*-gene product, encodes a Ser/Thr kinase protein and conveys resistance to strains of *Pseudomonas syringae* pv tomato (*Pst*) carrying the avirulence gene *avrPto* (Martin *et al.*, 1993). A possible direct interaction between *Pto-avrPto* is likely to activate a phosphorylation cascade (Tang *et al.*, 1996). *Pto* function requires the NB-LRR protein *Prf* (Salmeron *et al.*, 1996) and these two gene products are thought to function in combination as a bi-partite switch (Rathjen, personal communication). *Class 3*: Another tomato *R*-gene, the tomato *Cf-9* encodes a single pass transmembrane protein with an extracellular LRR domain (Jones *et al.*, 1994). *Class 4*: The *Xa21* from rice conveys resistance to the race 6 of *Xanthomonas oryzae* pv oryzae. *Xa21* encodes a transmembrane receptor with a large extracellular LRR domain and an intracellular protein kinase domain (Song *et al.*, 1995). *Class 5*: In this groups is the the recently cloned *RPW8* gene from *Arabidopsis*, which has no homology to any other previously described class of *R-gene* and encodes a small putative transmembrane coiled-coil protein (Xiao *et al.*, 2001).

The gene-for-gene model predicted a direct interaction between receptor (product of an *R* gene) and elicitor (*Avr* protein). Because this direct interaction was only demonstrated in two cases (Tang *et al.*, 1996; Jia *et al.*, 2000) another model has been proposed (the guard cell hypothesis) in which the *R* protein interact with another plant protein (the guardee) activating resistance (Van der Biezen and Jones, 1998). In this case, the guardee is targeted by the *Avr* protein and resistance is activated when the *R* protein detects *Avr* binding to or modification of the targeted protein. Evidence based on *Pto, AvrPto* and Fen interaction (PTO the target, Fen the guardee) supporting the second hypothesis was found for the *Arabidopsis Rpm1-avrRpm1* and *Rps2-avrRps2* interactions (Mackey *et al.*, 2002), and also between *Rpm1-avrRps2* (Mackey *et al.*, 2003). In addition it was shown that RPS2 and RIN4 physically interact in *Arabidopsis* and that delivery of avrRPS2 in the plant releases RIN4 and thus indirectly activates RPS2 (Axtell and Staskawicz, 2003; Mackey *et al.*, 2003). Recently, it was demonstrated that avrRps2 is delivered into the plant cells as an inactive protein, which is then activated by the plant *CPR1,* a single-domain cyclophilin

peptidyl-prolyl cis-trans isomerase (PPIase) (Coaker *et al.*, 2005). Nevertheless, it seems that the deployment of one or another recognition system is likely to be on a case-by-case basis.

## 1.2 Plant defence signalling Pathways

The plant defence response is mediated through a complex network of signalling pathways, with frequent crosstalk and antagonism. Activation of one signalling pathway or another depends on the nature of the invading pathogen. Thus, biotrophic pathogens are believed to activate a signalling pathway that is SA-dependent, while necrotrophic pathogens are responsible for activating a SA-independent and JA/ET-dependent (Thomma *et al.*, 1998).

### 1.2.1 Salicylic acid (SA)-dependent disease resistance

SA has been implicated in many aspects of the plant defence response against invading pathogens. However, the mechanisms by which SA accumulates and modulates defence activation are still not well characterized, although it appears that SA acts as a signal amplifier (Shirasu *et al.*, 1997). SA rapidly accumulates both locally and systemically following attempted pathogen attack (Malamy *et al.*, 1990; Metraux *et al.*, 1990; Ryals *et al.*, 1996). Recognition of an elicitor (pathogen avirulence gene product) by the corresponding *R* gene leads to a rapid response at the site of infection named the hypersensitive response -HR (Jones and Dangl, 1996). HR, a mechanism deployed by host plants to restrict pathogen spread, precedes the activation of a systemic resistance against a broad spectrum of pathogens termed systemic acquired resistance (SAR) (Uknes *et al.*, 1992). Salicylic acid has long been recognised as one of the essential molecules for the induction of SAR (Gaffney *et al.*, 1993; Lawton *et al.*, 1995).

Pathogen induced accumulation of SA correlates with induction of SAR marker genes such as *PR1*, *PR2* and *PR5* (Metraux *et al.*, 1990; Ryals *et al.*, 1996). Moreover, exogenous application of SA or its chemical analog 2,6-dichloroisonicotinic acid (INA) (Ward *et al.*,

1991) or benzotiadazole (BTH) (Gorlach *et al.*, 1996) also cues defence gene induction and SAR activation. Moreover, transgenic *nahG* plants, that constitutively express a salicylate hydroxylase gene, are unable to accumulate SA. The active salicylate hydoxylase enzyme degrades SA into catechol (Gaffney *et al.*, 1993). These plants are not only depleted in SA accumulation, but also are unable to activate SAR. Depletion of SA accumulation also compromises gene-for-gene resistance (Delaney *et al.*, 1994). Grafting experiments using transgenic *nahG* plants demonstrated that SA is not the translocating SAR-inducing signal as previously speculated, even though reciprocal graft experiments showed a requirement for SA in challenged tissue (Vernooij *et al.*, 1994). The *non-expresser of PR1 (npr1) Arabidopsis* mutant is non-responsive to SA and shows reduced expression of PR proteins and enhanced susceptibility to the fungal pathogen *Hyaloperonospora parasitica* and the bacterial pathogen *Pseudomonas syringae* pv *tomato* DC3000 (Cao *et al.*, 1998). *NPR1* encodes a protein with a BTB/BOZ domain and an ankyrin repeat domain, which are involved in protein-protein interactions. *NPR1* was shown to translocate to the nucleus, following SA accumulation, where it interacts with leucine zipper (bZIP) transcription factors (Spoel *et al.*, 2003). bZIP transcription factors are involved in SA-dependent *PR* gene expression. bZIP transcription factors have been shown to specifically bind sis-elements in the *PR1* promoter and to be responsible for SA-mediated gene induction (Lebel *et al.*, 1998).

Moreover, mutations in the *SID2* (*Salicylic-acid induction defficient2*) gene of *Arabidopsis* abolish SA accumulation and compromise resistance against a range of biotrophic pathogens and also the non-host fungal pathogen *Uromyces vignae* (Nawrath and Metraux, 1999; Mellersh and Heath, 2003). *SID2* encodes a putative chloroplast-localized isochorismate synthase (ICS) which catalyzes the conversion of chorismate to isochorismate. By analogy to bacterial SA biosynthesis it is assumed isochorismate is converted to SA by the enzyme isochorismate pyruvate lyase. Exogenous application of SA restores resistance confirming involvement of *SID2* in SA biosynthesis. Furthermore, expression of *SID2* is induced by pathogen attack and also in tissue expressing SAR (Nawrath and Metraux, 1999; Wildermuth *et al.*, 2001).

A series of other mutants affected in SA-dependent resistance have been uncovered. The mutants *eds1*, *pad4* and *ndr1* (non-race specific disease resistance) are examples of mutants compromised in specific or basal resistance. Both *EDS1* and *PAD4* expression are enhanced by exogenous application of SA as part of a positive feedback loop important for signal amplification, while *ndr1* mutation blocks ROS-dependent SA accumulation (Zhou *et al.*, 1998; Falk *et al.*, 1999; Jirage *et al.*, 1999; Shapiro and Zhang, 2001; Shirano *et al.*, 2002; Shah, 2003; Xiao *et al.*, 2003). Both *EDS1* and *PAD4* encode plant specific lipase-like proteins, while *NDR1* encodes a glycosylphosphatidylinositol (GPI) anchored protein (Century *et al.*, 1997; Falk *et al.*, 1999; Jirage *et al.*, 1999). involving the processing of ROI-derived signals around the site of infection (Feys *et al.*, 2001; Rusterucci *et al.*, 2001).

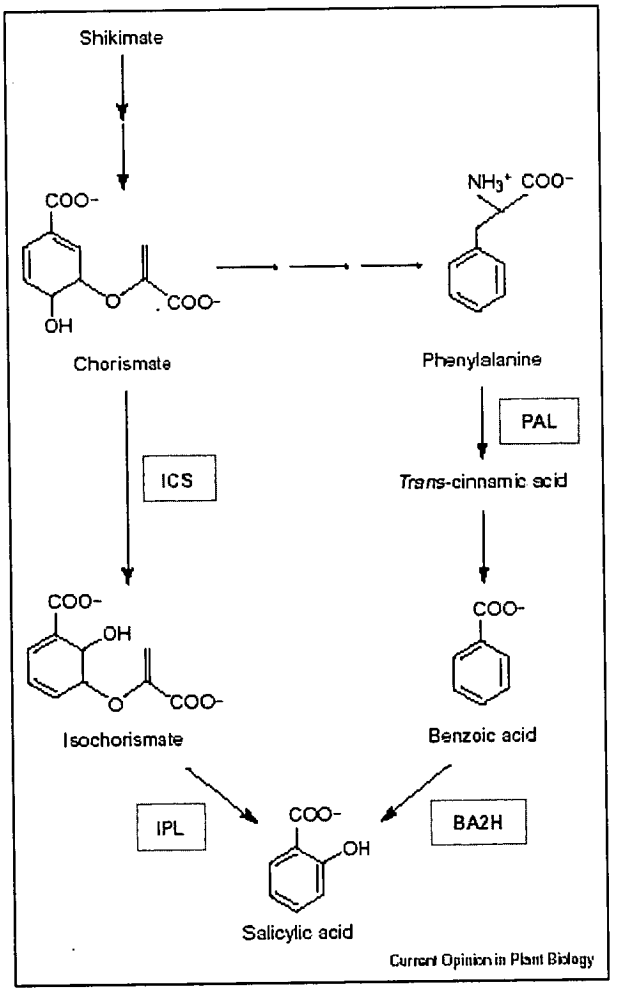


**Figure 1.2.1 Proposed pathways for the biosynthesis of SA in plants.** The shikimate pathway provides chorismate, which can be converted into SA. The main chorismate pool occurs in the chloroplast. The Arabidopsis *SID2* gene encodes a predicted ICS that has a putative plastid-transit sequence. The *SID2*-encoded ICS is proposed to catalyze the conversion of chorismate to isochorismate, presumably in the chloroplast. By analogy to the mechanism of SA biosynthesis in bacteria, it is suggested that an IPL catalyzes the conversion of isochorismate to SA. An alternative pathway that has been studied in tobacco synthesizes SA from phenylalanine via benzoic acid. Phenylalanine ammonia lyase (PAL) catalyzes the first step in this pathway, which is the conversion of phenylalanine to trans-cinnamic acid. Trans-cinnamic acid is subsequently converted into benzoic acid. A benzoic-acid-2- hydroxylase (BA2H) catalyzes the final step, the conversion of benzoic acid to SA. **Source** (Shah, 2003)

EDS1 and PAD4 proteins interact *in vivo* with each other in soluble cell extracts of healthy plants, showing a pre-existing EDS1-PAD4 interaction, with the amount of this complex increasing with pathogen attack (Feys *et al.*, 2001). The EDS1-PAD4 complex is shown to play a central role in basal resistance to invasive biotrophic and hemi-biotrophic pathogens and to be required for TIR-NB-LRR protein-triggered resistance in different plant species (Aarts *et al.*, 1998; Feys *et al.*, 2001; Liu *et al.*, 2002; Peart *et al.*, 2002; Hu *et al.*, 2005; Wiermer *et al.*, 2005). *EDS1* acts in early stages of TIR-NB-LRR resistance siganlling, upstream of the oxidative burst, and in combination with *PAD4,* exerts a key role in SA accumulation and potentiation of disease resistance involving the processing of ROI-derived signals around the site of infection (Feys *et al.*, 2001; Rusterucci *et al.*, 2001).

Another component of the EDS1-PAD4 interaction complex, SAG101, was recently identified in healthy leaf extracts from tagged EDS1 using an affinity-purification approach (Wiermer *et al.*, 2005). Analysis of *Arabidopsis sag101* and *pad4* double mutants revealed involvement of *SAG101* in TIR-NB-LRR *R*-gene mediated resistance and basal protection. Moreover, these double mutants supported growth of a non-host powdery mildew even more extensively than that allowed by *eds1* plants (Wiermer *et al.*, 2005). These results suggest that, besides a fundamental role in *R*-gene mediated defence *EDS1-PAD4*, also play an important role in basal and non-host disease resistance.

Mutant screens identified several genes that negatively regulate the *EDS1* pathway. Analyses of double mutants with *lesion simulating disease1* (*lsd1*), a gene encoding a zing finger protein acting as a ROI modulator, showed a dependency for *EDS1-PAD4* in run away cell death (Mateo *et al.*, 2004). Involvement of *LSD1* in plant cell death will be further discussed in the next session. Inactivation of *MAP Kinase 4* (*MAP4*) showed this gene negatively regulates SA accumulation and resistance mediated through the *EDS1-PAD4* signalling pathway. *AtMPK4* also positively regulates activation of the JA dependent defence response. Therefore *AtMPK4* maybe a node between the two signalling networks (Petersen *et al.*, 2000).

In addition to mutants compromised in SA signalling, some mutants constitutively expressing *PR* genes and displaying enhanced resistance against virulent strains of *Pseudomonas syringae* and *Hyaloperonospora parasitica* (Noco2) have been identified.

The recessive mutants *cpr1* and *cpr5* (constitutive expresser of *PR* genes) and the dominant *cpr6* are resistant to biotrophic pathogens (Bowling *et al.*, 1994; Bowling *et al.*, 1997; Clarke *et al.*, 1998). The *cpr5* mutant also forms spontaneous lesions in the absence of pathogens (Boch *et al.*, 1998). Interestingly, *cpr5* and *cpr6*, but not *cpr1*, also constitutively activate expression of the JA/ET-dependent gene *PDF1.2* (Bowling *et al.*, 1997; Boch *et al.*, 1998; Clarke *et al.*, 1998), suggesting possible crosstalk between SA-dependent and JA/E-dependent signalling patways. Disease resistance phenotypes of *cpr1* and *cpr6* were shown to be *EDS1* dependent while an *eds1* null mutation only partially compromised *cpr5*-mediated resistance (Clarke *et al.*, 2001). These results suggest that *EDS1* is a necessary downstream component of *cpr1* and *cpr6* mediated disease resistance and a more complex relationship exists between *cpr5* and *EDS1*.

### 1.2.2 Oxidative burst and hypersensitive response in plant defence

Following pathogen recognition one the earliest defence responses is the generation and accumulation of reactive oxygen species ROS [superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$)] at site of infection (Doke *et al.*, 1996), leading to a localized cell death, the so-called 'the hypersensitive response-HR' (Jones and Dangl, 1996; Loake, 2001). Similarly to animal defence responses against pathogen challenge, nitric oxide (NO) also accumulates during plant infection (Delledonne *et al.*, 1998). Moreover, it was recently shown that balanced accumulation of NO and ROS is fundamental for development of the HR (Delledonne *et al.*, 2001). Furthermore, HR is only triggered after interaction of NO with $H_2O_2$ generated from $O_2^-$ by superoxide dismutase and not directly with $O_2^-$ as was speculated before. During HR, dismutation of $O_2^-$ by superoxide dismutase reduces loss of NO by reaction with $O_2^-$ to trigger programmed cell death through NO/ $H_2O_2$ interaction. Thus, $O_2^-$ is the primary ROS signal for pathogen induction of glutathione S-transferase, and the rates of production and dismutation of $O_2^-$ during the oxidative burst is fundamental for the establishment of the HR (Delledonne *et al.*, 2001).

Genomic approaches have been deployed for the identification of genes from several plant species involved in the hypersensitive response. Interestingly, some mutant genes resulted

in spontaneous lesions formation even in healthy plants. The recessive null mutant *lsd1* shows typical HR lesions when challenged by avirulent pathogens, however, subsequent spontaneous runaway cell death (RCD) is started from neighbouring cells of HR lesions ending with complete collapse of leaves (Dietrich *et al.*, 1994). In association with lesion formation *lsd1* mutant plants also exhibit enhanced resistance against several normally virulent pathogens. *LSD1* gene was found to encode a zinc finger protein with homology to the GATA-type transcription factors and is believed to function as a negative regulator of basal resistance and programmed cell death and thereby establishes the boundaries to the plant HR (Dietrich *et al.*, 1994; Dietrich *et al.*, 1997). Spontaneous lesions in *lsd1* can be induced by provision of $O_2^-$ (Jabs *et al.*, 1996) or exogenous application of SA onto healthy *lsd1* plants (Dietrich *et al.*, 1994).

The involvement of *EDS1*, *NDR1* and *PAD4* in the establishment of cell death in *lsd1* was accessed by analysing the response of double mutants *lsd1ds1; lsd1ndr1* and *lsd1pad4* to strains of the bacterial pathogen *Pst* carrying the avirulence genes *avrRPS4* or *avrRPM1* (Rusterucci *et al.*, 2001). *avrRPS4* is recognised by *RPS4* and resistance mediated through an *EDS1-PAD4* dependent signalling pathway. In contrast, *avrRPM1* is recognised by RPM1 through the *NDR1* dependent signalling pathway (Aarts *et al.*, 1998; Gassmann *et al.*, 1999). It was shown that cell death in *lsd1*, in response to avirulent strains of *Pst*, was completely abolished by *eds1* or *pad4* mutations, but only partially supressed by the *ndr1* mutation. In further investigations it was shown that ROS accumulation does not depend on *EDS1* or *PAD4* genes, but was significantly affected by the *ndr1* mutation (Rusterucci *et al.*, 2001). These results were rather surprising because this novel function of *EDS1*and *PAD4* in RCD is initiated through a *R*-gene that does not require *EDS1* or *PAD4* for disease resistance.

### 1.2.3 Jasmonic Acid (JA) dependent defence responses

JA acid and its derivatives are compounds known for modulating several aspects of plant growth and development. JA and related lipids have been shown to be involved in root growth, anther dehiscence, pollen development, abscission, senescence and also activation

of plant defence responses against biotic and abiotic stresses (Creelman and Mullet, 1997; Turner *et al.*, 2002; Loake and Nurmberg, 2003). This family of fatty acids are derived from oxygenated linolenic acid (LA) through the octadecanoic pathway (Vick and Zimmerman, 1984) (Figure 1). Jasmonates share high similarity in structure with that of animal eicosinoids, a derived polyunsaturated fatty acid signalling molecule produced during tissue inflammation (Mueller, 1997). Despite this similarity in structure, inhibitors of animal phospholipase $A_2$ ($PLA_2$), a key enzyme for synthesis of animal prostaglandins, did not inhibit the accumulation of JA after wounding of potato tuber (Kòda and Kikuta, 1994), suggesting some diversity between JA and eicosinoids biosynthesis.

Elucidation of the biosynthetic pathway and understanding of JA function was greatly enhanced after JA was found to play a central role in the plant defence response (Schaller *et al.*, 2004). The JA concentration *in planta* rapidly increases in response to environmental stimuli (Gundlach *et al.*, 1992; Creelman and Mullet, 1995; Lehmann *et al.*, 1995). Because the enzymes lipoxygenase (LOX), allene oxide synthase (AOS), and allene oxide cyclase (AOC) are shown to be located in the chloroplast it is assumed that the first steps in the biosynthesis of jasmonates are performed in that organelle (Bell *et al.*, 1995; Maucher *et al.*, 2000; Froehlich *et al.*, 2001). However, despite the reactions downstream of linolenic acid being characterised, the identity of the membrane source of the linolenic acid precursor is still unclear, as there seems to be more than one precursor (Turner *et al.*, 2002; Schaller *et al.*, 2004). Recent work suggests that developmentally regulated JA biosynthesis in *Arabidopsis* is mediated through a pathway that differs from, but overlaps with the biosynthetic pathway activated for defence (Bell *et al.*, 1995; Creelman and Mullet, 1997; He *et al.*, 2002).

In the biosynthesis of jasmonates, assuming a more general pathway and by analogy with mammalian eicosanoid, firstly the unsaturated fatty acid linolenic acid is released from the cellular membrane by a phospholipase (PLA) enzyme Figure 1.2.3a). Requirement of a PLA for releasing LA from membrane lipids was confirmed by characterization of the *Arabidopsis* mutant *dad1* (Ishiguro *et al.*, 2001). As expected, *dad1* mutant plants, are male sterile and exogenous application of JA restored their fertility. *DAD1* was found to encode a lipase that hydrolyses phospholipids, suggesting *DAD1* is a phospholipase A1. *DAD1*

possess an N-terminal chloroplast transit peptide and was shown to accumulate in chloroplasts, however, it does not seem to be required for defence as wounding induced a 100 fold increase in JA accumulation in *dad1* plants (Schaller *et al.*, 2004). Additionally, antisense suppression of a phospholipase D (PLD) compromised wound-induced JA accumulation in *Arabidopsis* and tomato, but did not affect fertility, suggesting PLD is not required for JA biosynthesis in stamen development (Zien *et al.*, 2001). Since PLA does not release fatty acids (substrates for the octadecanoic pathway), suppression of PLD by antisense is unlikely the direct cause of reduced JA accumulation. PLD is probably engaged in phosphatidic acid (PA) synthesis, which might be a key messenger for hydrolysis of lipids by other lipases (Wang, 2004). Other lipases related to *DAD1* have been identified, and at least one might be involved in the wound-induced JA pathway (Ishiguro *et al.*, 2001).

Activation of a mitogen-activated protein kinase in tobacco plants (*WIPK*) and *Arabidopsis* (*MAPK4*) occurs in minutes after wounding (Seo *et al.*, 1999; Ichimura *et al.*, 2000). *WIPK* is required for triggering the jasmonate-mediated signal transduction cascade in wild-type tobacco plants, but is not involved in SA-mediated defence response to wounding, since wounded plants accumulated SA ad *PR1* gene transcripts (SA-dependent) at normal levels (Seo *et al.*, 1995; Seo *et al.*, 1999). The *mpk4* mutant plants are stunted and show enhanced accumulation of SA and are unable to express the JA-dependent genes *PDF1.2* and *Thi2.1* in response to JA treatment, suggesting that MPK4 suppress SA biosynthesis and promotes JA signalling (Niki *et al.*, 1998).

Once released, LA is then converted into 13-hydroperoxylinolenic acid by the enzyme 13-LOX, which catalyses the incorporation of molecular oxygen into the polyunsaturated chain forming hydroperoxides (Brash, 1999; Feussner and Wasternack, 2002). In plants, linoleic acid (18:2) and linolenic acid (18:3) are oxygenated at positions C-9 or C-13, and the derivative hydroperoxide are further metabolized into oxylipins (Vick and Zimmerman, 1983; Feussner and Wasternack, 2002). LOX comprise a family of at least eight genes in soybean and six in *Arabidopsis*, which are involved in many aspects of plant growth and development (Feussner and Wasternack, 2002).

The next step in the synthesis of jasmonates is the dehydratation of 13-hydroperoxylinolenic acid by allene oxide synthase (AOS) followed by the action of allene oxide cyclase (AOC) resulting in the formation of 12-oxo-phytodienoic acid (12-oxo-PDA) (Vick and Zimmerman, 1978; Baertschi *et al.*, 1988; Crombie and Morgan, 1988; Hamberg and Fahlstadius, 1990; Simpson and Gardner, 1995). AOC shows a more specific substrate specificity compared to AOS, adding additional specificity to the octadecanoic pathway (Ziegler *et al.*, 1997). Like JA, 12-oxo-PDA has also been found to modulate gene expression in response to wounding and insect attack (Stintzi *et al.*, 2001). These authors obtained an *Arabidopsis* mutant (*opr3*) unable to synthesize JA. *OPR3* encodes a 12-oxo-phytodienoate (OPDA) reductase enzyme, which converts 12-Oxo-PDA into JA. 12-Oxo-PDA was found to accumulate by wounding the plants or by foliar damage caused by herbivorous insects. Additionally, *opr3 Arabidopsis* mutant plants, in contrast to JA-insensitive mutant *coi1* and the *fad3fad7fad8* JA deficient mutants, were resistant to the dipteran *Bradysia impatiens* and the necrotrophic fungus *A. brassicicola*. Moreover, mutation in *opr3* did not affect expression of *PDF1.2* in response to *A. brassicicola* challenge. *PDF1.2* encodes a plant defensin with known antifungal activity in vitro and JA-dependent expression (Reymond *et al.*, 2000).

The 12-oxo-PDA then has its double bond in the cyclopentanone ring reduced by OPDA reductase (OPR) forming 3-oxo-2-(2'-pentenyl) cyclopentenone-1-octadecanoic acid (Schaller *et al.*, 2000), followed by three cycles of β-oxidation to form JA (Vick and Zimmerman, 1983, 1984; Strassner *et al.*, 2002) (Figure 1.2.3a,b). The chemical nature of OPDA allows the formation of four different stereoisomers, however, the enzymes AOS and AOC result in the formation of only 9S, 13S-OPDA (Hause *et al.*, 2000) - the precursor of the bioactive 3R,7S-JA. This conversion is still not well characterized.

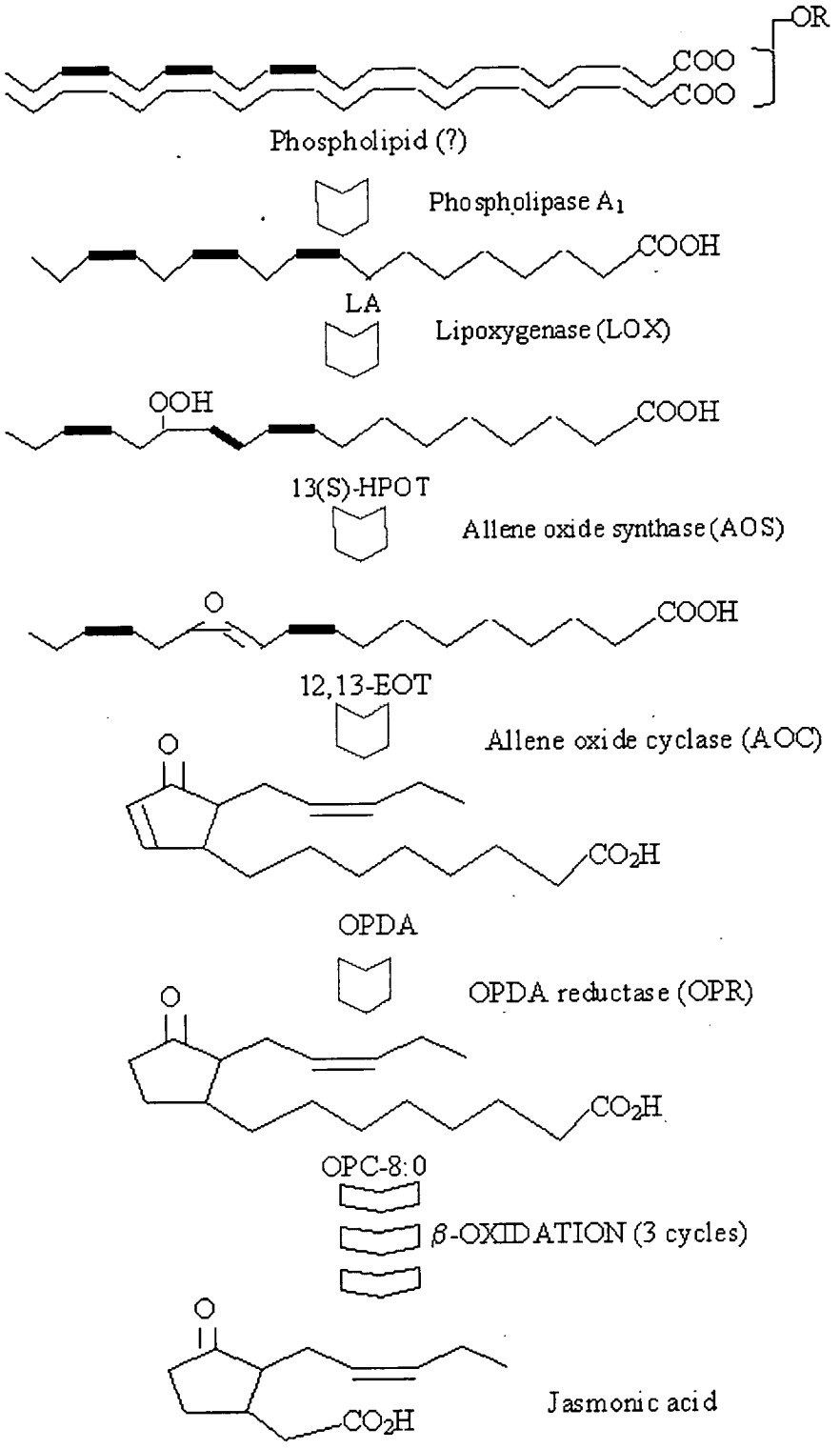


**Figure 1.2.3a JA Biosynthesis of jasmonic acid and related jasmonates** (The Vick and Zimmerman pathway). See text for details.

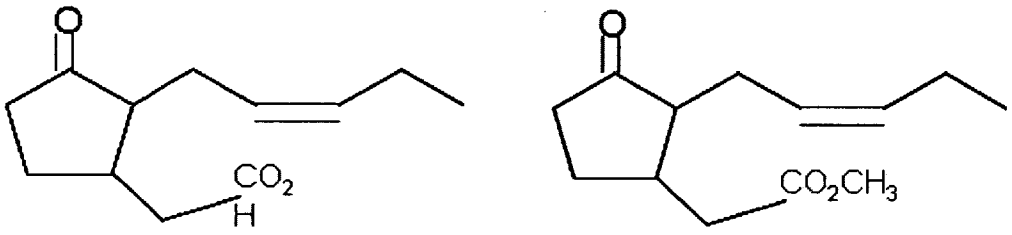


**Figure 1.2.3b Structure of jasmonic acid (JA) and methyl jasmonate (Me-JA).**

The commercially available synthetic Me-JA used experimentally is produced through methylation of JA. Recently, a gene (JMT) encoding a methyl-transferase (S-adenosyl-methionine:jasmonic acid carboxyl methyltransferase) has been identified (Seo *et al.*, 2001). This gene, like other genes in JA biosynthesis is promptly induced by Me-JA, resulting in 'de novo' synthesis of Me-JA. Several studies on plant-pathogen interactions have highlighted important roles for JA in plant insect and disease resistance. However, the transduction pathway is still not well understood. The first evidence for the involvement of JA in plant defence responses were published in 1990 by Farmer and Ryan, when they showed that JA and Me-JA induce expression of proteinase inhibitor in response to herbivorous insects (Farmer and Ryan, 1990). Following this observation it was shown that endogenous concentrations of JA and its precursor LA increased significantly in plant tissue after treatment with elicitors or following wounding (Mueller *et al.*, 1993; Conconi *et al.*, 1996). Initially, JA accumulates at the challenged region followed by a systemic increase. This group of compounds also induce expression of genes encoding antifungal proteins such as *PDF1.2* (Penninckx *et al.*, 1996), thionin (Becker and Apel, 1992) and osmotin (Chaudhry *et al.*, 1994). In *Arabidopsis* JA and Me-JA also modulate the expression of the basic pathogenesis related proteins *PR3* and *PR4*, which are are regulated independently of SA (Thomma *et al.*, 1998). Additionally, jasmonates modulate activation of genes encoding cell wall proteins and genes involved in phytoalexin biosynthesis (Gundlach *et al.*, 1992), which play important roles in plant defence. Moreover, exogenous application of Me-JA to *Arabidopsis* induces resistance against the necrotrophic pathogens *A. brassicicola*, *B. cinerea* and *Plectosphaerella cucumerina* (Thomma *et al.*, 2000). A

similar observation was made in potato plants, which had their resistance against *Phytophthora infestans* enhanced after exogenous application of Me-JA (Cohen *et al.*, 1993). Therefore, jasmonates act as multifunctional activators of plant defence responses.

Like JA and Me-JA, other intermidiates, or related compounds, in JA biosynthesis also play similar roles in plant defence responses cued by insects, necrotrophic pathogens or wounding (Stintzi *et al.*, 2001). In the biosynthesis of jasmonates through the oxylipin pathway aldehydes and alcohols are also synthesized and collaborate in the activation of the defence system against insect attack, affecting their fecundity (Hildebrand *et al.*, 1993).

A series of mutants affected in JA biosynthsesis and signal transduction have been identified in several plants species. For example, in tomato, a mutant (*jl5*), which is unable to convert 13-hydroxylinolenic acid into 12-oxo-PDA is more susceptible to *Manduca sexta* (Howe *et al.*, 1996). In *Arabidopsis*, the mutant *coi1* affected in signal transduction downstream of JA is insentive to JA and is much more susceptible to *A. brassicicola* and *B. cinerea* (Thomma *et al.*, 1998; Xie *et al.*, 1998). Moreover, this mutant is incapable of inducing *PDF1.2* and/or *thionin2.1* expression either after wounding or challenge by the necrotrophic pathogen *A. brassicicola* (Penninckx *et al.*, 1996). *COI1* was found to encode an F-box protein, and suggested to be involved in ubiquitin-mediated protein degradation in JA signalling pathway (Xie *et al.*, 1998). This hypothesis is supported by the fact that COI1 is present in a functional SKIP-CULLIN-F-box-type E3 ubiquitin ligase complex. Interestingly, male sterility of tomato *coi1* homologue (*jai1*) is caused by a maternal defect in seed maturation, which is associated with loss of JA-mediated accumulation of proteinase inhibitor in reproductive tissues. Similarly to *Arabidopsis coi1*, the tomato *jai1* mutant plants were also unable to express JA-dependent genes and were more susceptible to at least two spider mite species and in addition showed abnormal development of glandular thrichomes (Li *et al.*, 2004b).

In contrast to the strong phenotype of *coi1*, mutations in *JAR1* (jasmonic acid resistant 1) did not affect fertility and only partially compromised sensitivity to JA, suggesting *JAR1* is involved in some, but not all, JA-regulated responses (Staswick *et al.*, 1992; Staswick *et al.*, 1998). *Jar1* mutant plants are compromised in resistance against root rot caused by *Phythium* species (Staswick *et al.*, 1998). *JAR1* was found to encode a JA-conjugating

enzyme. Thin-layer chromatography and gas chromatography–mass spectrometry (GC-MS) analysis demonstrated that this enzyme forms JA-amido conjugates with several amino acids (Staswick *et al.*, 2002; Staswick and Tiryaki, 2004). Neither *coi1* nor *jar1* compromised resistance to the biotrophic pathogen *Hyaloperonospoa parasitica*, in contrast to the mutants defective in the SA signalling pathway, including *nahG*, *npr1* and *eds1* (Penninckx *et al.*, 1996; Thomma *et al.*, 1998).

### 1.2.4 Involvement of Ethylene in plant defence

ET is a phytohormone that, like jasmonates, is involved in many different processes of plant growth and development. ET is engaged in seed germination, cell growth and differentiation, senescense and abcission, and fruit ripening (Ecker, 1995; Johnson and Ecker, 1998). The involvement of ET in defence has been the subject of controversy for a long while, and only recently conclusive evidence was produced (Knoester *et al.*, 1998; Hoffman *et al.*, 1999). However, the role of ethylene can be dramatically different, depending on pathogen and plant species. Plants deficient in ethylene signalling may show either increased resistance or enhanced susceptibility (Wang *et al.*, 2002). Transgenic tobacco plants expressing a dominant-negative mutant allele of the *Arabidopsis* ethylene receptor gene *ETR1* displayed enhanced susceptibility to the soilborn fungus *Pythium irregulare* (Knoester *et al.*, 1998). Production of ET is stimulated by pathogen challenge (Penninckx *et al.*, 1998), and this rise in ET concentration modulates the expression of defence response genes (Memelink *et al.*, 1990; Knoester *et al.*, 1998; Berrocal-Lobo *et al.*, 2002).

When seedlings of *Arabidopsis* are grown in the dark in the presence of ET, root and hypocotyls elongation are inhibited, there is a radial swelling of the hypocotyls and an exaggeration of the curvature of the apical hook, termed the triple response (Guzman and Ecker, 1990; Kieber, 1997). This response of *Arabidopsis* seedlings to exogenous application of ethylene has allowed the identification of several classes of mutants impaired in the response to this hormone (Bleecker *et al.*, 1988; Guzman and Ecker, 1990; Hoffman

*et al.*, 1999). Mutants constitutively displaying the triple response can be originated from ET overproduction or by constitutive activation of hormone biosynthesis.

The first scientific evidence of the existence of an ET/JA dependent signalling pathway came from analysis of *PDF1.2* expression in plants challenged by *A. brassicicola*. *PDF1.2* was induced both at the site of infection and systemically (Penninckx *et al.*, 1998). Transgenic *Arabidopsis nahG* plants or *npr1* mutants were not compromised in *PDF1.2* expression, showing SA is not required for induction of *PDF1.2* gene expression (Cao *et al.*, 1994; Shah *et al.*, 1997). On the other hand, induction of *PDF1.2* is blocked in *coi1*, *ein2*, and *etr1* mutants (Penninckx *et al.*, 1996; Penninckx *et al.*, 1998; Thomma *et al.*, 1998). A single mutation in either *coi1*, *ein2* or *etr1* was sufficient to disrupt *PDF1.2* gene expression. In this case ET works in association with JA to modulate gene expression.

In contrast, other experiments have presented evidence that JA and ET individually are sufficient to induce resistance against *A. brassicicola* and *B. cinerea*, respectively (Thomma *et al.*, 1998; Berrocal-Lobo *et al.*, 2002). Despite the role of ET in the activation of *PDF1.2* in response to *A. brassicicola*, resistance to this pathogen was not affected by the *ein2* mutation (Thomma *et al.*, 1998). Moreover, despite the existence of positive co-operation between JA and ET, overexpression of an ethylene-response-factor (*ERF1*) is sufficient to confer resistance against *B. cinerea* and *Plectosphaerella cucumerina* but reduces SA-mediated tolerance against the bacterial virulent pathogen *Pst* (Berrocal-Lobo *et al.*, 2002). However, it worth noting that a positive co-operation between SA and ET was observed in the plant response to *P. cucumerina*. In addition, resistance to the soilborne fungi *Fusarium oxysporum* sp. *conglutinans* and *F. oxysporum* sp. *Lycopersici* in *Arabidopsis* is also mediated through the *ERF1* expression (Berrocal-Lobo and Molina, 2004). *ERF1* transcripts can be rapidly induced by *B. cinerea*, ethylene, jasmonate or synergistically by both molecules (Berrocal-Lobo *et al.*, 2002; Lorenzo *et al.*, 2003). It was also shown that, both JA and ET intact signalling pathways are required simultaneously to activate *ERF1*, and that mutations blocking either pathway blocked *ERF1* expression (Lorenzo *et al.*, 2003). Furthermore, *35S:ERF1* expression can rescue the defence response defects of *coi1* (*coronatine insensitive1*) and *ein2* (*ethylene insensitive2*), suggesting it is a downstream component of both jasmonate and ethylene signalling pathways. It is believed, this

transcription factor acts downstream of the intersection between ethylene and jasmonate pathways, functioning as a key element in the integration of both signals for the regulation of defence response genes (Lorenzo *et al.*, 2003).

*In vitro* DNA binding studies revealed that primary ethylene response elements in the promoter of *ERF1* are directly bound by homodimers of the *EIN3* protein family (Solano *et al.*, 1998). *EIN3* encodes a nuclear localized protein that belongs to a multigene family in *Arabidopsis* (Chao *et al.*, 1997). From six members of this family, at least three of them (EIN3, EIL1 and EIL2) are involved in ethylene signal transduction. Overexpression of *EIN3* in a *ein2* null mutant background results in constitutive activation of ethylene responses, similarly to overexpression of *EIN2CEND*, confirming that *EIN3* acts downstream of *EIN2* or proteins are functionally equivalent (Wang *et al.*, 2002).

Additionally, it has been demonstrated that the activation of one or another defence response is preferentially dependent on the type of pathogen involved. For example, *nahG* plants or *npr1* mutants show increased susceptibility to the biotrophic pathogen *H. parasitica* and *Pst*, but are not affected in their resistance against necrotrophic fungi. In contrast, mutants impaired in JA or ET signalling such as *coi1* and *ein2* are much more susceptible to the necrotrophic fungus *B. cinerea* (Thomma *et al.*, 1998).

### 1.2.5 Role of phytoalexins in disease resistance

The accumulation of phytoalexins is a conspicuous defence response of plants in response to attempted pathogen ingress (Zhao and Last, 1996; Zhao *et al.*, 1998). Camalexin is the only phytoalexin found in *Arabidopsis* to date (Hansen and Halkier, 2005) and its accumulation is induced by pathogens such as *Pst* DC3000 (Zhao and Last, 1996), *A. brassicicola* (Thomma *et al.*, 1999b), and *B. cinerea* (Tierens *et al.*, 2002; Ferrari *et al.*, 2003). However, the antimicrobial activities of this compound are still not well elucidated, and they may vary according to pathogen species (Thomma *et al.*, 1999c). Camalexin was shown to have *in vitro* antifungal activity and to be required for local resistance against *B. cinerea* (Ferrari *et al.*, 2003). On the other hand, *Arabidopsis pad3* plants, deficient in

camalexin accumulation, are markedly more susceptible to *A. brassicicola* but are not affected in resistance against *B. cinerea* (Thomma *et al.*, 1999b). Likewise, mutation in the *PAD3* gene did not affect growth of virulent *Pst* DC3000 (Glazebrook and Ausubel, 1994). *PAD3* encodes a cytochrome P450 mono-oxygenase CYP71B715, a key enzyme in the camalexin biosynthesis (Zhou *et al.*, 1999). In contrast, two other phytoalexin deficient mutants, *pad1* and *pad2,* showed enhanced susceptibility to virulent *P. syringae* DC3000, however, no correlation between R gene-mediated resistance and camalexin accumulation was encountered (Glazebrook and Ausubel, 1994).

Camalexin induction is not triggered by exogenous application of either SA, ET or JA and camalexin synthesis was not significantly compromised by mutations that compromised the perception of these defence-related signalling molecules (Thomma *et al.*, 1999c). Nevertheless, SA was shown to be required for accumulation of camalexin in response to pathogen attack (Zhao and Last, 1996). Moreover, it was recently shown that local resistance to *B. cinerea* requires JA, ET and SA signalling pathways (Ferrari *et al.*, 2003). In this case, local resistance against *B. cinerea* is believed to be mediated through SA synthesized via phenylalanine ammonia lyase (PAL) rather than the isochorismate (ICS) pathway, with camalexin playing a role in limiting lesion development. In addition, the *Arabidopsis* gain-of-function mutant *acd6-1* shows constitutively high levels of SA and camalexin (Vanacker *et al.*, 2001). These plants also display enhanced resistance against *Pst* DC3000 and reduced stature, phenotypes which can be partially suppressed by blocking SA perception or its accumulation, by the *npr1* and *pad4* mutations, respectively (Rate et al, 1999; Lu et al 20003). Interestingly, *bos3* plants which are susceptible to *B. cinerea,* show higher accumulation of camalexin in response to this pathogen and show enhanced resistance to *Pst* DC3000 (Veronese *et al.*, 2004). This could suggest a positive correlation between camalexin accumulation and resistance to the bacterial pathogen *Pst* DC3000, however, the authors only measured camalexin accumulation in response to *B. cinerea* and not *Pst* DC3000

### 1.2.6 Crosstalk between signalling pathways

JA and SA-dependent signalling pathways are activated in response to distinct groups of pathogens and some studies provide evidence for the synergistic interaction between them. Simultaneous treatment of tobacco plants with JA and SA resulted in significantly higher expression of the basic *PR-1* gene (*PR-1b*) compared to independent treatments (Xu *et al.*, 1994). Further, data for this synergistic interaction comes from microarray experiments, where a subset of genes was shown to be upregulated either by JA and SA (Schenk *et al.*, 2000; Li *et al.*, 2004a). In addition, basal resistance to the bacterial pathogens *Pst* and *Xanthomonas campestris* pv. *armoraciae* were found to be affected either in *nahG* or JA and ET-responsive mutants (Pieterse *et al.*, 1998; Ton *et al.*, 2002). However, a series of other studies suggest a more pronounced antagonistic interaction between SA and JA (Schenk *et al.*, 2000; Glazebrook *et al.*, 2003; Spoel *et al.*, 2003). Thus, it seems that plants are able to prioritize which defence response will be deployed, according to the type of pathogen detected.

For example, SAR activation was shown to suppress JA-mediated resistance in plants, prioritizing therefore SA-dependent response over JA induced resistance against the herbivorous insect *Spodoptera exigua* (Felton and Korth, 2000). In addition, SA was shown to suppress JA-mediated gene expression (PenaCortes H, 1993; Doares *et al.*, 1995; Gupta *et al.*, 2000). Furthermore, exogenous application of aspirin (acetyl salicylate- a non-steroidal anti-inflamatory drug-NSAID) disrupted the biosynthesis of prostaglandins in animal cells and JA in plants. Aspirin acetylates the enzyme cyclooxygenase preventing formation of prostaglandins and inhibit the activity of hydroxyperoxide-dehydrase, which catalyses the conversion of 13-HPLA into 12-oxo-pda in plants (PenaCortes H, 1993). However, it is worth noting that the acetylated form of SA (acetylsalicylic acid) does not occur in plants and that no inhibitory effect of SA over allene oxide synthase activity was observed in either *Arabidopsis* or flax plants (Harms *et al.*, 1998; Laudert and Weiler, 1998). Nevertheless, the negative effect of SA over JA-mediated defence gene expression has been shown in several studies (Doares *et al.*, 1995; Harms *et al.*, 1998; Gupta *et al.*, 2000).

Recent studies shown that either a positive co-operation or a negative interaction between SA and ET is possible, depending on the plant and or pathogen species (O'Donnell *et al.*, 2001; Berrocal-Lobo *et al.*, 2002; Berrocal-Lobo and Molina, 2004). In tomato plants, depletion of ET accumulation in the transgenic ACC deaminase line (*acd*) or its perception by the ethelyne-insensitive mutant *nr* result in lower accumulation of SA upon infection with *Xanthomonas campestris* pv. *Vesicatoria* and consequently reduced disease symproms (O'Donnell *et al.*, 2001). Activation of ethylene responses in *Arabidopsis* by *ERF1* overexpression results in enhanced resistance against the necrotrophic pathogen *B. cinerea* and in counterpart reduced SA-mediated tolerance to *Pst* (Berrocal-Lobo *et al.*, 2002). On the other hand, in this same study it was demonstrated that *ERF1*-mediated resistance to *P. cucumerina* was benefited by a positive co-operation between these two defence signalling pathways. Moreover, *Arabidopsis* resistance against *F. oxysporum*, also mediated through *ERF1* expression, requires ET, JA, and SA signalling pathways, together with a functional *NPR1* gene, altought it is independent of *PAD4* and *EDS1* (Berrocal-Lobo and Molina, 2004). Altogether, these results confirm that both positive and negative interaction between SA and ET can be established.

Together with ET, JA is required for the expression of the *PDF1.2* gene and induction of induced systemic resistance (ISR) in response to the non-pathogenic rhizobacteria *Pseudomonas fluorescens* (Penninckx *et al.*, 1998; van Wees *et al.*, 1999). Loss-of-function mutations in the *COI1* gene blocked induction of *PDF1.2* gene expression by ET, while mutations in the *EIN2* gene suppressed induction by JA (Penninckx *et al.*, 1998). Like SAR, induction of ISR confers resistance against a broad spectrum of pathogens like *F. oxysporum* and *Pst* (Pieterse *et al.*, 1998; Pieterse and van Loon, 1999), however, activation of ISR is SA-independent. Transgenic *Arabidopsis nahG* plants, depleted in the accumulation of SA, are incapable of mounting SAR, but are still able to activate ISR. On the other hand, mutants insensitive to JA or ET, like *etr1* and *jar1*, fail in the establishment of ISR (Pieterse *et al.*, 1996; Pieterse *et al.*, 1998). Hence, it appears that JA and ET work in combination to establish ISR. Our knowledge about this defence mechanism is still unclear, however, in contrast to SAR, ISR may be mediated via super-expression of defence genes when the plant is challenged by virulent pathogens.

## 1.3 Non-host disease resistance

The prevalent type of plant disease resistance in nature, and perhaps the most important is 'non-host resistance'. Resistance shown by an entire plant species to a specific parasite or pathovar is known as non-host resistance (Heath, 1987). Non-host resistance is effective against a broad spectrum of pathogens, and in contrast to gene-for-gene resistance, is durable, which makes it much valuable for agricultural applications (Holub, 2001). In spite of its natural and economical importance non-host resistance is still poorly understood. Non-host resistance can be divided into two types: type I- is when the interaction between a pathogen and a non-host plant does not produce any visible symptoms (necrosis) and type II- is always associated with rapid localized cell death (HR) (Mysore and Ryu, 2004). Whether type I or II will be triggered will depend on both plant and pathogen.

Recently, it has been suggested that plants respond in a similar fashion to host and non-host pathogens, and that signalling components can be shared between these two type of resistance (Thordal-Christensen, 2003). Therefore, the difference between host and non-host resistance may reside in the solidity of recognition leading to resistance. Similarly to race specific resistance, inducible defence mechanisms and signalling molecules have been shown to play a fundamental role against several non-host pathogens. For example, disruption of actin filaments (a key component of the cytoskeleton) by exogenous application of cytochalasins, specific inhibitors of actin polymerisation, resulted in loss of resistance in several plant species against different non-host fungi (Kobayashi *et al.*, 1997a). Likewise, simultaneous loss of actin and *EDS1* gene function in *Arabidopsis* compromised resistance against non-host powdery mildew (*Blumeria graminis* f. sp. *tritici*) (Yun *et al.*, 2003). Saponins, secondary metabolites with antimicrobial activity, constitutively produced by some plants species and also induced by pathogens are also suggested to play a role in non-host resistance (Papadopoulou *et al.*, 1999).

Beyond pre-existing barriers, attacking pathogens have to deal with plant inducible defence mechanisms and signalling molecules. General elicitors can be released during attempted ingression of both host or non-host interaction, activating new barriers against the invading parasite. Examples of such general elicitors are the *Flagellins,* proteins from the bacterial flagella, and oligomers of chitin and glucans released during the enzymatic degradation of

cell-wall polymers during the infection process (Thordal-Christensen, 2003). Recognition of the conserved 22-amino acid motif (flg22) by *Arabidopsis* plants is mediated through a signalling pathway that depends on a protein recognition complex containing the FLS2 leucine-rich repeat (LRR) receptor kinase (Gomez-Gomez *et al.*, 2001). Interaction between flg22 and FLS2 leads to accumulation of *'in planta'* accumulation of ROS and activation of a mitogen-activated protein cascade, in addition to induction of defence pathogenesis-related genes (Felix *et al.*, 1999; Gomez-Gomez *et al.*, 1999; Nuhse *et al.*, 2000; Asai *et al.*, 2002). The release of general elicitors (oligomers) is indispensable for the pathogens and plants are believed to exploit them during recognition, similarly to the animal recognition of 'pathogen-associated molecular patterns' (PAMPs) (Parker, 2003; Thordal-Christensen, 2003). Recognition of such elicitors activate the production a series of antimicrobial proteins and phytoalexins.

Evidence for the involvement of camalexin (an indole phytoalexin with low molecular weight and antimicrobial activity), ET, and SA in resistance to non-host pathogens has been produced (Knoester *et al.*, 1998; Thomma *et al.*, 1998; Mellersh and Heath, 2003). The *sid2* mutant and *nahG* transgenic *Arabidopsis* plants, which are depleted in SA accumulation (Gaffney *et al.*, 1993; Wildermuth *et al.*, 2001), lack resistance against non-host fungus *Uromyces vignae* (Mellersh and Heath, 2003). *SID2* encodes the enzyme isochorismate synthase involved in SA biosynthesis (Wildermuth *et al.*, 2001). *NahG* plants are also susceptible to the non-host bacterium *Pseudomonas phaseolicola*, however, accumulation of catechol in addition to depletion of SA contributes to this susceptible phenotype (van Wees and Glazebrook, 2003). Further evidence for the involvement of SA in non-host resistance comes from experiments with WIPK (wound-induced protein kinase) and SIPK (salicylic acid-induced protein kinase) (Sharma *et al.*, 2003), when it was shown that silencing these two genes in *Nicotiana benthamiana* compromises resistance against the non-host bacterial pathogen *Pseudomonas cichorii*.

The first gene (*NHO1*) directly involved in resistance against the non-host bacterial pathogen *Pseudomonas syringae* pv *phaseolicola* was recently cloned (Kang *et al.*, 2003) and shown to encode a glycerol kinase, which converts glycerol into glycerol 3-phosphate. Glycerol 3-phosphate can be directed into three different metabolic pathways: glycolysis,

gluconeogenesis, and synthesis of phospholipids. Therefore, susceptibility of *nho1* towards non-host pathogens may be due to a deficiency in G3P which affects one of these metabolic pathways and consequently shortage of secondary metabolites such as sugars or lipids.

### 1.4 *Botrytis cinerea*, a rotting pathogen

*Botrytis cinerea* Pers., anamorph of *Botryotinia fuckeliana* (De Bary) Whetzel, the causal agent of grey mould disease, is a member of the family *(Moniliaceae/Sclerotiniaceae)* (Inoperculate Discomycetes) (Agrios, 1997). This deuteromicota fungus produces multinucleate, heterokariotic asexual conidia and is a pathogen with worldwide distribution and able to attack over 200 plant species, including agronomic crops such as strawberry, tomato, pepper, kiwi fruit and petunia. Due to its broad spectrum of hosts, *Botrytis* is the most common imperfect fungus causing fruit and general diseases on plants, causing blossom blights and fruit rots but also damping-off, stem cankers or rots, leaf spots, and tuber, corm, bulb, and root rots of many vegetables, flowers, small fruits, and other fruit trees (Agrios, 1997).

Despite recent advances in crop protection, control of grey mould is still mainly dependent on fungicides such as imidazoles and dicarboximides. However, control efficiency is seriously affected by the appearance of resistant strains (Baraldi, 2002). Resistance to fungicide can result in significant crop losses (Michailides and Elmer, 2000). The sexual form, *Botryotinia fuckeliana* (de Bary) Whetz is rarely found in nature and in the laboratory is very laboriously obtained, therefore sexual reproduction is very unlikely to be the main source of variation (Faretra *et al.*, 1988). Analysis of genetic diversity between field strains has shown a large variability in DNA content with frequent occurrence of aneuploids and polyploids (Buttner *et al.*, 1994). Thus, frequent mutations and aneuploidy are possibly the main generators of genetic variability in this species, and consequently the source of resistance to fungicide. In more recent work in the filamentous fungi *Colletotrichum lindemuthianum*, it was demonstrated that genetic material was exchanged between isolates, through structures named CATs - conidial anastomosis tubes (Roca *et al.*, 2003), this could be an alternative mechanism deployed by fungal species for genetic recombination.

In contrast to biotrophic pathogens, necrotrophic pathogens do not require host living cells to survive, in fact, they kill host cells prior to penetration and thrive on nutrients from them. Moreover, this group of fungi can remain active after tissues have died, an advantage in relation to the biotrophic pathogens and saprophytic microrganisms. Direct penetration of intact plants using structures such as appressorium has been reported (Rijkenberg *et al.*, 1980; Williamson *et al.*, 1995). However, there is far more evidence for the involvement of reactive oxygen species (ROS) and cell wall degrading enzymes (CWDE) in pathogenicity of *Botrytis* than for the involvement of appressorium-like structures (Viterbo *et al.*, 1993; Tiedemann, 1997).

### 1.4.1 Role of Reactive Oxygen Species on Virulence of *B. cinerea*

One of the first responses of plants against invading pathogens is the generation and accumulation of reactive oxygen species (ROS) [superoxide ($O_2^-$) and hydrogen peroxide ($H_2O_2$)] at the site of infection (Grant and Loake, 2000). This oxidative burst leads to the so-called hypersensitive response (HR). HR mimics programmed cell death in mammals and is deployed by plants in attempt to restrict pathogen spread (Jones and Dangl, 1996).

While HR is one of the most efficient mechanism of defence against a broad spectrum of biotrophic pathogens it has been shown to facilitate plant infection by the necrotrophic pathogen *B. cinerea* (Govrin and Levine, 2000). In this study they showed that the oxidative burst and cell death was in fact induced by the pathogen and not by the host as one would expect. Moreover, it was shown that the degree of pathogenicity of both *B. cinerea* and *Sclerotinia sclerotiorum* was directly dependent on the level of generation and accumulation of $O_2^-$ and $H_2O_2$.

One of the first suggestions of the involvement of ROS in pathogenicity of *B. cinerea* dates from 1980s, when the secretion of several sugar oxidases into the inoculation medium, releasing phytotoxic levels of $H_2O_2$ which could potentially kill host cells prior to pathogen penetration (Edlich *et al.*, 1989). This observation was in agreement with previous findings that spores of *Botrytis* depend on the presence of sugars in the inoculum or on the host

surface (plant-surface exudates) for successful germination. Even though oxidative enzymes were not purified these observation were confirmed by the inhibitory effect of several antioxidants on infection.

A positive correlation for *in situ* accumulation of ROS and aggressiveness of six isolates of *Botrytis* was also reported on bean leaf disks floating on diluted malt broth medium inoculated with conidia of this pathogen (Tiedemann, 1997). That was the first report of direct detection of toxic superoxides and hydrogen peroxides *in situ* during infection by this pathogen. In this study it was reported that the more aggressive isolates induced peaks of $O_2^-$ accumulation within 24h and $H_2O_2$ within 48h post-inoculation (hpi). However, such an increase in ROS was not detected for non-aggressive strains. Moreover, scavengers of ROS, catalase and D-mannitol, significantly reduced severity of infection. Furthermore, this author reported suppression of plant peroxidase activity by all isolates tested, with stronger suppression by the more virulent isolates, suggesting a role for peroxidases in plant resistance as scavengers of harmful ROS.

Further observations of ROS functioning as virulence factors in *B. cinerea* were performed by analysis of potential $H_2O_2$ generators (Rolke *et al.*, 2004). A Cu-Zn-superoxide dismutase gene and a putative glucose oxidase gene (named *bcsod1* and *bcgod1*, respectively) were cloned and their potential function in pathogenicity investigated. It was reported that only mutations in the *bcsod1*(*Δbcsod1*) significantly delayed development of symptoms, suggesting Cu-Zn-SOD-activity could play an important role in virulence of this necrotrophic species.

### 1.4.2 Cell Wall Degrading Enzymes (*Attack Enzymes*)

The presence of cell wall degrading enzymes (CWDEs) (also called *attack enzymes)* during the different stages of infection suggests these enzymes play a major role in pathogenicity. Several studies have been undertaken in order to prove involvement of appressorium-like structures in pathogenicity of the necrotrophic fungus *B. cinerea.* In spite of the evidence for the contribution of these penetration structures in the infection of *Botrytis* a requirement

for CWDE could be ruled out by the authors. A more recent study suggests an appressorium is required for physical penetration of intact leaves of bean, tomato or rose but not wounded plants (Gourgues *et al.*, 2004), but again they were unable to demonstrate that CWDE were not required. Therefore, if appressorium-like structures are needed for plant infection they work in association with secreted *attack enzymes*.

A common feature of the symptoms caused by these pathogens is that infected tissues are rotted, due the action of degrading enzymes. Despite large variability in the ploidy, associated with phenotypic instability of isolates, it has been possible to establish the function of some proteins secreted by *Botrytis* during pathogenesis (Edlich *et al.*, 1989; Staples and Mayer, 1995; ten Have, 2002). Some of the more commonly secreted enzymes are pectin methyltransferase (Valette-Collet *et al.*, 2003), polygalacturonase (Wubben *et al.*, 1999; ten Have *et al.*, 2001), carboxypeptidase (Viterbo *et al.*, 1993), β-glucosidase (Sasaki and Nagayama, 1994), laccases (Schouten *et al.*, 2002) and aspartate proteinase (ten Have *et al.*, 2004).

When *B. cinerea* penetrates the anticlinal wall it subsequently grows into and through the middle lamella, which consists mostly of pectin. The host range of *B. cinera*, albeit wide, is restricted to plants with high pectin contents in the cell wall, i.e., dicotyledons and corroliferous monocot species. Graminaceae contain a low amount of pectins and they are typically non-hosts (ten Have, 2002).

It appears that some of these enzymes are secreted during the early stages of the interaction, initiating the infection process, while a second group of enzymes would be synthesized later enhancing pathogen spread and macerating plant tissue (Staples and Mayer, 1995). Other secreted proteins are also speculated to act in protecting the fungus against secondary metabolites produced by the plant during infection. In this case, pectinases such as pectin methylesterases (PME) act at first instance by deesterifying pectin, producing, methanol + polygalacturonic acid (PGA), allowing subsequent function of polygalacturonases (PG), which hydrolyse glycoside bonds between galacturonans, and pectate lyases (PL). This breaks PGA into oligogalacturonides by β-elimination. Pectin lyases (PLN) can also

depolymerise native pectin by breaking bounds between methylated galacturonide residues via β-elimination (Valette-Collet *et al.*, 2003).

Endopolygalacturonases cannot cleave highly methylated pectin, while methylesterases can demethylate pectin without affecting the polymer chain. Thus, concerted and sequential action of these two enzymes is essential. This will cleave the polymer chain into oligomers and ultimately into di- or monomers (ten Have, 2002). Concerted and sequential secretion of these enzymes is regulated by the susbtrate available and by environmental conditions such as pH and host condition.

A detailed *in vitro* study of a *Botrytis* endopolygalactuonase gene family (*Bcpg1-6)* revealed different features for each of its six members (ten Have *et al.*, 2001). They noticed a significant variation in terms of basal expression level between all six genes, being higher for *Bcpg1* and *Bcpg2* (at least 10 times higher for *Bcpg1* than all other transcripts). While *Bcpg4* and *Bcpg6* were induced by galacturonic acid, glucose only repressed expression of *Bcpg4.* On the other hand, *Bcpg3* was not responsive to carbon source and induced by low pH. Regulation of *Bcpg5* could not be discriminated. This large battery of pectinases confers an enormous advantage to a fungus with such a wide range of host species. Similarly, the expression pattern of these pectinases on four different hosts (tomato, broad bean, apple and courgette) varied according to the stage of infection. However, the functionality of such a flexible pectin degradation machinery may be revealed by analysing knockout mutants for these genes. Deletions in *Bcpg1* resulted in reduced virulence on leaves and tomato fruits as well as on apple (ten Have *et al.*, 1998). Other pectinases secreted by *Botrytis* have been reported and/or cloned but little is known about their function in pathogenicity

The role of proteinases as virulence factors on infections by *B. cinerea* is becoming more evident. Proteinases might be used by *Botrytis* to degrade plant cell wall proteins or antifungal proteins secreted by the host (ten Have *et al.*, 2004). A proteinase that has been the subject for different studies is aspartate proteinase (AP) (Movahedi and Heale, 1990b, 1990a; Manteau *et al.*, 2003; ten Have *et al.*, 2004). This enzyme is present in ungerminated spores, and is produced during germination before pectic enzyme

components, both in culture and in host-infected tissue (Staples and Mayer, 1995). Moreover, cell death was detected in *B. cinerea*-infected carrot root before pectic enzymes or necrosis-inducing glycoprotein or polysaccharide production, supporting the view that AP may be involved since early stages of infection (Movahedi *et al.*, 1991). While several types of proteinases are secreted by pathogens, AP is particularly significant because it was the only type of proteinase activity in fluid obtained from *B. cinerea*-infected tissue of apple, pepper, tomato and courgettes (ten Have *et al.*, 2004). In this study, five *B. cinerea* genes encoding AP were cloned and transcript of all five genes were detected in infected plant tissue, suggesting that at least part of AP activity *in planta* originated from the pathogen.

### 1.4.3 Detoxifying secreted enzymes and plant defence

Similarly to those enzymes synthesized for host attack, *B. cinerea* also secretes a range of enzymes in response to activated defence mechanisms in the plant host, with the purpose of detoxification. Closely associated with the oxidative burst is the secretion of a series of laccases (Deighton *et al.*, 1999). Laccase, a polyphenoloxidase reduces oxygen to water accompanied by the oxidation of a phenolic substrate. The full spectrum of laccase substrates is unknown, but includes polyphenols, methoxy-substituted phenols and diamines, but not tyrosine (Staples and Mayer, 1995). These enzymes are believed to function as detoxifying enzymes to protect the fungus against toxic metabolites, by the oxidative polymerization of lignins and oxidation of phytoalexins. A role for laccase in pathogenicity of *Botrytis* has been reported almost twenty years ago (Barnun *et al.*, 1988). Treatment of growing cultures of the fungus with EDTA suppressed laccase synthesis without suppressing fungal growth, with effects reversed by copper application. Likewise, pathogenesis on cucumber fruits was significantly reduced by EDTA treatment. It is postulated one of the possible mechanisms of resistance against *Botrytis* is by repressing laccase secretion. This speculation is due to the observation that cucurbitacin, a cyclic triterpenoid produced by *Cucumis prophetarium* and *Ecbollium elaterium* affected laccase synthesis (Barnun *et al.*, 1988; Barnun and Mayer, 1989). However, cucurbitacin neither suppresses fungal growth nor blocks the action of attack enzymes. Tannins from the skin of

grape berries are capable of inhibiting these laccases, but are unlikely to have a significant reduction on maceration of ripening fruits, since phytoalexin levels decline during ripening (Staples and Mayer, 1995). Therefore, effective resistance against *B. cinerea* should take into account all these observations.

Like laccase, benzyl alcohol oxidase (BAO) is also secreted by *B. cinerea* in response to the plant defence mechanism. BAO is speculated to function by degrading the by-products of cell wall degradation and or the biodegradation of lignified compounds (Goetghebeur *et al.*, 1993). Similarly to laccase, BAO enzyme induction by *B. cinerea* occurs in specific response to the defence mechanism of the plant host.

Mutations in an ATP-binding cassette (ABC) transporter BcatrB gene resulted in increased sensitivity to the phytoalexin resveratrol and the fungicide fenpiclonil (Schoonbeek *et al.*, 2001). Moreover, virulence of ΔBcatrB mutants on grape vine leaves was slightly reduced. ABC superfamily genes encode membrane-bound proteins with an ATP-binding cassette (Higgins, 1992). These proteins transport a wide range of compounds over various membranes via ATP binding (Senior *et al.*, 1995). Disruption of the *Magnaporthe grisea* ABC1 gene resulted in reduced virulence of this pathogen on rice plants (Urban *et al.*, 1999), confirming the involvement of ABC transporters in pathogenicity. Thus, these membrane transporters might protect a pathogen from plant defence compounds.

In response to pathogen attack, plants express a variety of pathogenesis related proteins, also called PR-proteins, which possess strong antifungal and other antimicrobial activity. Some of them inhibit spore releases and germination, whereas others are associated with strengthening of host cell wall and its outgrowths and papillae. For example, β-1,3-glucanase and chitinase, degrade the chitin-supported structure of the cell walls of several but not all pathogenic fungi, whereas lysozymes degrade the glucosamine and muranic acid components of bacterial cell walls (Agrios, 1997). Expression of pathogenesis related proteins will be discussed in more detail later on together with defence signalling molecules.

Oligogalacturonides, the end-products of CWDEs act as elicitors of plant defence mechanisms (Cervone *et al.*, 1989). They are called endogenous elicitors and believed to be

non-specific. In tomato plants, oligogalacturonide concentration was significantly increased by the action of feeding insects or mechanical wounding (Bergey *et al.*, 1996). Moreover, galacturonides with a degree of polymerization between 4-6 are reported to induce ET production in tomato plants (Simpson *et al.*, 1998). Polygalacturonase inhibing proteins (PGIPs) are produced by plants in order to inhibit microbial endopolygalacturonases. Specificity of PGIPs differ between plant species as well as within a single species, depending on type of attacking pathogen (Desiderio *et al.*, 1997). Transgenic tomato plants over expressing a PGIP gene from pear showed increased resistance against *B. cinerea* (Powell *et al.*, 2000). On the other hand, when a gene from pear, encoding a polygalacturonase inhibiting protein (PGIP-1) was over expressed in tomato plants using CaMV35S promoter, no significant increase in resistance against *B. cinerea, Fusarium oxysporum* f.sp. *lycopersici* or *A. solani* was observed (Desiderio *et al.*, 1997). Moreover, in this study was shown that PGIP-1 expressed in tomato and *N. benthamiana* only partially inhibited crude PG preparations from *F. oxysporum* f.sp. *Lycopersici, B. cinerea* and *A. solani.*

The presence of conserved leucine-rich repeat domains in PGIPs suggests some sort of coevolution between PGIPs from plants and PG from pathogens (Stotz *et al.*, 2000), similar to the gene-for-gene concept (Flor, 1971; DeWit, 1997).

### 1.4.4 *Arabidopsis* mutants affected in resistance against *B. cinerea*

The deployment of *Arabidopsis-B.cinerea* model pathosystem started to reveal some important components of the defence signalling pathway against necrotrophic pathogens. Mutant *Arabidopsis* plants conveying enhanced susceptibility towards *B. cinerea* (*BOS* loci) have been isolated (Mengiste *et al.*, 2003; Veronese *et al.*, 2004). The *Botrytis Susceptible 1* (*BOS1*), isolated from a T-DNA population, also display enhanced susceptibility to *A. brassicicola* and impaired tolerance to drought, salinity and oxidative stresses (Mengiste *et al.*, 2003). Conversely, in response to the biotrophic pathogens *Pst* and *H. parasitica bos1* plants show enhanced disease symptoms, but unaltered pathogen growth. *BOS1* was shown to encode an R2R3MYB transcription factor protein and is

suggested to act by modulating response to signals, via the reactive oxygen species generated during biotic and abiotic stresses (Mengiste *et al.*, 2003).

In addition to the *bos1* mutant, another three *Botrytis Susceptible Loci* (*bos2, bos3* and *bos4*) (Veronese *et al.*, 2004) and a *Botrytis-Induced serine/threonine protein kinase* (*BIK1*) (Veronese, 2004) have been identified. Besides the enhanced susceptibility to *B. cinerea,* the mutants *bos3* and *bos4* also show increased susceptibility to *A. brassicicola.* In contrast, *bos2* seems to be specifically required for resistance against *B. cinerea.* Interestingly, mutation in *BOS4* also compromised resistance against a strain of *P. syringae* carrying the avirulence gene *avrRpm1*, while *bos3* shows increased resistance against virulent and avirulent strains of *Pst* and *H. parasitica Noco2.* The expression kinetics of the *PR1* gene in the different *bos* mutants seemed to inversely associated with susceptibility to *B. cinerea,* with the highest expression in *bos3*, the most susceptible line (Veronese *et al.*, 2004). Transcription of the *BIK1* gene is not induced by the exogenous application of Me-JA, ET or SA and its transcriptional activation by *B. cinerea* does not require the accumulation or transduction signalling of these hormones. Loss-of-function in the *BIK1* gene conversely resulted in enhanced susceptibility to *B. cinerea* and increased resistance to a virulent strain of the bacterial pathogen *P. syringae*, while resistance to the avirulent strain *avrRpm1* was unaffected, suggesting *BIK1* is required for basal but not R gene mediated resistance. The presence of a plant N-myristoylation motif in the open reading frame of *BIK1* suggests membrane localization, where *BIK1* may figure as a general receptor for necrotrophic pathogens, functioning at early stages of pathogen recognition or transduction of pathogen response (Veronese, 2004).

The *Arabidopsis ocp3* (*overexpresser of cationic protein 3*) was recently isolated and its role in disease resistance investigated (Coego *et al.*, 2005). Recessive mutations in *OCP3* convey enhanced resistance against the necrotrophic pathogens *B. cinerea* and *Plectosphaerella cucumerina*, while resistance against the virulent pathogens *H. parasitica* and *Pst* is not affected. The *OCP3* gene was cloned and shown to encode a homeodomain transcription factor that is constitutively expressed in healthy plants but repressed in response to infection with necrotrophic fungi (Coego *et al.*, 2005). Healthy *ocp3* plants show increased accumulation of $H_2O_2$ and constitutively express the ROI inducible *Gst1* (*Glutathione S-transferase1*) and JA- dependent *PDF1.2* genes, while SA-dependent

expression of *PR-1* still remains. Analysis of double mutants *ocp3coi1, ocp3npr1* and *ocp3ein2* suggest resistance against necrotrophic pathogens is JA-dependent, mediated through *COI,* but does require neither SA nor ET, through *NPR1* or *EIN2* perception.

# 2 Materials and Methods

## 2.1 Plant material and growth conditions

*Arabidopsis thaliana* (*Arabidopsis*) seeds of ecotype Columbia (Col-0) and *Landsberg erecta* (L*er*) were used. All *Arabidopsis* transgenic and mutant lines used were in a Col-0 or L*er* background (table 2.1).

Table 2.1 *Arabidopsis* lines used

| ***Arabidopsis* accessions** | | | |
|---|---|---|---|
| Accession | Phenotype | Reference | Source |
| Col-0 | wild-type | | NASC |
| L*er* | wild-type | | NASC |
| *as1* (Ler) | *Asymetric leaves* | (Byrne *et al.*, 2000) | Hudson, Edinburgh |
| *as1* (Col-0) | *Asymetric leaves* | (Redei, 1965) | Hudson, Edinburgh |
| *NahG* | Salicylate hydroxylase transgenic | (Lawton *et al.*, 1995) | Novartis, USA |
| *ein2-1* | Ethylene insensitive | (Guzman and Ecker, 1990) | NASC |
| *etr1* | Ethylene insensitive | (Bleecker *et al.*, 1988) | NASC |
| *coi1-1* | Jasmonate insensitive | (Feys *et al.*, 1994) | Turner, University of East Anglia |
| *as2* | Asymetric leaves | (Ori *et al.*, 2000) | Hudson, Edinburgh |
| *KN1* | Lobed leaves | (Hay *et al.*, 2003) | Tsantis, Oxford Univ. |

**Antirrhinum accessions**

| Accession | Phenotype | Reference | Source |
|---|---|---|---|
| Sippe 50 | Wild type | | Hudson, Edinburgh Univ. & IPK inst. Germany |
| *PHAN* | asymmetric leaves | (Waites *et al.*, 1998) | Hudson, Edinburgh Univ. & IPK inst. Germany |

**Tobacco accessions (*Nicotiana silvestris*)**

| Accession | Phenotype | Reference | Source |
|---|---|---|---|
| *Nicotiana silvestris* | Wild type | (McHale and Koning, 2004) | Koning, Connecticut-USA |
| *AF18B.1* | *Asymetric leaves* | (McHale and Koning, 2004) | Koning, Connecticut-USA |

Seeds were placed on soil and allowed to vernalise for 48 hours at 4°C after which they were transferred into growth rooms. Plants were grown under 8-hours of light at 22°C and 16-hours of dark at 18°C. For aseptic growth, seeds were sterilised with 10% commercial bleach (v/v) for 20 minutes, washed 4 times in distilled water and maintained for 48 hours in the dark at 4°C to improve germination uniformity. Seeds were subsequently transferred to MS plates containing MS basal salts supplemented with 1% (w/v) sucrose and 1% (w/v) agar. All chemicals employed were purchased from Sigma-Aldrich UK or unless stated otherwise. Petri dishes were transferred to a growth chamber with 8-hours of light at 22°C and 16-hours of dark at 18°C.

### 2.1.1 Chemical treatments

A 20 mM methyl jasmonate (Me-JA) stock solution was prepared in 0.1% (v/v) ethanol. A 1:40 dilution, containing 0.01% (v/v) Silwet (Union Carbide, UK), was used to paint buds of *AOS* antisense plants in order to rescue seed production. For sensitivity of *Arabidopsis* to jasmonate, seeds were sown on MS plates containing 150 μM Me-JA and seedlings root length measured seven to ten days after germination.

To transiently express *KN1* a conditional expression system was employed in a Col-0 background (Hay *et al.*, 2003). This consisted of a C-terminal fusion of the *KN1* cDNA with the steroid binding domain of the rat glucocorticoid receptor from pBI-ΔGR. A line containing an empty gene cassette was utilized as a control. *35S::KN1-GR* and *35S::-GR* transgenic lines were treated with dexamethasone (DEX) as described previously (Grant *et al.*, 2003). Briefly, a 1mM DEX stock solution was prepared in ethanol and a 1:1000 dilution, containing 0.01% (v/v) Silwet, was used to homogenously spray plant leaves.

The *Arabidopsis* activationally tagged seeds were sown in flats and transformants selected by spraying seedlings twice with a 150mg/l BASTA herbicide solution (Agrevo, Germany). Seedlings were first sprayed one week after germination, and again four days later. Resistant plants were visually identified one week post herbicide treatment.

### 2.1.2 Generation of double mutants

Crosses were performed using pollen from homozygous *ibr1* plants to fertilise flowers of the male sterile *coi1* (Feys *et al.*, 1994) and *aos* (Loake lab) plants. Unopened flowers of homozygous *ibr1* plants were pollinated with pollen from the mutants *etr1* (Bleecker *et al.*, 1988), *ein2* (Guzman and Ecker, 1990), and *adr1* (Grant *et al.*, 2003). Since *ibr1* phenotype is conferred by a single recessive mutation, successful crosses were easily identified by lack of *ibr1* phenotype in the $F_1$ generation. Double mutants between *ibr1* and ethylene signalling mutants were identified in the $F_2$ and $F_3$ generations by the morphological phenotype and response to the ethylene precursor 1-amino-cyclopropane-1-carboxilic acid (ACC). $F_2$ seeds from a crossing between *ibr1* and *coi1* were sown on MS plates containing

150μM Me-JA and JA insensitive seedlings were transferred onto soil prior to selection for *ibr1* phenotype. *Ibr1aos* double mutants were selected by the *ibr1* phenotype and male sterility conferred by *aos antisense*, and rescued by painting buds with a 500μM Me-JA solution.

## 2.2 Pathogen growth and disease resistance assays

***Pseudomonas syringae*** pv tomato DC3000 (*Pst*DC3000) (Whalen *et al.*, 1991) was grown in King's broth (KB) liquid media supplemented with 50 mg/l rifampicin. Four-week old soil-grown *Arabidopsis* plants were infected with a *Pst* DC3000 suspension in 10 mM $MgCl_2$ by infiltrating the abaxial side of the leaf with a 1 mL syringe (Cao *et al.*, 1994). Three leaves per plant and six to ten plants per line were infiltrated. After three days, leaves were harvested for analysis of bacterial growth. 100mg of leaf tissue were ground in 1000 μl 10 mM $MgCl_2$ using a mortar and pestle. Serial dilutions were made from the resulting bacterial suspension, and 100 μl of each dilution was used to inoculate KB medium plates containing 50 mg/l rifampicin. The plates were incubated at 30°C for 2 days, and the number of bacterial colonies for each sample was recorded. Bacterial counts were statistically analysed using the Student T test (Mini-tab version 13).

***P. syringae*** pv tomato DC3000 isolates carrying the avirulent genes *avrB* and *avrRps4*, respectively, were grown on Kings Broth supplemented with 50 mg/l rifampicin and 50 mg/l kanamycin. Liquid cultures were grown on a shaker at 30°C, and cells were pelleted by centrifugation and re-suspended for plant inoculation in 10 mM $MgCl_2$ at. For inoculations, 10 μl of the PstDC3000 (*avrB*) solution were forced under the abaxial epidermis using a 1 ml syringe. Successful inoculations were visualised by the appearance of a watery area under the epidermis.

***Pseudomonas fluorescens*** *2-79* (Thara *et al.*, 1999) was grown on KB liquid media supplemented with 50 mg/l rifampicin. Four-week-old soil-grown plants were infected with a *P. fluorescens* suspension in 10 mM $MgCl_2$ by syringae infiltration, as described above

for *Pst* DC3000 infection. Measurements of bacterial growth and statistical analysis were carried out as described for *Pst* DC3000 infection.

***Botrytis cinerea*** isolated from strawberry plants was grown on oat meal medium for 7-10 days. Spores were collected by washing the colonies with sterile water, passed through a cheese cloth tissue to remove mycellium and centrifuged at 3,500 rpm for 10 minutes to pellet the spores. The pellet was resuspended in 1mL of ½ potato dextrose broth (PDB) medium, and dilutions thereafter used to determine spores concentration using a haemacytometer chamber and a light microscope. For testing resistance against *B. cineria*, four to five week-old *Arabidopsis* plants were sprayed with a conidial suspension containing 5 x $10^5$ spores/mL resuspended in 1/2 PDB until droplets ran off, or drop inoculated by applying a 5 µL drop of spore suspension on surface of three leaves per plant. Two months old *Antirrhinum* plants were inoculated by applying a single 10 µL drop of spore suspension on surface of three leaves of each plant and twelve plants per line. Experiments were repeated three times. Inoculated plants were incubated at 100% relative humidity for 4-6 days before being examined for typical necrotic lesions caused by *B. cineria* infection. Scoring was as follows: 0 = no necrotic lesions, 1 = plants showing small-dry lesions, 2 = plants showing a mix of small and medium size lesion, 3 = plants showing medium size and spreading lesions, 4 = plants showing predominantly spreading lesions, 5 = plants showing predominantly wide necrotic lesions. Plants in different replicates were assigned a disease index as follows: $D.I.=\sum i.j/n$, where i = Infection class, j = the number of plants scored for that infection class and n = the total number of plants in the replicate (Epple *et al.*, 1997). Twenty plants were infected for each line.

***A. brassicicola*** (MUCL20297; Mycotheque Universite Catholique de Louvain, Louvain-la-Neuve, Belgium) (Thomma *et al.*, 1999b) was grown on potato carrot and agar (PCA) medium. Spores were collected as for *B. cinerea* and the spore density adjusted to $5x10^5$ spores/ml in sterile water. Plant inoculation was done in a similar fashion to *Botrytis*, except that spore drop inoculation was performed on detached leaves. Four-week old *Arabidopsis* plants were either spray or drop inoculated with a spore suspension. Leaves from two months old *Antirrhinum* plants were inoculated by applying a single 10 µL drop

of spores suspension on surface of each leaf (three leaves per plant and twelve plants per line).

***Hyaloperonospora parasitica*** Noco2 (Parker *et al.*, 1993) and Cala2 (Holub *et al.*, 1994) were maintained as previously reported (Bowling *et al.*, 1994). Typically, 3-week-old soil-grown plants were infected by spraying a solution of $1x10^6$ conidiospores per ml. Plants were maintained in humid conditions for 10 days and results were taken at this point. Infected plants were scored by counting the number of conidiophores per leaf. Three to eight leaves per plant were scored and three to six plants per line were examined.

## 2.3 DNA and RNA blot analyses

### 2.3.1 Southern blot analysis

DNA (10 µg) isolated from *ebs1* mutant line was digested overnight using the restriction enzymes *ApaI*, *BamHI*, *EcoRI*, *KpnI*, *SacI*, *SpeI*, *TaqI* and *XhoI*, (Promega, UK). Digested samples were run through a 0.8% agarose gel and transferred onto a nylon membrane (Amersham, UK) according to the supplier instructions. The membrane was pre-hybridised for 1 hour at 42°C and hybridised sequentially with two probes (described below). Probes were labelled with $\alpha$-$^{32}$P-dCTP by random priming using the Prime-a-Gene® labelling system (Amersham, UK). Hybridization was run over-night at 65°C according to the instructions of the supplier (Promega, UK). Blots were washed twice for 30 min each at 65°C in 4 X SSC (saline sodium·citrate), 1% (w/v) SDS, which was followed by two washes at 65°C in 4 X SSC, 0.5% (w/v) SDS. Blots were exposed to X-Omat-AR™ imaging film (Kodak) for an appropriate time period. Blots were stripped by incubation in boiling 0.1% (w/v) SDS and washing in 0.5 X SSC for 30 min at room temperature, before hybridization with a subsequent probe (Sambrook *et al.*, 1989).

Two DNA fragments were employed as probes: a 339 bp fragment corresponding to the CaMV35S enhancer region and a 610 bp fragment corresponding to the BAR gene. Probes were obtained by PCR using primers 5'-GATCCCCAACATGGTGGAGCACG-3' and 5'-

TAGATATCACATCAATCCACTTGC-3' (CaMV35S enhancer); 5'-GAAGTCCAGCTG CCAGAAAC-3' and 5'-CACCAAATCGACTCTAGCCA-3'(BAR gene).

### 2.3.2 Northern blot analysis

Total RNA was extracted from *Arabidopsis* leaves harvested from four-week old plants using the Trizol protocol www.science.siu.edu/nickrent/PLB420/DNA.Technique/Trizol.method.html). In summary, leaf tissue (approximately 0.1g) was ground inside of a 1.5 ml microcentrifuge tube (kept in liquid nitrogen), using an electric micropestle, followed by the addition of 200 μL of Trizol solution. In sequence, tubes were vortexed vigorously until the sample was completely dispersed and 40 μL chloroform added into each tube and vortexed again for another 15 seconds. After 2-3 minutes standing at room temperature, tubes were centrifuged at 10,000g for 10 minutes and the aqueous phase (upper phase) transferred into a new tube. Isopropanol and 0.8 M sodium citrate/1.2 M NaCl, 1/2 volume of the aqueous phase each was added into each tube and mixed by inverting the tubes gently. Tubes were let to stand for 10 minutes before another centrifugation cycle at 10,000g for 10 min. Pellet was washed in 75% ethanol (v/v) once, followed by an extra centrifugation step at 10,000g for 5 min. The pellet was dried at room temperature and then resuspended in 50 μL DEPC-treated water. Alternatively, RNA extraction was carried out using an RNA kit (Qiagen, CA, USA) according to the instructions of the supplier. The absorbance of each sample was measured at 260 nm, and used to calculate the concentration of RNA. Samples (10 μg) were separated on formaldehyde-agarose gels (Sambrook *et al.*, 1989), transferred to a Hybond™-N hybidization membrane according to the instructions of the supplier (Amersham, UK) and hybridized with the relevant probes (described below). Dextran sulphate (10% w/v) was included in the pre-hybridization / hybridization solution in order to enhance efficient binding of the probe (Sambrook *et al.*, 1989). Blots were washed twice for 30 min each at 65°C in 4 X SSC, 1% (w/v) SDS, which was followed by two washes at 65°C in 4 X SSC, 0.5% (w/v) SDS. Blots were exposed to X-Omat-AR™ imaging film (Kodak) for an appropriate period. Blots were stripped by incubation in boiling 0.1% (w/v)

SDS and washing in 0.5 X SSC for 30 min at room temperature, before hybridization with a subsequent probe .

Probes were prepared by amplification of appropriate sequences using PCR and directly purified using a kit (Quiagen, UK) (Table2.2). Alternatively, probes were generated from plasmids by digestion with relevant restriction enzymes and purified from the gel using a gel purification kit purchased from Quiagen (Table2.2). Sequences for the PCR primers and templates used for each probe are reported. Probes were labelled with $\alpha$-$^{32}$P-dCTP by random priming using the Prime-a-Gene® labelling system (Promega, UK).

**Table 2.2 DNA probes employed for northern blot analysis.**

| Gene | Template | Forward primer | Reverse primer | RE |
|---|---|---|---|---|
| *AS1* | Genomic DNA | GCCTATTGACGAG AGTAAGTAC | CCACAAGCTCTGAC AAGAACAC | |
| *PR-1* | *TA-PR1* | CTGCAGACTCATA CACTCTGG | TATGTACGTGTGTA TGCATGATC | - |
| *PDF1.2* | genomic DNA | TCATGGCTAAGTT TGCTTCC | AATACACACGATTT AGCACC | - |
| *PR-3* | Genomic DNA | GCCACGTCCACAC TCCAATC | CTACAGCACCAGA CGGACCA | - |
| *PR-4* | Genomic DNA | ATACTTGCTCCGC CATGC | ACAACAATGCGGT CGTGAAG | - |
| *Botrytis ActinA* | pSK-ActinA | ---------- | ---------- | *EcoRI* *HindIII* |

## 2.4 Biochemical Analysis

### 2.4.1 Ethylene emission

For measurements of ethylene emission, 4-week-old plants were spray inoculated with *B. cinerea* spores and samples collected 48 h post-inoculation. Leaves from infected and mock plants were carefully detached by cutting the petiole with a sharp blade, weighed and placed into a 20 mL capsule containing 1 mL water with petioles immersed in the water. Three replications for each treatment were analysed, and each sampled composed of leaves from six plants. Capsules were sealed and left to stand in the growth room for 24 hours. A 1 mL gas sample removed from the headspace was subjected to gas chromatography using a HP5980 series II gas chromatograph with a flame ionisation detector (Hewlett Packard, Palo Alto, CA) equipped with a Quadrex BTR-CW column (50m length, 0.32mm diameter) and run at 80°C. These experiments were performed in collaboration with Dr. Peter Morris (Heriot Watt University).

### 2.4.2 Jasmonic acid

Free endogenous JA was quantified by gas chromatography/mass spectrometry. Quantification procedure was carried out by Chemistry Department, University of Edinburgh. The stable isotope-$2H_2$-JA (90.4% total JA), was employed as an internal standard. The samples were extracted three times with 70% methanol. The combined extract was then passed through a $C_{18}$ solid phase extraction cartridge (pre-conditioned, in turn in methanol and 70% methanol) and washed with 70% methanol. The combined eluate and washings were vacuum concentrated, diluted with 2.5 ml of water and acidified with 0.15 ml concentrated hydrochloric acid. The aqueous phase was extracted with chloroform and concentrated to 0. 2ml before transfer to a 0.2 ml autosampler vial. Samples were evaporated to dryness and derivatized with hexane and N-methyltrimethylsilyl-trifluoroacetamide and analysed on a Micromass GC-TOF mass spectrometer in accurate mass acquisition mode. Endogenous JA was quantified on the basis of the ratio of the JA: $^2H_2$-JA integrated peak areas for the molecular ions (m and z 282.1651 and 284.1777

respectively). For each sample, three sample preparation replicates were each analysed in triplicate.

### 2.4.3 Salicylic acid

Free and conjugated endogenous SA levels were determined by HPLC analysis, as described previously (Aboul-Soud *et al.*, 2004) with minor modifications. Essentially 200 mg of leaf tissue per sample was collected and promptly frozen in liquid nitrogen. Samples were then ground in liquid nitrogen using a mortar and pestle and pulverized tissue transferred to a Falcon tube, followed by the addition of 1 mL of 90% methanol and vortexed for 1 minute. It is important that sample does not thaw before adding MeOH. The sample was then transfered to a 2 mL microtube, centrifuged at 15000g for 5 minutes and supernatant transferred to a new tube. Pellet was resuspended in 1 mL 100% methanol, centrifuged and two supernatant pooled together and dried in a speed vacuum centrifuge at medium temperature. Each pellet was then resuspended in 1 mL 5% of 5% trichloroacetic acid, followed by the addition of 1 mL of ethylacetate:cyclopentane:isopropanol (50:50:1) and vortexed for 1 minute. The organic phase was transferred to a new tube. The aqueous phase was reextracted with another 1 mL of the organic the 50:50:1 mix and the two supernatant pooled together and evaporated under heat in the vacuum centrifuge. The aqueous phase was then acidified to pH 1, boiled for half hour to release conjugated SA and extracted with the organic mix twice. The two supernatant were pooled together and dried in the vacuum centrifuge. The residues were dissolved in 200 µL of 50% methanol, filtered and subjected to HPLC analysis.

### 2.4.4 Camalexin accumulation

Camalexin was extracted as previously (Glazebrook and Ausubel, 1994). Essentially, leaf disks (50 mg FW) were incubated in 1.7 mL of methanol (80%) at 65°C for 20 min. Leaf tissue was removed, and methanol extracts subjected to evaporation under reduced pressure (speedvac) untill the volume was reduced to approximately 500 µL. Samples were

extracted with 2x 150μL of chloroform, and removal of the chloroform phases (the green lower phases) into clean tubes. Chloroform was then evaporated under reduced pressure and residues disolved in 200 μL of 50% methanol. After filteration, samples were quantified by using high-performance liquid chromatography (HPLC) (Mert-Turk *et al.*, 2003; Veronese *et al.*, 2004) with a Dionex HPLC system equipped with AS50 autosampler, AS50 thermal compartment, GS50 gradient pump, PDA-100UV-is detector and RF2000 fluorescence detector. The analytical column used was a 250 x 4.6mm Phenomenex Luna 5μ C-18 (2) and a variable wavelength spectrofluorometric detector (excitation 318 nm/emission 385 nm) used. Quantification was performed using a *chromaleon* chromatography software by integration of peak area and a calibration curve of camalexin standards. The eluents used were: Time 0 - 5 min 60% MeOH to 70% MeOH; time 5-10 min Isocratic 70% MeOH and flow rate 1 ml/min at column temperature of 35 degrees Celsius. Camalexin was quantified by comparing peak areas with camalexin standards (1μg/ml) (provided by Dr Jane Glazebrook, University of Minnesota-Twin cities, MN, USA) with respect to retention time (~9 min) and UV absorption spectra.

## 2.5 Histochemical analyses

### 2.5.1 Trypan blue staining

Trypan blue staining was employed to examine dead plant cells. Leaves were stained by boiling for 5 minutes in alcoholic lactophenol trypan blue (20 ml of ethanol, 10 ml of phenol, 10 ml of water, 10 ml of lactic acid and 10 mg of trypan blue). Stained leaves were cleared in chloral hydrate (2.5 g in 1 mL of water) overnight at constant shaking and then mounted under coverslips in 60% glycerol (Bowling *et al.*, 1997).

Hydrogen peroxide ($H_2O_2$) production was detected by histochemical staining using 3,3-diaminobenzidine (DAB) as described previously (Thordal-Christensen *et al.*, 1997). Leaves were stained by treatment with a solution of 1 mg/ml DAB over-night with constant shaking. Leaves were then cleared by boiling in an acetic acid/glycerol/ethanol (1/1/3 v/v/v) solution and then mounted under coverslips in 60% glycerol.

### 2.5.2 Nitroblue tetrazolium (NBT) stain

$O_2^-$ accumulation in planta was visualised by NBT staining (Jabs *et al.*, 1996). Leaves were immersed in 10 mM $KH_2PO_4$ buffer (pH 7.8) containing 600 μM NBT for 2 hours in the dark. The stain was removed and chlorophyll cleared by boiling in 96% (v/v) ethanol for 10 minutes.

### 2.5.3 Diaminobenzidine (DAB) staining

$H_2O_2$ accumulation in planta was visualised by DAB staining (Yun *et al.*, 2003). DAB is rapidly absorbed by the plant and is polymerised locally in the presence of $H_2O_2$ and peroxidase giving a visible brown stain. Leaves were placed in 50 ml tubes, covered with 0.1% (w/v) 3,3-diaminobenzidine solution and incubated overnight. The stain was removed and the chlorophyll cleared by boiling in 96% (v/v) ethanol for 10 minutes.

## 2.6 Abiotic stress treatments

### 2.6.1 Drought stress assay

For drought stress assay, seedlings from both lines were transplanted into soil, in trays with 72 plants, with rows of each line planted in sequence, in order to provide the most similar growth condition to both lines. Plants were grown for about three weeks when they were not watered for 12 days and re-watered at day 13 (Kang *et al.*, 2002). Approximately 144 plants per line were used in each experiment, which was repeated once.

### 2.6.2 Salt stress assay

To examine potential tolerance to salinity, 3-week-old soil grown plants were irrigated with an increasing concentration of salt (NaCl) every 4 days. The concentrations of sequentially

applied sodium chloride were 50 mM, 100 mM, 150 mM, 200 mM and 400 mM (Song *et al.*, 2003).

## 2.7 Homology searches and sequence analyses

The bioinformatic tools from web-sites presented here (Table 2.7) were used for sequence search and analysis. Instructions were followed as detailed at each site. Homology searches in *Arabidopsis* were carried out using BLAST and WU-BLAST2 at the TAIR web-site, whereas plant homology searches were conducted using the MIPS and TIGR databases.

**Table 2.7 Web-sites used for sequence search and analysis.**

| Name | Function | Web-address |
|---|---|---|
| BLAST | Homology search in *Arabidopsis* | http://www.*Arabidopsis*.org/Blast |
| WU-BLAST2 | Homology search in *Arabidopsis* | http://www.*Arabidopsis*.org/wublast/index2.jsp |
| Multiple Expectation Maximisation for Motif Elicitation (MEME) | Alignment of multiple peptide sequences | http://www.ebi.ac.uk/clustalw/index.html |
| The Institute for Genomics Research (TIGR) | Plant Sequence Database and BLAST search | http://www.tigr.org/tdb/euk/ |

## 2.8 PCR based methods

### 2.8.1 TAIL-PCR

Genomic DNA from *Arabidopsis* tagged lines was extracted using the CTAB protocol (Dellaporta, 1983) and TAIL-PCR performed as described previously (Singer and Burke, 2003) with suggested modifications (Sessions *et al.*, 2002). The specific and arbitrary degenerated primers used for TAIL-PCR are listd in table 2.8.1.

**Table 2.8.1- Arbitrary degenerated primers for TAIL-PCR**

| Primer | Sequence |
|---|---|
| AD1 | NTCGASTWTSGWGTT |
| AD2 | NGTCGASWGANAWGANA |
| AD3 | TGWGNAGSANCASAGA |
| AD4 | WGTNAGWANGANAGA |
| AD5 | AGWGNAGWANCAWAGG |
| AD6 | STTGNTASTNCTNTGG |

Key to symbols: R=A+G, Y=C+T, M=A+C, K=G+T, S=G+C, W=A+t, H=A+T+C, B=g+T+C, D=G+A+T, N=A+C+G+T, V=G+A+C

### 2.8.2 RT-PCR

RNA was extracted and the concentration determined as before. RNA was DNAase (Promega) treated by adding 1 µl of DNAase per µg of RNA in 1X Promega DNase buffer and incubating at 37°C for 30 minutes. The reaction was stopped by adding 1X Promega

stop buffer and incubating at 65 °C for 10 minutes. RT-PCR was carried out using QIAGEN One Step RT-PCR kits according to the manufacturer's instructions. The PCR program was as follows; Reverse Transcription for 30 minutes at 50 °C, PCR activation 15 minutes at 95 °C, 35 cycles of 1 minute at 94°C, 1 minute at 64 °C, 1 minute at 72°C and final extension 10 minutes at 72 °C. The primers used were as shown in table 2.8.2.

**Table 2.8.2 Primers used for RT-PCR**

| Gene | Forward primer | Reverse primer | Product |
|---|---|---|---|
| *At*2G37630 *ASI* | GCCTATTGACGAGAGT AAGTAC | CCACAAGCTCTGACA AGAACAC | 0.4 kb |
| *At*3G26650 (GAPDH) | CTTGCTCCCTTTGTCAA AGTTCTT | CGCGTCTAGCAACCT CTGGT | 0.11 kb |

## 2.9 Mapping of *ibr2* using SSLP markers

In order to generate an $F_2$ mapping population, *ibr2* plants were crossed to L*er*. Homozygous $F_2$ plants expressing the mutant phenotype were selected for mapping, which was performed using single sequence length polymorphism (SSLP) protocol (Bell and Ecker, 1994). This is an approach based on PCR of polymorphic genomic DNA sequences between Col-0 and L*er*. The selection of markers was based on The *Arabidopsis* Information Resource (TAIR) (http://www.*Arabidopsis*.org/).

Genomic DNA was extracted from leaf tissue using a CTAB DNA extraction protocol (Dellaporta, 1983). Approximately 100 mg leaf tissue from each plant were ground in 0.4 ml DNA extraction buffer with 0.2% 2-mercaptoethanol. Samples were incubated at 60°C for 30 min followed by extraction with an equal volume of chloroform. After centrifugation (4000g, 15 min), the DNA was precipitated from the resulting supernatant by the addition of an equal volume of isopropanol. After a further centrifugation step (13000g,

20 min), the DNA pellet was washed in 70% (v/v) ethanol and finally dissolved in 60 µl of water.

The markers used in the present project are listed on Table 7.5. All primers were manufactured by Sigma (UK). PCR reactions were performed using 10X buffer, magnesium chloride, *Taq* polymerase (Promega) and deoxynucleotides from Sigma. The PCR reactions were carried out in 20 µl volume containing 1-2µl of the DNA miniprep (approximately 10-20 ng DNA), 5 pmol of each primer, 200 µM each of four deoxynucleotides, 2 mM magnesium chloride and 1 unit of *Taq* polymerase. PCR reactions were carried out in a PTC- 200 Peltier thermal cycler. PCR conditions were as follow: 94°C for 3min, followed by another 34 cycles of (94°C for 1min, 58°C for 1min, and 72°C for 1min and 30 seconds), 72°C for 10min, and cooled down to 4°C. 5 µl loading dye was added to each tube prior to electrophoresis. Agarose gel concentrations optimized for each marker are listed in Table 2.2. Col-0 and L*er* samples were included on each gel as controls and run in adjacent lanes in order to visualize polymorphisms. A 1 kb and or a 100bp DNA ladder (New England Biolabs) were also included on each gel in order to determine the size of different PCR products.

# 3 Results

## 3.1 Mutagenesis – the source of genetic variability

Mutagenesis is a vital tool for the elucidation of gene function and understanding the genetic mechanisms underlying the phenotypic expression of characters. Creation of mutants for studying host-pathogen interactions has gained significant attention, especially during the last few decades. Mutagens have been routinely used for this purpose, however, most mutagens employed until recently produced predominantly loss-of-function (knockout) mutants (Weigel *et al.*, 2000). Sequencing of *Arabidopsis* and other eukaryote genomes revealed the existence of two classes of genes that are unlikely to emerge from classical loss-of-function screens (Miklos and Rubin, 1996). Some genes are present with several copies in the genome which are very similar in sequence and function, and are therefore unlikely to be uncovered by loss-of-function screens. A second group of genes, encoding proteins that function at multiple stages of the plant life cycle, and play a fundamental role during embryogenesis are also difficult to be identified by classical screening, because loss-of-function is frequently lethal. The deployment of gain-of-function techniques such as activation tagging has rendered significant progress for studying these two classes of genes (Tani *et al.*, 2004).

## 3.2 Activation tagging strategy

Deployment of the activation tagging technique in the identification of genes has been undertaken for several species with diverse purposes (Tani *et al.*, 2004). Genes have been uncovered in *Arabidopsis* (Hua and Meyerowitz, 1998; Borevitz *et al.*, 2000; van der Graaff *et al.*, 2000; Berrocal-Lobo *et al.*, 2002), in *Catharanthus roseus* (van der Fits and Memelink, 2000), in petunia (Zubko *et al.*, 2002) and in *Drosophila* (Schneuwly *et al.*, 1987).

A landmark technique for alteration of gene expression was created by Walden and co-workers (Hayashi *et al.*, 1992). They created a construct carrying four copies of the

enhancer element of the constitutively expressed promoter of the Cauliflower mosaic virus (CaMV) *35S* gene. This construct can enhance gene transcription in either side of insert. This technique was termed activation tagging. Considering that *Agrobacterium* randomly inserts the T-DNA construct in the plant genome, activation tagging can generate either gain-of-function or for loss-of-function mutants. Overexpressed genes almost always occurred immediately adjacent to the inserted CaMV*35S* enhancers, at distances varying from 0.38-3.6 kb (Weigel *et al.*, 2000). Moreover, they noted that in at least one case, the CaMV*35S* enhancers led to an enhancement of the endogenous expression pattern rather than to constitutive gene expression, suggesting that the enhancers alone act differentially from the complete CaMV*35S* promoter. The enhancement promoted by these elements indicated that activation tagging can overcome the stringent genetic controls regulating accumulation of specific natural products during plant developent (Borevitz *et al.*, 2000).

In *Arabidopsis*, activation tagging is particularly easy and efficient. This plant is easily transformed by *Agrobacterium tumefaciens* and simple and robust methods have been developed (Bechtold *et al.*, 1993; Clough and Bent, 1998). A recently cloned mutant (*adr1*) shows constitutive expression of a series of key defence marker genes, elevated levels of SA and enhanced resistance against the biotrophic pathogens *Hyaloperonospora parasitica* and *Erysiphe cichoracearum*, (Grant *et al.*, 2003). Interestingly, *adr1* plants are not affected in either JA or ET pathways, and consequently show unaltered resistance against the necrotrophic pathogen *B. cinerea*. In addition to disease resistance, *adr1* mutant plants also display enhanced tolerance to water deficit and conversely sensitivity to heat and salinity stresses (Chini *et al.*, 2004). Analysis of a series of *adr1* double mutants suggested that *adr1*-mediated resistance against *H. parasitica* is SA-dependent, while resistance against *E. cichoracearum* was both SA-dependent and partially NPR1-dependent (Chini, 2004; Chini *et al.*, 2004). *ADR1* was shown to encode a protein with homology to subdomains of protein kinases, a nucleotide binding domain and leucine rich repeats (Grant *et al.*, 2003). Sequence analysis revealed that *ADR1* is a member of a small atypical *Arabidopsis* sub-class containing four *CC-NBS-LRR* genes. In addition, homologous genes were uncovered in many phylogenetically distant and agronomically important plant species; their sequence analysis revealed a number of consensus motifs unique and distinctive for the ADR1 family (Chini, 2004).

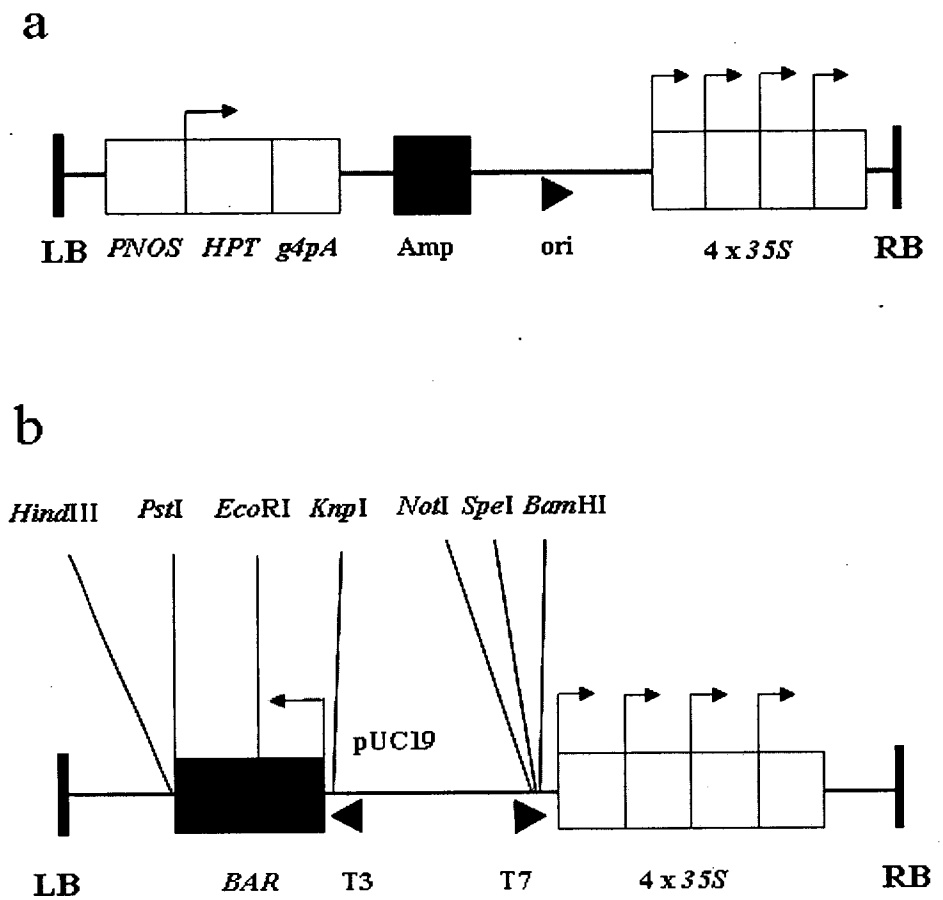


**Figure 3.1 Molecular architecture of the activation tagging vectors pVICE n4HPT and pSKI015 (a)** pVICE n4HPT contains four copies of the CaMV 35S enhancer sequence adjacent to the right T-DNA -border sequence (RB), which potentially enhance expression of genes neighbouring both the left border (LB) and RB sequences. The construct harbours an ampicillin gene (Amp) and an origin of replication (ori) which allows the isolation of flanking sequences by plasmid rescue in *Escherichia coli.* A hygromycin resistance gene (HPT) fused with the nopaline synthase promoter (PNOS) and the poly A sequences of gene 4 of the *A. tumefaciens* T-DNA -(g4pA) provides a selectable marker for successful transformation. (**b**) The activation tagging vector pSKI015 (Weigel *et al.*, 2000) derived from pVICE 4HPT. BAR: glufosinate resistance gene. The construct harbours an ampicillin gene (Amp) and an origin of replication (ori) which allows the isolation of flanking sequences by plasmid rescue in *E. coli.* The restriction enzyme sites indicated can be employed to rescue pUC19 and adjacent plant sequences from transformed plants. The restriction sites *NotI, SpeI* and *BamHI* can be used to rescue plant sequences adjacent to the left T-DNA -border. In a similar fashion, *KpnI, PstI, EcoRI* and *HindIII* sites can be used to rescue plant sequences adjacent to the right T-DNA –border. **Source: (Tani *et al.*, 2004)**

Overexpression of the *constitutive disease resistance* (*cdr1*) gene in *Arabidopsis* by a T-DNA insertion resulted in enhanced resistance of *cdr1* plants against the bacterial pathogen *Pseudomonas syringae* (Xia *et al.*, 2004). *CDR1* encodes an apoplastic aspartic protease. *Cdr1* plants also display a dwarf phenotype and develop spontaneous micro-lesions. Development of spontaneous lesion is frequently reported in constitutive disease resistance mutants (Maleck *et al.*, 2002; Pilloff *et al.*, 2002). Genetic analysis of *cdr1 nahG* plants revealed that all phenotypes conveyed by *cdr1* were SA-dependent. Activation tagging and its applicability in studying gene function has been reviewed recently (Tani *et al.*, 2004).

Previously, two activationally tagged populations, one in a *PR1:Luc* and another in a *PDF1.2:Luc* background were generated by floral dip of Col-0 *Arabidopsis* plants using the transformation vector pSK1015 (Fig. 3.1) (Grant *et al.*, 2003; Tani *et al.*, 2004) and were the subject of this study. Due to virulence of the *B. cinerea* strain used in our experiments and the risk of loosing susceptible lines during the screening process, $T_1$ plants were not inoculated with this pathogen. Instead, they were, in collaboration with Andrea Chini and Eleanor Gilroy, the subject of parallel screens for either resistance or susceptibility to the virulent bacterial pathogen *Pseudomonas syringae* pv *tomato* (*Pst* DC3000). Screening primary transformants will identify dominant activation tagged mutations, and since *Pst* is a non-systemic pathogen there was little risk of loosing mutant lines. Four week-old plants were infiltrated with a bacterial suspension ($1x10^6$ cfu/mL for resistance; and $1x10^4$ cfu/mL for susceptibility) and mutants exhibiting either enhanced resistance or susceptibility were identified 3-4 days post-inoculation by comparing symptom development with wild type Col-0 and transgenic *nahG* control plants. Mutants showing an abnormal morphological phenotype were also selected, transplanted to an individual pot and seeds collected. Some phenotypic abnormality, such as stunting and spontaneous lesions, are sometimes associated with disease resistance (Boch *et al.*, 1998; Grant *et al.*, 2003). From this screening for *Pst* a few mutant lines were isolated and some of them characterized (Chini, 2004; Gilroy, 2005). The remaining $T_1$ plants were grown in flats, typically 72 plants per flat, and seeds from each flat pooled together and used for screening lines displaying enhanced resistance or susceptibility towards *B. cinerea*.

### 3.3 Screening for altered resistance to *Botrytis cinerea*

To uncover *Arabidopsis* mutants expressing increased resistance against *B. cinerea*, we screened a $T_2$ population of approximately 60,000 activation tagged T-DNA lines (Grant *et al.*, 2003; Tani *et al.*, 2004) by spray inoculation of a *B. cinerea* spore suspension. We obtained four lines which differed in their response to *B. cinerea* compared to wild-type Col-0 plants. A sample of 432 plants was taken as representative for each $T_2$ pool. This number should cover most possible dominant and recessive mutant lines. In the $T_2$ generation 3/4 of plants are expected to be resistant to the herbicide Basta (amonium glufosinate) whilst the 25% remaining are eliminated by the herbicide. With the purpose of avoiding plant stress during transplantation, seeds are poured on each soil pot and sprayed with Basta one-week later.

Four-week old plants were spray inoculated with a spore suspension, covered with a polyetylene lid and wrapped with Sellotape to keep humidity around 100%, which is a requirement for *B. cinerea* spore germination and tissue penetration. Wild type Col-0 and *coi1* mutant plants were used as controls. Five days post-inoculation the level of infection was evaluated and mutants showing enhanced resistance or susceptibility against *B. cinerea* were selected by comparing the level of infection in relation to the Col-0 and *coi1* control plants. Four mutant lines, two showing *INCREASED BOTRYTIS RESISTANCE* (*IBR*) and two mutants displaying *ENHANCED BOTRYTIS SUSCEPTIBILITY* (*EBS*) were selected and characterization of three of these lines is described here.

## 3.4 Discussion

The employment of *Arabidopsis* as a model system has rendered significant insights into the genetic mechanisms governing the interaction between host and pathogens. However, most of the progress achieved was from studying the interaction of biotrophic pathogens with their host plants, while interactions involving plant-necrotrophic pathogens still remain poorly understood. In an attempt to identify some components of resistance of plants against *B. cinerea* we screened two *Arabidopsis* T-DNA mutagenised populations (Grant *et al.*, 2003; Tani *et al.*, 2004; Murray *et al.*, 2005) and isolated mutant lines which are affected in resistance against this pathogen. The two resistant lines were termed *Increased Botrytis Resistance 1 (IBR1)* and *IBR2,* while the two susceptible mutants were termed *Enhanced Botrytis Resistance 1* (*ebs1*) and *ebs2*, respectively. Three of these lines were characterized and will be presented later in this study, two of which have a T-DNA insert closely linked to the mutation and another line that is not tagged. Lack of co-segregation between gene and T-DNA tag has been documented. For an activation tagging population, recombination frequencies of 25 and 63% for left and right border have been reported, respectively (Sessions *et al.*, 2002). In agreement, it has been shown that only about 17% of mutant phenotypes obtained in the first generation ($T_1$) re-appeared in the next generation, confirming instability of the insertion in a large proportion of mutants (Ichikawa *et al.*, 2003). It has been suggested that for some populations co-segregation between T-DNA tag and phenotype can be as low as 20% (Budziszewski *et al.*, 2001).

For our T-DNA mutagenized population a transformation rate of 0.31% was noted. This percentage of transformation is similar to what has been previously reported (Clough and Bent, 1998). Approximately 15% of all primary transformants were male sterile, preventing further analysis. These numbers are in agreement with those from similar studies (Maldonado *et al.*, 2002; Sessions *et al.*, 2002). However, in our experimental conditions less than 5% of all morphological phenotypes present in the primary trasnformants reappeared in the next generation, significantly lower than previous reports (Maldonado *et al.*, 2002; Ichikawa *et al.*, 2003). Integration into the plant genome is often accompanied by general DNA "irritation", such as deletion and rearrangements of genomic fragments unrelated to the integration. This "irritation" can frequently result in unforced mutation and

consequently phenotypes that do not co-segregate with the gene tag (Nacry *et al.*, 1998). These data could explain the low frequency of $T_2$ plants inheriting the mutant phenotype spotted in the primary generation. Nevertheless, activation tagging is an efficient tool for gene discovery and apart from the results obtained in this study, an extensive number of mutants identified by using this technique could be listed here. In plant disease resistance, several tagged mutants were recently isolated (Grant *et al.*, 2003; Xia *et al.*, 2004; Murray *et al.*, 2005). Thus, in spite of the instability of the T-DNA insertion in several lines, the benefits of working with a mutant line generated by activation tagging justify the employment of this technique for studying gene function.

## 4 Characterization of *ibr1* mutant

### 4.1 *Ibr1* plants exhibit increased resistance against necrotrophic fungi

One of these lines, termed *INCREASED BOTRYTIS RESISTANCE1* (*IBR1*), exhibited strikingly reduced susceptibility to *B. cinerea* infection compared to wild-type plants (Fig. 4.1.1, a-b). Furthermore, trypan blue staining employed to monitor cell death following *B. cinerea* inoculation revealed that leaf lesion development was significantly reduced in the *ibr1* line compared to wild-type plants (Fig. 4.1.1, k-r). This line also had lobed leaves, which possessed a shorter petiole and broader midvein. Neither *B. cinerea* resistance nor lobed leaves were a characteristic of $F_1$ plants derived from a backcross of the *ibr1* line with their wild-type Col-0 parent. Out of a total of 746 $F_2$ plants scored, 176 plants expressed increased resistance against *B. cinerea*, consistent with a 1:3 segregation ratio ($X^2 = 0.68$) for this phenotype. Thus, suggesting that *ibr1* was a single recessive mutation. Interestingly, the lobed leaf phenotype co-segregated with *B. cinerea* resistance. All 176 *B. cinerea* resistant, lobed leaved plants expressed resistance against Basta, suggesting the *ibr1* mutation was tightly linked to the T-DNA insertion. We employed TAIL-PCR (Singer and Burke, 2003) to amplify DNA sequences flanking the inserted T-DNA. This analysis revealed that the T-DNA was inserted 521 bp upstream of an open reading frame encoding a MYB-related regulator (MYB91), termed *ASSYMETRIC LEAVES* (*AS*) *1* (Redei, 1965). A series of alleles of *as1* have been identified and following a conventional nomenclature our mutant line was named *as1-4*. One pathway involved in reprogramming gene expression during plant leaf development requires *Arabidopsis AS1* and its orthologs *PHANTASTICA* (*PHAN*) in *Antirrhinum* and *ROUGH SHEATH* (*RS*) 2 in maize (Freeling *et al.*, 1992; Waites *et al.*, 1998). These genes are members of a small unique MYB-related gene family ($R_2R_3$-MYB) (Stracke *et al.*, 2001). The presence of lobed leaves in *as1-4* plants suggested the cognate mutation may be an allele of *as1*. All $F_1$ progeny resulting from a cross between *as1-4* and a Col-0 line possessing an X-ray-induced mutation, *as1-1* (Redei, 1965), exhibited the characteristic phenotype of *as1* plants, indicating that *as1-4* was a mutant allele of *AS1*. Furthermore, RT-PCR analysis of *AS1* gene expression in *as1-4* plants revealed a complete absence of *AS1* transcripts (Fig. 4.1.1, j), suggesting this mutation was a null allele. To confirm these findings, we challenged *as1-4* and also a L*er*

line containing an introgressed *as1-1* allele with *B. cinerea*. Both of these *as1* lines exhibited increased resistance against *B. cinerea* (Fig. 4.1.1, a-d).

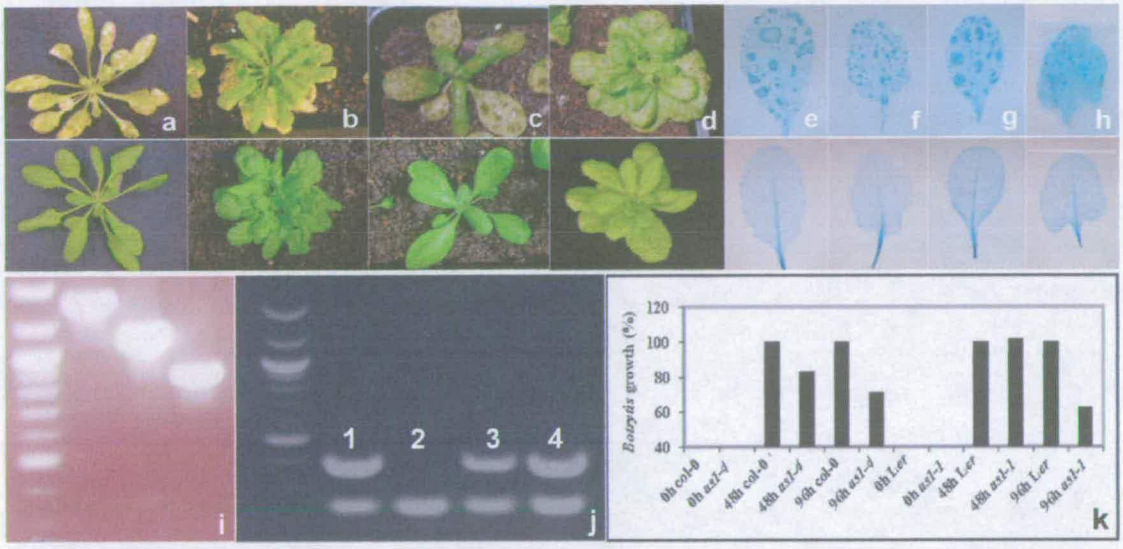


**Figure 4.1.1** ***As1*** **plants show increased resistance to** ***B. cinerea.*** 4-week old plants were spray inoculated and disease scored 6 dpi. Upper panel *B. cinerea* infected and lower panel respective mock plants. (**a**) Col-0, (**b**) *as1-4*, (**c**) L*er* and (**d**) *as1-1*. Upper right, trypan blue stained leaves of (**e**) Col-0, (**f**) *as1-4*, (**g**) L*er* and (**h**) *as1-1* infected plants with *B. cinerea*. Lower, (**i**) TAIL-PCR amplified *as1-4* flanking T-DNA sequence, (**j**) RT-PCR amplification of *Arabidopsis AS1* in (1- Col-0, 2-*as1-4*, 3-*as1*L*er*, 4-*as1*Col-0 and actin1 genes, lower and upper row, respectively. (**k**) Relative growth of *B. cinerea* in the given lines, measured by amount of actin transcripts accumulated at different times post-infection.

To examine if the apparent resistance observed in *as1* plants was due to a decrease in pathogen growth or reduced symptom development, we monitored the expression of the *B. cinerea* actin gene. The amount of transcript accumulation has been shown to directly correlate with the extent of *B. cinerea* growth (Benito *et al.*, 1998). The level of transcripts correlated with lesion size. The amount of actin transcripts were on average, 20% higher in wild-type than in *as1-4* mutant plants at 48 hpi, and up to 2-fold greater at 4 dpi, suggesting *B. cinerea* growth is decreased in this mutant line (Fig. 4.1.1 i).

To investigate if *as1* conveys resistance against other necrotrophic fungi, *as1-1* and *as1-4* plants were challenged with *A. brassicicola*. Compared to wild-type plants, the *as1-4* and *as1-1* lines exhibited significantly increased resistance against *A. brassicicola* (Fig. 4.1.2, a-g). The size of *A. brassicicola* lesions on *as1-4* and *as1-1* plants were significantly reduced compared to the wild-type line (Fig. 4.1.2g).

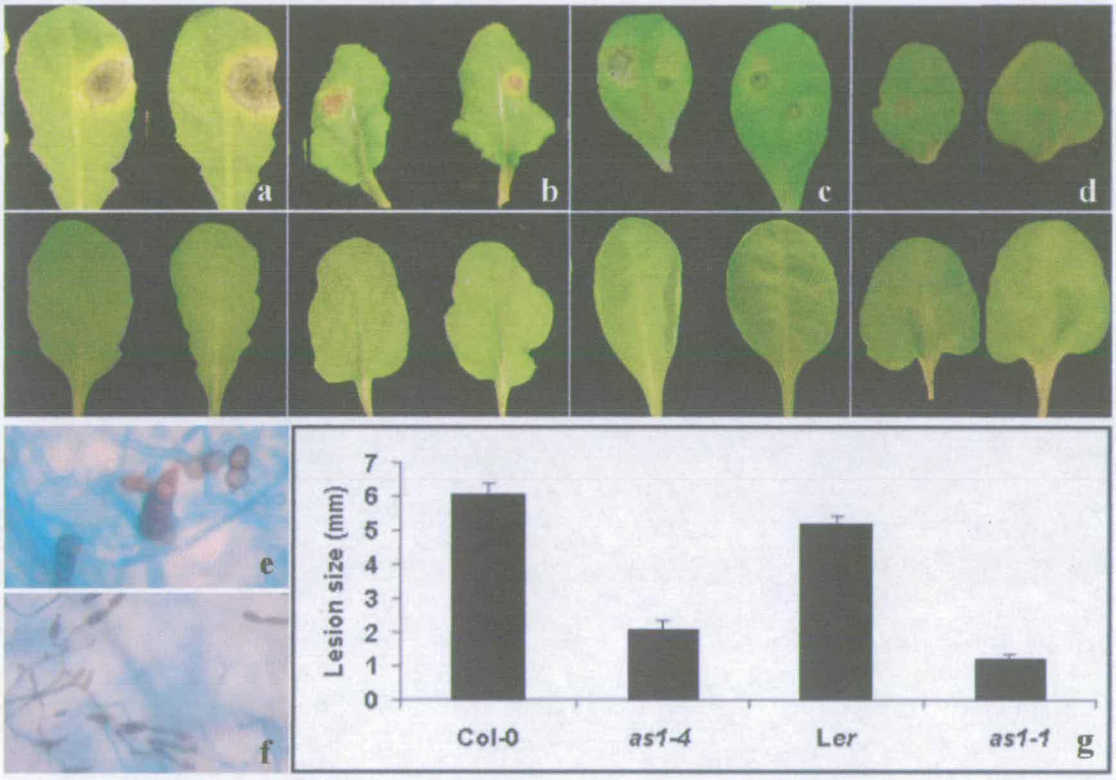


**Figure 4.1.2 *As1* plants show increased resistance against *A. brassicicola*.** Plants were drop incoculated and disease symptoms scored 6dpi. Upper (a-d) typical necrotric lesion on leaves of Col-0, *as1-4*, L*er* and *as1-1*, with respective mock leaves on the row below. (e) detail of spore germination on Col-0 plants, (f) *Alternaria* sporulation on wild type plants and (g) lesion size on given lines at 6 dpi.

## 4.2 Neither *as2* nor conditional *KNOX* gene expression conveys enhanced *B. cinerea* resistance

Plants possessing loss-of-function mutations in *AS2* are phenotypically similar to *as1* plants (Ori *et al.*, 2000). *AS2*, which encodes a member of the LOB family of plant specific proteins (LBD6), is thought to function in the same genetic pathway as *AS1* (Xu *et al.*, 2003). We therefore investigated if loss of *AS2* function also resulted in enhanced resistance to *B. cinerea*. While disease development in *as2* plants was similar to that observed in wild-type Col-0 plants, the *as1-4* and *as1-1* lines again showed increased resistance against this pathogen (Fig. 4.2). Both *AS1* and *AS2* are required for the repression in leaves of certain *KNOX* (*KNOTTED1-like homeobox*) genes, such as *KNAT1*, *KNAT2* and *KNAT6* (Semiarti *et al.*, 2001; Xu *et al.*, 2003). Furthermore, misexpression of *KN1* has been shown to elaborate lobed leaf morphology in *Arabidopsis* similar to that established in *as1* and *as2* plants. We therefore examined if misexpression of *KN1* could convey enhanced resistance to *B. cinerea*. For this experiment, a conditional *KN1* allele (*35S::KN1-GR*) was utilized, which encoded an inducible fusion between KN1 and the rat glucocorticoid receptor (GR) expressed under the control of the cauliflower mosaic virus (CaMV) *35S* promoter (Hay *et al.*, 2003). The *KN1* glucocorticoid receptor fusion protein was not detected in leaves in the absence of DEX-induction and the application of DEX to a transgenic line containing an empty vector failed to establish a lobed leaf morphology (Hay *et al.*, 2003). Plant lines were treated with 1 μM DEX prior to inoculation with *B. cinerea*. Conditional *KN1* expression failed to increase resistance against *B. cinerea*, unlike the *as1-4* and *as1-1* lines which exhibited significant resistance against this pathogen (Fig. 4.2).

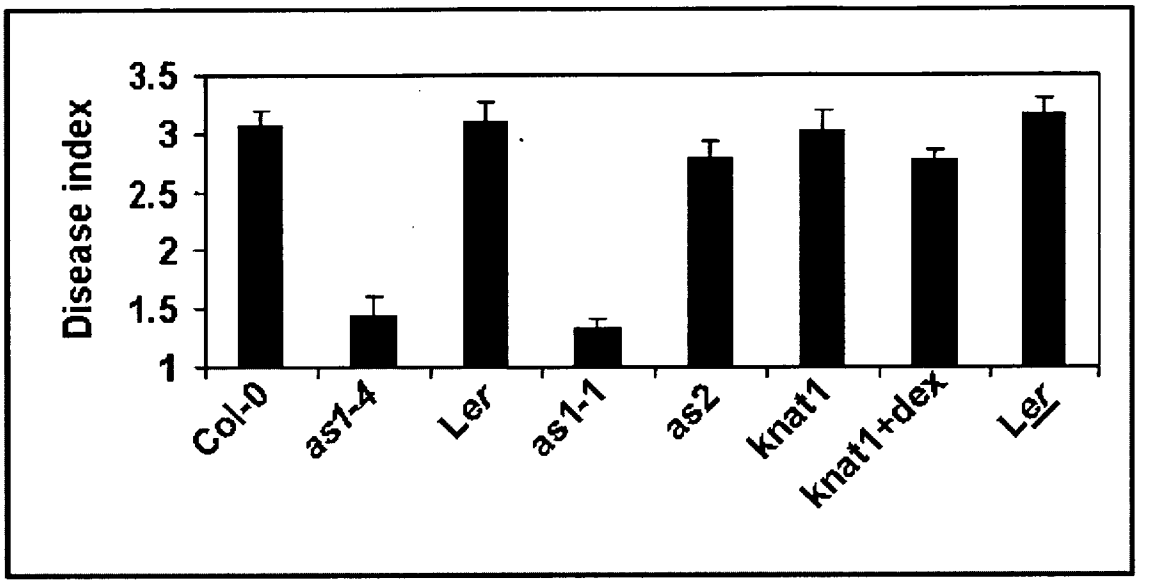


**Figure 4.2 Disease index for *B. cinerea* infected plants**. Neither *as2* nor conditional *Knat1* are affected in resistance. The *Knat1* line corresponds to the Knotted 1 like gene fused to a glucocorticoid inducible promoter. L*er* is the ecotype from which L*er* was originated.

### 4.3 *AS1* is required for basal and non-host resistance against bacterial pathogens

To explore whether the increased basal resistance expressed in *as1* plants could provide protection against other pathogens in addition to necrotrophic fungi, the *as1-4* and *as1-1* mutant lines were challenged with the virulent bacterial pathogen *Pseudomonas syringae* pv. *tomato* (*Pst*) (DC3000) (Whalen *et al.*, 1991). Following leaf inoculation the *as1-4* and *as1-1* lines exhibited significantly enhanced disease symptom development at 4 dpi compared to that observed on wild-type plants. Quantification of the number of bacteria in infected leaves revealed that *Pst*DC3000 grew to levels 1 log greater in the *as1-4* and *as1-1* lines compared to wild-type plants (Fig. 4.3A). Interestingly *as1-1* and *as1-4* did not exhibit increased susceptibility against *Erysiphe cichoracearum* and *B. gramninis* f. sp. *tritici* (Fig. 4.3C,D).

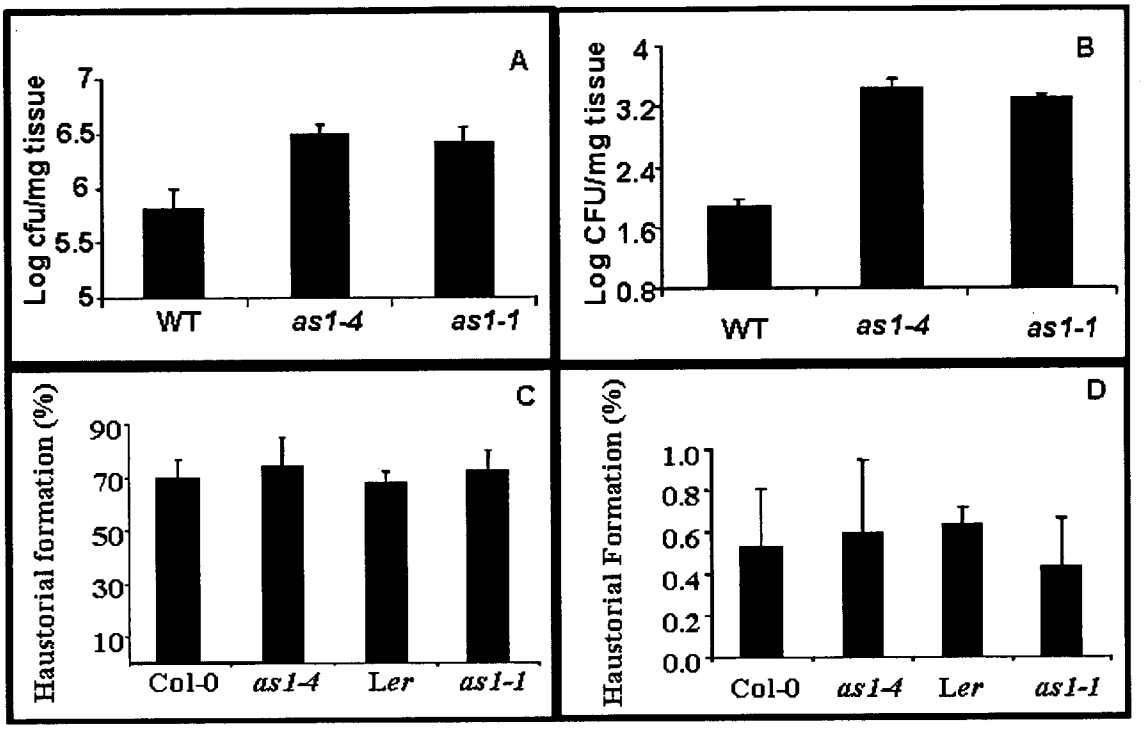


**Figure 4.3 *IBR1* is required for basal and non-host resistance against bacterial pathogen.** (A) growth of virulent *P. syringae* pv tomato (DC3000), (B) growth of the non-host pathogen *P. fluorescens 2-79*. Plants were infiltrated with a bacterial suspension and leaf samples collected 3 and 4 dpi for *Pst* and *P.f.2-79*, respectively. (C and D) *as1* and *as1-4* plants are not affected in resistance against *E. cichoracearum* and *B. graminis tritici*. Plants were inoculated by dusting fungal spores over the leaves of plants and haustorial formation scored 10 days later.

We also tested the response of *as1-4* and *as1-1* plants to *P. fluorescens* 2-79, which is not ordinarily pathogenic on *Arabidopsis* (Thara *et al.*, 1999). Interestingly, the numbers of *P. fluorescens* 2-79 bacteria in the leaves of the *ibr1* and *as1-1* lines was 2.3 logs greater than that found in wild-type plants (Fig. 4.3B). Collectively, these data implicate a role for *IBR1* in both basal and non-host resistance against specific microbial pathogens.

## 4.4 *AS1* is required for *R* gene-mediated disease resistance

Avirulence genes encode protein products that are recognised directly or indirectly only by a R gene (Flor, 1971; Staskawicz, 2001). Specific recognition results in induction of defence gene expression and the inhibition of pathogen growth. However, if the plant host does not possess the cognate R gene, the pathogen will cause disease (Staskawicz, 2001). Following the enhanced susceptibility of *as1-4* to virulent *Pst* DC3000, we investigated whether mutations in *AS1* compromised *R* gene-mediated resistance against the avirulent strains *avrB* and *avrRPS4*. Four-week old plants were inoculated with a bacterial suspension ($1x10^6$ cfu/mL) and leaf samples were collected four days later for bacterial growth assay. Wild type *Arabidopsis* plants are able to recognise the avirulence genes *avrB* and *avrRps4* (Bisgrove *et al.*, 1994; Hinsch and Staskawicz, 1996). Recognition of bacteria expressing the avirulence genes *avrB* and *avrRPS4* is mediated by the *RPM1* and *RPS4* genes, respectively (Bisgrove *et al.*, 1994; Hinsch and Staskawicz, 1996).

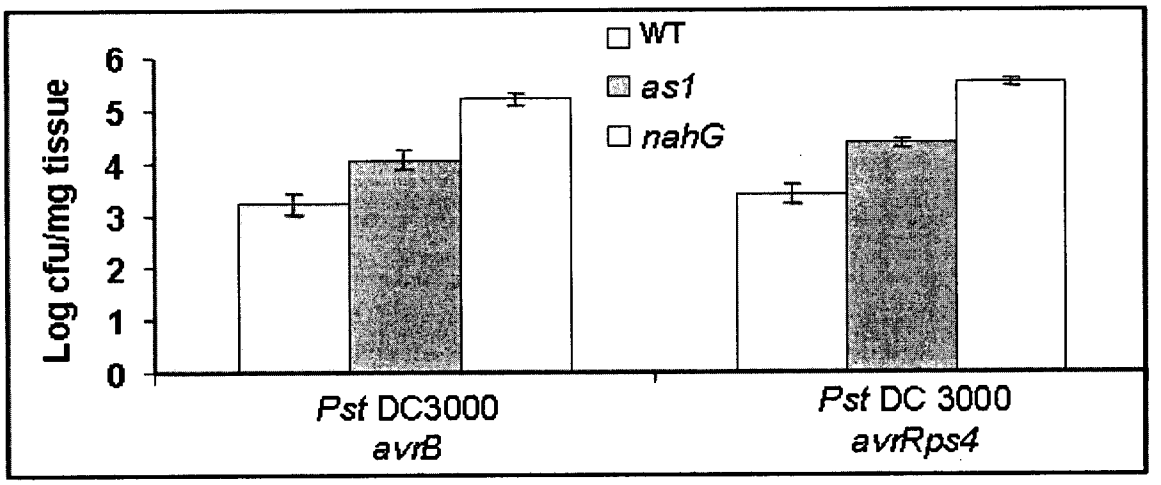


**Figure 4.4 *As1* is required for *R* gene-mediated resistance.** Plants were syringe inoculated with *Pst* DC3000 carrying either avirulence gene *avrB* or *avrRps4* and leaf samples collected 4dpi for bacterial growth assay. *As1* supported significantly higher bacterial growth than wild type plants. Histogram bars represent the average between Col-0 and *Ler* and *as1-1* and *as1-4* lines, respectively. The error bars represent the standard error of mean (SE).

The growth of avirulent strains was significantly lower in both wild type and *as1* accessions in comparison to growth of virulent *Pst.* However, *as1-1* and *as1-4* plants

supported up to eight times more bacterial growth than wild type plants when infected with *avrB*, while in *nahG* plants bacteria grew to a level twenty times greater (Fig. 4.4). Likewise, growth of *Pst* DC3000 (*avrRps4*) was 10 and 20 fold higher in *as1* and *nahG* than in wild type plants, respectively (Fig. 4.5). These results suggest that *R* gene-mediated resistance against *Pst* DC3000 expressing either *avrB* or *avrRps4* is partially compromised by *as1*.

## 4.5 Drought and salinity stress tolerance are not affected in *as1*

In nature plants are frequently exposed to biotic (e.g. pathogens, pests, nematodes) and abiotic stresses (e.g. high/low temperature, drought, salinity). To deal with these adversities plants have evolved complex signalling networks that perceive external signals and integrate diverse defence mechanisms at the molecular level (Bohnert *et al.*, 1995). From perception of external stimuli to activation and establishment of defence, a complex interplay between signalling networks is required (Yang *et al.*, 1997). The accumulating evidence suggests some components of plant defence against pathogens are often involved in response against abiotic stresses such as drought tolerance and salinity tolerance (Mengiste *et al.*, 2003; Anderson *et al.*, 2004; Chini *et al.*, 2004). The gain-of-function mutant *adr1* for example, in addition to enhanced resistance against a series of virulent pathogens also displays increased tolerance to water deficit (Grant *et al.*, 2003; Chini *et al.*, 2004), thus, suggesting multiple roles for *ADR1* in biotic and abiotic stress responses. Similarly, loss-of-function in *BOS1*, which encodes a MYB transcription factor from *Arabidopsis*, conveys enhanced susceptibility towards the necrotrophic pathogen *B. cinerea* and *A. brassicicola,* in addition to impaired tolerance to water deficit, salinity and oxidative stress (Mengiste *et al.*, 2003).

In order to identify any possible role for *AS1* in abiotic tolerance, wild type and *as1* plants were subjected to drought and salinity stresses. Seedlings from both lines were transplanted onto soil, in trays with 72 plants, with rows of each line planted in sequence, in order to provide the most similar growth condition to both lines. Plants were grown for about three weeks prior to treatments. Both wild type and *as1* plants were severely withered following

a twelve days water withdrawal. Trays were watered and plant recovery recorded three days later. No significant difference between *as1* and wild-type lines were detected (Fig. 4.5a).

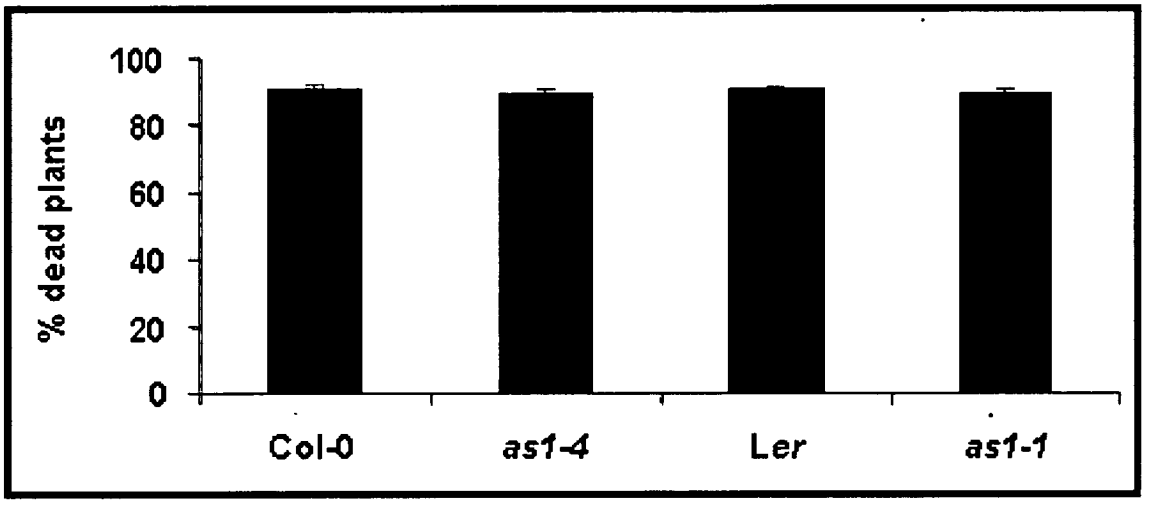


**Figure 4.5.a *As1* plants are not affected in drought tolerance**. Three-week old plants were exposed to water deficit for two weeks and impact of drought stress is presented as the (%) of dead plants following 14 days water withdrawal.

Another set of three week-old *as1* and wild type plants were subjected to salinity stress. Trays were watered every four days with either a 50 mM, 100 mM NaCl, 150 mM, 200 mM or 400 mM NaCl and development and death of plants monitored for another three weeks. No significant difference could be detected between *as1* and wild-type plants for any of the NaCl concentrations used. Irrigation of plants with a 100 mM NaCl solution resulted in a weak reduction in growth compared to untreated plants, with a progressive inhibition of growth at 150 NaCl and a large proportion of dead plants after three weeks of receiving 200 mM NaCl (Fig. 4.5b).

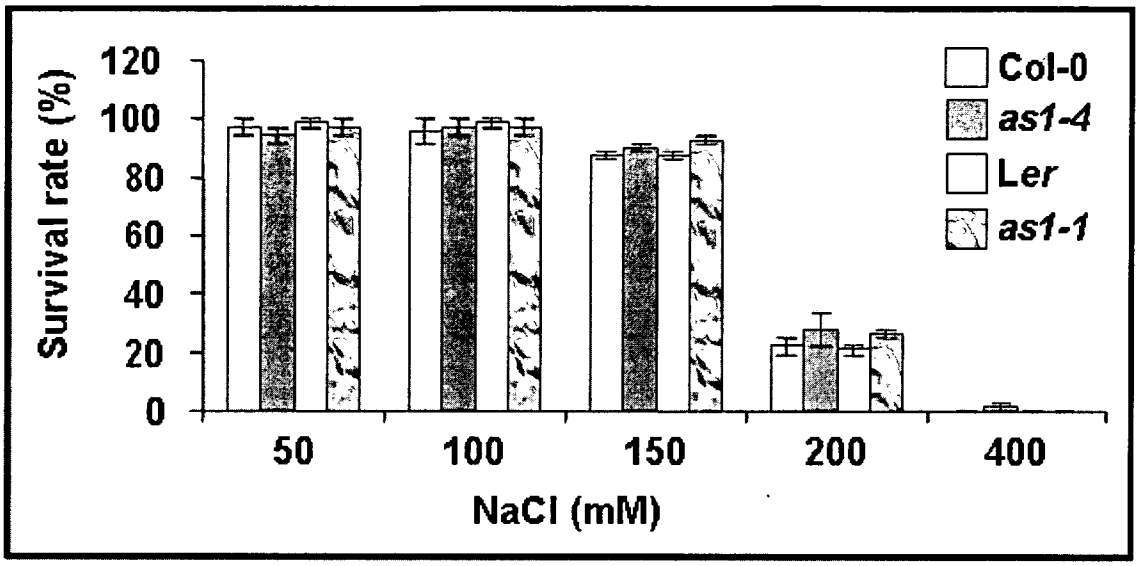


**Fig. 4.5b Loss of *AS1* function did not affect salinity stress tolerance.** Three-week old plants were irrigated every four days with a range of NaCl concentrations, as shown, for a period of three weeks and number of survival plants counted. The impact of NaCl stress was scored as the % of plants surviving the treatment at the NaCl concentrations indicated.

### 4.6 *As1*-mediated disease resistance is conserved in *Antirrhinum*

The *Antirrhinum PHAN* gene is the ortholog of *Arabidopsis AS1* (Waites *et al.*, 1998). We therefore determined if *IBR1* function was conserved between *Antirrhinum* and *Arabidopsis*. Both wild-type controls and *Antirrhinum phan* plants were inoculated with *B. cinerea* and scored for resistance at 10 dpi. Wild-type *Antirrhinum* plants showed strong disease development by 10 dpi (Fig. 4.6, A-B). In contrast, *phan* plants exhibited a significantly reduced level of infection (Fig 4.6C-D). The response of these lines to *B. cinerea* was quantified following a number of independent experiments using a disease index (Fig. 4.6E). In the absence of *PHAN Antirrhinum* plants therefore show increased disease resistance against the necrotrophic pathogen *B. cinerea.*

We also inoculated wild-type and *phan* plants with *Pst*DC3000 to examine whether basal resistance against this bacterial pathogen was compromised. The leaves of *phan* plants supported the growth of seven-folds more *Pst*DC3000 than that found in wild-type

*Antirrhinum* leaves (Fig. 4.6E). Consequently, we determined the response of these plants to *P. flourescens* 2-79. This bacterium grew to a level two logs greater in the mutant *phan* line than in wild-type plants (Fig. 4.6E). In sum, these data show that *PHAN* function is also required for both basal protection and non-host resistance in *Antirrhinum*.

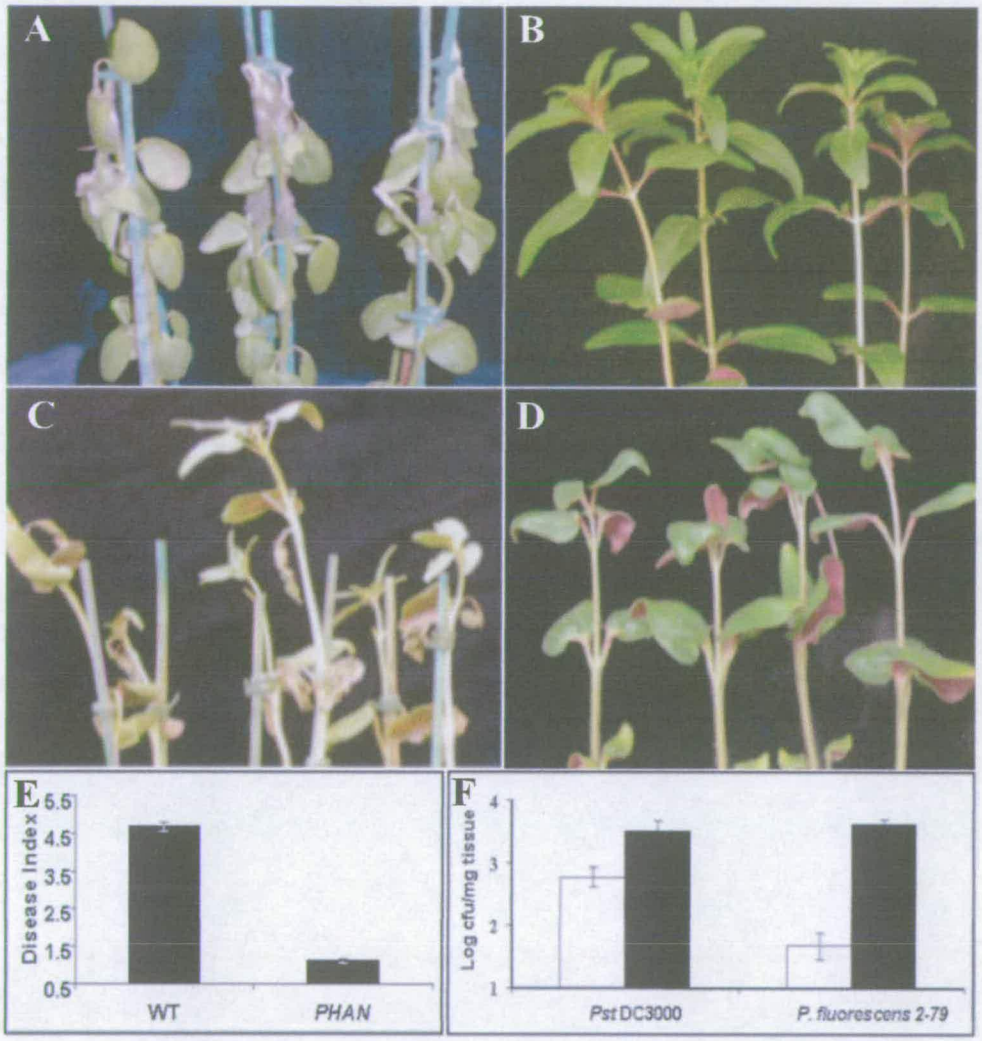


**Figure 4.6 *AS1* function is conserved in *Antirrhinum*.** Upper, leaves were drop inoculated with a 10 µL suspension of spores from *B. cinerea* containing 5x105 spores/mL and disease severity assayed 10 dpi. **(A,B)** wild type plants infected and mock plants, respectively. **(C,D)** *Phantastica* plants 10 dpi with *B. cinerea* and respective unchallenged plants. **(E)** disease index for *B. cinerea* infected plants based on symptoms development. (**F**) growth of virulent *Pst* DC3000 and non-host *P. fluorescens 2-79*, respectively. Experiments were repeated three times and error bars represent standard error of mean (SE).

### 4.7 *AS1* function is conserved in *Nicotiana silvestris*

In addition to *Arabidopsis* and A*ntirrhinum* plants, we also investigated the resistance of a *Nicotiana silvestris* transgenic line constitutively expressing an antisense *N. tabaccum PHAN* gene (*NtPHAN*) (McHale and Koning, 2004). Antisense *NtPHAN* leaves show ectopic expression of the *NTH20*, a knox gene, and consequently misregulation of knox gene expression resulted in a series of developmental abnormalities (McHale and Koning, 2004). Antisense plants infected with either of *Pst* DC3000, *Pst* DC3000 (*avrB*), or the non-host pathogen *P. fluorescens*2-79, supported increased bacterial growth compared to wild type tobacco plants (Fig. 4.7, A-B). In constrast, when infected with the necrotrophic pathogen *B. cinerea,* lesion development was at least four times smaller on antisense than on wild type plants (Fig. 4.7, C-D). Altogether, these results suggest, *AS1* function is conserved in at least three evolutionary diverse plant species.

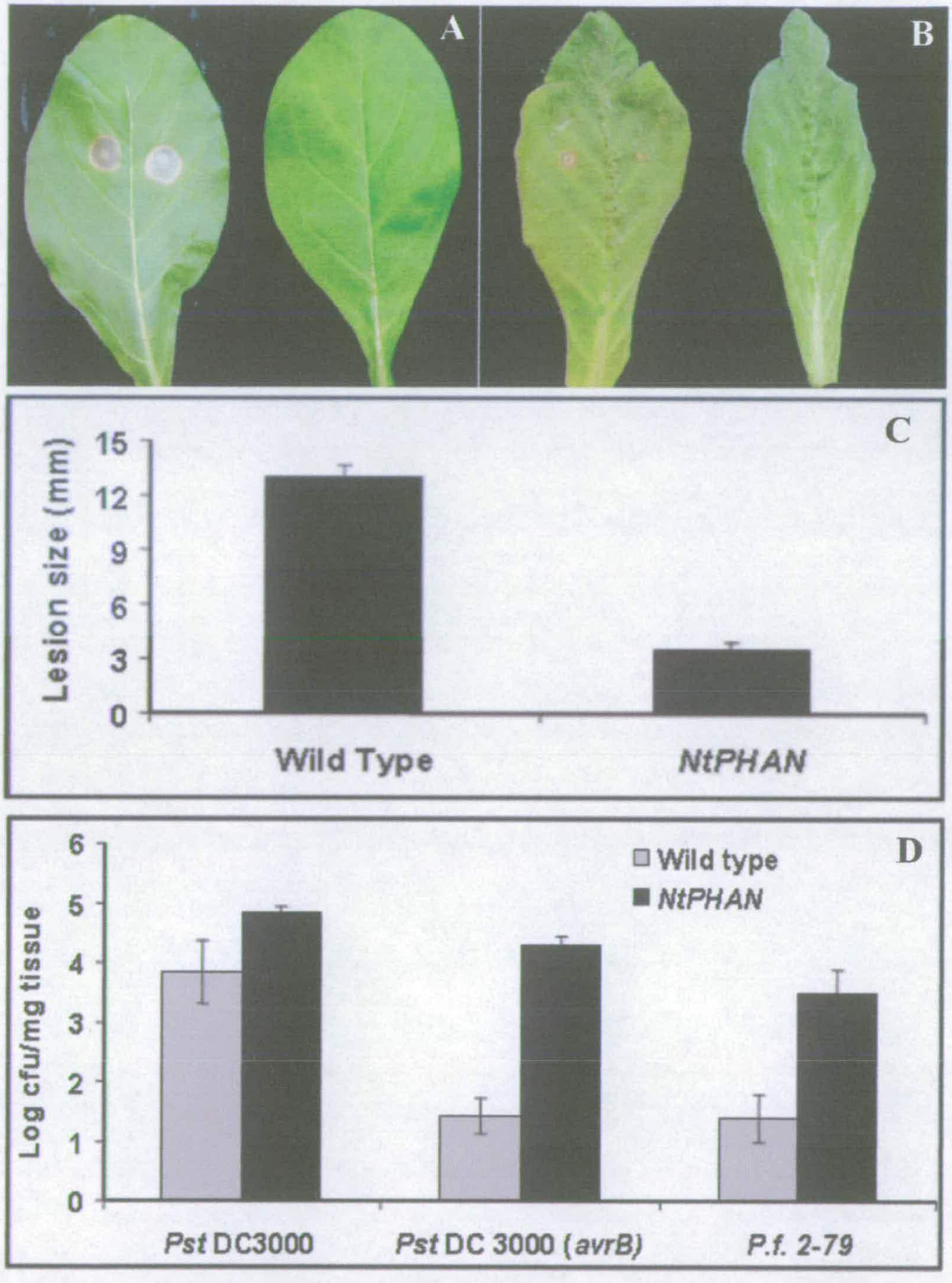


**Figure 4.7 *As1* function is conserved in tobacco**. **(A, B)** leaves of wild type and antisense *NtPHAN* lines 5 dpi with *B. cinerea*. **(C)** lesion size for the given lines infected with *B. cinerea* and **(D)** growth of virulent *Pst* DC3000, *Pst* DC3000 (*avrB*) and non-host bacterial *P. fluorescens 2-79*. Experiments were repeated twice with similar results. Error bars represent the standard error (SE).

### 4.8 JA/ET-dependent defence gene expression is potentiated in *as1* plants.

The misexpression of defence-related genes often correlates with heightened disease resistance (Bowling *et al.*, 1997; Murray *et al.*, 2002; Grant *et al.*, 2003). We therefore monitored the expression of key defence marker genes in *as1-4* and *as1-1* plants challenged with *B. cinerea*. In wild-type plants, transcripts of the JA/ET-dependent gene *PDF1.2* (Penninckx *et al.*, 1996) were first detected, at a relatively low level, at 48 hpi (Fig. 4.8). The amount of *PDF1.2* transcripts had increased by 72 hpi and was greater still at 96 hpi. In *as1-4* and *as1-1* plants, however, the level of *PDF1.2* gene expression was significantly greater at both 48 and 72 hpi compared to wild-type plants. This trend was even more striking when the expression of the ET-dependent *PR3* gene (Verburg and Huynh, 1991) was examined. In wild-type plants no transcripts for *PR3* were detected up to 72 hpi (Fig. 4.8a). In contrast, a significant amount of *PR3* transcripts had accrued at 48 hpi, in *as1* plants with transcript accumulation peaking for this gene at 72 hpi. The expression pattern of the SA-dependent gene *PR1* (Uknes *et al.*, 1992) was also examined. The accumulation of *PR1* transcripts in either *as1-4* or *as-1-1* plants was congruent with that observed for wild-type plants (Fig. 4.8b).

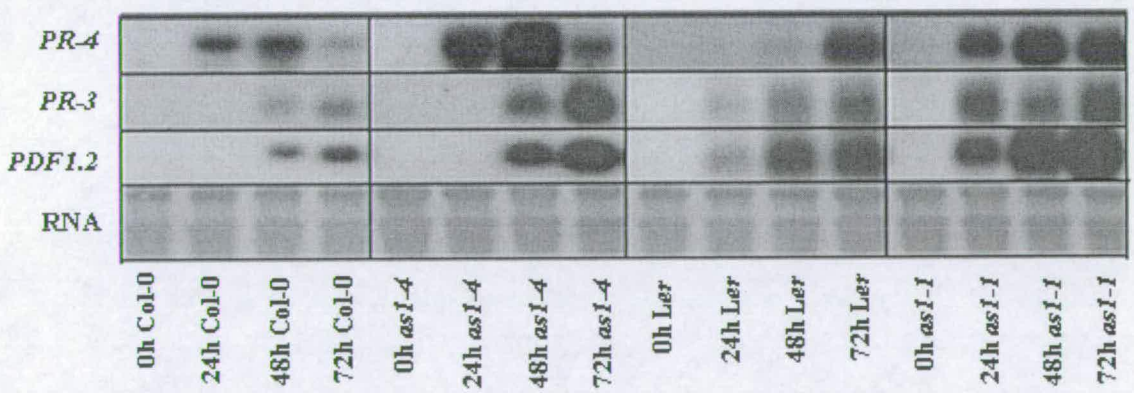


**Figure 4.8 Induced expression of JA/ET-dependent defense genes.** As*1* exhibited accelerated and enhanced expression of defence marker genes in response to *B. cinerea* challenge when compared to wild type plants. Plants were spray inoculated and leaf samples collected at given time points. Northern blot analysis were repeated twice on each ecotype with similar results.

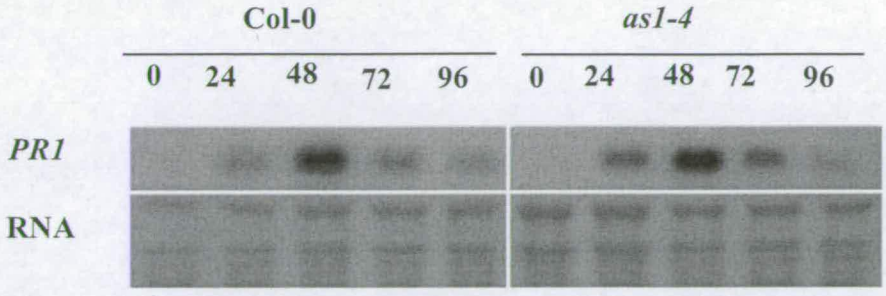


**Figure 4.8b Induced expression of PR-1 gene**. The expression of the SA-dependent *PR-1* gene is not affected in *as1* plants in response to PstDC3000 challenge when compared to wild type plants. Plants were syringae infiltrated with a bacterial suspension containing $1x10^6$ colony forming unit/mL. Northern blot analysis were repeated twice on each ecotype with similar results.

To examine if the potentiated expression of JA/ET-dependent genes in *as1* plants was due to changes in the biosynthesis of JA, ET or SA we determined the concentrations of these molecules in *as1-4* and *as1-1* plants and respective wild type, which will be presented in the next chapter.

## 4.9 Discussion

In spite of several screens aiming to uncover new components of resistance against necrotrophic pathogens, only a few mutants have been characterized so far (Mengiste *et al.*, 2003; Veronese, 2004; Veronese *et al.*, 2004). We screened *Arabidopsis* T-DNA mutagenised populations for resistance against *B. cinerea* and the characterization of a mutant line displaying enhanced resistance against this pathogen is discussed here. By TAIL-PCR the mutant gene was isolated and shown to be allelic to the *asymetric leaves 1* (*as1*) (Redei, 1965). Following the conventional gene nomenclature and the number of *AS1* alleles already published, the new mutant allele was named *as1-4*. In addition to enhanced resistance against *B. cinerea, as1* and *as1-4* also showed increased resistance against *A. brassicicola*, another necrotrophic pathogen, suggesting that *AS1* may have a general function in resistance against necrotrophic pathogens. Interestingly, however, is the fact that *A. brassicicola* is frequently regarded as a non-host pathogen of *Arabidopsis* (Penninckx *et al.*, 1996; Thomma *et al.*, 1999b). Under our experimental conditions *A. brassicicola* routinely completed its life cycle on *Arabidopsis*. On figure 4.9 we show in details, different stages of *A. brassicicola* infected Col-0 plants, leading to '*in planta*' fungal sporulation. These results demonstrate that *A. brassicicola* is therefore able to complete its life cycle on wild type *Arabidpopsis* plants, suggesting *Arabidopsis* is not a non-host for *A. brassicicola.* This conflict of information may be due to variations in the experimental conditions used in the different laboratories, or because the period between inoculation and evaluation was not long enough for the pathogen to develop.

The increased resistance of *as1* and *as1-4* to the necrotrophic pathogens may be explained by the enhanced expression of JA-ET-dependent gene expression in comparison to challenged wild type plants. Misexpression of JA/ET-dependent defence genes is often associated with resistance to necrotrophic pathogens (Thomma *et al.*, 1998; Berrocal-Lobo *et al.*, 2002). Conversely, pathogen-induced expression of the SA-dependent gene *PR-1* was not altered in either of the two *as1* mutants when compared to wild type plants. Accumulating data highlight the existence of a mutual antagonism between the SA- and JA/ET-dependent defence signalling pathways, normally involved in resistance against biotrophic and necrotrophic pathogens, respectively. Thus, one defence signalling pathway

is believed to be activated to the detriment of the other pathway. Despite this long lasting speculation, and as far as we are aware, only one mutant identified so far (*bik1*) corroborates, at least in part, with this theory (Veronese, 2004). The mutant *bik1* plants are more susceptible to *B. cinerea* and *A. brassicicola* but exhibit increased resistance to *Pst* DC3000. While susceptibility of *bik1* towards necrotrophic pathogens seems to correlate with attenuated expression of the JA/ET-dependent *PDF1.1* gene, increased resistance against *Pst* is mounted with normal induction of the SA-dependent *PR-1* gene. To elucidate the possible impact of *as1* in the induced accumulation of SA, JA, ET and the phytoalexin, camalexin, the levels of these molecules were investigated. The results of this analysis is presented in chapter 5.

In contrast to the increased resistance against necrotrophic pathogens, *as1-1* and *as1-4* plants showed impaired resistance against virulent and avirulent strains of the hemi-biotrophic pathogen *Pst* DC3000 as well as of *P. fluorescens*, which is a non-host pathogen of *Arabidopsis*. These results suggest *as1* is impaired in basal, *R* gene-mediated and also non-host disease resistance. Moreover, it shows that *as1* conveys resistance against necrotrophic fungal pathogens and susceptibility towards host and non-host bacterial pathogens. Significantly *as1* plants did not exhibit increased susceptibility against *E. cichoracearum* and *B.graminis* f. sp. *tritici*. Thus, the susceptibility of *as1* plants is specific to bacterial pathogens. The establishment of both host and non-host resistance was shown to require a functional SA signalling pathway (Malamy and Klessig, 1992; Rairdan *et al.*, 2001; Rairdan and Delaney, 2002; Mellersh and Heath, 2003; Sharma *et al.*, 2003). Several mutants compromised in basal and *R* gene-mediated resistance against biotrophic pathogens have been isolated, however, to date, only two mutants, *nho1* and *atgsnor*, compromised in non-host resistance have been characterized (Lu *et al.*, 2001; Feechan *et al.*, 2005). Loss of *NHO1* function, a glycerol kinase (Kang *et al.*, 2003), also impaired resistance against *B. cinerea,* but did not compromise defence against *Pst* DC3000 (Lu *et al.*, 2001). Similarly, *atgsnor1* plants are impaired in basal and non-host resistance, but unaffected in resistance against *B. cinerea*. Thus, *as1* is, to our knowledge, the first mutant, which simultaneously exhibits increased resistance to necrotrophic and enhanced susceptibility to hemi-biotrophic, and non-host bacterial pathogens.

The normal expression of genes in response to pathogen attack suggests that *as1* does not affect the activation of the SA-dependent defence signalling pathway. Therefore, *AS1* is unlikely to represent a direct component of the SA-dependent signaling pathway. We hypothesize that *AS1* may function at different stages of the defence response or that it could play distinct roles in the establishment of disease resistance. One possibility is that *AS1* is a negative regulator of JA-dependent defence against necrotrophic pathogens and simultaneously negatively regulates an unknown susceptibility factor for bacterial pathogens. Thus, following this hypothesis, loss-of-function in the *AS1* gene would result in enhanced activation of the JA-mediated defence response and conversely increased susceptibility against bacterial pathogens, due to increased expression of a susceptibility factor. *AS1* may function by suppressing the expression of one or more plant proteins that are requierd for the pathogen to successfully infect the host (Ham *et al.*, 1999). Plants are unlikely to develop and maintain genes that are required for susceptibility, but during the course of co-evolution, pathogens may have evolved mechanisms to subvert the plant cellular machinery. For example in the acquisition of nutrients and other factors for pathogenesis. The most well known susceptibility factors correspond to plant metabolites that serve as chemoatractants or cues for the induction of genes involved in pathogenesis (Stachel *et al.*, 1986; Stachel and Zambryski, 1986a; Vogel *et al.*, 2002). One example of a susceptibility factor uncovered recently is the *PMR6* gene in *Arabidopsis* (Vogel *et al.*, 2002). The *PMR6* gene encodes a pectate lyase-like protein and mutations in this gene resulted in increased accumulation of pectin inside the plant cell wall, and consequently enhanced resistance to the pathogen *E. cichoracearum*. The *pmr6*-mediated resistance requires neither SA nor the ability to perceive JA or ET.

The involvement of *AS1* in controlling disease resistance or susceptibility against distinct groups of pathogens was conserved in three evolutionary diverse plant species. Therefore, suggesting the function of *AS1* in disease resistance and susceptibility is relatively ancient.

## 5 Measurement of defence signalling molecules in *as1* plants

The effective establishment of disease resistance depends upon the concentration and synergistic and antagonistic interactions between the different signalling molecules SA, JA and ET (and respective analogues and derivatives). As previously shown, the *as1* mutation conveys resistance to necrotrophic pathogens and susceptibility to host and non-host bacterial pathogens. Therefore, we investigated the accumulation of SA, JA and ET in response to pathogen infection in *as1* plants.

### 5.1 Mutation in *AS1* partially compromises SA accumulation

The plant hormone SA is a key regulator of plant defence against biotrophic and hemi-biotrophic pathogens, as well as a potent inducer of systemic acquired resistance (Delaney *et al.*, 1994; Ryals *et al.*, 1996; Shirasu *et al.*, 1997; Delaney, 2000). Negative crosstalk involving SA and other signalling molecules have been reported (Doares *et al.*, 1995; Harms *et al.*, 1998; Thaler *et al.*, 2002). Mutations that compromise SA biosynthesis or its perception result in enhanced susceptibility to a range of biotrophic pathogens (Cao *et al.*, 1994; Delaney *et al.*, 1994; Vernooij *et al.*, 1994; Cao *et al.*, 1998). In addition, disruption of SA accumulation or transduction has been shown to compromise *R* gene-mediated resistance (Delaney *et al.*, 1994).

The accumulation of free SA (SA) and its conjugate β-glucoside (SAG) in *as1-4* and *as1-1* mutants and the respective wild type parental lines (Col-0, L*er*), in response to challenge with *Pst* DC3000 carrying the avirulence gene *avrB* (Bisgrove *et al.*, 1994) was analyzed (Fig. 5.1). Comparable amounts of SA were found in all four untreated lines. In contrast, both wild type lines accumulated significantly greater levels of SA β-glucoside (SAG) than the two mutant lines. SAG content found in uninfected Col-0 plants was up to 35% higher than in *as1-4* plants, while untreated L*er* plants accumulated about four times more SAG than the mutant *as1-1*. When comparing *Pst* DC3000 (*avrB*) infected plants, again no significant difference was detected for free SA between Col-0 and *as1-4*, while up to two fold more SAg was found in Col-0 plants compared to *as1-4*. The effect of *as1* mutation in

the pathogen-induced SA accumulation is more pronounced in the *Ler* background. Pathogen challenged *as1-1* mutant plants accumulated less than 35% and 50% of SA and SAG found in L*er* infected plants, respectively. Interestingly, both *as1-4* and *as1-1* mutant plants displayed similar level of resistance or susceptibility to all pathogens tested.

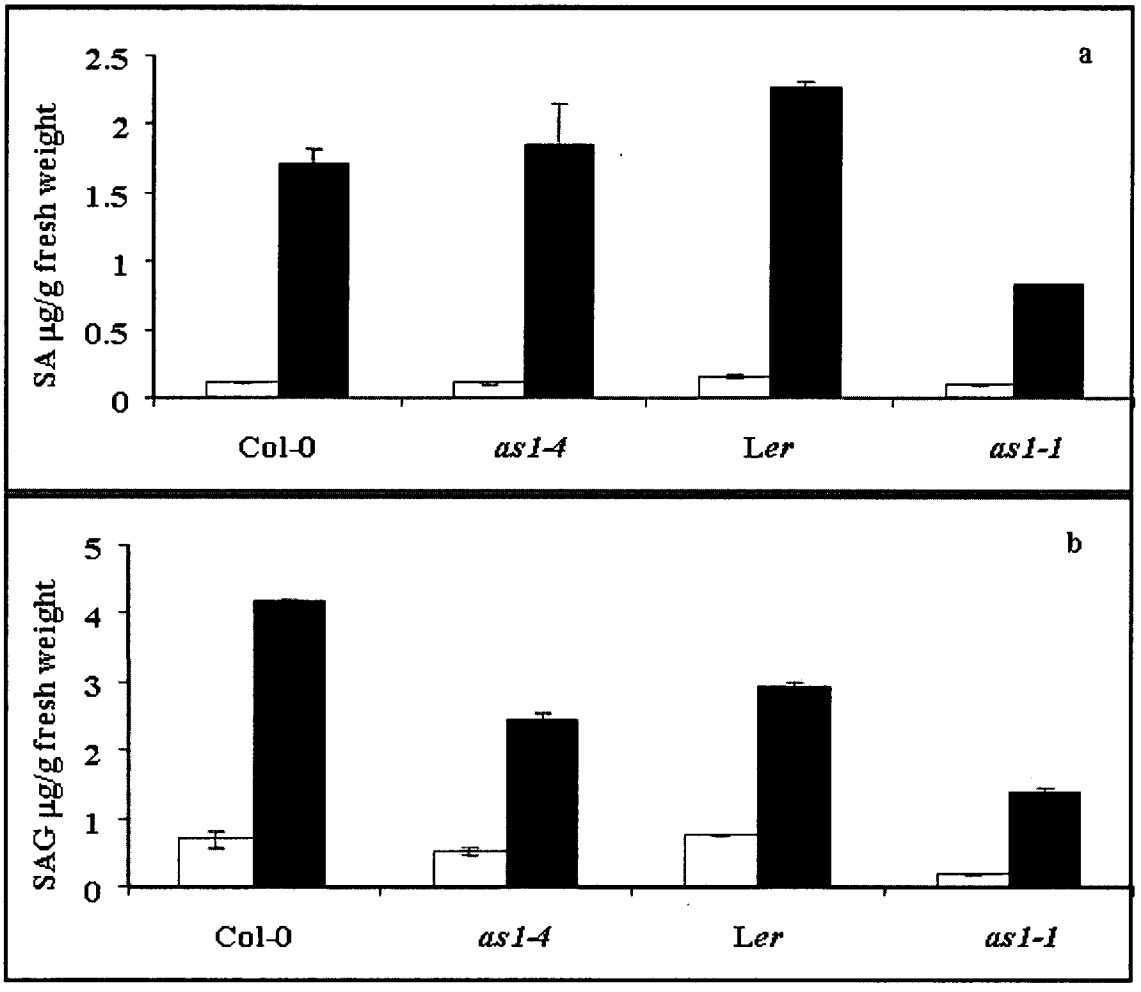


**Figure 5.1 Pathogen induced accumulation of SA is not significantly affected in *as1* plants**. Four-week old plants were syringe inoculated with avirulent strain of *Pst (avrB)* and samples collected 48 hpi. Empty bars represent untreated plants and filled bars represent plants challenged with *Pst* DC3000 (*avrB*). (**a**) *as1* plants synthesize SA in response to pathogen attack, only *as1* line accumulates less SA than wt, and (**b**) amounts of β-glucoside, both *as1* lines accumulate less SAG than respective wild type. Experiments were repeat thre times with similar results. The error bars represent standard errors (SE).

## 5.3 *as1* plants show higher basal levels of hormone ethylene

The plant growth regulator ethylene is involved in many aspects of plant growth and development and accumulating evidence suggests this hormone also plays a fundamental role in defence against necrotrophic pathogens (Xu *et al.*, 1994; Solano and Ecker, 1998; Berrocal-Lobo *et al.*, 2002). After finding enhanced expression of JA/ET-mediated defence genes in *as1* lines in comparison to wild type plants, we investigated ET accumulation in *as1* plants in response to infection with *B. cinerea* (Fig. 5.3). Four-week old *as1* and wild type plants were spray inoculated with a spore suspension and samples collected 48 hpi. Petioles of infected and mock inoculated plants were cut using a sharp razor blade, and placed into a 20mL capsule containing 1 mL of water with petioles immersed in the water. Three replications per treatment were collected and samples accommodated under growth conditions used for growing *Arabidopsis* plants. Both wild type and *as1* plants released similar amounts of ET in response to *Botrytis* infection. However, mock infected *as1* plants showed significantly higher basal ET content in comparison to wild type. Untreated *as1-1* and *as1-4* plants displayed two-fold more ET than wild type plants. This higher basal ET concentrations was not enough to confer constitutive expression of ET-dependent marker genes, but may account for at least part of the accelerated expression of such genes, leading to enhanced resistance against *B. cinerea*. Moreover, the increased accumulation of ET in *as1* plants did not result in premature senescence, what is a characteristic of many plants that accumulate higher amounts of ET.

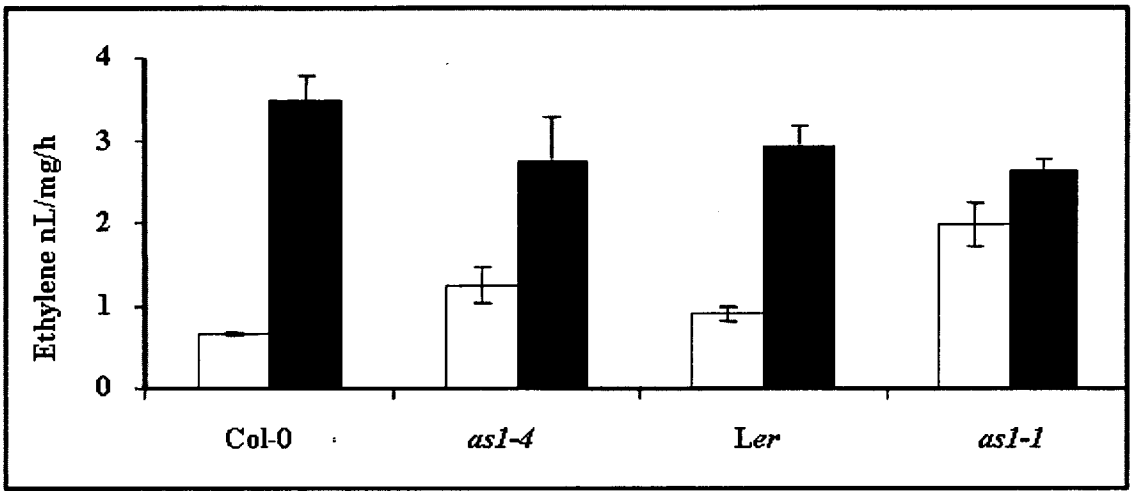


**Figure 5.3 *As1* plants present higher basal levels of the hormone ET.** Four-week old plants were spray inoculated with *B. cinerea* and leaf samples collected 2 dpi. Three replications were collected per treatment. Empty histogram bars represent untreated plants and full bars represent plants challenged with *B. cinerea.* Error bars represent standard error (SE).

## 5.4 Camalexin accumulation upon pathogen infection is not affected by *as1*

In an attempt to establish a link between *as1* susceptibility to *Pst* DC3000 and a compromised defence response, we investigated accumulation of the antimicrobial phytoalexin camalexin upon infection of wild type and *as1* plants (Fig. 5.4). Four-week old plants were infiltrated with a bacterial suspension ($1x10^6$ cfu/mL) and leaf samples collected at 48 hpi. Camalexin was extracted as previously described (Glazebrook and Ausubel, 1994) and quantified by HPLC analysis (Veronese *et al.*, 2004). The amounts of camalexin increased from undetectable to over 4 μg/g of fresh tissue in both wild type and *as1* plants, in response to pathogen infection. In the Col-0 background, camalexin content was up to 60% higher in *as1-4* mutants. Interestingly, L*er* plants accumulated up to 54% more camalexin upon infection with *Pst* DC3000 than *as1-1* plants. These results suggest *as1* may differentially affect camalexin accumulation in the different backgrounds. However, camelexin levels are also shown to vary between *Arabidopsis* accessions, as reported previously (Denby *et al.*, 2004). Nevertheless, the susceptibility of *as1* plants to

*Pst* DC3000 was confirmed in both backgrounds, and therefore altered camalexin accumulation does not seem to be responsible for enhanced susceptibility of *as1* plants towards *Pst*.

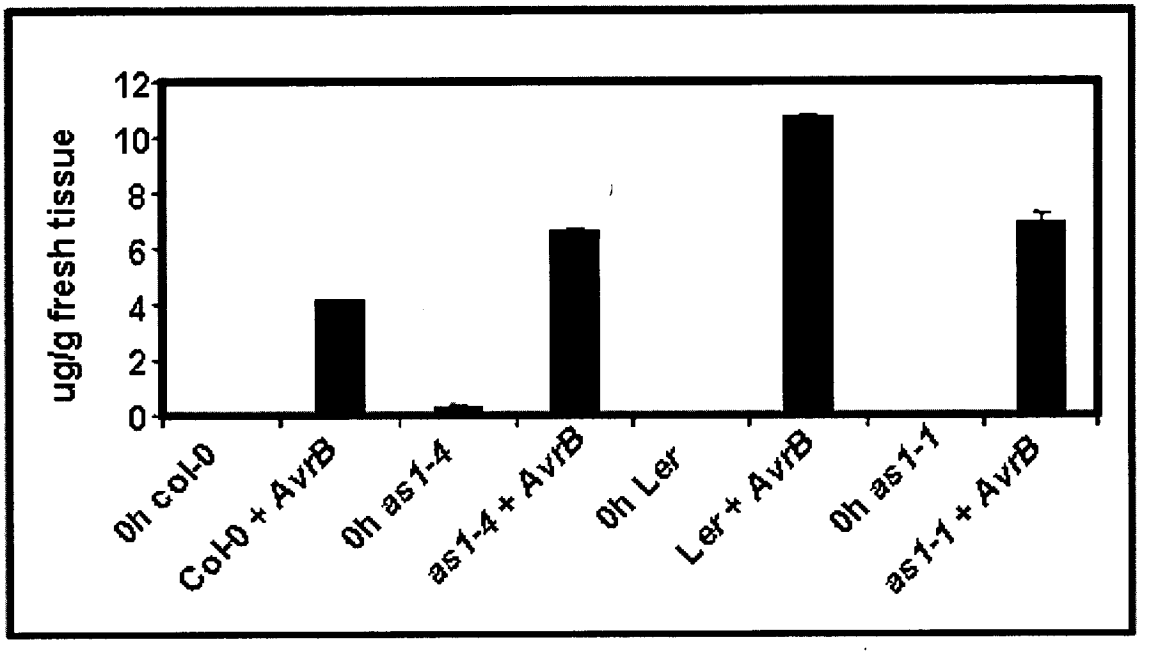


**Figure 5.4 *Pst* DC3000 (*avrB*) induced accumulation of camalexin**. No significant difference could be detected in camalexin accumulation in wild type and *as1* plants inoculated with a strain of *Pst* DC3000 expressing the avirulence gene *avrB*. Samples were collected 0 and 48hpi. Experiment was repeated twice with similar results. Histogram bars represent the average and error bars represent the standard error (SE).

## 5.5 Discussion

As described in the previous chapter, *as1* plants showed increased resistance against necrotrophic pathogens and susceptibility towards host and non-host bacterial pathogens. The establishment of defence against host and non-host pathogens is believed to require a functional SA signalling pathway (Vernooij *et al.*, 1994; Cao *et al.*, 1998), while resistance against necrotrophic is mediated through a JA/ET dependent pathway (Thomma *et al.*, 1998; Glazebrook *et al.*, 2003). In this chapter we analysed the induced accumulation of the signalling molecules as well as the phytoalexin camalexin, in response to challenge with different pathogens. The role of these signalling molecules in the defence response is proven to be complex and a natural antagonism between the different signalling pathways has been suggested (Glazebrook *et al.*, 2003; Spoel *et al.*, 2003; Glazebrook, 2005).

The defence metabolite determination in *as1* plants presented here suggests that neither impaired resistance against bacterial pathogens nor increased resistance against necrotrophic pathogens is due to altered accumulation of any of these signalling molecules in response to pathogen challenge. Determination of JA and ET accumulation in reponse to *B. cinerea* challenge revealed no difference between wild type and *as1* plants. The only slight difference noted was in the basal level of ET. Nevertheless, *as1* plants do not display accelerated senescence, which is a characteristic of many plants that accumulate higher levels of ET. It has been previously shown that constitutive expression of the ethylene receptor factor 1 (*ERF1*) resulted in constitutive expression of ET-dependent defence marker genes and enhanced resistance to different species of necrotrophic fungi (Berrocal-Lobo *et al.*, 2002). Therefore, the potentiated JA/ET-dependent gene expression observed in *as1-4* and *as1-1* could be due to increased baseline concentration of ET.

The accumulation of the plant hormone SA in *as1* plants inoculated with a strain of *Pst*DC3000 expressing the avirulence gene *avrB* was not greatly different from those levels determined for wild-type challenged plants. The amounts of SAG were lower in the two *as1* lines comparing to the respective wild types plants. SA is required for the induction of *PR-1* gene expression (Malamy *et al.*, 1990) and has long been recognised as one of the essential molecules for the establishment of defence against biotrophic pathogens (Malamy

and Klessig, 1992; Shah, 2003) as well as against non-host pathogens (Mellersh and Heath, 2003; Sharma *et al.*, 2003). Despite the differences in SA accumulation, pathogen-induced expression of the SA-dependent *PR-1* gene was not different between *as1* and wild type plants. These results suggest that the enhanced susceptibility of *as1* towards bacterial pathogens is not due a defect in the SA-dependent pathway.

Previous studies have reported the involvement of camalexin in host and non-host resistance, as well as in resistance against the necrotrophic pathogen *A. brassicicola* (Knoester *et al.*, 1998; Thomma *et al.*, 1998; Mellersh and Heath, 2003). Analysis of camalexin accumulation in response to either *Pst*DC3000 or *Pst*DC3000 (*avrB*) revealed that in both wild type and *as1* plants camalexin levels increased in response to pathogen challenge. Higher levels of camalexin in L*er* and *as1-1* than in Col-0 and *as1-4* were detected, suggesting variation in camalexin accumulation between the different ecotypes, which is in agreement with previous reports (Denby *et al.*, 2004).

Altogether, these results support our hypothesis that *AS1* may function as a negative regulator of a susceptibility factor and also as a repressor of the JA-dependent defence marker gene expression.

## 6 Analysis of *as1* double mutants

In an attempt to identify other components engaged in the *as1* resistance we generated a series of *as1* double mutants. This is a particularly powerful approach to uncover the basis of *as1*-mediated resistance against *B. cinerea*. For example, this strategy has been utilized for the *Arabidopsis adr1*, *cpr1* and *cpr5* (Bowling *et al.*, 1997; Clarke *et al.*, 2001; Chini *et al.*, 2004). We therefore examined the response of *as1aos, as1coi1, as1ein2* and *as1adr1* double lines against *B. cinerea*. The *aos* is a transgenic line expressing an antisense transcript of the allene oxide synthase (AOS) gene required for JA biosynthesis. *COI1* is required for the activation of JA-mediated signalling (Feys *et al.*, 1994), while *EIN2* is a key component of the ET signalling pathway (Guzman and Ecker, 1990; Hua and Meyerowitz, 1998; Thomma *et al.*, 1999a). *Adr1* is a gain-of-function mutant conferring resistance to a series of virulent pathogens (Grant *et al.*, 2003). The mutant lines *coi1* and *ein2* are recessive while *aos* and *adr1* are dominant. Details of these genes and their function in disease resistance was described in chapter 1.

### 6.1 Response of double mutants to *B. cinerea* challenge

Selection of *as1ein2* double mutants was performed on MS plates supplemented with the ET precursor aminocarboxilic acid (ACC) at a final concentration of 10 μM. $F_2$ seedlings were grown in the dark in the presence of ACC for 7 days and those lines not displaying the triple response (due to mutation in *EIN2* gene) were transferred onto soil and double mutants selected by the morphological phenotype of *as1*. Infection with *B. cinerea* resulted in enhanced disease symptoms in *as1ein2* double mutants in comparison to wild type and *as1* plants (Fig. 6), suggesting that resistance of *as1* against *B. cinerea* requires a functional ET signalling pathway. Resistance of *Arabidopsis* plants against this necrotroph was previously shown to be compromised by the *ein2* mutation (Thomma *et al.*, 1999a). In contrast, the double mutants *as1adr1* exhibited increased resistance to *B. cinerea* compared to *as1* (Fig. 6) suggesting an additive effect of both genes in resistance against this pathogen. Interestingly, however, was the fact that resistance of *adr1* alone was not

significantly different from that of wild type plants infected with *B. cinerea* (Grant *et al.*, 2003; Chini, 2004).

*As1aos* planta were identified in $F_2$ by the morphological phenotype of *as1* and male sterility conferred by the *aos* antisense transgene, which were then rescued by exogenous application of Me-JA at buds of conditionally sterile plants. Homozygous *ibr1/ibr1aos/aos* plants were confirmed in $F_3$. Blocking JA accumulation by the *aos* antisense resulted in enhanced susceptibility of *as1aos* double mutant lines when infected with the necrotrophic pathogen *B. cinerea* (Fig 6).

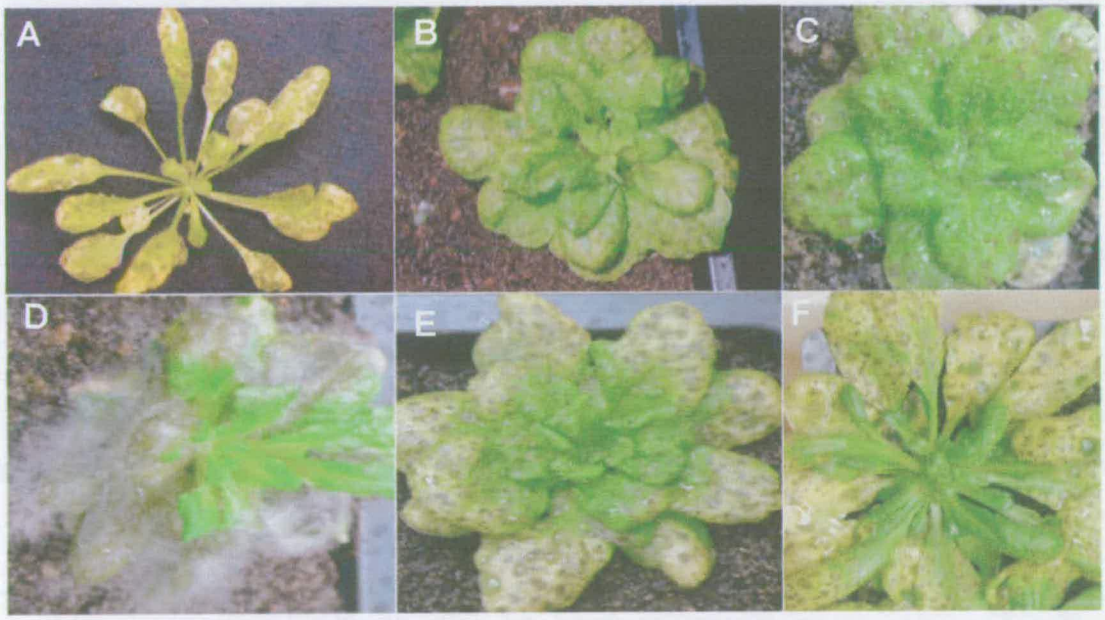


**Figure 6.1 *As1*-mediated resistance against necrotrophic pathogens is JA/ET dependent**. Disease symptoms on wild type, *as1* and double mutants challenged with *B. cinerea*. Plants were spray inoculated with a spore suspension and pictures taken 6dpi. (A) wild type Col-0, (B) *as1/as1*, (C) *as1/adr1*, (D) *aos/as1*, (E) *as1/ein2*, (F) wild type L*er*. Experiment was repeated twice with similar results.

Interestingly, no double mutants between *as1* and *coi1* could be identified in the $F_2$ generation. For selection, $F_2$ seeds were surface sterilized and placed on MS+Me-JA plates and insensitive seedlings (*coi1*) transferred to soil. A total of 1,763 $F_2$ Me-JA insensitive seedlings were selected, but none showed lobed leaves, a characteristic feature of *as1*. There are two possible explanations for the absence of double mutants between these two

lines: (1) The physical distance between these two genes, *coi1* and *ibr1,* precludes frequent recombination; or (2) the null mutation in *coi1* suppresses *as1*. *COI1* and *AS1* are located on chromosome 2 at positions 16679040-16682826 and 15788693-15790476, respectively, which means these two genes are separated from each other about 888.56kbp. Assuming that in *Arabidopsis*, on average, a 1% of recombination frequency corresponds to approximately 250 kbp (Jander *et al.*, 2002), an estimated 3-4% recombination rate would be expected between *COI1* and *AS1*. Thus, 0.04% of the total segregating population would be expected to carry both recessive phenotypes. However, if the segregating population is firstly subjected to selection on Me-JA, our chances of finding a double mutants is increased by four fold, even though the proportion of double mutants is still small. Thus, from 1,763 plants already selected for insensitivity to Me-JA only approximately 3 of them are also expected to possess lobed leaves. However, no plants selected for Me-JA insensitivity displayed the *as1* phenotype. Therefore, to confirm if *coi1* does indeed suppress *as1*, two different strategies were adopted to silence the expression of *COI1* in *ibr1* plants.

An antisense construct driven by the CaMV35S promoter was engineered and used to transform homozygous *as1* plants. In addition a hairpin forming method was also employed (Hamilton and Baulcombe, 1999). For the antisense mediated gene silencing a 300 bp region was PCR amplified and cloned into vector *pJIT166*, containing 2x 35S promoter and terminator, using the enzymes *XbaI* and *NcoI*. The pJIT166+*COI1* was sub-cloned into vector pGreenII using enzymes *SpeI* and *SacI*. Positive clones were confirmed by PCR and enzyme digestion (data not shown). The resulting vector was named *pJIT166+pGreen0049+Coi1* (Fig. 6.1) and *as1* plants were transformed by the deploying of the *Agrobacterium* mediated transformation method, termed floral dip (Clough and Bent, 1998). For the hairpin vector a 351 bp *COI1* coding sequence was PCR amplified and inserted into vector *pBluescript2 minus* using enzymes *XhoI* and *HindIII*, followed by the insertion of a 160 bp intron sequence with enzymes *HindIII* and *BamHI* and another copy of the 351 bp coding sequence inserted in the reverse orienatation with enzymes *BamHI* and *SpeI*. All cloning steps were performed in *E. coli* and positive transformants confirmed by both PCR reaction and enzymatic digestion. The pBluescript vector containing the three *COI1* sequences was sub-cloned into vector pGreenII already containing 2x 35S promoter

and CaMV terminator, obtained from vector *pJIT166*. The resulting vector, was named pJIT166Green0049Coi1 (Fig 6.2) and transfected into homozygous *as1* plants by *Agrobacterium* mediated transformation (Clough and Bent, 1998).

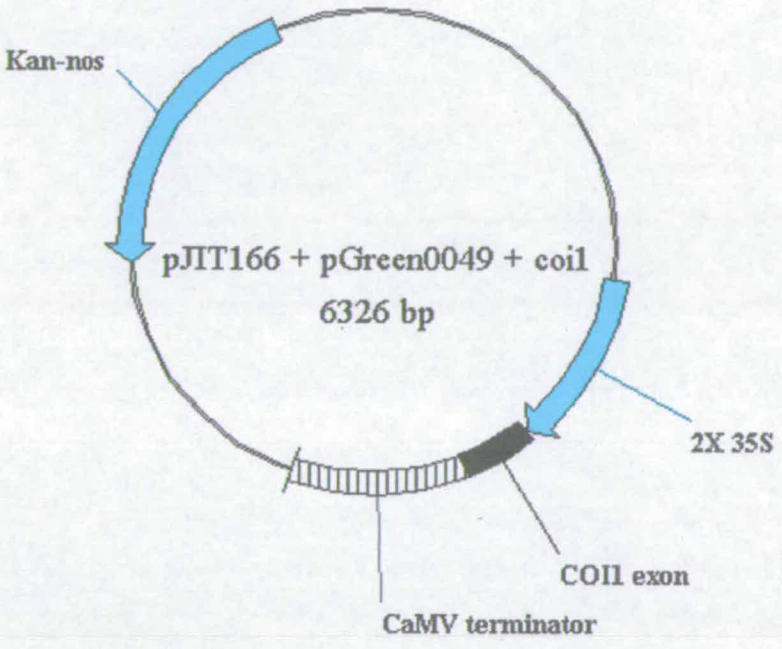


**Figure 6.2 Molecular architecture of the silencing vector pJIT166+pGreen0049+Coi1**. The pJIT166Green0049Coi1 contains 2x CaMV 35S promoter sequence adjacent to the *COI1* exon, followed by the CaMV terminator sequence. Promoter and terminator sequence were inserted from the vector pJIT166 into vector pG0049. The construct harbours a kanamycin resistance gene (KAN) fused with the nopaline synthase promoter (PNOS) and the poly A sequences of gene 4 of the A. tumefaciens.

The transformation rate obtained was very low comparing to previous reports using this method (Grant *et al.*, 2003; Tani *et al.*, 2004). From approximately 10,000 $T_1$ screened seeds, only 8 transformants were selected as kanamycin resistant, and none of them showed the wild type morphological phenotype, expected due to *coi1* suppression of *as1*. However, none of these lines showed the male sterility, a feature of *coi1* plants, suggesting therefore silencing of *COI1* was not achieved, at least not at level able to suppress *as1* or to cause male sterility.

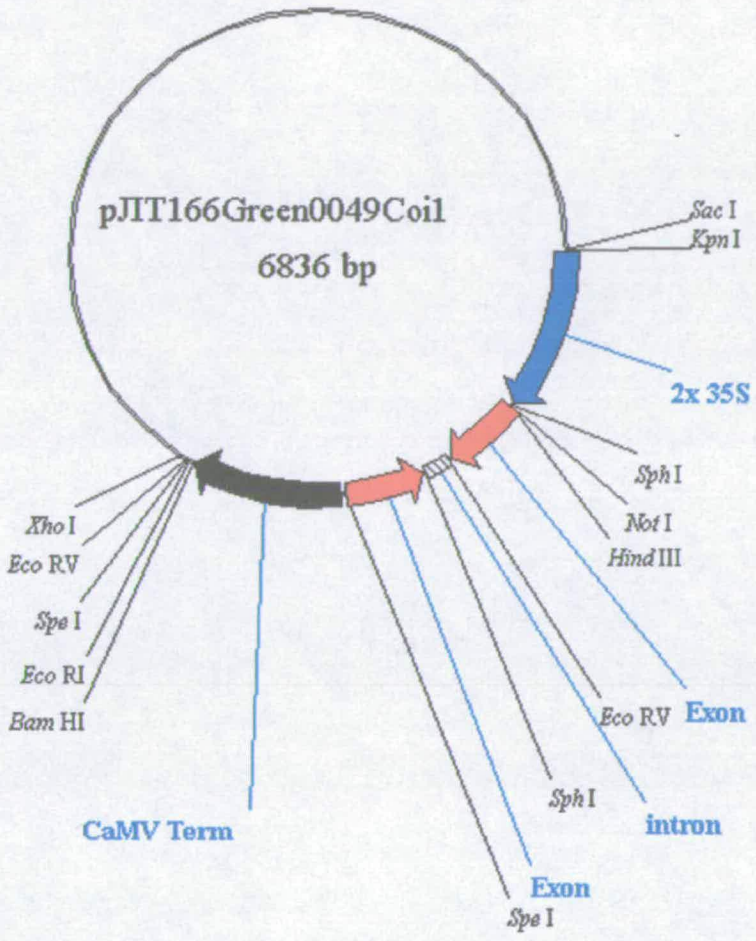


**Fig 6.3 Molecular architecture of the silencing vector pJIT166Green0049Coi1. The pJIT166Green0049Coi1** contains 2x CaMV 35S promoter sequence adjacent to the *COI1* exons inserted in opposite orientations and separated by a short intron sequence, followed by the CaMV terminator sequence. Promoter and terminator sequence were inserted from the vector pJIT166 vector pG0049. The construct harbours a kanamycin resistance gene (KAN) fused with the nopaline synthase promoter (PNOS) and the poly A sequences of gene 4 of *A. tumefaciens*.The exon-intron-exon sequence under transcriptional activation of the 35S promoter will form the hairpin which will function as a sign for RNA degradation.

## 6.2 Discussion

To determine the individual contribution of the JA and ET signaling pathways in the *as1*-mediated resistance, the response of *as1/ein2, aos/as1, coi1/as1*. and *as1/adr1* double mutants to infection with *B. cinerea* started to be investigated and the preliminary results are discussed in this chapter.

The results present here strongly suggest that *as1*-mediated resistance against *B. cinerea* is dependent on both JA and ET signaling pathways. While increased symptoms in *as1/ein2* and *aos/as1* double mutants show a requirement of ET perception and JA synthesis, the absence of *coi1/as1* double mutants in the $F_2$ population prevented us from specifying whether resistance is *COI1*-dependent or independent. A possibility for the absence of double mutants is that the physical distance between these two genes, *coi1* and *ibr1,* precludes frequent recombination. However, based on their physical location and the average recombination rate in *Arabidopsis*, it is estimated that approximately a 4% recombination frequency would occur between *coi1* and *as1*. We checked over 1700 F2 plants insensitive to Me-JA but none showed the typical lobed leaves phenotype, instead of about 70 double recombinants that were expected. Therefore, we speculate that a null mutation in *coi1* suppresses *as1*.

Suppression of *as1* by *coi1* may involve the ubiquitin-mediated protein degradation in which *COI1* was shown to take part. *COI1* encodes one of at least 694 *Arabidopsis* F-box proteins, the largest class of plant proteins to date (Gagne *et al.*, 2002). In *Arabidopsis* F-box proteins interact with the SKP1-like, ASK1 and ASK2, and cullin proteins to form the SCF ubiquitin-ligase complex, for the ubiquitylation of substrate proteins targeted for degradation (Bai *et al.*, 1996). It is estimated that approximately 5% of all *Arabidopsis* genes are involved in the ubiquitin/26S pathway, making it one of the most elaborate regulatory mechanisms in plants (Vierstra, 2003). A more extensive discussion about the possible involvement of the $SCF^{COI1}$ complex in suppression of *as1* and consequently resistance against necrotrophic pathogens will be presented in the last chapter of this study.

The additive effect of *adr1* in the *as1*-mediated resistance was rather unexpected because it was previously shown that *adr1/adr1* plants were unaffected in resistance against *B.*

*cinerea* (Grant *et al.*, 2003). *Adr1* conveys resistance to a broad spectrum of biotrophic and hemibiotrophic pathogens, in addition to increased tolerance to water deficit (Grant *et al.*, 2003; Chini *et al.*, 2004). The defence metabolite profiling showed that untreated *adr1* plants constitutively express the defence marker genes *PR-1, GST-1* and *PDF1.2*. Misexpression of *PR-1* and *GST-1* genes in *adr1* correlates with higher levels of endogenous SA and spontaneous HR formation, while constitutive expression of *PDF1.2* is not associated with changes in JA or ET accumulation, which is in agreement with other studies showing that *PDF1.2* expression alone is not enough to convey resistance against *B. cinerea* (Ferrari *et al.*, 2003; Mengiste *et al.*, 2003). However, combined overexpression of *PDF1.2* by *adr1* with resistance conveyed by *as1* may explain the enhanced resistance of *as1/adr1* double mutants against *B. cinerea*. Since no direct relationship between the conveyed *B. cinerea* resistance and the SA signaling pathway was established in *as1* plants, it is unlikely that high accumulation of endogenous SA in *adr1* could antagonize resistance in *as1/adr1* plants against *B. cinerea*. Moreover, no antagonism between SA- and JA/ET signaling pathways was observed in *adr1* plants (Grant *et al.*, 2003; Chini *et al.*, 2004).

## 7 Characterization of *ibr2*

Screening of our activationaly tagged population identified another *B. cinerea* resistant line termed *INCREASED BOTRYTIS RESISTANCE 2* (*IBR2*). This line was partially characterized and will be documented here. Since *ibr2* was not tagged, a map-based cloning strategy was adopted. This is an indirect approach, based on recombination rate between mutant phenotype and molecular markers in the genome. A molecular marker consists of a gene (or DNA sequence) for which two phenotypically distinguishable alleles are known. By this approach, the physical position of a mutated gene, on a given chromosome, is defined by narrowing down the genetic interval containing the mutation (Lukowitz *et al.*, 2000; Jander *et al.*, 2002).

In order to elucidate the map position of a mutation, the mutant plant has to be crossed with plants from another divergent accession. In *Arabidopsis*, the most commonly used accessions are Columbia and Landsberg *erecta*. It is estimated that these two accessions differ from four to eleven positions for every 1000 bp in their genome (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). Currently, Single Sequence Length Polymorphism (SSLP) and Cleaved Amplified Polymorphic Sequences (CAPS), are the most widely used molecular markers in map based cloning (Konieczny and Ausubel, 1993; Bell and Ecker, 1994). SSLP markers typically exploit the variability of short repetitive sequences (80 to 250 bp). The main advantages of SSLPs markers are: 1- They are codominant, that means that both chromosomes may be genotyped and 2- they are PCR based and can be visualised on an agarose gel. Therefore, they are easy to use and relatively inexpensive (Lukowitz *et al.*, 2000).

The CAPS markers exploit polymorphic restriction sites, amplified by PCR. They are more costly and can not be visualized directly after PCR, but they require enzyme digestion prior to visualization (Lukowitz *et al.*, 2000). Comparison of Col-0 genomic sequence (*Arabidopsis* genome initiative- AGI) and shotgun sequences of *Ler* (Cereon-Monsanto database) allowed the identification of two main groups of polymorphisms between the two ecotypes: 1- Polymorphisms due to single nucleotide polymorphism changes (SNP), and 2- Insertion-deletion (InDel) of DNA sequences. These polymorphic sequences have great

applicability as markers for mapping purpose. InDels, when used for mapping are easily visualized on agarose gels, and preference should be given to InDels of longer sequences. In *Arabidopsis* it is predicted one InDel will be present every 6.6 kbp, while SNPs are found in higher density. With this saturation of markers (InDels & SNPs) one should be able to map a given mutation down to a few thousand base pairs. However, it should be noted that markers are not evenly distributed along all chromosomes (Jander *et al.*, 2002).

### 7.1 *Ibr2* plants are resistant to *Botrytis cinerea*

The *ibr2* plants, similarly to *ibr1* also show significantly reduced susceptibility to *B. cinerea* when compared to wild type plants (Fig. 7.1, a-c). This mutant line also had twisted leaves and roots, resembling the morphological phenotype of the *Arabidopsis* mutants *lefty1* and *lefty2* (Thitamadee *et al.*, 2002). F1 plants, derived from a backcross between homozygous *ibr2* and wild type plants, showed twisted leaves and increased resistance to *B. cinerea*, suggesting that these phenotypes are dominant. In the F2 generation, from 210 plants, 59 were wild type while 151 plants displayed the mutant twisted leaf phenotype co-segregating with *B. cinerea* resistance. However, the morphological phenotype could be separated into two groups, one group with 102 plants displaying an intermediate twisting of leaves and a smaller group (49 plants) showing a stronger morphological phenotype (Fig.7.1 d-g). These numbers showed no significant deviation from the ratio 1:2:1 (typical of a single codominant gene) by chi-square test with two degrees of freedom ($\chi^2$= 1.63).

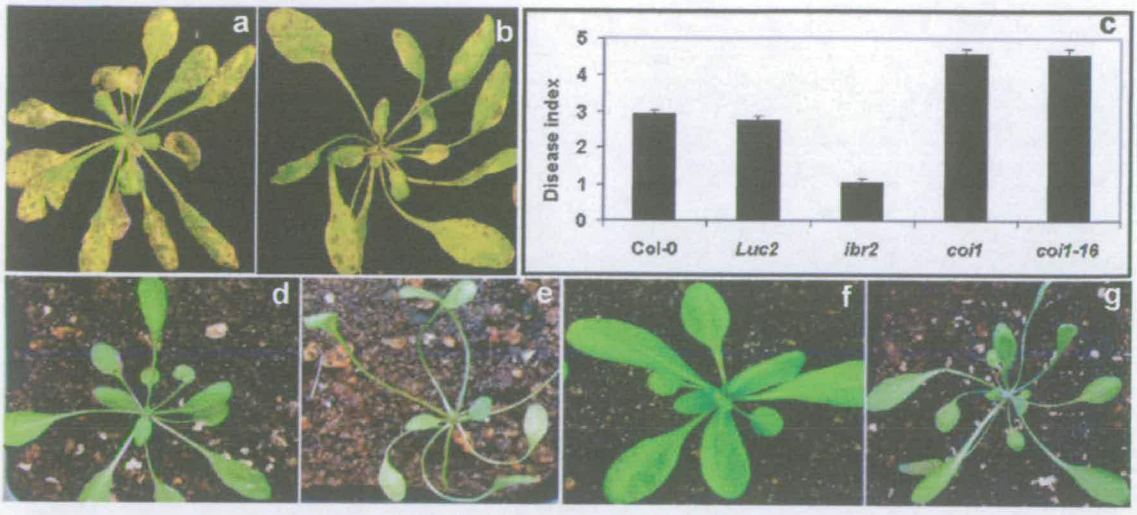


**Figure 7.1- *Ibr2* shows enhanced resistance against *B. cinerea*.** Plants were spray inoculated and symptoms scored 5 days post-inoculation. (**a**) wild Col-0 five days post-inoculation, (**b**) *ibr2* five days post-inoculation, (**c**) disease index based on visual evaluation of disease symptoms, (**d**) wild type Col-0, (**e**) homozygous *ibr2*, (**f**) wild type L*er*, (**g**) heterozygous *ibr2*.

While the twisted leaf phenotype correlated with *B. cinerea* resistance there was no association between these traits and resistance to Basta, suggesting *ibr2* was not tagged. Frequent rearrangements in the T-DNA insert and flanking genomic DNA has been reported (De Neve *et al.*, 1997; Krizkova and Hrouda, 1998; De Buck *et al.*, 1999; Sessions *et al.*, 2002). Unstable insertion of T-DNA results in rearrangement and splicing of foreign material. The alternative strategy in this case was therefore map based cloning (Lukowitz *et al.*, 2000). Mapping of *ibr2* was made easier by the fact that the morphological phenotype and *B. cinerea* resistance co-segregate.

## 7.2 *Ibr2* results in enhanced expression of defence marker genes

Inoculation of wild type *Arabidopsis* plants with *B. cinerea* results in the induced expression of series of defence marker genes (Thomma *et al.*, 1998; Thomma *et al.*, 1999b). Necrotrophic pathogens are normally responsible for the induction of an array of marker genes which are essentially SA-independent and JA/ET dependent (Thomma *et al.*,

1998). The JA-dependent expression of *PDF1.2* (plant defensin), *PR-3* (basic chitinase) as well as *PR-4* (acidic hevein-like protein) is associated with induced resistance against the necrotrophic fungi *B. cinerea*, *Alternaria brassicicola* and *Fusarium oxysporum* f.sp. *methiolae* (Penninckx *et al.*, 1996; Bohlmann *et al.*, 1998; Thomma *et al.*, 1998; Berrocal-Lobo *et al.*, 2002; Berrocal-Lobo and Molina, 2004). Conversely, mutant plants impaired in JA-signalling are more susceptible to necrotrophic pathogens (Staswick *et al.*, 1998; Vijayan *et al.*, 1998; Thomma *et al.*, 2000). Purified *Arabidopsis PDF1.2* and *PR-4* proteins were shown to possess antifungal activity *in vitro* (Verburg and Huynh, 1991; Penninckx *et al.*, 1996). Interestingly, *Arabidopsis PR-4* shows high homology to tobacco CBP-20, but antifungal activity has only been demonstrated on growth of *Thrichoderma reesei* (Verburg and Huynh, 1991). The tobacco protein CBP-20 acts by causing the lysis of germ tubes and/or inhibiting growth of *Thricoderma viride* and *Fusarium solani*. In addition, CBP-20 and tobacco class I chitinase act synergistically to inhibit growth of *F. solani*, and together with a tobacco class I β-glucanase act against *F. solani* and *Alternaria radicina* (Ponstein *et al.*, 1994). PR-3 proteins are endochitinases that cleave cell wall chitin polymers in situ, resulting in a weakened cell wall and rendering fungal cells osmotically sensitive. In addition, it was found that *PR-2* (β-glucanases) acts synergistically with *PR-3* for inhibiting fungal growth, both in vitro and *in planta* (Jach *et al.*, 1995).

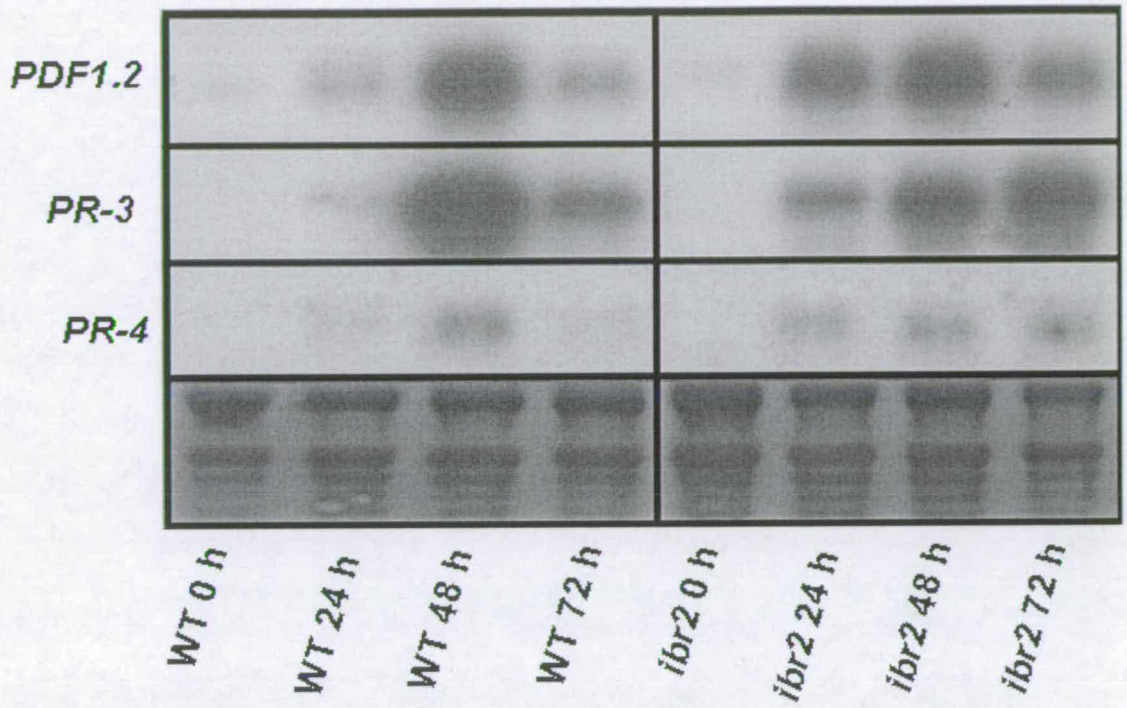


**Figure 7.2** *B. cinerea* induced defence gene expression in wild type and *ibr2* mutant *Arabidopsis* plants. *Ibr2* plants show accelerated expression of defence marker genes.

Therefore, we monitored (by northern blot analysis) the expression of key defence marker genes in *ibr2* in comparison to wild type plants, after infection with *B. cinerea*. In wild type plants, transcripts of *PDF1.2* were first detected, in a very low level at 24 h post-inoculation (hpi) (Fig. 7.2). The amount of transcripts detected, increased significantly by 48 hpi, and gene expression remained high at 72 hpi. Interestingly, transcription of *PDF1.2* was accelerated in *ibr2* plants in comparison to the wild type line. At 24 hpi *PDF1.2* transcripts were significantly increased in *ibr2*. Similar results were found for the *PR-3* marker gene. As can be seen on Fig.7.2, transcripts of *PR-3* were also induced at earlier time points than in wild type Col-0 plants. Again, the expression at 72 hpi was still greater in the mutant plants. Interestingly, for the *PR-4* gene, a much lower level of transcripts were found for both wild type and *ibr2* plants. Thus, resistance of *ibr2* plants maybe due to accelerated expression of the JA-ET dependent defence genes *PDF1.2* and *PR-3*.

### 7.3 *Ibr2* does not affect resistance against bacterial pathogens

In addition to the necrotrophic pathogens *B. cinerea* and *A. brassicicola*, *ibr2* mutant plants were also tested for resistance against virulent and avirulent strains of *Pst* DC 3000 and also *P. fluorescens 2-79*, a non-host pathogen of *Arabidopsis*. Four-week old wild type and *ibr2* plants were inoculated with either *Pst* DC3000, *Pst* expressing *avrB* or *P. fluorescens 2-79* and disease symptoms were monitored daily until five days post-infection (dpi). Both wild type and *ibr2* plants inoculated with virulent *Pst* DC3000 started to show chlorotic lesions at 4 dpi, with no visible difference between them, suggesting *ibr2* did not affect resistance against this pathogen. Very weak chlorotic lesions were seen on both lines when infected with *Pst* DC300 (*avrB*), while no symptoms were produced in response to *P. fluorescens 2-79* (data not shown). To confirm *ibr2* plants are not affected in resistance against these pathogens, another set of plants were infected and leaf samples collected at 3 dpi for bacterial growth assay experiments. This time point coincides with peaks of *Pst* growth in *Arabidopsis* (Whalen *et al.*, 1991; Delaney *et al.*, 1994). As can be seen in Figure 7.3, both wild type and mutant plants supported growth of virulent *Pst* DC3000 to a similar extent, confirming resistance against this strain of *Pseudomonas* was not affected by *ibr2*. Likewise, *Pst* DC3000 (*avrB*) and *P. fluorescens* strains grew to similar levels in both wild type and *ibr2* infected plants (Fig. 7.3). These results suggest that *ibr2* does not have a major impact on basal resistance against *Pst* DC3000 and non-host resistance against *P. fluorescens 2-79*.

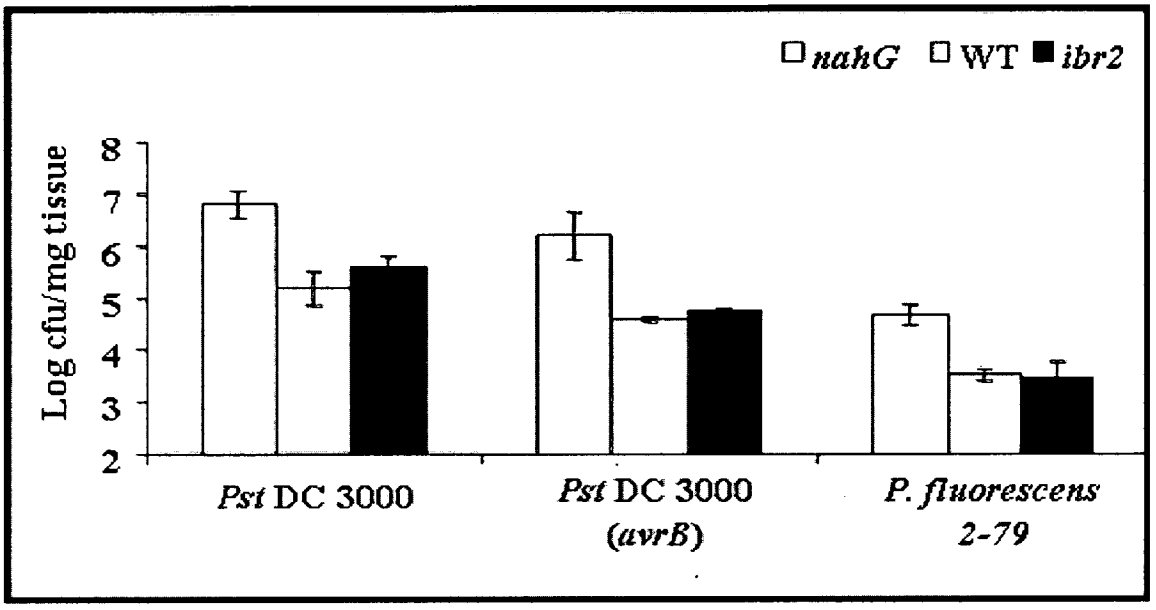


**Figure 7.3 *Ibr2* is not affected in resistance against bacterial pathogens.** Plants were challenged with virulent *Pst* DC3000, *Pst* DC 3000 (*avrB*) or *P. fluorescens 2-79*. Col-0 wild-type plants and a *nahG* transgenic line were included as controls. Pathogen growth was analysed 3 days after challenge. The experiment was repeated 3 times; error bars represent standard errors.

## 7.4 Seed yield is reduced in *ibr2* plants

Mutations leading to enhanced resistance to biotic and/or abiotic stresses are frequently associated with altered morphology and development penalties (Frye and Innes, 1998; Maleck *et al.*, 2002; Pilloff *et al.*, 2002; Grant *et al.*, 2003). Therefore, we investigated whether *ibr2*, besides enhancing resistance to *B. cinerea* also affected seed yield. Seeds from homozygous and heterozygous *ibr2* plants, together with wild type Col-0 and the transgenic *luc2* line were collected and weighed (Fig. 7.4). No significant difference was observed in the amount of seeds produced by wild type, *luc2* and heterozygous *ibr2* plants. On the other hand, homozygous *ibr2* plants exhibited a reduction in seed yield compared to the other three lines examined. It is worth noting that it was not always easy to distinguish between homo and heterozygous plants by looking at morphological or disease resistance phenotype. Thus, to benefit from resistance conveyed by *ibr2* in the absence of a seed yield penalty, heterozygous and not homozygous plants should be used. However, for practical

applications, such a measure is not always possible. For example, in self-pollinated crop species it would be almost impossible to maintain the heterozygous condition of a gene. An elegant alternative is to make use of an inducible promoter to control the expression of a gene of interest (Aoyama and Chua, 1997). Transient gene expression has been shown to be an efficient method to establish resistance (Chini *et al.*, 2004).

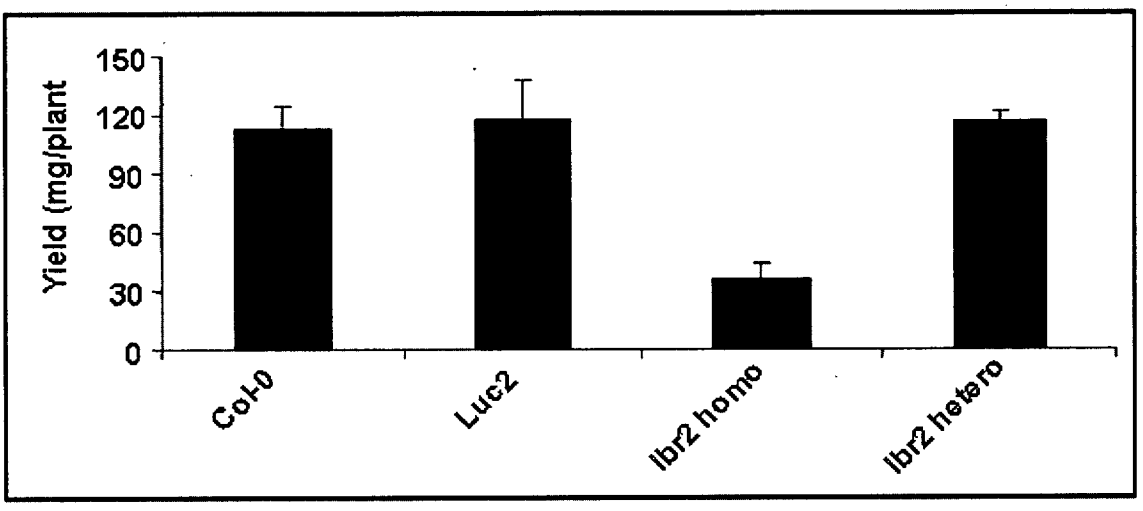


**Figure 7.4 Seed yield is reduced in the *ibr2* mutant**. Seeds from thirty plants per line were harvested and weighed. Data were analysed using the software Minitab 13. Histogram bars represent the mean ± standard error (SE).

## 7.5 Map based cloning of *ibr2* - Bulked segregant analysis (BSA)

In an attempt to identify *ibr2,* a map based cloning strategy was adopted (Lukowitz *et al.*, 2000). The *ibr2* line was crossed with L*er* plants, and F1 left to self-pollinate in order to create the mapping population (F2). In F1, the mutant allele is in the Col-0 background, that is, the parental chromosome. Once obtained the segregant population, the next step was to find the rough chromosomal location of the *ibr2* mutation, that is, to identify the chromosome and region in which the mutant allele is located. Linkage between morphological phenotype and molecular markers, distributed along the five chromosomes, was determined by PCR amplification and visualization of corresponding polymorphic DNA sequences. Usually, recombination frequency less than 30% in a small population is accepted as evidence of linkage between the mutation and the tested marker (Ponce *et al.*,

1999). The unit adopted for genetic distance is the centimorgan (cM). A 1% recombination frequency between a marker and the mutant phenotype corresponds to 1 cM. In *Arabidopsis*, on average, 1cM equals approximately 250 kbp (Jander *et al.*, 2002). The molecular markers used are listed in Table 7.5, alternatively they can be found at (http://www.*Arabidopsis*.org).

**Table 7.5** SSLP markers on chromosome 5 used with respective location and recombination frequency

| **Marker** | MBK20 | MYH9 | Nga151 | Nga106 | MPI7 | MRN17 | Nga139 | F14123 | T26D22 | AthS0191 |
|---|---|---|---|---|---|---|---|---|---|---|
| **Locat. (kbp)** | 2476 | 3122 | 4670 | 5397 | 5929 | 7659 | 8428 | 9935 | 13000 | 15022 |
| **Nº plants** | 50 | 48 | 51 | 50 | 51 | 50 | 51 | 50 | 52 | 51 |
| **Hom.(Col-0)** | 34 | 34 | 51 | 43 | 45 | 45 | 47 | 42 | 43 | 37 |
| **Homo (L*er*)** | 0 | 0 | 44 | 0 | 0 | 0 | 0 | 1 | 1 | 1 |
| **Heteroz.** | 16 | 14 | 0 | 7 | 6 | 5 | 4 | 7 | 8 | 13 |
| **Recomb. Freq (%)** | **16** | **14.58** | **7** | **7.00** | **5.88** | **5.00** | **3.92** | **9.00** | **9.62** | **14.71** |

In order to reduce the number of PCR reactions for rough mapping, a bulk segregant analysis (BSA) method was utilised (Michelmore *et al.*, 1991). For BSA analysis, pools of DNA samples from 37 *ibr2* homozygous F2 plants, rather than individuals, were used as template for the PCR reactions with 12 SSLP markers evenly spaced along the genome (Fig. 7.5). The recombination frequencies between each marker and the *ibr2* mutation were calculated as the number of chromosomes containing the L*er* allele divided by the total number of chromosomes examined. That means the number of heterozygous individuals plus twice the number of individuals which are homozygous for the L*er* allele divided by the total number of alleles. Once linkage to a marker was established, fine mapping was carried out with further SSLP markers chosen from TAIR database. When markers were not available, they were designed based on polymorphism/allele database deposited at TAIR centre.

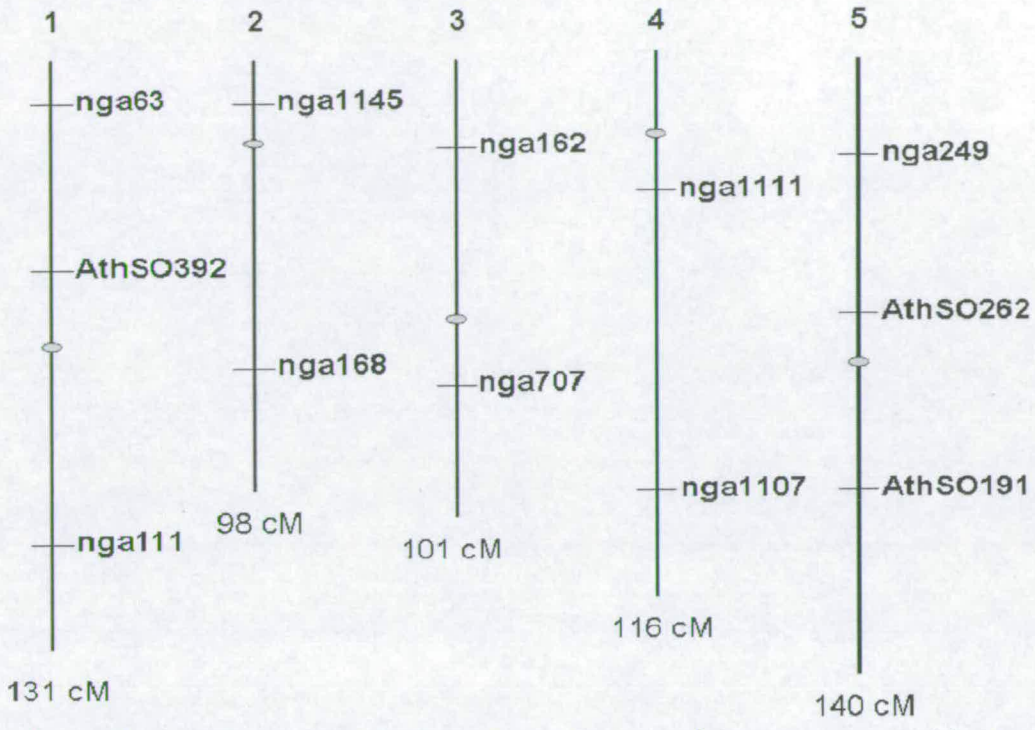


**Figure 7.5-** SSLP markers distributed over the *Arabidopsis* genome used for rough mappinig of *ibr2*

### 7.6 The *ibr2* mutation is linked to chromosome 5

The result of BSA showed a linkage between *ibr2* and the marker nga249 (Fig. 7.6.1), which is located on chromosome 5, around 2,770 kbp (Fig.7.5).

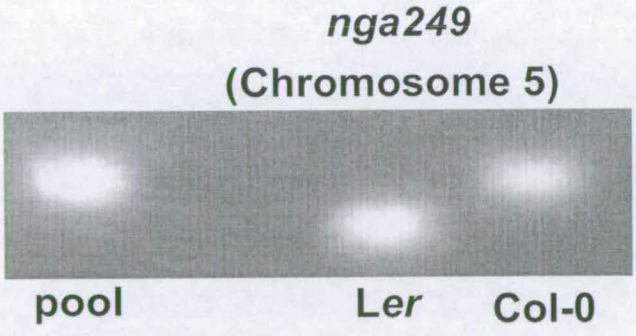


**Figure 7.6.1** Results of bulked segregant analysis. A single band for pooled DNA, at size of Col-0 band, indicates the mutation is linked to marker nga249

To confirm the linkage between nga249 and the *ibr2* mutation, DNA from each of 37 plants was tested individually for this marker. Due to difficulties in visualizing differences between PCR products for DNA from homozygous and heterozygous Col-0 plants for that chromosome location, we decided to no longer use nga249 for this project. Instead, two new markers (MBK20 and MYH9), were designed for that region, and tested with DNA from 52 individual plants. The marker MBK20 is located at position 2,476kbp on chromosome 5 while MYH9 lays on position 3,122 kbp (Fig. 7.6.2). A 13% and 11.6% recombination frequency between these markers and *ibr2* was recorded, respectively. These results suggest *ibr2* is on chromosome 5 closer to marker MYH9. In sequence, we chose four other markers, nga151 (4,670 kbp), nga106 (5,397 kbp), nga139 (8,428 kbp) and AthSO191 (15,022 kbp), and carried out PCR with DNA from the same 52 plants. Gel electrophoresis showed recombination frequencies of 6.86 % for nga151, 7% for nga106, 3.92 % for nga139 and 14.71 % for AthSO191. These results suggest the mutation is closely linked to nga139. Another two markers, F14I23 (9,935 kbp) and T26D22 (13,000 kbp) were also examined. Testing these two new markers with the same 52 DNA samples revealed 9 and 9.62% recombination frequencies for F14I23 and T26D22, respectively (Table 7.5), confirming *ibr2* is close to nga139.

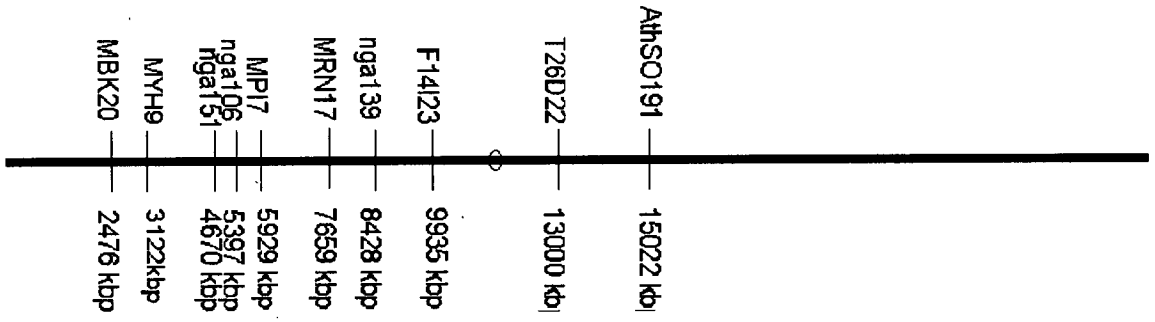


**Figure 7.6.2-** Markers on chromosome 5 used for fine mapping of *ibr2*. High density agarose gels were used to analyze PCR products for markers nga249, MYH9 and AthS0191.

Based on these recombination rates we assumed that *ibr2* should be in between nga106 and nga139 markers, but closer to nga139. Thus, two additional markers, MPI7 (5,929kbp) and MRN17 (7,659kbp) were designed, and each of them tested with DNA from those 52 plants plus another 156 new F2 individuals. A total of 42 and 39 heterozygous were recorded for markers MPI7 and MRN17, respectively (Table 7.6.1*). The number of recombinants was far different from what we expected. Therefore we decided to test these DNA samples for other markers and we found that some plants were heterozygous for all markers used. Thus, we assumed, those plants were mistakenly selected as homozygous. Despite heterozygous mutant plants showing an intermediate phenotype between homozygous and wild type it is not always easy to distinguish between them, especially at early stages of development. Therefore, those plants were discarded.

**Table 7.6.1** Recombination frequency after mistakenly selected plants are discarded.

| | Marker location (Kbp) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | MBK20 | MYH9 | Nga151 | Nga106 | MPI7 | MRN17 | Nga139 | F14123 | T26D22 | AthS0191 |
| **Plant** | 2476 | 3122 | 4670 | 5397 | 5929 | 7659 | 8428 | 9935 | 13000 | 15022 |
| 1 | C | C | C | C | C | C | C | C | **H** | **H** |
| 2 | C | C | C | C | C | C | C | C | C | **H** |
| 3 | C | C | C | C | C | C | C | C | C | C |
| 4 | C | C | C | C | C | C | C | C | C | C |
| 5 | C | C | C | C | C | C | C | C | C | C |
| 6 | C | C | C | C | C | C | C | C | C | C |
| 7 | C | C | C | C | C | C | C | C | C | C |
| 8 | C | C | C | C | C | C | C | C | C | C |
| 9 | C | C | C | C | C | C | C | C | C | C |
| 10 | C | C | C | C | C | C | C | C | **H** | **H** |
| 11 | **H** | **H** | **H** | **H** | **H** | **H** | C | C | C | C |
| 12* | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** |
| 13* | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** |
| 14 | C | C | C | C | C | C | C | C | C | C |
| 15 | C | C | C | C | C | C | C | C | C | C |
| 16 | C | C | C | C | C | C | C | C | C | C |
| 17* | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** | C | C |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 18 | C | | C | C | C | C | C | C | C | C |
| 19 | C | C | C | C | C | C | C | C | C | C |
| 20 | C | C | C | C | C | C | C | **H** | **H** | **H** |
| 21 | C | C | C | C | C | C | C | C | C | C |
| 22 | C | C | C | C | C | C | C | C | C | **H** |
| 23 | C | C | C | C | C | C | C | C | C | C |
| 24 | C | C | C | C | C | C | C | C | C | C |
| 25 | C | | C | C | C | C | C | C | C | C |
| 26 | **H** | **H** | C | C | C | C | C | C | C | C |
| 27 | C | C | C | C | C | C | C | C | C | C |
| 28 | C | C | C | C | C | C | C | C | C | C |
| 29 | **H** | C | C | C | C | C | C | C | C | C |
| 30 | C | C | C | C | C | C | C | C | C | C |
| 31 | C | C | C | C | C | C | C | L | L | L |
| 32 | C | C | C | C | C | C | C | C | C | C |
| 33 | C | C | C | C | C | C | C | C | C | C |
| 34 | **H** | **H** | C | C | C | C | C | C | C | C |
| 35 | **H** | **H** | C | C | C | C | C | C | C | C |
| 36 | C | C | C | C | C | C | C | C | C | C |
| 37 | **H** | C | C | C | C | C | C | C | C | C |
| 38* | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** | **H** |
| 39 | **H** | **H** | **H** | **H** | **H** | C | C | C | C | **H** |
| 40 | **H** | **H** | C | C | C | C | C | C | C | **H** |
| 41 | **H** | **H** | C | C | C | C | C | C | C | C |
| 42 | C | | | | | | | | C | |
| 43 | C | C | C | C | C | C | C | C | C | C |
| 44 | C | C | C | C | C | C | C | C | C | H |
| 45 | **H** | **H** | C | C | C | C | C | C | C | C |
| 46 | C | C | C | C | C | C | C | C | C | C |
| 47 | **H** | **H** | C | C | C | C | C | C | C | C |
| 48 | C | C | C | C | C | C | C | C | C | **H** |
| 49 | C | C | C | C | C | C | C | **H** | **H** | **H** |
| 50 | **H** | **H** | **H** | **H** | C | C | C | **H** | **H** | **H** |
| 51 | | | C | C | C | C | C | C | C | C |
| 52 | | | C | C | C | C | C | C | C | C |
| 53* | | | | **H** | **H** | **H** | **H** | **H** | | |
| 54 | | | | **H** | **H** | **H** | **H** | **H** | | |
| 55 | | | | C | C | C | C | C | | |

| | | | | | |
|---|---|---|---|---|---|
| 56 | **H** | **H** | C | C | C |
| 57 | **H** | **H** | C | C | C |
| 58 | C | C | C | C | C |
| 59 | C | C | C | C | C |
| 60 | C | C | C | C | C |
| 61 | C | C | C | C | C |
| 62 | C | C | C | C | C |
| 63* | **H** | **H** | **H** | **H** | **H** |
| 64 | C | C | C | C | C |
| 65 | **H** | **H** | C | C | C |
| 67 | C | C | **H** | **H** | **H** |
| 68 | C | C | C | C | C |
| 69 | **H** | **H** | **H** | L | L |
| 70 | C | C | C | C | C |
| 71 | C | C | C | C | C |
| 72 | C | C | C | C | C |
| 73 | **H** | **H** | C | C | C |
| 74 | C | C | C | C | C |
| 75* | **H** | **H** | **H** | **H** | **H** |
| 76* | **H** | **H** | **H** | **H** | **H** |
| 77 | C | C | C | C | C |
| 78 | C | C | C | C | C |
| 79 | C | C | C | C | C |
| 80 | C | C | C | C | C |
| 81 | **H** | **H** | C | C | C |
| 82 | C | C | C | C | C |
| 83 | C | C | C | C | C |
| 84 | C | C | C | C | C |
| 85 | C | C | C | C | C |
| 86 | C | C | C | C | C |
| 87 | C | C | C | C | C |
| 88 | C | C | C | C | C |
| 89 | C | C | C | C | C |
| 90 | C | C | C | C | C |
| 91* | **H** | **H** | **H** | **H** | **H** |
| 92 | | C | C | **H** | L |
| 93* | **H** | **H** | **H** | **H** | **H** |
| 94 | C | C | C | C | C |

| | | | | | |
|---|---|---|---|---|---|
| 95 | C | C | C | C | C |
| 96 | C | C | C | C | C |
| 97* | **H** | **H** | **H** | **H** | **H** |
| 98* | **H** | **H** | **H** | **H** | **H** |
| 99 | C | C | C | C | C |
| 100 | C | C | C | C | C |
| 101 | C | C | C | C | C |
| 102 | C | C | **H** | **H** | **H** |
| 103 | C | C | C | C | C |
| 104 | C | C | C | C | C |
| 105 | C | C | C | C | |
| 106 | C | C | **H** | **H** | |
| 107 | C | C | C | C | |
| 108 | C | C | C | C | |
| 109 | C | C | C | C | |
| 110* | **H** | **H** | **H** | **H** | |
| 111 | C | C | C | C | |
| 112 | C | C | C | C | |
| 113* | **H** | **H** | **H** | **H** | |
| 114 | C | C | C | C | |
| 115 | C | C | C | C | |
| 116 | C | C | C | C | |
| 117 | **H** | **H** | C | C | |
| 118 | C | C | C | **H** | |
| 119 | C | C | C | C | |
| 120 | C | C | C | C | |
| 121 | C | C | C | C | |
| 122* | **H** | **H** | **H** | **H** | |
| 123 | C | C | C | C | |
| 124 | C | C | C | C | |
| 125 | C | C | C | C | |
| 126 | C | C | C | C | |
| 127 | C | C | C | C | |
| 128 | C | C | C | C | |
| 129* | **H** | **H** | **H** | **H** | |
| 130* | **H** | **H** | **H** | **H** | |
| 131 | C | C | C | C | |
| 132 | C | C | C | C | |

| | | | | |
|---|---|---|---|---|
| 133* | **H** | **H** | **H** | **H** |
| 134 | C | C | C | C |
| 135 | C | C | C | C |
| 136 | C | C | C | C |
| 137 | C | C | C | C |
| 138 | C | C | C | C |
| 139* | **H** | **H** | **H** | **H** |
| 140 | C | C | C | C |
| 141 | C | C | C | C |
| 142 | C | C | C | C |
| 143* | **H** | **H** | **H** | **H** |
| 144 | C | C | C | C |
| 145 | C | C | C | C |
| 146 | C | C | C | C |
| 147 | C | C | C | C |
| 148* | **H** | **H** | **H** | **H** |
| 149* | **H** | **H** | **H** | **H** |
| 150* | **H** | **H** | **H** | **H** |
| 151* | **H** | **H** | **H** | **H** |
| 152 | C | C | C | C |
| 153 | C | C | C | C |
| 154 | C | C | C | C |
| 155 | C | C | C | C |
| 156 | C | C | C | C |
| 157 | C | C | C | C |
| 158 | C | C | C | C |
| 159 | C | C | C | C |
| 160* | **H** | **H** | **H** | **H** |
| 161 | C | C | C | C |
| 162 | C | C | C | C |
| 163* | **H** | **H** | **H** | **H** |
| 164 | C | C | C | C |
| 165 | C | C | C | C |
| 166 | C | C | C | C |
| 167 | C | C | C | C |
| 168 | C | C | C | C |
| 169 | C | C | C | C |
| 170 | C | C | C | C |

| | | | | |
|---|---|---|---|---|
| 171 | C | C | C | C |
| 172 | C | C | C | C |
| 173 | C | C | C | C |
| 174 | C | C | C | C |
| 175 | C | C | C | C |
| 176* | **H** | **H** | **H** | **H** |
| 177 | C | C | C | C |
| 178* | **H** | **H** | **H** | **H** |
| 179 | **H** | **H** | C | C |
| 180 | C | C | C | C |
| 181* | **H** | **H** | **H** | |
| 182 | C | C | C | C |
| 183 | C | C | C | C |
| 184 | C | C | C | C |
| 185 | C | C | C | C |
| 186* | **H** | **H** | **H** | **H** |
| 187 | C | C | C | C |
| 188 | C | C | C | C |
| 189 | C | C | C | C |
| 190 | C | C | C | C |
| 191 | C | C | C | C |
| 192 | C | | **H** | **H** |
| 193 | C | C | C | C |
| 194 | C | C | C | C |
| 195 | C | C | C | C |
| 196 | C | C | C | C |
| 197 | C | C | C | C |
| 198 | C | C | C | C |
| 199 | C | C | C | C |
| 200 | C | C | C | C |
| 201 | C | C | C | C |
| 202 | C | C | C | C |
| 203 | C | C | C | C |
| 204 | C | C | C | C |
| 205* | **H** | **H** | **H** | **H** |
| 206 | C | C | C | C |
| 207 | C | C | C | C |
| 208 | C | C | | C |

| Samples | 50 | 48 | 51 | 204 | 206 | 205 | 206 | 102 | 52 | 51 |
|---|---|---|---|---|---|---|---|---|---|---|
| H. Col- | 34 | 33 | 44 | 161 | 164 | 166 | 167 | 80 | 43 | 37 |
| H.*Ler* | 0 | 0 | 0 | 0 | 0 | 0 | 1 | 3 | 1 | 1 |
| Heteroz. | 16 | 14 | 7 | 43 | 42 | 39 | 38 | 19 | 8 | 13 |
| Rec. Freq. (%) | 16 | 14.58 | 6.86 | 10.53 | 10.19 | 9.51 | 9.71 | 12.25 | 9.62 | 14.71 |

The new recombination frequencies obtained were 2.92% for marker nga106 marker, 2.60% for MPI7, 1.74% for MRN17 and 2.01% for nga139 (Table 7.6.2). These new numbers suggest that *ibr2* mutation is located between markers MPI7 and MRN17. Considering that 1% of recombination is equivalent to 1 cM, which corresponds to approximately 250 kbp, then 2.6% recombination would be equal to aproximately a distance of around 650 kbp away from MPI7. This marker being located at position 5929 kbp, the mutation is expected to be around 6579 kbp. However, when taking recombination frequency for MRN17 into account, which is located at 7659 kbp, this suggest that the mutation should be at 1.74 cM or 436 kbp away from this marker, that is at position 7223 kbp. These estimates are based on average recombination frequencies for *Arabidopsis thaliana*, however, the ratio between genetic and physical distance is by no means constant and it varies with respect to position on the chromosome as well as with respect to different mapping populations (Lukowitz 2000).

**Table 7.6.2-** Recombination frequency after mistakenly selected plants are discarded.

| | **Marker location (Kbp)** | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **MBK20** | **MYH9** | **Nga151** | **Nga106** | **MPI7** | **MRN17** | **Nga139** | **F14123** | **T26D22** | **AthS0191** |
| **Plant** | **2,476** | **3,122** | **4,670** | **5,397** | **5,929** | **7,659** | **8,428** | **9,935** | **13,000** | **15,022** |
| 1 | C | C | C | C | C | C | C | C | H | H |
| 2 | C | C | C | C | C | C | C | C | C | H |
| 10 | C | C | C | C | C | C | C | C | H | H |
| 11 | H | H | H | H | H | H | C | C | C | C |
| 20 | C | C | C | C | C | C | C | H | H | H |

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| 26 | H | H | C | C | C | C | C | C | C | C |
| 29 | H | C | C | C | C | C | C | C | C | C |
| 31 | C | C | C | C | C | C | C | L | L | L |
| 34 | H | H | C | C | C | C | C | C | C | C |
| 35 | H | H | C | C | C | C | C | C | C | C |
| 37 | H | C | C | C | C | C | C | C | C | C |
| 39 | H | H | H | H | H | C | C | C | C | H |
| 40 | H | H | C | C | C | C | C | C | C | H |
| 41 | H | H | C | C | C | C | C | C | C | C |
| 44 | C | C | C | C | C | C | C | C | C | H |
| 45 | H | H | C | C | C | C | C | C | C | C |
| 47 | H | H | C | C | C | C | C | C | C | C |
| 48 | C | C | C | C | C | C | C | C | C | H |
| 49 | C | C | C | C | C | C | C | C | C | H |
| 50 | H | H | H | H | C | C | C | C | C | H |
| 56 | | | | H | H | C | C | C | | |
| 57 | | | | H | H | C | C | C | | |
| 65 | | | | H | H | C | C | C | | |
| 66 | | | | C | C | H | H | H | | |
| 73 | | | | H | H | C | C | C | | |
| 81 | | | | H | H | C | C | C | | |
| 85 | | | | C | C | C | C | H | | |
| 92 | | | | | C | C | H | L | | |
| 102 | | | | C | C | H | H | H | | |
| 106 | | | | C | C | H | H | | | |
| 117 | | | | H | H | C | C | | | |
| 118 | | | | C | C | C | C | | | |
| 134 | | | | C | C | H | H | | | |
| 179 | | | | H | H | C | C | | | |
| 192 | | | | C | C | H | H | | | |

| **Samples** | 46 | 43 | 47 | 171 | 173 | 172 | 174 | 88 | 48 | 47 |
|---|---|---|---|---|---|---|---|---|---|---|
| **H. Col-0** | 34 | 33 | 44 | 161 | 164 | 166 | 167 | 80 | 42 | 36 |
| **H.L*er*** | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 2 | 1 | 1 |
| **Heteroz.** | 12 | 10 | 3 | 10 | 9 | 6 | 7 | 6 | 5 | 10 |
| **Rec. Freq. (%)** | **13.04** | **11.63** | **3.19** | **2.92** | **2.6** | **1.74** | **2.01** | **5.68** | **7.29** | **12.77** |

Presuming that the mutant gene lies in between positions 5929kbp and 7659kbp on chromosome 5, we consulted the *Arabidopsis* database (www.tair.org) and two genes encoding alpha-tubulin proteins, TUA3 (AT5G19770) and TUA5 (AT5G19780), were selected as the most likely candidates. Despite the mapped region containing more that 160 genes, the two alpha-tubulins were selected because *ibr2* resembles the morphological phenotype of *lefty1* and *lefy2* plants (Thitamadee, 2002). Considering that *ibr2* was isolated from an activation tagged population, some sort of genomic recombination is likely to have occurred around the insertion site. Often introgression of foreign DNA sequences in the genome causes big insertions or deletions at the site. Thus, in an attempt to identify any possible large insertion/deletion we designed specific primers to amplify both genes in full. Amplified PCR products for wild type and mutant *ibr2* plants were visualized in gel electrophoresis (Fig. 7.6.3). But unfortunately, no visible difference between the amplified sequences could be detected. It is worth noting that amplified sequences were large, and therefore a relatively small deletion/insertion is unlikely to be detected.

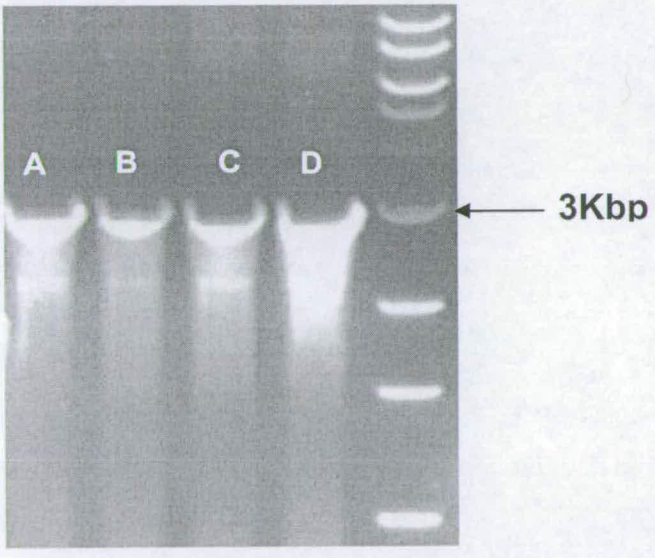


**Figure 7.6.3** PCR amplified *TUA3* and *TUA5* genes. (A-B) PCR product for *TUA3* in wild type Col-0 and *ibr2*, and (C-D) Amplified *TUA5* gene from Co-0 and *ibr2*, respectively. No visible difference can be detected between PCR products.

## 7.7 Discussion

In this chapter we described the isolation and partial characterization of *ibr2,* an *Arabidopsis* mutant that shows increased resistance against *B. cinerea* but unaffected defence against other pathogens tested. Previously, mutants specifically affected in resistance against this pathogen have been reported (Veronese *et al.*, 2004).

Segregation analysis of the $F_1$ and $F_2$ populations between *ibr2* and wild type plants revealed that *ibr2* gene is not tagged. Subsequently, *ibr2* was mapped to an interval of approximately 644 kbp on chromosome 5. Despite the relatively large numbers of molecular markers in the *Arabidopsis* TAIR database, in some parts of the genome the low saturation of markers presently available is still limiting mapping progress (Jander *et al.*, 2002). The absence of markers in that region on chromosome 5 stopped us from narrowing down the interval containing *ibr2*. Nevertheless, based on the morphological phenotype of *ibr2* we selected two candidate genes out of the approximately 160 genes present in the 644 kbp region. We selected as candidates two alpha-tubulin encoding genes *TUA3* (At5G19770) and *TUA5* (At5G19780), which are closely located to the predict location of *ibr2*, based on the recombination frequency of the last two markers tested. *TUA3* is located at position 6682 kbp and encodes a tubulin alpha-3/alpha-5 chain, while *TUA5* is located at 6687 kbp and encodes an isoform of an alpha tubulin. *TUA5* is closely related to *TUA3* suggesting a relatively recent duplication. These two candidate genes were selected because *ibr2* resembles the morphological phenotype of two alpha-tubulin mutants, *lefty1* and *lefty2*, previously described (Thitamadee *et al.*, 2002) (Fig. 7.7). *Lefty1* resulted from a single nucleotide exchange imposed in the α-tubulin *TUA6*, which encodes a tubulin alpha-6 chain and is located on chromosome 4. Likewise, *Lefty2* is due a single nucleotide substitution in the tubulin alpha-2/alpha-4 chain (*TUA4*) encoding gene, located on chromosome 1.

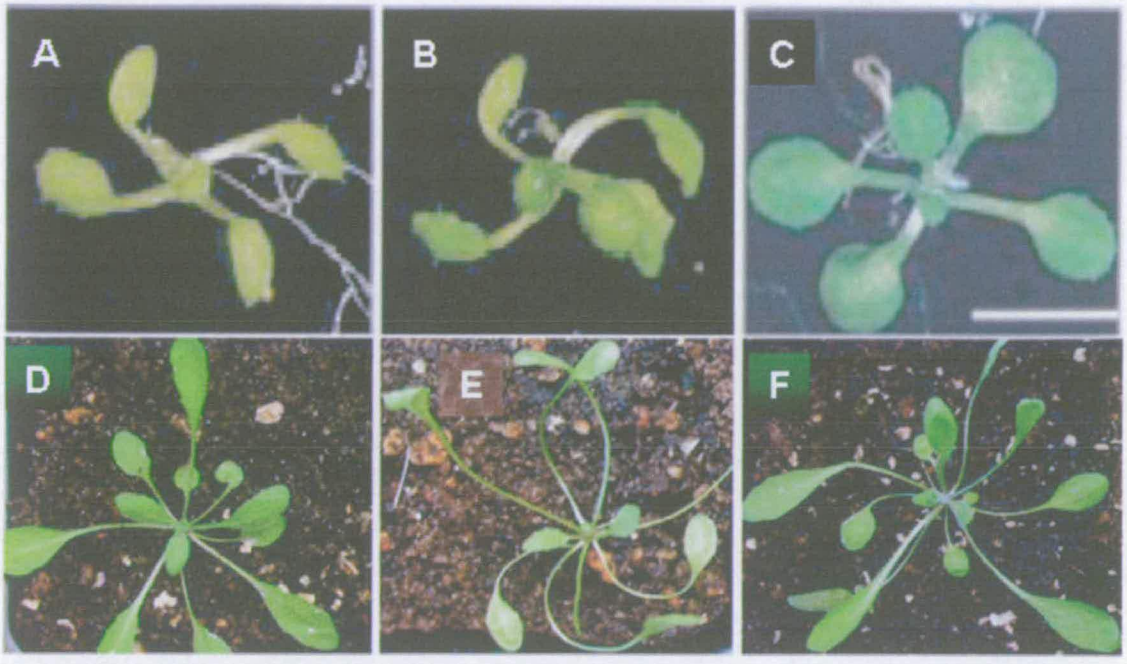


**Figure 7.7** *Ibr2* resembles the *lefty1* and l*efty2* morphological phenotype. Upper row, **(A-C)** seedlings of *lefty1, lefty2* and wild type L*er* grown on MS plates (paper). Lower, **(D-F)** soil grown wild type Col-0, homozygous and heterozygous *ibr2* plants, respectively.

Considering the *ibr2* phenotype, we speculate that it is rather likely a mutation in the coding sequence of *TUA3* or *TUA5* changing the protein configuration, rather than a mutation in the promoter region which could modulate gene expression. Moreover, in agreement with our speculation are the phenotypes shown by *lefty1* and *lefty2*, which are caused by mutations in the nucleotide sequences (Thitamadee *et al.*, 2002). Looking at the kinetics of JA/ET-dependent defence gene expression in *ibr2* plants in response to *B. cinerea* infection revealed that transcription of these genes is slightly accelerated in comparison to wild type plants. However, expression doesn't seem to be stronger than in wild type challenged plants. Because alpha-tubulins are key components of the cytoskeleton, we speculate that the increased resistance of *ibr2* plants against *B cinerea* may possible be due changes in the structure of either *TUA3* or *TUA5*.

Tubulins are key components of the plant cytoskeleton and play fundamental roles in cellular morphogenesis and response to biotic and abiotic stresses. The plant microtubules

are composed of heterodimers made up of one α–tubulin and one β–tubulin subunit of similar molecular weight. These dimers assemble into hollow cylinders, composed of 13 columns, forming the so-called pro-filament (Breviario and Nick, 2000). The half-life time of a microtubule is estimated in approximately 1 minute (Moore *et al.*, 1997), giving the impression of an everchanging river, whose direction and width are defined by the relation between tubulin dimers and heterodimer dissociation (Breviario and Nick, 2000). Plant microtubules have been shown to play a major role in resistance against non-host fungal pathogens (Kobayashi *et al.*, 1997b; Kobayashi *et al.*, 1997a; Yun *et al.*, 2003) and to be used by some viruses for cell-cell movement (Heinlein *et al.*, 1995). In addition, microtubules are shown to be involved in cold acclimation (Abdrakhamanova *et al.*, 2003). Changing the protein structure or conformation of an alpha-tubulin may possibly alter its interaction with a toxin specifically secreted by *B. cinerea*. Thus, altering the structure of an alpha-tubulin and perhaps also the cytoskeleton may result in reduced sensitivity to a phytotoxin produced by *B. cinerea*.

In order to elucidate the involvement of *ibr2* in plant disease resistance further characterization of the response of *ibr2* plants to necrotrophic, biotrophic as well as non-host pathogens is required. In addition, a series of double mutants are being created which will help us to establish a possible involvement of the JA/ET and SA-dependent signalling pathways in the resistance mediated by *ibr2*. The sequencing of the *ibr2* gene sequence and comparative analysis between wild type and mutant genes may provide conclusive evidence for the role of tubulins in plant defence.

The necrotrophic fungus *B. cinera* is one of the most devastating pathogens in greenhouses, especially on crops such as tomato, strawberry and petunias. Thus, isolating a gene conveying resistance against this pathogen could result in significant savings in pre- and post-harvest diseases. The transfer of genes from one species to another is becoming more straightforward, and several transgenic plant varieties are nowadays commercially available for farmers (James, 2004). The *ibr2* mutant shows enhanced resistance against *B. cinerea,* however, it has a significant negative impact on plant yield. An alternative to overcome the negative effect of *ibr2* on seed yield is to create transgenic plants expressing this gene under an inducible promoter system. Many agricultural biotech companies have developed

their own proprietry inducible gene expression systems for transgenic crops. This stategy has been shown to be effective in the generation of conditional alleles that convey disease resistance (Chini *et al.*, 2004).

## 8 Characterization of a mutant susceptible to *B. cinerea*

In addition to the mutants showing increased *Botrytis* resistance, two other lines displaying enhanced susceptibility towards this pathogen have also been isolated and partially characterized. One of these lines, termed *actiated disease susceptibility* (*ads1*) has been described previously (Chini, 2004) and therefore will not be presented here. The *ads1* line besides exhibiting increased susceptibility to *B. cinerea* also showed impaired resistance against the virulent and non-host bacterial pathogens *Pst* DC3000 and *P. fluorescens 2-79*, respectively. The *ADS1* gene has recently been identified as a MATE-efflux protein (Sun, Chini, Nurmberg and Loake, unpublished data). The second line, termed *enhanced botrytis susceptibility 1* (*ebs1*) was also partially characterized and is documented here.

### 8.1 *Ebs1* plants exhibit enhanced susceptibility towards *B. cinerea*

Infection of *ebs1* plants with the necrotrophic pathogen *B. cinerea* resulted in significantly increased symptom development in comparison to wild type Col-0 plants (Fig. 8.1, a-b). Differential disease development was even more evident at 8 dpi, when *ebs1* plants were completely colonised by the fungus, while mycelium spread was significantly less on Col-0 plants (Fig. 8.1, c-d). These experiments were repeat four times with consistent results. Staining of *B. cinerea* infected leaves with lactophenol trypan blue confirmed that lesion development was significantly increased in *ebs1* plants compared to wild type plants (Fig. 8.1, e-f). *ebs1* plants also exhibited reduced chlorophyll accumulation and slightly reduced stature when compared to wild type Col-0 plants. All $F_1$ plants resulting from a backcrossing between *ebs1* and wild type plants showed reduced chlorphyl accumulation, *B. cinerea* susceptibility and insensitive to the herbicide ammonium glufosinate (Basta). In the $F_2$ generation, from a total of 885 plants 678 displayed reduced chlorophyll accumulation and resistance to Basta herbicide, while 207 plants had a wild type phenotype and were Basta susceptible. These numbers showed no significant deviation from a 3:1 segregation hypothesis, based on the $X^2$ test ($X^2$=1.22). To confirm that susceptibility to *B. cinerea* co-segregated with Basta resistance, four trays containing 256 $F_2$ plants resistant to Basta and exhibiting reduced accumulation of chlorophyl, together with another tray

containing *coi1* and Col-0 plants were spray inoculated with *B. cinerea*. All plants exhibiting Basta resistance were significantly more susceptible, showing large necrotic lesions similar to those seen on *coi1* plants, suggesting that the reduced accumulation of chlorophyll, susceptibility to *B. cinerea* and Basta resistance co-segregate. Also, *ebs1* is therefore tightly linked to the T-DNA tag.

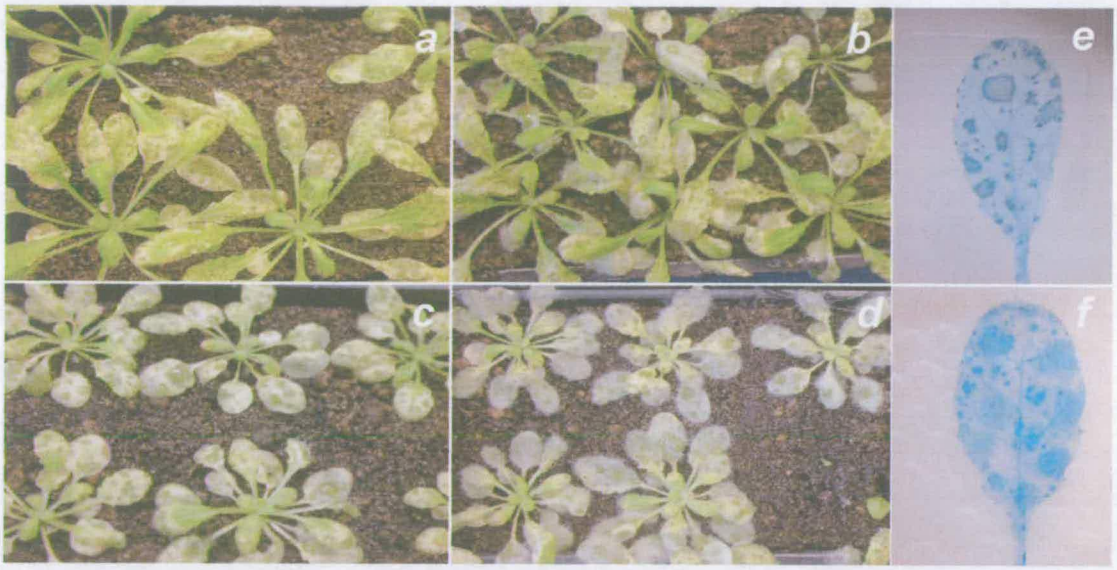


**Figure 8.1 *Ebs1* plants show enhanced susceptibility towards *B. cinerea*.** (**A**) and (**B**) Col-0 and (**C**) and (**D**) *ebs1* plants 5 and 8 days post-infection, respectively. Trypan blue stained leaves of Col-0 (**e**) and *ebs1* plants (**f**), collected 5 dpi with *B. cinerea*.

## 8.2 Strategies employed to isolate the *ebs1* mutant gene

Once susceptibility of *ebs1* was confirmed in independent experiments, we attempted to identify *EBS1*. A Southern blot analysis was performed in order to confirm the presence of a single T-DNA insertion. Genomic DNA of *ebs1* was isolated, purified and digested with 8 restriction enzymes, whose recognition site was not present in the probe DNA sequence. A 370 bp PCR product of the *35S* enhancer element was used as a probe. Results showed only one specific band for every restriction enzyme employed, thus confirming the presence of a single T-DNA insertion in *ebs1*. A single T-DNA insertion was also confirmed when the same membrane was probed with 700 bp PCR product corresponding specifically to the *BAR* gene. The *BAR* gene is located adjacent to the left border of the T-

DNA and confers resistance to Basta. We employed TAIL-PCR (Liu *et al.*, 1995; Liu and Whittier, 1995) to amplify DNA sequences flanking the inserted T-DNA, but our success was partial. Successful amplification was obtained when using arbitrary degenerate (*AD*) primer 1 together with specific primers for the left border, however, only T-DNA sequence was recovered. *Agrobacterium* insertion of a T-DNA plasmid into the plant genome is usually accompanied by the splicing of left and right borders (Bechtold *et al.*, 1993; Clough and Bent, 1998). However, sequencing of *ebs1* TAIL-PCR products revealed an extra fragment of the left border of the inserted T-DNA tag. We then designed new primers based on this region (outside left border) and used these together with the *AD1* primer to perform a new round of TAIL-PCR. Unfortunately, another left border sequence was amplified. An extra set of primers were designed, however, no appropriate sequence for designing primers is present in the last amplified sequence and consequently no success was obtained in the next round of PCR amplification. Another strategy, termed inverse PCR (Yuanxin *et al.*, 2003) was also employed but again negative results were obtained.

After failing with TAIL-PCR and inverse PCR, a plasmid rescue strategy was adopted (Perucho *et al.*, 1980). The plasmid rescue experiments were repeated several times using the restriction enzymes *EcoRI*, *HindIII*, *KpnI*, *SpeI* and *TaqI*, but only false-positive transformants were obtained. Due to time limitations we were unable to carry on with the experiments to clone *ebs1*.

### 8.3 *Ebs1* plants respond normally to exogenous application of Me-JA

Mutant plants depleted in the accumulation of JA (Howe *et al.*, 1996) or impaired in its perception (Feys *et al.*, 1994) are often more susceptible to necrotrophic pathogens (Thomma *et al.*, 1998), chewing insects (Howe *et al.*, 1996) and wounding (Koda and Kikuta, 1994). The *Arabidopsis coi1* mutant is insensitive to JA and therefore root growth is not affected in this mutant by exogenous application of JA or its methyl ester Me-JA. In addition, *coi1* plants are susceptible to necrotrophic pathogens (Thomma *et al.*, 1998) and are also male sterile. To investigate whether *ebs1* plants are compromised in the JA response, seeds from wild type Col-0, *coi1* and *ebs1* were sown on MS plates supplemented with a range of different concentrations of Me-JA. Roots of wild type and *coi1* seedlings

grown on MS medium without Me-JA were 20.8 mm long at 9 days after sowing, while the average length of *ebs1* was only 11.9 mm. When germinated on medium supplemented with increasing concentrations of Me-JA a gradual root inhibition was observed on both wild type and *ebs1* seedlings, while seedlings from the *coi1* mutant were insensitive at the Me-JA concentrations tested (Fig.8.3).

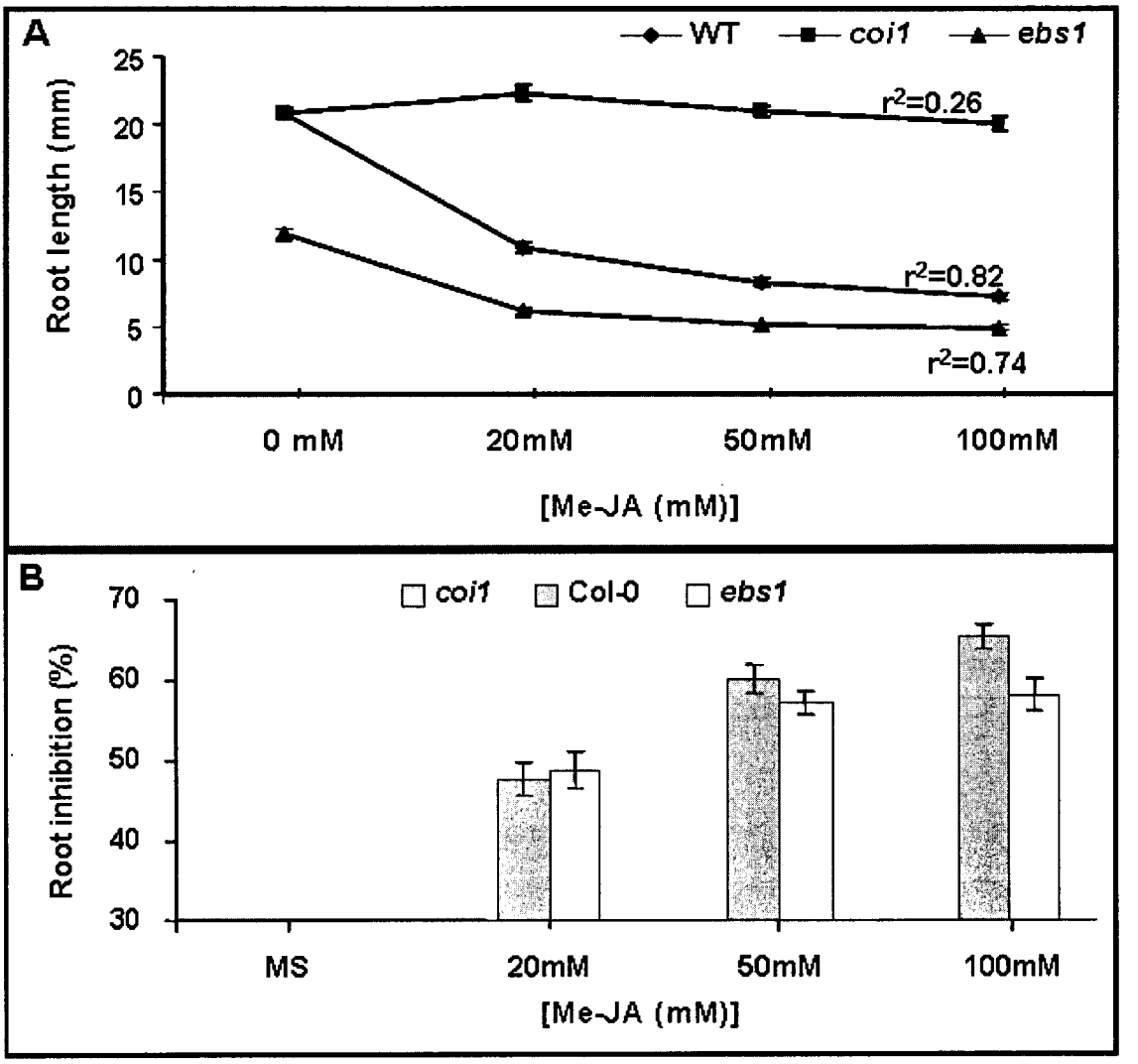


**Figure 8.3 *Ebs1* and wilt type seedlings show similar response to exogenous Me-JA.** **(A)** Effect of increasing concentration of Me-JA on root length of Col-0, *coi1* and *ebs1* seedlings. Root length was measured for of at least thirty seedlings from each line per treatment. **(B)** inhibition of root elongation in response to different concentrations of Me-JA.

Addition of Me-JA to a final concentration of 20 μM promoted a 47.6% and 48.7% reduction in the root length of Col-0 and *ebs1* seedlings, respectively. Root elongation was even further reduced when seedlings were exposed to a 50μM Me-JA, in this case, root inhibition was up to 60% and 58% in wild type and *ebs1*, respectively. The effect of Me-JA concentration of 100 μM and above was not different from that of 50 μM. The association between increased concentration of Me-JA and inhibition of root length was tested by the estimated coefficient of determination between these two variables (Fig. 8.3). The higher the $r^2$ the more associated the two variables are. As was expected, for Col-0 and *ebs1* over 73% and 81% of variability in root length is due to the increase in the concentration of Me-JA, while for *coi1* the estimated $r^2$ suggests little association exists between concentration of Me-JA and root length.

## 8.4 Resistance to virulent and avirulents strains of *Pst* is not affected in *ebs1*

To investigate whether mutation in the *ebs1* gene affected resistance against other pathogens, four-week old wild type and *ebs1* plants were inoculated with virulent and avirulent strains of the bacterial pathogens *Pst* DC3000. Leaf inoculation with virulent *Pst* DC3000 resulted in the development of weak chloroctic lesions at 4 dpi in both wild type and *ebs1* lines, with no significant difference between them. On the other hand, leaves of *nahG* plants were completely infected at this time point. To confirm *ebs1* is not affected in resistance towards *Pst*DC3000, bacterial growth was determined. Again, no significant difference was detected between wild type and *ebs1* plants (Fig. 8.4). In addition, *ebs1* and wild type plants were also inoculated with two strains of *Pst* DC3000 carrying the avirulent genes *avrB* and *avrRps4*, respectively. Both lines were resistant to *Pst* DC3000 expressing either of these two avirulence genes. As expected, Col-0 plants supported less growth of avirulent strains than of virulent *Pst* DC3000. Growth of *Pst* DC3000 expressing either *avrB* or *avrRps4* was similar in wild type and *ebs1* plants (Fig.8.4).

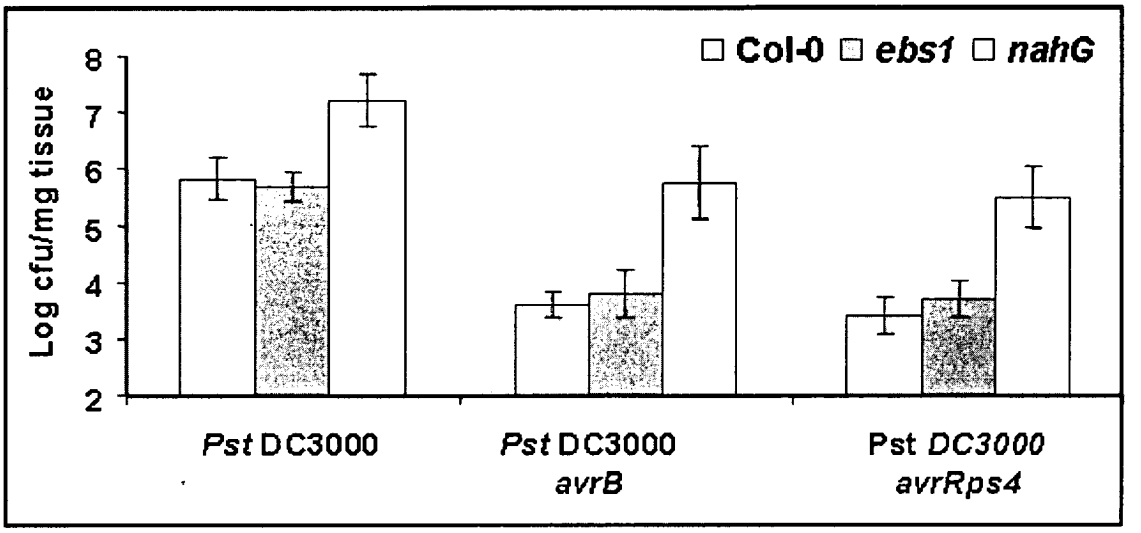


**Figure 8.4** *Ebs1* plants are not affected in resistance against virulent or avirulent strains of *Pst* DC3000. Four-week old plants were inoculated ($1x10^6$ cfu/mL) and leaf samples collected at 4 dpi for a bacterial growth assay.

## 8.5 Discussion

In this chapter we presented the partial characterization of *ebs1*, a dominant T-DNA generated *Arabidopsis* mutant showing enhanced susceptibility to the necrotrophic pathogen *B. cinerea*. Due to time limitations we only partially characterized this line. Following inoculation with *B. cinerea* the *ebs1* line exhibited disease symptoms similar to those of *coi1* plants. On the other hand, resistance against virulent and avirulent strains of the hemi-biotrophic bacterial pathogen *Pst* DC3000 was unaffected in *ebs1* plants. Thus, *EBS1* seems to be a component of the defence response specific to necrotrophic pathogens, or perhaps more specifically against *B. cinerea*. Recently, other *Arabidopsis* mutants compromised in resistance against *B. cinerea* have been identified (Veronese *et al.*, 2004). We investigated the transcriptional activation of the *PDF1.2* gene in response to infection with *B. cinerea* but no difference could be detected between *ebs1* and wild type plants. *PDF1.2* is a marker for plant defence responses against necrotrophic pathogens and its expression was shown to require both JA and ET signalling pathways (Thomma *et al.*, 1999a). Moreover, both wild type and *ebs1* seedlings responded similarly to the exogenous application of Me-JA, suggesting *ebs1* did not compromise either the synthesis or perception of this signalling molecule. Susceptibility of *Arabidopsis* mutants against *B. cinerea* has been demonstrated despite normal expression of *PDF1.2*, suggesting expression of this gene may not be sufficient to convey resistance against *B. cinerea* (Ferrari *et al.*, 2003; Mengiste *et al.*, 2003).

Since we were only able to partially characterize the response of *ebs1* to pathogen challenge and because the cloning of the *EBS1* gene was not accomplished, it is difficult to hypothesize how *ebs1* might affect resistance against *B. cinerea* and perhaps responses against other biotic and/or abiotic stresses. Thus, investigating the synthesis and response of *ebs1* to other signalling molecules, such as ET and SA, as well as the response of *ebs1* and double mutants to challenge with other pathogens could provide fundamental information about the roles of *ebs1* in disease resistance. Furthermore, the cloning of *EBS1* and analysis of the kinetics of its transcriptional activation in response to pathogen challenge is fundamental.

# 9 General Discussion

Despite significant damage and losses caused by the necrotrophic pathogens in both pre- and post-harvest, our knowledge about the genetic mechanisms controlling resistance against these pathogens is still poorly understood. During this study four independent *Arabidopsis* mutants altered in resistance against *B. cinerea* were isolated. In this concluding chapter the results from one of these lines, *as1,* will be discussed since the other mutants require further characterization. Firstly, I will attempt to integrate our understanding of the involvement of *AS1* in leaf development and disease resistance. Secondly, I will ascribe a putative mechanism of defence controlled by the *AS1* gene. Finally, I suggest some strategies that could be used for the identification of other components of the *AS1*-mediated disease resistance.

## *i* *AS1* functions independently of *ER, AS2* and *KNOX* genes to regulate disease resistance & susceptibility

*As1* has been extensively studied because of its central function in leaf development. Here we show that *AS1* also plays a significant role in the plant defence response against several pathogen species. Moreover, our data show that *as1* establishes disease resistance independently from the *AS1*-dependent pathway that regulates leaf development. Furthermore, by analysing *AS1* orthologs in *Antirrhinum* and *Nicotiana silvestris,* we demonstrate that *AS1* function in disease resistance is conserved in at least three evolutionarily diverse plant species.

*AS1* belongs to the *R2R3* subfamily of MYB-domain genes containing putative transcription factors. Members of this gene family are involved in many aspects of plant secondary metabolism, as well as in the identity and fate of plants cells and the defence response against biotic and abiotic stresses (Stracke *et al.*, 2001; Mengiste *et al.*, 2003). For normal plant growth, a tight regulation of meristem cell division and differentiation is required. The mechanisms involved are still relatively obscure but it is suggested that a highly conserved class of homeobox genes related to *KNOTTED1* in maize are involved

(Byrne *et al.*, 2000). *AS1* negatively regulates the homeobox genes *KNAT1* and *KNAT2* and is in turn negatively regulated by the homeobox gene *SHOOT MERISTEMLESS* (Byrne *et al.*, 2000). *AS1* function in plant development is conserved between plant species with a divergence time of over 200 million years (Waites *et al.*, 1998; Byrne *et al.*, 2000; Theodoris *et al.*, 2003; McHale and Koning, 2004). Despite the extensive characterization of *AS1*, there is still no evidence that *AS1* functions by direct DNA binding, a conspicuous feature of the *MYB*-domain. It has been shown recently that *AS1* interacts physically with *AS2* in the regulation of the adaxial-abaxial polarity of leaves (Xu *et al.*, 2003). Loss-of-function in the *AS1* and *AS2* genes result in plants with a similar phenotype, while independently overexpression of these two genes results in dramatically different plant morphology. Plants constitutively overexpressing *AS2* develop narrow leaves with lamina curled upwards. In contrast, *35S::AS1* plants display reduced stature with normally shaped leaves (Xu *et al.*, 2003). Furthermore, it was demonstrated that in addition to their regulatory function in leaf polarity, *AS1* and *AS2* have separate functions in flower development, suggesting that *AS1* plays multi-functions in plant development (Xu *et al.*, 2003).

The increased *B. cinerea* resistance specifically shown by *as1-1* and *as1*-4, but not by the *as2* mutant or the inducible *KNAT1* line suggests that *AS1* plays distinct roles in development and disease resistance/susceptibility. The independent function of *AS1* in disease resistance & susceptibility is a significant surprise. *AS1* may interact with different cofactors in the regulation of distinct and independent signalling pathways. The *ERECTA* gene encodes a leucine-rich repeat receptor-like kinase (LRR-RLK) (Torii *et al.*, 1996) and is believed to function in the same developmental pathway with *AS1* and *AS2*. *ERECTA* was recently shown to act as a QTL in resistance against the plant pathogens *Ralstonia solanacearum* and *Plectosphaerella cucumerina* (Godiard *et al.*, 2003; Llorente *et al.*, 2005). Interestingly, resistance against *B. cinerea* or *Pst*DC3000 was unaffected by *erecta (Llorente et al., 2005)*, suggesting that *ERECTA* is only required for resistance against a sub-set of pathogen species. In concordance, our results showed that *as1,* but not *erecta* plants, are affected in resistance against *Pst*DC3000 and *B. cinerea*, suggesting that in addition to their function in leaf development, *AS1* and *erecta* also have separate roles in plant disease resistance.

Mutation in the *AS1* gene affected resistance against at least four different pathogen species. While resistant to *B. cinera* and *A. brassicicola, as1* plants show enhanced susceptibility against virulent and avirulent strains of *Pst*DC3000, in addition to susceptibility to *P. fluorescens 2-79*, a non-host pathogen of *Arabidopsis*. Conversely, resistance against the pathogens *B. graminis* f. sp. *tritici* and *E. cichoracearum* was not compromised in these mutant plants, suggesting that *AS1* is involved is resistance against some, but not all pathogens. While the resistance of *as1* against necrotrophic pathogens may be explained by the accelerated expression of JA/ET-dependent defence genes, susceptibility to the different strains of bacterial pathogens occurred in the light of normal *PR-1* expression and SA accumulation. Susceptibility against a wide range of biotrophic and hemi-biotrophic pathogens, including *Pst* DC3000, are often associated with defects in SA accumulation/perception and with blocked *PR* gene expression (Malamy and Klessig, 1992; Cao *et al.*, 1994; Clarke *et al.*, 1998). However, there have been cases in which normal defence gene expression was not enough to convey disease resistance (Ferrari *et al.*, 2003; Mengiste *et al.*, 2003; Veronese, 2004) and examples of resistant mutants, whose resistance is activated independently of either SA- or JA-dependent signalling pathways (Kim and Delaney, 2002; Vogel *et al.*, 2002). Our experimental data demonstrate that enhanced susceptibility of *as1-1* and *as1-4* plants towards bacterial pathogens does not result from defects in SA-dependent signalling.

In addition to *as1*, a number of *Arabidopsis* mutants compromised in resistance against bacterial pathogens have been identified. However, similarly to *as1,* only transgenic *nahG* plants are shown to be compromised in resistance against such a broad-spectrum of bacterial pathogens. Transgenic *nahG* plants, constitutively expressing a salicylate hydroxylase gene, besides depleted SA accumulation also show enhanced levels of catechol, a bioproduct from the SA degradation, which acts to suppress defence responses. *nahG* plants are compromised in basal defence, *R* gene-mediated resistance, and non-host resistance (Delaney *et al.*, 1994; Friedrich *et al.*, 1995; Lawton *et al.*, 1995). In contrast to *as1*, *nahG* plants are not affected in resistance against necrotrophic pathogens. In addition to transgenic *nahG* plants, other mutants such as *eds1, npr1, ndr1* are also compromised in resistance against virulent strains of *Pst*DC3000 (Cao *et al.*, 1994; Parker *et al.*, 1996), but in contrast to *as1,* the *eds1* plants retain the ability to recognize *Pst*DC3000 expressing the

avirulence gene *avrB* (Parker *et al.*, 1996) while *ndr1* is still able to recognize *Pst*DC3000 carrying *avrRps4* (Aarts *et al.*, 1998).

In contrast to the relatively large number of mutants affected in resistance against bacterial pathogens, only a few cases of increased resistance against *B. cinerea* have been reported so far. These are, an *Arabidopsis* plant overexpressing an ET response factor (*ERF1*) (Berrocal-Lobo *et al.*, 2002) , a JA-insensitive line (*jin1*) (Lorenzo *et al.*, 2004) and an overexpresser of a cationic peroxidase gene (Coego *et al.*, 2005). Interestingly, *35S::ERF1* expression was shown to rescue the defence response defects of *coi1* and *ein2,* mutants compromised in JA and ET perception, respectively, suggesting *ERF1* acts downstream of these two components integrating the JA and ET signalling pathways (Lorenzo *et al.*, 2003). Surprisingly, loss-of-function in the *JIN1* gene, which encodes a MYC-transcription factor, result in enhanced expression of JA-induced genes and reduced susceptibility towards necrotrophic pathogens. Interestingly, *jin1* seems to prevent the activation of JA-dependent genes activated in response to insects, herbivores and mechanical damage and conversely enhances the expression of pathogen-induced JA-dependent gene expression (Lorenzo *et al.*, 2004). In addition, overexpression of a cationic peroxidase led to enhanced accumulation of $H_2O_2$ and constitutive expression of the *GST1* and *PDF1.2* genes and consequently enhanced resistance against *B. cinerea* and *P. cucumerina* (Coego *et al.*, 2005). Interestingly, one of the potential mechanisms of virulence by *B. cinerea* is to induce *in planta* accumulation of reactive oxygen species.

### *ii* Putative roles of *AS1* in disease resistance

As shown by the normal pathogen-induced accumulation of SA and expression of the SA-dependent gene *PR-1*, susceptibility of *as1* plants do not seem to be due to a defect in these plants to activate the SA-dependent defence signaling pathway. Furthermore, *AS1* is unlikely to encode a direct component of defence because otherwise either accumulation or perception of SA, and consequently *PR-1* expression should be affected. Moreover, susceptibility towards bacterial pathogens do not seem to be directly associated with resistance against necrotrophic pathogens. Rather, *AS1* may function by simultaneously

affecting defence signalling pathways and susceptibility to pathogens. For example, as a negative regulator of JA-mediated defence gene expression and also negatively regulating the synthesis of a susceptibility factor. Susceptibility factors are synthesized by the plant and are integral to its biology (Stachel *et al.*, 1986; Stachel and Zambryski, 1986b; Vogel *et al.*, 2002), and pathogens are expected to have evolved mechanisms to exploit these factors during pathogenesis.

Despite evidence to suggest nuclear localization (Theodoris *et al.*, 2003), *AS1* may be involved in the genetic mechanisms controlling the synthesis of compounds metabolized in different parts of the cell. For example, *AS1* may negatively regulate the expression of a protein that is required for either the biosynthesis or transport of an apoplastic metabolite that is required by *Pst*DC3000 for effective host colonization. Support for this hypothesis is provided by the recent identification of *ads1* (Sun, Chini, Gilroy, Nurmberg and Loake, unpublished data). An activation tagged allele of *ADS1* which resulted in constitutive over-expression of this gene establishes increased susceptibility to *Pst*DC3000. *ADS1* encodes a MATE efflux protein which may modify the composition of the apoplast, promoting colonization by *Pst*DC3000. Alternatively, *as1* may negatively regulate an unknown defence response (s) which contribute to the establishment of increased resistance against *B. cinerea*.

As demonstrated previously, during infection of *Arabidopsis*, the bacterial pathogen *Pst* delivers the effector protein *AvrRpt2* into the plant cell, through the type III secretion system, in the inactive form. After delivery, *AvrRpt2* undergoes N-terminal processing and causes elimination of *Arabidopsis RIN4* (Mudgett and Staskawicz, 1999; Jin *et al.*, 2003), indirectly activating *RPS2*. *RPS2* and *RIN4* physically interact in *Arabidopsis* and delivery of *AvrRpt2* results in elimination of *RIN4* and indirect activation of *RPS2*. However, *RIN4* is not the only substrate for *AvrRpt2*, in fact, it was recently shown that *AvrRpt2* substrate recognition only requires a 7-aa consensus sequence (VPxFGxW), which is present in at least 19 different *Arabidopsis* proteins (Chisholm *et al.*, 2005). In *N. benthamiana* transient co-expression experiments showed that several of these genes are cleaved by *AvrRpt2*. In addition, it was suggested that *AvrRpt2* may also cleave variations of the (x)PxFGxW aa sequence. This relatively degenerated consensus, suggests that *AvrRpt2* may eliminate a

large number of host factors during the infection process. Searching the AS1 protein sequence revealed the absence of *AvrRpt2* cleavage site, suggesting AS1 is not a direct target of the bacterial effector target. However, *AS1* may encode a regulator of one of these factors that are targeted by the effectors protein.

Compromised resistance of *as1/ein2* and *as1/aos* against *B. cinerea* implied a requirement for a JA/ET signalling pathways in the *as1*-mediated resistance against necrotrophic pathogens. This is in agreement with previously published data about the involvement of JA and ET in defence against necrotrophic pathogens. Mutations in *EIN2* were shown to compromise resistance against *B. cinerea*. In addition, *ein2* was shown to block *PDF1.2* expression in response to *A. brassicicola,* however, resistance against this pathogen was not compromised (Thomma *et al.*, 1999a). Activation of *PDF1.2* was not monitored in the double mutant neither was resistance against *A. brassicicola*, however it is likely that this double mutant is compromised in the expression of *PDF1.2*. Blocking JA synthesis in the *aos/as1* double mutants had a greater negative effect on resistance than did blocking the perception of ET, suggesting the JA signalling pathway plays a more important role in *as1*-mediated resistance than the ET pathway. It is believed that these two signalling pathways function in association during the activation of the defence response (Penninckx *et al.*, 1996; Penninckx *et al.*, 1998). In contrast, other experiments have presented evidence that JA and ET individually are sufficient to induce resistance against *A. brassicicola* and *B. cinerea*, respectively (Thomma *et al.*, 1998; Berrocal-Lobo *et al.*, 2002; Ellis *et al.*, 2002).

While synthesis of JA was shown to be necessary for establishing *as1*-dependent resistance against *B. cinerea* it is still uncertain whether resistance is activated in a *COI1*-dependent manner because *coi1as1* double mutants were absent in the $F_2$ population. The absence of this double mutant may be because *coi1* suppresses the *as1* phenotype. It is worth noting that blocking JA synthesis in *as1* plants by the *aos* antisense did not suppress the lobed leaves phenotype, suggesting that suppression of *as1* is mediated through the SCF complex rather than synthesis of JA. *COI1* encodes a F-box protein (Xie *et al.*, 1998) with homology to the auxin receptor *TIR1* (Dharmasiri *et al.*, 2005). F-box proteins are present in the eukaryote kingdom and function as receptors that recruit regulatory proteins as substrates for ubiquitin-mediated destruction in the proteasome. F-box proteins associate with SKP1,

cullin and Rbx proteins to form an E3 ubiquitin known as the SCF complex (Bai *et al.*, 1996; Devoto *et al.*, 2002; Xu *et al.*, 2002). TIR1 and COI1 are shown to interact *in vivo* with the plant SKP1-like, ASK1 and ASK2 (Gray *et al.*, 1999; Xu *et al.*, 2002). COI1 forms two complexes separately with ASK1 and ASK2, and these two complexes may have separate functions but overlapping functions in the JA signaling pathway (Xu *et al.*, 2002).

The *Arabidopsis ASK1* is highly expressed in dividing cells, such as those in meristems and organ primordia (Porat *et al.*, 1998). Accumulating data suggest SKP1 can interact with different F-box proteins, regulating different plant processes (Devoto and Turner, 2003). Analysis of several alleles of the JA resistant mutant *jar1*, and also auxin resistant plant lines revealed that the JA- and auxin act through a common signalling intermediate, which may also affect response to other hormones (Devoto and Turner, 2003). This observation might also be supported by the fact that $SCF^{COI1}$ shares components with $SCF^{TIR1}$ (Devoto *et al.*, 2002). Interestingly, loss-of-function in maize *ROUGH SEATH2* (ortholog of *Arabidopsis AS1*) compromised polar auxin transport (Tsiantis *et al.*, 1999). This resulted in increased local auxin concentrations, which in turn may affect the JA-dependent signalling pathway. By analogy, loss-of-function in *AS1* in *Arabidopsis* may result in higher levels of local auxin and consequently perturbed regulation of the JA/ET-dependent signalling pathway, promoting resistance against necrotrophic pathogens. Furthermore, auxin has also been shown to positively regulate ET mediated responses in root growth of *Arabidopsis* plants. Feeding experiments using low doses of auxin indicated that certain levels of auxin are required to sensitize the cells to ET (Rahman *et al.*, 2001). A simplified diagram representing our suggested mechanism of resistance regulated by *AS1* is presented below.

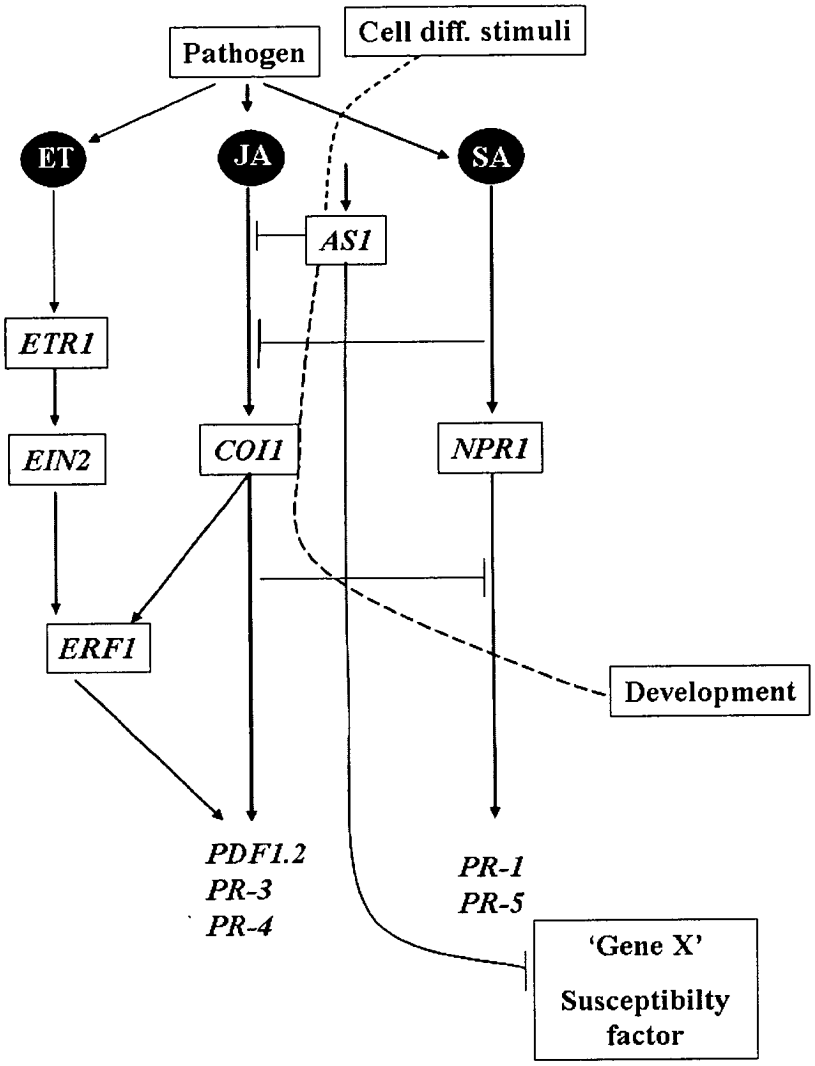


**Figure 9 Proposed model of *as1*-mediated disease resistance.** This model integrates *AS1* into distinct signalling pathways regulating plant development and disease resistance. We suggest that *AS1* simultaneously negatively regulates the JA/ET expression of defence genes and repress the expression of a susceptibility factor (gene X). While functional *COI1* seems to be required together with *AS1* for leaf development, resistance and development seem to be regulated separately.

### *iii* Strategies for the identification of new components of *as1*-mediated disease resistance & susceptibility

In order to elucidate the function of *AS1* in disease resistance some key experiments are required. The defence response of transgenic lines transiently overexpressing the *AS1* gene under the control of an inducible promoter (e.g. Glucocorticord - DEX inducible system) (Aoyama and Chua, 1997), as well as a comparative analysis of the transcript profiles of transgenic pathogen-infected and healthy lines, using an *Arabidopsis* microarray chip such as the Affymetrix, could provide fundamental insights into *as1*-mediated disease resistance. The deployment of inducible lines (*35S::AS1::Gr*) has potential benefits over the use of lines constitutively overexpressing the *AS1* gene. (1) The role of *AS1* in disease resistance can be examined in the absence of the interference caused by the developmental phenotype of *as1* knockout or constitutive overexpresser. This developmental interference can mask the effect of *AS1* in disease resistance. (2) Using Affymetrix GeneChips for comparison between the inducible system and the knockout line, we can look for genes that are up- and down-regulated at early stages following pathogen infection, respectively. These genes are potentially primary targets of *AS1* and possibly directly involved in the *as1*-mediated disease resistance. (3) Time course pathogen assays using the inducible lines can reveal genes that are sequentially transcribed in response to pathogen attack.

The promoter region of genes with consistent differences in their pattern of expression (up- and down-regulation) will be analysed in an attempt to identify conserved motifs (cis-elements). The proteins potentially targeted for binding can then be identified by an electophoretic mobility sift assay (EMSA) and specificity of these elements can be further investigated by coordinated deletions in the consensus sequence. This methodology recently rendered the elucidation of new functions of the *NPR1* gene (Wang *et al.*, 2005). These authors analyzed the transcript profiles of *npr1* and *35S::NPR1::Gr* lines, sequentially treated with SA and DEX, and identified a group of genes that have different pattern of expression in the two lines. Analysis of these genes resulted in the identification of the TIL cis-element, which is involved in the SA-induction of secretion-related genes via *NPR1*. Thus, besides its function in *PR-1* expression and SAR activation, *NPR1* also prepares the cells for *PR-1* secretion by making more components of the secretory

machinery. The transcription factor that controls TLI is still unknown, but it is unlikely a TGA because TIL is distinct from TGA-binding *as-1* element. Members of the TGA/OBF family of basic leucine zipper (bZIP) transcription factors have been implicated in the activation of SA-responsive genes, and two of these TGA factors were shown to interact with *NPR1* in vitro (Zhou *et al.*, 2000). Therefore, Wang and co-workers speculate that *NPR1* regulates secretion-related and *PR* genes through different transcription factors and cis-elements.

In addition, we could also attempt to identify specific targets for *AS1*-binding. Previous experiments using the yeast two-hybrid system failed to reveal any *AS1* direct binding factor (Andrew Hudson, *personal communication*). However, a new methodology for determining the DNA-binding specificity of transcription factors, termed bacterial one-hybrid system (B1H), was introduced recently (Meng *et al.*, 2005). This system is similar to yeast one-hybrid system, but it is advantageous over the yeast systems because transformation efficiency is much higher in bacteria, allowing screening of libraries with much higher complexity. Moreover, only basic molecular techniques are required, what makes it readily available. The B1H system contains the transcription factor expression vector, a library of randomized binding sites in the receptor vector and the bacterial selection strain. The DNA-binding domain is expressed as fusion to the α-subunit of RNA polymerase. The reporter vector contains restriction sites for introducing a library of randomized oligonucleotides upstream of the promoter of two reporters, the yeast *HIS3* and *URA3* genes. If a DNA-binding domain (bait) recognizes a target site (prey) in the reporter vector, it will recruit RNA polymerase to the promoter and activate transcription of both reporter genes. Selection of active promoters can be performed by addition of 3-amino-triazole (3-AT), a competitor of *HIS3,* into the growth medium, while addition of 5-fluoro-orotic acid (5-FOA), converted into a toxic compound by the uracyl biosynthesis, will select against active promoters. Selection for increased levels of HIS3 will isolate vectors harbouring a binding site for the bait. Vectors containing DNA sequences that activate the promoter independent of the bait (self-activation) can be eliminated by selection against URA3 expression. Thus, recognition of sequences for the bait can be isolated from the library of prey by a combination of positive selection in the presence of the bait and the negative selection in the absence of the bait (Meng *et al.*, 2005). Therefore, this new system

constitutes of a robust technique to be used to determine the specificity of transcription factors and could be used to further search for proteins targeted by *ASI* in response to pathogen infection.

## 10 Bibliography

**Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J., and Parker, J.E.** (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95,** 10306-10311.

**Abdrakhamanova, A., Wang, Q.Y., Khokhlova, L., and Nick, P.** (2003). Is microtubule disassembly a trigger for cold acclimation? Plant Cell Physiol. **44,** 676-686.

**Aboul-Soud, M.A.M., Cook, K., and Loake, G.J.** (2004). Measurement of salicylic acid by a high-performance liquid chromatography procedure based on ion-exchange. Chromatographia **59,** 129-133.

**Agrios, G.N.** (1997). Plant Pathology (4th Ed). Academic Press. San Diego, Calafornia.

**Anderson, J.P., Badruzsaufari, E., Schenk, P.M., Manners, J.M., Desmond, O.J., Ehlert, C., Maclean, D.J., Ebert, P.R., and Kazan, K.** (2004). Antagonistic interaction between abscisic acid and jasmonate-ethylene signaling pathways modulates defense gene expression and disease resistance in *Arabidopsis*. Plant Cell **16,** 3460-3479.

**Aoyama, T., and Chua, N.H.** (1997). A glucocorticoid-mediated transcriptional induction system in transgenic plants. Plant J. **11,** 605-612.

**Asai, T., Tena, G., Plotnikova, J., Willmann, M.R., Chiu, W.L., Gomez-Gomez, L., Boller, T., Ausubel, F.M., and Sheen, J.** (2002). MAP kinase signalling cascade in *Arabidopsis* innate immunity. Nature **415,** 977-983.

**Axtell, M.J., and Staskawicz, B.J.** (2003). Initiation of RPS2-specified disease resistance in *Arabidopsis* is coupled to the AvrRpt2-directed elimination of RIN4. Cell **112,** 369-377.

**Baertschi, S.W., Ingram, C.D., Harris, T.M., and Brash, A.R.** (1988). Absolute configuration of cis-12-oxophytodienoic acid of flaxseed: implications for the mechanism of biosynthesis from the 13(S)-hydroperoxide of linolenic acid. Biochemistry **27,** 18-24.

**Bai, C., Sen, P., Hofmann, K., Ma, L., Goebl, M., Harper, J.W., and Elledge, S.J.** (1996). SKP1 connects cell cycle regulators to the ubiquitin proteolysis machinery through a novel motif, the F-box. Cell **86,** 263-274.

**Baraldi, E.B., E. Chierici, E. Trufelli, B. Luiselli, D.** (2002). Genetic diversity between *Botrytis cinerea* isolates from unstored and cold stored kiwi fruit. J. Phytopath. **150,** 629-635.

**Barnun, N., and Mayer, A.M.** (1989). Cucurbitacins - repressors of induction of laccase formation. Phytochemistry **28,** 1369-1371.

**Barnun, N., Lev, A.T., Harel, E., and Mayer, A.M.** (1988). Repression of laccase formation in *Botrytis cinerea* and its possible relation to phytopathogenicity. Phytochemistry **27,** 2505-2509.

**Bechtold, N., Ellis, J., and Pelletier, G.** (1993). In-planta *Agrobacterium*-mediated gene-transfer by infiltration of adult *Arabidopsis thaliana* plants. Comptes Rendus De L Academie Des Sciences Serie Iii-Sciences De La Vie-Life Sciences **316,** 1194-1199.

**Becker, W., and Apel, K.** (1992). Isolation and characterization of a cDNA clone encoding a novel jasmonate-induced protein of barley (*Hordeum vulgare* L.). Plant Mol. Biol. **19,** 1065-1067.

**Bell, C.J., and Ecker, J.R.** (1994). Assignment of 30 microsatellite loci to the linkage map of *Arabidopsis*. Genomics **19,** 137-144.

**Bell, E., Creelman, R.A., and Mullet, J.E.** (1995). A chloroplast lipoxygenase is required for wound-induced jasmonic acid accumulation in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **92,** 8675-8679.

**Benito, E.P., ten Have, A., van't Klooster, J.W., and van Kan, J.A.L.** (1998). Fungal and plant gene expression during synchronized infection of tomato leaves by *Botrytis cinerea*. Eur. J. Plant Path. **104,** 207-220.

**Bergey, D.R., Howe, G.A., and Ryan, C.A.** (1996). Polypeptide signaling for plant defensive genes exhibits analogies to defense signaling in animals. Proc. Natl. Acad. Sci. USA **93,** 12053-12058.

**Berrocal-Lobo, M., and Molina, A.** (2004). Ethylene response factor 1 mediates *Arabidopsis* resistance to the soilborne fungus *Fusarium oxysporum*. Mol. Plant Microbe-Interact. **17,** 763-770.

**Berrocal-Lobo, M., Molina, A., and Solano, R.** (2002). Constitutive expression of *ETHYLENE-RESPONSE-FACTOR1* in *Arabidopsis* confers resistance to several necrotrophic fungi. Plant J. **29,** 23-32.

**Bisgrove, S.R., Simonich, M.T., Smith, N.M., Sattler, A., and Innes, R.W.** (1994). A disease resistance gene in *Arabidopsis* with specificity for two different pathogen avirulence genes. Plant Cell **6,** 927-933.

**Bleecker, A.B., Estelle, M.A., Somerville, C., and Kende, H.** (1988). Insensitivity to ethylene conferred by a dominant mutation in *Arabidopsis thaliana*. Science **241,** 1086-1089.

**Boch, J., Verbsky, M.L., Robertson, T.L., Larkin, J.C., and Kunkel, B.N.** (1998). Analysis of resistance gene-mediated defense responses in *Arabidopsis thaliana* plants carrying a mutation in CPR5. Mol. Plant-Microbe Interact. **11,** 1196-1206.

**Bohlmann, H., Vignutelli, A., Hilpert, B., Miersch, O., Wasternack, C., and Apel, K.** (1998). Wounding and chemicals induce expression of the *Arabidopsis thaliana* gene *Thi2.1*, encoding a fungal defense thionin, via the octadecanoid pathway. FEBS Lett. **437,** 281-286.

**Bohnert, H.J., Nelson, D.E., and Jensen, R.G.** (1995). Adaptations to Environmental Stresses. Plant Cell **7,** 1099-1111.

**Borevitz, J.O., Xia, Y., Blount, J., Dixon, R.A., and Lamb, C.** (2000). Activation tagging identifies a conserved MYB regulator of phenylpropanoid biosynthesis. Plant Cell **12,** 2383-2394.

**Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X.** (1997). The *cpr5* mutant of *Arabidopsis* expresses both *NPR1*-dependent and *NPR1*-independent resistance. Plant Cell **9,** 1573-1584.

**Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F., and Dong, X.** (1994). A mutation in *Arabidopsis* that leads to constitutive expression of systemic acquired resistance. Plant Cell **6,** 1845-1857.

**Brash, A.R.** (1999). Lipoxygenases: occurrence, functions, catalysis, and acquisition of substrate. J. Biol. Chem. **274,** 23679-23682.

**Breviario, D., and Nick, P.** (2000). Plant tubulins: a melting pot for basic questions and promising applications. Transgenic Res. **9,** 383-393.

**Budziszewski, G.J., Lewis, S.P., Glover, L.W., Reineke, J., Jones, G., Ziemnik, L.S., Lonowski, J., Nyfeler, B., Aux, G., Zhou, Q., McElver, J., Patton, D.A., Martienssen, R., Grossniklaus, U., Ma, H., Law, M., and Levin, J.Z.** (2001). *Arabidopsis* genes essential for seedling viability: isolation of insertional mutants and molecular cloning. Genetics **159,** 1765-1778.

**Buttner, P., Koch, F., Voigt, K., Quidde, T., Risch, S., Blaich, R., Bruckner, B., and Tudzynski, P.** (1994). Variations in ploidy among isolates of *Botrytis cinerea*: implications for genetic and molecular analyses. Curr. Genet. **25,** 445-450.

**Byrne, M.E., Barley, R., Curtis, M., Arroyo, J.M., Dunham, M., Hudson, A., and Martienssen, R.A.** (2000). *Asymmetric leaves1* mediates leaf patterning and stem cell function in *Arabidopsis*. Nature **408,** 967-971.

**Cao, H., Li, X., and Dong, X.** (1998). Generation of broad-spectrum disease resistance by overexpression of an essential regulatory gene in systemic acquired resistance. Proc. Natl. Acad. Sci. USA **95,** 6531-6536.

**Cao, H., Bowling, S.A., Gordon, A.S., and Dong, X.** (1994). Characterization of an *Arabidopsis* mutant that is nonresponsive to inducers of systemic acquired resistance. Plant Cell **6,** 1583-1592.

**Century, K.S., Shapiro, A.D., Repetti, P.P., Dahlbeck, D., Holub, E., and Staskawicz, B.J.** (1997). *NDR1*, a pathogen-induced component required for *Arabidopsis* disease resistance. Science **278,** 1963-1965.

**Cervone, F., Hahn, M.G., Delorenzo, G., Darvill, A., and Albersheim, P.** (1989). Host-Pathogen Interactions.33. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defense responses. Plant Physiol. **90,** 542-548.

**Chao, Q., Rothenberg, M., Solano, R., Roman, G., Terzaghi, W., and Ecker, J.R.** (1997). Activation of the ethylene gas response pathway in *Arabidopsis* by the nuclear protein *ETHYLENE-INSENSITIVE3* and related proteins. Cell **89,** 1133-1144.

**Chaudhry, B., Muller-Uri, F., Cameron-Mills, V., Gough, S., Simpson, D., Skriver, K., and Mundy, J.** (1994). The barley 60 kDa jasmonate-induced protein (JIP60) is a novel ribosome-inactivating protein. Plant J. **6,** 815-824.

**Chini, A.** (2004). Genomic analysis and characterisation of *adr1*, an activated *Arabidopsis* mutant overexpressing a CC-NBS-LRR gene that confers disease resistance and drought tolerance. Thesis, 158.

**Chini, A., Grant, J.J., Seki, M., Shinozaki, K., and Loake, G.J.** (2004). Drought tolerance established by enhanced expression of the CC-NBS-LRR gene, *ADR1*, requires salicylic acid, *EDS1* and *ABI1*. Plant J. **38,** 810-822.

**Chisholm, S.T., Dahlbeck, D., Krishnamurthy, N., Day, B., Sjolander, K., and Staskawicz, B.J.** (2005). Molecular characterization of proteolytic cleavage sites of the *Pseudomonas syringae* effector *AvrRpt2*. Proc. Natl. Acad. Sci. USA **102,** 2087-2092.

**Clarke, J.D., Liu, Y., Klessig, D.F., and Dong, X.** (1998). Uncoupling *PR* gene expression from *NPR1* and bacterial resistance: characterization of the dominant *Arabidopsis cpr6-1* mutant. Plant Cell **10,** 557-569.

**Clarke, J.D., Aarts, N., Feys, B.J., Dong, X., and Parker, J.E.** (2001). Constitutive disease resistance requires *EDS1* in the *Arabidopsis* mutants *cpr1* and *cpr6* and is partially *EDS1*-dependent in *cpr5*. Plant J. **26,** 409-420.

**Clough, S.J., and Bent, A.F.** (1998). Floral dip: a simplified method for *Agrobacterium*-mediated transformation of *Arabidopsis thaliana*. Plant J. **16,** 735-743.

**Coaker, G., Falick, A., and Staskawicz, B.** (2005). Activation of a phytopathogenic bacterial effector protein by a eukaryotic cyclophilin. Science **308,** 548-550.

**Coego, A., Ramirez, V., Gil, M.J., Flors, V., Mauch-Mani, B., and Vera, P.** (2005). An *Arabidopsis* homeodomain transcription factor, *OVEREXPRESSOR OF CATIONIC*

*PEROXIDASE 3*, mediates resistance to infection by necrotrophic pathogens. Plant Cell **17,** 2123-2137.

**Cohen, Y., Gisi, U., and Niderman, T.** (1993). Local and systemic protection against *Phytophthora infestans* induced in potato and tomato plants by jasmonic acid and jasmonic methyl-ester. Phytopathology **83,** 1054-1062.

**Conconi, A., Miquel, M., Browse, J.A., and Ryan, C.A.** (1996). Intracellular levels of free linolenic and linoleic acids increase in tomato leaves in response to wounding. Plant Physiol. **111,** 797-803.

**Creelman, R.A., and Mullet, J.E.** (1995). Jasmonic acid distribution and action in plants: regulation during development and response to biotic and abiotic stress. Proc. Natl. Acad. Sci. USA **92,** 4114-4119.

**Creelman, R.A., and Mullet, J.E.** (1997). Biosynthesis and action of jasmonates in plants. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 355-381.

**Crombie, L., and Morgan, D.O.** (1988). Formation of acyclic alpha-ketols and gamma-jetols and 12-oxophytodienoic acid from linolenic acid 13-hydroperoxide by a flax enzyme preparation - evidence for a single enzyme leading to a common allene epoxide intermediate. J. Chem. Soc.-Chem. Comm., 558-560.

**Dangl, J.L., and Jones, J.D.** (2001). Plant pathogens and integrated defence responses to infection. Nature **411,** 826-833.

**Dangl, J.L., Dietrich, R.A., and Richberg, M.H.** (1996). Death don't have no mercy: Cell death programs in plant-microbe interactions. Plant Cell **8,** 1793-1807.

**De Buck, S., Jacobs, A., Van Montagu, M., and Depicker, A.** (1999). The DNA sequences of T-DNA junctions suggest that complex T-DNA loci are formed by a recombination process resembling T-DNA integration. Plant J. **20,** 295-304.

**De Neve, M., De Buck, S., Jacobs, A., Van Montagu, M., and Depicker, A.** (1997). T-DNA integration patterns in co-transformed plant cells suggest that T-DNA repeats originate from co-integration of separate T-DNAs. Plant J. **11,** 15-29.

**Deighton, N., Muckenschnabel, I.I., Goodman, B.A., and Williamson, B.** (1999). Lipid peroxidation and the oxidative burst associated with infection of *capsicum annuum* by *Botrytis cinerea*. Plant J. **20,** 485-492.

**Delaney, T.P.** (2000). New mutants provide clues into regulation of systemic acquired resistance. Trends Plant Sci. **5,** 49-51.

**Delaney, T.P., Uknes, S., Vernooij, B., Friedrich, L., Weymann, K., Negrotto, D., Gaffney, T., Gutrella, M., Kessmann, H., Ward, E., and Ryals, J.** (1994). A central role of salicylic acid in plant disease resistance. Science **266,** 1247-1250.

**Dellaporta, S.L.W., J.; Hicks, J.B.** (1983). A plant DNA mini preparation: version II. Plant Mol. Biol. Rep. **1,** 19-21.

**Delledonne, M., Xia, Y., Dixon, R.A., and Lamb, C.** (1998). Nitric oxide functions as a signal in plant disease resistance. Nature **394,** 585-588.

**Delledonne, M., Zeier, J., Marocco, A., and Lamb, C.** (2001). Signal interactions between nitric oxide and reactive oxygen intermediates in the plant hypersensitive disease resistance response. Proc. Natl. Acad. Sci. USA **98,** 13454-13459.

**Denby, K.J., Kumar, P., and Kliebenstein, D.J.** (2004). Identification of *Botrytis cinerea* susceptibility loci in *Arabidopsis thaliana*. Plant J. **38,** 473-486.

**Desiderio, A., Aracri, B., Leckie, F., Mattei, B., Salvi, G., Tigelaar, H., Van Roekel, J.S., Baulcombe, D.C., Melchers, L.S., De Lorenzo, G., and Cervone, F.** (1997). Polygalacturonase-inhibiting proteins (PGIPs) with different specificities are expressed in *Phaseolus vulgaris*. Mol. Plant-Microbe Interact. **10,** 852-860.

**Devoto, A., and Turner, J.G.** (2003). Regulation of jasmonate-mediated plant responses in *Arabidopsis*. Ann. Bot. (Lond) **92,** 329-337.

**Devoto, A., Nieto-Rostro, M., Xie, D., Ellis, C., Harmston, R., Patrick, E., Davis, J., Sherratt, L., Coleman, M., and Turner, J.G.** (2002). *COI1* links jasmonate signalling and fertility to the SCF ubiquitin-ligase complex in *Arabidopsis*. Plant J. **32,** 457-466.

**DeWit, P.J.G.M.** (1997). Pathogen avirulence and plant resistance: a key role for recognition. Trends Plant Sci. **2,** 452-458.

**Dharmasiri, N., Dharmasiri, S., and Estelle, M.** (2005). The F-box protein TIR1 is an auxin receptor. Nature **435,** 441-445.

**Dietrich, R.A., Richberg, M.H., Schmidt, R., Dean, C., and Dangl, J.L.** (1997). A novel zinc finger protein is encoded by the *Arabidopsis LSD1* gene and functions as a negative regulator of plant cell death. Cell **88,** 685-694.

**Dietrich, R.A., Delaney, T.P., Uknes, S.J., Ward, E.R., Ryals, J.A., and Dangl, J.L.** (1994). *Arabidopsis* mutants simulating disease resistance response. Cell **77,** 565-577.

**Doares, S.H., Narvaez-Vasquez, J., Conconi, A., and Ryan, C.A.** (1995). Salicylic acid inhibits synthesis of proteinase inhibitors in tomato leaves induced by systemin and jasmonic acid. Plant Physiol. **108,** 1741-1746.

**Doke, N., Miura, Y., Sanchez, L.M., Park, H.J., Noritake, T., Yoshioka, H., and Kawakita, K.** (1996). The oxidative burst protects plants against pathogen attack: Mechanism and role as an emergency signal for plant bio-defence - A review. Gene **179,** 45-51.

**Ecker, J.R.** (1995). The ethylene signal transduction pathway in plants. Science **268,** 667-675.

**Edlich, W., Lorenz, G., Lyr, H., Nega, E., and Pommer, E.H.** (1989). New aspects on the infection mechanism of *Botrytis cinerea* Pers. Neth. J. Plant Path. **95,** 53-62.

**Ellis, C., Karafyllidis, I., and Turner, J.G.** (2002). Constitutive activation of jasmonate signaling in an *Arabidopsis* mutant correlates with enhanced resistance to *Erysiphe cichoracearum*, *Pseudomonas syringae*, and *Myzus persicae*. Mol. Plant-Microbe Interact. **15,** 1025-1030.

**Epple, P., Apel, K., and Bohlmann, H.** (1997). Overexpression of an endogenous *thionin* enhances resistance of *Arabidopsis* against *Fusarium oxysporum*. Plant Cell **9,** 509-520.

**Falk, A., Feys, B.J., Frost, L.N., Jones, J.D., Daniels, M.J., and Parker, J.E.** (1999). *EDS1*, an essential component of *R* gene-mediated disease resistance in *Arabidopsis* has homology to eukaryotic lipases. Proc. Natl. Acad. Sci. USA **96,** 3292-3297.

**Faretra, F., Antonacci, E., and Pollastro, S.** (1988). Sexual behavior and mating system of *Botryotinia fuckeliana*, teleomorph of *Botrytis cinerea*. J. Gen. Microb. **134,** 2543-2550.

**Farmer, E.E., and Ryan, C.A.** (1990). Interplant communication: airborne methyl jasmonate induces synthesis of proteinase inhibitors in plant leaves. Proc. Natl. Acad. Sci. USA **87,** 7713-7716.

**Feechan, A., Kwon, E., Yun, B.W., Wang, Y., Pallas, J.A., and Loake, G.J.** (2005). A central role for S-nitrosothiols in plant disease resistance. Proc. Natl. Acad. Sci. USA **102,** 8054-8059.

**Felix, G., Duran, J.D., Volko, S., and Boller, T.** (1999). Plants have a sensitive perception system for the most conserved domain of bacterial flagellin. Plant J. **18,** 265-276.

**Felton, G.W., and Korth, K.L.** (2000). Trade-offs between pathogen and herbivore resistance. Curr. Opin. Plant Biol. **3,** 309-314.

**Ferrari, S., Plotnikova, J.M., De Lorenzo, G., and Ausubel, F.M.** (2003). *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2*, but not *SID2*, *EDS5* or *PAD4*. Plant J. **35,** 193-205.

**Feussner, I., and Wasternack, C.** (2002). The lipoxygenase pathway. Annu. Rev. Plant Biol. **53,** 275-297.

**Feys, B., Benedetti, C.E., Penfold, C.N., and Turner, J.G.** (1994). *Arabidopsis* mutants selected for resistance to the phytotoxin coronatine are male sterile, insensitive to methyl jasmonate, and resistant to a bacterial pathogen. Plant Cell **6,** 751-759.

**Feys, B.J., Moisan, L.J., Newman, M.A., and Parker, J.E.** (2001). Direct interaction between the *Arabidopsis* disease resistance signaling proteins, EDS1 and PAD4. Embo J. **20,** 5400-5411.

**Flor, H.H.** (1971). Current status of the gene-for-gene concept. Annu. Rev. Phytopath. **9,** 275-296.

**Freeling, M., Bertrand-Garcia, R., and Sinha, N.** (1992). Maize mutants and variants altering developmental time and their heterochronic interactions. Bioessays **14,** 227-236.

**Friedrich, L., Vernooij, B., Gaffney, T., Morse, A., and Ryals, J.** (1995). Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. Plant Mol. Biol. **29,** 959-968.

**Froehlich, J.E., Itoh, A., and Howe, G.A.** (2001). Tomato allene oxide synthase and fatty acid hydroperoxide lyase, two cytochrome P450s involved in oxylipin metabolism, are targeted to different membranes of chloroplast envelope. Plant Physiol. **125,** 306-317.

**Frye, C.A., and Innes, R.W.** (1998). An *Arabidopsis* mutant with enhanced resistance to powdery mildew. Plant Cell **10,** 947-956.

**Gaffney, T., Friedrich, L., Vernooij, B., Negrotto, D., Nye, G., Uknes, S., Ward, E., Kessmann, H., and Ryals, J.** (1993). Requirement of salicylic acid for the induction of systemic acquired resistance. Science **261,** 754-756.

**Gagne, J.M., Downes, B.P., Shiu, S.H., Durski, A.M., and Vierstra, R.D.** (2002). The F-box subunit of the SCF E3 complex is encoded by a diverse superfamily of genes in *Arabidopsis*. Proc. Natl. Acad. Sci. USA **99,** 11519-11524.

**Gassmann, W., Hinsch, M.E., and Staskawicz, B.J.** (1999). The *Arabidopsis RPS4* bacterial-resistance gene is a member of the TIR-NBS-LRR family of disease-resistance genes. Plant J. **20,** 265-277.

**Gilroy, E.M.** (2005). Utilising model systems and crop species to discover and characterise novel genes in plant disease resistance. Thesis **1,** 169.

**Glazebrook, J.** (2001). Genes controlling expression of defense responses in *Arabidopsis*--2001 status. Curr. Opin. Plant Biol. **4,** 301-308.

**Glazebrook, J.** (2005). Contrasting mechanisms of defense against biotrophic and necrotrophic pathogens. Annu. Rev. Phytopathol. **43,** 205-227.

**Glazebrook, J., and Ausubel, F.M.** (1994). Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proc. Natl. Acad. Sci. USA **91,** 8955-8959.

**Glazebrook, J., Chen, W., Estes, B., Chang, H.S., Nawrath, C., Metraux, J.P., Zhu, T., and Katagiri, F.** (2003). Topology of the network integrating salicylate and jasmonate signal transduction derived from global expression phenotyping. Plant J. **34,** 217-228.

**Godiard, L., Sauviac, L., Torii, K.U., Grenon, O., Mangin, B., Grimsley, N.H., and Marco, Y.** (2003). ERECTA, an LRR receptor-like kinase protein controlling development pleiotropically affects resistance to bacterial wilt. Plant J. **36,** 353-365.

**Goetghebeur, M., Brun, S., Galzy, P., and Nicolas, M.** (1993). Benzyl alcohol oxidase and laccase synthesis in *Botrytis cinerea*. Biosc. Biotech. Bioch. **57,** 1380-1381.

**Gomez-Gomez, L., Felix, G., and Boller, T.** (1999). A single locus determines sensitivity to bacterial flagellin in *Arabidopsis thaliana*. Plant J. **18,** 277-284.

**Gomez-Gomez, L., Bauer, Z., and Boller, T.** (2001). Both the extracellular leucine-rich repeat domain and the kinase activity of FSL2 are required for flagellin binding and signaling in *Arabidopsis*. Plant Cell **13,** 1155-1163.

**Gorlach, J., Volrath, S., Knauf-Beiter, G., Hengy, G., Beckhove, U., Kogel, K.H., Oostendorp, M., Staub, T., Ward, E., Kessmann, H., and Ryals, J.** (1996). Benzothiadiazole, a novel class of inducers of systemic acquired resistance, activates gene expression and disease resistance in wheat. Plant Cell **8,** 629-643.

**Gourgues, M., Brunet-Simon, A., Lebrun, M.H., and Levis, C.** (2004). The tetraspanin *BcPls1* is required for appressorium-mediated penetration of *Botrytis cinerea* into host plant leaves. Mol. Microbiol. **51,** 619-629.

**Govrin, E.M., and Levine, A.** (2000). The hypersensitive response facilitates plant infection by the necrotrophic pathogen *Botrytis cinerea*. Curr. Biol. **10,** 751-757.

**Grant, J.J., and Loake, G.J.** (2000). Role of reactive oxygen intermediates and cognate redox signaling in disease resistance. Plant Physiol. **124,** 21-29.

**Grant, J.J., Chini, A., Basu, D., and Loake, G.J.** (2003). Targeted activation tagging of the *Arabidopsis* NBS-LRR gene, *ADR1*, conveys resistance to virulent pathogens. Mol. Plant-Microbe Interact. **16,** 669-680.

**Gray, W.M., del Pozo, J.C., Walker, L., Hobbie, L., Risseeuw, E., Banks, T., Crosby, W.L., Yang, M., Ma, H., and Estelle, M.** (1999). Identification of an SCF ubiquitin-ligase complex required for auxin response in *Arabidopsis thaliana*. Genes Dev. **13,** 1678-1691.

**Gundlach, H., Muller, M.J., Kutchan, T.M., and Zenk, M.H.** (1992). Jasmonic acid is a signal transducer in elicitor-induced plant cell cultures. Proc. Natl. Acad. Sci. USA **89,** 2389-2393.

**Gupta, V., Willits, M.G., and Glazebrook, J.** (2000). *Arabidopsis thaliana EDS4* contributes to salicylic acid (SA)-dependent expression of defense responses: evidence

for inhibition of jasmonic acid signaling by SA. Mol. Plant-Microbe Interact. **13,** 503-511.

**Guzman, P., and Ecker, J.R.** (1990). Exploiting the triple response of *Arabidopsis* to identify ethylene-related mutants. Plant Cell **2,** 513-523.

**Ham, B.K., Lee, T.H., You, J.S., Nam, Y.W., Kim, J.K., and Paek, K.H.** (1999). Isolation of a putative tobacco host factor interacting with cucumber mosaic virus-encoded 2b protein by yeast two-hybrid screening. Mol. Cells **9,** 548-555.

**Hamberg, M., and Fahlstadius, P.** (1990). Allene Oxide Cyclase - a new enzyme in plant lipid metabolism. Arch. Bioch. Biophys. **276,** 518-526.

**Hamilton, A.J., and Baulcombe, D.C.** (1999). A species of small antisense RNA in posttranscriptional gene silencing in plants. Science **286,** 950-952.

**Hansen, B., and Halkier, B.** (2005). New insight into the biosynthesis and regulation of indole compounds in Arabidopsis thaliana. Planta **221,** 603-606.

**Harms, K., Ramirez, I.I., and Pena-Cortes, H.** (1998). Inhibition of wound-induced accumulation of allene oxide synthase transcripts in flax leaves by aspirin and salicylic acid. Plant Physiol. **118,** 1057-1065.

**Hause, B., Stenzel, I., Miersch, O., Maucher, H., Kramell, R., Ziegler, J., and Wasternack, C.** (2000). Tissue-specific oxylipin signature of tomato flowers: allene oxide cyclase is highly expressed in distinct flower organs and vascular bundles. Plant J. **24,** 113-126.

**Hay, A., Jackson, D., Ori, N., and Hake, S.** (2003). Analysis of the competence to respond to KNOTTED1 activity in *Arabidopsis* leaves using a steroid induction system. Plant Physiol. **131,** 1671-1680.

**Hayashi, H., Czaja, I., Lubenow, H., Schell, J., and Walden, R.** (1992). Activation of a plant gene by T-DNA tagging: auxin-independent growth in vitro. Science **258,** 1350-1353.

**He, Y., Fukushige, H., Hildebrand, D.F., and Gan, S.** (2002). Evidence supporting a role of jasmonic acid in *Arabidopsis* leaf senescence. Plant Physiol. **128,** 876-884.

**Heath, M.C.** (1987). Evolution of plant resistance and susceptibility to fungal invaders. Can. J. Plant Pathol. **9,** 389-397.

**Heinlein, M., Epel, B.L., Padgett, H.S., and Beachy, R.N.** (1995). Interaction of tobamovirus movement proteins with the plant cytoskeleton. Science **270,** 1983-1985.

**Higgins, C.F.** (1992). ABC transporters: from microorganisms to man. Annu. Rev. Cell Biol. **8,** 67-113.

**Hildebrand, D.F., Brown, G.C., Jackson, D.M., and Hamiltonkemp, T.R.** (1993). Effects of some seaf-smitted solatile sompounds on aphid population increase. J. Chem. Ecol. **19,** 1875-1887.

**Hinsch, M., and Staskawicz, B.** (1996). Identification of a new *Arabidopsis* disease resistance locus, *RPs4*, and cloning of the corresponding avirulence gene, *avrRps4*, from *Pseudomonas syringae* pv. *pisi*. Mol. Plant-Microbe Interact. **9,** 55-61.

**Hoffman, T., Schmidt, J.S., Zheng, X., and Bent, A.F.** (1999). Isolation of ethylene-insensitive soybean mutants that are altered in pathogen susceptibility and gene-for-gene disease resistance. Plant Physiol. **119,** 935-950.

**Holt, B.F., 3rd, Hubert, D.A., and Dangl, J.L.** (2003). Resistance gene signaling in plants--complex similarities to animal innate immunity. Curr. Opin. Immunol. **15,** 20-25.

**Holub, E.B.** (2001). The arms race is ancient history in *Arabidopsis*, the wildflower. Nat. Rev. Genet. **2,** 516-527.

**Holub, E.B., Beynon, L.J., and Crute, I.R.** (1994). Phenotypic and genotypic characterization of interactions between isolates of *Peronospora parasitica* and accessions of *Arabidopsis thaliana*. Mol. Plant-Microbe Interact. **7,** 223-239.

**Howe, G.A., Lightner, J., Browse, J., and Ryan, C.A.** (1996). An octadecanoid pathway mutant (JL5) of tomato is compromised in signaling for defense against insect attack. Plant Cell **8,** 2067-2077.

**Hu, G., deHart, A.K., Li, Y., Ustach, C., Handley, V., Navarre, R., Hwang, C.F., Aegerter, B.J., Williamson, V.M., and Baker, B.** (2005). *EDS1* in tomato is required for resistance mediated by TIR-class R genes and the receptor-like R gene Ve. Plant J. **42,** 376-391.

**Hua, J., and Meyerowitz, E.M.** (1998). Ethylene responses are negatively regulated by a receptor gene family in *Arabidopsis thaliana*. Cell **94,** 261-271.

**Huang, J., Pray, C., and Rozelle, S.** (2002a). Enhancing the crops to feed the poor. Nature **418,** 678-684.

**Huang, J., Rozelle, S., Pray, C., and Wang, Q.** (2002b). Plant biotechnology in China. Science **295,** 674-676.

**Ichikawa, T., Nakazawa, M., Kawashima, M., Muto, S., Gohda, K., Suzuki, K., Ishikawa, A., Kobayashi, H., Yoshizumi, T., Tsumoto, Y., Tsuhara, Y., Iizumi, H., Goto, Y., and Matsui, M.** (2003). Sequence database of 1172 T-DNA insertion sites in *Arabidopsis* activation-tagging lines that showed phenotypes in T1 generation. Plant J. **36,** 421-429.

**Ichimura, K., Mizoguchi, T., Yoshida, R., Yuasa, T., and Shinozaki, K.** (2000). Various abiotic stresses rapidly activate *Arabidopsis* MAP kinases ATMPK4 and ATMPK6. Plant J. **24,** 655-665.

**Ishiguro, S., Kawai-Oda, A., Ueda, J., Nishida, I., and Okada, K.** (2001). The DEFECTIVE IN ANTHER DEHISCIENCE gene encodes a novel phospholipase A1 catalyzing the initial step of jasmonic acid biosynthesis, which synchronizes pollen maturation, anther dehiscence, and flower opening in *Arabidopsis*. Plant Cell **13,** 2191-2209.

**Jabs, T., Dietrich, R.A., and Dangl, J.L.** (1996). Initiation of runaway cell death in an *Arabidopsis* mutant by extracellular superoxide. Science **273,** 1853-1856.

**Jach, G., Gornhardt, B., Mundy, J., Logemann, J., Pinsdorf, E., Leah, R., Schell, J., and Maas, C.** (1995). Enhanced quantitative resistance against fungal disease by combinatorial expression of different barley antifungal proteins in transgenic tobacco. Plant J. **8,** 97-109.

**James, C.** (2004). Preview: Global Status of Commercialized Biotech/GM Crops:2004. ISAAA Briefs **32,** 1-11.

**Jander, G., Norris, S.R., Rounsley, S.D., Bush, D.F., Levin, I.M., and Last, R.L.** (2002). *Arabidopsis* map-based cloning in the post-genome era. Plant Physiol. **129,** 440-450.

**Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P., and Valent, B.** (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. Embo J. **19,** 4004-4014.

**Jin, P., Wood, M.D., Wu, Y., Xie, Z., and Katagiri, F.** (2003). Cleavage of the *Pseudomonas syringae* type III effector *AvrRpt2* requires a host factor(s) common among eukaryotes and is important for *AvrRpt2* localization in the host cell. Plant Physiol. **133,** 1072-1082.

**Jirage, D., Tootle, T.L., Reuber, T.L., Frost, L.N., Feys, B.J., Parker, J.E., Ausubel, F.M., and Glazebrook, J.** (1999). *Arabidopsis thaliana PAD4* encodes a lipase-like gene that is important for salicylic acid signaling. Proc. Natl. Acad. Sci. USA **96,** 13583-13588.

**Johnson, P.R., and Ecker, J.R.** (1998). The ethylene gas signal transduction pathway: a molecular perspective. Annu. Rev. Genet. **32,** 227-254.

**Jones, A.M., and Dangl, J.L.** (1996). Logjam at the Styx: Programmed cell death in plants. Trends Plant Sci. **1,** 114-119.

**Jones, D.A., and Jones, J.D.** (1996). The role of leucine rich repeat proteins in plant defences. Adv. Bot. Res. Incorporat. Adv. Plant Path. **24,** 89-167.

**Jones, D.A., Thomas, C.M., Hammond-Kosack, K.E., Balint-Kurti, P.J., and Jones, J.D.** (1994). Isolation of the tomato *Cf-9* gene for resistance to *Cladosporium fulvum* by transposon tagging. Science **266,** 789-793.

**Jones, J.D.** (2001). Putting knowledge of plant disease resistance genes to work. Curr. Opin. Plant Biol. **4,** 281-287.

**Kachroo, P., Shanklin, J., Shah, J., Whittle, E.J., and Klessig, D.F.** (2001). A fatty acid desaturase modulates the activation of defense signaling pathways in plants. Proc. Natl. Acad. Sci. USA **98,** 9448-9453.

**Kang, J.Y., Choi, H.I., Im, M.Y., and Kim, S.Y.** (2002). *Arabidopsis* basic leucine zipper proteins that mediate stress-responsive abscisic acid signaling. Plant Cell **14,** 343-357.

**Kang, L., Li, J., Zhao, T., Xiao, F., Tang, X., Thilmony, R., He, S., and Zhou, J.M.** (2003). Interplay of the *Arabidopsis* nonhost resistance gene *NHO1* with bacterial virulence. Proc. Natl. Acad. Sci. USA **100,** 3519-3524.

**Kieber, J.J.** (1997). The ethylene response pathway in *Arabidopsis*. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 277-296.

**Kim, H.S., and Delaney, T.P.** (2002). *Arabidopsis* SON1 is an F-box protein that regulates a novel induced defense response independent of both salicylic acid and systemic acquired resistance. Plant Cell **14,** 1469-1482.

**Knoester, M., van Loon, L.C., van den Heuvel, J., Hennig, J., Bol, J.F., and Linthorst, H.J.M.** (1998). Ethylene-insensitive tobacco lacks nonhost resistance against soil-borne fungi. Proc. Natl. Acad. Sci. USA **95,** 1933-1937.

**Kobayashi, Y., Yamada, M., Kobayashi, I., and Kunoh, H.** (1997a). Actin microfilaments are required for the expression of nonhost resistance in higher plants. Plant Cell Physiol. **38,** 725-733.

**Kobayashi, Y., Kobayashi, I., Funaki, Y., Fujimoto, S., Takemoto, T., and Kunoh, H.** (1997b). Dynamic reorganization of microfilaments and microtubules is necessary for the expression of non-host resistance in barley coleoptile cells. Plant J. **11,** 525-537.

**Kobe, B., and Kajava, A.V.** (2001). The leucine-rich repeat as a protein recognition motif. Curr. Opin. Struct. Biol. **11,** 725-732.

**Koda, Y., and Kikuta, Y.** (1994). Wound-induced accumulation of jasmonic acid in tissues of potato tubers. Plant Cell Physiol. **35,** 751-756.

**Konieczny, A., and Ausubel, F.M.** (1993). A procedure for mapping *Arabidopsis* mutations using co-dominant ecotype-specific PCR-based markers. Plant J. **4,** 403-410.

**Krizkova, L., and Hrouda, M.** (1998). Direct repeats of T-DNA integrated in tobacco chromosome: characterization of junction regions. Plant J. **16,** 673-680.

**Laudert, D., and Weiler, E.W.** (1998). Allene oxide synthase: a major control point in *Arabidopsis thaliana* octadecanoid signalling. Plant J. **15,** 675-684.

**Lawton, K., Weymann, K., Friedrich, L., Vernooij, B., Uknes, S., and Ryals, J.** (1995). Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Mol. Plant-Microbe Interact. **8,** 863-870.

**Lebel, E., Heifetz, P., Thorne, L., Uknes, S., Ryals, J., and Ward, E.** (1998). Functional analysis of regulatory sequences controlling *PR-1* gene expression in *Arabidopsis*. Plant J. **16,** 223-233.

**Lehmann, J., Atzorn, R., Bruckner, C., Reinbothe, S., Leopold, J., Wasternack, C., and Parthier, B.** (1995). Accumulation of jasmonate, abscisic-acid, specific transcripts and proteins in osmotically stressed barley leaf segments. Planta **197,** 156-162.

**Li, J., Brader, G., and Palva, E.T.** (2004a). The WRKY70 transcription factor: a node of convergence for jasmonate-mediated and salicylate-mediated signals in plant defense. Plant Cell **16,** 319-331.

**Li, L., Zhao, Y., McCaig, B.C., Wingerd, B.A., Wang, J., Whalon, M.E., Pichersky, E., and Howe, G.A.** (2004b). The tomato homolog of *CORONATINE-INSENSITIVE1* is required for the maternal control of seed maturation, jasmonate-signaled defense responses, and glandular trichome development. Plant Cell **16,** 126-143.

**Liu, Y., Schiff, M., Marathe, R., and Dinesh-Kumar, S.P.** (2002). Tobacco *Rar1*, *EDS1* and *NPR1/NIM1* like genes are required for N-mediated resistance to tobacco mosaic virus. Plant J. **30,** 415-429.

**Liu, Y.G., and Whittier, R.F.** (1995). Thermal asymmetric interlaced PCR: automatable amplification and sequencing of insert end fragments from P1 and YAC clones for chromosome walking. Genomics **25,** 674-681.

**Liu, Y.G., Mitsukawa, N., Oosumi, T., and Whittier, R.F.** (1995). Efficient isolation and mapping of *Arabidopsis thaliana* T-DNA insert junctions by thermal asymmetric interlaced PCR. Plant J. **8,** 457-463.

**Llorente, F., Alonso-Blanco, C., Sanchez-Rodriguez, C., Jorda, L., and Molina, A.** (2005). *ERECTA* receptor-like kinase and heterotrimeric G protein from *Arabidopsis* are required for resistance to the necrotrophic fungus *Plectosphaerella cucumerina*. Plant J. **43,** 165-180.

**Loake, G.** (2001). Plant cell death: unmasking the gatekeepers. Curr. Biol. **11,** R1028-1031.

**Loake, G., and Nurmberg, P.** (2003). Role of Jasmonates in Growth, Development and Defence. (Ed. Murphy, D.M and Thomson, W.).

**Lorenzo, O., Piqueras, R., Sanchez-Serrano, J.J., and Solano, R.** (2003). *ETHYLENE RESPONSE FACTOR1* integrates signals from ethylene and jasmonate pathways in plant defense. Plant Cell **15,** 165-178.

**Lorenzo, O., Chico, J.M., Sanchez-Serrano, J.J., and Solano, R.** (2004). JASMONATE-INSENSITIVE1 encodes a MYC transcription factor essential to discriminate between different jasmonate-regulated defense responses in *Arabidopsis*. Plant Cell **16,** 1938-1950.

**Lu, M., Tang, X., and Zhou, J.M.** (2001). *Arabidopsis NHO1* is required for general resistance against *Pseudomonas* bacteria. Plant Cell **13,** 437-447.

**Lukowitz, W., Gillmor, C.S., and Scheible, W.R.** (2000). Positional cloning in *Arabidopsis*. Why it feels good to have a genome initiative working for you. Plant Physiol. **123,** 795-805.

**Mackey, D., Holt, B.F., Wiig, A., and Dangl, J.L.** (2002). RIN4 interacts with *Pseudomonas syringae* type III effector molecules and is required for RPM1-mediated resistance in *Arabidopsis*. Cell **108,** 743-754.

**Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R., and Dangl, J.L.** (2003). *Arabidopsis* RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. Cell **112,** 379-389.

**Malamy, J., and Klessig, D.F.** (1992). Salicylic acid and plant disease resistance. Plant J. **2,** 643-654.

**Malamy, J., Carr, J.P., Klessig, D.F., and Raskin, I.** (1990). Salicylic acid: a likely endogenous signal in the resistance of tobacco to viral infection. Science **250,** 1002-1004.

**Maldonado, A.M., Doerner, P., Dixon, R.A., Lamb, C.J., and Cameron, R.K.** (2002). A putative lipid transfer protein involved in systemic resistance signalling in *Arabidopsis*. Nature **419,** 399-403.

**Maleck, K., Neuenschwander, U., Cade, R.M., Dietrich, R.A., Dangl, J.L., and Ryals, J.A.** (2002). Isolation and characterization of broad-spectrum disease-resistant *Arabidopsis* mutants. Genetics **160,** 1661-1671.

**Manteau, S., Abouna, S., Lambert, B., and Legendre, L.** (2003). Differential regulation by ambient pH of putative virulence factor secretion by the phytopathogenic fungus *Botrytis cinerea*. Fems Microb. Ecol. **43,** 359-366.

**Martin, G.B., Brommonschenkel, S.H., Chunwongse, J., Frary, A., Ganal, M.W., Spivey, R., Wu, T., Earle, E.D., and Tanksley, S.D.** (1993). Map-based cloning of a protein kinase gene conferring disease resistance in tomato. Science **262,** 1432-1436.

**Mateo, A., Muhlenbock, P., Rusterucci, C., Chang, C.C., Miszalski, Z., Karpinska, B., Parker, J.E., Mullineaux, P.M., and Karpinski, S.** (2004). *LESION SIMULATING DISEASE1* is required for acclimation to conditions that promote excess excitation energy. Plant Physiol. **136,** 2818-2830.

**Maucher, H., Hause, B., Feussner, I., Ziegler, J., and Wasternack, C.** (2000). Allene oxide synthases of barley (*Hordeum vulgare* cv. Salome): tissue specific regulation in seedling development. Plant J. **21,** 199-213.

**McDowell, J.M., and Woffenden, B.J.** (2003). Plant disease resistance genes: recent insights and potential applications. Trends Biotechnol. **21,** 178-183.

**McHale, N.A., and Koning, R.E.** (2004). PHANTASTICA regulates development of the adaxial mesophyll in nicotiana leaves. Plant Cell **16,** 1251-1262.

**Mellersh, D.G., and Heath, M.C.** (2003). An investigation into the involvement of defense signaling pathways in components of the nonhost resistance of *Arabidopsis thaliana* to rust fungi also reveals a model system for studying rust fungal compatibility. Mol. Plant-Microbe Interact. **16,** 398-404.

**Memelink, J., Linthorst, H.J., Schilperoort, R.A., and Hoge, J.H.** (1990). Tobacco genes encoding acidic and basic isoforms of pathogenesis-related proteins display different expression patterns. Plant Mol. Biol. **14,** 119-126.

**Meng, X., Brodsky, M.H., and Wolfe, S.A.** (2005). A bacterial one-hybrid system for determining the DNA-binding specificity of transcription factors. Nat. Biotechnol. **23,** 988-994.

**Mengiste, T., Chen, X., Salmeron, J., and Dietrich, R.** (2003). The *BOTRYTIS SUSCEPTIBLE1* gene encodes an R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. Plant Cell **15,** 2551-2565.

**Mert-Turk, F., Bennett, M.H., Mansfield, J.W., and Holub, E.B.** (2003). Camalexin accumulation in *Arabidopsis thaliana* following abiotic elicitation or inoculation with virulent or avirulent *Hyaloperonospora parasitica*. Physiol. Mol. Plant Pathol. **62,** 137-145.

**Metraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Blum, W., and Inverardi, B.** (1990). Increase in salicylic acid at the onset of sytemic acquired resistance in cucumber. Science **250,** 1004-1005.

**Michailides, T.J., and Elmer, P.A.G.** (2000). *Botrytis* gray mold of kiwifruit caused by *Botrytis cinerea* in the United States and New Zealand. Plant Disease **84,** 208-223.

**Michelmore, R.W., Paran, I., and Kesseli, R.V.** (1991). Identification of markers linked to disease-resistance genes by bulked segregant analysis: a rapid method to detect

markers in specific genomic regions by using segregating populations. Proc. Natl. Acad. Sci. USA **88,** 9828-9832.

**Miklos, G.L., and Rubin, G.M.** (1996). The role of the genome project in determining gene function: insights from model organisms. Cell **86,** 521-529.

**Moore, R.C., Zhang, M., Cassimeris, L., and Cyr, R.J.** (1997). In vitro assembled plant microtubules exhibit a high state of dynamic instability. Cell. Motil. Cytoskeleton **38,** 278-286.

**Movahedi, S., and Heale, J.B.** (1990a). Purification and characterization of an aspartic proteinase secreted by *Botrytis cinerea* Pers Ex Pers in culture and in infected carrots. Physiol. Mol. Plant Pathol. **36,** 289-302.

**Movahedi, S., and Heale, J.B.** (1990b). The roles of aspartic proteinase and endo-pectin lyase enzymes in the primary stages of infection and pathogenesis of various host tissues by different isolates of *Botrytis cinerea* Pers Ex Pers. Physiol. Mol. Plant Pathol. **36,** 303-324.

**Movahedi, S., Norey, C.G., Kay, J., and Heale, J.B.** (1991). Infection and pathogenesis of cash crops by *Botrytis cinerea*: primary role of an aspartic proteinase. Adv. Exp. Med. Biol. **306,** 213-216.

**Mudgett, M.B., and Staskawicz, B.J.** (1999). Characterization of the *Pseudomonas syringae* pv. *tomato* AvrRpt2 protein: demonstration of secretion and processing during bacterial pathogenesis. Mol. Microbiol. **32,** 927-941.

**Mueller, M.J.** (1997). Enzymes involved in jasmonic acid biosynthesis. Physiol. Plantarum **100,** 653-663.

**Mueller, M.J., Brodschelm, W., Spannagl, E., and Zenk, M.H.** (1993). Signaling in the elicitation process is mediated through the octadecanoid pathway leading to jasmonic acid. Proc. Natl. Acad. Sci. USA **90,** 7490-7494.

**Murray, S.L., Thomson, C., Chini, A., Read, N.D., and Loake, G.J.** (2002). Characterization of a novel, defense-related *Arabidopsis* mutant, *cir1*, isolated by luciferase imaging. Mol. Plant-Microbe Interact. **15,** 557-566.

**Murray, S.L., Adams, N., Kliebenstein, D.J., Loake, G.J., and Denby, K.J.** (2005). A constitutive PR-1: luciferase expression screen identifies *Arabidopsis* mutants with differential disease resistance to both biotrophic and necrotrophic pathogens. Mol. Plant Pathol. **6,** 31-41.

**Mysore, K.S., and Ryu, C.M.** (2004). Nonhost resistance: how much do we know? Trends Plant Sci. **9,** 97-104.

**Nacry, P., Camilleri, C., Courtial, B., Caboche, M., and Bouchez, D.** (1998). Major chromosomal rearrangements induced by T-DNA transformation in Arabidopsis. Genetics **149,** 641-650.

**Nawrath, C., and Metraux, J.P.** (1999). Salicylic acid induction-deficient mutants of *Arabidopsis* express *PR-2* and *PR-5* and accumulate high levels of camalexin after pathogen inoculation. Plant Cell **11,** 1393-1404.

**Niki, T., Mitsuhara, I., Seo, S., Ohtsubo, N., and Ohashi, Y.** (1998). Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (*PR*) protein genes in wounded mature tobacco leaves. Plant Cell Physiol. **39,** 500-507.

**Nimchuk, Z., Rohmer, L., Chang, J.H., and Dangl, J.L.** (2001). Knowing the dancer from the dance: R-gene products and their interactions with other proteins from host and pathogen. Curr. Opin. Plant Biol. **4,** 288-294.

**Nuhse, T.S., Peck, S.C., Hirt, H., and Boller, T.** (2000). Microbial elicitors induce activation and dual phosphorylation of the *Arabidopsis thaliana* MAPK 6. J. Biol. Chem. **275,** 7521-7526.

**O'Donnell, P.J., Jones, J.B., Antoine, F.R., Ciardi, J., and Klee, H.J.** (2001). Ethylene-dependent salicylic acid regulates an expanded cell death response to a plant pathogen. Plant J. **25,** 315-323.

**Ori, N., Eshed, Y., Chuck, G., Bowman, J.L., and Hake, S.** (2000). Mechanisms that control *knox* gene expression in the *Arabidopsis* shoot. Development **127,** 5523-5532.

**Papadopoulou, K., Melton, R.E., Leggett, M., Daniels, M.J., and Osbourn, A.E.** (1999). Compromised disease resistance in saponin-deficient plants. Proc. Natl. Acad. Sci. USA **96,** 12923-12928.

**Parker, J.E.** (2003). Plant recognition of microbial patterns. Trends Plant Sci. **8,** 245-247.

**Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D., and Daniels, M.J.** (1996). Characterization of *eds1*, a mutation in *Arabidopsis* suppressing resistance to *Peronospora parasitica* specified by several different *RPP* genes. Plant Cell **8,** 2033-2046.

**Parker, J.E., Szabo, V., Staskawicz, B.J., Lister, C., Dean, C., Daniels, M.J., and Jones, J.D.G.** (1993). Phenotypic characterization and molecular mapping of the *Arabidopsis thaliana* locus *Rpp5*, determining disease resistance to *Peronospora parasitica*. Plant J. **4,** 821-831.

**Peart, J.R., Cook, G., Feys, B.J., Parker, J.E., and Baulcombe, D.C.** (2002). An *EDS1* orthologue is required for N-mediated resistance against tobacco mosaic virus. Plant J. **29,** 569-579.

**PenaCortes H, A.T., Prat S, Weiller EW, Willmitzer L.** (1993). Aspirin prevents wound-induced gene-exression in tomato leaves by blocking jasmonic acid biosynthesis. (New York: Springer Verlag).

**Penninckx, I.A., Thomma, B.P., Buchala, A., Metraux, J.P., and Broekaert, W.F.** (1998). Concomitant activation of jasmonate and ethylene response pathways is required for induction of a plant defensin gene in *Arabidopsis*. Plant Cell **10,** 2103-2113.

**Penninckx, I.A., Eggermont, K., Terras, F.R., Thomma, B.P., De Samblanx, G.W., Buchala, A., Metraux, J.P., Manners, J.M., and Broekaert, W.F.** (1996). Pathogen-induced systemic activation of a plant defensin gene in *Arabidopsis* follows a salicylic acid-independent pathway. Plant Cell **8,** 2309-2323.

**Perucho, M., Hanahan, D., Lipsich, L., and Wigler, M.** (1980). Isolation of the chicken thymidine kinase gene by plasmid rescue. Nature **285,** 207-210.

**Petersen, M., Brodersen, P., Naested, H., Andreasson, E., Lindhart, U., Johansen, B., Nielsen, H.B., Lacy, M., Austin, M.J., Parker, J.E., Sharma, S.B., Klessig, D.F., Martienssen, R., Mattsson, O., Jensen, A.B., and Mundy, J.** (2000). *Arabidopsis* map kinase 4 negatively regulates systemic acquired resistance. Cell **103,** 1111-1120.

**Pieterse, C.M., and van Loon, L.C.** (1999). Salicylic acid-independent plant defence pathways. Trends Plant Sci. **4,** 52-58.

**Pieterse, C.M., van Wees, S.C., Hoffland, E., van Pelt, J.A., and van Loon, L.C.** (1996). Systemic resistance in *Arabidopsis* induced by biocontrol bacteria is independent of salicylic acid accumulation and pathogenesis-related gene expression. Plant Cell **8,** 1225-1237.

**Pieterse, C.M., van Wees, S.C., van Pelt, J.A., Knoester, M., Laan, R., Gerrits, H., Weisbeek, P.J., and van Loon, L.C.** (1998). A novel signaling pathway controlling induced systemic resistance in *Arabidopsis*. Plant Cell **10,** 1571-1580.

**Pilloff, R.K., Devadas, S.K., Enyedi, A., and Raina, R.** (2002). The *Arabidopsis* gain-of-function mutant *dll1* spontaneously develops lesions mimicking cell death associated with disease. Plant J. **30,** 61-70.

**Ponce, M.R., Robles, P., and Micol, J.L.** (1999). High-throughput genetic mapping in *Arabidopsis thaliana*. Mol. Gen. Genet. **261,** 408-415.

**Ponstein, A.S., Bres-Vloemans, S.A., Sela-Buurlage, M.B., van den Elzen, P.J., Melchers, L.S., and Cornelissen, B.J.** (1994). A novel pathogen- and wound-inducible tobacco (*Nicotiana tabacum*) protein with antifungal activity. Plant Physiol. **104,** 109-118.

**Porat, R., Lu, P., and O'Neill, S.D.** (1998). *Arabidopsis SKP1*, a homologue of a cell cycle regulator gene, is predominantly expressed in meristematic cells. Planta **204,** 345-351.

**Powell, A.L., van Kan, J., ten Have, A., Visser, J., Greve, L.C., Bennett, A.B., and Labavitch, J.M.** (2000). Transgenic expression of pear PGIP in tomato limits fungal colonization. Mol. Plant-Microbe Interact. **13,** 942-950.

**Rahman, A., Amakawa, T., Goto, N., and Tsurumi, S.** (2001). Auxin is a positive regulator for ethylene-mediated response in the growth of *Arabidopsis* roots. Plant Cell Physiol. **42,** 301-307.

**Rairdan, G.J., and Delaney, T.P.** (2002). Role of salicylic acid and *NIM1/NPR1* in race-specific resistance in *Arabidopsis*. Genetics **161,** 803-811.

**Rairdan, G.J., Donofrio, N.M., and Delaney, T.P.** (2001). Salicylic acid and NIM1/NPR1-independent gene induction by incompatible *Peronospora parasitica* in *Arabidopsis*. Mol. Plant-Microbe Interact. **14,** 1235-1246.

**Redei, G.P.** (1965). Non-mendelian megagametogenesis in *Arabidopsis*. Genetics **51,** 857-&.

**Reymond, P., Weber, H., Damond, M., and Farmer, E.E.** (2000). Differential gene expression in response to mechanical wounding and insect feeding in *Arabidopsis*. Plant Cell **12,** 707-720.

**Rijkenberg, F.H.J., Deleeuw, G.T.N., Traas, J., and Verhoeff, K.** (1980). Host-parasite relationships of *Botrytis cinerea* on immature tomato fruits. Acta Bot. Nederl. **29,** 215-215.

**Roca, M.G., Davide, L.C., Mendes-Costa, M.C., and Wheals, A.** (2003). Conidial anastomosis tubes in *Colletotrichum*. Fungal Genet. Biol. **40,** 138-145.

**Rolke, Y., Liu, S.J., Quidde, T., Williamson, B., Schouten, A., Weltring, K.M., Siewers, V., Tenberge, K.B., Tudzynski, B., and Tudzynski, P.** (2004). Functional analysis of H2O2-generating systems in *Botrytis cinerea*: the major Cu-Zn-superoxide dismutase (BCSOD1) contributes to virulence on French bean, whereas a glucose oxidase (BCGOD1) is dispensable. Mol. Plant Pathol. **5,** 17-27.

**Rusterucci, C., Aviv, D.H., Holt, B.F., 3rd, Dangl, J.L., and Parker, J.E.** (2001). The disease resistance signaling components *EDS1* and *PAD4* are essential regulators of the cell death pathway controlled by *LSD1* in *Arabidopsis*. Plant Cell **13,** 2211-2224.

**Ryals, J.A., Neuenschwander, U.H., Willits, M.G., Molina, A., Steiner, H.Y., and Hunt, M.D.** (1996). Systemic Acquired Resistance. Plant Cell **8,** 1809-1819.

**Salmeron, J.M., Oldroyd, G.E., Rommens, C.M., Scofield, S.R., Kim, H.S., Lavelle, D.T., Dahlbeck, D., and Staskawicz, B.J.** (1996). Tomato *Prf* is a member of the

leucine-rich repeat class of plant disease resistance genes and lies embedded within the *Pto* kinase gene cluster. Cell **86,** 123-133.

**Sambrook, J., Fritish, E.F., and Manaiatis, T.** (1989). Molecular Cloning- A labratory Manual. Cold Spring Harbor Laboratory Press, USA.

**Sasaki, I., and Nagayama, H.** (1994). Beta-glucosidase from *Botrytis cinerea* - Its relation to the pathogenicity of this fungus. Biosc. Biotech. Bioch. **58,** 616-620.

**Schaller, F., Schaller, A., and Stintzi, A.** (2004). Biosynthesis and metabolism of jasmonates. J. Plant Growth Reg. **23,** 179-199.

**Schaller, F., Biesgen, C., Mussig, C., Altmann, T., and Weiler, E.W.** (2000). 12-Oxophytodienoate reductase 3 (OPR3) is the isoenzyme involved in jasmonate biosynthesis. Planta **210,** 979-984.

**Schenk, P.M., Kazan, K., Wilson, I., Anderson, J.P., Richmond, T., Somerville, S.C., and Manners, J.M.** (2000). Coordinated plant defense responses in *Arabidopsis* revealed by microarray analysis. Proc. Natl. Acad. Sci. USA **97,** 11655-11660.

**Schneuwly, S., Klemenz, R., and Gehring, W.J.** (1987). Redesigning the body plan of *Drosophila* by ectopic expression of the homoeotic gene *Antennapedia.* Nature **325,** 816-818.

**Schoonbeek, H., Del Sorbo, G., and De Waard, M.A.** (2001). The ABC transporter *BcatrB* affects the sensitivity of *Botrytis cinerea* to the phytoalexin resveratrol and the fungicide fenpiclonil. Mol. Plant-Microbe Interact. **14,** 562-571.

**Schouten, A., Wagemakers, L., Stefanato, F.L., van der Kaaij, R.M., and van Kan, J.A.** (2002). Resveratrol acts as a natural profungicide and induces self-intoxication by a specific laccase. Mol. Microbiol. **43,** 883-894.

**Semiarti, E., Ueno, Y., Tsukaya, H., Iwakawa, H., Machida, C., and Machida, Y.** (2001). The *ASYMMETRIC LEAVES2* gene of *Arabidopsis thaliana* regulates formation of a symmetric lamina, establishment of venation and repression of meristem-related homeobox genes in leaves. Development **128,** 1771-1783.

**Senior, A.E., al-Shawi, M.K., and Urbatsch, I.L.** (1995). The catalytic cycle of P-glycoprotein. FEBS Lett. **377,** 285-289.

**Seo, H.S., Song, J.T., Cheong, J.J., Lee, Y.H., Lee, Y.W., Hwang, I., Lee, J.S., and Choi, Y.D.** (2001). Jasmonic acid carboxyl methyltransferase: a key enzyme for jasmonate-regulated plant responses. Proc. Natl. Acad. Sci. USA **98,** 4788-4793.

**Seo, S., Sano, H., and Ohashi, Y.** (1999). Jasmonate-based wound signal transduction requires activation of WIPK, a tobacco mitogen-activated protein kinase. Plant Cell **11,** 289-298.

**Seo, S., Okamoto, M., Seto, H., Ishizuka, K., Sano, H., and Ohashi, Y.** (1995). Tobacco MAP kinase: a possible mediator in wound signal transduction pathways. Science **270,** 1988-1992.

**Sessions, A., Burke, E., Presting, G., Aux, G., McElver, J., Patton, D., Dietrich, B., Ho, P., Bacwaden, J., Ko, C., Clarke, J.D., Cotton, D., Bullis, D., Snell, J., Miguel, T., Hutchison, D., Kimmerly, B., Mitzel, T., Katagiri, F., Glazebrook, J., Law, M., and Goff, S.A.** (2002). A high-throughput *Arabidopsis* reverse genetics system. Plant Cell **14,** 2985-2994.

**Shah, J.** (2003). The salicylic acid loop in plant defense. Curr. Opin. Plant Biol. **6,** 365-371.

**Shah, J., Tsui, F., and Klessig, D.F.** (1997). Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. Mol. Plant-Microbe Interact. **10,** 69-78.

**Shapiro, A.D., and Zhang, C.** (2001). The role of NDR1 in avirulence gene-directed signaling and control of programmed cell death in *Arabidopsis*. Plant Physiol. **127,** 1089-1101.

**Sharma, P.C., Ito, A., Shimizu, T., Terauchi, R., Kamoun, S., and Saitoh, H.** (2003). Virus-induced silencing of *WIPK* and *SIPK* genes reduces resistance to a bacterial pathogen, but has no effect on the INF1-induced hypersensitive response (HR) in *Nicotiana benthamiana*. Mol. Genet. Genomics **269,** 583-591.

**Shirano, Y., Kachroo, P., Shah, J., and Klessig, D.F.** (2002). A gain-of-function mutation in an *Arabidopsis* Toll Interleukin1 receptor-nucleotide binding site-leucine-rich repeat type R gene triggers defense responses and results in enhanced disease resistance. Plant Cell **14,** 3149-3162.

**Shirasu, K., Nakajima, H., Rajasekhar, V.K., Dixon, R.A., and Lamb, C.** (1997). Salicylic acid potentiates an agonist-dependent gain control that amplifies pathogen signals in the activation of defense mechanisms. Plant Cell **9,** 261-270.

**Simpson, S.D., Ashford, D.A., Harvey, D.J., and Bowles, D.J.** (1998). Short chain oligogalacturonides induce ethylene production and expression of the gene encoding aminocyclopropane 1-carboxylic acid oxidase in tomato plants. Glycobiology **8,** 579-583.

**Simpson, T.D., and Gardner, H.W.** (1995). Allene oxide synthase and allene oxide cyclase, enzymes of the jasmonic acid pathway, localized in glycine max tissues. Plant Physiol. **108,** 199-202.

**Singer, T., and Burke, E.** (2003). High-throughput TAIL-PCR as a tool to identify DNA flanking insertions. Meth. Mol. Biol. **236,** 241-272.

**Solano, R., and Ecker, J.R.** (1998). Ethylene gas: perception, signaling and response. Curr. Opin. Plant Biol. **1,** 393-398.

**Solano, R., Stepanova, A., Chao, Q., and Ecker, J.R.** (1998). Nuclear events in ethylene signaling: a transcriptional cascade mediated by *ETHYLENE-INSENSITIVE3* and *ETHYLENE-RESPONSE-FACTOR1*. Genes Dev. **12,** 3703-3714.

**Song, W.Y., Sohn, E.J., Martinoia, E., Lee, Y.J., Yang, Y.Y., Jasinski, M., Forestier, C., Hwang, I., and Lee, Y.** (2003). Engineering tolerance and accumulation of lead and cadmium in transgenic plants. Nat. Biotechnol. **21,** 914-919.

**Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., and et al.** (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, *Xa21*. Science **270,** 1804-1806.

**Spoel, S.H., Koornneef, A., Claessens, S.M.C., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Metraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X.N., and Pieterse, C.M.J.** (2003). NPR1 modulates cross-talk between salicylate- and jasmonate-dependent defense pathways through a novel function in the cytosol. Plant Cell **15,** 760-770.

**Stachel, S.E., and Zambryski, P.C.** (1986a). *virA* and *virG* control the plant-induced activation of the T-DNA transfer process of *A. tumefaciens*. Cell **46,** 325-333.

**Stachel, S.E., and Zambryski, P.C.** (1986b). *Agrobacterium tumefaciens* and the susceptible plant cell: a novel adaptation of extracellular recognition and DNA conjugation. Cell **47,** 155-157.

**Stachel, S.E., Nester, E.W., and Zambryski, P.C.** (1986). A plant cell factor induces *Agrobacterium tumefaciens* vir gene expression. Proc. Natl. Acad. Sci. USA **83,** 379-383.

**Staples, R.C., and Mayer, A.M.** (1995). Putative virulence factors of *Botrytis cinerea* acting as a wound pathogen. Fems Microb. Lett. **134,** 1-7.

**Staskawicz, B.J.** (2001). Genetics of plant-pathogen interactions specifying plant disease resistance. Plant Physiol. **125,** 73-76.

**Staswick, P.E., and Tiryaki, I.** (2004). The oxylipin signal jasmonic acid is activated by an enzyme that conjugates it to isoleucine in *Arabidopsis*. Plant Cell **16,** 2117-2127.

**Staswick, P.E., Su, W., and Howell, S.H.** (1992). Methyl jasmonate inhibition of root growth and induction of a leaf protein are decreased in an *Arabidopsis thaliana* mutant. Proc. Natl. Acad. Sci. USA **89,** 6837-6840.

**Staswick, P.E., Yuen, G.Y., and Lehman, C.C.** (1998). Jasmonate signaling mutants of *Arabidopsis* are susceptible to the soil fungus *Pythium irregulare*. Plant J. **15,** 747-754.

**Staswick, P.E., Tiryaki, I., and Rowe, M.L.** (2002). Jasmonate response locus *JAR1* and several related *Arabidopsis* genes encode enzymes of the firefly luciferase superfamily that show activity on jasmonic, salicylic, and indole-3-acetic acids in an assay for adenylation. Plant Cell **14,** 1405-1415.

**Stintzi, A., Weber, H., Reymond, P., Browse, J., and Farmer, E.E.** (2001). Plant defense in the absence of jasmonic acid: the role of cyclopentenones. Proc. Natl. Acad. Sci. USA **98,** 12837-12842.

**Stotz, H.U., Bishop, J.G., Bergmann, C.W., Koch, M., Albersheim, P., Darvill, A.G., and Labavitch, J.M.** (2000). Identification of target amino acids that affect interactions of fungal polygalacturonases and their plant inhibitors. Physiol. Mol. Plant Pathol. **56,** 117-130.

**Stracke, R., Werber, M., and Weisshaar, B.** (2001). The R2R3-MYB gene family in *Arabidopsis thaliana*. Curr. Opin. Plant Biol. **4,** 447-456.

**Strassner, J., Schaller, F., Frick, U.B., Howe, G.A., Weiler, E.W., Amrhein, N., Macheroux, P., and Schaller, A.** (2002). Characterization and cDNA-microarray expression analysis of 12-oxophytodienoate reductases reveals differential roles for octadecanoid biosynthesis in the local versus the systemic wound response. Plant J. **32,** 585-601.

**Tameling, W.I.L., Elzinga, S.D.J., Darmin, P.S., Vossen, J.H., Takken, F.L.W., Haring, M.A., and Cornelissen, B.J.C.** (2002). The tomato R gene products I-2 and Mi-1 are functional ATP binding proteins with ATPase activity. Plant Cell **14,** 2929-2939.

**Tang, X.Y., Frederick, R.D., Zhou, J.M., Halterman, D.A., Jia, Y.L., and Martin, G.B.** (1996). Initiation of plant disease resistance by physical interaction of AvrPto and Pto kinase. Science **274,** 2060-2063.

**Tani, H., Chen, X., Nurmberg, P., Grant, J.J., SantaMaria, M., Chini, A., Gilroy, E., Birch, P.R., and Loake, G.J.** (2004). Activation tagging in plants: a tool for gene discovery. Funct. Integr. Genomics **4,** 258-266.

**ten Have, A., Mulder, W., Visser, J., and van Kan, J.A.** (1998). The endopolygalacturonase gene *Bcpg1* is required for full virulence of *Botrytis cinerea*. Mol. Plant-Microbe Interact. **11,** 1009-1016.

**ten Have, A., Breuil, W.O., Wubben, J.P., Visser, J., and van Kan, J.A.** (2001). *Botrytis cinerea* endopolygalacturonase genes are differentially expressed in various plant tissues. Fungal Genet. Biol. **33,** 97-105.

**ten Have, A., Dekkers, E., Kay, J., Phylip, L.H., and van Kan, J.A.** (2004). An aspartic proteinase gene family in the filamentous fungus *Botrytis cinerea* contains members with novel features. Microbiology **150,** 2475-2489.

**ten Have, A., Tenberge, K., Benen, J.A.E., Tudzynski, P., Visser, J. & van Kan, J.A.L.** (2002). The contribution of cell wall degrading enzymes to pathogenesis of fungal plant pathogens. The Mycota: Application in Agriculture **XI,** 341-358.

**Thaler, J.S., Karban, R., Ullman, D.E., Boege, K., and Bostock, R.M.** (2002). Cross-talk between jasmonate and salicylate plant defense pathways: effects on several plant parasites. Oecologia **131,** 227-235.

**Thara, V.K., Tang, X., Gu, Y.Q., Martin, G.B., and Zhou, J.M.** (1999). *Pseudomonas syringae* pv tomato induces the expression of tomato EREBP-like genes *pti4* and *pti5* independent of ethylene, salicylate and jasmonate. Plant J. **20,** 475-483.

**Theodoris, G., Inada, N., and Freeling, M.** (2003). Conservation and molecular dissection of *ROUGH SHEATH2* and *ASYMMETRIC LEAVES1* function in leaf development. Proc. Natl. Acad. Sci. USA **100,** 6837-6842.

**Thitamadee, S., Tuchihara, K., and Hashimoto, T.** (2002). Microtubule basis for left-handed helical growth in *Arabidopsis*. Nature **417,** 193-196.

**Thomma, B., Eggermont, K., Penninckx, I., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broekaert, W.F.** (1998). Separate jasmonate-dependent and salicylate-dependent defense-response pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. USA **95,** 15107-15111.

**Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F.** (1999a). Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiol. **121,** 1093-1102.

**Thomma, B.P., Nelissen, I., Eggermont, K., and Broekaert, W.F.** (1999b). Deficiency in phytoalexin production causes enhanced susceptibility of *Arabidopsis thaliana* to the fungus *Alternaria brassicicola*. Plant J. **19,** 163-171.

**Thomma, B.P.H.J., Tadesse, Y.S.H., Jacobs, M., and Broekaert, W.F.** (1999c). Disturbed correlation between fungal biomass and beta-glucuronidase activity in infections of *Arabidopsis thaliana* with transgenic *Alternaria brassicicola*. Plant Science **148,** 31-36.

**Thomma, B.P.H.J., Eggermont, K., Broekaert, W.F., and Cammue, B.P.A.** (2000). Disease development of several fungi on *Arabidopsis* can be reduced by treatment with methyl jasmonate. Plant Physiol. Biochem. **38,** 421-427.

**Thordal-Christensen, H.** (2003). Fresh insights into processes of nonhost resistance. Curr. Opin. Plant. Biol. **6,** 351-357.

**Thordal-Christensen, H., Zhang, Z.G., Wei, Y.D., and Collige, D.B.** (1997). Subcellular localization of $H_2O_2$ in plants. $H_2O_2$ accomolation in papille and hypersensitive response during the barley powdery mildew interaction. Plant J. **11,** 1187-1194.

**Tiedemann, A.V.** (1997). Evidence for a primary role of active oxigen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. Physiol. Mol. Plant Pathol. **50,** 151-166.

**Tierens, K.F., Thomma, B.P., Bari, R.P., Garmier, M., Eggermont, K., Brouwer, M., Penninckx, I.A., Broekaert, W.F., and Cammue, B.P.** (2002). *Esa1*, an *Arabidopsis* mutant with enhanced susceptibility to a range of necrotrophic fungal pathogens, shows a distorted induction of defense responses by reactive oxygen generating compounds. Plant J. **29,** 131-140.

**Ton, J., Van Pelt, J.A., Van Loon, L.C., and Pieterse, C.M.** (2002). Differential effectiveness of salicylate-dependent and jasmonate/ethylene-dependent induced resistance in *Arabidopsis*. Mol. Plant-Microbe Interact. **15,** 27-34.

**Torii, K.U., Mitsukawa, N., Oosumi, T., Matsuura, Y., Yokoyama, R., Whittier, R.F., and Komeda, Y.** (1996). The *Arabidopsis ERECTA* gene encodes a putative receptor protein kinase with extracellular leucine-rich repeats. Plant Cell **8,** 735-746.

**Tsiantis, M., Brown, M.I., Skibinski, G., and Langdale, J.A.** (1999). Disruption of auxin transport is associated with aberrant leaf development in maize. Plant Physiol. **121,** 1163-1168.

**Turner, J.G., Ellis, C., and Devoto, A.** (2002). The jasmonate signal pathway. Plant Cell **14 Suppl,** S153-164.

**Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E., and Ryals, J.** (1992). Acquired resistance in *Arabidopsis*. Plant Cell **4,** 645-656.

**Urban, M., Bhargava, T., and Hamer, J.E.** (1999). An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. Embo J. **18,** 512-521.

**Valette-Collet, O., Cimerman, A., Reignault, P., Levis, C., and Boccara, M.** (2003). Disruption of *Botrytis cinerea* pectin methylesterase gene *Bcpme1* reduces virulence on several host plants. Mol. Plant-Microbe Interact. **16,** 360-367.

**Van der Biezen, E.A., and Jones, J.D.** (1998). Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem. Sci. **23,** 454-456.

**van der Fits, L., and Memelink, J.** (2000). ORCA3, a jasmonate-responsive transcriptional regulator of plant primary and secondary metabolism. Science **289,** 295-297.

**van der Graaff, E., Dulk-Ras, A.D., Hooykaas, P.J., and Keller, B.** (2000). Activation tagging of the *LEAFY PETIOLE* gene affects leaf petiole development in *Arabidopsis thaliana*. Development **127,** 4971-4980.

**van Wees, S.C., and Glazebrook, J.** (2003). Loss of non-host resistance of *Arabidopsis NahG* to *Pseudomonas syringae* pv. *phaseolicola* is due to degradation products of salicylic acid. Plant J. **33,** 733-742.

**van Wees, S.C., Luijendijk, M., Smoorenburg, I., van Loon, L.C., and Pieterse, C.M.** (1999). Rhizobacteria-mediated induced systemic resistance (ISR) in *Arabidopsis* is not associated with a direct effect on expression of known defense-related genes but stimulates the expression of the jasmonate-inducible gene *Atvsp* upon challenge. Plant Mol. Biol. **41,** 537-549.

**Vanacker, H., Lu, H., Rate, D.N., and Greenberg, J.T.** (2001). A role for salicylic acid and *NPR1* in regulating cell growth in *Arabidopsis*. Plant J. **28,** 209-216.

**Verburg, J.G., and Huynh, Q.K.** (1991). Purification and characterization of an antifungal chitinase from *Arabidopsis thaliana*. Plant Physiol. **95,** 450-455.

**Vernooij, B., Friedrich, L., Morse, A., Reist, R., Kolditz-Jawhar, R., Ward, E., Uknes, S., Kessmann, H., and Ryals, J.** (1994). Salicylic acid is not the translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell **6,** 959-965.

**Veronese, P., Chen, X., Bluhm, B., Salmeron, J., Dietrich, R., and Mengiste, T.** (2004). The *BOS* loci of *Arabidopsis* are required for resistance to *Botrytis cinerea* infection. Plant J. **40,** 558-574.

**Veronese, P., Ouaked, F., Hirt, H., Mengiste, T.** (2004). The *BIK1* gene of *Arabidopsis* encodes a protein kinase required for resistance to *Botrytis cinerea*. Abstract Book of the 15th International Conference on *Arabidopsis* Research **1,** T05-081.

**Vick, B., and Zimmerman, D.** (1978). Substrate specificity for plant biosynthesis of fatty acids containing a cyclopentenone ring. Plant Physiol. **61,** 70-70.

**Vick, B.A., and Zimmerman, D.C.** (1983). The biosynthesis of jasmonic acid - a physiological role for plant lipoxygenase. J. Amer. Oil Chem. Soc. **60,** 706-706.

**Vick, B.A., and Zimmerman, D.C.** (1984). Biosynthesis of jasmonic acid by several plant species. Plant Physiol. **75,** 458-461.

**Vierstra, R.D.** (2003). The ubiquitin/26S proteasome pathway, the complex last chapter in the life of many plant proteins. Trends Plant Sci. **8,** 135-142.

**Vijayan, P., Shockey, J., Levesque, C.A., Cook, R.J., and Browse, J.** (1998). A role for jasmonate in pathogen defense of *Arabidopsis*. Proc. Natl. Acad. Sci. USA **95,** 7209-7214.

**Viterbo, A., Yagen, B., and Mayer, A.M.** (1993). Cucurbitacins, attack enzymes and laccase in *Botrytis cinerea*. Phytochemistry **32,** 61-65.

**Vogel, J.P., Raab, T.K., Schiff, C., and Somerville, S.C.** (2002). *PMR6*, a pectate lyase-like gene required for powdery mildew susceptibility in *Arabidopsis*. Plant Cell **14,** 2095-2106.

**Waites, R., Selvadurai, H.R., Oliver, I.R., and Hudson, A.** (1998). The *PHANTASTICA* gene encodes a MYB transcription factor involved in growth and dorsoventrality of lateral organs in *Antirrhinum*. Cell **93,** 779-789.

**Wang, D., Weaver, N.D., Kesarwani, M., and Dong, X.N.** (2005). Induction of protein secretory pathway is required for systemic acquired resistance. Science **308,** 1036-1040.

**Wang, K.L., Li, H., and Ecker, J.R.** (2002). Ethylene biosynthesis and signaling networks. Plant Cell **14 Suppl,** S131-151.

**Wang, X.** (2004). Lipid signaling. Curr. Opin. Plant Biol. **7,** 329-336.

**Ward, E.R., Uknes, S.J., Williams, S.C., Dincher, S.S., Wiederhold, D.L., Alexander, D.C., Ahl-Goy, P., Metraux, J.P., and Ryals, J.A.** (1991). Coordinate gene activity in response to agents that induce systemic acquired resistance. Plant Cell **3,** 1085-1094.

**Weigel, D., Ahn, J.H., Blazquez, M.A., Borevitz, J.O., Christensen, S.K., Fankhauser, C., Ferrandiz, C., Kardailsky, I., Malancharuvil, E.J., Neff, M.M., Nguyen, J.T., Sato, S., Wang, Z.Y., Xia, Y., Dixon, R.A., Harrison, M.J., Lamb, C.J., Yanofsky, M.F., and Chory, J.** (2000). Activation tagging in *Arabidopsis*. Plant Physiol. **122,** 1003-1013.

**Whalen, M.C., Innes, R.W., Bent, A.F., and Staskawicz, B.J.** (1991). Identification of *Pseudomonas syringae* pathogens of *Arabidopsis* and a bacterial locus determining avirulence on both *Arabidopsis* and soybean. Plant Cell **3,** 49-59.

**Wiermer, M., Feys, B.J., and Parker, J.E.** (2005). Plant immunity: the *EDS1* regulatory node. Curr. Opin. Plant Biol. **8,** 383-389.

**Wildermuth, M.C., Dewdney, J., Wu, G., and Ausubel, F.M.** (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414,** 562-565.

**Williamson, B., Duncan, G.H., Harrison, J.G., Harding, L.A., Elad, Y., and Zimand, G.** (1995). Effect of humidity on infection of rose Petals by dry-inoculated conidia of *Botrytis cinerea*. Mycol. Res. **99,** 1303-1310.

**Wubben, J.P., Mulder, W., ten Have, A., van Kan, J.A., and Visser, J.** (1999). Cloning and partial characterization of endopolygalacturonase genes from *Botrytis cinerea*. Appl. Environ. Microbiol. **65,** 1596-1602.

**Xia, Y., Suzuki, H., Borevitz, J., Blount, J., Guo, Z., Patel, K., Dixon, R.A., and Lamb, C.** (2004). An extracellular aspartic protease functions in *Arabidopsis* disease resistance signaling. Embo J.

**Xiao, S., Ellwood, S., Calis, O., Patrick, E., Li, T., Coleman, M., and Turner, J.G.** (2001). Broad-spectrum mildew resistance in *Arabidopsis thaliana* mediated by *RPW8*. Science **291,** 118-120.

**Xiao, S.Y., Brown, S., Patrick, E., Brearley, C., and Turner, J.G.** (2003). Enhanced transcription of the *Arabidopsis* disease resistance genes *RPW8.1* and *RPW8.2* via a salicylic acid-dependent amplification circuit is required for hypersensitive cell death. Plant Cell **15,** 33-45.

**Xie, D.X., Feys, B.F., James, S., Nieto-Rostro, M., and Turner, J.G.** (1998). COI1: An Arabidopsis gene required for jasmonate-regulated defense and fertility. Science **280,** 1091-1094.

**Xu, L., Xu, Y., Dong, A., Sun, Y., Pi, L., Xu, Y., and Huang, H.** (2003). Novel *as1* and *as2* defects in leaf adaxial-abaxial polarity reveal the requirement for *ASYMMETRIC LEAVES1* and *2* and *ERECTA* functions in specifying leaf adaxial identity. Development **130,** 4097-4107.

**Xu, L., Liu, F., Lechner, E., Genschik, P., Crosby, W.L., Ma, H., Peng, W., Huang, D., and Xie, D.** (2002). The SCF(COI1) ubiquitin-ligase complexes are required for jasmonate response in *Arabidopsis*. Plant Cell **14,** 1919-1935.

**Xu, Y., Chang, P., Liu, D., Narasimhan, M.L., Raghothama, K.G., Hasegawa, P.M., and Bressan, R.A.** (1994). Plant defense genes are synergistically induced by ethylene and methyl jasmonate. Plant Cell **6,** 1077-1085.

**Yang, Y., Shah, J., and Klessig, D.F.** (1997). Signal perception and transduction in plant defense responses. Genes Dev. **11,** 1621-1639.

**Yuanxin, Y., Chengcai, A., Li, L., Jiayu, G., Guihong, T., and Zhangliang, C.** (2003). T-linker-specific ligation PCR (T-linker PCR): an advanced PCR technique for chromosome walking or for isolation of tagged DNA ends. Nucleic Acids Res. **31,** e68.

**Yun, B.W., Atkinson, H.A., Gaborit, C., Greenland, A., Read, N.D., Pallas, J.A., and Loake, G.J.** (2003). Loss of actin cytoskeletal function and EDS1 activity, in combination, severely compromises non-host resistance in *Arabidopsis* against wheat powdery mildew. Plant J. **34,** 768-777.

**Zhao, J., and Last, R.L.** (1996). Coordinate regulation of the tryptophan biosynthetic pathway and indolic phytoalexin accumulation in *Arabidopsis*. Plant Cell **8,** 2235-2244.

**Zhao, J., Williams, C.C., and Last, R.L.** (1998). Induction of *Arabidopsis* tryptophan pathway enzymes and camalexin by amino acid starvation, oxidative stress, and an abiotic elicitor. Plant Cell **10,** 359-370.

**Zhou, J.M., Trifa, Y., Silva, H., Pontier, D., Lam, E., Shah, J., and Klessig, D.F.** (2000). NPR1 differentially interacts with members of the TGA/OBF family of

transcription factors that bind an element of the PR-1 gene required for induction by salicylic acid. Mol. Plant-Microbe Interact. **13,** 191-202.

**Zhou, N., Tootle, T.L., and Glazebrook, J.** (1999). *Arabidopsis PAD3*, a gene required for camalexin biosynthesis, encodes a putative cytochrome P450 monooxygenase. Plant Cell **11,** 2419-2428.

**Zhou, N., Tootle, T.L., Tsui, F., Klessig, D.F., and Glazebrook, J.** (1998). PAD4 functions upstream from salicylic acid to control defense responses in *Arabidopsis*. Plant Cell **10,** 1021-1030.

**Ziegler, J., Hamberg, M., Miersch, O., and Parthier, B.** (1997). Purification and characterization of allene oxide cyclase from dry corn seeds. Plant Physiol. **114,** 565-573.

**Zien, C.A., Wang, C., Wang, X., and Welti, R.** (2001). In vivo substrates and the contribution of the common phospholipase D, PLDalpha, to wound-induced metabolism of lipids in *Arabidopsis*. Biochim. Biophys. Acta **1530,** 236-248.

**Zubko, E., Adams, C.J., Machaekova, I., Malbeck, J., Scollan, C., and Meyer, P.** (2002). Activation tagging identifies a gene from *Petunia hybrida* responsible for the production of active cytokinins in plants. Plant J. **29,** 797-808.