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Cuticular defects or wounding lead to full immunity to a major plant pathogen

THÈSE

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ABSTRACT

The cuticular coat on the aerial parts of plants is considered as a physical barrier that prevents water loss and protects against prospective invaders, excess irradiance or xenobiotics. The hydrophobic cuticular layer is mainly composed of cutin, a complex polymer rich in esterified fatty acid derivatives associated and coated with waxes. It is the first line of defence microbes come in contact with, through which cutinase-producing pathogens can penetrate [1, 2]. The cuticle breakdown products act as powerful signals for pathogens; they were shown to induce germination and appressorium formation [3, 4] and to trigger expression of the cutinase gene [5]. It is unknown, however, if plants can react to changes in the structure of the cuticle. This was tested by overexpressing a fungal cutinase of the pathogen *Fusarium solani* f.sp. *pisi* in *Arabidopsis thaliana* (CUTE plants) [6]. CUTE plants have a modified ultrastructure of the cuticle that lead to an increased permeability and to organ fusions, highlighting the importance of the cuticle for plant growth and development.

In this work, we show that degradation of the cuticular layer leads to full immunity to *Botrytis cinerea*, an ubiquitous fungal pathogen causing important damages to many crop plants. This powerful defence is independent of the known defence signalling routes involving salicylic acid, ethylene or jasmonic acid. Moreover, the strong resistance to *B. cinerea* is accompanied by the diffusion of a fungitoxic activity and changes in gene transcription. The fungitoxic activity was shown to diffuse from CUTE leaves, to have a presumed size of 1000 to 3500 Da and might be of proteinaceous nature.

Further insights in the changes in gene expression after inoculation with *B. cinerea* were obtained by genome-wide microarray analysis. The expression of several members of the lipid transfer protein (LTP), peroxidase (PER) and protease inhibitor (PI) gene families was found to be strongly enhanced in CUTE plants compared to wild-type (WT) plants. The involvement of those genes in the defence against *B. cinerea* was demonstrated by overexpressing them in susceptible WT plants, which led to increased resistance. In addition, different mutants with cuticular defects, like *lacerata* [7] and *bodyguard* [8], also displayed a strong resistance to *B. cinerea*, associated with the diffusion of the fungitoxic activity and the priming of the *LTP*, *PER* and *PI* genes. Modification of the cuticle can thus activate a multi-layered resistance syndrome that reveals a novel defence pathway leading to complete resistance to *B. cinerea*.

Full immunity to *B. cinerea* was also observed in *A. thaliana* following wounding of the leaf. The resistance was delimited at the wound site and was not associated with salicylic acid-, ethylene- or jasmonic acid defence responses. The fungitoxic phytoalexin camalexin was found to be involved in this resistance, since camalexin-deficient mutants were susceptible after wounding and inoculation. Wounding was shown to prime camalexin accumulation after inoculation with *B. cinerea*. Glutathione was also found to be required for the resistance, as

mutants deficient in glutathione biosynthesis showed susceptibility to *B. cinerea* after wounding, indicating that basal levels of glutathione are needed for the wound-induced resistance. These results demonstrate how an abiotic stress can induce full immunity to the virulent fungus *B. cinerea*.

1. Kolattukudy, P.E. (1985). Enzymatic penetration of the plant cuticle by fungal pathogens. Annu. Rev. Phytopathol. 23, 223-250.
2. Köller, W., Yao, C., Trail, F., and Parker, D.M: (1995). Role of cutinase in the invasion of plants. Can. J. Bot. 73, 1109-1118.
3. Gilbert, R.D., Johnson, A.M., and Dean, R.A. (1996). Chemical signals responsible for appressorium formation in rice blast fungus *Magnaporthe grisea*. Physiol. Mol. Plant Pathol. 48, 335-346.
4. Francis, S.A., Dewey, F.M., and Gurr, S.J. (1996). The role of cutinase in germling development and infection by *Erysiphe graminis* f.sp. *hordei*. Physiol. Mol. Plant Pathol. 49, 201-211.
5. Kolattukudy, P.E., Rogers, L.M., Li, D., Hwang, C.S., and Flaishman, M.A. (1995). Surface signaling in pathogenesis. Proc. Natl. Acad. Sci. 92, 4080-4087.
6. Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Métraux, J.-P., and Nawrath, C. (2000). Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell 12, 721-738.
7. Wellesen, K., Durst, F., Pinot, F., Benveniste, I., Nettesheim, K., Wisman, E., Steiner-Lange, S., Saedler, H., and Yephremov, A. (2001). Functional analysis of the *LACERATA* gene of *Arabidopsis* provides evidence for different roles of fatty acid ω-hydroxylation in development. Proc. Natl. Acad. Sci. 98, 9694-9699.
8. Kurdyukov, S., Faust, A., Nawrath, C., Bär, S., Voisin, D., Efremova, N., Franke, R., Schreiber, L., Saedler, H., Métraux, J.-P., and Yephremov, A. (2006). The epidermis-specific extracellular *BODYGUARD* controls cuticle development and morphogenesis in *Arabidopsis*. Plant Cell 18, 321-339.

RESUME

Toutes les parties aériennes des plantes sont recouvertes par la cuticule, une barrière physique qui empêche l'évaporation de l'eau et protège contre les microorganismes, l'excès de radiations et les xénobiotiques. Cette couche cuticulaire est principalement composée de cutine, un polymère complexe de dérivés d'acides gras inter-estérifiés, associé et recouvert de cires. Elle représente la première ligne de défense avec laquelle les microorganismes sont en contact, que certains pathogènes producteurs de cutinase peuvent cependant traverser [1, 2]. Les produits de dégradation de la cuticule servent de signaux pour les pathogènes. Ainsi, on a montré qu'ils pouvaient induire la germination et la differentiation de l'appressorium chez certains champignons [3, 4], ainsi que l'expression du gène de la cutinase [5]. On ignore cependant si les plantes réagissent elles aussi à des modifications de la structure de leur cuticule. Ceci a été testé à l'aide de plantes d'*Arabidopsis thaliana* transgéniques exprimant une cutinase fongique du pathogène *Fusarium solani* f.sp. *pisi* (plantes CUTE) [6]. La structure de la cuticule des plantes CUTE est perturbée, menant à une augmentation de la perméabilité cuticulaire et à la formation de fusions d'organes, mettant ainsi en évidence l'importance de la cuticule pour la croissance et le développement des plantes.

Ce travail montre que la dégradation de la couche cuticulaire induit une résistance totale contre *Botrytis cinerea*, un champignon très répandu causant d'importants dommages à de nombreuses plantes de culture. Cette forte immunité est indépendante des voies connues de signalisation de défense impliquant l'acide salicylique, l'éthylène ou l'acide jasmonique. De plus, la résistance à *B. cinerea* est accompagnée par la diffusion d'une activité fongitoxique et par des changements d'expression génique. L'activité fongitoxique est capable de diffuser à travers la couche cuticulaire plus perméable des plantes CUTE, aurait une taille de 1000 à 3500 Da et semble être de nature protéique.

Des informations plus approfondies sur les changements d'expression génique provoqués par *B. cinerea* ont été obtenues par l'analyse de puces d'ADN de tout le génôme d'*A*. *thaliana*. Le niveau d'expression de plusieurs membres des familles de gènes codant pour des protéines de transfert de lipides (LTP), des peroxidases (PER) et des inhibiteurs de protéases (PI) s'est révélé plus intense dans les plantes CUTE que dans les plantes de type sauvage. La surexpression de ces gènes dans des plantes de type sauvage normalement susceptibles a procuré une augmentation de la résistance à *B. cinerea*, démontrant ainsi le rôle de ces gènes dans la résistance contre *B. cinerea*. De plus, les mutants *lacerata* [7] et *bodyguard* [8], présentant des cuticules défectueuses, montrent également une résistance accrue à *B. cinerea*, associée à la diffusion de l'activité fongitoxique et au conditionnement de l'expression des gènes *LTP*, *PER* et *PI*. Ainsi, cette dernière observation renforce l'idée que des

modifications cuticulaires puissent activer un syndrome de résistance à plusieurs niveaux, révélant une nouvelle voie de défense menant à une immunité totale contre *B. cinerea*.

Une forte immunité contre *B. cinerea* a également été obtenue en appliquant une blessure sur les feuilles d'*A*. *thaliana*. Cette résistance est limitée au site de la blessure et n'est pas associée aux voies de défense dépendantes de l'acide salicylique, de l'éthylène ou de l'acide jasmonique. La camalexine, principale phytoalexine d'*A*. *thaliana*, est impliquée dans cette résistance, puisque des mutants déficients en camalexine sont moins protégés par la blessure contre *B. cinerea*. Il a également été montré que la blessure conditionne la plante à accumuler de la camalexine après inoculation avec *B. cinerea*. L'instauration de la résistance induite par la blessure nécessite aussi un taux normal de glutathion, puisque des mutants déficients dans la biosynthèse de glutathion sont moins protégés par la blessure que des plantes de type sauvage. Ces résultats démontrent comment l'application d'un stress abiotique peut induire une immunité totale contre le pathogène *B. cinerea*.

1. Kolattukudy, P.E. (1985). Enzymatic penetration of the plant cuticle by fungal pathogens. Annu. Rev. Phytopathol. 23, 223-250.
2. Köller, W., Yao, C., Trail, F., and Parker, D.M: (1995). Role of cutinase in the invasion of plants. Can. J. Bot. 73, 1109-1118.
3. Gilbert, R.D., Johnson, A.M., and Dean, R.A. (1996). Chemical signals responsible for appressorium formation in rice blast fungus *Magnaporthe grisea*. Physiol. Mol. Plant Pathol. 48, 335-346.
4. Francis, S.A., Dewey, F.M., and Gurr, S.J. (1996). The role of cutinase in germling development and infection by *Erysiphe graminis* f.sp. *hordei*. Physiol. Mol. Plant Pathol. 49, 201-211.
5. Kolattukudy, P.E., Rogers, L.M., Li, D., Hwang, C.S., and Flaishman, M.A. (1995). Surface signaling in pathogenesis. Proc. Natl. Acad. Sci. 92, 4080-4087.
6. Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Métraux, J.-P., and Nawrath, C. (2000). Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell 12, 721-738.
7. Wellesen, K., Durst, F., Pinot, F., Benveniste, I., Nettesheim, K., Wisman, E., Steiner-Lange, S., Saedler, H., and Yephremov, A. (2001). Functional analysis of the *LACERATA* gene of *Arabidopsis* provides evidence for different roles of fatty acid ω-hydroxylation in development. Proc. Natl. Acad. Sci. 98, 9694-9699.
8. Kurdyukov, S., Faust, A., Nawrath, C., Bär, S., Voisin, D., Efremova, N., Franke, R., Schreiber, L., Saedler, H., Métraux, J.-P., and Yephremov, A. (2006). The epidermis-specific extracellular *BODYGUARD* controls cuticle development and morphogenesis in *Arabidopsis*. Plant Cell 18, 321-339.

CONTENTS

ABBREVIATIONS

avr	avirulence factor
CUTE	transgenic plants expressing a fungal cutinase constitutively
dpi	days post-inoculation
ET	ethylene
JA	jasmonate
hpi	hours post-inoculation
HR	hypersensitive response
MAPK	mitogen-activated protein kinase
MWCO	molecular weight cut-off
NO	nitric oxide
PAMP	pathogen-associated molecular pattern
PGIP	polygalacturonase-inhibiting protein
PR	pathogenesis-related protein
R	resistance factor
ROI	reactive oxygen intermediate
SA	salicylic acid
SAR	systemic acquired resistance
WT	wild-type plants

I. General Introduction

Plants are attacked by a broad range of microbes like bacteria, fungi, oomycetes, viruses, nematodes or insects. They have elaborated a multi-layered system of defence, comprising physical and chemical barriers, which are either preformed or induced after the infection. The hydrophobic cuticular layer covering all aerial plant organs is the first line of defence microbes have to overcome. Some pathogens, and in particular necrotrophic fungi like *Botrytis cinerea*, produce enzymes that can degrade this plant barrier. At the very beginning of the infection process, plants are able to perceive the presence of a pathogen by sensing the degradation of their cuticular layer and to switch on a complex programme of defence. This work focuses on the defence responses induced in *Arabidopsis thaliana* upon degradation of its leaf surface, either enzymatically by the action of fungal cutinases that breach the cuticle, or mechanically by wounding.

I.1 Types of plant resistance

The most common case in nature occurs when a plant encounters a pathogen to which it is not a host. This resistance shown by an entire plant species to a specific parasite or pathogen is known as non-host resistance, and is expressed by every plant towards the majority of potentially pathogenic microbes (Heath, 1985). The non-host resistance may be determined by preformed barriers; but it often depends on active responses following recognition of the pathogen or its activities as it attempts to penetrate the plant (Jones and Takemoto, 2004).

Some pathogens are known to exhibit a total lack of specialization and infect a wide host range, as exemplified by most necrotrophic fungi or bacteria that kill colonized plant tissues. Basal resistance inhibits pathogen spread after successful infection and onset of disease (Dangl and Jones, 2001). The induction of basal resistance to invasive pathogens is a crucial protection layer. The numerous *Arabidopsis* mutants that are compromised in basal defences to virulent pathogens points to the involvement of many genes in maintaining this resistance layer and to the existence of numerous potential targets that the pathogen might disable to promote disease (Hammond-Kosack and Parker, 2003; Wiermer et al., 2005). Thus, disease results either from the failure of the recognition event or the ability of the pathogen to avoid or overcome the resistance response. Treatments with abiotic or biotic stimuli prior to inoculation can enhance this basal resistance, this is referred to as induced resistance. In many cases, induced resistance is not only expressed locally at the site of treatment but also systemically, in other parts of the plant. This is termed systemic induced resistance or systemic acquired resistance.

During evolution specific plant species resistance was overcome by individual pathogens strains (or races) by the acquisition of virulence factors, which enabled them to either evade or

suppress plant defence mechanisms. As a consequence, individual plant cultivars have evolved resistance genes that recognize specific pathogen strains and allow the plant to resist infection (Nürnberger et al., 2004). This type of resistance is called gene-for-gene resistance, R-gene-mediated resistance or cultivar-specific resistance. Many plant-pathogen interactions, especially those involving biotrophs, are governed by specific interactions between the products of pathogen *avr* (avirulence) gene loci and alleles of the corresponding products of a plant disease resistance (*R*) locus. When corresponding *R* and *avr* genes are present in both host and pathogen, the resistance is triggered (incompatibility). If either gene is inactive or absent, disease occurs (compatibility) (Flor, 1971).

The spectrum of reactions elicited in plants undergoing either type of resistance is complex but nevertheless strikingly similar (Nürnberger et al., 2004). The most common expression of host resistance, and a frequent expression of non-host resistance, is the hypersensitive response (HR), a rapid death of cells at the infection site that is associated with defence gene activation (Goodman and Novacky, 1994). Specific R-mediated innate immunity is superimposed onto one or more basal defence pathways. Genetic overlap between specific and basal resistance responses suggests that one function of R-mediated signalling might more rapidly and effectively activate defence mechanisms that are shared by both pathways (Dangl and Jones, 2001).

I.2 Preformed defences

The plant surface is the first line of defence that microbes come in contact with. The epidermal cell layer is covered on all aerial parts with a hydrophobic cuticle mainly composed of wax and cutin. The plant cuticle has multiple roles, including the regulation of epidermal permeability, non-stomatal water loss and protection against insects, pathogens, UV light and frost. The cuticular layer is made of cutin and intra- and epicuticular waxes. The cutin polymer is mainly composed of 16 and 18 carbon interesterified hydroxy and epoxy-hydroxy fatty acids (Nawrath, 2002). Waxes consist of very-long-chain fatty acids, alcohols, aldehydes, alkanes, ketones, and esters (Kunst and Samuels, 2003). The cuticle represents a strong barrier for microbes that do not produce enzymes to degrade it. Such microbes might use natural openings like stomata or wounds to enter the plant. However, some phytopathogenic fungi are able to penetrate through the cuticle by secreting cutinases (Soliday et al., 1984).

The cell wall of epidermal cells also represents a barrier for phytopathogenic fungi. The high molecular weight polysaccharides that compose the cell wall are cross-linked by ionic and covalent bonds into a network that resists physical penetration (Carpita and McCann, 2000). Plant cell walls are highly hydrated and gel-like. They are mainly composed of cellulose (β-1,4-

linked glucose) and hemicellulose, of pectin in dicotyledons (mostly polygalacturonic acid) and of structural proteins. Some pathogens, like necrotrophic fungi, are able to directly penetrate the cell wall by producing various cell-wall degrading enzymes that relax the cell wall structure and finally lead to tissue maceration (Vorwerk et al., 2004).

Cells walls are a reservoir of secondary metabolites and antimicrobial proteins. Phytoanticipins are low-molecular weight, antimicrobial compounds that are present in plants before challenge by microorganisms (Van Etten et al., 1994). Such antimicrobial compounds, like phenolics, tannins or saponins, may determine the host range of some fungal pathogens (Morrissey and Osbourn, 1999). In contrast, antimicrobial compounds that accumulate upon pathogen stress are called phytoalexins. Preformed antimicrobial proteins can act as inhibitors of pathogenic enzymes, like proteinase inhibitors (Shewry and Lucas, 1997) or polygalacturonase-inhibiting proteins (PGIPs) (De Lorenzo et al., 2001; De Lorenzo, 2002). They also include hydrolytic enzymes that may cause the breakdown of pathogen cell-wall components, like for example chitinases (Robinson et al., 1997) and glucanases (Beffa and al., 1996).

I.3 Perception of the pathogen

I.3.1 Elicitors

The timely perception of the pathogen by the plant is central to the activation of defence responses. Plants have complex innate mechanisms to recognize pathogenic microorganisms. The pathogen must either penetrate the plant cell wall and / or the membrane, or locally diffuse effectors or elicitors that are perceived by the host (Gòmez-Gòmez, 2004). An elicitor can be broadly defined as a biotic or an abiotic treatment that induces defence reactions in plants. An abiotic elicitor may be of physical or chemical nature, while a biotic elicitor is derived from a biological source. Elicitors from a variety of different plant pathogenic microbes have been characterized and shown to trigger defence responses in intact plants or cultured plant cells.

Microbe-associated hydrolytic enzyme activities have been found to release elicitors of plant defence by limited degradation of the plant cell wall, such as oligogalacturonides (Shibuya and Minami, 2001). Thus, plants do not only recognize and respond to exogenous pathogen-derived signals but also to endogenous plant-derived structures.

Pathogen elicitors fall into two broad categories, specific and non-specific elicitors. The specific elicitors are so-called avr factors that are unique for a particular pathogen strain. Avr factors are produced by pathogens and function in host cells invasion, colony formation, avoidance of host immune responses, or in the adjustment to new nutrient sources (Van der Biezen and Jones, 1998). Many pathogen avr proteins are pathogenicity factors with virulence

effector functions that can actively or passively suppress host defences (Jones and Takemoto, 2004).

The non-specific elicitors are constitutively present in the pathogen. They are essential and therefore very conserved within a class of microbes. This broader, more basal, plant perception system mediates the activation of plant defence responses in a non-cultivar specific manner (Boller, 1995). These non-specific elicitors might be the prime inducers of defence responses in non-host plant-pathogen interactions (Heath, 2000). Elicitors like surface-derived structural molecules from pathogens, such as fungal cell-wall constituents (chitin, glucan, protein and glycoprotein), bacterial lipopolysaccharide or flagellin (Gòmez-Gòmez and Boller, 2002; Erbs and Newman, 2003; Montesano et al., 2003) are conceptually similar to PAMPs (pathogen-associated molecular patterns) described for mammals and *Drosophila* (Parker, 2003). However, non-pathogens also synthesize these molecules, so “pathogen-associated” is a misnomer and “microbe-associated” would be a more precise term (Ausubel, 2005).

I.3.2 Plant receptors

Many R genes have been identified in model and crop species. Despite the wide range of pathogen taxa and their presumed pathogenicity effector molecules, R genes encode only five classes of proteins (Dangl and Jones, 2001). The most important resistance proteins include the (NBS)-LRR proteins (nucleotide-binding site leucine-rich repeat proteins) and, to a lesser extent, LRR-RLK proteins (LRR-receptor-like kinases) and membrane-anchored LRR-RLP proteins (LRR-receptors-like proteins) (Martin et al., 2003). Whereas the NBS-LRRs are involved in cytosolic perception, the LRR-RLKs and LRR-RLPs are involved in extracytosolic perception of various ligands, including pathogen molecules (Jones and Takemoto, 2004).

Direct interactions between avr factors and R receptors have rarely been shown (Deslandes et al., 2003). Dangl and Jones (2001) postulated the “guard hypothesis”; it proposes other factors that participate in the recognition of the pathogen avirulence factor. The interaction between an R protein and its cognate avr determinant is mediated by a host protein that is the target for the effector function of the avr determinant, and under R-protein surveillance for such interference (Van der Biezen and Jones, 1998). Plant receptors for general elicitors might represent a subclass of R proteins that are capable of recognizing PAMPs rather than race-specific pathogen effectors (Parker, 2003).

I.4 Inducible defences

Upon pathogen detection, plants activate a number of defences. Many inducible defence responses are involved in the expression of both host and non-host resistance (Heath, 2000) (Figure 1). The earliest events following pathogen perception are calcium influx, alkalinization of the extracellular space, production of reactive oxygen intermediates (ROIs) and nitric oxide (NO), cell-wall cross-linking, protein kinase activation and transcriptional reprogramming. Transcriptional reprogramming establishes the “effector” arm of the plant innate immune system (Dangl and Jones, 2001). Resistance mechanisms induced by recognition of general and specific elicitors share similar signal transduction pathways. The MAPK (mitogen-activated protein kinase) cascade (see below) is a convergence point leading to both types of resistance (Zhang and Klessig, 2001).

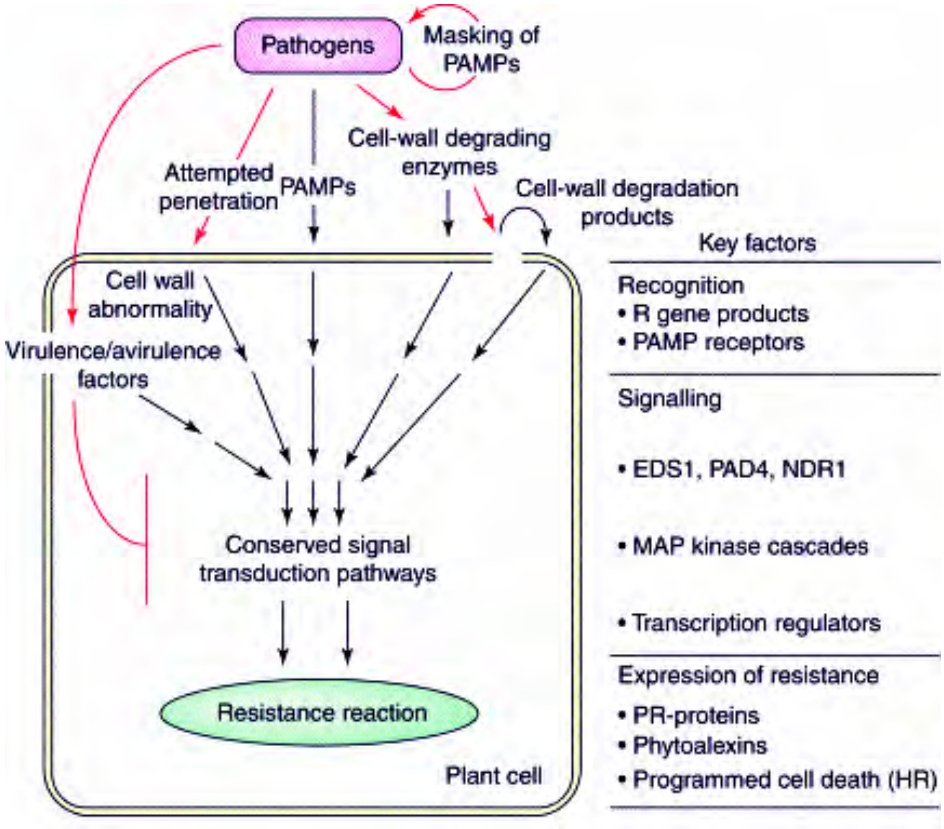

Figure 1. Plant signalling responses induced by various pathogen elicitors. This model illustrates the possible integration of non-host and host-specific resistance signalling pathways. Red arrows indicate pathogen strategies for infection and black arrows indicate plant signalling for resistance (modified from Jones and Takemoto, 2004).

I.4.1 Early events

The early responses to pathogen invasion have been studied with plant cell cultures challenged with pathogens or purified elicitors. The earliest detectable cellular events are ion fluxes across the plasma membrane. An increase in cytosolic calcium was shown specifically in

gene-for-gene interactions (Grant et al., 2000). This phenomenon preceeds the burst of oxygen metabolism that produces ROIs, such as O_2^- and H_2O_2 (Lamb and Dixon, 1997). Cells of many plant species have the potential to cause an oxidative burst as the very initial reaction leading to induced resistance (Doke, 1983). H_2O_2 can have two functions in plants: at low concentrations, it acts as a signalling molecule (Karpinsky et al., 1999; Dat et al., 2000), and at high concentrations as inducer of cell death (Dat et al., 2003). Indeed, exogenous application of ROIs can induce cell death in plants (Levine et al., 1996). However ROIs, although necessary, are not sufficient to trigger cell death and must therefore require accomplices. Studies have provided evidence that NO interacts with ROIs to induce HR and defence gene expression (McDowell and Dangl, 2000). ROIs can also be directly toxic to pathogens.

Other plant responses associated with pathogen defence result from allosteric enzyme activation initiating cell-wall reinforcement by oxidative cross-linking of cell-wall components (Lamb and Dixon, 1997) and apposition of callose (Stone and Clarke, 1992). H_2O_2 acts as a substrate for the rapid oxidative cross-linking of cell-wall proteins and lignification, making cell walls more difficult to penetrate. Localized appositions of callose (β-1,3-glucan) in the cell wall beneath fungal penetration sites are called papillae.

MAPKs link stimuli that are activated by external sensors to cellular responses. MAPK cascades are minimally composed of three kinase modules, MAPKKK, MAPKK and MAPK, which are linked in various ways to upstream receptors and downstream targets, and are activated by phosphorylation. The phylogenetic analysis of potential MAPK cascades in *Arabidopsis* has revealed bewildering complexity (Jonak et al., 2002). MAPK kinase cascades are implicated in signalling defence responses under biotic stress like pathogen invasion, and abiotic stresses like wounding, high salinity, high or low osmolarity, extreme temperature, drought, ozone, ROIs, and ultraviolet irradiation. A subset of plant responses to biotic and abiotic stresses is shared, such as the generation of ROIs and the activation of defence genes. MAPKs are likely to be one of the converging points in the defence-signalling network (Figure 1) (Zhang and Klessig, 2001).

I.4.2 Late events

Plant defence mechanisms include processes that require gene transcription and protein synthesis, like the HR, phytoalexin synthesis, lignification, synthesis of pathogenesis-related proteins (PR proteins) and systemic acquired resistance (SAR). These mechanisms are therefore slower. The most prominent plant defence response is the frequently observed, highly localized, hypersensitive cell death, or HR. The HR is the death of a limited number of cells in the immediate vicinity of the challenge, while the whole plant remains healthy. The HR is fundamental to resistance in the majority of situations in which a plant is challenged with an

avirulent organism. Such a reaction is sufficient in itself to prevent colonization of a plant by biotrophs because it deprives the pathogen of access to nutrient sources present in living cells (Cohn et al., 2001). However, necrotrophic pathogens are able to feed and live on dead plant tissues and can take advantage of the HR (Govrin and Levine, 2000)

Phytoalexins are low-molecular-weight antimicrobial compounds that accumulate in plants after attempted pathogen invasion (Paxton, 1981). Phytoalexins have been extensively studied, but their role in disease resistance is primarily supported by correlative or circumstantial evidence (Hammerschmidt, 1999). The major phytoalexin in *Arabidopsis* is camalexin (Tsuji et al., 1991; Glazebrook and Ausubel, 1994), which accumulates after both biotic and abiotic stress (Zhao and Last, 1996; Zhao et al., 1998). Several *pad* mutants (*phytoalexin-deficient*) with defects in camalexin production have been isolated (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997) The *pad3* mutant is likely to have a defect in the biosynthetic pathway of camalexin (Zhou et al., 1999)

Oligomers of chitin derived from enzymatic degradation of fungal chitin by plant endochitinases were found to induce lignin synthesis (Vander et al., 1998). Lignification is thought to reinforce physically the cell walls, to increase cell-wall resistance to degradation by pathogenic enzymes and to set up an impermeable barrier to the flow of nutrients and toxins (Humphreys and Chapple, 2002).

Plant defence mechanisms include the transcriptional activation of pathogenesis-related genes, such as the production of lytic enzymes (chitinases, glucanases, and proteases) or anti-microbial proteins (defensins) (Kombrink and Somssich, 1997). PR proteins can be defined as “proteins encoded by plants which are induced in tissue infected by pathogens as well as systemically and are associated with the development of SAR” (Van Loon and Van Strien, 1999). Several PR proteins including PR-1, β-1,3-glucanases (PR-2), chitinases (PR-3, PR-4), and osmotin (PR-5) show antimicrobial activities *in vitro* (Sticher et al., 1997). Their antimicrobial activity is synergistic (Mauch et al., 1988). The importance of PR proteins in plant resistance was shown by overexpression in various plants (Datta et al., 1999).

Activation of local responses at the point of infection can be followed by establishment of secondary immunity throughout the plant, called SAR, which is long lasting and effective against a broad spectrum of pathogens (Ryals et al., 1996). The onset of SAR is accompanied by a local and systemic increase in the endogenous levels of salicylic acid (SA) (Malamy et al., 1990; Métraux et al., 1990) and the activation of a large set of genes, including those encoding PR proteins (Van Loon and Van Strien, 1999). SAR is also able to induce cellular defence responses more rapidly or to a greater degree than in non-induced plants. This process, called priming, leads to the enhanced expression (or potentiation) of defence-related genes once pathogen infection occurs (Conrath et al., 2002).

I.4.3 Defence signalling pathways

Several signal molecules establish molecular bridges between the earliest responses and later ones, as well as between localized responses such as HR and more generalized responses such as SAR (Reignault and Sancholle, 2005). Three endogenous plant signalling molecules are involved in plant defence, namely SA, jasmonic acid (JA) and ethylene (ET) (Dong, 1998; Thomma et al., 2001). These molecules are involved in two major defence signalling pathways: an SA-dependent pathway and an SA-independent pathway that involves JA and ET (Kunkel and Brooks, 2002). The study of cellular transduction mechanisms involving these molecules is facilitated due to discovery of *Arabidopsis* mutants in the signalling pathways.

I.4.3.1 SA-mediated defences

SA levels increase in infected plant tissues, and exogenous application of SA results in enhanced resistance to a broad range of pathogens (Ryals et al., 1996). SA is required for gene-for-gene resistance, for the induction of local defences that contain the growth of virulent pathogens, and for the establishment of SAR (Delaney et al., 1994; Nawrath and Métraux, 1999). The SA-dependent pathway was studied using transgenic *Arabidopsis* plants expressing an SA hydroxylase that degrades SA to catechol (NahG plants) (Delaney et al., 1994). These NahG plants are blocked in the expression of PR-1, PR-2 and PR-5, that contribute to the resistance against virulent *Pseudomonas syringae* pv *tomato* and *Hyaloperonospora parasitica* (Delaney et al., 1994). Mutations in *eds1* (*enhanced disease susceptibility 1*), *eds4*, or *pad4* (*phytoalexin deficient 4*) reduce SA levels in infected leaves (Zhou et al., 1998; Gupta et al., 2000; Feys et al., 2001). Mutations in *eds5* and *sid2* (*SA induction deficient 2*) block SA synthesis (Nawrath and Métraux, 1999; Wildermuth et al., 2001). *NPR1* (*NON-EXPRESSOR OF PR1*) functions downstream of SA and is required for *PR* gene expression (Cao et al., 1997; Fan and Dong, 2002). In general, pathogens that are controlled by SA-dependent responses colonize the apoplast and multiply within the host tissue for several days before causing plant cell death and tissue damage (Kunkel and Brooks, 2002).

I.4.3.2 JA-mediated defences

JA is a fatty-acid-derived molecule. Expression of thionin (*Thi2.1*) (Epple et al., 1995), defensin (*PDF1.2*) (Penninckx et al., 1996) and hevein-like protein (*HEL*, *PR-4*) (Norman-Setterblad et al., 2000) is controlled via the JA-signalling pathway. The *fad3 fad7 fad8* triple mutant (*fatty acid desaturase*) is impaired in JA production (Vijayan et al., 1998). The *coi1* (*coronatine insensitive 1*) (Xie et al., 1998) and the *jar1* (*JA resistant 1*) (Staswick et al., 1992) mutants are impaired in the perception of JA. All these mutants have an increased susceptibility

to the fungi *Alternaria brassicicola*, *Botrytis cinerea*, and *Plectosphaerella cucumerina* (Thomma et al., 1998), the oomycete *Pythium* sp. (Staswick et al., 1998) and the bacteria *Erwinia carotovora* (Norman-Setterblad et al., 2000). The *jin1* (*JA insensitive 1*) mutant has been recently discovered (Lorenzo et al., 2004). *JIN1* encodes the bHLH-leucine-zipper transcription factor AtMYC2, which is nuclear-localised and rapidly upregulated by JA and abscisic acid in a COI1-dependent manner. *jin1* mutants show increased resistance to necrotrophic pathogens. Thus, JA-mediated defences are generally required for resistance against necrotrophic pathogens.

I.4.3.3 ET-mediated defences

ET-signalling is required in addition to JA to induce the expression of *Thi2.1*, *PDF1.2*, and *HEL*. Indeed, expression of these genes is blocked in the *ein2* mutant (*ethylene insensitive 2*) (Penninckx et al., 1998; Norman-Setterblad et al., 2000). The *ein2* mutant is affected in a membrane-associated signal transduction component of the ethylene response (McGrath and Ecker, 1998) and is more sensitive to *B. cinerea* (Thomma et al., 1999b) and *E. carotovora* (Norman-Setterblad et al., 2000). The *etr1* (*ethylene resistant 1*) mutant is affected in the *ETR1* gene encoding an ethylene receptor (Chang et al., 1993). The constitutive expression of *ETHYLENE RESPONSE FACTOR 1* (*ERF1*), a downstream component of the ET-signalling pathway (Solano et al., 1998), increases resistance to *B. cinerea* and *P. cucumerina* (Berrocal-Lobo et al., 2002). The role of ET in plant defence is controversial, as it contributes to resistance in some interactions, but promotes disease in others (Kunkel and Brooks, 2002). The pattern of altered pathogen responses for *ein2* generally parallels the patterns observed for the *coi1* and *jar1* mutants. Therefore ET and JA are often placed together in a single SA-independent pathway (Kunkel and Brooks, 2002).

I.4.3.4 Cross-talk

The different signalling pathways have different degrees of efficacy in limiting the growth of discrete pathogens (Figure 2). The defence signalling pathways do not work independently; positive and negative cross-talk has been observed between the pathways leading to an intricate plant defence network (Genoud and Métraux, 1999; Pieterse and Van Loon, 1999; Feys and Parker, 2000). JA and ET were shown in different studies to work in concert, like for the expression of *PDF1.2* in response to *A. brassicicola* (Penninckx et al., 1996). Therefore, there are positive interactions between the JA- and the ET-signalling pathways, and the transcription factor ERF1 (ETHYLENE RESPONSE FACTOR 1) was suggested to integrate the signals from both pathways (Lorenzo et al., 2003).

Evidence for an antagonistic effect of SA on JA signalling came from the *eds4* and *pad4* mutants, which are impaired in SA accumulation but exhibit enhanced responses to inducers of JA-dependent gene expression (Gupta et al., 2000). NPR1 has been identified in the cross-talk between SA and JA (Spoel et al., 2003). There is also evidence that JA antagonizes SA signalling. For example, the *coi1* mutant shows enhanced expression of SA-dependent defences and enhanced resistance to *P. syringae* (Feys et al., 1994; Kloek et al., 2001). Some data also suggest positive and negative interactions between the SA- and the ET pathways (Kunkel and Brooks, 2002).

Thus, separate signalling pathways may have evolved to allow plants to fine-tune their defence responses, to use the appropriate combination of defences against specific pathogens according to their infection strategies. Antagonistic interactions between the pathways might ensure that inappropriate defences are not activated (Kunkel and Brooks, 2002).

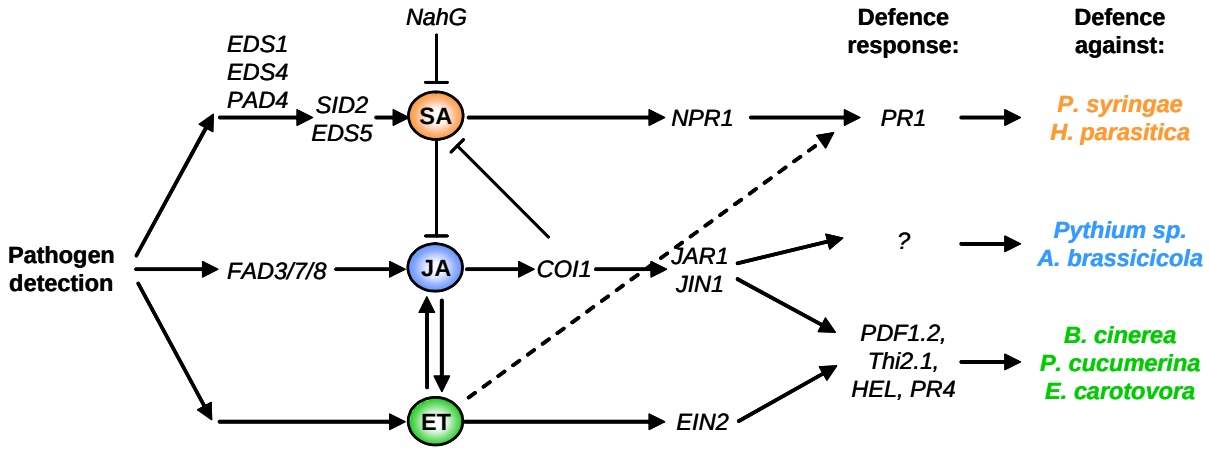

Figure 2. Cross-talk between signalling pathways (modified from Kunkel and Brooks, 2002).

I.5 Interaction between *Arabidopsis* and *Botrytis cinerea*

I.5.1 Botrytis cinerea

The Ascomycete fungi *Botrytis* spp. are important plant pathogens, and in particular *B. cinerea,* the causal agent of grey mould. While some *Botrytis* species are restricted to specific plants, like *B. alii* on *Allium* spp, *B. cinerea* is a non-specialised necrotroph developing on more than 230 possible hosts, including agronomically important crops like ornamentals, vegetables, corns, fruits, etc. (Jarvis, 1977) (Figure 3a). *B. cinerea* can also provoke important damages to foodstuffs during transport and cold storage. It is known to induce one of the most important fungal diseases on plants worldwide, namely the grey mould of grapes (Figure 3b).

B. cinerea produces grey mycelium and long, branched conidiophores bearing grape-like clusters of conidia (Figure 3c). Conidia are considered as the main dissemination units and can be propagated by wind and rain. The fungus rarely produces a *Botryotinia* perfect stage in which ascospores are formed in an apothecium. It overwinters in the soil, as mycelium in plant debris or as sclerotia, and requires cool, damp weather (18-23°C) for best growth and establishment of infection. Thus, the life cycle of *B. cinerea* includes a saprophytic phase, where it can grow on dead plant material, and a pathogenic phase (Figure 4).

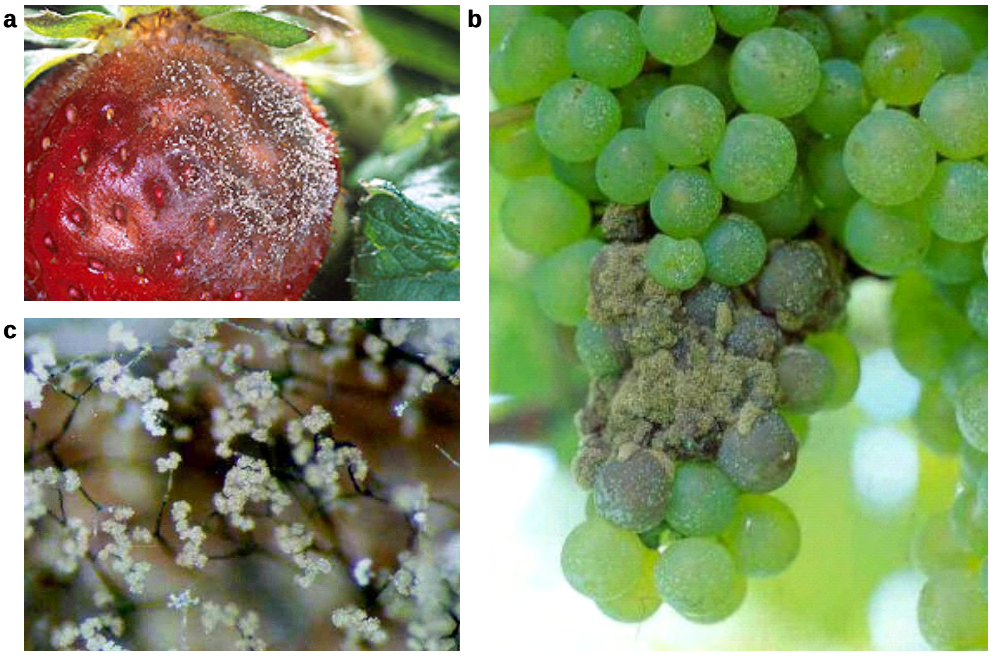

Figure 3. Disease symptoms caused by *B. cinerea* on strawberry (a) and grape (b). Clusters of conidia on hyphae can be visualised with a binocular (c) (source: internet).

An infection starts with the attachment of conidia to the host surface. Under favourable conditions conidia produce a germ tube that penetrates the host surface. After penetration the underlying cells are killed and the fungus establishes a primary lesion in which necrosis and defence responses may occur. In some cases this is the onset of a period of quiescence during which the fungus does not grow. At a certain stage the defence barriers are breached and the fungus starts to grow, resulting in a rapid maceration of plant tissue (Prins et al., 2000). In the field, *B. cinerea* often takes advantage of wounded plant parts to start an infection, but it can also directly penetrate through the plant cuticle. Under our laboratory conditions, addition of nutrients to the inoculum and almost saturated relative humidity enable *B. cinerea* to directly penetrate *Arabidopsis* leaves. Tissue maceration can be observed after two to three days and *B. cinerea* completes its life cycle in less than a week.

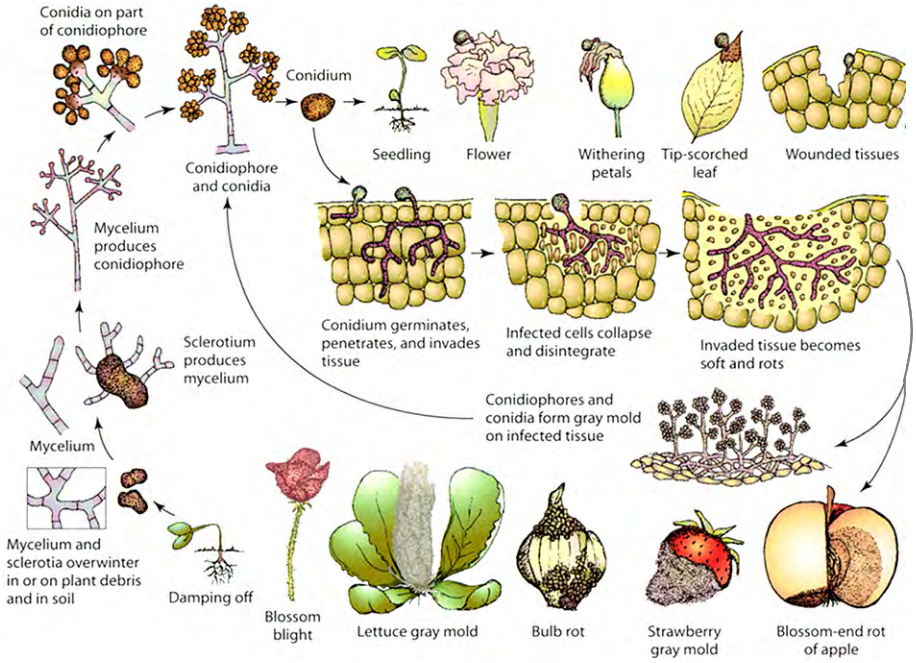

Figure 4. Disease cycle of *Botrytis* grey mold diseases (Agrios, 2005).

I.5.2 Pathogenicity factors of *B. cinerea*

Conidia of *B. cinerea* are able to adhere to plant surfaces by secreting an extracellular matrix, to germinate and to penetrate cuticles (McKeen, 1974; Doss et al., 1993). Several cutinolytic enzymes have been characterised and purified from culture filtrates of *B. cinerea* (Salinas et al., 1986; Salinas, 1992; Gindro and Pezet, 1999). The cutinase gene (*cutA*) was cloned and found to be expressed during conidia germination and induced by 16C fatty acids (Van der Vlugt-Bergmans et al., 1997). However, a *B. cinerea* strain mutated in the *cutA* gene is as virulent as the wild type strain on gerbera and tomato, leading to the conclusion that this cutinase is not essential for infection (Van Kan et al., 1997).

Other enzymes with cutinolytic activity have been discovered, like esterases and lipases (Salinas, 1992; Comménil et al., 1995; Comménil et al., 1999). Antibodies raised against the *B. cinerea* lipase prevented the infection of tomato leaves (Comménil et al., 1998). However, a recent study demonstrated that *B. cinerea lip1* mutants and even *lip1cutA* double mutants retained full pathogenicity in various host systems (Reis et al., 2005). These authors suggest therefore that *B. cinerea* breaches the host cuticle mainly by physical forces rather than by enzymatic dissolution, and that it can not be excluded that there are other, yet uncharacterized, secreted cutinolytic enzymes involved in the penetration process.

Furthermore, *B. cinerea* produces cell-wall degrading enzymes that provoke the lysis of the middle lamellae and the breaking-down of the tissues (Prins et al., 2000). It also produces

toxic levels of reactive oxygen intermediates (Deighton et al., 1999; Muckenschnabel et al., 2002) and toxins like oxalic acid or botrydial (Rebordinos et al., 1996; Tiedemann, 1997; Colmenares et al., 2002), that result in the death and maceration of tissue, leading to plant decay. Many enzymes are expressed by *B. cinerea* during infection of plant tissues and some of them have been shown to have essential roles for its virulence.

Endopolygalacturonases (*Bcpg*) are enzymes that degrade the polygalacturonic acid of the cell wall pectin. Six *Bcpg* were shown to be differentially expressed in various plant tissues (Ten Have et al., 2001) and *Bcpg1* is required for full virulence of *B. cinerea* on tomato leaves and fruits and on apple fruits (Ten Have et al., 1998). Moreover, deletion of the *Bcpg2* gene resulted in a strong reduction in virulence on tomato and broad bean (Kars et al., 2005). Likewise, disruption of the gene coding for a pectin methylesterase (*Bcpme1*) reduced *B. cinerea* virulence on apple fruits and on leaves of grapevine and *Arabidopsis* (Valette-Collet et al., 2003), which was not the case for the gene coding for the glutathione S-transferase 1 (*Bcgst1*) (Prins et al., 2000). The two subunits of the protein Gα (*Bcg*) were found to be expressed at very early stages in infected bean leaves, and a mutation in the *bcg1* gene reduces fungal pathogenicity (Schoulze Gronover et al., 2001). Among others, exopolygalacturonases (Rha et al., 2001) and aspartic proteinases (Movahedi et al., 1991; Ten Have et al., 2004) are also expressed during plant infection. Killing the plant tissue not only facilitates *B. cinerea* to penetrate and colonize its host, it also provides precious carbon sources for its growth. Thus, *B. cinerea* possess a very large array of weapons, probably providing it the ability to grow on a broad host range.

I.5.3 Defence responses of *Arabidopsis* after inoculation with *B. cinerea*

The generation of an oxidative burst is one of the earliest plant response to pathogen attack, that triggers HR. The HR deprives pathogens of food and the collapse of life-sustaining host cells is usually considered as a major barrier to many biotrophic pathogens (Torres et al., 2002). Necrotrophs such as *Botrytis* or *Sclerotinia* can utilize dead tissue and might use the HR to their advantage for a better colonization of the host. Indeed, the susceptibility to *B. cinerea* correlates positively with the levels of O_2^- or H_2O_2 produced (Govrin and Levine, 2000). The *Arabidopsis* MAPK kinase pathway is activated by bacteria, fungi, and ROIs, suggesting that signalling events initiated by diverse pathogens and external signals converge into a conserved MAPK cascade (Nühse et al., 2000; Asai et al., 2002). The kinase activities of MPK3 and MPK6 are induced during infection by *B. cinerea* in *Arabidopsis* (Veronese et al., 2005).

The ET- and JA-dependent pathway determines defences to necrotrophic pathogens in *Arabidopsis* and the expression of a set of *PR* genes that include the *PR-3*, *PR-4* or *PDF1.2* (Penninckx et al., 1996; Penninckx et al., 1998). *jar1* and *coi1*, the JA perception mutants, and

the triple *fad* mutant impaired in JA biosynthesis are characterised by an increased susceptibility to *B. cinerea* (Thomma et al., 1999b; Ferrari et al., 2003a). JA was also found to be important for *B. cinerea* resistance in tomato, as demonstrated by the increased susceptibility of the JA-biosynthesis mutant *defenceless* (Diaz et al., 2002). The *Arabidopsis* ET-insensitive mutant *ein2* has enhanced susceptibility to *B. cinerea* (Thomma et al., 1999b). *Arabidopsis* plants overexpressing the *ETHYLENE RESPONSE FACTOR 1*, a gene involved in the ET-dependent expression of proteins such as chitinase or defensins, exhibit increased resistance to *B. cinerea*, accompanied by constitutive expression of *PDF1.2* (Berrocal-Lobo et al., 2002). Furthermore, application of ET or JA reduces disease severity caused by *B. cinerea* in *Arabidopsis* and tomato (Thomma et al., 1999b; Diaz et al., 2002). Thus, JA and ET synthesis and signalling are required for both local and systemic resistance to *B. cinerea* (Thomma et al., 1999b; Ferrari et al., 2003a).

Mutants impaired in SA signalling, such as *npr1* and *pad4*, have no effect on resistance to *B. cinerea*, whereas NahG plants that do not accumulate SA display enhanced disease symptoms (Ferrari et al., 2003a). Moreover, exogenous application of SA decreased lesion size and plants treated with a phenylalanine-ammonia lyase (PAL) inhibitor showed enhanced symptoms, leading to the idea that SA synthesized via PAL, and not via isochorismate synthase (ICS), mediates lesion development (Ferrari et al., 2003a). The non-protein amino acid β-aminobutyric acid (BABA), an inducer of resistance in numerous plants, was found to protect *Arabidopsis* against *B. cinerea*. BABA-treated plants displayed a potentiated mRNA accumulation of *PR-1* after infection and the protection was no longer effective in plants impaired in the SAR transduction pathway (Zimmerli et al., 2001). This indicates that the SAR-signalling-pathway contributes to restrict *B. cinerea* infection. However, Govrin and Levine (2002) demonstrated that *B. cinerea* can induce the expression of SAR marker genes without resulting in the enhanced resistance characteristic of SAR. Furthermore, chemical or biological activation of SAR is not effective against *B. cinerea*. This suggests that SA may be required only for local resistance of *Arabidopsis* to *B. cinerea*.

The *pad3* and *pad2* mutants isolated for their defect in camalexin accumulation upon pathogen treatment (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997; Zhou et al., 1999) display an enhanced susceptibility to *B. cinerea*, suggesting that camalexin plays a major role in resistance to *B. cinerea* (Ferrari et al., 2003a; Denby et al., 2004). Camalexin was shown to have a direct toxic effect against *B. cinerea* (Ferrari et al., 2003a; Denby et al., 2004) but earlier observations did not support such a conclusion (Thomma et al., 1999a). Different *B. cinerea* isolates were shown to differ in their camalexin tolerance (Kliebenstein et al., 2005) and the production of camalexin was also found to vary greatly among different *Arabidopsis* ecotypes (Denby et al., 2004). This could explain the contradictory results among the different studies. SA, ET or JA application alone does not trigger camalexin accumulation (Thomma et al.,

1999a), but SA is required for camalexin accumulation in response to pathogen infection (Zhao and Last, 1996). Camalexin has been shown to accumulate after treatments with ROI-inducing compounds (Zhao et al., 1998; Tierens et al., 2002).

PGIPs are present in the plant cell walls and specifically inhibit endopolygalacturonases (PGs) of fungi. The two genes coding for PGIPs in *Arabidopsis* are induced in response to *B. cinerea* infection. Overexpression of either *PGIP* leads to a moderate reduction in the size of lesions caused by *B. cinerea* (Ferrari et al., 2003b). Inhibition of PGs by PGIPs might cause the accumulation of oligogalacturonides, which serve as elicitors of plant defence responses (Cervone et al., 1989).

I.5.3.1 Recently described mutants with altered responses to *B. cinerea*

Several mutants with enhanced susceptibility or resistance to *B. cinerea* have been discovered in the past few years. The *ssi2* plants, mutated in the gene encoding a stearoyl-acyl carrier protein desaturase, are blocked in the expression of *PDF1.2* and show enhanced susceptibility to *B. cinerea* (Kachroo et al., 2001). Enhanced susceptibility to necrotrophic (*Alternaria, Botrytis, Plectosphaerella*) but not to biotrophic pathogens (*Hyaloperonospora, Pseudomonas*) was observed in *esa1* (*enhanced susceptibility to Alternaria 1*) mutants. These plants show a delayed expression of *PDF1.2* and of camalexin accumulation upon pathogen inoculation (Tierens et al., 2002).

The *bos1* mutant (*botrytis-susceptible 1*) contains a T-DNA insertion in a gene encoding a R2R3MYB transcription factor. *bos1* is more susceptible than wild-type (WT) plants to *Botrytis* and *Alternaria*, but accumulates WT levels of *PDF1.2* (Mengiste et al., 2003). Infection with *B. cinerea* induces *BOS1* in WT plants but not in *coi1*, suggesting an interaction between BOS1 and the JA pathway. Three other *bos* mutants were described, that are more susceptible to *B. cinerea* and are affected in the regulation of *PDF1.2*. One of these (*bos2*) seems to be specifically linked to the establishment of the resistance to *B. cinerea*, while the other two (*bos3* and *bos4*) are also impaired in defences to *Alternaria.* Camalexin production is reduced in the *bos2* and *bos4* mutants but is enhanced in the *bos3* mutant (Veronese et al., 2005).

The *ups1* (*underinducer after pathogen and stress 1*) mutant was isolated on the basis of its reduced expression of phosphoribosylanthranilate transferase, an enzyme involved in the biosynthesis of tryptophan. This mutant displays reduced levels of camalexin and a lower expression of SA- and JA- / ET-dependent defence genes after infection with *B. cinerea*, but is not more sensitive to *B. cinerea* than WT plants (Denby et al., 2005). The *ocp3* (*overexpressor of cationic peroxidase 3*) mutant is more resistant to the necrotrophs *B. cinerea* and *P. cucumerina*. *ocp3* plants display increased accumulation of H_2O_2 and constitutive expression of *PDF1.2* and *GST1*. The *OCP3* gene codes for a transcription factor that is constitutively expressed in healthy plants, but repressed during infection by necrotrophic pathogens (Coego

et al., 2005). The *bik1* (*botrytis-induced kinase* 1) mutant is defective in a Ser/Thr kinase induced during *B. cinerea* infection. *bik1* is more sensitive to *B. cinerea* and to *A. brassicicola*. It accumulates higher levels of SA but not camalexin after *B. cinerea* infection, while the expression of *PDF1.2* is reduced (Veronese et al., 2005).

I.5.3.2 Status of *Arabidopsis* disease resistance against *B. cinerea*

Despite the fact that the ET- and JA-dependent pathway has been found to determine the resistance to necrotrophic pathogens, there is not always a clear correlation between the levels of expression of the *PDF1.2* marker gene and the level of susceptibility to *B. cinerea* (Ferrari et al., 2003a). For example, in the *ssi2*, *esa1*, *bos2, bos3, bos4 and bik1* mutants, enhanced susceptibility to *B. cinerea* correlates with a delayed or reduced expression of *PDF1.2*, whereas increased expression of *PDF1.2* results in higher resistance in the *ocp3* mutant. However, susceptible responses to *B. cinerea* have been observed during normal activation of *PDF1.2*, as in the *bos1* mutant for example. This suggests that *PDF1.2* may not be sufficient to limit pathogen growth.

The role of camalexin in the resistance to *B. cinerea* is the source of much discussion. Camalexin-deficient mutants like *pad3* and *pad2* show an enhanced susceptibility to *B. cinerea*, suggesting that camalexin plays a major role. Likewise, in addition to the *bos2* and *bos4* mutants, the *esa1* mutant shows an increased susceptibility to *B. cinerea*, which correlates with a delayed induction of camalexin. However, *bos3* accumulates as much camalexin as WT plants, despite its enhanced susceptibility, and *ups1* is impaired in camalexin accumulation, although its response to *B. cinerea* is not altered. As already mentioned, *B. cinerea* isolates seem to vary in their sensitivity to camalexin, which could explain the divergent results observed by research teams working with different *B. cinerea* strains.

Resistance to *B. cinerea* might therefore be the result of a combined action of several antifungal proteins and yet unknown factors that are induced along with *PDF1.2* and the production of camalexin. Moreover, *B. cinerea* is able to avoid plant defences in different ways. For example, isolates of *B. cinerea* were found to detoxify bean phytoalexins, the grapevine phytoalexin resveratrol, or the tomato α–tomatin (Osbourn, 1999). *B. cinerea* is also able to produce ROIs in the host tissue, which correlates with the occurrence of host tissue damage (Tiedemann, 1997). These high levels of ROIs might induce plant cell death to its own advantage. Thus, in contrast to the responses to biotrophic pathogens governed by gene-for-gene interactions, resistance to necrotrophic pathogens like *B. cinerea* is determined by multiple host factors, which might be a logical consequence of the arsenal of virulence factors used by the fungus.

I.6 Role of the cuticle in plant defence

I.6.1 Structure and function of the cuticle

The hydrophobic cuticle coating cell walls of the epidermis of all aerial plant organs is the major barrier to protect plants against invading microbes, in addition to its role as permeation barrier for solutes, gases and water. The cuticular membrane can be subdivided into the cuticular layer of the cell wall and the cuticle proper. The cuticle proper can be easily detached in some plant species and its chemical composition is well known (Kolattukudy, 2001). Its major component is the polyester cutin. Analysis of the cutin composition of *Arabidopsis* has been performed only recently, mainly because its cuticle is extremely thin in comparison with other plants cuticles (Nawrath, 2002). The chemical composition of *Arabidopsis* cutin is different from other plants (Xiao et al., 2004; Bonaventure et al., 2005; Franke et al., 2005). It is characterised by the presence of C16 and C18 fatty acids bearing ω- and mid-chain hydroxyl groups (Kolattukudy, 2001). In addition, α,ω-dicarboxylic fatty acids and 2-hydroxy fatty acids were also identified in *Arabidopsis* cutin (Franke et al., 2005). Ultrastructural studies by transmission electron microscopy revealed that the cuticular membrane of *Arabidopsis* leaves covers the leaf epidermis as a very fine electron opaque layer (20 nm) that represents only a small fraction of the outer extracellular matrix of the epidermal cell. The cuticular membrane close to the veins tends to be slightly thicker (30 nm) (Franke et al., 2005).

Mutagenised plant populations were screened for cuticular defects to find mutants, as tools to study the cuticle functions. The cuticle structure is conventionally analysed by transmission electron microscopy (TEM). A number of defects can be associated with improper cuticle formation in *Arabidopsis* mutants: poor growth and performance, sensitivity to low humidity, increased sensitivity to chemicals such as pesticides and herbicides, morphological irregularities in the shapes of organs and single cells, altered resistance to pathogens, distorted cell differentiation, illicit cell-cell interactions and cell death (Yephremov and Schreiber, 2005). Thus, the cuticle plays an essential role for the normal development of the plant and for the quality of the interface between the plant and its external environment (Sieber et al., 2000).

I.6.2 Transgenic plants with a degraded cuticle

An indirect way to analyse the cuticle function is to disrupt it by expressing a cutin-degrading enzyme. Cutinases are enzymes produced by phytopathogenic fungi during infection of plants (Kolattukudy, 1985; Kolattukudy et al., 1995). Transgenic *Arabidopsis* plants were generated that overexpress a cutinase from *Fusarium solani* f.sp. *pisi* and therefore degrade their cutin *in situ* (Sieber et al., 2000). These constitutively cutinase-expressing plants (so-called CUTE plants) show an altered ultrastructure of the cuticle and an enhanced permeability of the

cuticle to solutes, in addition to ectopic pollen germination on leaves and strong organ fusions (Figure 5). The pleiotropic phenotype of CUTE plants overlaps the phenotypes of the mutants with defective cuticles. CUTE plants have no impairment in wax deposition and it was therefore assumed that the cutinase expressed in these plants specifically degrades the cutin polymer. Organ fusions occur early in organ development, and alterations in the shape of organs are observed when fused organs grow in different directions.

Figure 5. Organ fusions are observed in CUTE plants, as shown on rosette leaves (b) in comparison to WT leaves (a), and on inflorescence (d) in comparison to WT plants (c) (pictures c and d: Sieber et al., 2000).

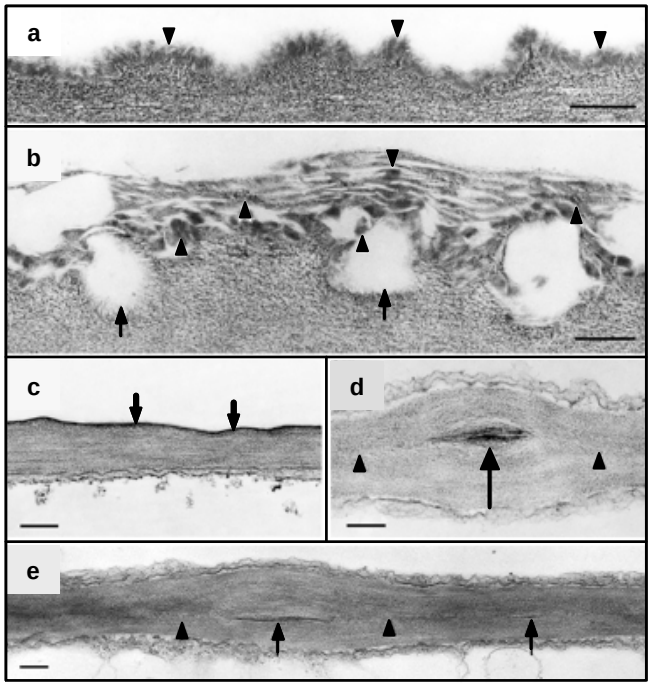

Figure 6. (a) Ultrastructure of the cuticle of the stem (a, b; bar = 200 nm) and leaf (c, d, e; bar = 250 nm) of CUTE and WT plants. The outer wall of a WT epidermal cell has a cuticle of uniform structure (arrowheads) overlaying the cell wall polysaccharides. **(b)** The contact zone between the cell wall polysaccharides and the cuticle of CUTE epidermal cells is interrupted (arrows), and amorphous material of cuticular origin is interspersed with polysaccharide microfibrils in a loosely structured cuticle (arrowheads). **(c)** The cuticle in WT leaves is thin and electron-dense. **(d, e)** The fusion zone between CUTE leaves is characterised by stretches of a direct contact of the polysaccharides (arrowheads) of the two epidermal cells and the local occurrence of interspersed cuticular material (arrows) (Sieber et al., 2000).

The cuticle in stems of WT plants is an electron-opaque, amorphous layer of uniform structure (Figure 6a). The cuticle of stems of CUTE plants is of uneven thickness and shows a loose structure and polysaccharide-like material in the outer layers (Figure 6b) whereas the cuticle in leaves of WT plants is a thin but very electron-dense layer (Figure 6c). In fusion zones of CUTE leaves, a direct contact between cell-wall polysaccharides of the two epidermal cell layers is observed, but stretches of small amounts of cuticular material also occur (Figure 6d, e). Fibrillar pectic polysaccharides are deposited in the fusion zones (Sieber et al., 2000).

I.6.3 Mechanical and chemical role of the cuticle in defence

It has long been recognised that the plant cuticle is the first barrier to be overcome by fungal pathogens (Köller et al., 1991). Many pathogenic fungi find their way via stomata or other openings, while some penetrate the cuticle directly. In some cases, direct penetration is facilitated by fungal cutinase loosening the cuticular matrix (Kolattukudy, 1985; Köller, 1995). Some fungi may also penetrate by physical force alone, even if cutinase is present (Stahl and Schäfer, 1992). Cuticle thickness has been demonstrated to have an effect on the ability of some pathogens to successfully penetrate the host cell (Gevens and Nicholson, 2000). For example, *Venturia inaequalis* penetrates only the thin cuticle of juvenile leaves, but fails to penetrate older leaves where the cuticle is thicker (Nicholson et al., 1973).

Cuticle components also have a role in pathogenesis signalling. Surface wax of avocado plants susceptible to *Colletotrichum gloeosporioides* selectively induces the germination and appressorium formation of the pathogen, whereas that from non-hosts did not induce pathogen development (Podila et al., 1993). Degradation of the cutin polymer by fungal cutinases during infection releases cutin monomers. Cutin monomers have been found to stimulate germination and appressorium formation in *Magnaporthe grisea* (Gilbert et al., 1996) and differentiation of appressorial germ tube in *Erysiphe graminis* conidia (Francis et al., 1996). Cutin monomers induce the expression of the cutinase gene in many fungi (Kolattukudy et al., 1995). However, it was also demonstrated that cutin monomers can be toxic to fungi. Transgenic tomato plants expressing a yeast Δ-9 desaturase gene display changes in their fatty acid and cutin monomer profiles. Cutin monomers from these trangenic plants inhibited the germination of *E. polygoni* spores (Wang et al., 2000). Thus, the cuticle should be thought as a layer of hydrophobic material that, over time, changes in structural morphology as well as chemistry, and is not a “ hard” barrier of material that simply blocks fungal ingress (Gevens and Nicholson, 2000).

There is evidence that cutin monomers might also act as signals in plants during pathogen attack, eliciting defence responses. They have been shown to induce medium alkalinisation and ethylene production of suspension-cultured potato cells (Schweizer et al., 1996a) and H_2O_2 production in cucumber hypocotyls (Fauth et al., 1998; Kauss et al., 1999).

Indeed, application of specific cutin monomers leads to increased resistance to *E. graminis* f.sp. *hordei* and *M. grisea* in barley and rice respectively (Namai et al., 1993; Schweizer et al., 1994; Schweizer et al., 1996b).

I.7 Aim of the work

The cuticle is a barrier that pathogens have to overcome to reach plant cells and their nutrients. Plants have probably evolved sophisticated sensing mechanisms to detect the very early steps of pathogen invasion. We are interested in the role of cuticle defects to trigger innate immunity in *Arabidopsis*. As model of study, we focused on CUTE plants that degrade their own cuticle by secreting a fungal cutinase and their responses upon pathogen inoculation. CUTE plants were found to be totally resistant to the necrotrophic pathogen *B. cinerea* (Chassot and Métraux, 2005). The aim of this work was to analyse the mode of resistance of CUTE plants (Chapters II, III, IV) and other mutants with defects in cuticle ultrastructure (Chapter V) against *B. cinerea*. In addition to the specific enzymatic cuticle degradation of CUTE plants, disruption of the leaf integrity was also performed by wounding. Induced defence responses by this abiotic stimulus in correlation with resistance to *B. cinerea* were investigated (Chapter VI).

I.8 References

Agrios, G.N. (2005). Plant Pathology (5th edition). Elsevier Academic Press.

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.

Ausubel, F.M. (2005). Are innate immune signaling pathways in plants and animals conserved? Nature Immunol. **6,** 673-679.

Beffa, R.S. et al. (1996). Dicreased susceptibility to viral disease of beta-1,3-glucanase-deficient plants generated by antisense transformation. Plant Cell **8,** 1001-1111.

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.

Bonaventure, G., Beisson, F., Ohlrogge, J., and Pollard, M. (2005). Analysis of the aliphatic monomer composition of polyesters associated with *Arabidopsis* epidermis: occurrence of octdeca-*cis*-6, *cis*-9-diene-1,18-dioate as the major component. Plant J. **40,** 902-930.

Cao, H., Glazebrook, J., Clarke, J.D., Volko, S., and Dong, X. (1997). The *Arabidopsis NPR1* gene that controls systemic acquired resistance encodes a novel protein containing ankyrin repeats. Cell **88,** 57-63.

Carpita, N.C., and McCann, M. (2000). The cell wall. In: Biochemistry and Molecular Biology of Plants. Buchanan B. et al., American Society of Plant Physiologists.**,** 52-108.

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

Chang, C., Kwok, S.F., Bleecker, A.B., and Meyerowitz, E.M. (1993). Arabidopsis ethylene-response gene *etr1*: similarity of product to two-component regulators. Science **262,** 539-544.

Chassot, C., and Métraux, J.P. (2005). The cuticle as source of signals for plant defense. Plant Biosystems **139,** 28-31.

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.

Cohn, J., Sessa, G., and Martin, G.B. (2001). Innate immunity in plants. Curr. Opin. Immunol. **13,** 55-62.

Colmenares, A.J., Aleu, J., Duran-Patron, R., Collado, I.G., and Hernandez-Galan, R. (2002). The putative role of botrydial and related metabolites in the infection mechanism of *Botrytis cinerea*. J. Chem. Ecol. **28,** 997-1005.

Comménil, P., Belingheri, L., and Dehorter, B. (1998). Antilipase antibodies prevent infection of tomato leaves by *Botrytis cinerea*. Physiol. Mol. Plant Pathol. **52,** 1-14.

Comménil, P., Belingheri, L., Sancholle, M., and Dehorter, B. (1995). Purification and properties of an extracellular lipase from the fungus *Botrytis cinerea*. Lipids **30,** 351-356.

Comménil, P., Belingheri, L., Bauw, G., and Dehorter, B. (1999). Molecular characterization of a lipase induced in *Botrytis cinerea* by components of grape berry cuticle. Physiol. Mol. Plant Pathol. **55,** 37-43.

Conrath, U., Pieterse, C.M., and Mauch-Mani, B. (2002). Priming in plant pathogen interactions. Trends Plant Sci. **7,** 210-216.

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

Dat, J., Vandenabeele, S., Vranova, E., Van Montagu, M., Inzé, D., and Van Bresegem, F. (2000). Dual action of the active oxygen species during plant stress responses. Cell. Mol. Life Sci. **57,** 779-795.

Dat, J., Pellinen, R., Beeckman, T., Kangasjärvi, J., Langebartels, C., Inzé, D., and Van Breusegem, F. (2003). Changes in hydrogen peroxide homeostasis trigger an active cell death process in tobacco. Plant J. **33,** 621-632.

Datta, K., Mutukrishnan, S., and Datta, S.K. (1999). Expression and function of PR-protein genes in trangenic plants. In: Pathogenesis-related proteins in plants, SK Datta & S Mutukrishnan, eds (Baton Roca: CRC press)**,** 261-277.

De Lorenzo, G., D'Ovidio, R., and Cervone, F. (2001). The role of polygalacturonase-inhibiting proteins (PGIPs) in defense against pathogenic fungi. Annu. Rev. Phytopathol. **39,** 313-335.

De Lorenzo, G., and Ferrari, S. (2002). Polygalacturonase-inhibiting proteins in defense against phytopathogenic fungi. Curr. Opin. Plant Biol. **5,** 295-299.

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., Uknes, S., Vernooij, B., and al., e. (1994). A central role of salicylic acid in plant disease resistance. Science **266,** 1247-1249.

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

Denby, K.J., Jason, L.J., Murray, S.L., and Last, R.L. (2005). *ups1*, an *Arabidopsis thaliana* camalexin accumulation mutant defective in multiple defence signalling pathways. Plant J. **41,** 673-684.

Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S., and Marco, Y. (2003). Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proc. Natl. Acad. Sci. **100,** 8024-8029.

Diaz, J., Ten Have, A., and Van Kan, J.A.L. (2002). The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. Plant Physiol. **129,** 1341-1351.

Doke, N. (1983). Involvement of superoxide anion generation in the hypersensitive response of potato tuber tissues to infection with an incompatible race of *Phytophthora infestans* and to the hyphal wall components. Physiol. Plant Pathol. **23,** 345-357.

Dong, X.N. (1998). SA, JA, ethylene, and disease resistance in plants. Curr. Opin. Plant Biol. **1,** 316-323.

Doss, R.P., Potter, S.W., Chastagner, G.A., and Christian, J.K. (1993). Adhesion of nongerminated *Botrytis cinerea* conidia to several substrata. Appl. Environ. Microbiol. **59,** 1789-1791.

Epple, P., Apel, K., and Bohlmann, H. (1995). An *Arabidopsis thaliana* thionin gene is inducible via a signal transduction pathway different from that for pathogenesis-related proteins. Plant Physiol. **109,** 813-820.

Erbs, G., and Newman, M.A. (2003). The role of lipopolyssacharides in induction of plant defence responses. Mol. Plant Pathol. **4,** 421-425.

Fan, W., and Dong, X. (2002). In vivo interaction between NPR1 and transcription factor TGA2 leads to salicylic acid-mediated gene activation in Arabidopsis. Plant Cell **14,** 1377-1389.

Fauth, M., Shweizer, P., Buchala, A., Markstadter, C., Riederer, M., Kato, T., and Kauss, H. (1998). Cutin monomers and surface wax constituents elicit H_2O_2 in conditioned cucumber hypocotyl segments and enhance the activity of other H_2O_2 elicitors. Plant Physiol. **117,** 1373-1380.

Ferrari, S., Plotnikova, J.M., De Lorenzo, G., and Ausubel, F.M. (2003a). *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.

Ferrari, S., Vairo, D., Ausubel, F.M., Cervone, F., and De Lorenzo, G. (2003b). Tandemly duplicated *Arabidopsis* genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. Plant Cell **15,** 93-106.

Feys, B.J., and Parker, J. (2000). Interplay of signaling pathways in plant disease resistance. Trends Genet. **16,** 449-455.

Feys, B.J., 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. (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. Phytopathol. **9,** 275-296.

Francis, S.A., Dewey, F.M., and Gurr, S.J. (1996). The role of cutinase in germling development and infection by *Erysiphe graminis* f.sp. *hordei*. Physiol. Mol. Plant Pathol. **49,** 201-211.

Franke, R., Briesen, I., Wojciechowski, T., Faust, A., Yephremov, A., Nawrath, C., and Schreiber, L. (2005). Apoplastic polyesters in Arabidopsis surface tissues - a typical suberin and a particular cutin. Phytochem. **66,** 2643-2658.

Genoud, T., and Métraux, J.P. (1999). Crosstalk in plant cell signaling: structure and function of the genetic network. Trends Plant Sci. **4,** 503-507.

Gevens, A., and Nicholson, R.L. (2000). Cutin composition: a subtle role for fungal cutinase? Physiol. Mol. Plant Pathol. **57,** 43-45.

Gilbert, R.D., Johnson, A.M., and Dean, R.A. (1996). Chemical signals responsible for appressorium formation in rice blast fungus *Magnaporte grisea*. Physiol. Mol. Plant Pathol. **48,** 335-346.

Gindro, K., and Pezet, R. (1999). Purification and characterization of a 40.8 kDa cutinase in ungerminated conidia of *Botrytis cinerea*. FEMS Microbiol. Letters **171,** 239-243.

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. **91,** 8955-8959.

Glazebrook, J., Rogers, E.E., and Ausubel, F.M. (1997). Use of *Arabidopsis* for genetic dissection of plant defence responses. Annu. Rev. Genet. **31,** 547-569.

Gòmez-Gòmez, L. (2004). Plant perception systems for pathogen recognition and defence. Mol. Immunol. **41,** 1055-1062.

Gòmez-Gòmez, L., and Boller, T. (2002). Flagellin perception: a paradigm for innate immunity. Trends Plant Sci. **7,** 251-256.

Goodman, R.N., and Novacky, A.J. (1994). The Hypersensitive Reaction in Plants to Pathogens. St Paul: APS Press.

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

Govrin, E.M., and Levine, A. (2002). Infection of *Arabidopsis* with a necrotrophic pathogen, *Botrytis cinerea*, elicits various defense responses but does not induce systemic acquired resistance (SAR). Plant Mol. Biol. **48,** 267-276.

Grant, M., Brown, I., Adams, S., Knight, M., Ainslie, A., and Mansfield, J. (2000). The RPM1 plant disease resistance gene facilitates a rapid and sustained increase in cytosolic calcium that is necessary for the oxidative burst and hypersensitive cell death. Plant J. **23,** 441-450.

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.

Hammerschmidt, R. (1999). Phytoalexins: what have we learned after 60 years? Annu. Rev. Phytopathol. **37,** 285-306.

Hammond-Kosack, K.E., and Parker, J.E. (2003). Deciphering plant pathogen communication: fresh perspectives for molecular resistance breeding. Curr. Opin. Biotechnol. **14,** 177-193.

Heath, M.C. (1985). Implications of nonhost resistance for understanding host-parasite interactions. Genetic Basis of Biochemical Mechanisms of Plant Disease**,** 25-42.

Heath, M.C. (2000). Nonhost resistance and nonspecific plant defenses. Curr. Opin. Plant Biol. **3,** 315-319.

Humphreys, J.M., and Chapple, C. (2002). Rewriting the lignin roadmap. Curr. Plant Biol.

Jarvis, W.R. (1977). *Botryotinia* and *Botrytis* species: taxonomy, physiology, and pathogenicity: a guide to the litterature. Canada Department of Agriculture, Harrow **15,** 195 pages.

Jonak, C., Ökrész, L., Bögre, L., and Hirt, H. (2002). Complexity, cross talk and integration of plant MAP kinase signalling. Curr. Opin. Plant Biol. **5,** 415-424.

Jones, D.A., and Takemoto, D. (2004). Plant innate immunity - direct and indirect recognition of general and specific pathogen-associated molecules. Curr. Opin. Immunol. **16,** 48-62.

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. **98,** 9448-9453.

Karpinsky, S., Reynolds, H., Karpinska, B., Wingsle, G., Creissen, G., and Mullineaux, P. (1999). Systemic signaling and acclimation in response to excess excitation energy in Arabidopsis. Science **284,** 654-657.

Kars, I., Krooshof, G.H., Wagemakers, L., Joosten, R., Benen, J.A., and Van Kan, J.A. (2005). Necrotizing activity of five *Botrytis cinerea* endopolygalacturonases produced in *Pichia pastoris*. Plant J. **43,** 213-225.

Kauss, H., Fauth, M., Merten, A., and Jeblick, W. (1999). Cucumber hypocotyls respond to cutin monomers via both an inducible and a constitutive H_2O_2-generating system. Plant Physiol. **120,** 1175-1182.

Kliebenstein, D.J., Rowe, H.C., and Denby, K.J. (2005). Secondary metabolites influence *Arabidopsis* / *Botrytis* interactions: variation in host production and pathogen sensitivity. Plant J. **44,** 25-36.

Kloek, A.P., Verbsky, M.L., Sharma, S.B., Schoelz, J.E., Vogel, J., Klessig, D.F., and Kunkel, B.N. (2001). Resistance to *Pseudomonas syringae* conferred by an *Arabidopsis thaliana* coronative-insensitive (*coi1*) mutation occurs through two distinct mechanisms. Plant J. **26,** 509-522.

Kolattukudy, P.E. (1985). Enzymatic penetration of the plant cuticle by fungal pathogens. Annu. Rev. Phytopathol. **23,** 223-250.

Kolattukudy, P.E. (2001). Polyesters in higher plants. In: Advances in Biochemical Engineering Biotechnology: Biopolyesters, W. Babel and A. Steinbüchel, eds (Berlin: Springer-Verlag)**,** 1-49.

Kolattukudy, P.E., Rogers, L.M., Li, D., Hwang, C.S., and Flaishman, M.A. (1995). Surface signaling in pathogenesis. Proc. Natl. Acad. Sci. **92,** 4080-4087.

Köller, W., Yao, C., Trail, F., and Parker, D.M. (1991). The plant cuticle: a barrier to be overcome by fungal plant pathogens. Cole GT, Hoch HC, eds. The fungal spore in disease initiation in plants and animals. New York: Plenum**,** 219-246.

Köller, W., Yao, C., Trail, F., and Parker, D.M. (1995). Role of cutinase in the invasion of plants. Can. J. Bot. **73,** 1109-1118.

Kombrink, E., and Somssich, I.E. (1997). Pathogenesis-related proteins and plant defense. In: Carroll GC, Tudzynski P, eds. Plant Relationships. Part A. Berlin-Heidelberg: Springer Verlag**,** 107-128.

Kunkel, B.N., and Brooks, D.M. (2002). Cross talk between signaling pathways in pathogen defense. Curr. Opin. Plant Biol. **5,** 325-331.

Kunst, L., and Samuels, A.L. (2003). Biosynthesis and secretion of plant cuticular wax. Prog. Lipid Res. **42,** 51-80.

Lamb, C., and Dixon, R.A. (1997). The oxidative burst in plant disease resistance. Annu. Rev. Plant Physiol. Plant Mol. Biol. **48,** 251-275.

Levine, A., Pennell, R.I., Alvarez, M.E., Palmer, R., and Lamb, C. (1996). Calcium-mediated apoptosis in a plant hypersensitive disease resistance response. Curr. Biol. **6,** 427-437.

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 defence responses in Arabidopsis. Plant Cell **16,** 1938-1950.

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

Martin, G.B., Bogdanove, A.J., and Sessa, G. (2003). Understanding the functions of plant disease resistance proteins. Annu. Rev. Plant Biol. **54,** 23-61.

Mauch, F., Mauch-Mani, B., and Boller, T. (1988). Antifungal hydrolases in pea tissue. 2. Inhibition of fungal growth by combinations of chitinase and beta-1,3-glucanase. Plant Physiol. **88,** 936-942.

McDowell, J.M., and Dangl, J.L. (2000). Signal transduction in the plant immune response. Trends Biochem. Sci. **25,** 79-82.

McGrath, R.B., and Ecker, J.R. (1998). Ethylene signalling in *Arabidopsis*: events from the membrane to the nucleus. Plant Physiol. Biochem. **36,** 103-113.

McKeen, W.E. (1974). Mode of penetration of epidermal cell walls of *Vicia faba* by *Botrytis cinerea*. Phytopathol. **64,** 461-467.

Mengiste, T., Chen, X., Salmeron, J.M., and Dietrich, R.A. (2003). The *BOS1* gene encodes a R2R3MYB transcription factor protein that is required for biotic and abiotic stress responses in *Arabidopsis*. Plant Cell **15,** 2551-2565.

Métraux, J.P., Signer, H., Ryals, J., Ward, E., Wyss-Benz, M., Gaudin, J., Raschdorf, K., Schmid, E., Blum, W., and Inverardi, B. (1990). Increase in salicylic acid at the onset of systemic acquired resistance in cucumber. Science **8,** 2309-2323.

Montesano, M., Brader, G., and Palva, E.T. (2003). Pathogen derived elicitors: searching for receptors in plants. Mol. Plant Pathol. **4,** 73-79.

Morrissey, J.P., and Osbourn, A.E. (1999). Fungal resistance to plant antibiotics as a mechanism of pathogenesis. Microbiol. Mol. Biol. Rev. **63,** 708.

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.

Muckenschnabel, I., Goodman, B.A., Williamson, B., Lyon, G.D., and Deighton, N. (2002). Infection of leaves of *Arabidopsis thaliana* by *Botrytis cinerea*: changes in ascorbic acid, free radicals and lipid peroxidation products. J. Exp. Bot. **53,** 207-214.

Namai, T., Kato, T., Yamaguchi, Y., and Hirukawa, T. (1993). Antirice blast activity and resistance induction of C-18 oxygenated fatty acids. Biosci. Biotechnol. Biochem. **57,** 611-613.

Nawrath, C. (2002). The biopolymers cutin and suberin. In: The Arabidopsis Book, Somerville CR, Meyerowitz EM, eds (Rockville, MD: American Society of Plant Biologists).

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

Nicholson, R.L., VanScoyoc, S., Kuc, J., and Williams, E.B. (1973). Response of detached apple leaves to *Venturia inaequalis*. Phytopathol. **63,** 649-650.

Norman-Setterblad, C., Vidal, S., and Palva, E.T. (2000). Interacting signal pathways control defense gene expression in *Arabidopsis* in response to cell wall-degrading enzymes from *Erwinia carotovora*. Mol. Plant Microbe Interact. **13,** 430-438.

Nühse, T.S., Peck, S.C., Hirt, H., and Boller, T. (2000). Microbial elicitors induce activation and dual phosphorylation of the *Arabidopsis thaliana* MAPK6. J. Biol. Chem. **275,** 7521-7526.

Nürnberger, T., Brunner, F., Kemmerling, B., and Piater, L. (2004). Innate immunity in plants and animals: striking similarities and obvious differences. Immunol. Rev. **198,** 249-266.

Osbourn, A.E. (1999). Antimicrobial phytoprotectants and fungal pathogens: a commentary. Fungal Genet. Biol. **26,** 163-168.

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

Paxton, J. (1981). Phytoalexins - a working redefinition. J. Phytopathol. **101,** 106-109.

Penninckx, I.A., Thomma, B.P., De Samblanx, G.W., Buchala, A., Métraux, 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.M.A., Eggermont, K., Terras, F.R.G., Thomma, G.P.H.J., De Samblanx, G.W., Buchala, A., Métraux, 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.

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

Podila, G.K., Rogers, L.M., and Kolattukudy, P.E. (1993). Chemical signals from avocado surface wax trigger germination and appressorium formation in *Colletotrichum gloeosporioides*. Plant Physiol. **103,** 267-272.

Prins, T.W., Wagemakers, L., Schouten, A., and Van Kan, J.A. (2000). Cloning and characterization of a glutathione S-transferase homologue from the plant pathogenic fungus *Botrytis cinerea*. Mol. Plant Pathol. **1,** 169-178.

Prins, T.W., Tudzynski, P., van Tiedemann, A., Tudzynski, B., Ten Have, A., Hansen, M.E., Tenberge, K., and Van Kan, J.A.L. (2000). Infection strategies of *Botrytis cinerea* and related necrotrophic pathogens. In: Fungal Pathology, J.W. Kronstad, ed (Dordrecht, The Netherlands: Kluwer Academic Publishers)**,** 33-64.

Rebordinos, L., Cantoral, J.M., Prieto, M.V., Hanson, J.R., and Collado, I.G. (1996). The phytotoxic activity of some metabolites of *Botrytis cinerea*. Phytochem. **42,** 383-387.

Reignault, P., and Sancholle, M. (2005). Plant-pathogen interactions: will the understanding of common mechanisms lead to the unification of concepts? C. R. Biologies 328**,** 821-833.

Reis, H., Pfiffi, S., and Hahn, M. (2005). Molecular and functional characterization of a secreted lipase from *Botrytis cinerea*. Mol. Plant Pathol. **6,** 257-267.

Rha, R., Park, H.J., Kim, M.O., Chung, Y.R., Lee, C.W., and Kim, J.W. (2001). Expression of exo-polygalacturonase in *Botrytis cinerea*. FEMS Microbiol. Letters **210,** 105-109.

Robinson, S.P., Jacobs, A.K., and Dry, I.B. (1997). A class IV chitinase is highly expressed in grape berries during ripening. Plant Physiol. **114,** 771-778.

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.

Salinas, J., Warnaar, F., and Verhoeff, K. (1986). Production of cutin hydrolyzing enzymes by *Botrytis cinerea* in vitro. J. Phytopathol. **116,** 299-307.

Salinas, J.C. (1992). Function of cutinolytic enzymes in the infection of gerbera flowers by *Botrytis cinerea.* Ph.D. thesis, University of Utrecht, The Netherlands.

Schoulze-Gronover, C., Kasulke, D., Tudzynski, P., and Tudzynski, B. (2001). The role of G protein alpha subunits in the infection process of the gray mold fungus *Botrytis cinerea*. Mol. Plant Microbe Interact. **14,** 1293-1302.

Schweizer, P., Jeanguénat, A., Mösinger, E., and Métraux, J.P. (1994). Plant protection by free cutin monomers in two cereal pathosystems. Adv. Mol. Genet. Plant Microbe Interact. M.J. Daniels, J.A. Downie and A.E. Osbourn, eds. (Kluwer Academic Publishers, Dordrecht, The Netherlands)**,** 371-374.

Schweizer, P., Felix, G., Buchala, A., Müller, C., and Métraux, J.P. (1996a). Perception of free cutin monomers by plant cells. Plant J. **10,** 331-341.

Schweizer, P., Jeanguénat, Whitacre, D., A., Métraux, J.P., and Mösinger, E. (1996b). Induction of resistance in barley against *Erysiphe graminis* f.sp. *hordei* by free cutin monomers. Physiol. Mol. Plant Pathol. **49,** 103-120.

Shewry, P.R., and Lucas, J.A. (1997). Plant proteins that confer resistance to pests and pathogens. Advances in Botanical Research **26,** 135-192.

Shibuya, N., and Minami, E. (2001). Oligosaccharide signalling for defence responses in plant. Physiol. Mol. Plant Pathol. **59,** 223-333.

Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Métraux, J.P., and Nawrath, C. (2000). Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell **12,** 721-738.

Solano, R., Stepanova, A., Chao, Q.M., 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.

Soliday, C.L., Flurkey, W.H., Okita, T.W., and Kolattukudy, P.E. (1984). Cloning and structure determination of complementary DNA for cutinase: an enzyme involved in fungal penetration for plants. Proc. Natl. Acad. Sci. **81,** 3939-3943.

Spoel, S.H., Koorneef, A., Clessens, S.M.C., Korzelius, J.P., Van Pelt, J.A., Mueller, M.J., Buchala, A.J., Métraux, J.P., Brown, R., Kazan, K., Van Loon, L.C., Dong, X., 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.

Stahl, D.J., and Schäfer, W. (1992). Cutinase is not required for fungal pathogenicity on pea. Plant Cell **4,** 621-629.

Staswick, P.E., Su, W.P., 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. **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.

Sticher, L., Mauch-Mani, B., and Métraux, J.P. (1997). Systemic acquired resistance. Annu. Rev. Phytopathol. **35,** 235-270.

Stone, B.A., and Clarke, A.E. (1992). Chemistry and biology of (1-3)-b-D-glucan. In: Victoria, Australia: La Trobe University Press.

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. Microbiol. **150,** 2475-2489.

Thomma, B., Penninckx, I., Broekaert, W.F., and Cammue, B.P.A. (2001). The complexity of disease signaling in *Arabidopsis*. Curr. Opin. Immunol. **13,** 63-68.

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

Thomma, B.P., Eggermont, K., Tierens, K.F., and Broekaert, W.F. (1999b). 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.H.J., Eggermont, K., Penninckx, I.A.M.A., Mauch-Mani, B., Vogelsang, R., Cammue, B.P.A., and Broedaert, W.F. (1998). Separate jasmonate-dependent and salicylate-dependent defense pathways in *Arabidopsis* are essential for resistance to distinct microbial pathogens. Proc. Natl. Acad. Sci. **95,** 15107-15111.

Tiedemann, A.v. (1997). Evidence for a primary role of active oxygen species in induction of host cell death during infection of 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.

Torres, M.A., Dangl, J.L., and Jones, J.D. (2002). *Arabidopsis* gp91phox homologues AtrbohD and AtrbohF are required for accumulation of reactive oxygen intermediates in the plant defense response. Proc. Natl. Acad. Sci. **99,** 517-522.

Tsuji, J., Jackson, E.P., Gage, D.A., Hammerschmidt, R., and Sommerville, S.C. (1991). Phytoalexin accumulation in *Arabidopsis thaliana* during the hypersensitive reaction to *Pseudomonas syringae* pv *syringae*. Plant Physiol. **98,** 1304-1309.

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,** 454-546.

Van der Vlugt-Bergmans, C.J.B., Wagemakers, C.A.M., and Van Kan, J.A.L. (1997). Cloning and expression of the cutinase A gene of *Botrytis cinerea*. Mol. Plant Microbe Interact. **10,** 21-29.

Van Etten, H.D., Mansfield, J.W., Bailey, J.A., and Farmer, E.E. (1994). Two classes of plant antibiotics: Phytoalexins versus "Phytoanticipins". Plant Cell **6,** 1191-1192.

Van Kan, J.A.L., Van't Klosster, J.W., Wagemakers, C.A.M., Dees, D.C.T., and Van der Vlugt-Bergmans, C.J.B. (1997). Cutinase A of *Botrytis cinerea* is expressed, but not essential, during penetration of gerbera and tomato. Mol. Plant Microbe Interact. **10,** 30-38.

Van Loon, L.C., and Van Strien, E.A. (1999). The families of pathogenesis-related proteins, their activities, and comparative analysis of PR-1 type proteins. Physiol. Mol. Plant Pathol. **55,** 85-97.

Vander, P.V., Domard, A., Eddine El Gueddari, N., and Moerschbacher, B.M. (1998). Comparison of the ability of partially N-acetylated chitosans and chitooligosaccharides to elicit resistance reactions in wheat leaves. Plant Physiol. **118,** 1353-1359.

Veronese, P., Nakagami, H., Bluhm, B., Abuqamar, S., Chen, X., Salmeon, J., Dietrich, R.A., Hirt, H., and Mengiste, T. (2005). The membrane-anchored BOTRYTIS-INDUCED KINASE1 plays distinct roles in *Arabidopsis* resistance to necrotrophic and biotrophic pathogens. Plant Cell **18,** 257-273.

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. **95,** 7209-7214.

Vorwerk, S., Somerville, S., and Somerville, C. (2004). The role of plant cell wall polysaccharide composition in disease resistance. Trends in Plant Science **9,** 203-209.

Wang, C., Chin, C.K., and Gianfagna, T. (2000). Relationship between cutin monomers and tomato resistance to powdery mildew infection. Physiol. Mol. Plant Pathol. **57,** 55-61.

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.

Xiao, F., Goodwin, M.S., Xiao, Y., Sun, Z., Baker, D., Tang, X., Jenks, M.A., and Zhou, J.M. (2004). *Arabidopsis* CYP86A2 represses *Pseudomonas syringae* type III genes and is required for cuticle development. EMBO J. **23,** 2903-2913.

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.

Yephremov, A., and Schreiber, L. (2005). The dark side of the cell wall: molecular genetics of plant cuticle. Plant Biosystems **139,** 74-79.

Zhang, S., and Klessig, D.F. (2001). MAPK cascades in plant defense signaling. Trends Plant Sci. **6,** 520-527.

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, N., Tootle, T.L., and Glazebrook, J. (1999). *Arabidopsis PAD3*, a gene required for camalexin biosynthesis, encodes a putative cytochrome P_{450} 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.

Zimmerli, L., Métraux, J.P., and Mauch-Mani, B. (2001). beta-amino butyric acid-induced resistance of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*. Plant Physiol. **126,** 517-523.

II. The cuticle as a source of signals for plant defence

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REVIEW ARTICLE

The cuticle as source of signals for plant defense

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Abstract
The cuticle is a physical barrier that prevents water loss and protects against irradiation, xenobiotics and pathogens. This article reviews some of the studies showing that the cuticle is also a source of signals used by invading fungi to activate pathogenic responses or by plants to induce defense mechanisms.

Key words: *Innate immunity, signalling, defence mechanisms, defence network*

Introduction

The aerial parts of higher plants are covered with a conspicuous waxy layer. This cuticle is an effective barrier to water or solute loss while remaining translucent for optimal light absorption and providing protection against excessive irradiation, xenobiotics or prospective invaders (Kerstiens, 1996; Kolattukudy, 1985; Riederer & Schreiber, 2001). In addition, mutants or transgenic plants with abnormal cuticles show organ-fusion phenotypes, indicating a possible function of cuticles in delimiting organs during development (Nawrath, 2002; Sieber et al., 2000; Wellesen et al., 2001; Yephremov et al., 1999).

The cuticular matrix is principally made up of cutin, a chloroform-insoluble complex polymer, consisting mostly of C16 or C18 ω-hydroxylated esterified fatty acids (Heredia, 2003). Recently, dicarboxylic acid monomers such as octadeca-cis-6, cis-9-diene-1,18-dioate, octadec-cis-9-ene-1,18-dioate and hexadeca-1,16-dioate were also observed (Bonaventure et al., 2004). An etherified polymethylenic core material highly refractory to hydrolysis termed cutan has been isolated from some species (Heredia, 2003; Jeffree, 1996). In addition, chloroform-soluble waxes and polysaccharides are associated with this matrix. The waxes comprise long-chain aliphatics such as alcohols, aldehydes or fatty acids combined with terpenoids or simple phenolics (Barthlott et al., 1998; Moire et al., 1999; Riederer & Schreiber, 2001). The elucidation of the chemical structure of these complex polymer networks has been notoriously difficult. Nevertheless, several models of the cuticle have been described (Heredia, 2003; Jeffree, 1996; Kolattukudy, 1985). Non-invasive Fourier-transformation infrared spectroscopy has yielded information on the crosslink architecture of the cuticular layer (Ramirez et al., 1992). The chemical synthesis of a polymer identical to natural cutin was reported recently (Benitez et al., 2004). Transmission electron microscopy has provided a lamellar view of the cuticle with a fine wax sheet covering the cuticular layer (Jeffree, 1996; Kolattukudy, 1985; Nawrath, 2002). Autofluorescence of phenolics and flavonoids in plant cuticles made it possible to obtain three-dimensional images of cuticular membranes using confocal laser scanning microscopy (Fernandez et al., 1999). The self-assembly of wax into crystalline micro- and nanostructures from an underlying wax film was observed using atom force microscopy (Koch et al., 2004).

A series of hydroxylation and epoxydation steps was proposed for the biosynthesis of cutin monomers (Kolattukudy, 1985). The enzyme system catalyzing such reactions has remained elusive for a long time. A novel pathway was later discovered involving lipoxygenation, peroxydation and epoxydation. The critical enzyme is a cytochrome P450-dependent peroxygenase associated with a membrane-bound epoxide hydrolase (Blée & Schuber, 1993). The physiological relevance of this biosynthetic pathway was recently confirmed *in planta* (Lequeu et al.,

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2003). The cutin monomers are postulated to be transported to the membrane via vesicles and their movement through the cell wall was hypothesized be assisted by lipid transfer proteins (Hollenbach et al., 1997; Pyee & Kolattukudy 1995)

The cuticle as a source of signals for pathogens

Besides its role as a multifunctional barrier, the cuticle is a source of signals perceived by invading organisms. The first indication was provided by observations on the induction of *Fusarium solani* pv *pisi* cutinase by cutin monomers (Lin et al., 1978). The production of cutinase by *F. solani* pv *pisi* only occurs when cutin is present in the medium (Woloshuk et al., 1984). Thus, fungal spores landing on plants might sense the presence of cutin monomers on the cuticle and induce high levels of cutinase required for penetration. In this model proposed by Kolattukudy (1985), cutin monomers act like a plant signal for the fungus and much work was dedicated to understanding how this signal acts in the pathogen. The induction of fungal cutinase by cutin monomers was shown to result from transcriptional activation (Woloshuk & Kolattukudy, 1986). A G-rich palindromic element essential for induction by cutin monomers is in the promoter of the cutinase gene and was shown to be a binding site for a transcription factor (Kamper et al., 1994). Cuticular components are also perceived by fungi to induce developmental processes. Cutin monomers released during the early stages of infection can induce the germination and appressorium in the rice blast fungus *Magnaporthe grisea* (Gilbert et al., 1996) and appressorial tube formation in *Erysiphe graminis* (Francis et al., 1996).

Surface waxes from avocado induced germination and appressorium formation of the avocado pathogen *Colletotrichum gloeosporioides*, while waxes from other plants were not effective. *Colletotrichum* species pathogenic on other plants were not affected and waxes of other plants did not induce differentiation in *C. gloeosporioides* (Podila et al., 1993). The signal transduction pathway induced by cutin monomers and leading to appressorium formation in *Colletotrichum* involves a protein kinase homologous to the mammalian protein kinase C (PKC). Treatments with inhibitors of PKC or replacement of the PKC-like gene in the fungus interfered with the ability to form appressoria (Dickman et al., 2003).

In summary, cuticular products have been repeatedly shown to be possible signals perceived and used by invading fungi to activate pathogenic responses.

The cuticle as a source of signals for the plant

Plants perceive molecular patterns from pathogens or molecules generated during the interaction between plants and pathogens. Components of the plant cell wall released by pathogens during the early stages of an infection can act as elicitors of defense responses in the plant (Boller, 1995; Ebel & Cosio, 1994). For example, pectin fragments released by fungal pectinases are sensed by plants and induce defense responses (Ryan & Farmer, 1991). Plants also accumulate pectinase-inhibiting proteins (PGIPs) in the cell wall that can protect the plant from the action of pathoenzymes. The action of PGIPs might also lead to short-chained pectic oligomers with elicitor activity (Cervone et al., 1989). Plants might also perceive components of the cuticle. Theoretically, such components would be among the first elicitors to be released during the penetration of a pathogen. This hypothesis was tested in barley and rice by applying cutin monomers to plants and testing the resistance respectively to *Erysiphe graminis* and *Magnaporthe grisea* (Schweizer et al., 1994; 1996a). Two monomers of the C_{18} family were effective in protection while exhibiting no direct fungicidal effect. The effect of cutin monomers on defense mechanisms was determined in suspension-cultured potato cells where they induced medium alkalinization, ethylene production and accumulation of defense-related genes (Schweizer et al., 1996b). Interestingly, the most active compound was n,16-hydroxypalmitic acid (n = 8, 9 or 10), a major component of the potato cuticle. In etiolated and conditioned cucumber hypocotyls, cutin monomers from hydrolysates of cucumber, apple, and tomato cutin elicited H_2O_2 production (Fauth et al., 1998).

A novel activity of fungal cutinases was observed by supplementing spores of *Rhizoctonia solani* with purified cutinase from *Venturia inaequalis* (or from *Fusarium solani*). Such inoculum droplets lead to a decrease in web blight symptoms on bean when compared to drops containing spores alone. This surprising effect of cutinase resides in the lipolytic esterase activity of the cutinase and can be mimicked by other enzymes with similar activity, like microbial lipases and non-specific esterases. The disease-prevention activity of cutinase seems to be independent of known resistance responses (Parker & Köller, 1998).

The relevance of the cuticle as a source of signals was further tested in plants overexpressing a cutinase gene from *Fusarium solani* pv *pisi* under the control of the CaMV35S promoter and targeted to the cell wall (so-called CUTE plants; see Sieber et al., 2000). In this conference, we have presented the first results from such studies. CUTE plants have a normal layer of wax but the cuticle structure appears partly absent. They exhibit enhanced permeability to solutes and to some extent present fusion of the flower organs (Sieber et al., 2000). CUTE plants with normal

overall leaf morphology but with an altered cuticle were further tested to determine the level of resistance to pathogens. No difference was observed between CUTE and wild-type plants when infected with the obligate biotrophs *Erysiphe cichoracearum* and *Hyaloperonospora parasitica* as well as the non-obligate biotroph *Phytophthora brassicae*. CUTE plants were found to be similarly susceptible to the non-host *Blumeria graminis*. However, they show a strong resistance to the necrotrophic fungus *Botrytis cinerea*. No direct effect of *Fusarium solani* f.sp. *pisi* cutinase could be observed against *B. cinerea*. The hypothesis was tested whether CUTE plants could sense cuticular defects by inducing or potentiating known defense genes. Neither *PR1, PR3, PR5* nor *PDF1.2* showed any induction, making their possible involvement in the resistance unlikely. One gene coding for the Arabidopsis PGIP protein was found to be activated in CUTE plants. Further work is now going on in our laboratory to understand the basis of cutinase-induced resistance in Arabidopsis.

In summary, evidence from various experimental approaches indicates a possible sensing by plants of cuticular degradation products. Such signals might be another information used to activate innate defense responses.

Conclusion

The view of the cuticle as a passive barrier on the plant surface has to be expanded to include its function as a source of signals for invading pathogens and for the induction of innate immune responses in plants. This raises some intriguing questions for future research and points towards possible applications.

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References

Barthlott W, Neinhuis C, Cutler D, Ditsch F, Meusel I, Theisen I, Wilhelmi H. 1998. Classification and terminology of plant epicuticular waxes. Bot J Linn Soc 126:237–260.

Benitez JJ, Matas AJ, Heredia A. 2004. Molecular characterization of the plant biopolyester cutin by AFM and spectroscopic techniques. J Struct Biol 147:179–184.

Blée E, Schuber F. 1993. Biosynthesis of cutin monomers – Involvement of a lipoxygenase peroxygenase pathway. Plant J 4(1):113–123.

Boller T. 1995. Chemoperception of microbial signals in plant cells. Ann Rev Plant Physiol Plant Mol Biol 46:189–214.

Bonaventure G, Beisson F, Ohlrogge J, Pollard M. 2004. Analysis of the aliphatic monomer composition of polyesters associated with Arabidopsis epidermis: occurrence of octadeca-cis-6, cis-9-diene-1,18-dioate as the major component. Plant J 40:920–930.

Cervone F, Hahn MG, Delorenzo G, Darvill A, Albersheim P. 1989. Host–Pathogen Interactions 33. A plant protein converts a fungal pathogenesis factor into an elicitor of plant defence responses. Plant Physiol 90:542–548.

Dickman MB, Ha YS, Yang Z, Adams B, Huang C. 2003. A protein kinase from Colletotrichum trifolii is induced by plant cutin and is required for appressorium formation. Mol Plant-Microbe Interact 16:411–421.

Ebel J, Cosio EG. 1994. Elicitors of plant defence responses. In: *Int. Review of Cytology* Vol. 148, Jeon KW, Jarvik J, editors. San Diego: Academic Press Inc. pp 1–36.

Fauth M, Schweizer P, Buchala A, Markstadter C, Riederer M, Kato T, Kauss H. 1998. Cutin monomers and surface wax constituents elicit H_2O_2 in conditioned cucumber hypocotyl segments and enhance the activity of other H_2O_2 elicitors. Plant Physiol 117:1373–1380.

Fernandez S, Osorio S, Heredia A. 1999. Monitoring and visualising plant cuticles by confocal laser scanning microscopy. Plant Physiol Biochem 37:789–794.

Francis SA, Dewey FM, Gurr SJ. 1996. The role of cutinase in germling development and infection by Erysiphe graminis f sp hordei. Physiol Mol Plant Pathol 49:201–211.

Gilbert RD, Johnson AM, Dean RA. 1996. Chemical signals responsible for appressorium formation in the rice blast fungus Magnaporthe grisea. Physiol Mol Plant Pathol 48: 335–346.

Heredia A. 2003. Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer. Biochim Biophys Acta Gen Sub 1620:1–7.

Hollenbach B, Schreiber L, Hartung W, Dietz KJ. 1997. Cadmium leads to stimulated expression of the lipid transfer protein genes in barley: implications for the involvement of lipid transfer proteins in wax assembly. Planta 203:9–19.

Jeffree CE. 1996. Structure and ontogeny of plant cuticles: an integrated functional approach. In: Kerstiens G, editor. Plant cuticles: an integrated functional approach. Oxford: BIOS Scientific Publishers. pp 33–82.

Kamper JT, Kamper U, Rogers LM, Kolattukudy PE. 1994. Identification of regulatory elements in the cutinase promoter from Fusarium solani f.sp. pisi (Nectria haematococca). J Biol Chem 269: 9195–9204.

Kerstiens GE. 1996. Plant cuticle: An integrated functional approach. Oxford: BIOS Scientific Publishers.

Koch K, Neinhuis C, Ensikat HJ, Barthlott W. 2004. Self-assembly of epicuticular waxes on living plant surfaces imaged by atomic force microscopy (AFM). J Exp Bot 55:711–718.

Kolattukudy PE. 1985. Enzymatic penetration of the plant cuticle by fungal pathogens. Ann Rev Phytopathol 23:223–250.

Lequeu J, Fauconnier M-L, Chammai A, Bronner R, Blee E. 2003. Formation of plant cuticle: evidence for the occurrence of the peroxygenase pathway. Plant J 36:155–164.

Lin TS, Kolattukudy PE. 1978. Induction of a bio-polyester hydrolase (cutinase) by low-levels of cutin monomers in Fusarium solani f.sp. pisi. J Bacteriol 133:942–951.

Moire L, Schmutz A, Buchala A, Stark RE, Ryser U. 1999. Glycerol is a suberin monomer. New experimental evidence for an old hypothesis. Plant Physiol 119:1137–1146.

Nawrath C. 2002. The biopolymers cutin and suberin. In: Somerville CR, Meyerowitz EM, editors. The Arabidopsis Book. Rockville, MD: American Society of Plant Biologists.

Parker DM, Köller W. 1998. Cutinase and other lipolytic esterases protect bean leaves from infection by Rhizoctonia solani. Mol Plant-Microbe Interact 11:514–522.

Podila GK, Rogers LM, Kolattukudy PE. 1993. Chemical signals from avocado surface wax trigger germination and appressorium formation in Colletotrichum gloeosporioides. Plant Physiol 103:267–272.

Pyee J, Kolattukudy PE. 1995. The gene for the major cuticular wax-associated protein and 3 homologous genes from broccoli (Brassica oleracea) and their expression patterns. Plant J 7:49–59.

Ramirez FJ, Luque P, Heredia A, Bukovac MJ. 1992. Fourier-transform IR study of enzymatically isolated tomato fruit cuticular membrane. Biopolymers 32:1425–1429.

Riederer M, Schreiber L. 2001. Protecting against water loss: analysis of the barrier properties of plant cuticles. J Exp Bot 52:2023–2032.

Ryan CA, Farmer EE. 1991. Oligosaccharide signals in plants – a current assessment. Ann Rev Plant Physiol Plant Mol Biol 42:651–674.

Schweizer P, Jeanguénat A, Métraux JP, Mösinger E. 1994. Plant protection by free cutin monomers in two cereal pathosystems. In: Daniels MJ, Downie JA, Osbourn AE, editors. Advances in Molecular Genetics of Plant-Microbe Interactions. Dordrecht: Kluwer. pp 371–374.

Schweizer P, Jeanguenat A, Whitacre D, Métraux JP, Mösinger E. 1996a. Induction of resistance in barley against Erysiphe graminis f sp hordei by free cutin monomers. Physiol Mol Plant Path 49:103–120.

Schweizer P, Felix G, Buchala A, Muller C, Métraux JP. 1996b. Perception of free cutin monomers by plant cells. Plant J 10:331–341.

Sieber P, Schorderet M, Ryser U, Buchala A, Kolattukudy P, Métraux JP, Nawrath C. 2000. Transgenic Arabidopsis plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell 12:721–737.

Wellesen K, Durst F, Pinot F, Benveniste I, Nettesheim K, Wisman E, Steiner-Lange S, Saedler H, Yephremov A. 2001. Functional analysis of the LACERATA gene of Arabidopsis provides evidence for different robes of fatty acid ω-hydroxylation in development. Proc Nat Acad Sci USA 98:9694–9699.

Woloshuk CP, Soliday CL. Kolattukudy PE. 1984. Cutinase induction in germinating spores of Fusarium-solani f.sp pisi. Phytopathology 74:832–832.

Woloshuk CP, Kolattukudy PE. 1986. Mechanism by which contact with plant cuticle triggers cutinase gene-expression in the spores of Fusarium solani f.sp. pisi. Proc Nat Acad Sci USA 83:1704–1708.

Yephremov A, Wisman E, Huijser P, Huijser C, Wellesen K, Saedler H. 1999. Characterization of the FIDDLEHEAD gene of Arabidopsis reveals a link between adhesion response and cell differentiation in the epidermis. Plant Cell 11:2187–2201.

III. Cuticular defects lead to full immunity to a major plant pathogen

III.1 Article

Cuticular defects lead to full immunity to a major plant pathogen

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Aerial parts of land plants are covered with a cuticle made of cutin, a complex polymer rich in esterified fatty acid derivatives, associated and coated with waxes. The cuticle forms a protection against water loss, irradiation, xenobiotics, and is involved in the delimitation of organs during development [1, 2]. It is assumed that the cuticle also constitutes a physical barrier to microbial invaders, through which cutinase-producing pathogens can penetrate. The cuticle breakdown products act as powerful signals for pathogens [1]. It is unknown, however, if plants can react to changes in the structure of the cuticle. Here we show that degradation of the cuticular layer by the expression of a fungal cutinase in *Arabidopsis* plants (CUTE plants) leads to full immunity to *Botrytis cinerea*, an ubiquitous fungal pathogen causing important damages to many crop plants. This powerful defence is independent of the known defence signalling routes involving salicylic acid, ethylene or jasmonic acid and is accompanied by the diffusion of a fungitoxic substance and changes in gene transcription. After inoculation with *B. cinerea*, several members of the lipid transfer protein, peroxidase, and protease inhibitor gene families were induced in CUTE plants and in *bodyguard*, a mutant with defects in the cuticle structure [3], that shows a similar resistant phenotype. Our results demonstrate the involvement of those genes in the defence against *B. cinerea* in *Arabidopsis*, since their overexpression in susceptible wild type plants led to increased resistance. Modification of the cuticle can thus activate a multi-layered resistance syndrome that reveals a novel defence pathway and adds to our knowledge on plant innate immunity.

III.1.1 Results and Discussion

The importance of the cuticular layer for plant growth and development was investigated previously in transgenic *Arabidopsis* plants constitutively expressing a fungal cutinase gene from *Fusarium solani* f.sp. *pisi* fused with a signal sequence for secretion (CUTE plants) [4]. Surprisingly, CUTE plants were completely resistant to the virulent necrotrophic pathogen *B.*

cinerea, which causes soft rot lesions on wild type (WT) plants (Fig. 1a, b), while the disease symptoms after inoculation with other necrotrophic fungi were unchanged (data not shown). Similarly, a strong protection was observed in the cuticle-defective mutant *bodyguard* (*bdg*), that shares a number of morphological characteristics with CUTE plants [3] (Fig. 1a, b). Plants expressing the cutinase gene driven by a dexamethasone-inducible promoter [5] (so-called DEX-CUTE plants) also exhibited resistance (Supplementary Fig. 1a, b). Protection was completely lost in *Arabidopsis* transformed with a non-functional *F. solani* cutinase gene containing a point mutation in the catalytic site [6] (data not shown; see Methods). Resistance similar to that in CUTE and *bdg* was also observed in WT plants after application of purified *F. solani* cutinase (Fig. 1b), the direct effect of which was harmless to *B. cinerea* (data not shown; see Methods). Taken together, these observations support the notion that plants can react to cuticular defects, presumably by the action of endogenous signals or elicitors.

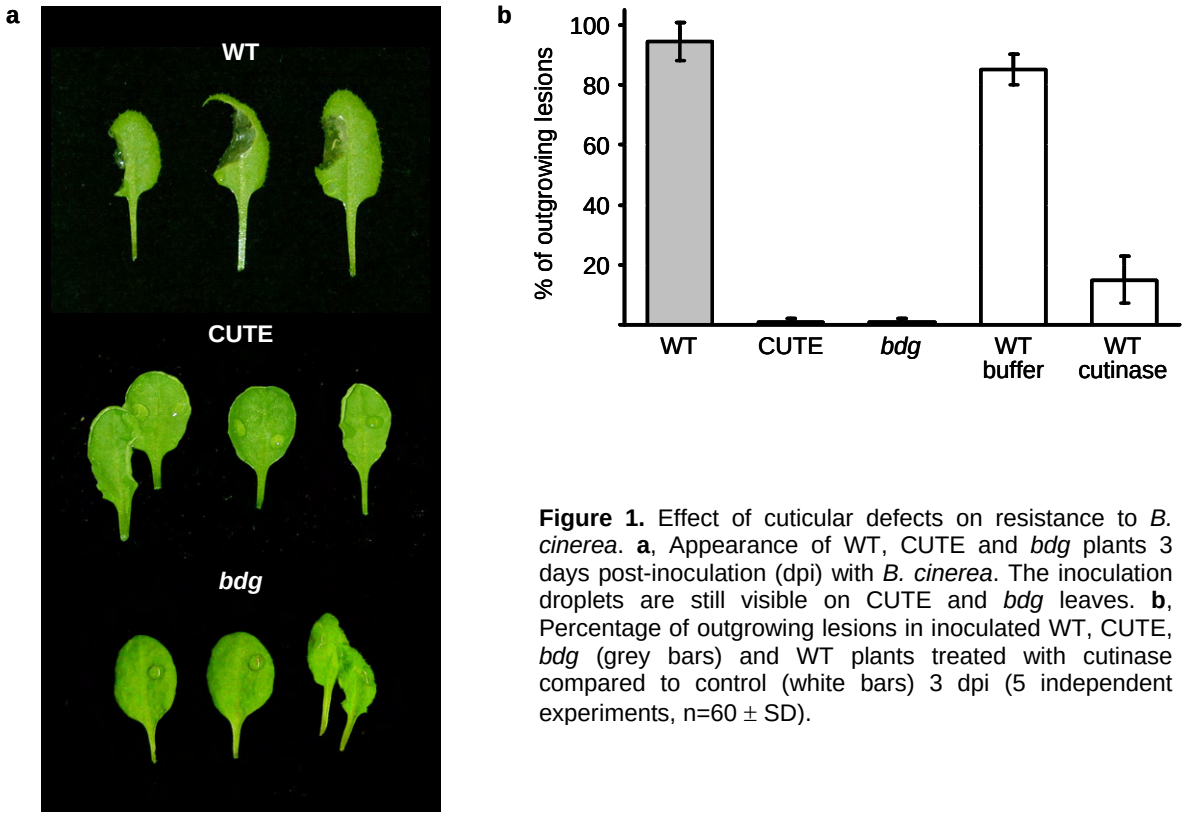


Figure 1. Effect of cuticular defects on resistance to *B. cinerea*. **a**, Appearance of WT, CUTE and *bdg* plants 3 days post-inoculation (dpi) with *B. cinerea*. The inoculation droplets are still visible on CUTE and *bdg* leaves. **b**, Percentage of outgrowing lesions in inoculated WT, CUTE, *bdg* (grey bars) and WT plants treated with cutinase compared to control (white bars) 3 dpi (5 independent experiments, n=60 ± SD).

Hyphal growth of *B. cinerea* was inhibited after spore germination on the plant surface of the resistant plants and penetration did not take place (data not shown). A fungitoxic activity diffusing from the leaf surface was observed in CUTE and *bdg* plants (Fig. 2). The fungitoxic activity was demonstrated in both *in-vitro* and *in-vivo* assays. The early arrest of fungal growth in the resistant plants might therefore be related to the secreted fungitoxic compound(s), the chemical nature of which remains to be determined. This provides a first powerful defence layer against invasion by *B. cinerea.*

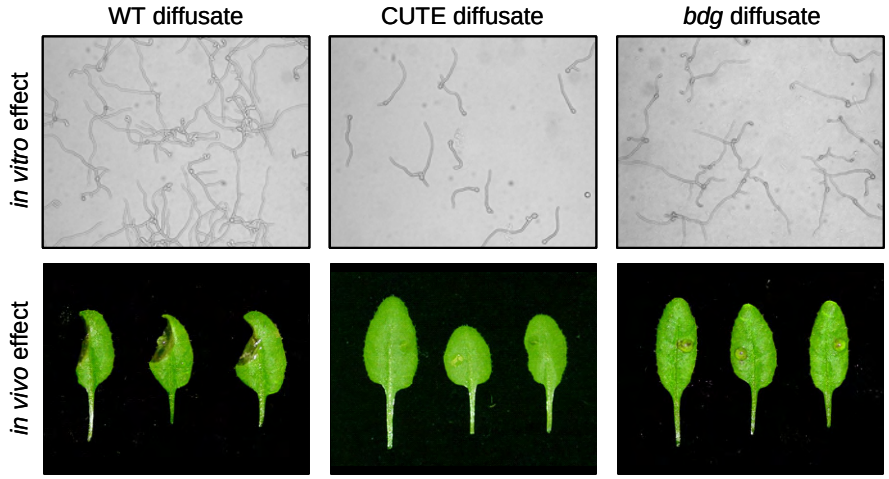


Figure 2. Fungitoxic activity diffusing from the leaf surface in plants with cuticular defects. *In vitro* fungitoxic activity of CUTE and *bdg* exudates to *B. cinerea*, in comparison to WT diffusate (upper pictures) (10 independent experiments, typical examples are shown). *In-vivo* activity of diffusates, shown by the appearance of WT plants 3 dpi with *B. cinerea* mixed with diffusates of WT, CUTE and *bdg* plants (lower pictures) (10 independent experiments, n=60).

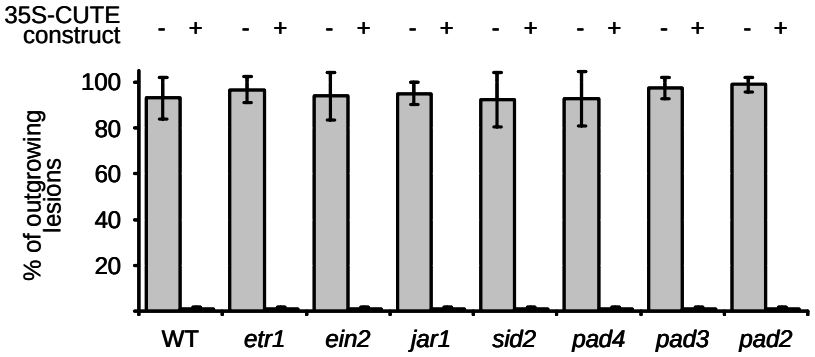


Figure 3. Resistance to *B. cinerea* in signalling mutants with cuticular defects. Percentage of outgrowing lesions in various defence signalling mutants overexpressing the cutinase of *F. solani* f.sp. *pisi* (35S-CUTE construct) in comparison to untransformed controls 3 dpi with *B. cinerea* (3 independent experiments, n=24 ± SD).

We also tested the involvement of the major signalling routes commonly associated with plant defence reactions and involving the hormones salicylic acid (SA), ethylene (ET) or jasmonic acid (JA). Resistance to *B. cinerea* was shown previously to be associated mainly with the JA- and ET- signalling pathways, and with SA and camalexin for local responses [7, 8, 9, 10]. Mutants defective in the SA- (*sid2, pad4)* [11, 12], the ET- (*ein2, etr1*) [13, 14], or the JA- (*jar1*) [15] pathways were transformed with the *F. solani* cutinase under the control of a constitutive promoter and all transformants displayed full resistance, comparable to the original CUTE plants (Fig. 3). Therefore, resistance of CUTE plants is independent of pathways involving these signals. Furthermore, overexpression of the *F. solani* cutinase in the *pad2* and *pad3* [16] mutants

impaired in the production of the fungitoxic phytoalexin camalexin, resulted in fully immune plants, indicating that camalexin is not involved (Fig. 3). Moreover, CUTE plants show no induction of known defence genes such as pathogenesis-related proteins (*PRs*) or plant defensin (*PDF1.2*) during infection with *B. cinerea*, unlike infected WT plants (Supplementary Fig. 2a). In addition, the production of reactive oxygen species was absent in CUTE plants (Supplementary Fig. 2b). The induction of these responses in WT plants correlates with the massive development of symptoms.

Further insights in the changes associated with resistance of CUTE plants were obtained by genome-wide gene expression studies using Affymetrix microarrays. Gene expression was analysed 12 and 30 h after inoculation with *B. cinerea* (data available on internet). Interesting candidate genes were selected both on the basis of their earlier and higher induction after inoculation with *B. cinerea* in CUTE compared to WT plants. These genes were also induced in infected WT plants, albeit to a lower level, indicating that they might be part of an attempted but insufficient defence response against the virulent pathogen. Moreover, the cuticle-defective mutant *bdg* [3] also showed enhanced expression of the same genes as in CUTE plants after inoculation with *B. cinerea*, providing further support that these genes are involved in the response associated with cuticular defects (Supplementary Fig. 3). Among the candidates filling these criteria, genes were selected on their conspicuous affiliation to large gene families (Supplementary Table 1) and the microarray data were confirmed using Real-time RT-PCR (data not shown). The possible implication of such genes was tested by constitutive overexpression in WT plants. Resistance to *B. cinerea* was observed in transformed plants overexpressing closely-related members of the lipid transfer protein (LTP) family (At4g12470, At4g12480, At4g12490) [17], of the class III peroxidase (PER) family (At2g37130, At5g39580, At5g64120) [18] and of the proteinase inhibitor (PI) family (At2g38870, At2g43510) [19] (Fig. 4). Overexpression of other candidate genes like At4g23600, At2g43590, or At2g50200 did not provide significant protection against *B. cinerea*, in addition to two other genes (At2g38530, At3g20470) with different expression patterns (Fig. 4). Interestingly, a considerable increase in the expression of the selected genes was also observed in both CUTE and *bdg* plants after mock inoculation and was further enhanced in the presence of *B. cinerea*, indicating that the signalling pathways for their induction are complex with pathogen-dependent and pathogen-independent elements. Taken together, our data support that the combined action of the products of the *LTP*, *PER* and *PI* genes contributes to the resistance of cuticle-defective plants possibly forming a second protective shield against infection with *B. cinerea*. Thus, defective cuticles might provide cues that condition the plant for a better defence towards *B. cinerea* upon perception of different environmental stimuli.

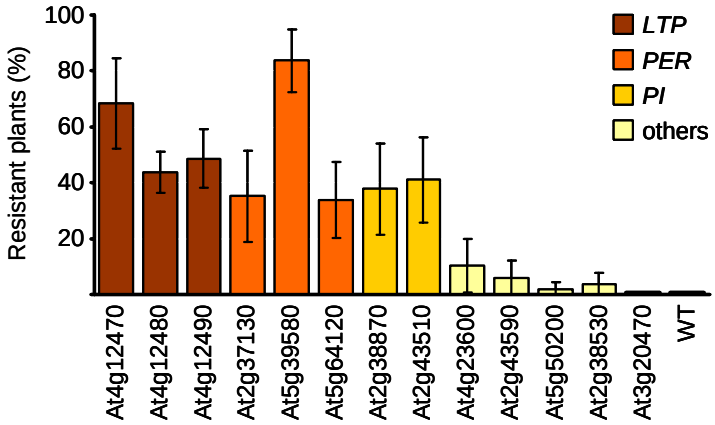
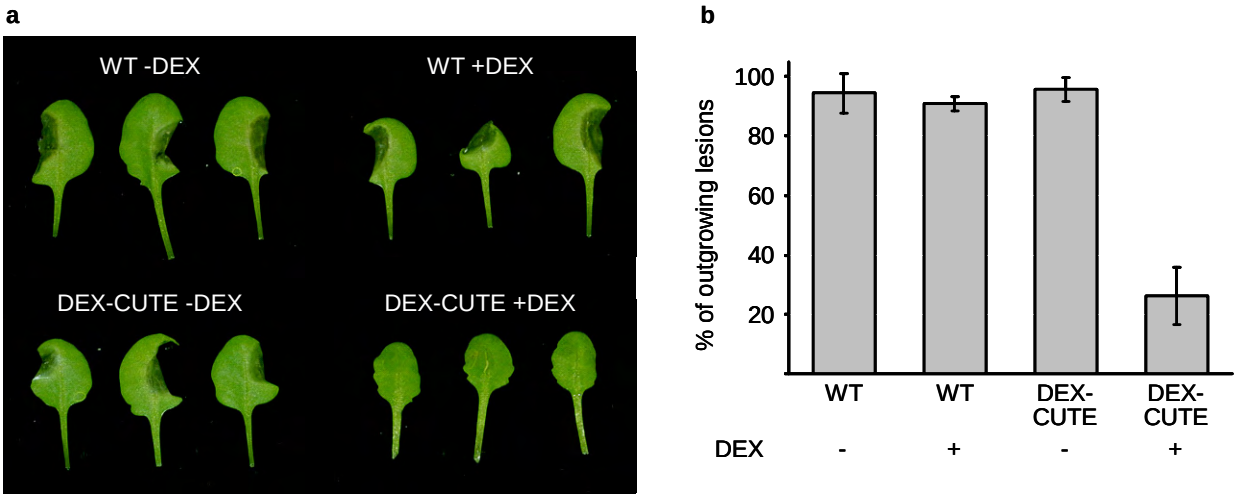


Figure 4. Effect of overexpression of candidate genes identified in microarrays on the resistance to *B. cinerea.* Each bar represents the mean of the percentage of independent primary transformants of each selected gene (indicated by the gene locus number) exhibiting full resistance (4 independent experiments, n=60 ± SD). Resistance was tested on 3 leaves per transformant. The resistance to *B. cinerea* conferred by the overexpression of the *LTP*, *PER* and *PI* genes was further confirmed in transformants of the second generation.

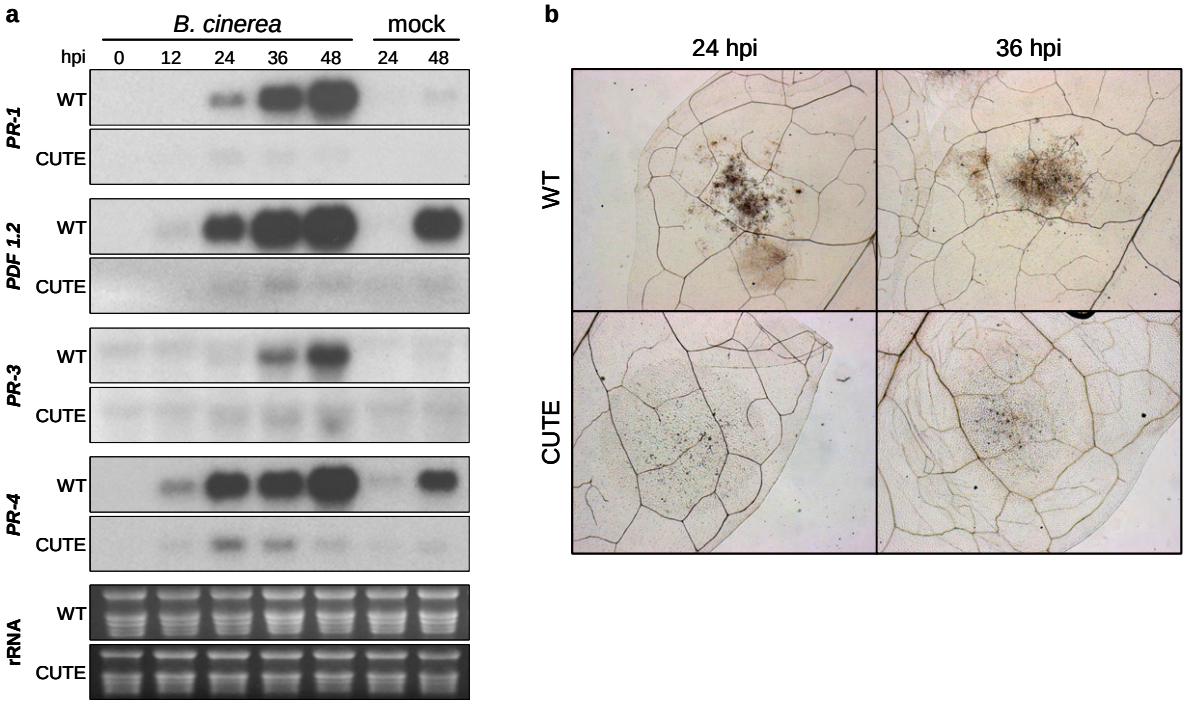
Generally, LTPs are known to be associated with the formation of the cutin and suberin layers and to inhibit fungal growth [20, 21, 22]. For example, heterologous expression of a LTP from pepper in *Arabidopsis* was recently reported to increase the resistance to *B. cinerea* [23]. The biochemical function of the three *LTP* genes identified remains unknown but our data provide the first experimental support for their implication in resistance to *B. cinerea* in *Arabidopsis*. Members of the class III *PER* gene family encode proteins involved in cell-wall lignification and crosslinking, H_2O_2 generation or detoxification, and in responses to wounding and pathogens [18]. PER activity was associated with crosslinking of phenolic acids at infection sites of *B. allii* in onion cell walls [24]. In bean leaves, aggressive isolates of *B. cinerea* suppressed the PER activity compared to non-virulent isolates, supporting a role for PER in plant resistance as scavengers of harmful active oxygen species [25]. Our results are in agreement with these observations and provide a new biological function for a group of hitherto undescribed *PER* genes in plants in relation to *B. cinerea* resistance. *B. cinerea* secretes aspartic proteases during the early stages of infection that are likely to play a primordial role in pathogenesis [26, 27]. Proteinase inhibitors produced in many plants, including trypsin inhibitors, can inhibit *B. cinerea* proteases *in vitro* [19], and overexpression of proteinase inhibitors from *Nicotiana alata* in tobacco was recently shown to protect against *B. cinerea* [28]. The data presented here corroborate these findings and support a role for *PI* genes in the defence of *Arabidopsis* against *B. cinerea.*

In summary, *Arabidopsis* plants displaying cuticular defects induce changes leading to complete resistance to *B. cinerea*. Immunity results from a multi-layered defence that is independent of SA, ET, JA, and involves an effective diffusible fungitoxic activity. In addition, the expression of genes such as *LTP*, *PER*, *PI* that are naturally induced by *B. cinerea* in WT plants, is strongly enhanced in plants with cuticular defects. The resistance syndrome presented is the first example by which a species normally susceptible to a necrotrophic fungus establishes full immunity. These results increase our knowledge on plant defences and highlight a novel mechanism that might possibly be exploited to protect plants against this ubiquitous fungal pathogen.

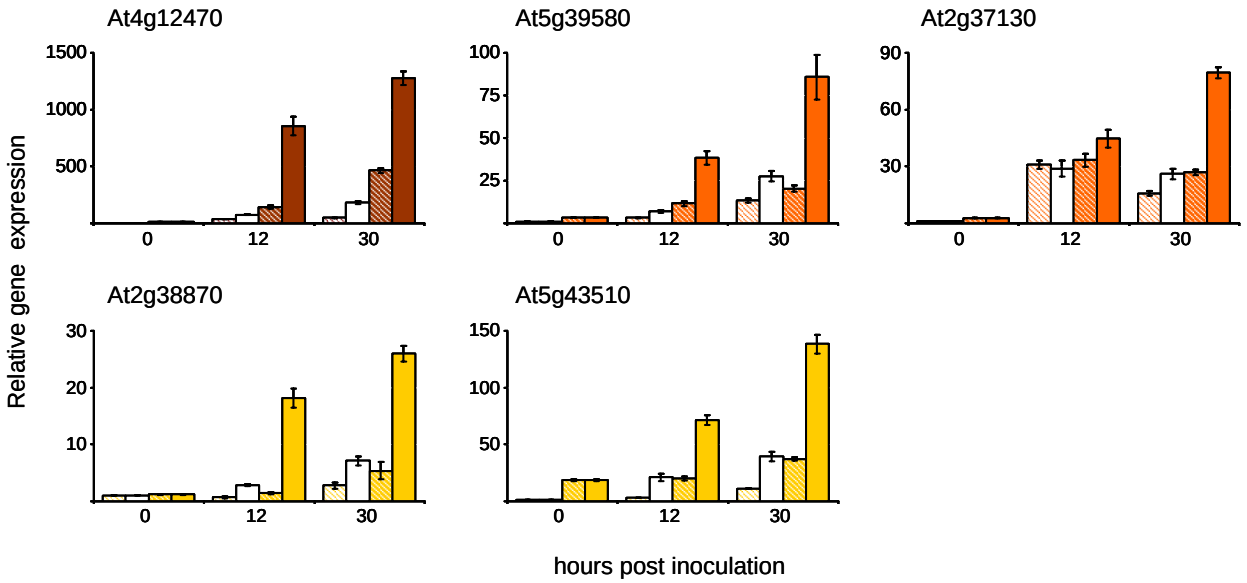
III.1.2 Supplementary Figures



Supplementary Figure 1. Effect of inducible cutinase expression on resistance to *B. cinerea*. **a**, Appearance of DEX-CUTE plants induced with DEX 3 dpi with *B. cinerea* compared to controls. **b**, Percentage of outgrowing lesions in DEX-CUTE plants induced with DEX 3 dpi compared to controls (5 independent experiments, n=60 ± SD). Dexamethasone (Sigma) was dissolved in ethanol at 30 mM and diluted into H_2O + 0.01% Tween20 (Fluka) to a final concentration of 0.03 mM. Two to 3 weeks after transplanting, DEX-CUTE and WT plants were sprayed twice a day with the DEX solution and again 4 days later. Plants were infected with *B. cinerea* 3 days after the second treatment. After spraying of DEX, trays were covered overnight with a lid. Induction of cutinase was confirmed by an enzymatic assay [4].



Supplementary Figure 2. Defense reactions in WT and CUTE plants after inoculation with *B. cinerea*. **a**, Gene expression analysis (Northern blot) of *PR-1*, *PDF1.2*, *PR-3* and *PR-4* in WT and CUTE plants 0, 12, 24, 36 and 48 hpi with *B. cinerea*. 5 μg of RNA per sample was loaded. The experiment was repeated 2 times with similar results. **b**, Production of H_2O_2 in infected WT and CUTE plants. The presence of H_2O_2 was visualised by 3,3'-diaminobenzidine (DAB) [33] staining 24 and 36 hpi with *B. cinerea*. Detached leaves were immersed in 1 mg ml^{-1} DAB-HCl pH 3.8 (Sigma) and vacuum-infiltrated. After overnight incubation, leaves were bleached in 4 mg ml^{-1} chloralhydrate and observed with a microscope. The experiment was carried out on 32 plants and repeated 2 times. Typical examples are shown.



Supplementary Figure 3. Priming of genes in the mutant *bdg* after inoculation with *B. cinerea*. Relative expression of the genes At4g12470, At5g39580, At2g37130, At2g38870 and At2g43510 in WT (bars with white background) and *bdg* plants (bars with coloured background), mock (dashed bars) or *B. cinerea* inoculated (full bars). Each bar represents the mean of triplicate samples ± SE (WT 0 is arbitrary defined as 1). The experiment was repeated 2 times with similar results.

		WT	CUTE	WT				CUTE			
	hpi	0		12		30		12		30	
	treatments	-	-	mock	*Bc*	mock	*Bc*	mock	*Bc*	mock	*Bc*
At4g12470	pEARLI 1-like protein *	5.5	46.3	208	2056.2	2265.5	5257.8	4721.7	8208.7	6431.5	8498.4
At4g12480	pEARLI 1 *	1.5	3.2	14.2	144.3	113.9	1270.1	202.2	1264	2515.8	2686
At4g12490	pEARLI 1-like protein *	19.8	100.9	41.5	101.2	90.2	6223.1	138.9	610.8	1983.3	6087.4
At2g37130	peroxidase 21	45.2	194.4	52.2	135	167.6	802.8	1753.2	1663.6	4110	1828.6
At5g39580	peroxidase ATP24a	22.8	26.4	28.6	108.1	15.8	435.6	114.7	734.5	453.7	968
At5g64120	peroxidase	46.2	100	137	972.1	1132.6	4592.1	3755.2	6654.7	6429.1	7745.5
At2g38870	protease inhibitor	231	482.4	189.2	989.8	588.9	6103.3	3205.3	4098.1	5187.1	6975.2
At2g43510	trypsin inhibitor	29.6	329.2	16.9	108.8	72.2	1132.3	607.4	1167.6	2225.1	1921.4
At4g23600	tyrosine transaminase	193.7	4217.9	597.9	1286.7	335.6	1318.4	3576.5	3167.7	1005.6	729.1
At2g43590	chitinase	18.3	2.3	39	1167.5	34.2	164	116	2530.8	289.8	1396.8
At5g50200	wound-responsive protein 3	43.5	74.4	64.5	202.8	241.9	824.1	542.8	647.8	762.2	1162.8
At2g38530	non-specific lipid-transfer protein 2	7.5	204	335.7	348.1	580.5	4.4	2331.4	1893.5	9572.8	5288.4
At3g20470	glycine-rich protein	66.9	750.7	110.4	52.5	206.9	5.2	1122.3	408.3	1219.4	503.9

* lipid-tranfer family / protease inhibitor / seed storage family protein

Supplementary Table 1. Selected candidate genes identified in microarrays. Microarray expression values (raw data) of WT and CUTE plants at 0, 12 and 30 hpi with *B. cinerea* (*Bc* = *B. cinerea*) and with mock solution.

III.1.3 Material and Methods

Plant material

Plants were grown on a pasteurized soil mix of humus / perlite (3:1) under a 12 h light and 12 h dark cycle, with a night temperature of 16 to 18°C and a day temperature of 20 to 22°C (60 to 70% humidity). WT plants are the *Arabidopsis* accession Col-0, obtained from the Arabidopsis Biological Reasearch Center (Columbus, OH). The Arabidopsis mutant *sid2* was *sid2-1* [12]*; pad3* was *pad3-1, pad2* was *pad2-1* and *pad4* was *pad4-1* [11, 16]*; etr1* was *etr1-1* and *ein2* was *ein2-1* [13, 14]; *jar1* was *jar1-1* [15]. The mutant *bdg* was *bdg-1* [3].

Inoculation with *B. cinerea*

B. cinerea strains B05.10 [29] and BMM [30] provided by J. van Kan and B. Mauch-Mani were grown on 1x PDA (Potato Dextrose Agar, 39 g l^{-1}, Difco). Spores were harvested in water and filtered through glass wool to remove hyphae. Spore concentration was adjusted to 5 x 10^4 spores ml^{-1} in ¼ PDB (Potato Dextrose Broth, 6 g l^{-1}, Difco) for inoculation. Leaves were inoculated with 5 µl droplets of spore suspension to evaluate the symptoms. The level of protection was estimated by the potential of *B. cinerea* to cause soft rot symptoms extending beyond the inoculation site (outgrowing lesions). The spore suspension was sprayed on whole plants for microarray and real-time RT-PCR experiments. Control plants were inoculated with ¼ PDB (mock). The inoculated plants were kept under a water-sprayed transparent lid to maintain high humidity. Both *B. cinerea* strains gave similar results for all experiments carried out.

***In-vitro* effect of cutinase and application of cutinase on leaves**

Purified cutinase from *F. solani*, kindly provided by M. Van der Burg-Koorevaar (UNILEVER Vlaardingen, The Netherlands), was diluted in 10 mM Na-acetate pH 5.2. The *in-vitro B. cinerea* growth assay was performed in a final volume of 12 µl. Nine µl of the cutinase solution were mixed with 3 µl of *B. cinerea* spores in PDB to a final concentration of 1000, 100 or 10 µg ml^{-1} of cutinase and 5 x 10^4 spores ml^{-1} in ¼ PDB, and deposited on a microscope glass slide. After incubation under high humidity conditions for ca. 16 h fungal growth was observed under the microscope. For cutinase application on WT plants, 5 µl droplets of 100 µg ml^{-1} cutinase or buffer were deposited on leaves and incubated for 3 days under high humidity to prevent evaporation of the droplets. Droplets were removed and replaced by a droplet of *B. cinerea* spores.

***In vitro* and *in vivo* effect of leaf diffusates**

Five µl droplets of ¼ PDB were incubated for 18 h on WT, CUTE and *bdg* leaves. Leaf diffusates were collected. Nine µl of the exudate solution was mixed with 3 µl of *B. cinerea* spores in H_2O to a final concentration of 5 x 10^4 spores ml^{-1} and deposited on a microscope glass slide. Fungal growth was observed under the microscope after incubation under high humidity conditions for ca. 16 h. *In vivo* tests were performed by inoculating WT *Arabidopsis* leaves with the leaf diffusates mixed with *B. cinerea* spores. Disease symptoms were evaluated 3 days later.

Plant transformation

Arabidopsis Col-0 plants, *sid2* [12], *ein2* [13, 14], *etr1* [13, 14], *jar-1* [15], *pad2, pad3* and *pad4* mutants [11, 16] were transformed with a *F. solani* cutinase as previously described [4]. The presence of the 35S-CUTE construct was confirmed by the morphological phenotype typical for CUTE plants.

DEX-CUTE plants were generated using the glucocorticoid-inducible system [5].The *F. solani* cutinase gene fused with the tobacco chitinase A signal sequence were excised from pMMB7066:SS:CUT (see [4]) with SstI/XbaI and cohesive ends were filled in with T4 DNA polymerase (New England Biolabs). The pTA7002 binary vector [5] was opened using the XhoI site and prepared for blunt-end ligation with the SS-CUT insert. This construct was transformed in *Agrobacterium tumefaciens* pGV3101. WT plants were transformed by vacuum infiltration and several independent homozygous lines were analyzed.

The mutated cutinase gene from *F. pisi* [6] was amplified from the pET-16b using the oligonucleotides 5'-CUT (5'-TGCTAGCGCTGGTAGAACAACTCG-3'; NheI site underlined) and 3'-CUT (5'-TAGGTACCTCAAGCAGAACCACG-3'; KpnI site underlined). The gene was cloned behind the signal sequence of the tobacco chitinase A in the pPMB7066:SS:CUT (see [4]) previously digested with NheI/KpnI. The fusion construct was then cloned in the pART7/pART27 vector system [31] and WT plants were transformed as previously described [4].

Candidate genes selected from the microarray analysis were overexpressed in WT plants by using the pART7/pART27 vector system [31]. Gene coding sequences were amplified by PCR on cDNA from infected plants, using the following primers: 5'-At4g12470 (5'-CCTTACAACACCGAATATAAC-3') and 3'-At4g12470 (5'-

ATCGCATCGTATGCATAATG-3'); 5'-At4g12480 (5'-TCAAAGACACTGAATAAATCC-3') and 3'-At4g12480 (5'-TCACATCGTATGCATAGCTG-3'); 5'-At4g12490 (5'-CACTCAAACATTCTCCATAAC-3') and 3'-At4g12490 (5'-GTGCGTCGTATGTGTAATTG-3'); 5'-At2g37130 (5'-AGAGAGAGAGAGCAATGGC-3') and 3'-At2g37130 (5'-CAAGGACCATCAATATTAGTTC-3'); 5'-At5g39580 (5'-AAAAAATGGGCTTGGTCCG-3') and 3'-At5g39580 (5'-TAGCCATAAAAACTTAATCACTG-3'); 5'-At5g64120 (5'-ATGGGTTTGGTTAGATCATTG-3') and 3'-At5g64120 (5'-TTTATCACGACAGATTCTAATC-3'); 5'-At2g43510 (5'-AGAAAATGGCAAAGGCTATC-3') and 3'-At2g43510 (5'-GCGATTGCTTTAGATTTTACTG-3'); 5'-At2g38870 (5'-CATACAAATACATCAGAAGAC-3') and 3'-At2g39970 (5'-CTTTTTCATTATGAATATAGAAAT-3'); 5'-At4g23600 (5'-ATGACTCTGGGATGCAAA-3') and 3'-At4g23600 (5'-TTACTTAACACCATTGACGTCT-3'); 5'-At2g43590 (5'-ATGGCTTTCACAAAAATCTC-3') and 3'-At2g43590 (5'-TTAGCAACTAAGGTTAGGA-3'); 5'-At5g50200 (5'-ATGGCGATCCAGAAGAT-3') and 3'-At5g50200 (5'-TCATTTGCTTTGCTCTATCTTG-3'); 5'-At2g38530 (5'-ATGGCTGGAGTGATGAAGT-3') and 3'-At2g38530 (5'-CTTCATTTGACCGTCGCT-3'); 5'-At3g20470 (5'-ATGGCTTCCAAGTCACTCT-3') and 3'-At3g20470 (5'-TCAATGATGTCCACCACC-3'). The coding sequences were cloned in the pGEM®-T Easy Vector (Promega) and excised with EcoRI, or with SacI/SacII and blunt-ended. The fragments were cloned in the pART7 vector opened with EcoRI or with SmaI and the orientation was checked by PCR using a 5'-primer annealing upstream of the insertion site (5'-ATCCCACTATCCTTCGCAA-3') and a 3'-primer annealing in the insert. The constructs were cloned in the pART27 vector and *Arabidopsis* Col-0 plants were transformed as previously described [4]. Overexpression levels of the transgenes in primary transformants were confirmed using RT-PCR. Enhanced resistance to *B. cinerea* exhibited by primary transformants and overexpression of the transgenes were confirmed on 5 independent lines of the second generation for the *LTP*, *PER* and *PI* genes.

Microarray analysis

Whole genome expression analysis using 20k Affymetrix microarrays (Paradigm Genetics Inc.) was performed on WT and CUTE plant samples uninoculated, mock-inoculated or inoculated with *B. cinerea*, 12 and 30 hpi. Genes were selected using a program called FiRe [32] with the following criteria: among the genes induced by *B. cinerea* in WT plants (>2 fold), genes overexpressed (>2 fold) in CUTE plants were selected (induced genes with expression values <100 were eliminated). For confirmation of the microarray data, RNA was prepared using the TRIzol® reagent (Molecular Research Center, Inc., Invitrogen) and retrotranscribed in cDNA (Omniscript® RT kit, Qiagen). Real-time PCR was performed using the Absolute QPCR SYBR Green Mix (ABgene). Microarray expression values were confirmed in two independent experiments. Analysis of gene expression in *bdg* and WT after inoculation with *B. cinerea* was performed by Real-time RT-PCR. Gene expression values were normalised with the expression of the plant actin 2 gene.

Acknowledgements

Financial support of the Swiss National Science Foundation (Grant 3100A0–104224) is gratefully acknowledged. This work was also supported in part by the National Center for Competence in Research (NCCR). We thank T. Grau for her contribution in the development of DEX-CUTE plants; P. Kolattukudy, L. Rogers for providing the *Fusarium* cutinase gene containing a mutation in the active site. O. Lamotte, D. Reinhardt, H.-J. Schoonbeek and L. Sticher are thanked for critical reading of the manuscript.

III.1.4 References

1. Kolattukudy, P.E. Enzymatic penetration of the plant cuticle by fungal pathogens. Annual Review of Phytopathology 23, 223-250 (1985).

2. Nawrath, C. In the Arabidopsis Book: eds. Somerville, C.R. & Meyerowitz, E.M., American Society of Plant Biologists, Rockville, MD (2002).

3. Kurdyukov, S. et al. The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in *Arabidopsis*. Plant Cell 18, 321-339 (2006).

4. Sieber, P. et al. Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell 12, 721-737 (2000).

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

6. Rogers, L.M., Flaishman, M.A. & Kolattukudy, P.E. Cutinase gene disruption in *Fusarium solani* f.sp. *pisi* decreases its virulence on pea. Plant Cell 6, 935-945 (1994).

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

8. Kliebenstein, D.J., Rowe, H.C. & Denby, K.J. Secondary metabolites influence *Arabidopsis*/*Botrytis* interactions: variation in host production and pathogen sensitivity. Plant Journal 44, 25-36 (2005).

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

10. Thomma, B., Eggermont, K., Tierens, K. & Broekaert, W.F. Requirement of functional *ethylene-insensitive 2* gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiology 121, 1093-1101 (1999).

11. Glazebrook, J. & Ausubel, F.M. Isolation of phytoalexin-deficient mutants of *Arabidopsis thaliana* and characterization of their interactions with bacterial pathogens. Proceedings of the National Academy of Sciences of the United States of America 91, 8955-8959 (1994).

12. Nawrath, C. & Metraux, J.P. 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 (1999).

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

14. Schaller, G.E. & Bleecker, A.B. Ethylene-binding sites generated in yeast expressing the *Arabidopsis ETR1* gene. Science 270, 1809-1811 (1995).

15. Staswick, P.E., Tiryaki, I. & Rowe, M.L. 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 (2002).

16. Glazebrook, J. et al. Phytoalexin-deficient mutants of *Arabidopsis* reveal that *PAD4* encodes a regulatory factor and that four *PAD* genes contribute to downy mildew resistance. Genetics 146, 381-392 (1997).

17. Arondel, V., Vergnolle, C., Cantrel, C. & Kader, J.C. Lipid transfer proteins are encoded by a small multigene family in *Arabidopsis thaliana*. Plant Science 157, 1-12 (2000).

18. Tognolli, M., Penel, C., Greppin, H. & Simon, P. Analysis and expression of the class III peroxidase large gene family in *Arabidopsis thaliana*. Gene 288, 129 (2002).

19. Dunaevskii, Y.E. et al. Proteinase inhibitors as antistress proteins in higher plants. Applied Biochemistry And Microbiology 41, 344-348 (2005).

20. Heredia, A. Biophysical and biochemical characteristics of cutin, a plant barrier biopolymer. Biochimica et Biophysica Acta-General Subjects 1620, 1-7 (2003).

21. Blein, J.-P., Coutos-Thevenot, P., Marion, D. & Ponchet, M. From elicitins to lipid-transfer proteins: a new insight in cell signalling involved in plant defence mechanisms. Trends in Plant Science 7, 293-296 (2002).

22. Segura, A., Moreno, M. & Garciaolmedo, F. Purification and antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach. Febs Letters 332, 243-246 (1993).

23. Jung, H.W., Kim, K.D. & Hwang, B.K. Identification of pathogen-responsive regions in the promoter of a pepper lipid transfer protein gene (*CALTP1*) and the enhanced resistance of the *CALTP1* transgenic *Arabidopsis* against pathogen and environmental stresses. Planta 221, 361-373 (2005).

24. McLusky, S.R. et al. Cell wall alterations and localized accumulation of feruloyl-3'-methoxytyramine in onion epidermis at sites of attempted penetration by *Botrytis allii* are associated with actin polarisation, peroxidase activity and suppression of flavonoid biosynthesis. Plant Journal 17, 523-534 (1999).

25. von Tiedemann, A. Evidence for a primary role of active oxygen species in induction of host cell death during infection of bean leaves with *Botrytis cinerea*. Physiological and Molecular Plant Pathology 50, 151-166 (1997).

26. Movahedi, S. & Heale, J.B. 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. Physiological and Molecular Plant Pathology 36, 303-324 (1990).

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

28. Charity, J.A. et al. Pest and disease protection conferred by expression of barley beta-hordothionin and *Nicotiana alata* proteinase inhibitor genes in transgenic tobacco. Functional Plant Biology 32, 35-44 (2005).

29. Buttner, P. et al. Variations in ploidy among isolates of *Botrytis cinerea* - Implications for genetic and molecular analyses. Current Genetics 25, 445-450 (1994).

30. Zimmerli, L., Metraux, J.P. & Mauch-Mani, B. beta-aminobutyric acid-induced protection of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*. Plant Physiology 126, 517-523 (2001).

31. Gleave, A.P. A versatile binary vector system with a T-DNA organizational-structure conducive to efficient Integration of cloned DNA into the plant genome. Plant Molecular Biology 20, 1203-1207 (1992).

32. Garcion, C. et al. FiRe and microarrays: a fast answer to burning questions. submitted (2006).

33. Thordal-Christensen, H., Zhang, Z., Wei, Y. & Collinge, D.B. Subcellular localization of H_2O_2 in plants. H_2O_2 accumulation in papillae and hypersensitive response during the barley powdery mildew interaction. Plant Journal 11, 1187-1194 (1997).

III.2 Supplementary data

III.2.1 Results and Discussion

B. cinerea growth can be visualised histochemically using Trypan blue, a dye that stains fungal cells and dead plant cells. *B. cinerea* was observed 12, 24, 36 and 48 hours post-inoculation (hpi) and was found to germinate on CUTE plants without further hyphal growth, extensive fungal growth took place after germination on WT plants (Figure 1).

We tested whether *B. cinerea* attempts to penetrate CUTE leaves by analysing microscopically semi-thin sections of infected leaves. Samples collected at 12, 24 and 30 hpi were fixed and embedded. Transverse sections of 5 μm were stained with FITC-WGA (fluorescein isothiocyanate labelled wheat germ agglutinin) and CW (calcofluor white) (Meyberg, 1988). FITC-WGA exclusively stains fungal cell walls, while CW stains both fungal and plant cell walls. This double staining allows specific detection of fungal hyphae and visualisation of plant cell walls. Figure 2 shows sections through hyphae (in green) and plant cells (in blue) in WT and CUTE leaves. *B. cinerea* is visible at the surface of WT plants at 12 and 24 hpi and hyphae are found within plant tissues at 30 hpi. However, no fungal hyphae can be observed within CUTE tissues at similar time points, strongly suggesting that fungal growth already stops at the surface of CUTE leaves.

CUTE plants were found to be fully resistant to the necrotrophic fungus *B. cinerea*. Interactions with other pathogens were also tested. No difference was observed between CUTE and WT plants when infected with the obligate biotrophs *Erysiphe cichoracearum* and *Hyaloperonospora parasitica,* the non-obligate biotroph *Phytophthora porri*, as well as the non-host *Blumeria graminis* (data not shown). Testing other necrotrophic fungi like *Plectosphaerella cucumerina*, *Alternaria brassicicola* and *Sclerotinia sclerotiorum* also revealed no increased resistance of CUTE plants (Figure 3). Thus, the defence reactions mounted by CUTE plants appear to be quite specific to *B. cinerea*.

The resistance of CUTE plants to *B. cinerea* is provided by the expression of the cutinase enzyme of *F. solani* f.sp. *pisi*. To test whether the cutinase itself might be directly toxic to *B. cinerea*, spores were incubated with different concentrations of the enzyme (Figure 4a). No inhibition of fungal growth could be observed *in vitro*, leading to the conclusion that the resistance is triggered by the action of the cutinase on the plant and its subsequent release of unknown compounds. Moreover, overexpression of an inactive cutinase containing a mutation in the catalytic site (Rogers et al., 1994) did not provide resistance in transformed *Arabidospis* plants (data not shown).

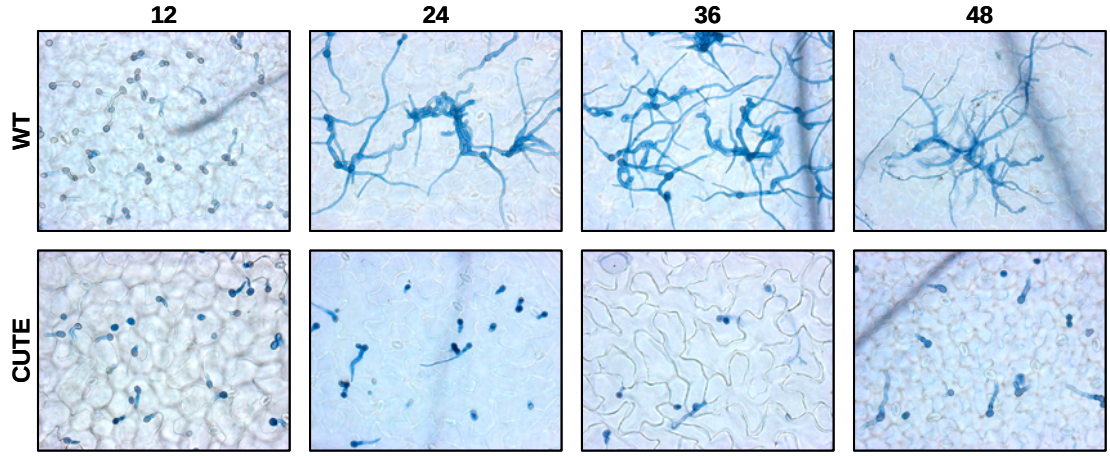


Figure 1. Trypan blue staining of WT and CUTE leaves 12, 24, 36 and 48 hpi with *B. cinerea*.

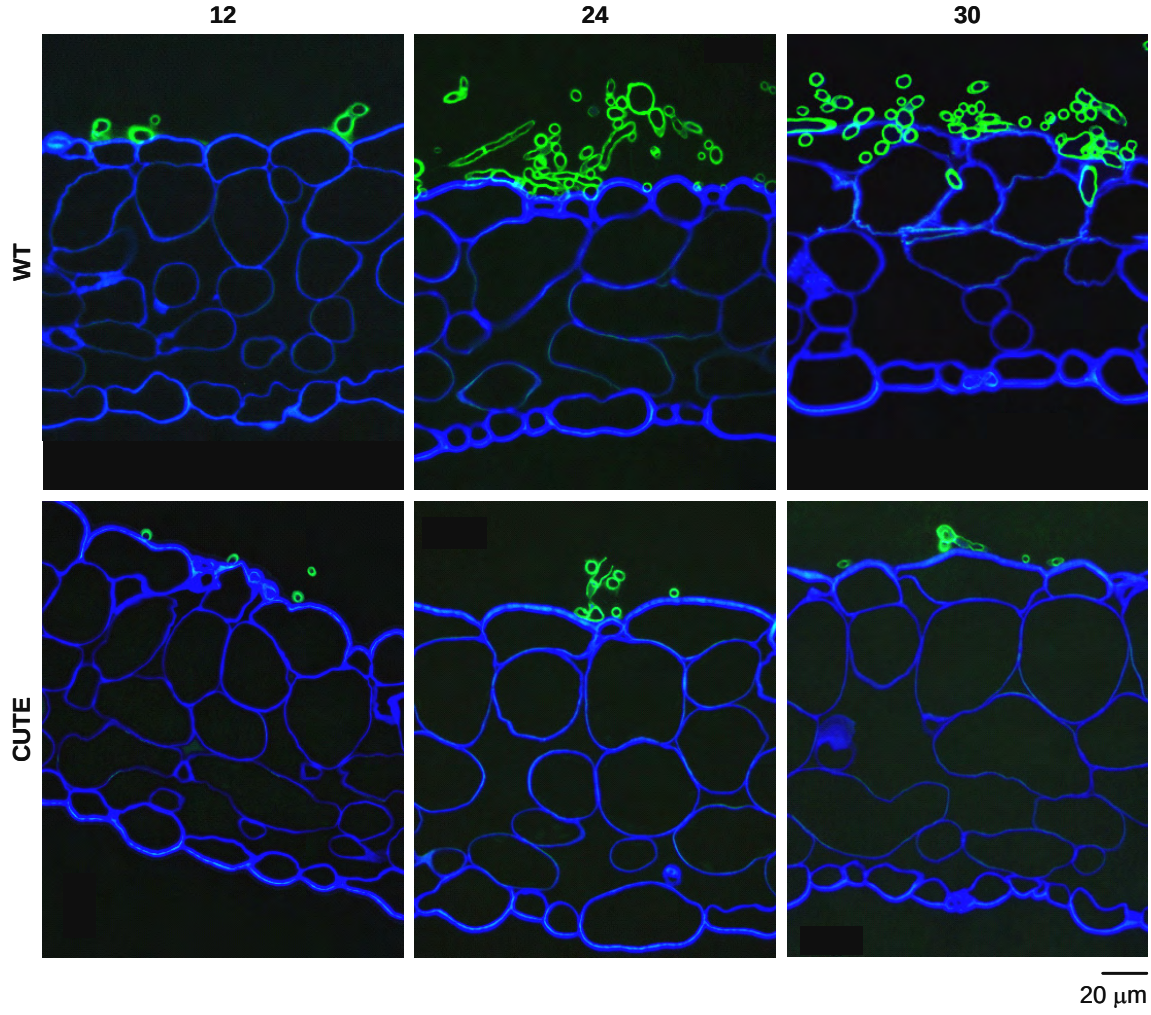


Figure 2. FITC-WGA staining of sections through WT and CUTE leaves 12, 24 and 30 hpi with *B. cinerea*.

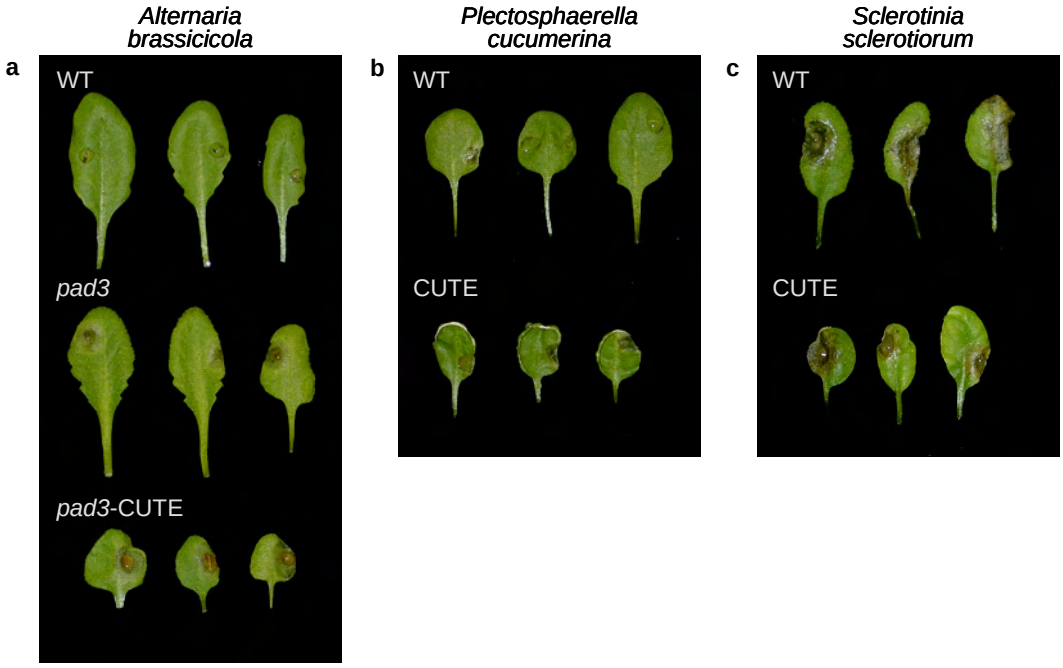


Figure 3. Disease symptoms of WT and CUTE plants inoculated with different necrotrophic fungi. **a.** WT, *pad3* and *pad3*-CUTE plants inoculated with *A. brassicicola*, 4 dpi. WT Col-0 plants are resistant while *pad3* is susceptible as *pad3*-CUTE plants. **b.** WT and CUTE plants inoculated with *P. cucumerina*, 5 dpi. **c.** WT and CUTE plants inoculated with *S. sclerotiorum*, 3 dpi.

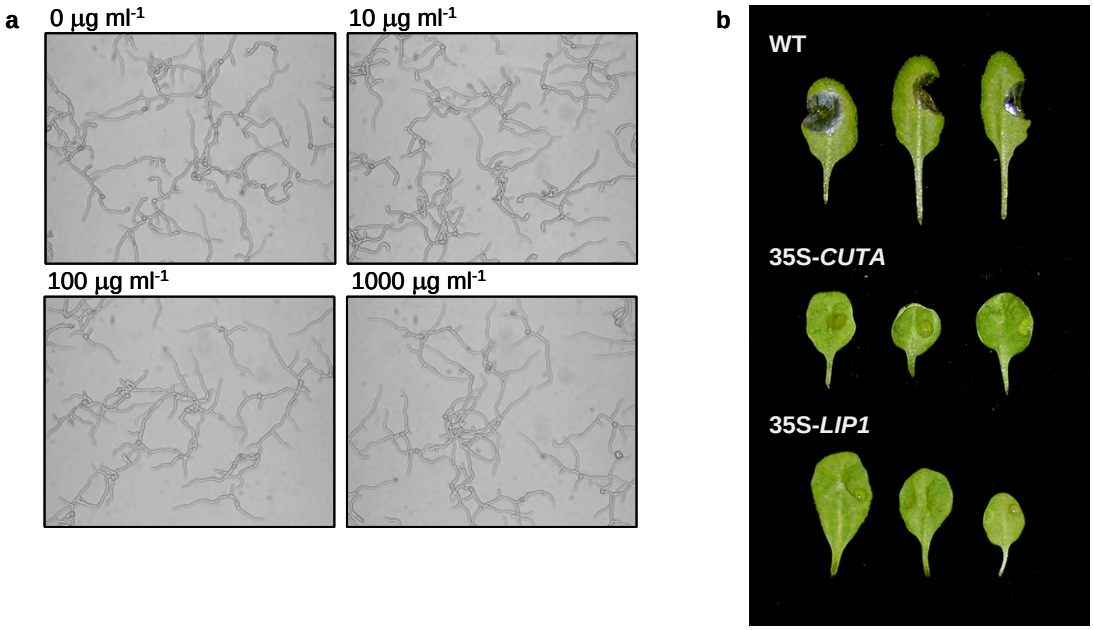


Figure 4. a. Toxicity of the *F. solani* cutinase to *B. cinerea* spores incubated with different concentrations of the enzyme (microscope pictures). **b.** Effect of overexpression of *B. cinerea* cutinase A or lipase 1 on resistance to *B. cinerea*. Symptoms of WT, 35S-*CUTA* and 35S-*LIP1* plants inoculated with *B. cinerea*, 3 dpi.

The protection of CUTE plants might be specifically induced by the cutinase of *F. solani* f.sp. *pisi*. To test this, we isolated the cDNAs from *B. cinerea* strain BMM coding for the cutinase A (CUTA) (Van der Vlugt-Bergmans et al., 1997) and the cutinolytic lipase 1 (LIP1) (Reis et al., 2005). WT plants constitutively overexpressing these genes (35S-*CUTA* and 35S-*LIP1* plants) have a phenotype similar to CUTE plants, with respect to plant growth and development. Moreover, 35S-*CUTA* and 35S-*LIP1* plants are just as resistant to *B. cinerea* (Figure 4b) as CUTE plants (Figure 4b). Thus, the phenotype and the resistance of CUTE plants are not specific to the cutinase enzyme of *F. solani* f.sp. *pisi*. This raises the question of why the cutinase produced during infection by *B. cinerea* does not induce resistance. In WT plants, the rapidly growing fungus can apparently overcome the effect of cutinase produced *in planta*. The quantity of the cutinase produced might be insufficient to trigger resistance. The timing of cutinase production might also explain this phenomenon, as CUTE plants already contain high amounts of cutinase before infection with *B. cinerea*. Moreover, a *B. cinerea* strain mutated in both the *CUTA* and the *LIP1* genes was recently shown to be as virulent as the WT strain (Reis et al., 2005), supporting the minor role of these two cutinolytic enzymes during infection with *B. cinerea*.

The absence of disease development in CUTE plants might be related to a lack of host recognition by *B. cinerea* due to the degraded CUTE surface. The hypothesis was tested by monitoring the expression of some fungal genes typically associated with pathogenesis, coding for a cutinase (*BccutA*) (Van Kan et al., 1997), an exopolygalacturonase (*Bcpgx*) (Rha et al., 2001), an endopolygalacturonase (*Bcpg1*) (Ten Have et al., 1998), a pectin methyl esterase (*Bcpme1*) (Valette-Collet et al., 2003), an aspartic proteinase (*Bcap1*) (Ten Have et al., 2004), a glutathione-S-transferase (*Bcgst1*) (Prins et al., 2000), and a G protein α subunit 2 (*Bcg2*) (Gronover et al., 2001). Gene expression in *B. cinerea* was quantified 12, 24, 36 and 48 hpi on WT and CUTE plants (Figure 5). No major differences in the expression of *BccutA*, *Bcpgx*, *Bcpme1*, *Bcgst1* and *Bcg2* genes were noticeable in *B. cinerea* inoculated on susceptible and resistant plants. However, the expression of *Bcpg1* and *Bcap1* was strongly reduced on CUTE plants in comparison to WT plants. Thus, *B. cinerea* germinating on CUTE leaves expresses at least part of the pathogenesis-associated genes similarly when growing on susceptible plants. The two genes that are expressed differentially might be important determinants for the reduced pathogenicity of the fungus on CUTE leaves, but this cannot be deduced from this experiment. We can nevertheless conclude that the fungal pathogenic behaviour is not completely blocked on CUTE plants by the absence of surface recognition.

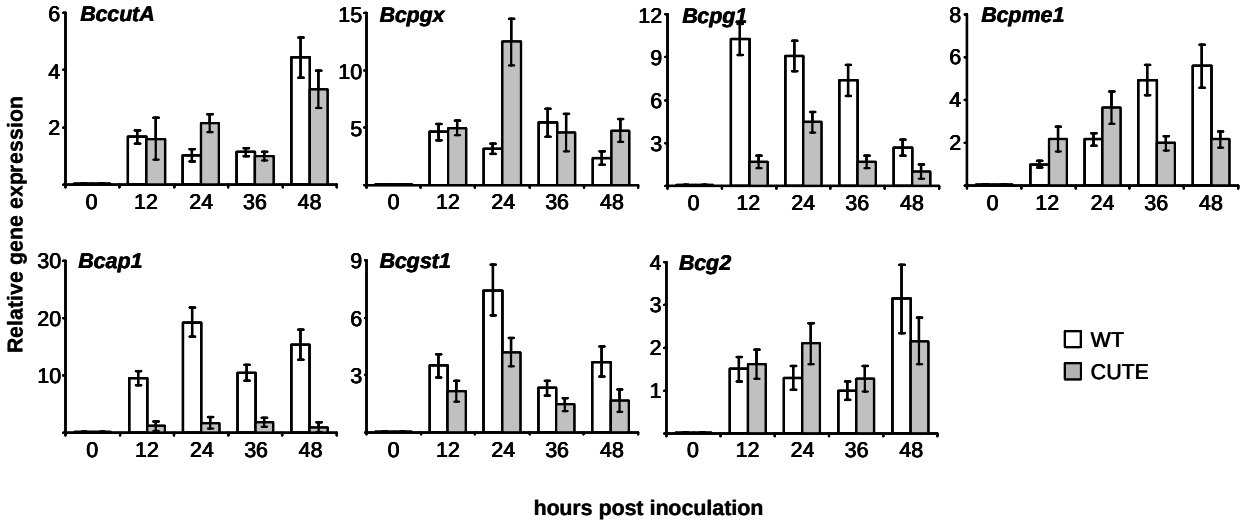


Figure 5. Expression of *B. cinerea* pathogenesis-associated genes in WT and CUTE plants. WT (white bars); CUTE (grey bars). Mean relative expression (in fold change) of 2 independent experiments (± SD). Each value in one experiment is the mean of triplicate samples (± SE).

Polygalacturonase-inhibiting proteins (PGIPs) are defence proteins present in the plant cell walls that specifically inhibit endopolygalacturonases (PGs) of fungi. A direct role for PGIPs in defence against *B. cinerea* was shown by the transgenic expression of a pear PGIP in tomato that resulted in a reduction of symptoms (Powell et al., 2000). Moreover, overexpression of both *AtPGIP1* and *AtPGIP2* genes in *Arabidopsis* led to increased resistance to *B. cinerea* (Ferrari et al., 2003). Therefore, the implication of PGIPs in the resistance of CUTE plants to *B. cinerea* was investigated. The PGIP activity of uninduced WT and CUTE extracts was measured *in vitro* (Figure 6a). PG isolated from *B. cinerea* was able to degrade the pectin-containing medium and to produce a halo representing the extent of pectin degradation (a). The reduction in the diameter of the halo confirmed the presence of PGIP activity in both extracts of *AtPGIP1* and *AtPGIP2* overexpressing plants (b, c), as already observed by Ferrari et al. (2003). Interestingly, CUTE extracts contained a strong PGIP activity (e), in comparison to WT extracts (d), meaning that CUTE plants produce PGIPs prior to infection. This was confirmed by gene expression analysis of *AtPGIP1* and *AtPGIP2* in WT and CUTE plants during infection with *B. cinerea* (Figure 6b). *AtPGIP2* was found to be constitutively expressed in CUTE plants, while its expression was detectable 36 hpi in WT plants. However, *AtPGIP1* expression was strongly induced after 24 hpi in WT plants, but was not induced in CUTE plants. The endogeneous PGIP activity of CUTE plants might therefore result from the presence of AtPGIP2. Antisense *Arabidopsis* plants with reduced PGIP expression (AsPGIP plants, kindly provided by G. de Lorenzo) were described to be more sensitive to *B. cinerea* (personal communication).

Overexpression of the *F. solani* cutinase construct in AsPGIP plants still provided full immunity against the fungus (Figure 6c). This suggests that PGIPs could play a role but are not essential for the resistance of cutinase-expressing plants to *B. cinerea*.

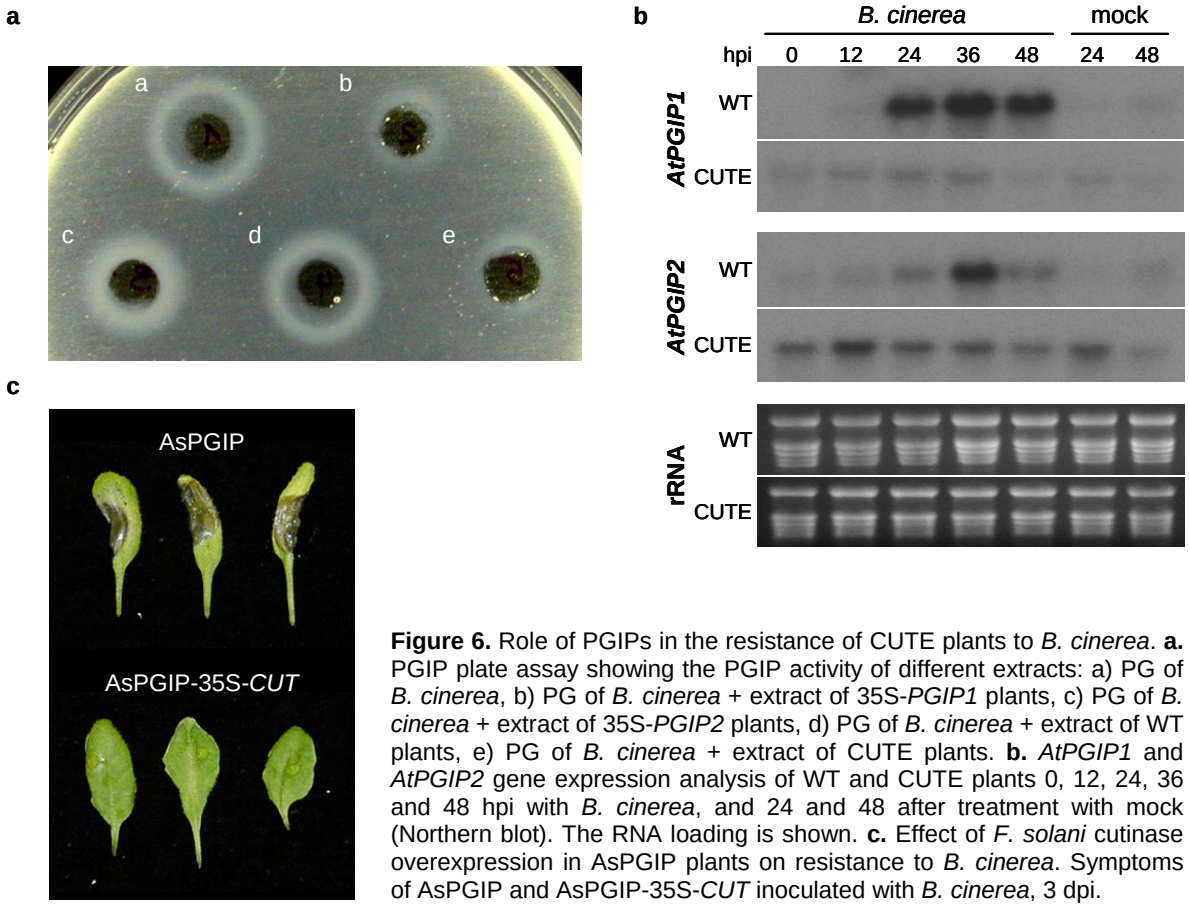


Figure 6. Role of PGIPs in the resistance of CUTE plants to *B. cinerea*. **a.** PGIP plate assay showing the PGIP activity of different extracts: a) PG of *B. cinerea*, b) PG of *B. cinerea* + extract of 35S-*PGIP1* plants, c) PG of *B. cinerea* + extract of 35S-*PGIP2* plants, d) PG of *B. cinerea* + extract of WT plants, e) PG of *B. cinerea* + extract of CUTE plants. **b.** *AtPGIP1* and *AtPGIP2* gene expression analysis of WT and CUTE plants 0, 12, 24, 36 and 48 hpi with *B. cinerea*, and 24 and 48 after treatment with mock (Northern blot). The RNA loading is shown. **c.** Effect of *F. solani* cutinase overexpression in AsPGIP plants on resistance to *B. cinerea*. Symptoms of AsPGIP and AsPGIP-35S-*CUT* inoculated with *B. cinerea*, 3 dpi.

Further insights in the changes associated with resistance of CUTE plants were obtained by genome-wide gene expression studies using Affymetrix microarrays. Gene expression was analysed 12 and 30 hpi with *B. cinerea*. Interesting candidate genes were selected both on the basis of their earlier and higher induction after inoculation with *B. cinerea* in CUTE compared to WT plants. The possible implication of such genes was tested by constitutive overexpression in WT plants. Resistance to *B. cinerea* was observed in transformed plants overexpressing closely-related members of the lipid transfer protein (LTP) family (At4g12470, At4g12480, At4g12490), of the class III peroxidase (PER) family (At2g37130, At5g39580, At5g64120) and of the proteinase inhibitor (PI) family (At2g38870, At2g43510). Overexpression of other candidate genes like At4g23600, At2g43590, or At2g50200 did not provide significant protection against *B. cinerea*, in addition to two other genes (At2g38530, At3g20470) with different expression patterns (see III.1). The presence of the transgene was controlled in 10 independent primary transformants (T1 generation) by RT-PCR (data not shown).

Transformants of the second generation (T2 generation) were analysed for the transgenes that conferred resistance to *B. cinerea* in the T1 generation. Seeds were collected from individual T1 plants and 24 T2 plants per line were grown. The percentage of outgrowing lesions was calculated on the 24 T2 plants for each line. The level of expression of the transgene was quantified by Real-time RT-PCR on a pool of one leaf of each of the 24 T2 plants. Here one example of the *LTP*, *PER* and *PI* overexpressing plants is shown: T2 lines overexpressing At4g12470, At5h39580 and At2g43510 (Figure 7). In some cases the level of overexpression of the transgene correlates with an increased resistance to *B. cinerea*, as for *LTP*-overexpressing lines.

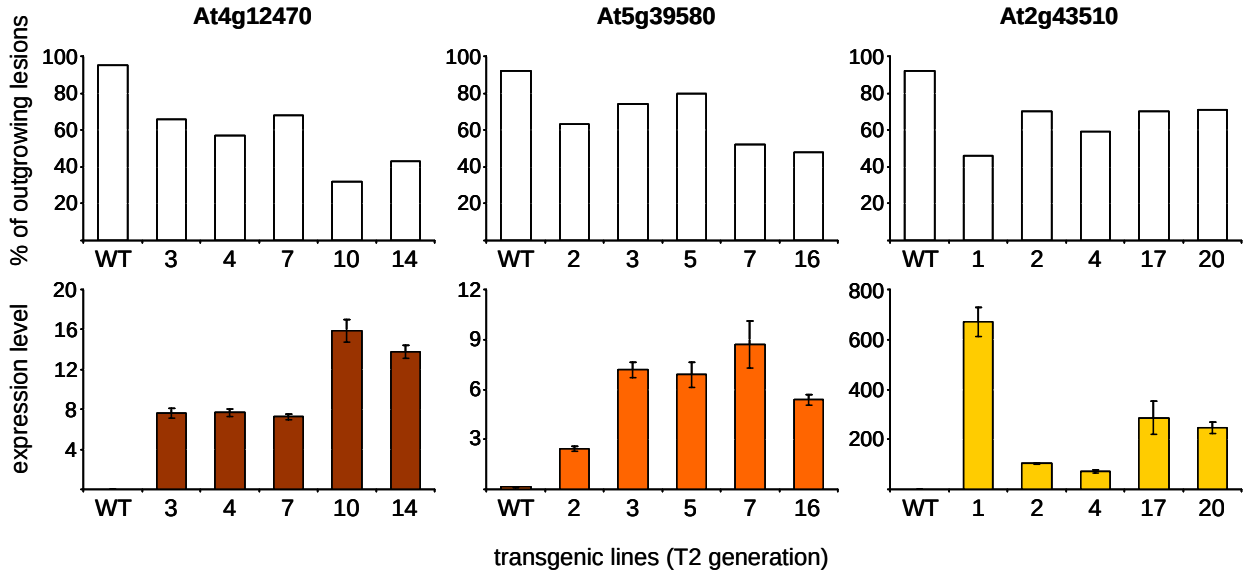


Figure 7. Analysis of transgenic lines overexpressing the *LTP* At4g12470, the *PER* At5g39580 and the *PI* At2g43510. The percentage of outgrowing lesions was counted on 24 T2 plants per line, 3 dpi with *B. cinerea* (upper graphs). The relative gene expression was quantified on a pool of one leaf per T2 plants for each line. Bars are mean of triplicate samples (± SE).

MAPK cascades link stimuli that are activated by external sensors to cellular responses. Different MAPKs are involved during defence signalling. The induced resistance of CUTE plants could be the consequence of an increased or earlier activation of MAPKs during infection, or of the activation of a different set of MAPKs from WT plants leading to an efficient defence response. An in-gel kinase assay was performed on protein extracts of WT (one plant) and CUTE (four individual plants) plants (Figure 8; carried out by David Lecourieux, personnal communication). Extracts from CUTE plants show a stronger MAPK activity than the WT extract. An *in-vitro* kinase assay made after immunoprecipitation with specific MAPK antibodies revealed a stronger activity of MAPK6 and MAPK4 in CUTE plants (Figure 8). This experiment

was not repeated and therefore no premature conclusions should be drawn. However, it opens new avenues to understand the resistance mechanisms of CUTE plants.

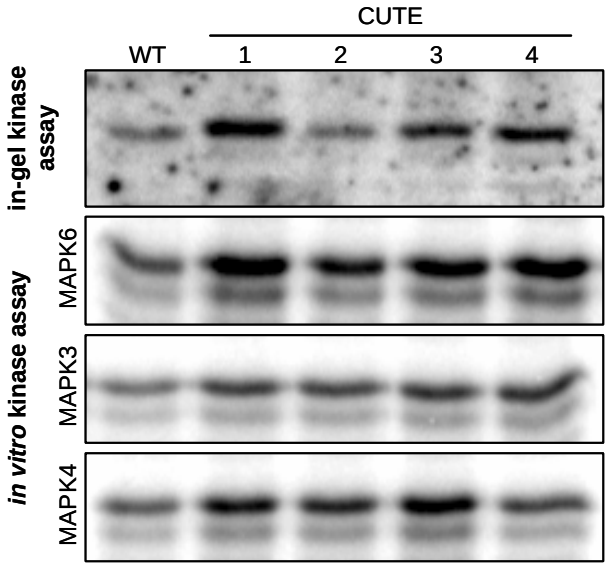


Figure 8. In-gel kinase assay of uninduced WT and CUTE plants (1st picture). *In-vitro* kinase assay of WT and CUTE plants after immunoprecipitation with antibodies that bind to MAPK6, MAPK3 or MAPK4 (2nd, 3rd and 4th pictures).

III.2.2 Material and Methods

Staining of fungal hyphae

To visualise growth of *B. cinerea* under the microscope, inoculated leaves were stained with lactophenol-trypan blue (Nawrath and Métraux, 1999). To visualise penetration of hyphae in leaves, small pieces of leaves cut around the inoculation site were fixed for 1h30 to 2h in 2% glutaraldehyde and 0.05 M Na-cacodylate pH 7.0. The material was washed 6 times 10 min in 0.05 M Na-cacodylate and fixed in 1% osmium tetroxide and 0.05 M Na-cacodylate overnight at 4°C. The samples were washed, dehydrated in a graded series of acetone and embedded by the method of Spurr (1969). Semi-thin sections of 5 μm were fixed on glass slides by heating to 95°C for 2h. The resin was removed by exposing the sections to 2% NaOH in absolute ethanol for 5 min (Meyberg, 1988). After washing in ethanol and rehydration, the sections were immersed in H_2O for 10 min. The sections were then incubated with 50 μg ml^{-1} FITC-WGA (excitation: 450-490 nm; emission: 550 nm) in 10 mM phosphate buffer for 2h. The sections were washed in distilled water and additionnally stained for 5 min with 0.1% calcofluor white. Samples were observed with a fluorescence microscope.

Inoculation of WT and CUTE plants with different pathogens

Alternaria brassicicola and *Plectosphaerella cucumerina* were grown on 1x PDA (Potato Dextrose Agar, 39 g l^{-1}, Difco) at room temperature. Spores were harvested in water and filtered through glass wool to remove hyphaes. The spore concentration was adjusted to 5 x 10^4 spores ml^{-1} in ¼ PDB (Potato Dextrose Broth, 6 g l^{-1}, Difco) for inoculation. Leaves were inoculated with 5 μl droplets of spore suspension and symptoms were evaluated after 4-5 days. *Sclerotinia sclerotiorum* was grown on 1x PDA at 18°C. An agar plug was transferred to 20 ml of 1x PDB liquid medium and incubated for 48h at 22°C, 180 rpm. The culture medium was removed by centrifugation and mycelium aggregates were resuspended in 20 ml ¼ PDB and homogenised. Leaves were inoculated with 5 μl droplets of mycelium fragments and symptoms were evaluated after 2-3 days.

In-vitro* effect of cutinase on *B. cinerea

Purified cutinase from *F. solani*, kindly provided by M. Van der Burg-Koorevaar (UNILEVER Vlaardingen, The Netherlands), was diluted in 10 mM Na-acetate pH 5.2. The *in-vitro B. cinerea* growth assay was performed in a final volume of 12 μl. Nine μl of the cutinase solution were mixed to 3 μl of *B. cinerea* spores in PDB to a final concentration of 1000, 100 or 10 μg ml $^{-1}$ of cutinase and 4 x 10^5 spores ml $^{-1}$ in ¼ PDB, and deposited on a microscope glass slide. After incubation under high humidity conditions for ca. 16h, fungal growth was observed under the microscope.

***B. cinerea* cutinase and lipase overexpression in WT plants**

The *CUTA* (accession number Z69264) (van der Vlugt-Bergmans et al., 1997) and *LIP1* (accession number AY738714) (Reis et al., 2005) genes from *B. cinerea* strain BMM were amplified by PCR on cDNA from liquid cultures using the oligonucleotides 5'-cutA (5'-ACTGCTAGCGCTGCTCCAACAGGTTCC-3'; NheI site underlined), 3'-cutA (5'-GATGAGCTCCTACAATCCGGCTGC-3'; SacI site underlined), 5'-lip1 (5'-ACTGCTAGCGCTCTTCCAGTCGAAAA TGC-3'; NheI site underlined), and 3'-lip1 (5'-GATGAGCTCTCAAATATAAAAGCTGGGC-3'; SacI site underlined). Their putative signal sequence was not amplified. The genes were cloned in the pART27 binary vector (Gleave, 1992). WT plants were transformed as previously described (Sieber et al., 2000). Independent transformed plants were collected for further analysis. The morphology of the plant confirmed the presence of the cutinase or the lipase genes.

Monitoring pathogen gene expression using real-time RT-PCR

WT and CUTE plants were inoculated with *B. cinerea* spores and leaves were harvested 12, 24, 36 and 48 hpi. RNA was extracted using the RNeasy® Plant Mini Kit (Qiagen) and retrotranscribed in cDNA (Omniscript® RT kit, Qiagen). Real-time PCR was performed using the Absolute QPCR SYBR Green Mix (ABgene). Gene expression values were normalised with the *B. cinerea* actin (*BcactA*) expression. Primers used: 5'-actA (5'-AATGTGTAAGGCCGGTTTCG-3') and 3'-actA (5'-CTTCTCCATATCATCCCAGTTG-3'); 5'-cutA (5'-AATCTGCTCTTGGGTCATCTTC-3') and 3'-cutA (5'-AGCTGGATAAGGTTGATGTGAC-3'); 5'-bcpg1 (5'-TTCAACTTCTCTCAATGGCCTC-3') and 3'-bcpg1 (5'-AGTCAAGTCAAGAGTAGTACC-3'); 5'-bcpme1 (5'-CTTTCCTTTGCGGCCACTCTTC-3') and 3'-bcpme1 (5'-AGGTGTAGTGGTAGTGGTGGAG-3'); 5'-pgx (5'-CAACGCCACAGGACGATTCATG-3') and 3'-pgx (5'-GAAGTGCGCTCATAATGGCTGG-3'); 5'-ap1 (5'-CACTAAACCTGGACCATACTTC-3') and 3'-ap1 (5'-ATTTTGTTGGTCTTCTGCTGGG-3'); 5'-gst1 (5'-TGGGTCTTTCCTACGAGGTAC-3') and 3'-gst1 (5'-TGACCTTGCATGGGACCTACAC-3'); 5'-g2 (5'-CCACAGAGATGAGAAGGTTATG-3') and 3'-g2 (5'-TGTCATTCTCAAGCCATCCAAG-3').

PGIP assay

PG activity was measured using an agarose diffusion assay in plates containing 100 mM Na-acetate pH 4.6, 0.5% polygalacturonic acid (? degree of polymerisation) and 0.8% agarose. WT and CUTE leaves were homogenised with 2 ml g^{-1} tissue of 1M NaCl and 20 mM Na-acetate pH 4.7. After 1h of shaking at 4°C, the homogenate was centrifuged and the supernatant filtered through Miracloth®. Extracts from plants overexpressing *PGIP1* and *PGIP2* were kindly provided by Giulia de Lorenzo (University of Rome). A solution containing *B. cinerea* PG (provided by Giulia de Lorenzo) was mixed with 2.5 μl of the plant extract and added to 0.5 cm wells in the plates. Plates were incubated for 18-36h at 30°C, and the halo caused by PG activity was visualised after 5 min of treatment with 6N HCl.

Cutinase overexpression in AsPGIP plants

Arabidopsis AsPGIP plants (kindly provided by G. de Lorenzo) were transformed with the *F. solani* cutinase gene as previously described (Sieber et al., 2000). Independent transformed plants were collected for further analysis based to their antibiotic resistance and on the typical developmental phenotype of CUTE plants. Twenty-four primary transformants were used for quantification of resistance to *B. cinerea*.

Analysis of plants overexpressing genes selected from the microarray data

Overexpression of the transgenes in primary transformants (T1 plants) was evaluated in 10 of 60 primary transformants. RNA was prepared using the TRIzol® reagent (Molecular Research Center, Inc., Invitrogen) and retrotranscribed in cDNA (Omniscript® RT kit, Qiagen). Presence of the transgene was evaluated by RT-PCR. Quantification of transgene expression level in transformants of the second generation (T2 plants) was performed by real-time RT-PCR using the Absolute QPCR SYBR Green Mix (ABgene). Gene expression values were normalised with the expression of the plant *actin 2* gene.

III.2.3 References

Ferrari, S., Vairo, D., Ausubel, F.M., Cervone, F., and De Lorenzo, G. (2003). Tandemly duplicated *Arabidopsis* genes that encode polygalacturonase-inhibiting proteins are regulated coordinately by different signal transduction pathways in response to fungal infection. Plant Cell **15,** 93-106.

Gleave, A.P. (1992). A versatile binary vector system with a T-DNA organizational-structure conducive to efficient integration of cloned DNA into the plant genome. Plant Mol. Biol. **20,** 1203-1207.

Gronover, C.S., Kasulke, D., Tudzynski, P., and Tudzynski, B. (2001). The role of G protein alpha subunits in the infection process of the gray mold fungus *Botrytis cinerea*. Mol. Plant Microbe Inter. **14,** 1293-1302.

Meyberg. (1988). Selective staining of fungal hyphae in parasitic and symbiotic plant-fungus associations. Histochemistry **88,** 197-199.

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

Powell, A.L.T., 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.

Prins, T.W., Wagemakers, L., Schouten, A., and Van Kan, J.A. (2000). Cloning and characterization of a glutathione S-transferase homologue from the plant pathogenic fungus *Botrytis cinerea*. Mol. Plant Pathol. **1,** 169-178.

Reis, H., Pfiffi, S., and Hahn, M. (2005). Molecular and functional characterization of a secreted lipase from *Botrytis cinerea*. Mol. Plant Pathol. **6,** 257-267.

Rha, R., Park, H.J., Kim, M.O., Chung, Y.R., Lee, C.W., and Kim, J.W. (2001). Expression of exo-polygalacturonase in *Botrytis cinerea*. FEMS Microbiol. Letters **210,** 105-109.

Rogers, L.M., Flaishman, M.A., and Kolattukudy, P.E. (1994). Cutinase gene disruption in *Fusarium solani* f.sp. *pisi* decreases its virulence on pea. Plant Cell **6,** 935-945.

Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Métraux, J.P., and Nawrath, C. (2000). Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell **12,** 721-738.

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., 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. Microbiol. **150,** 2475-2489.

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 Vlugt-Bergmans, C.J.B., Wagemakers, C.A.M., and Van Kan, J.A.L. (1997). Cloning and expression of the cutinase A gene of *Botrytis cinerea*. Mol. Plant Microbe Interact. **10,** 21-29.

Van Kan, J.A.L., Van't Klosster, J.W., Wagemakers, C.A.M., Dees, D.C.T., and Van der Vlugt-Bergmans, C.J.B. (1997). Cutinase A of *Botrytis cinerea* is expressed, but not essential, during penetration of gerbera and tomato. Mol. Plant Microbe Interact. **10,** 30-38.

IV. Cutinase-expressing plants and the diffusion of a fungitoxic compound

Aerial parts of plants are covered with a hydrophobic cuticular layer offering physical protection against water loss, irradiation, and mechanical protection against microbes. Many phytopathogenic fungi can breach this layer by secreting cutinases that degrade cutin, the main component of the cuticle. Transgenic *Arabidopsis* plants that overexpress a fungal cutinase from *Fusarium solani* f.sp. *pisi* targeted to the extracelluar space (CUTE plants) have a perturbed cuticle ultrastructure. Degradation of the cuticular layer leads among others to a phenotype with strongly increased permeability of the cuticle. Moreover, CUTE plants are fully resistant to the virulent necrotrophic fungus *Botrytis cinerea*. Here we show that a compound with strong fungitoxic activity diffuses from CUTE leaves and plays a role in the defence against *B. cinerea*. The active compound was shown to diffuse from CUTE leaves or from wild-type (WT) leaves digested with purified cutinase. The compound has a presumed size of 1000 to 3500 Da and might be of proteinaceous nature. It is also strongly fungitoxic against the closely-related fungus *Monilinia laxa*. Further fractionation of WT and CUTE diffusates revealed the presence of fungitoxic activity in untreated WT plants too, albeit in lower amount. These results report the discovery of a highly fungitoxic substance that might be naturally present in the extracellular matrix of *Arabidopsis* plants.

IV.1 Introduction

B. cinerea is a common plant pathogenic fungus that causes the gray mold disease of several crops. In response to pathogens, plants are generally able to mount a spectrum of defence responses that commonly confer resistance to a wide range of pathogens. Plants possess a range of tools against a *Botrytis* infection. These include pre-formed and induced plant defence compounds, like secondary metabolites, structural barriers and anti-fungal PR proteins (van Baarlen et al., 2004). Extracts of many plants have been shown to contain low-molecular-weight compounds which inhibit the growth of fungi *in vitro*. These compounds may be present in extracts from healthy unchallenged plants (preformed antimicrobial compounds or phytoanticipins), or may be found only in extracts of plants which have either been challenged with pathogens or stressed (phytoalexins) (Osbourn, 1999).

Trans-resveratrol, for example, is the most abundant stilbene in grapevine (Creasy and Creasy, 1998). Resveratrol displayed no immediate toxicity towards *B. cinerea* (Pezet and Pont, 1995). Long-term incubation of *B. cinerea* with resveratrol led, however, to inhibition of germination of conidia, as well as elongation of germ tubes and hyphae (Adrian et al., 1997). In many cases *Botrytis* species have adapted to antifungal compounds, which can be detoxified or secreted. *B. cinerea* possesses a large family of functional transporter genes, some of which confer protection to plant defence metabolites including resveratrol (Schoonbeck et al., 2001).

PR proteins represent a large array of proteins that are synthesised by the host plant after challenge with a pathogen or related situations. Infection with *B. cinerea* leads to induction of PR proteins in many plants (van Loon, 1985). Several PR proteins display some toxicity towards *B. cinerea in vitro*, e.g. a grape PR-like protein (chitinase) has a very high botryticidal activity (Derckel et al., 1998; Salzman et al., 1998). Legumes produce antifungal proteins including chitinases, ribosome-inactivating proteins, cyclophilin-like proteins, defensins, protease inhibitors, lectins, peroxidases and lysozymes (Wong and NG, 2005). Despite their antimicrobial activity *in vitro*, there is little evidence to support a potential role of PR proteins in effective plant disease resistance to *B. cinerea*. The homologous or heterologous expression of PR proteins in transgenic plants, or the infiltration of single PR proteins in leaves, has rarely led to any significant level of protection (van Baarlen et al., 2004).

Antifungal peptides inhibit the *in-vitro* growth of *B. cinerea* by targeting microbial membranes (Stotz et al., 2004). Overexpression of the lipid transfer protein Ace-AMP1 from onion increased resistance of scented geranium to *B. cinerea* (Bi et al., 1999). An antifungal peptide with a molecular mass of 6.5 kDa, limenin, was isolated from the seeds of shelf beans. It suppresses mycelial growth of *B. cinerea* and other fungi (Wong and NG, 2005). Vulgarinin, a broad-spectrum antifungal peptide was isolated from haricot beans, with a molecular mass of 7 kDa (Wong and NG, 2005). Others peptides with toxic activity against *B. cinerea* have been isolated from black pumkin seeds (Wang and NG, 2003), from green chickpea (Chu et al., 2003) or from Ceylon spinach seeds (Wang and NG, 2004).

Arabidopsis plants that constitutively express a fungal cutinase enzyme are fully immune to *B. cinerea*. Diffusates from leaves of CUTE plants were shown to have a strong fungistatic activity against *B. cinerea in vitro*, whereas diffusates from WT leaves allowed rapid hyphal growth. In this study, we report the characterisation of the fungitoxic activity.

IV.2 Results and Figures

CUTE plants are completely resistant to *B. cinerea*. The fungus germinates but stops growing at the surface of CUTE leaves, while it rapidly grows on susceptible WT plants and causes typical soft rot lesions. We hypothesised that a toxic substance could diffuse from CUTE leaves in the 5 µl inoculation droplets and lead to the early arrest of the growth of *B. cinerea in planta*. Five µl droplets of inoculation medium (1/4 potato dextrose broth or PDB) without spores were deposited on WT and CUTE leaves. The droplets were incubated during ca. 16 h under high humidity conditions. Incubated droplets or diffusates were collected and mixed with spores of *B. cinerea* and their fungitoxic activity was determined in an *in-vitro* growth assay (see Methods). In addition, this mixture was used to infect WT plants. We observed an effect of the

CUTE diffusate on *B. cinerea in vivo* (Figure 1). Spores germination was not inhibited, but hyphal growth was strongly slowed down, in comparison to the rapid growth of spores incubated with the WT diffusate. Moreover, infection of susceptible WT plants was completely inhibited when spores were in the presence of the CUTE diffusate (Figure 1). This absence of symptoms could also be observed on tomato plants inoculated with spores mixed with the CUTE diffusate (Figure 1). Therefore, we conclude that CUTE plant leaves contain a fungistatic compound that diffuses in droplets of inoculation medium and has an *in-vitro* and *in-vivo* effect against *B. cinerea*.

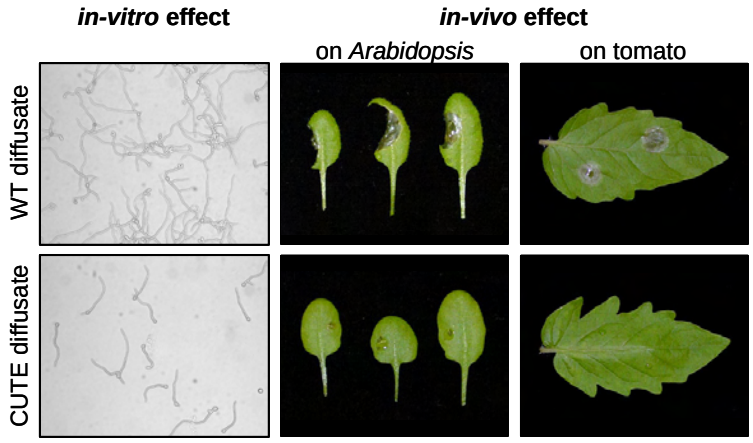


Figure 1. *In-vitro* and *in-vivo* effect of WT and CUTE diffusates (in ¼ PDB) on *B. cinerea*. CUTE diffusate strongly slows down hyphal growth *in vitro* (left pictures), as well as inhibits infection of *Arabidopsis* leaves (middle pictures) and tomato leaves (right pictures) *in vivo*. WT diffusate does not impair growth of *B. cinerea in vitro* or development of infection *in vivo*.

The PDB inoculation medium is made of potato extract (4 g l^{-1}) and glucose (20 g l^{-1}). It contains less than 20% NaCl and more than 90% of its components are smaller than 1000 Da. It is diluted 4 times in our inoculation conditions. In order to facilitate the future purification, different solutions that would allow the diffusion of the fungitoxic compound from CUTE leaves were tested. The fungitoxic compound did neither diffuse in H_20 nor in a glucose solution (5 g l^{-1}) (data not shown). However, the fungitoxic activity diffused in droplets of a 1M NaCl solution (ca. 5% NaCl) from CUTE leaves, while the WT diffusate allowed growth of *B. cinerea* (Figure 2). The presence of the fungitoxic activity in intercellular wash fluids (Figure 2) confirmed the hypothesis of an extracellular compound diffusing out of the leaves. NaCl seems to be needed to extract the compound(s). The active compound(s) could be bound to the extracellular matrix by ionic forces that are not loosened by H_20.

Application of purified cutinase of *F. solani* f.sp. *pisi* on WT leaves led to resistance to *B. cinerea* when the fungus was applied directly on the sites treated with cutinase (see III.1, data not shown). The fungitoxic compound was also found to diffuse in droplets of a solution containing cutinase, as shown on Figure 3. Indeed, incubation of droplets of a 100 µg ml^{-1} cutinase solution during 3 days on WT leaves released the fungitoxic compound. This was confirmed *in vitro* and *in vivo* on WT plants usually susceptible to *B. cinerea*. This phenomenon

was dependent on the quantity of cutinase applied, as a 10 μg ml^{-1} cutinase solution was less effective in releasing the fungitoxic compound, and on the duration of cutinase application, as an incubation time of 1 day was not enough to collect the fungitoxic compound (data not shown).

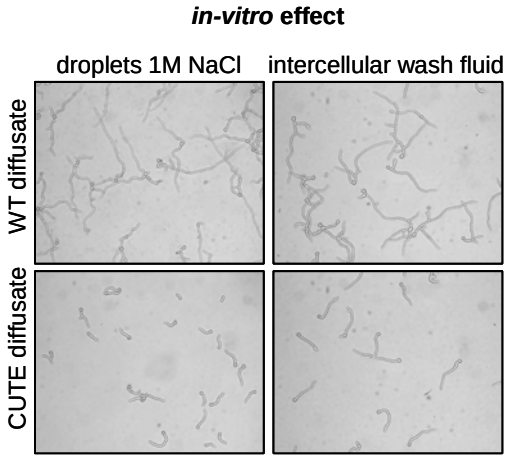


Figure 2. Presence of the fungitoxic compound 1M NaCl diffusates and in intercellular wash fluids. Droplets of 1M NaCl allowed the diffusion of the toxic compound (*in-vitro* effect). The toxic compound was shown to be present in the intercellular wash fluid of CUTE plants (*in-vitro* effect).

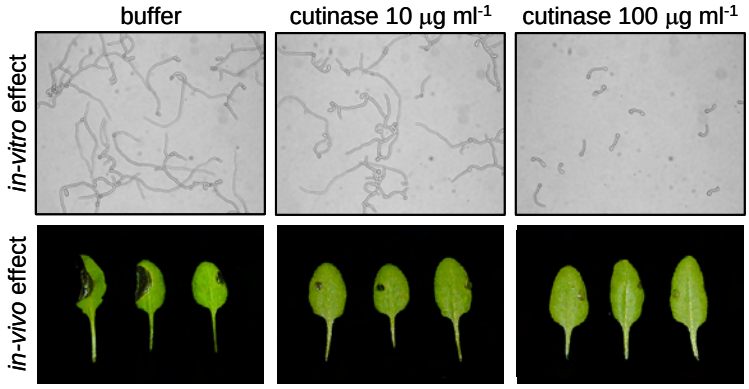


Figure 3. Diffusion of the fungitoxic activity from WT leaves treated with purified cutinase of *F. solani*. Droplets of buffer or of different amounts of cutinase were incubated for 3 days on WT leaves. Diffusates were tested *in vitro* and *in vivo* on *B. cinerea*. The fungitoxic compound diffused from WT leaves treated with cutinase.

CUTE plants are resistant to *B. cinerea* but not to other fungi and Oomycetes that were tested in our lab (see III.2). The WT and CUTE diffusates were incubated with spores of other fungi, to assess whether the fungitoxic activity was also specific to *B. cinerea in vitro*. The CUTE diffusate showed no toxic activity against spores of the necrotrophs *Plectosphaerella cucumerina* and *Alternaria brassicicola* (Figure 4). This fits the observation that CUTE plants are not resistant to *P. cucumerina*; *pad3*-CUTE plants are not resistant to *A. brassicicola* either (see III.2). However, the growth of spores of *Monilinia laxa* was inhibited by the CUTE diffusate (Figure 4). *M. laxa* is an Ascomycete which is phylogenetically very close to *B. cinerea*. It causes the brown rot of stone fruits; heavily infected fruits become mummified. Both *Botrytis* and *Monilinia* genera belong to the family of *Sclerotiniaceae*. This close linkage could explain the similar result obtained with the CUTE diffusate. However, *in-planta* evidence of a toxic activity against *M. laxa* could not be analysed, as the fungus is a pathogen restricted to stone fruits that does not infect *Arabidopsis*.

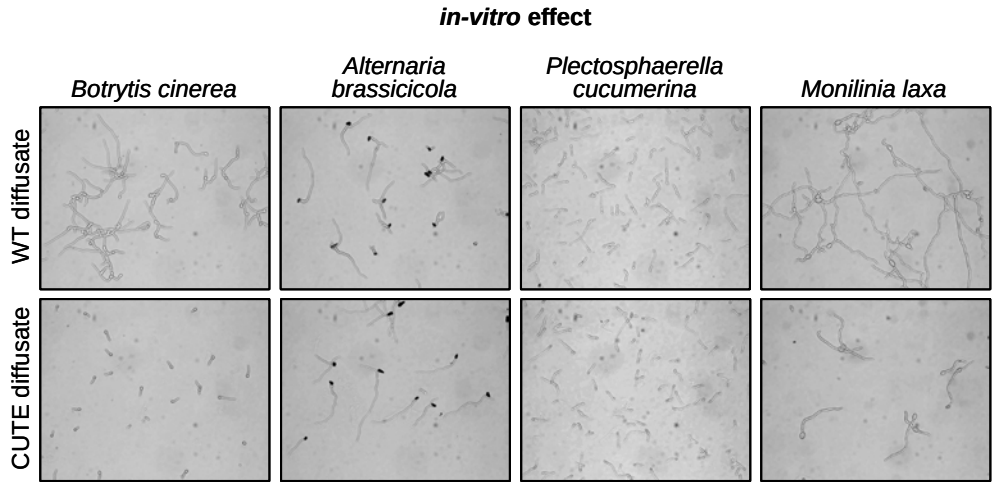


Figure 4. *In-vitro* effect of WT and CUTE diffusates on necrotrophic fungi. The CUTE diffusate had no fungitoxic activity on *A. brassicicola* and *P. cucumerina*, as no differences with the WT diffusate were observed. Hyphal growth of *B. cinerea* and *M. laxa* was slowed down by the CUTE diffusate, whereas the WT diffusate had no direct toxic effect.

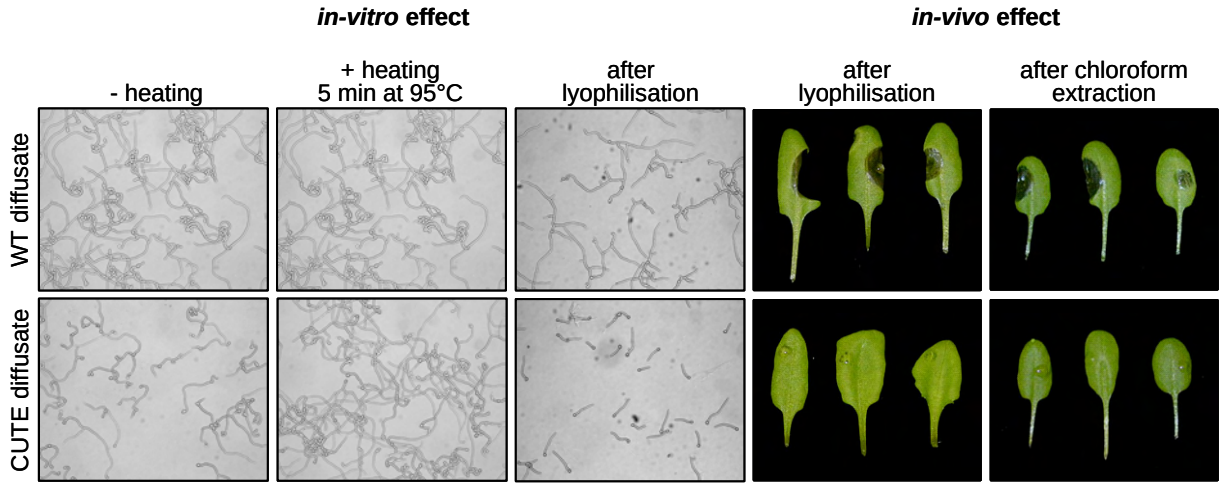


Figure 5. Characterisation of the fungitoxic compound. The fungitoxic compound was inactivated by heat treatment, as shown *in vitro*, compared to controls. It remained active after lyophilisation, as shown *in vitro* and *in vivo*, and was not removed by extraction with chloroform, as shown *in vivo*.

In an attempt to determine the chemical nature of the fungitoxic compound, several experiments were carried out on both WT and CUTE diffusates (in ¼ PDB). The toxicity of the CUTE diffusate was inactivated by heating for 5 minutes at 95°C *in vitro* (Figure 5). The fungitoxic activity was not removed by extraction with chloroform, suggesting that the compound is not of lipidic nature (Figure 5). The active compound resisted lyophilisation (Figure 5), a processus necessary for further purification. To evaluate the size of the active compound, WT and CUTE diffusates were dialysed with membranes of different size of pores: 1000 MWCO (molecular weight cut-off), 3500 MWCO, 8000 MWCO and 15'000 MWCO. The dialysates were

tested *in vitro* with spores of *B. cinerea* (Figure 6). The active compound was retained on the 1000 MWCO membrane, but passed through the 3500 MWCO membrane. Thus, the active CUTE compound is heat-sensitive, not soluble in chloroform, and has probably a size between 1000 and 3500 Da.

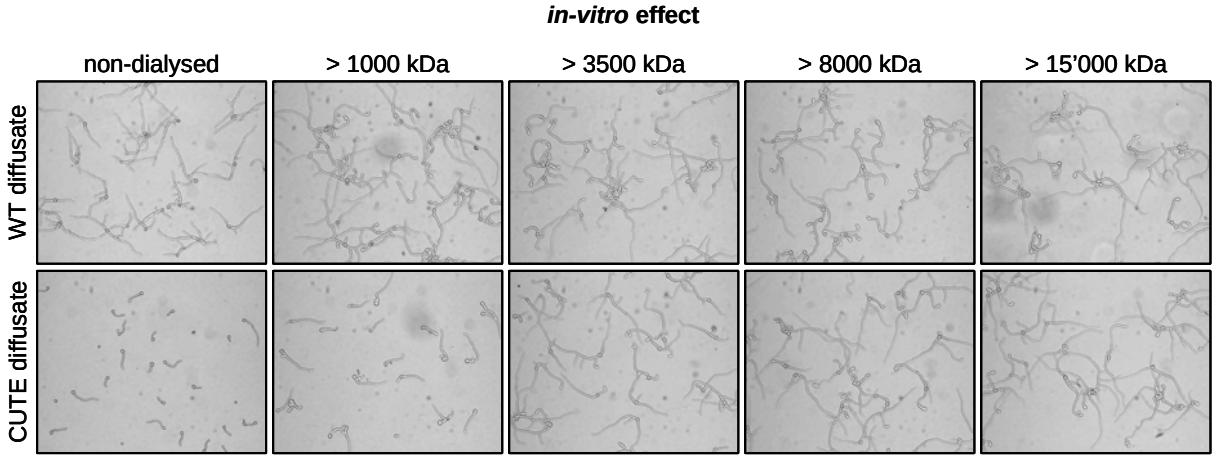


Figure 6. *In-vitro* effect of dialysed WT and CUTE diffusates. The activity of the CUTE diffusate was retained by the 1000 MWCO membrane, but not by the 3500, 8000 and 15'000 MWCO membranes. The active compound has an approximate molecular mass of 1000 to 3500 Da.

The WT and CUTE diffusates were treated with proteases, to test whether the active compound may be of proteinaceous nature. Pronase E, a mixture of endo- and exo-proteinases that cleaves almost any peptide bond, and proteinase K, an endolytic protease that cleaves peptide bonds at the carboxylic sides of aliphatic, aromatic or hydrophobic amino acids, were tested. Diffusates were treated with 1% of protease for 3 h at 37°C or overnight at room temperature. Treatment with pronase E decreased the fungitoxic activity of the CUTE diffusate after both incubation times, without having any effect on the WT diffusate (Figure 7). Treatment with proteinase K also decreased the fungitoxic activity, but after overnight incubation (Figure 7). These results suggest that the active compound diffusing from CUTE plants could be a small peptidic molecule.

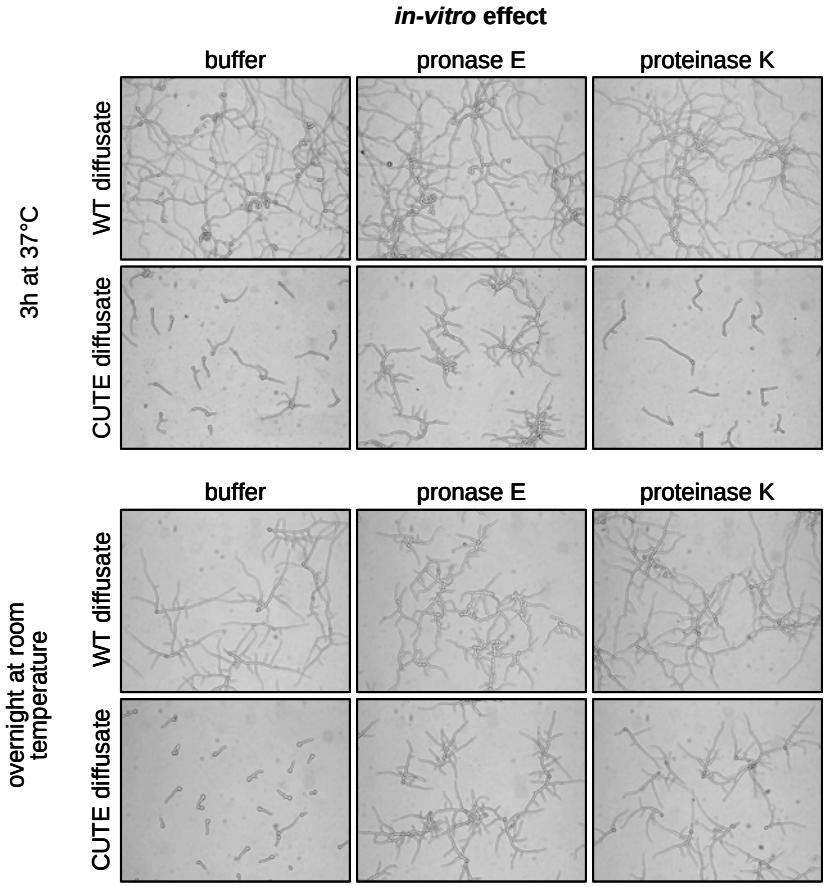


Figure 7. Treatment of WT and CUTE diffusates with proteases. WT and CUTE diffusates were incubated with buffer, with 1% pronase E or with 1% proteinase K, for 3 h at 37°C or overnight at room temperature. CUTE diffusate treated with both proteases lost (partially) its fungitoxic activity, compared to controls.

Diffusates (in ¼ PDB or 1M NaCl) of CUTE leaves were shown to have an *in-vitro* and an *in-vivo* effect against *B. cinerea*. We tested the *in-vitro* fungitoxic activity of 3 different protein extracts of WT and CUTE plants (Table 1, 3 first lines). CUTE material extracted with the 2 first solutions contained fungitoxic activity similar to the CUTE diffusate, while CUTE material extracted with the 3rd solution, similar to the WT extract, did not show any activity. Unlike the other solutions for extraction of plant proteins, the 3rd solution contained β-mercaptoethanol, a disulfide-reducing agent, which interferes with proteins and could thus inactivate or denature them. In addition, CUTE extract 2 had no PGIP activity but could still inhibit fungal growth, like the CUTE diffusate (Table 1, 5th line). CUTE extract 3 displayed cutinase activity, but no inhibition of fungal growth. This fits the data showing no fungal growth inhibition by the purified cutinase of *F. solani* f.sp. *pisi* (see III.2 and Table 1, 6th line). Moreover, the CUTE diffusate contained the fungitoxic compound, but no PGIP or cutinase activities were detectable (Table 1, 6th line). The presence of the cutinase enzyme in the different samples was also confirmed on a

Western blot (data not shown). Therefore, these results strengthen the hypothesis that the active compound might be of proteinaceous nature, and exclude the participation of the cutinase of *F. solani* f.sp. *pisi* or of PGIPs, as the fungitoxic activity is independent of the presence of PGIP or cutinase activities.

		in-vitro* effect on *B. cinerea		**PGIP activity**		**cutinase activity**	
Extracts	**Composition**	WT	CUTE	WT	CUTE	WT	CUTE
protein extract 1	Na-acetate 20 mM, 1M NaCl (pH 4.7)	-	+	-	+	-	+
protein extract 2	Tris HCl 50 mM (pH 7.5)	-	+	-	-	-	+
protein extract 3	Tris HCl 0.1 M, EDTA 5 mM, Triton 0.1%, β-mercaptoethanol 0.2% (pH 7.3)	-	-	-	-	-	+
IWF	Tris HCl 50 mM, 150 mM NaCl (pH 7.5)	-	+	-	+	-	+
diffusate	¼ PDB	-	+	-	-	-	-
cutinase *F. solani*	50 μg/ml in Na-acetate 10 mM (pH 5.2)	-	-	-	-	-	+

Table 1. Properties of different extracts of WT and CUTE plants. Different protein extracts (3 first lines), IWF, diffusates and purified cutinase of *F. solani* were tested: for their *in-vitro* effect on *B. cinerea*, for their PGIP activity and for their cutinase activity.

CUTE diffusate (in 1M NaCl) showing fungitoxic activity was further purified by gel filtration chromatography. The 35 resulting fractions were tested *in vitro* for their effect against *B. cinerea*. Five consecutive fractions superposed on a peak of absorption at 280 nm displayed full inhibition of fungal growth (Figure 8a, b). Fractions eluted before or after the active fractions were devoid of fungitoxic activity. As control, the inactive WT diffusate (in 1M NaCl) was purified on the column. Surprisingly, discrete fractions with fungitoxic activity were also found (Figure 9b). Fractions of WT and CUTE diffusates were compared after gel filtration chromatography performed the same day and under similar conditions. The number of fractions showing *in-vitro* fungitoxic activity was less important in the purified WT diffusate, compared to the purified CUTE diffusate (Figure 9a). Dilution series of active fractions of both WT and CUTE diffusates were carried out to estimate the fungitoxic activity. An active CUTE fraction diluted up to 8 times still showed full inhibition of fungal growth (data not shown), while an active WT fraction could only be diluted up to 4 times (data not shown). These results suggest that the fungitoxic substance is also present in WT diffusates, but in lower amounts.

The final aim of this study is the determination of the chemical structure of the active compound. Active fractions resulting from the gel filtration chromatography of CUTE diffusates were dialysed (500 MWCO), washed with ether to remove plastic traces and lyophilised, before analysis by mass spectrometry (MS). The chemical analysis did however not yet produce useful results.

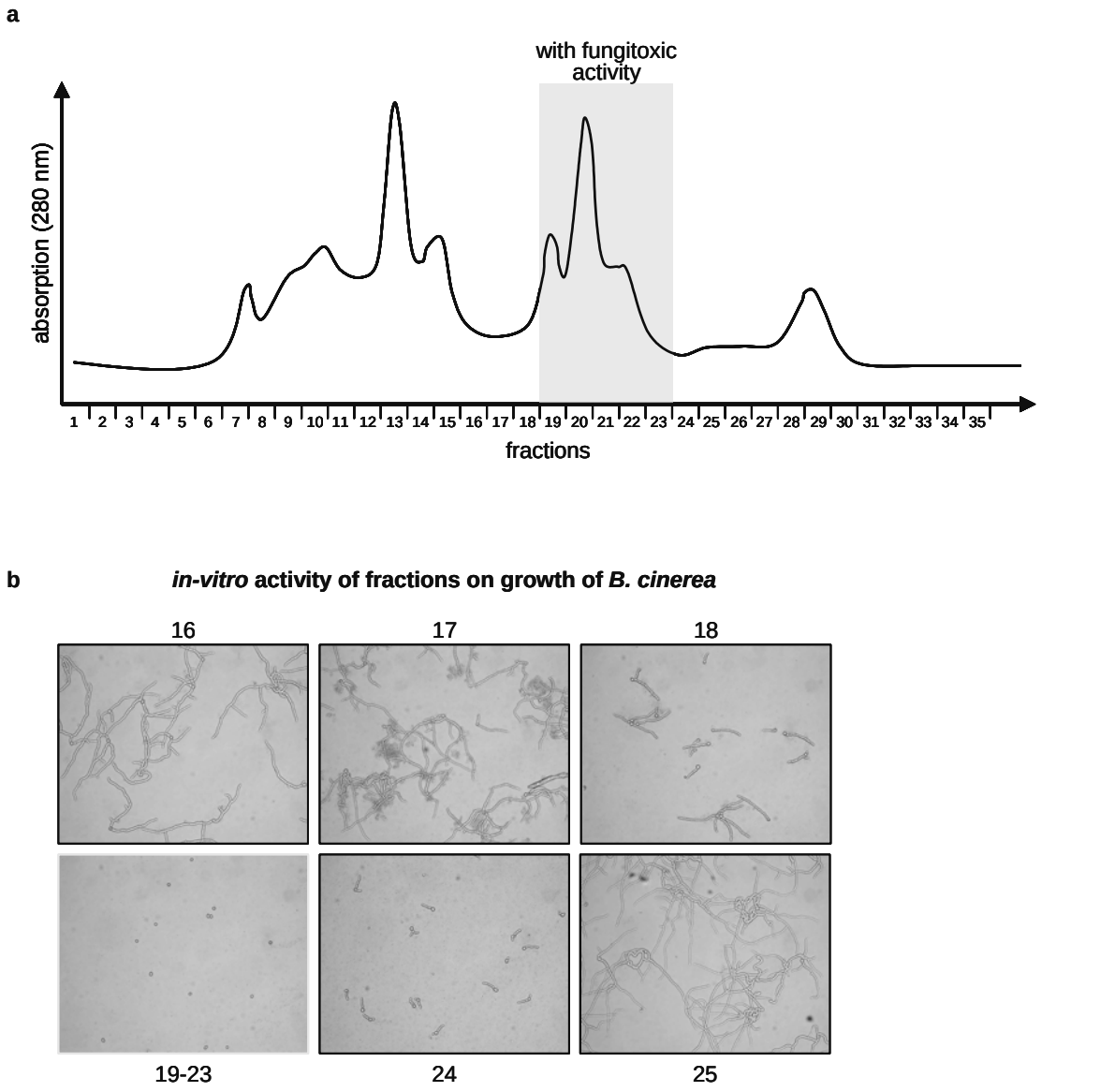


Figure 8. Gel filtration chromatography of CUTE diffusate. a. Absorption spectrum (280 nm) of CUTE diffusate eluted from the column. Fractions are numbered on the x-axis. The yellow box represents the fractions with strong fungitoxic activity against B. cinerea. b. Fractions were tested in vitro on B. cinerea. In-vitro effect of fractions 16 to 25 are shown.

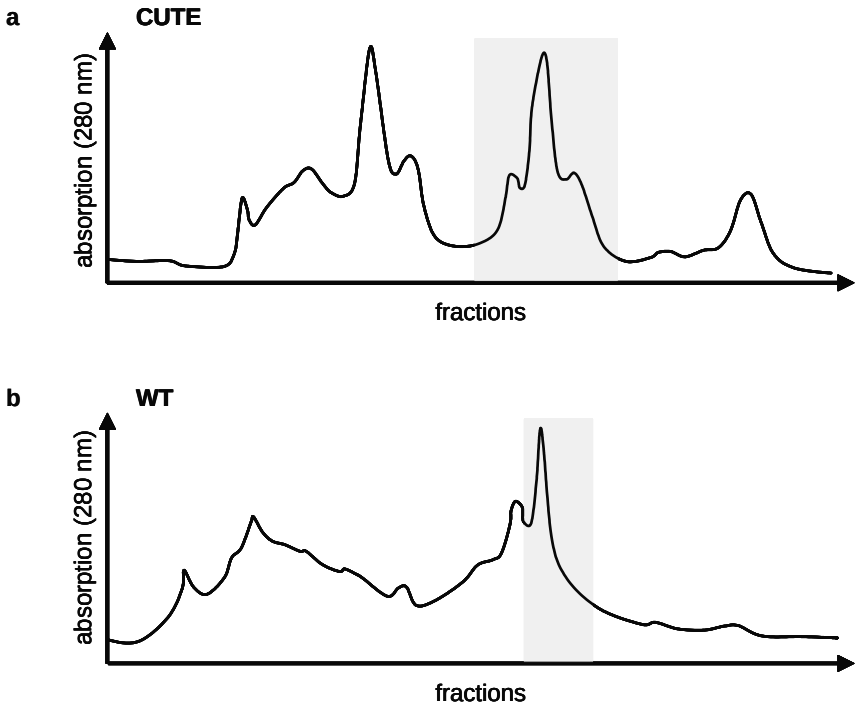


Figure 9. Gel filtration chromatography of CUTE and WT diffusates. **a.** Absorption spectrum (280 nm) of CUTE diffusate eluted from the column. The grey box represents the fractions with strong fungitoxic activity against *B. cinerea*. **b.** Absorption spectrum (280 nm) of WT diffusate eluted from the column. The grey box represents the fractions with strong fungitoxic activity against *B. cinerea*.

IV.3 Discussion

CUTE plants revealed the presence of an intercellular toxic compound in *Arabidopsis* Col-0 plants. This compound has a strong fungistatic activity against *B. cinerea* and *M. laxa in vitro*, and inhibits infection of WT plants *in vivo*. We highlight the existence of a natural fungitoxic compound that can easily diffuse from CUTE plants due to their enhanced cuticular permeability. The intact cuticle of WT plants serves as barrier to the flow of solutes but is not completely impermeable. This explains why the toxic activity could not be observed in the *in-vitro* assay of WT diffusates, but was found in purified fractions, therefore in lower amounts. In addition, external application of purified cutinase of *F. solani* on WT leaves degraded the cuticular layer and allowed the diffusion of the toxic compound. The amount of toxic compound present in diffusates is thus positively correlated with the permeability of the cuticle. The fungitoxic compound diffuses into droplets of ¼ PDB, or into droplets containing similar amounts of salt but not, however, in droplets of H_2O. NaCl is probably necessary to release the active molecule, that might be bound with ionic forces to the extracellular matrix. It is also possible that

the ¼ PDB or salt droplets induce reactions in the plant that lead to the production and secretion of the toxic compound.

We have shown that the expression of three *LTP* genes is primed in CUTE plants after inoculation with *B. cinerea*, compared to WT plants (see III.1). LTPs are small, basic, soluble proteins with eight cystein residues at conserved positions (Kader, 1996). They are characterized by their ability to transfer lipids between natural and artificial membranes *in vitro*, and may be involved in cuticle deposition (Kader, 1996). LTPs play also a role in plant defence. Purified LTP2 from barley applied on tobacco leaves eliminated symptoms caused by infiltration of *Pseudomonas syringae* pv *tabaci* (Molina and Garcia-Olmedo, 1997). Overexpression of the barley *LTP2* in transgenic tobacco and *Arabidopsis* enhanced tolerance to *P. syringae* pv *tabaci* and *P. syringae* pv *tomato*, respectively (Molina and Garcia-Olmedo, 1997). Three *LTP* genes were found to be strongly induced by pathogens in pepper (Jung et al., 2003). Overexpression of one of the pepper *LTP* genes in *Arabidopsis* led to enhanced resistance to *B. cinerea* and to *P. syringae* (Jung et al., 2005). Purified LTPs were shown to display antimicrobial activity. Two homogeneous LTPs were obtained from crude cell-wall preparations from leaves of *Arabidopsis* Col-0, as well as from spinach leaves. These LTPs showed toxic activity *in vitro* against the bacteria *Clavibacter michiganensis* and *P. solanacearum*, and the fungus *F. solani* (Segura et al., 1993). A LTP from *Vitis vinifera* displayed antifungal activity *in vitro*; it reduced mycelium growth of *B. cinerea*, but only in calcium-free medium (Gomes et al., 2003). Antifungal LTPs were also isolated from intercellular wash fluid of sugar beet leaves. They showed strong antifungal activity against *Cercospora beticola*, the causal agent of leaf spot disease in sugar beet (Nielsen et al., 1996; Kristensen et al., 2000).

The experiments carried out to characterise the nature of the active compound of CUTE and WT diffusates suggest that it might be of proteinaceous nature. The activity of the toxic compound was sensitive to heat and protease treatment. Hypothetically, the toxic compound could be a LTP. In *Arabidopsis*, 71 putative LTPs have been identified (Beisson et al., 2003) and they were found to be located extracellularly (Thoma et al., 1993). They all have eight cystein residues at conserved positions, but are highly divergent. Studies of the 3D structure of LTPs demonstrated that the protein has four α-helices cross-linked by four disulfide bridges (Shin et al., 1995). We have shown that extraction of CUTE plant material with a buffer containing the reducing agent β-mercaptoethanol eliminated the fungitoxic activity. Proteins with highly reactive thiol groups were demonstrated to form disulfide bridges with β-mercaptoethanol, thus interfering with protein-protein interactions or leading to protein denaturation (Begg and Speicher, 1999).

The fungitoxic compound diffusing from WT and CUTE leaves should be constitutively present in leaves. The three *LTP* genes isolated from the microarray analysis (see III.1, and Table 1) were shown to be induced by *B. cinerea*. At4g12470 and At4g12490 are more

expressed in uninduced CUTE plants that in uninduced WT plants (8 and 5 times, respectively), and could thus diffuse in the droplets. *LTP* genes were not only induced by *B. cinerea*, but also quite strongly by the mock treatment. In addition, the expression of the three *LTPs* was more strongly induced by mock treatment in CUTE plants than in WT plants. Since ¼ PDB or salt is needed to extract the active compound, we could hypothesize that salt induces responses in CUTE and WT plants, during the time of incubation of the droplets. CUTE plants would react stronger because of their higher cuticular permeability.

		WT	CUTE	WT				CUTE			
	hpi	0		12		30		12		30	
	treatments	-	-	mock	*Bc*	mock	*Bc*	mock	*Bc*	mock	*Bc*
At4g12470	pEARLI 1-like protein	5.5	46.3	208	2056.2	2265.5	5257.8	4721.7	8208.7	6431.5	8498.4
At4g12480	pEARLI 1	1.5	3.2	14.2	144.3	113.9	1270.1	202.2	1264	2515.8	2686
At4g12490	pEARLI 1-like protein	19.8	100.9	41.5	101.2	90.2	6223.1	138.9	610.8	1983.3	6087.4

Table 1. Microarray data of the three genes coding for pEARLI 1 or pEARLI 1-like proteins, that belong to the lipid transfer protein family.

Many *LTP* genes are induced by various abiotic stresses, like drought stress and/or water deficit (Trevino and O'Connell, 1998) and high salinity stress (Torres-Schumann et al., 1992). Expression of *LTPs* was induced in the leaves of pepper plants under drought and high salinity conditions (Jung et al., 2003). Transgenic *Arabidopsis* lines expressing the pepper gene *CALTPI* showed high levels of tolerance to NaCl and drought stresses (Jung et al., 2005). Thus, NaCl contained in the ¼ PDB inoculation medium might be responsible for the induction of the *LTP* genes in the mock samples. The droplets deposited on WT and CUTE leaves to collect the diffusates might induce reactions in the plant during the time of incubation. On the other hand, one could imagine that the application of liquid on leaves induces reactions. Changes in composition of the cell wall and membrane are known to occur under water stress conditions (Iraki et al., 1989). There is evidence that *LTP* transcripts accumulate under water stress in aerial plant parts such as leaves, stems and flowers (Torres-Schumann et al., 1992; Jung et al., 2003). LTPs might be involved in the repair of stress-induced damage in cells, as well as in inhibiting water loss via the assembly or deposition of the cell wall, membrane, and cuticular materials under water-stress conditions (Sterk et al., 1991).

We have not yet been able to determine the structure of the active compound that was isolated from *Arabidopsis* WT and CUTE diffusates, leaving all previous suggestions as hypothesis. The size of LTPs was found to be around 9 kDa (Kader, 1996). In our experiments, the active fungitoxic compound had approximately a size between 1000 and 3000 Da, which does not fit to the size of LTPs. However, we could isolate discrete fractions with strong fungitoxic activity by gel filtration chromatography. These fractions will be used for mass

spectrometry analysis, coupled to gas chromatography, or for direct sequencing of the amino acids, to confirm or infirm the presence of LTP.

IV.4 Material and Methods

Collection of leaf diffusates

Five µl droplets of ¼ PDB (Potato Dextrose Broth, 6 g l^{-1}, Difco) or 1M NaCl were deposited on leaves of WT or CUTE plants and the trays were covered to maintain high levels of humidity. Leaf diffusates were collected with a pipette tip after ca. 18 hours of incubation. For collection of bigger volumes of leaf diffusates, ca. 5 ml of ¼ PDB or 1M NaCl were poured in a Petri dish (8.5 cm in diameter). Leaves of WT and CUTE plants were deposited on the liquid (upper face on the liquid) to fill the surface. Petri dishes were sealed with parafilm and the liquid was collected after 18 hours of incubation at room temperature. Leaf diffusates were stored at -20°C.

Isolation of fungal spores

B. cinerea strains B05.10 and BMM, *Alternaria brassicicola* and *Plectosphaerella cucumerina* were grown on 1x PDA (Potato Dextrose Agar, 39 g l^{-1}, Difco) at room temperature. Spores were harvested in water and filtered through glass wool to remove hyphae. Spores of *Monilinia laxa* were isolated from infected mummified fruits (quince, prune). Spores were scratched with a pipet tip and mixed in H_2O. Concentration of spores was evaluated with a Thomma counting chamber.

***In-vitro* effect of leaf diffusates**

Nine µl of leaf diffusates were mixed with 3 µl of fungal spores to a final concentration of 5 x 10^4 spores ml^{-1} and deposited on a microscope glass slide. Fungal growth was observed under the microscope after incubation under high humidity conditions for ca. 16 h. Leaf diffusates extracted with ¼ PDB were mixed with spores in H_2O. Leaf exudates extracted with 1M NaCl were mixed with spores in 1x PDB.

***In-vivo* effect of leaf diffusates**

3x µl of leaf diffusates extracted with ¼ PDB were mixed with x µl of *B. cinerea* spores in H_2O to a final concentration of 5 x 10^4 spores ml^{-1}. Leaves were inoculated with 5 µl droplets of spores mixed with diffusates and symptoms were evaluated 3 dpi. Inoculated plants were kept under a water-sprayed transparent lid to maintain high humidity. Both *B. cinerea* strains gave similar results for all of the experiments carried out.

***In-vitro* and *in-vivo* effect of cutinase**

Purified cutinase from *F. solani*, kindly provided by M. Van der Burg-Koorevaar (UNILEVER Vlaardingen, The Netherlands), was diluted in 10 mM Na-acetate pH 5.2. Five µl droplets of buffer, 10 µg ml^{-1} cutinase solution or 100 µg ml^{-1} cutinase solution were deposited on WT leaves and incubated for 3 days under high humidity to prevent evaporation of the droplets. Droplets containing leaf exudates were collected. The *in-vitro* and *in-vivo* effects of these diffusates were tested as previously described with *B. cinerea* spores.

Plant extracts

Protein extract 1 (for PGIP assay, see III.2): WT and CUTE leaves were homogenised with 2 ml g^{-1} tissue of 1M NaCl and 20 mM Na-acetate pH 4.7. After 1h of shaking at 4°C, the homogenate was centrifuged 10 min (13'000 rpm, 4°C) and the supernatant filtered through Miracloth ®. *Protein extract 2*: WT and CUTE leaves were homogenised with 2 ml g^{-1} tissue of 50 mM Tris HCl pH 7.5. The homogenate was centrifuged (10 min, 13'000 rpm, 4°C) and the supernatant was collected. *Protein extract 3*: WT and CUTE leaves were homogenised with 2 ml g^{-1} tissue of 0.1 M Tris HCl pH 7.3, 5 mM EDTA, 0.1% Triton and 0.2% β-mercaptoethanol. The homogenate was centrifuged 10 min at 4°C and the supernatant was collected. The different protein extracts were stored at –20°C.

Intercellular wash fluid (IWF)

WT or CUTE leaves cut at the petiole were immersed in a buffer containing 50 mM Tris-HCl pH 7.5 and 150 mM NaCl. Leaves were vacuum-infiltrated and delicately dried on a towel. Infiltrated leaves were deposited in a syringe placed in a tube. The IWF was collected at the bottom of the tube after 20 min of centrifugation (150 g, 4°C). IWF were stored at –20°C.

Dialysis

WT and CUTE diffusates (in ¼ PDB) were dialysed with ready-to-use Spectra/Por® Float-A-Lyzer® tubes (Spectrum). Tubes for a dialysate volume of 300 µl were used (7 cm total length, 5 mm diameter), with MWCO (Molecular Weight Cut-Off) of 1000, 3500, 8000 and 15'000 Da. Samples were dialysed in 1 l of H_2O at 4°C during at least 24 hours (3 changes of H_2O). Fractions resulting from the gel filtration chromatography were lyophilised and dialysed with Spectra/Por® CE (Cellulose Ester) Membrane (500 MWCO, 16 mm flat width, 10 mm diameter, 0.81 ml/cm). The cellulose membrane was stored at 4°C in a preservative solution of 0.1% sodium azide.

Protease treatments

Diffusates of WT and CUTE plants (in ¼ PDB) were digested with pronase E (from *Streptomyces griseus*, Sigma) or proteinase K (from *Tritirachium album*, Sigma). Enzymes were dissolved in 50 mM Tris pH 8.0 at a stock concentration of 20 mg ml^{-1}. Enzymes were diluted 1:20 in leaf diffusates to a final concentration of 2.5 mM (1%). Samples were incubated 3h at 37°C or overnight at room temperature and checked for their *in-vitro* toxic effect against *B. cinerea*.

Gel filtration chromatography

The gel filtration chromatograph was performed on a column (length 250 mm, width 30 mm) with Bio-Gel P-4 (mesh 200-400). One ml of diffusate of WT or CUTE plants (in 1M NaCl) was injected on the column and eluted with 10 mM ammonium acetate pH 8.5. Thirty fractions of 6 ml were collected (180 ml in total). Each fraction was lyophilised, resuspended in 50 µl H_2O and tested for its *in-vitro* toxic activity against *B. cinerea*. Nine µl of each fraction was incubated with 3 µl of spores in 1x PDB as previously described.

IV.5 References

Adrian, M., Jeandet, P., Veneau, J., Weston, L.A., and Bessis, R. (1997). Biological activity of resveratrol, a stilbene compound from grapevines, against *Botrytis cinerea*, the causal agent of gray mold. J. of Chem. Ecol. **23,** 1689-1702.

Begg, G.E., and Speicher, D.W. (1999). Mass spectrometry detection and reduction of disulfide adducts between reducing agents and recombinant proteins with highly reactive cysteines. J. Biomol. Tech. **10,** 17-20.

Beisson, F., Koo, A.J.K., Ruuska, S., Schwender, J., Pollard, M., Thelen, J.J., Paddock, T., Salas, J.J., Savage, L., Milcamps, A., Mhaske, V.B., Cho, Y., and Ohlrogge, J.B. (2003). *Arabidopsis* genes involved in acyl lipid metabolism. A 2003 census of the candidates, a study of the distribution of expressed sequence tags in organs, and a web-based database. Plant Physiol. **132,** 681-697.

Bi, Y.M., Cammue, B.P.A., Goodwin, P.H., Krishna, R.S., and Saxena, P.K. (1999). Resistance to Botrytis cinerea in scented geranium transformed with a gene encoding that antimicrobial protein Ace-AMP1. Plant Cell Reports **18,** 835-840.

Chu, K.T., Liu, K.H., and NG, T.B. (2003). Cicerarin, a novel antifungal peptide from the green chickpea. Peptides **24,** 659-663.

Creasy, L.L., and Creasy, M.T. (1998). Grape chemistry and the significance of resveratrol: an overview. Pharmaceutical Biology **36,** 8-13.

Derckel, J.P., Audran, J.C., Haye, B., Lambert, B., and Legendre, L. (1998). Characterization, induction by wounding and salicylic acid, and activity against *Botrytis cinerea* of chitinases and beta-1,3-glucanases of ripening grape berries. Physiologia Plantarum **104,** 56-64.

Gomes, E., Sagot, E., Gaillard, C., Laquitaine, L., Poinssot, B., Sanejouand, Y.H., Delrot, S., and Coutos-Thevenot, P. (2003). Nonspecific lipid-transfer protein genes expression in grape (*Vitis* sp.) cells in response to fungal elicitor treatments. Mol. Plant Microbe Inter. **16,** 456-464.

Iraki, N.M., Singh, N., Bressen, R.A., and Carpita, N.C. (1989). Cell walls of tobacco cells and changes in composition associated with reduced growth upon adaptation to water and saline stress. Plant Physiol. **91,** 48-53.

Jung, H.W., Kim, K.D., and Hwang, B.K. (2003). Three pathogen-inducible genes encoding lipid transfer protein from pepper are differentially activated by pathogens, abiotic, and environmental stresses. Plant Cell Environ. **26,** 915-928.

Jung, H.W., Kim, K.D., and Hwang, B.K. (2005). Indentification of pathogen-responsive regions in the promoter of a pepper lipid transfer protein gene (*CALTPI*) and the enhanced resistance of the *CALTPI* transgenic *Arabidopsis* against pathogen and environmental stresses. Planta **221,** 361-373.

Kader, J.C. (1996). Lipid transfer proteins in plants. Annu. Rev. Plant Physiol. **47,** 627-654.

Kristensen, A.K., Brunstedt, J., Nielsen, K.K., Roepstorff, P., and Mikkelsen, J.D. (2000). Characterization of a new antifungal non-specific lipid transfer protein (NsLTP) from sugar beet leaves. Plant Science **155,** 31-40.

Molina, A., and Garcia-Olmedo, F. (1997). Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. Plant J. **12,** 669-675.

Nielsen, K.K., Neilsen, J.E., Madrid, S.M., and Mikkelsen, J.D. (1996). New antifungal proteins from sugar beet (*Beta vulgaris* L.) showing homology to non-specific lipid transfer proteins. Plant Mol. Biol. **31,** 539-552.

Osbourn, A.E. (1999). Antimicrobial phytoprotectants and fungal pathogens: a commentary. Fungal Genet. Biol. **26,** 163-168.

Pezet, R., and Pont, V. (1995). Mode of toxic action of Vitaceae stilbenes on fungal cells. In: Daniel M and Purkayastha RP (eds). Handbook of phytoalexin metabolism and action. Marcel Dekker Inc., New York, USA**,** 317-331.

Salzman, R.A., Tikhonova, I., Bordelon, B.P., Hasegawa, P.M., and Bressan, R.A. (1998). Coordinate accumulation of antifungal proteins and hexoses constitutes a developmentally controlled defence response during fruit ripening in grape. Plant Physiol. **117,** 465-472.

Schoonbeck, H.J., 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 Inter. **14,** 562-571.

Segura, A., Moreno, M., and Garcia-Olmedo, F. (1993). Purification and antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach. FEBS Letter **332,** 243-246.

Shin, D.H., Lee, J.Y., Hwang, B.K., Kim, K.K., and Suh, S.W. (1995). High-resolution crystal structure of the non-specific lipid-transfer protein from maize seedlings. Structure **3,** 189-199.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A., and De Vries, S.C. (1991). Cell-specific expression of the carrot *EP2* lipid transfer protein gene. Plant Cell **3,** 907-921.

Stotz, H.U., Elad, Y., Powell, A.L.T., and Labavitch, J.M. (2004). Innovative biological approaches to *Botrytis* suppression. Y. Elad et al. (eds), Botrytis: Biology, Pathology and Control (Kluwer Academic Publishers, Netherlands)**,** 369-392 (Chapter 20).

Thoma, S.L., Kaneko, Y., and Somerville, C. (1993). An *Arabidopsis* lipid transfer protein is a cell wall protein. Plant J. **3,** 427-437.

Torres-Schumann, S., Godoy, J.A., and Pintor-Toro, J.A. (1992). A probable lipid transfer protein gene is induced by NaCl in stems of tomato plants. Plant Mol. Biol. **18,** 749-757.

Trevino, M.B., and O'Connell, M.A. (1998). Three drought-responsive members of the nonspecific lipid-transfer protein gene family in *Lycopersicon pennellii* show different developmental patterns of expression. Plant Physiol. **116,** 1461-1468.

van Baarlen, P., Legendre, L., and van Kan, J.A.L. (2004). Plant defence compounds against *Botrytis* infection. Y. Elad et al. (eds), *Botrytis*: Biology, Pathology and Control (Kluwer Academic Publishers, Netherlands)**,** 143-161 (Chapter 9).

van Loon, L.C. (1985). Pathogenesis-related proteins. Plant Mol. Biol. **4,** 111-116.

Wang, H., and NG, T.B. (2004). Antifungal peptides, a heat shock protein-like peptide, and a serine-threonine kinase-like protein from Ceylon spinach seeds. Peptides **25,** 1209-1214.

Wang, H.X., and NG, T.B. (2003). Isolation of cucumoschin, a novel antifungal peptide abundant in arginine, glutamate and glycine residues from black pumpkin seeds. Peptides **24,** 969-972.

Wong, J.H., and NG, T.B. (2005). Limenin, a defensin-like peptide with multiple exploitable activities from shelf beans. J. of Peptide Science.

Wong, J.H., and NG, T.B. (2005). Vulgarinin, a broad-spectrum antifungal peptide from haricot beans (*Phaseolus vulgaris*). Int. J. Biochem. Cell Biol. **37,** 1626-1632.

V. Plants with defective cuticles share common characteristics

Aerial parts of plants are covered with a hydrophobic cuticle offering physical protection against water loss and irradiation, and mechanical protection against microbes. Transgenic *Arabidopsis* plants secreting a fungal cutinase from *F. solani* f.sp. *pisi* (CUTE plants) have a defective cuticle ultrastructure that leads to increased cuticular permeability, ectopic pollen germination and organ fusions. In addition, CUTE plants are fully resistant to the necrotrophic fungus *Botrytis cinerea*, whereas highly susceptible wild-type plants develop soft rot symptoms. Upon inoculation with *B. cinerea*, the resistance was shown to be correlated with a primed expression of genes coding for lipid transfer proteins (LTP), peroxidases (PER) and protease inhibitors (PI). In addition, a compound with strong fungistatic activity against *B. cinerea* diffuses from CUTE leaves. Different mutants impaired in cuticle biosynthesis or formation have been recently described. We present here two novel mutants, so-called *pec* mutants (*permeable cuticle* mutants), that were screened for their increased cuticular permeability. We show that *pec1* and *pec9* mutants, in addition to other mutants with cuticular defects, share common characteristics with CUTE plants. Most of the mutants displayed enhanced resistance to *B. cinerea* similar to that of CUTE plants, since priming of the *LTP*, *PER* and *PI* genes and diffusion of the fungitoxic compound could be observed. These results highlight a common mechanism involved in the defence against *B. cinerea* in *Arabidopsis* plants with cuticular defects.

V.1 Introduction

The hydrophobic cuticle coats the cell walls of epidermal cells of all aerial plant organs. It is the major barrier to protect plants against invading microbes, in addition to its role as permeation barrier for solutes, gases and water. The outermost layer of the cuticle is composed of wax, whereas the cuticle proper deposited beneath is seen in electron micrographs as a thin lamellate layer (Kolattukudy, 2001). The major constituent of the cuticle proper is the lipid poyester cutin, made of C16 and C18 fatty acid derivatives (Kolattukudy, 2001).

Mutagenised plant populations were screened for cuticular defects to find mutants, as tools to study the cuticle functions. Many mutants with reduced wax crystals on stems of *Arabidopsis* have been identified (Kornneef et al., 1989; McNevin et al., 1993; Jenks et al., 1996). For example, mutants like *wax1*, *cer10* or *cer13* (*ecer*iferum) have a reduced deposition of epicuticular wax, an altered leaf morphology and organ fusions (Jenks et al., 1996; Lolle et al., 1998). The *fdh* (*fiddlehead*) mutant is characterised by organ fusions in leaves and flower organs, pollen germination on leaves, and increased cuticle permeability (demonstrated by the chlorophyll leaching rate) (Lolle et al., 1992; Lolle et al., 1998; Lolle and Pruitt, 1999). The *FDH* gene may be involved in trichome initiation and codes for an enzyme of the *FATTY ACID*

ELONGATION family (Yephremov et al., 1999; Pruitt et al., 2000). The *shn* (*shine*) activation tag gain-of-function mutant shows a deep shiny green appearance, increased cuticle permeability, increased cuticular wax load and structure and altered epidermal differentiation compared with wild-type (WT) plants. *Arabidopsis* plants constitutively overexpressing the *SHN* gene (35S:*SHN*) show the same phenotype as the original activation tag line. SHN is an AP2/EREBP transcription factor and is suggested to be involved in the regulation of lipid biosynthesis (Aharoni et al., 2004).

Genetic studies of cutin synthesis are less developed, but some mutants have been recently characterised. The *lcr* (*lacerata*) mutant shows organ fusions and ectopic pollen germination. *lcr* has a defect in a gene coding for a cytochrome P450 monooxygenase, that can catalyse the formation of ω-hydroxy fatty acids in yeast and could be involved in cutin biosynthesis (Wellesen et al., 2001). The *wax2* mutant is deficient in both cutin and wax synthesis and shows an increased cuticular permeability and organ fusions. *wax2* has a thinner cuticle and a reduced wax load (Chen et al., 2003). The *lacs2* (*long-chain acyl-CoA synthetase*) mutant has a thinner cuticle than WT plants and shows a pleiotropic phenotype, such as reduced leaf size, plant growth and seed production, but no organ fusions. LACS2 might be required for cutin synthesis (Schnurr et al., 2004). The mutant *att1* (*aberrant induction of type three genes*) is impaired in a cytochrome P450 catalyzing fatty acid oxidation and has a reduced cutin content in comparison to WT plants. *att1* display increased resistance to a virulent strain of *P. syringae* and enhanced expression of the bacterial type III genes *avrPto* and *hrpL* (Xiao et al., 2004). The *ace*/*hth* (*adhesion of calyx edges* / *hothead*) mutant, deficient in fatty acid ω-alcohol dehydrogenase activity, shows a reduction in the levels of the major constituents of cuticular polyesters and cutin (Kurdyukov et al., 2006a). The *bdg* (*bodyguard*) mutant, which exhibits defects characteristic of the loss of cuticle structure, accumulates more cell wall-bound lipids and epicuticular waxes than WT plants. *BDG* is exclusively expressed in epidermal cells and might code for an extracellular synthase responsible for the formation of ester bonds in the cuticle (Kurdyukov et al., 2006b). Both *bdg* (in leaves) and *ace*/*hth* (in flowers) have a discontinuous or multilayered cuticle and show organ fusions (Krolikowski et al., 2003; Kurdyukov et al., 2006a; Kurdyukov et al., 2006b).

The cuticle structure is conventionally analysed by transmission electron microscopy (TEM). This requires laborious procedures, unsuitable for large-scale screening of mutants with defective cuticles. Therefore, a direct screening method based on the staining of plants with toluidine-blue was developed by Tanaka et al. (2004). In summary, a number of defects may be associated with improper cuticle formation in *Arabidopsis* mutants: poor growth and performance, sensitivity to low humidity, increased sensitivity to chemicals such as pesticides and herbicides, morphological irregularities in the shapes of organs and single cells, altered resistance to pathogens, distorted cell differentiation, illicit cell-cell interactions and cell death

(Yephremov and Schreiber, 2005). Thus, the cuticle plays an essential role for the normal development of the plant and for the quality of the interface between the plant and its external environment (Sieber et al., 2000).

Transgenic *Arabidopsis* plants overexpressing a fungal cutinase from *F. solani* f.sp. *pisi* (CUTE plants) display phenotypes similar to mutants with defective cuticles, like organ fusions, increased cuticular permeability and ectopic germination (Sieber et al., 2000). CUTE plants were shown to be totally resistant to the virulent necrotrophic fungus *B. cinerea* and were used as a tool to study the defence mechanisms linked to the sensing of cuticular defects. The expression of several genes coding for LTP, PER and PI was primed in CUTE plants, in comparison to WT plants. These genes play a role in the resistance against *B. cinerea* since their overexpression in WT plants led to increased protection to the fungus. In addition, a substance highly toxic to *B. cinerea* was found to diffuse from CUTE leaves. In this study, we show that many mutants with defects in cuticle structure and function are also fully resistant to *B. cinerea*. The mechanisms underlying this resistance are investigated.

V.2 Results and Figures

A double screening was performed on EMS-mutagenised plants (M2 plants) in order to isolate mutants with enhanced cuticular permeability, as well as mutants with increased resistance to *B. cinerea* strain BMM. The screening method for analysing the cuticular permeability came from an easy experiment with Calcofluor White (CW), a fluorescent dye used for the staining of fungi in inoculated leaves. CUTE leaves stained with CW showed a strong fluorescent background. Indeed, CW stains both fungal and plant cell walls; as CUTE plants have a more permeable cuticle, CW diffuses and binds to the plant cell wall cellulose. This observation gave rise to the “CW screening method” applied on M2 plants. CW staining was performed on one leaf per plant, two weeks after transplanting. M2 plants were grown for one to two more weeks and inoculated with *B. cinerea* strain BMM on three leaves per M2 plant.

The mutants that were strongly stained with CW were called *pec* for *permeable cuticle*, while mutants that were more resistant to *B. cinerea* than the WT controls were referred to as *bre* for *botrytis resistant*. *pec1* was isolated as strongly staining with CW, while *pec9*/*bre1* was first isolated as *B. cinerea* resistant (*bre1*), but then identified as strongly staining with CW too (*pec9*) and was renamed *pec9*. Both *pec1* and *pec9* have a normal growth habit, but display ultrastructural changes in their cuticular membrane, increased sensitivity to herbicides, and increased water loss in comparison to WT plants. *pec9* had a stronger phenotype than *pec1* in almost all experiments. Both mutants very rarely show organ fusions, but when organ fusions

occur, the cuticular membrane is disrupted or missing (Christiane Nawrath, personal communication).

Mapping localised *pec1-1* in a region of 30 kb potentially identical with At2g26910. Three T-DNA insertion lines (SALK) were identified that showed an identical phenotype: *pec1-2*, *pec1-3* and *pec1-4*. At2g26910 encodes an ABC-transporter called PDR4, belonging to the PDR family. One member of this family was characterised in tobacco as being capable of transporting the diterpene sclareol. The transport function of PEC1 still remains to be identified (Christiane Nawrath, personal communication).

pec9-1 was localised by mapping to the middle of chromosome I. A candidate gene approach led to the *lacs2-1* gene (At1g49430) (Schnurr et al., 2004). Indeed, *pec9-1* does not complement *lacs2-1*. Thus, *pec9-1* is allelic to *lacs2-1* and will be called *pec9-2*, or *lacs2-2*. *lacs2-2* does not complement the T-DNA insertion line (GABI) *lacs2-3* either. LACS2 is a long-chain acyl-CoA synthetase that has been found to have higher specific activity with ω-hydroxylated fatty acids than with normal fatty acids in *E. coli* (Schnurr et al., 2004). The analysis of residual bound lipids in *lacs2-2* and *lacs2-3* shows that all oxygenated fatty acids and their derivatives are reduced in the polyester, particularly the dicarboxylic acids (80% reduction). Thus, LACS2 is involved in a central step in cutin formation. Cutin monomers might be potentially exported as CoA-esters or esterified to glycerol before transport (Christiane Nawrath, personal communication).

Transgenic CUTE plants display a disrupted cuticle structure and are fully immune to *B. cinerea*. We tested whether *Arabidopsis* mutants with defects in cuticle structure were also resistant to *B. cinerea*. The mutants *bdg, lcr*, *ace*/*hth, pec1-1*, *pec1-2* (SALK line), *pec9-1* (=*lacs2-2*) and *pec9-2* (=*lacs2-3*, GABI line) were compared to the WT ecotype Col-0; the *wax2* mutant was compared to the WT ecotype C24; 35S-*SHN* was compared to the WT ecotype Ws. *lcr*, *bdg*, *pec1*, *pec1-2*, *pec9* and *pec9-2* were found to be strongly resistant to *B. cinerea*, in comparison to WT Col-0 plants that show soft rot symptoms 3 days post-inoculation (dpi) (Figure 1a). However, the mutant *ace*/*hth* was as susceptible as WT Col-0 plants (Figure 1). The WT C24 ecotype was less susceptible to *B. cinerea* than Col-0, but no enhanced resistance could be observed in the mutant *wax2* (Figure 1a). The overexpressing line 35S:*SHN* was fully resistant to *B. cinerea*, in comparison to the WT Ws plants (Figure 1a). Thus, all mutants that have defects in the cuticle structure, except *ace/hth*, have an enhanced resistance to *B. cinerea*, similar to CUTE plants. The level of resistance was also evaluated by counting the percentage of outgrowing lesions 3 dpi with *B. cinerea* (Figure 1b). *lcr* was the least resistant of all resistant mutants in the Col-0 background.

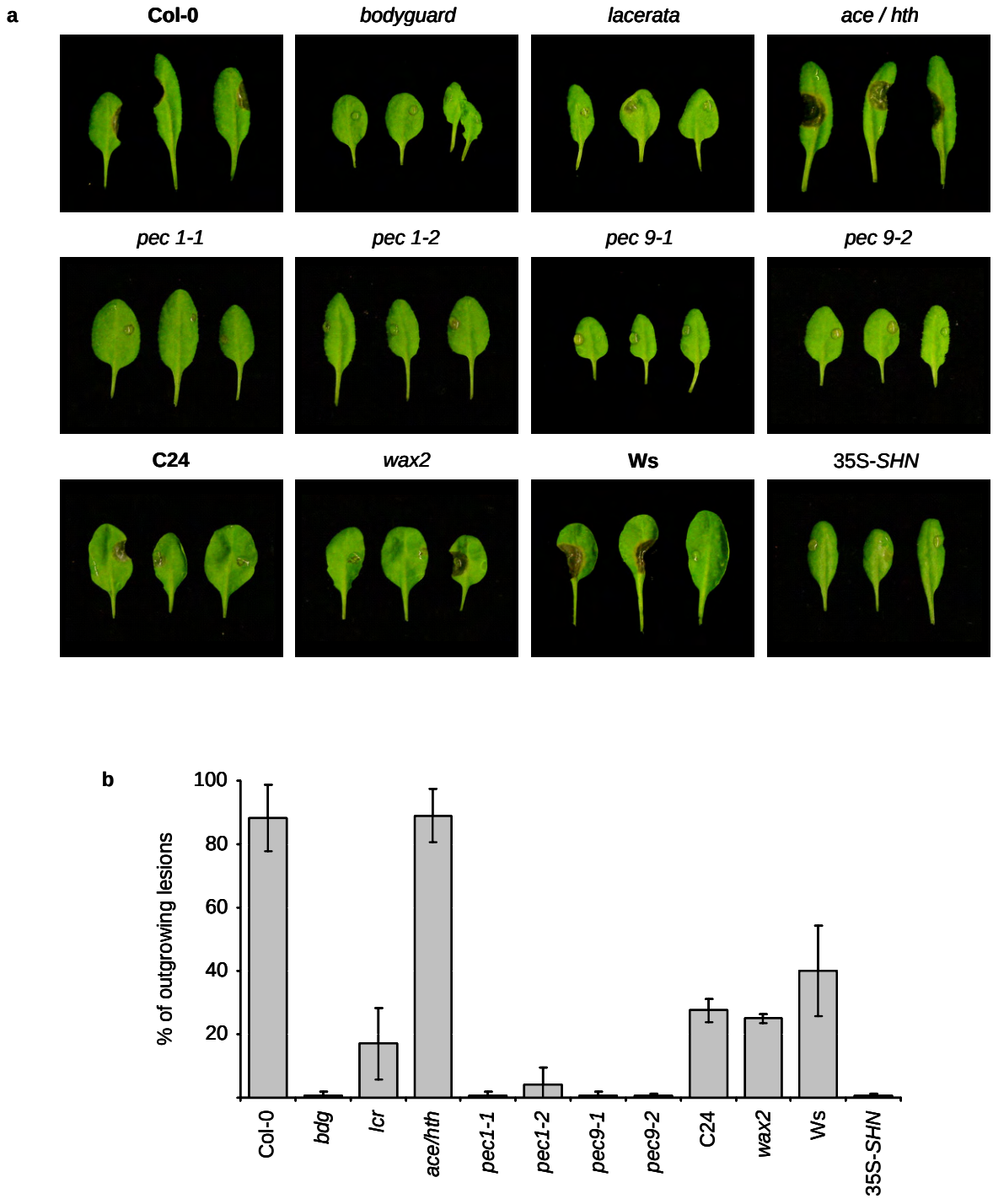


Figure 1. Resistance level of WT plants and mutants after infection with *B. cinerea*. **a.** Appearance of cuticle mutants 3 dpi with *B. cinerea*, compared to controls. **b.** Percentage of outgrowing lesions on cuticle mutants and controls, 3 dpi with *B. cinerea*.

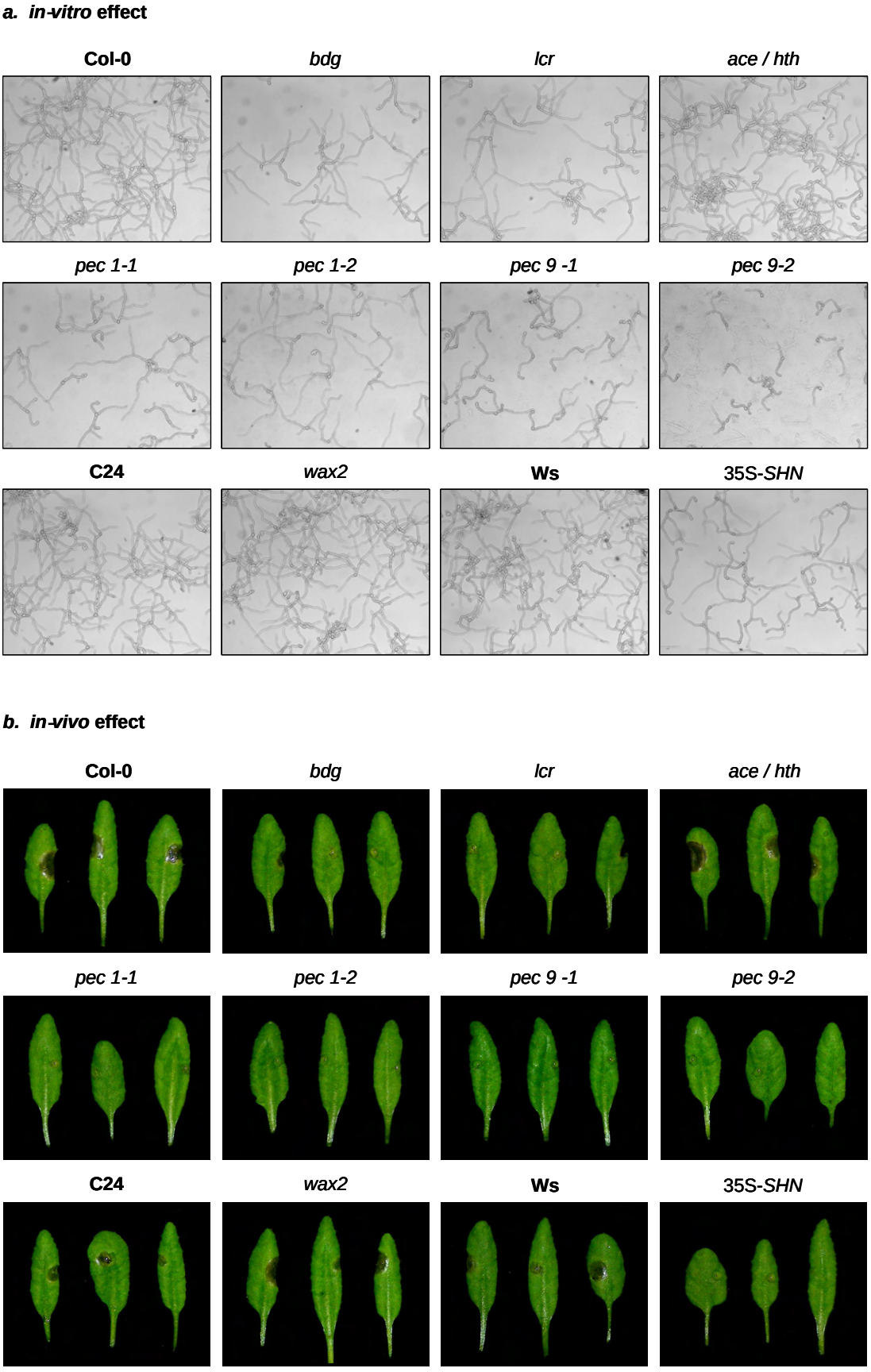


Figure 2. *In-vitro* (**a**) and *in-vivo* (**b**) effect of diffusates from WT plants and mutants on *B. cinerea*.

We have shown that a fungitoxic substance diffuses from leaves of CUTE and *bdg* plants (see III.1). In Chapter IV, we have hypothesised a natural fungitoxic compound that would be present in higher amounts in diffusates of CUTE plants because of their increased cuticular permeability. Leaf diffusates were collected for the different mutants with cuticular defects and WTs and tested *in vitro* for their fungitoxic activity against *B. cinerea* (Figure 2a). All mutants displaying enhanced resistance to the fungus (Figure 1) secreted the fungitoxic substance. The fungitoxic activity was also demonstrated in an *in-vivo* assay, where WT Col-0 plants were inoculated with *B. cinerea* spores mixed with the different diffusates (Figure 2b). However, the diffusates of *ace/hth* and *wax2* plants did not show any fungitoxic activity, although they have an enhanced cuticular permeability (Chen et al., 2003; Kurdyukov et al., 2006a). Analysis of transpiration rates of rosettes is commonly used as a measure of cuticle permeability. The transpiration rate of *ace/hth* was found to be higher than that of WT plants and similar to the transpiration rates of *lcr* and *pec1-2*. The transpiration rate of *bdg* was the highest, followed by *pec9-2* (Christiane Nawrath, personal communication). Thus, a fungitoxic activity can be recovered in leaf diffusates of most mutants that have defects in their cuticle, with the exception of a least one mutant where no fungitoxic activity was observed despite higher cuticle permeability. The reason for this peculiar behaviour needs to be further determined.

The resistance of CUTE plants to *B. cinerea* is related to the priming of *LTP*, *PER* and *PI* genes (see III.1). In a first experiment, expression of the *LTP* gene At4g12470, of the *PER* genes At5g39580 and At2g37130, and of the *PI* genes At2g43510 and At2g38870 was analysed in WT, *bdg* and *lcr* plants after inoculation with *B. cinerea*. Gene expression was analysed 12 and 30 hpi with *B. cinerea*, in comparison to mock-sprayed plants. The level of expression of these genes was higher in the *lcr* and *bdg* mutants after inoculation with *B. cinerea* than in WT plants at similar time points (Figure 3). Thus, like in CUTE plants, increased expression of the *LTP*, *PER* and *PI* genes was linked to the resistance of both mutants to *B. cinerea*.

The expression pattern of the *LTP*, *PER* and *PI* genes was also checked in the *pec* mutants after inoculation with *B. cinerea*, in comparison to inoculated WT Col-0 plants. *pec9-1*, *pec9-2* and *pec1-2* were analysed, in addition to *lcr* (as control for primed expression of the genes) and *ace/hth* (Figure 4). Expression of the selected genes was primed in the *lcr* mutant, except for At4g12470, whose induction in WT plants was also different from the previous experiment, and At2g37130. The expression pattern of the susceptible *ace/hth* mutant was quite similar to that of WT Col-0 plants, in agreement with the observation that *ace/hth* was as susceptible as WT plants to *B. cinerea*. The expression patterns of *pec9-2* and *pec9-1* however, were quite variable; the expression of At4g12470 and At2g37130 was primed upon inoculation with *B. cinerea* in *pec9-2* but not in *pec9-1*, while the expression of the other genes was not

primed. *pec1-2* did not show any increased expression of the selected genes after inoculation with *B. cinerea*, in comparison to WT plants.

These results suggest that the expression of these five genes is variable in the different mutants displaying strong resistance to *B. cinerea*, i.e. *bdg*, *lcr*, *pec9-1*, *pec9-2* and *pec1-2*. Although *lcr* and *bdg* showed a consistent priming of these genes like CUTE plants after inoculation with *B. cinerea*, the different results obtained with the *pec* mutants do not allow drawing strong correlations between the level of expression of these genes and the resistance to *B. cinerea*.

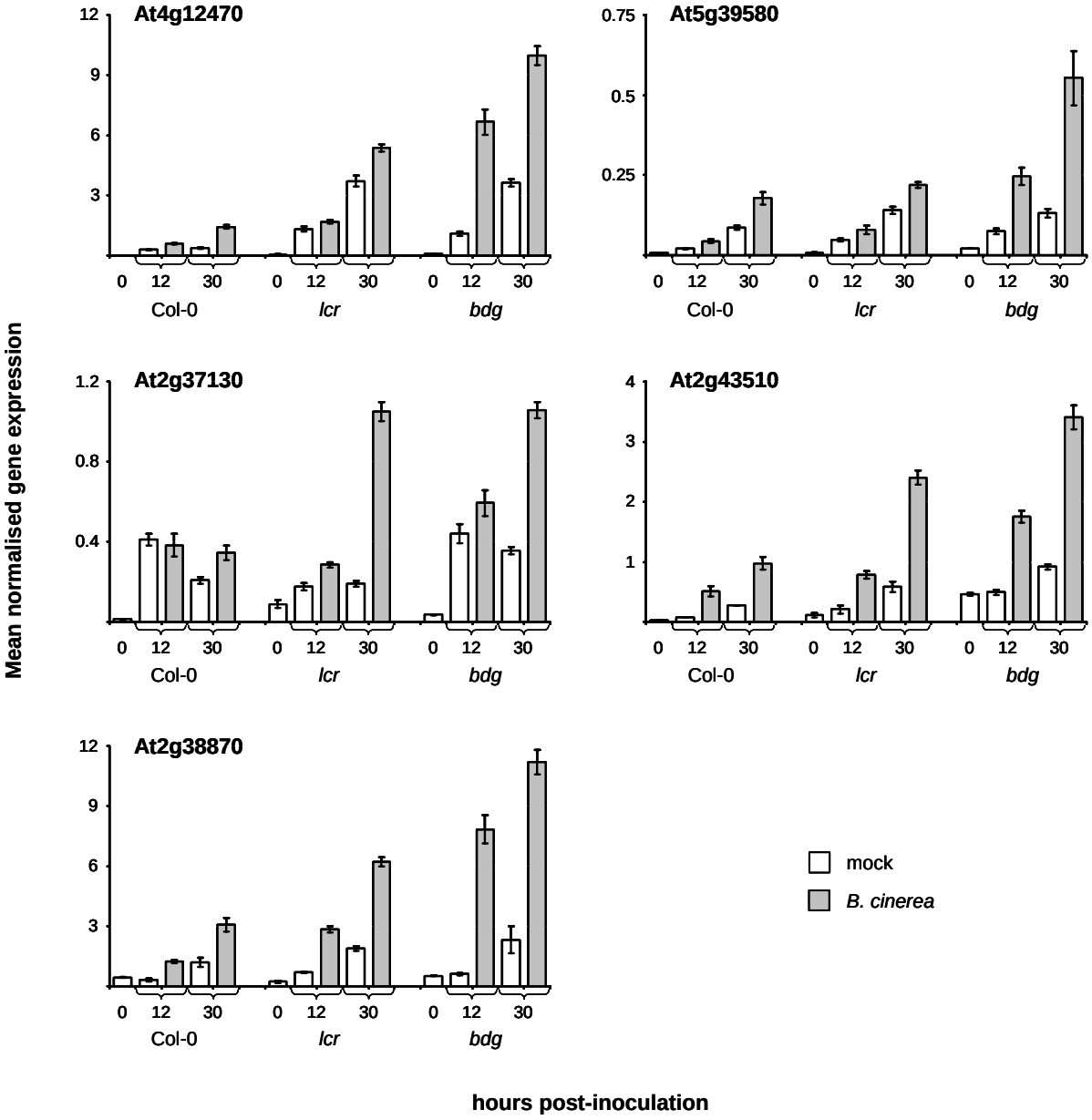
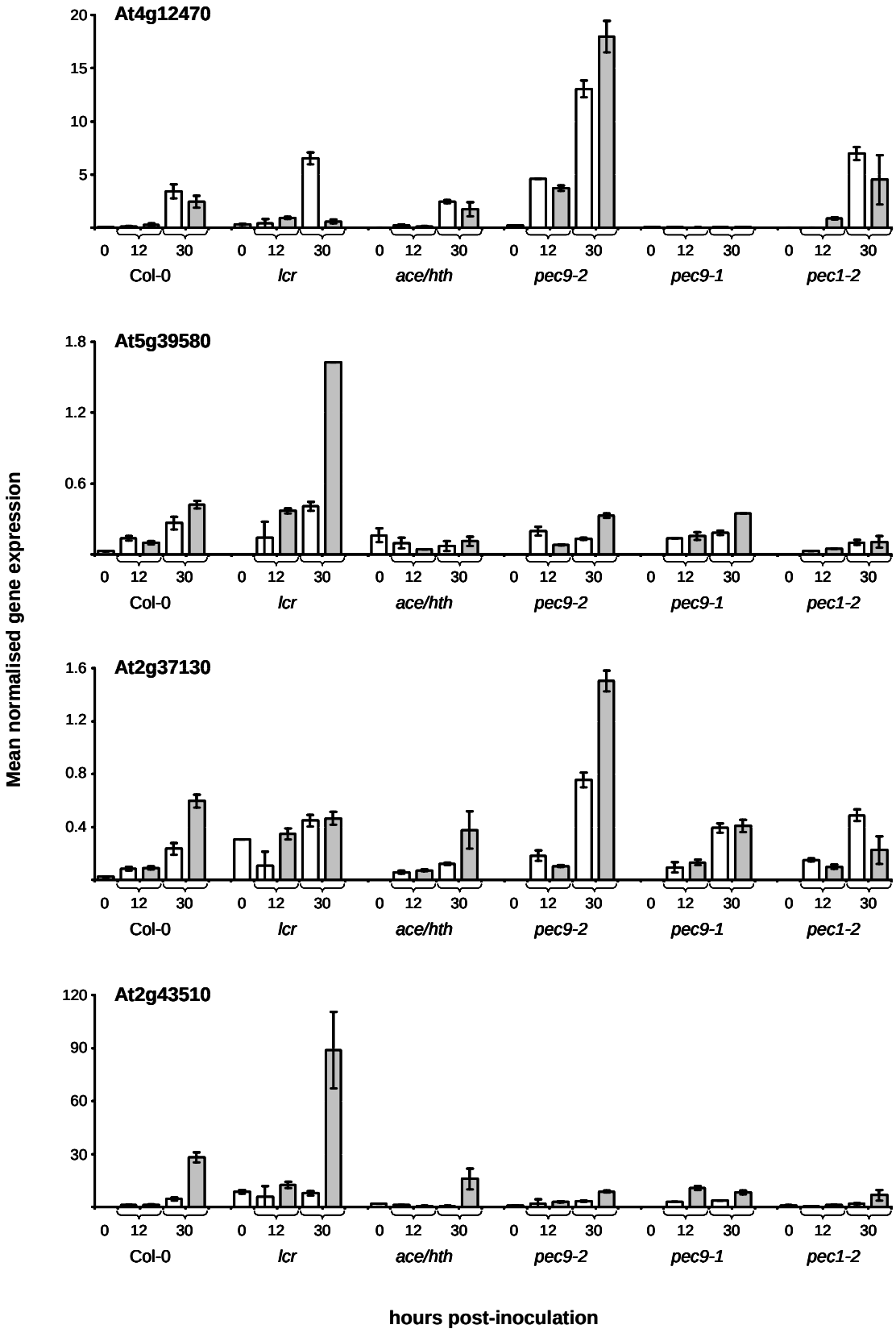


Figure 3. Priming of genes in the mutants *lcr* and *bdg* after inoculation with *B. cinerea*. Mean normalised gene expression of the genes At4g12470, At5g39580, At2g37130, At2g43510 and At2g38870 in WT and mutants, mock (white bars) or *B. cinerea* inoculated (grey bars). Each bar represents the mean of triplicate samples ± SE. The experiment was repeated one time with similar results.


At4g12470
At5g39580
At2g37130
At2g43510
Mean normalised gene expression
hours post-inoculation
0 12 30
Col-0
lcr
ace/hth
pec9-2
pec9-1
pec1-2

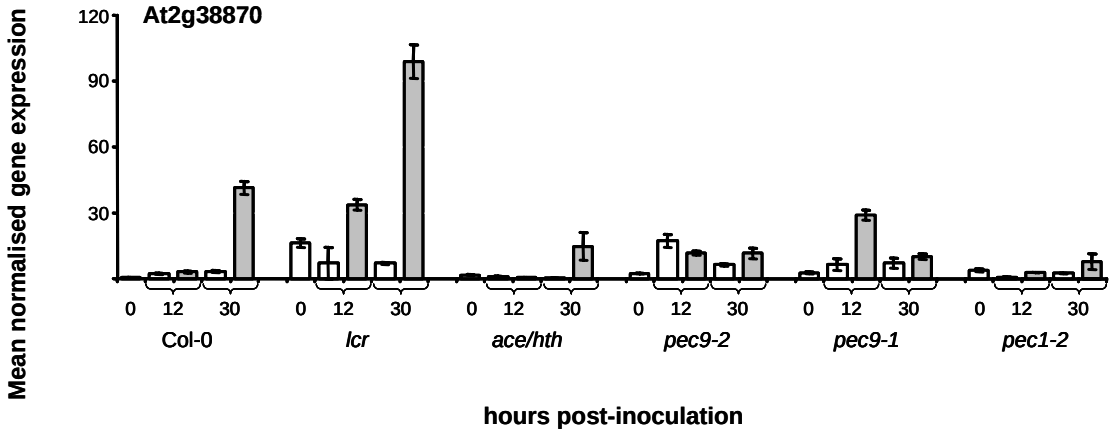


Figure 3. Priming of genes in the mutants *lcr*, *ace/hth*, *pec9-2*, *pec9-1* and *pec1-2* after inoculation with *B. cinerea*. Mean normalised gene expression of the genes At4g12470, At5g39580, At2g37130, At2g43510 and At2g38870 in WT and mutants, mock (white bars) or *B. cinerea* inoculated (grey bars). Each bar represents the mean of triplicate samples ± SE. The experiment was not repeated.

V.3 Discussion

CUTE plants have a modified cuticle, which is loosely structured and multilayered. Such perturbations lead to an enhanced cuticular permeability. CUTE plants, despite their degraded cuticle, display a total immunity against the virulent fungus *B. cinerea*. The increased resistance was not specific to the transgenic CUTE plants, but could also be observed in the *bdg* mutant, which exhibits cuticular defects (see III.1). This observation was extended to other mutants with cuticular defects, as shown in this study. Most mutants of Col-0 background tested, i.e. *bdg*, *lcr*, *pec1* and *pec9*, except *ace/hth*, were highly resistant to *B. cinerea* in comparison to WT plants. 35S-*SHN* plants were also fully resistant, in contrast to WT Ws plants. The *wax2* mutant, which displays phenotypes typical of cuticular defects, was however not resistant to *B. cinerea*, like *ace/hth* (Figure 1a).

Fungitoxic activity could be detected in all resistant mutants, while it was absent in the *ace/hth*, *wax2* and WT diffusates (Figure 2a). Thus, there was a strong correlation between the resistance to *B. cinerea* and the presence of fungitoxic activity in plant diffusates, strengthening the idea that the toxic compound plays an important role in the resistance linked to cuticular defects. This statement supports the hypothesis that the increased permeability of the cuticle might be directly related to the presence (or the amount of) the toxic compound. Indeed, all mutants described with cuticular defects display an enhanced cuticular permeability, which leads on one hand to increased sensitivity to external compounds like herbicides, and on the other hand to enhanced leaking of intercellular molecules. The rate at which chlorophyll can be extracted from rosette leaves is commonly used as a measure of cell-wall and cuticle

permeability. Indeed, the mutant *hth* was described to have an increased rate of chlorophyll extraction from rosette leaves (Lolle et al., 1998), but *ace/hth* did neither show any toxic activity in leaf diffusates, nor resistance to *B. cinerea*. Moreover, the cuticle in the mutant formed multilayered patterns marked by breaks, resembling the cuticular structure of CUTE plants (Kurdyukov et al., 2006a). The *wax2* mutant also showed increased epidermal permeability (Chen et al., 2003), but no toxic activity could be measured in its leaf diffusates (Figure 2a). Thus, diffusion of the toxic compound in inoculation droplets did not always correlate with increased cuticular permeability.

Although the relations between fusion phenotypes and cuticle properties remain controversial, it is commonly accepted that an ability to produce epidermal fusions is linked to cuticle defects because, beside fusions, this class of mutants is characterised by increased rate of chlorophyll extraction from rosette leaves and pollen germination on non-reproductive organs (Lolle et al., 1998). The *ace/hth* mutant shows a perturbed cuticle ultrastructure but normal levels of epicuticular wax, organ fusions in the inflorescence, increased rate of chlorophyll extraction from rosette leaves and pollen germination on non-reproductive organs. *ACE/HTH* is specifically expressed in the epidermis of all vegetative and generative organs, with stronger expression in epidermal cells of young and actively growing vegetative and floral organs (Kurdyukov 2006a). The lipid profile of mutant inflorescences showed differences compared to the WT, indicating a defect in the oxidation of long-chain ω-hydroxy fatty acids to ω-oxo fatty acids. Parallel experiments with leaf samples showed similar results but greater differences between WT and mutant plants were observed from the inflorescence samples (Kurdyukov 2006a). Likewise, the *wax2* mutant displays a disorganized cuticle ultrastructure, an altered visible wax deposition, organ fusions between aerial organs, increased rates of chlorophyll extraction and increased transpiration rates, in comparison to WT plants (Chen et al., 2003). In contrary to other mutants tested in this study, *wax2* has an altered visible wax deposition (Chen et al., 2003). Thus, *ace/hth* and *wax2*, despite their phenotype typical for cuticular defects, do not show increased resistance to *B. cinerea*. In contrast to *bdg* and *lcr* show strong organ fusions in leaves, *ace/hth* and *wax2* display fusions in floral organs. The cuticular defects might be therefore less important in leaves, explaining the susceptibility of inoculated leaves to *B. cinerea*.

The toxic compound in diffusates did not inhibit the germination of spores, nor the growth of hyphae, but significantly slowed down hyphal growth. It was also demonstrated that the fungitoxic compound had a strong *in-vivo* effect in preventing infection of normally susceptible WT Col-0 plants (Figure 2b). The direct fungitoxic activity might be sufficient to block infection of WT plants. However, the *in-vitro* toxic activity was strong but did not fully arrest fungal growth. Possibly, the toxic compound might also act as an elicitor and induce defence responses in WT plants, that lead to a complete stop of the infection. On the other hand, “normal” defence

reactions induced by the presence of *B. cinerea* could complete the direct effect of the compound on the pathogen and inhibit the infection. In addition, the participation of a third compound distinct from the toxic compound but with a similar molecular size (see IV.2) can not be excluded. It could be present in the diffusates and, parallel to the fungitoxic compound, act as inducer of defence reactions.

Upon inoculation with *B. cinerea*, the resistance of CUTE plants was shown to be correlated with an earlier and stronger expression of *LTP*, *PER* and *PI* genes, in comparison to WT plants. Primed expression of these genes was also observed in the *bdg* mutant (see III.1), suggesting that induction of these defence responses might be common to plants with cuticular defects that display an enhanced resistance to *B. cinerea*, similar to CUTE plants. Indeed, the expression of *LTP*, *PER* and *PI* genes was also primed in the *lcr* mutant (Figure 3). The expression pattern of the susceptible *ace/hth* mutant resembled that of WT Col-0 plants, fitting the observation that *ace/hth* was as susceptible as WT plants to *B. cinerea*. However, the expression patterns of *pec9-2* and *pec9-1* were quite variable; the expression of some genes was primed upon inoculation with *B. cinerea*, while expression of the other genes was not primed. Indeed, the background of *pec9-1* was found not to be Col-0, which may account for the differences observed between *pec9-1* and *pec9-2* (Christiane Nawrath, personal communication). *pec1-2* did not show any priming of the selected genes after inoculation with *B. cinerea*. The implication of the *LTP*, *PER* and *PI* in defence against *B. cinerea* was demonstrated in WT plants overexpressing these genes, which resulted in an increased resistance to the fungus. For the *pec* mutants, however, the strong resistance to *B. cinerea* did not correlate with a higher expression of these genes. This observation raises the suggestion that there are probably other yet unknown elements that are involved in the resistance induced by cuticular defects, acting together to build an effective defence against this virulent pathogen.

The full resistance of CUTE plants and other mutants with cuticular defects to *B. cinerea* highlights a multilayered defence taking place in *Arabidopsis* plants. Although most plants with defects in the cuticle ultrastructure share common characteristics like increased cuticular permeability, ectopic pollen germination, organ fusions and resistance to *B. cinerea*, it seems obvious that there are different classes of mutants. Indeed, all mutants described in this study show an enhanced permeability of the cuticle, whereas only some of them display strong organ fusions affecting leaves like CUTE plants, *bdg* and *lcr*. *ace/hth* and *wax2* have organ fusions in floral organs, whereas *pec1* and *pec9* do only rarely show organ fusions (Christiane Nawrath, personal communication). These differences could play a role in the resistance to *B. cinerea*, in the diffusion of the fungitoxic compound(s) and the expression of the *LTP*, *PER* and *PI* genes. The fungitoxic compound, the diffusion rate of which is probably influenced by the permeability of the cuticle, was found to play a major role in the defence response, since its presence was always correlated with resistance to *B. cinerea*. However, two mutants with increased cuticular

permeability did not show any fungitoxic activity in their leaf diffusates, indicating that there are probably other parameters affecting the presence of the fungitoxic compound. The priming of the *LTP*, *PER* and *PI* genes also revealed some discrepancies among the different resistant mutants.

In conclusion, CUTE plants represent a useful tool to study the plant responses involved in the defence against *B. cinerea* in *Arabidopsis* plants. CUTE plants enabled the discovery of new defence elements that were common to other mutants with cuticular defects. Thus, the cuticle integrity appears to play an important and complex role in the defence against pathogens. It remains to be understood how the virulent *B. cinerea* avoids these mechanisms on WT plants.

V.4 Material and Methods

Inoculation with *B. cinerea*

B. cinerea strains B05.10 (Buttner et al., 1994) and BMM (Zimmerli et al., 2001) provided by J. van Kan and B. Mauch-Mani were grown on 1x PDA (Potato Dextrose Agar, 39 g l^{-1}, Difco). Spores were harvested in water and filtered through glass wool to remove hyphae. Spore concentration was adjusted to 5 x 10^4 spores ml^{-1} in ¼ PDB (Potato Dextrose Broth, 6 g l^{-1}, Difco) for inoculation. Leaves were inoculated with 5 µl droplets of spore suspension to evaluate the symptoms. The level of protection was estimated by the potential of *B. cinerea* to cause soft rot symptoms extending beyond the inoculation site (outgrowing lesions). The spore suspension was sprayed on whole plants for microarray and real-time RT-PCR experiments. Control plants were inoculated with ¼ PDB (mock). The inoculated plants were kept under a water-sprayed transparent lid to maintain high humidity. Inoculated leaves were stained with lactophenol-trypan blue (Nawrath and Métraux, 1999) to visualize growth of *B. cinerea* under the microscope. Both *B. cinerea* strains gave similar results for all experiments carried out.

Collection of leaf diffusates

Five µl droplets of ¼ PDB (Potato Dextrose Broth, 6 g l^{-1}, Difco) were deposited on WT or CUTE leaves and trays were covered with a humid lid to maintain high levels of humidity. Leaf diffusates were collected with a pipette after 18 hours of incubation. Leaf diffusates were stored at –20°C.

***In-vitro* effect of leaf diffusates**

Nine µl of the leaf diffusate extracted with ¼ PDB was mixed with 3 µl of *B. cinerea* spores in H_2O to a final concentration of 5 x 10^4 spores ml^{-1} and deposited on a microscope glass slide. Fungal growth was observed under the microscope after incubation under high humidity conditions for ca. 14 hours.

***In-vivo* effect of leaf diffusates**

3x µl of the leaf diffusate extracted with ¼ PDB was mixed with x µl of *B. cinerea* spores in H_2O to a final concentration of 5 x 10^4 spores ml^{-1}. Leaves were inoculated with 5 µl droplets of the mixture and symptoms were evaluated after 3 days. The inoculated plants were kept under a water-sprayed transparent lid to maintain high humidity. Both *B. cinerea* strains gave similar results for all experiments carried out.

Gene expression analysis

RNA was prepared using the TRIzol® reagent (Molecular Research Center, Inc., Invitrogen) and retrotranscribed in cDNA (Omniscript® RT kit, Qiagen). Analysis of gene expression after inoculation with *B. cinerea* was performed by real-time RT-PCR (Absolute QPCR SYBR Green Mix, ABgene). The following primers were used: 5'-RTPCR-

4g12470 (5'-CCTTACAACACCGAATATAAC-3') and 3'-RTPCR-4g12470 (5'-GGACATTGGACCGGCTTG-3'); 5'-RTPCR-5g39580 (5'-TCCGATCATTTGCTTTGGTC-3') and 3'-RTPCR-5g39580 (5'-AATTGTCTCGGCATTAGGGC-3'); 5'-RTPCR-2g37130 (5'-TCTCCTCGGCTTCTTTTGT-3') and 3'-RTPCR-2g37130 (5'-TACGGCTGTGTTACCGTGTTT-3'); 5'-RTPCR-2g43510 (5'-AGAAATGGCAAAGGCTATC-3') and 3'-RTPCR-2g43510 (5'-GCGATTGCTTTAGATTTTACTG-3'); 5'-RTPCR-2g38870 (5'-CATCAAATACATCAGAAGAC-3') and 3'-RTPCR-2g38870 (5'-CTTTTTCATTATGAATATAGAAAT-3'). Gene expression values were normalised with the plant *actin 2* gene. The experiment was repeated 2 times with similar results.

V.5 References

Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., Van Arkel, G., and Pereira, A. (2004). The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. Plant Cell **16,** 2463-2480.

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.

Chen, X., Goodwin, M., Boroff, V.L., Liu, X., and Jenks, M.A. (2003). Cloning and characterization of the *WAX2* gene of *Arabidopsis* involved in cuticle membrane and wax production. Plant Cell **15,** 1170-1185.

Jenks, M.A., Rashotte, A.M., Tuttle, H.A., and Feldmann, K.A. (1996). Mutants in *Arabidopsis thaliana* altered in epicuticular wax and leaf morphology. Plant Physiol. **110,** 377-385.

Kolattukudy, P.E. (2001). Polyesters in higher plants. In: Advances in Biochemical Engineering Biotechnology: Biopolyesters, W. Babel and A. Steinbüchel, eds (Berlin: Springer-Verlag)**,** 1-49.

Kornneef, M., Hanhart, C.J., and Thiel, F. (1989). A genetic and phenotypic description of *eceriferum* (*cer*) mutants in *Arabidopsis thaliana*. J. Hered. **80,** 118-122.

Krolikowski, K.A., Victor, J.L., Wagler, T.N., Lolle, S.J., and Pruitt, R.E. (2003). Isolation and characterization of the *Arabidopsis* organ fusion gene *HOTHEAD*. Plant J. **35,** 501-511.

Kurdyukov, S., Faust, A., Tenkamp, S., Bär, S., Franke, B., Efremova, N., Tietjen, K., Schreiber, L., Saedler, H., and Yephremov, A. (2006a). Genetic and biochemical evidence for involvement of *HOTHEAD* in the biosynthesis of long chain a-,w-dicarboxylic fatty acids and formation of extracellular matrix. Planta **11,** 1-15.

Kurdyukov, S., Faust, A., Nawrath, C., Bär, S., Voisin, D., Efremova, N., Franke, R., Schreiber, L., Saedler, H., Métraux, J.P., and Yephremov, A. (2006b). The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in *Arabidopsis*. Plant Cell **18,** 321-339.

Lolle, S.J., and Pruitt, R.E. (1999). Epidermal cell interactions: case for local talk. Trends Plant Sci. **4,** 14-20.

Lolle, S.J., Cheung, A.Y., and Sussex, I.M. (1992). *Fiddlehead*: an *Arabidopsis* mutant constitutively expressing an organ fusion program that involves interactions between epidermal cells. Dev. Biol. **152,** 383-392.

Lolle, S.J., Hsu, W., and Pruitt, R.E. (1998). Genetic analysis of organ fusion in *Arabidopsis thaliana*. Genetics **149,** 607-619.

McNevin, J.P., Woodward, W., Hannoufa, A., Feldmann, K.A., and Lemieux, B. (1993). Isolation and characterization of *eceriferum* (*cer*) mutants induced by T-DNA insertions in *Arabidopsis thaliana*. Genome **36,** 610-618.

Nawrath, C., and Métraux, 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.

Pruitt, R.E., Vielle-Calzada, J.P., Ploense, S.E., Grossniklaus, U., and Lolle, S.J. (2000). *FIDDLEHEAD*, a gene required to suppress epidermal cell interactions in *Arabidopsis*, encodes a putative lipid biosynthetic enzyme. Proc. Natl. Acad. Sci. **97,** 1311-1316.

Schnurr, J., Shockey, J., and Browser, J. (2004). The acyl-CoA synthetase encoded by *LACS2* is essential for normal cuticle development in *Arabidopsis*. Plant Cell **16,** 629-642.

Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Métraux, J.P., and Nawrath, C. (2000). Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell **12,** 721-738.

Tanaka, T., Tanaka, H., Machida, C., Watanabe, M., and Machida, Y. (2004). A new method for rapid visualization of defects in leaf cuticle reveals five intrinsic patterns of surface defects in *Arabidopsis*. Plant J. **37,** 139-146.

Wellesen, K., Durst, F., Pinot, F., Benveniste, I., Nettesheim, K., Wisman, E., Steiner-Lange, S., Saedler, H., and Yephremov, A. (2001). Functional analysis of the *LACERATA* gene of *Arabidopsis* provides evidence for different roles of fatty acid w-hydroxylation in development. Proc. Natl. Acad. Sci. **98,** 9694-9699.

Xiao, F., Goodwin, M.S., Xiao, Y., Sun, Z., Baker, D., Tang, X., Jenks, M.A., and Zhou, J.M. (2004). *Arabidopsis* CYP86A2 represses *Pseudomonas syringae* type III genes and is required for cuticle development. EMBO J. **23,** 2903-2913.

Yephremov, A., and Schreiber, L. (2005). The dark side of the cell wall: molecular genetics of plant cuticle. Plant Biosystems **139,** 74-79.

Yephremov, A., Wisman, E., Huijser, P., Huijser, C., Wellesen, K., and Saedler, H. (1999). Characterization of the *FIDDLEHEAD* gene of *Arabidopsis* reveals a link between adhesion response and cell differentiation in the epidermis. Plant Cell **11,** 2187-2201.

Zimmerli, L., Métraux, J.P., and Mauch-Mani, B. (2001). beta-amino butyric acid-induced resistance of *Arabidopsis* against the necrotrophic fungus *Botrytis cinerea*. Plant Physiol. **126,** 517-523.

VI. Wound-induced resistance to *Botrytis cinerea*

VI.1 Article

Wound-induced resistance to *Botrytis cinerea*

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Full immunity to the soft rot pathogen *Botrytis cinerea* strains B05.10 and BMM was observed in *Arabidopsis thaliana* Col-0 following wounding of the leaf. The resistance was observed strictly at the delimited wound site produced using laboratory forceps or syringe needles. Wound-induced resistance was not associated with salicylic acid-, jasmonic acid- or ethylene-dependent defence responses. The phytoalexin camalexin was found to be involved in this defence response since the *pad2* and *pad3* camalexin-deficient mutants were susceptible after wounding and the *B. cinerea* strains were sensitive to this toxin. Wounding alone did not induce camalexin but primed its accumulation after inoculation with *B. cinerea*. Glutathione was also found to be required for the resistance, as mutants deficient in the γ-glutamylcysteine synthetase such as *pad2*, *rax1* or *cad2* showed susceptibility to *B. cinerea* after wounding indicating that basal levels of glutathione are required for the wound-induced resistance. In addition, expression of the gene encoding the glutathione-S-transferase 1 was primed by wounding in leaves inoculated with *B. cinerea*. Our results demonstrate how abiotic stress can induce full immunity to virulent strains of *B. cinerea*, a process that involves camalexin and glutathione.

VI.1.1 Introduction

Botrytis cinerea is an ubiquitous pre- and postharvest necrotrophic pathogen with a broad host range (Pezet et al., 2004) that causes substantial crop losses (Tournas, 2005). B. cinerea conidia penetrate through the cuticule and epidermal walls leading to the death of invaded cells. The mycelium spreads through dead tissue leading to tissue softening, rot or necrosis, depending on the invaded parts. *Botrytis* circumvents plant defenses in various ways. For instance, this fungus can degrade the cuticle and the plant cell wall (Commenil et al., 1998; Staples and Mayer, 1995; ten Have et al., 1998) and detoxify plant antifungal products (Gil-ad et al., 2000; Pezet et al., 1991; Kliebenstein et al., 2005). During the infection process, *B. cinerea* weakens or damages its host using reactive oxygen species (ROS) (Liu et al., 1998), toxins or oxalic acid that chelates cell-wall calcium and enhances its own endopolygalacturonase activity

(Prins et al., 2000; Reino et al., 2004). Targeted inactivation of fungal genes involved in cell-wall degradation (ten Have et al., 1998; Valette-Collet et al., 2003) support the biological relevance of the cell wall as a food source or as a general barrier against invading fungi. Further defence reactions induced during infection with *B. cinerea* involve cell-wall strengthening (McLusky et al., 1999; Stewart and Mansfield, 1985).

The defence reactions of the host plant to infection by *B. cinerea* have been studied in many plants. Oligogalacturonides protect grapevine against *B. cinerea*, presumably by eliciting defence responses such as increased chitinase and β-1,3-glucanase activities or the stimulation of an oxidative burst (Aziz et al., 2004). Changes in wyerone and wyeronic acid measured in *Vicia faba* were found to restrict growth of *B. cinerea* in broad bean (Mansfield and Hutson, 1980). The accumulation of the lettuce phytoalexin lettucenine correlates with resistance to *B. cinerea* (Bennett et al., 1994). Resistance to *B. cinerea* in *Arabidopsis* was recently described to involve the phytoalexin camalexin (Kliebenstein et al., 2005; Denby et al., 2004; Ferrari et al., 2003). The *pad2-1* and *pad3-1* mutants isolated for reduced accumulation of camalexin after inoculation with bacterial pathogens (Glazebrook et al., 1994) display a higher susceptibility to *B. cinerea* and camalexin has a direct toxic effect against *B. cinerea* (Denby et al., 2004, Ferrari et al., 2003). Earlier observations did not support such a conclusion (Thomma et al., 1999a), but different *B. cinerea* isolates were shown to differ in their camalexin tolerance (Kliebenstein et al., 2005). The production of camalexin was also found to vary greatly among different *Arabidopsis* ecotypes (Denby et al., 2004). This could explain the contradictory results among the different studies.

The development of a hypersensitive reaction is part of the defence responses of plants and the collapse of life-sustaining host cells is usually considered as a major barrier to many biotrophic pathogens. Necrotrophs such as *Botrytis* or *Alternaria* might use the hypersensitive cell death to their advantage for a better colonization of the host (Govrin and Levine, 2000; Lincoln et al., 2002; Van Baarlen et al., 2004). In *Arabidopsis*, distinct defence signalling pathways operate against discrete groups of pathogens. For instance, the salicylic acid (SA)-dependent pathway controls the expression of pathogenesis-related genes such as *PR1* together with defence to biotrophic pathogens such as *Hyaloperonospora parasitica*. The ethylene (ET)- and jasmonic acid (JA)-dependent pathway determines defence to necrotrophs and the expression of another set of *PR* genes that include the antimicrobial *PR-3* (chitinase), *PR-4* (hevein-like protein) or a plant defensin (*PDF1.2*) (Penninckx et al., 1996; Penninckx et al., 1998).

The plant defensins alone may determine resistance to various *Alternaria* species as demonstrated in transgenic plants constitutively overexpressing plant defensin genes (Parashina et al., 2000; Terras et al., 1995). While there is a strong case for *Alternaria* resistance linked to *PDF1.2* expression together with camalexin production, resistance to *B.*

cinerea might rather result from a combined action of several other antifungal proteins that are induced along with *PDF1.2* (Thomma et al., 1998; Thomma et al., 1999). Enhanced susceptibility to necrotrophic (*Alternaria*, *Botrytis*, *Plectosphaerella*) but not biotrophic pathogens (*Hyaloperonospora*, *Pseudomonas syringae*) was observed in *esa1* mutants. These plants show a delayed expression of *PDF1.2* and of camalexin accumulation upon pathogen inoculation (Tierens et al., 2002). Thus, *PDF1.2* is a good marker for responses against *B. cinerea*, but is not active itself. Resistance of tomato to *B. cinerea* is enhanced by ET treatments while an inhibitor of ET perception increases susceptibility (Diaz et al., 2002). A functional ET pathway is required for defence against *B. cinerea* in *Arabidopsis* (Thomma et al., 1999). *Arabidopsis* plants overexpressing the *ETHYLENE RESPONSE FACTOR 1*, a gene involved in the ET-dependent expression of proteins such as chitinase or defensins, exhibit increased resistance to necrotrophs (Berrocal-Lobo et al., 2002). Recently, Ferrari et al. (2003) proposed that the local resistance to *B. cinerea* requires ET-, JA-, and SA-signalling pathways as well as synthesis of camalexin.

Abiotic stimuli such as UV radiation, heat treatment or wounding have also been reported to be effective inducers of defenses against pathogens (Métraux and Durner, 2004). In particular, wounding of the plant surface creates a potential entry point for invading pathogens. Plants respond to this injury by localized defense responses including accumulation of phytoalexins (Kuc, 2000; Reymond et al., 2000) and antimicrobial proteins such as proteinase inhibitors or chitinase (Chang et al., 1995; Graham et al., 1986; Ryan, 1990). Genetic evidence demonstrates the involvement of octadecanoic acids as endogenous regulators for wound-induced resistance to insects or pathogens (Howe, 2004). However, a substantial number of wound-induced genes are expressed independently of JA perception (Reymond et al., 2000). Wounding and insect feeding also produce signals (hydraulic, electrical or chemical) that can propagate systemically. For example in tomato, wounding results in the production of systemin, a systemic signal involved in the activation of proteinase inhibitors (Howe, 2004). Green leafy volatiles or isoprenoids are produced after mechanical wounding or pathogen/herbivore attacks in higher plants. They are perceived by JA-dependent and -independent pathways in *Arabidopsis*. Treatment of *Arabidopsis* plants with such volatiles induces defence responses and increases resistance to *B. cinerea* (Kishimoto et al., 2005).

In this article, we describe a strong immunity of wounded *Arabidopsis* leaves in response to inoculation with *B. cinerea*. We have characterized the mechanisms involved in this defense and shown the implication of an early accumulation of camalexin after wounding. In addition we have documented the importance of glutathione in this process.

VI.1.2 Results

Under the experimental conditions described, both *B. cinerea* strains B05.10 and BMM were strongly pathogenic on *Arabidopsis* ecotype Col-0 as shown in Fig. 1a (first row). Wounding of *Arabidopsis* leaves with laboratory forceps and subsequent inoculation of the wound-site with *B. cinerea* led to a strong immunity compared to unwounded leaves (Fig 1a, middle row). The wound-induced protection could also be induced by puncturing several holes with a syringe needle. No outgrowing lesions were observed after placing a droplet of a *B. cinerea* spore suspension on such a wound site (Fig 1a, lower row). In our experience, sharp wound sites that contain intact cells able to induce defense reactions appeared to lead to a stronger protection. Indeed, a single puncture performed with the tip of a syringe needle was not sufficient to induce resistance against *B. cinerea*. Full protection was obtained when inoculation droplets were placed at sites enclosing 4 punctures or more. A wound site with a large area of dead cells allowed the propagation of the fungus due to its capacity to use dead tissue as a saprophytic base. Inoculation of *B. cinerea* spores distally from a wound site produced symptoms, indicating the absence of a systemic wound-induced protection comparable to those on non-wounded leaves (Fig. 1b). The level of protection was estimated by the potential of *B. cinerea* to cause soft rot symptoms extending beyond the inoculation site. On wild-type (WT) Col-0 plants most inoculation sites result in water-soaked lesions but a limited number of lesions do not spread beyond the primary infection site. Under our experimental conditions, the percentage of spreading lesions was the most adequate way to compare the level of resistance of different plants.

The dependency of wound-induced resistance on known signalling pathways for induced resistance was tested using several mutants as shown in Fig. 2. Wounding was performed on mutants affected in the ET perception (such as *ein2* and *etr1*), JA signalling (*jar1, coi1*) or SA accumulation (*sid2, pad4*). Inoculation of the wound sites with *B. cinerea* induced full resistance in all mutants compared to the corresponding WT plants, indicating that ET-, JA- and SA-signalling are not involved in the wound-induced resistance. Furthermore, we tested the phytoalexin-deficient mutants *pad2* and *pad3,* since camalexin has been previously implied in resistance to *B. cinerea*. Interestingly, protection induced by wounding was much less efficient on both *pad2* and *pad3* compared to WT plants. These results suggest that camalexin is involved in the wound-induced resistance to *B. cinerea.* In a recent study, camalexin was shown to have a variable impact on *B. cinerea*, depending on the pathogen isolate (Kliebenstein et al., 2005). Therefore we tested the sensitivity of the *B. cinerea* isolates B05.10 and BMM used in the studies presented here. *In-vitro* tests where camalexin was incorporated in the germination medium confirmed a strong sensitivity of the *B. cinerea* B05.10 and BMM strains to camalexin (data not shown).

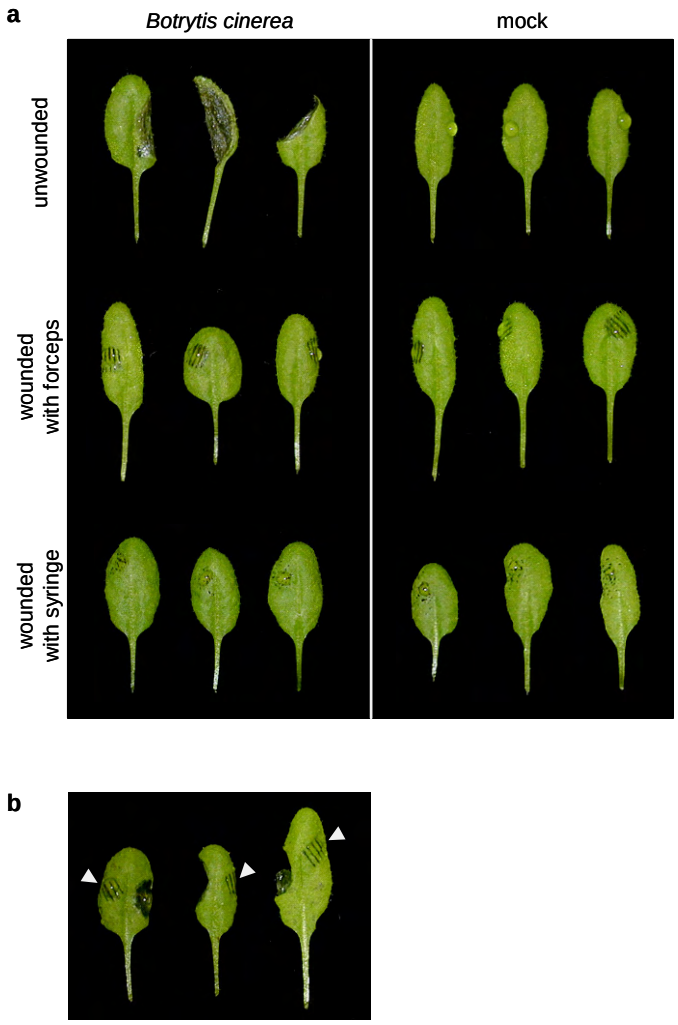

Figure 1. Wound-induced resistance of *Arabidopsis* against *B. cinerea*.
a. Disease symptoms on Col-0 WT plants 3 days after mock (right row) and inoculation with *B. cinerea* (5 x 10^4 spores ml^{-1} ¼ PDB) (left row). Plants were inoculated by depositing 5 µl spore suspension on unwounded leaves (upper row), directly after wounding with a forceps (middle row), or directly after puncturing several tiny holes with a syringe needle (lower row).
b. Leaves of WT plants were wounded with a forceps on one side of the central vein. Five µl droplets of *B. cinerea* spores (5 x 10^4 spores ml^{-1} ¼ PDB) were placed on the wounded site (arrow) and on the unwounded leaf half. Symptoms were evaluated 3 days after inoculation.

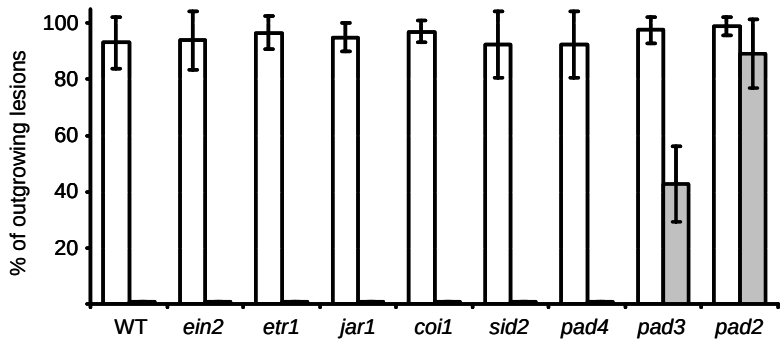

Figure 2. Percentage of outgrowing lesions after infection with *B. cinerea* on unwounded or forceps-wounded leaves. The resistance level of WT plants and different mutants to *B. cinerea* (5 x 10^4 spores ml^{-1} ¼ PDB) was evaluated as the percentage of outgrowing lesions. White bars: unwounded leaves; grey bars: wounded leaves. Symptoms were evaluated 3 days after inoculation. Bars represent the mean of 4 to 6 experiments (n=48 to 72, ±SD).

To further explore the importance of camalexin, we have determined the kinetics of camalexin accumulation in wounded WT plants after inoculation with *B. cinerea* (Fig. 3). The inoculation medium alone sprayed on wound sites had no effect on the accumulation of camalexin. The accumulation of camalexin in WT plants was triggered at 24 h after *B. cinerea* inoculation and increased drastically at 36 hours post-inoculation (hpi). Interestingly, in wounded and infected plants, camalexin was already detectable at 12 hpi. At 24 hpi, it has raised to a level 7-fold higher than that in unwounded *B. cinerea*-inoculated plants. The highest levels of camalexin were observed at 48 hpi in inoculated control plants, presumably due to the extensive disease development in the absence of wounding. Therefore, wounding primes camalexin accumulation after *B. cinerea* inoculation in WT plants.

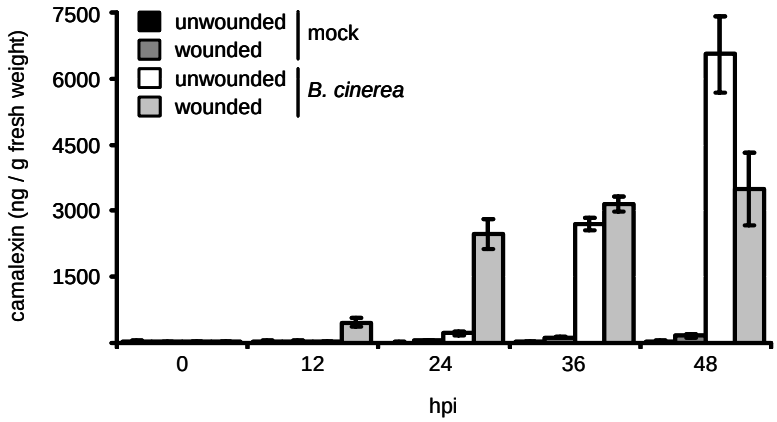

Figure 3. Priming of camalexin accumulation in wounded and infected WT plants.
WT plants were sprayed with the mock buffer (¼ PDB) (black bars), wounded and mock-sprayed (dark grey bars), inoculated with *B. cinerea* (5 x 10^4 spores ml^{-1} ¼ PDB) (white bars), and wounded and inoculated (light grey bars). Wounding was performed by puncturing the whole leaf surface with the « syringe stamp ». Three samples of leaves from 6 plants at least were collected at different time points after treatment. Camalexin was extracted and quantified by HPLC. Means and standard deviation of 3 replicates are shown. The experiment was repeated 2 times with similar results.

The wound-induced priming of camalexin was further analysed in mutants of the ET (*ein2*), JA (*jar1*) and SA (*pad4*) pathways used in the experiment described in Fig. 2. Camalexin was determined after 24 hpi, a time point where priming has taken place after wounding and inoculation in WT plants. As presented in Fig. 4, a strong induction of camalexin could be measured in the *ein2, jar1* and *pad4* mutants indicating wound-induced priming similar to that induced in WT plants. Both *pad3* and *pad2* showed an absence respectively an intermediate level of camalexin accumulation in response to *B. cinerea* in wounded leaves. Priming in *pad2* led to about 3 times less camalexin than in WT plants. The absence of camalexin in *pad3* agrees with published results describing PAD3 as a cytochrome P450 monooxygenase directly involved in camalexin biosynthesis (Zhou et al., 1999). Whereas *pad2* was the most susceptible of all mutants to *B. cinerea*, it did not display the lowest level of camalexin after wounding and

infection at 24 hpi. This suggests the participation of other components in the wound-induced resistance besides camalexin.

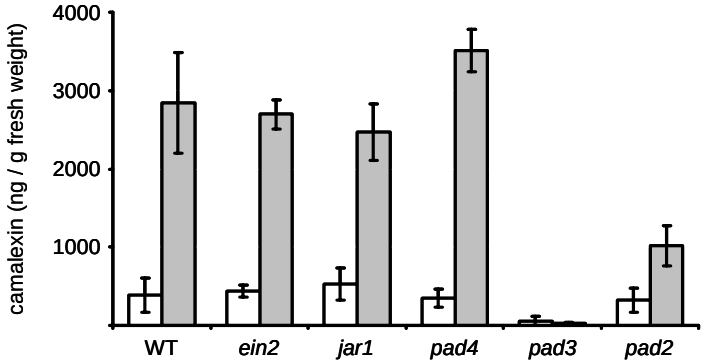

Figure 4. Wound-induced priming of camalexin in WT plants and various mutants.
Unwounded (white bars) and syringe-wounded plants (grey bars) were spray-inoculated with *B. cinerea* (5 x 10^4 spores ml^{-1} ¼ PDB). Three leaf samples were harvested 24 h after inoculation and camalexin was extracted. Means and standard deviation of 3 replicates are shown.

The *pad2* mutant has recently been shown to carry a mutation in *GSH1* encoding a γ-glutamylcysteine synthetase involved in the biosynthesis of glutathione (Parisy et al*.,* 2006). To find out whether a basal level of glutathione is required for the wound-induced resistance to *B. cinerea*, two other glutathione-deficient mutants *cad2-1* (*cadmium hypersensitive 2-1*) and *rax1-1* (*regulator of ASCORBATE PEROXIDASE 2 1-1*) were analysed. Both *cad2* and *rax1* carry point mutations in the *GSH1* gene (Cobbett et al, 1998; Ball et al., 2004). The level of glutathione was determined in *Arabidopsis* WT plants, in *pad2* as well as in *cad2, rax1,* and in *pad3* which was included as susceptible plant (Fig. 5a). In accordance with published results, the level of glutathione was strongly decreased in *pad2*, *rax1* and *cad2*, compared to WT plants (Parisy et al., 2006; Ball et al., 2004; Cobbett et al., 1998). The *pad3* mutant shows a basal level of glutathione comparable to WT plants. Interestingly, *rax1* and *cad2* show a strong reduction in the wound-induced protection to *B. cinerea*, confirming a possible implication of glutathione in this phenomenon (Fig. 5b). However, priming of camalexin accumulation 24 h after wounding and inoculation with *B. cinerea* in *cad2* and *rax1* is similar to WT plants (data not shown). Wound-induced accumulation of glutathione or wound-induced priming similar to that of camalexin could not be observed in WT plants (data not shown).

Glutathione is involved in the detoxification of organic compounds. Many xenobiotics as well as some metabolites like anthocyanins are conjugated with glutathione by a family of glutathione S-transferases (GST) and transported, possibly as conjugates, into the vacuole (Marrs, 1996). One of these GST's, GST1 (Greenberg et al., 1994), was selected previously as a robust molecular marker for the production of ROS and found to be expressed independently of SA, JA and ET after wounding and pathogen attack (Kishimoto et al., 2005; Vollenweider et al., 2000; Grant et al., 2000). We analysed *GST1* expression by real-time RT-PCR after wounding and inoculation with *B. cinerea* in WT plants (Fig. 6). *GST1* mRNA accumulation was

primed after wounding and inoculation (Fig. 3). At 24 hpi the increase in *GST1* expression induced by *B. cinerea* is larger than *GST1* expression induced by wounding and *B. cinerea*. However, *GST1* is also induced moderately by wounding alone and the expression peaks at 9 hpi and decreases thereafter. These results suggest that basal glutathione levels and expression of the stress-inducible *GST1* gene might play a role in the wound-induced resistance to *B. cinerea*, in addition to the accumulation of the phytoalexin camalexin.

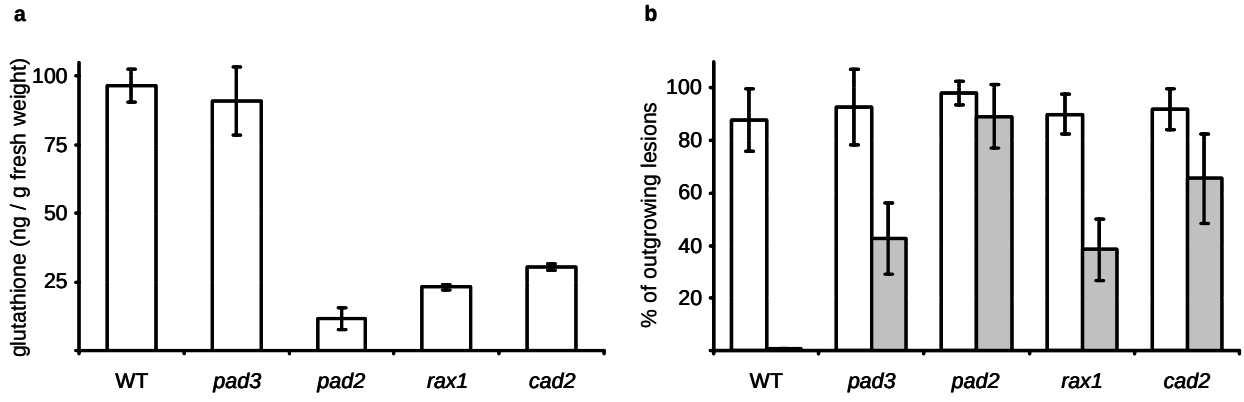

Figure 5. Wound-induced resistance to *B. cinerea* in mutants impaired in glutathione biosynthesis.
a. Level of total glutathione. Leaves of uninduced four-week old plants were analysed. Means and standard deviation from 3 independent measurements are shown. **b.** Percentage of outgrowing lesions after infection with *B. cinerea* (5 x 10^4 spores ml^{-1} ¼ PDB) on unwounded leaves and leaves wounded with a forceps. Symptoms were evaluated 3 days after inoculation. For each genotype, means and standard deviation from 3 independent experiments carried on 24 plants are shown.

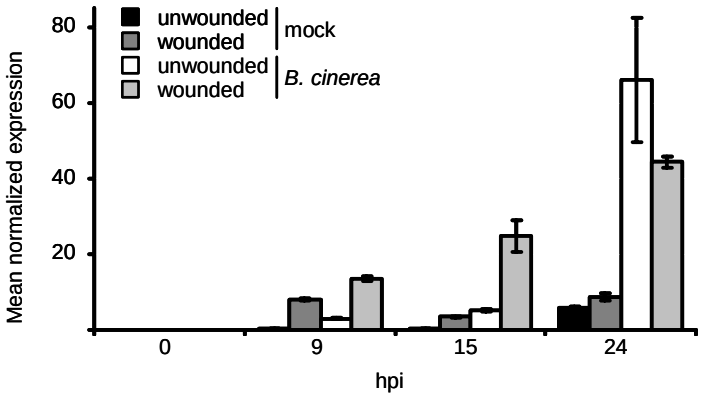

Figure 6. Priming of *GST1* mRNA accumulation.
WT plants were sprayed with the mock buffer (¼ PDB) (black bars), wounded and mock-sprayed (dark grey bars), inoculated with *B. cinerea* (5 x 10^4 spores ml^{-1} ¼ PDB) (white bars), and wounded and inoculated (light grey bars). Wounding was performed by puncturing the whole leaf surface with the « syringe stamp ». Leaves from 4 to 6 plants were harvested at different time points for each treatment, RNA was extracted and transformed in cDNAs. *GST1* transcript level was quantified by real-time PCR and normalized with the plant *ACTIN2* transcript. Data are expressed as mean normalized expression (no unit) of triplicate determinations. Standard error bars are represented. The experiment was repeated 2 times with similar results.

VI.1.3 Discussion

In general, wounding of plant surfaces offers an ideal entry point for many microorganisms invading a plant and a number of pathogenic species use such breaches to invade their host. Plants have evolved mechanisms to recognize and respond to injuries by activating various resistance mechanisms against microorganisms or insects (Kuc, 2000; Reymond et al., 2000). Results presented here extend these observations to the case of an infection with the necrotrophic fungus *B. cinerea*. Wounding was found to predispose the defence of the tissue to an infection by a necrotrophic pathogen localised at the wound site. This type of process is generally referred to as priming (Conrath et al*.,* 2002). Our experimental results suggest that sharply delimited wound surfaces are required to activate resistance responses, presumably offering an increased surface area for stress reactions to be induced.

In previous studies, basal resistance to *B. cinerea* was mainly associated with ET- and JA-dependent defence responses (Diaz et al., 2002; Thomma *et al*., 1998; 1999). The local resistance to *B. cinerea* was also reported to depend on SA and camalexin (Ferrari et al., 2003). The chemical inducer β-aminobutyric acid was shown to decrease *B. cinerea* lesion size and prime the accumulation of SA-dependent responses such as the accumulation of *PR1* mRNA while inhibiting ET- and JA-dependent responses (Zimmerli et al*.,* 2000). However, the wound-induced resistance to *B. cinerea* is independent of the JA-, ET- and SA- defence pathways, as shown with the different mutants presented in Fig. 2. This distinguishes our finding from previous studies. Moreover, the results presented here were carried under somewhat different conditions, since the leaf tissue was predisposed by wounding. Our observations complete these previous results and highlight the activation of a strong and localized immunity against *B. cinerea* at wound sites. The sensitivity to camalexin of *B. cinerea* isolates B05.10 and BMM used here, combined with the early accumulation of camalexin observed after wounding and *B. cinerea* infection as well as the decrease of wound-induced resistance observed in *pad3,* provides an explanation for the resistance observed in these plants. Under unwounded conditions, camalexin accumulation induced by the pathogen is likely to be too slow and the pathogen can outgrow the sites of high camalexin content. The accumulation of camalexin, unlike other metabolites such as sinapyl malate, flavonols, indole or aliphatic glucosides, was shown to remain confined to the necrotized infection area (Kliebenstein et al*.,* 2005), making it likely that the absence of systemic effect results from the absence of a systemic accumulation of camalexin.

The role of camalexin in the resistance to *B. cinerea* is the source of many discussions. Camalexin-deficient mutants like *pad3* and *pad2* show an enhanced susceptibility to *B. cinerea*, suggesting that camalexin plays a major role (Denby et al., 2004; Ferrari et al., 2003). The *esa1* mutant shows an increased susceptibility to *B. cinerea* which correlates with a delayed induction

of camalexin (Tierens et al., 2002). The *ups1* mutant however is impaired in camalexin accumulation, although its response to *B. cinerea* is not altered (Denby et al., 2005). The *bos* (*botrytis-susceptible*) mutants also highlight the discrepancy of the role of camalexin, as *bos2* has a reduced camalexin accumulation after infection with *B. cinerea* and *bos3* accumulates as much camalexin as WT plants, despite its enhanced susceptibility (Mengiste et al., 2003; Veronese et al., 2004). As reported recently (Kliebenstein et al*.,* 2005), *pad3,* a mutant in the biosynthesis of camalexin, is more susceptible to *B. cinerea* in our experiments. *B. cinerea* isolates might vary in their sensitivity to camalexin and camalexin production can vary depending on the ecotypes. The *B. cinerea* strains used in our experiments were both found to exhibit sensitivity to camalexin and all the plants used here belong to the *Arabidopsis* Col-0 ecotype that produces camalexin. The loss of resistance in *pad3* together with the wound-induced priming of camalexin, makes it reasonable to consider this metabolite a possible factor involved in resistance. Interestingly, wound-induced priming of camalexin is independent of ET, JA or SA signalling pathways (Fig. 2). The molecular basis of the wound-induced priming of camalexin offers a suitable experimental foundation for further studies.

The complete loss of wound-induced resistance in *pad2* combined with a higher camalexin content compared to *pad3* implies that the resistance of *pad2* must involve additional factors besides camalexin. This factor is likely to be glutathione, since *pad2* has recently been shown to bear a mutation in the gene encoding GSH1 (Parisy et al., submitted). Both *rax1* and *cad2* mutants also have lower glutathione contents (between 10% and 35% of the WT Col-0 plants) and show a reduction of resistance after wounding (Fig. 5). The level of wound-induced resistance in *pad2*, *rax1* and *cad2* is lower than in WT plants but somewhat variable despite a common defect in the *GSH1* gene. The *rax1* and *cad2* plants were found to present many differences in the expression profiles of defence and stress genes even although they bear a mutation in the same gene (Ball et al., 2004). Glutathione metabolism influences many cellular processes; for instance, it might participate in the detoxification of fungal products. Perhaps, plant cells also require glutathione to avoid excessive damage caused by the accumulation of phytoalexin, or to quench the oxidative stress inflicted by the pathogen. In tomato leaves, the content in total glutathione and GSH is decreased after *Botrytis* infection (Kuzniak and Sklodowska, 1999, 2001), but glutathione levels in *Phaseolus* leaves are little affected by *Botrytis* infection (Muckenschnabel et al., 2001). In our study, the WT level of glutathione might be sufficient since we observe no increase during the wound-induced resistance of WT plants (data not shown). Thus, WT glutathione levels represent one component of the wound-induced resistance to *B. cinerea*, besides the accumulation of camalexin and possibly other yet unknown mechanisms.

The glutathione S-transferase *GST1* gene is used as marker gene for pathogen- and wound-induced responses (Jabs et al., 1996; Kishimoto et al., 2005; Rushton et al., 2002;

Vollenweider et al., 2000). As a next logical step, we tested whether *GST1* expression might be affected by wounding. Our results show a wound-induced priming of *GST1* in infected WT plants (Fig. 6). It was also recently shown in the *ups1* mutant that a reduced *GST1* expression accompanies a lower camalexin accumulation (Denby et al., 2005). In addition, the mutant *ocp3* (overexpressor of cationic peroxidase 3) shows increased resistance to *B. cinerea* and constitutively expresses the *GST1* marker gene (Coego et al., 2005). Thus, resistance to *B. cinerea* has been linked to enhanced expression of *GST1* and accumulation of camalexin.

Summarizing, wounding of *Arabidopsis* Col-0 induces full immunity to *B. cinerea* that can be explained by the priming due to camalexin accumulation. In addition, glutathione is also likely to be involved in this process. These results add an interesting dimension to our understanding of plant defense against *B. cinerea.*

VI.1.4 Material and Methods

Plant maintenance

Plants were grown on a pasteurized soil mix of humus / perlite (3:1) under a 12 h light and 12 h dark cycle, with a night temperature of 16 to 18°C and a day temperature of 20 to 22°C (60 to 70% humidity). WT plants are the *Arabidopsis* accession Col-0 obtained from the Arabidopsis Biological Reasearch Center (Columbus, OH). The *Arabidopsis* mutant *eds5* was *eds5-3* (Nawrath and Métraux, 1999), *sid2* was *sid2-1* (Nawrath and Métraux, 1999) and *pad4* was *pad4-1* (Glazebrook and Ausubel, 1994;). The *etr1* mutant was *etr1-1* (Schaller and Bleecker, 1995) (Nottingham Arabidopsis Stock Center), *ein2* was *ein2-1* (Alonso *et al.*, 1999; Guzman and Ecker, 1990), *jar1* was *jar1-1* (Staswick *et al.*, 1992; Staswick *et al.*, 2002), *pad3* was *pad3-1* and *pad2* was *pad2-1* (Glazebrook and Ausubel, 1994; Glazebrook et al., 1997).

Culture of *B. cinerea* and infection method

B. cinerea strains B05.10 and BMM, provided by Jan van Kan et Brigitte Mauch-Mani, respectively, were grown on 1x PDA (Potato Dextrose Agar, 39 g l^{-1}, Difco). Spores were harvested in water and filtered through glass wool to remove hyphae. Spore concentration was adjusted to 5 x 10^4 spores ml $^{-1}$ in ¼ PDB (Potato Dextrose Broth, 6 g l^{-1}, Difco) for inoculation. Droplets of 5 µl of spore suspension were deposited on leaves for quantification of outgrowing lesions. The spore suspension was sprayed on whole plants for camalexin measurements or real-time RT-PCR experiments. The inoculated plants were kept under a water-sprayed transparent lid to maintain high humidity. Control plants were inoculated with ¼ PDB.

Wounding method

Wounding was performed using a sharp laboratory forceps or by puncturing several holes (ca. 10) with a syringe needle (27GA). For wounding of whole leaves, a self-made “syringe-stamp” made of syringe needles 1 mm apart was used. Wounded leaves were inoculated directly after treatment (up to 15 minutes).

Camalexin determination

Leaf material (ca. 200 mg) was collected and assayed for camalexin as previously described for SA (Meuwly and Métraux, 1993). Samples were frozen and ground with a glass rod. 2 ml of 70% ethanol and 200 ng of internal standard (ortho-anisic acid, 1 ng μl^{-1} in ethanol) were added. After homogenization (Polytron; Kinematica, Littau, Switzerland) and centrifugation, the supernatant was decanted into a fresh tube and the extraction was repeated with 2 ml of 90% methanol. Supernatants were pooled and evaporated under reduced pressure (Speed Vac, Buchler).

200 µl of 5% trichloroacetic acid was added to the remaining aqueous solution. After a brief centrifugation, the supernatant was transferred to a fresh tube and extracted with 2x 500 µl of ethyl acetate / cyclohexane (1:1). The pooled organic phases that contain the free phenols and camalexin were evaporated (Speed Vac) and resuspended in 200 µl (or more depending on the amount of plant tissue) of HPLC starting buffer (15% acetonitrile in 25 mM KH_2PO_4, pH 2.6). Chromatography was performed on a reverse phase HPLC column (ABZ+, 25 cm x 4.6 mm; Supelco, Buchs, Switzerland). The amount of camalexin was calculated in ng g^{-1} fresh weight, with reference to the amount of internal standard.

Glutathione extraction

Samples were extracted according to Harms et al. (2000) with some minor modifications (Parisy et al., 2006). Leaf material (ca. 200 mg) was collected, frozen and ground with a glass rod. Two ml of 0.1 N HCl and 50 µg of homoglutathione (internal standard) were added before homogenization (Polytron; Kinematica, Littau, Switzerland). Samples were centrifuged at 4°C (10 min, 13'000 g) and 120 µl of the supernatant was mixed to 200 µl of CHES buffer (0.2 M 2-N-cyclohexylamino-ethane sulfonic acid, pH 9.3). Ten µl of BMS (9 mM bis-2-mercaptoethylsulfone in 200 mM Tris-HCl and 5 mM EDTA, pH 8.0) were added to the samples to allow reduction of total disulphides for 40 min at room temperature. Free thiols were labeled for 15 min at room temperature in the dark with 15 µl of 15 mM monobromobimane in acetonitrile. The reaction was stopped with 250 µl of 15% HCl. The samples were kept on ice, centrifuged for 10 min and analyzed by HPLC column. The amount of total thiols was calculated in ng g^{-1} fresh weight, with reference to the amount of internal standard.

RNA extraction and real time RT-PCR

RNA was prepared using the TRIzol® reagent (Molecular Research Center, Inc., Invitrogen). 1 µg of RNA was retrotranscribed in cDNA (Omniscript® RT kit, Qiagen). Real-time PCR was performed using the Absolute QPCR SYBR Green Mix (ABgene). Gene expression values were normalised with the expression of the plant *ACTIN2* gene. Primers used: 5'-ACT2 (5'-AGCACCCTGTTCTTCTTACCGAG-3'); 3'-ACT2 (5'-GGCGACATACATAGCGGGAGAG-3'); 5'-GST1 (or *ATGSTF6*): (5'-ATCAAAGTTTTCGGTCACCCA-3'); 3'-GST1 (5'-TTTACCAAAGGGGTTGCGAAG-3').

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VI.1.5 References

Alonso, J.M., Hirayama, T., Roman, G., Nourizadeh, S. and Ecker, J.R. (1999) EIN2, a bifunctional transducer of ethylene and stress responses in *Arabidopsis*. *Science* **284**, 2148-2152.

Aziz, A., Heyraux, A., and Lambert, B. (2004). Oligogalacturonide signal transduction, induction of defense-related responses and protection of grapevine against *Botrytis cinerea*. *Planta* 218, 767-774.

Ball, L., Accotto, G.-P., Bechtold, Ul, Creissen, G., Funck, D., Jimenez, A., Kular, B., Leyland, N., Mejia-Carranza, J., Reynolds, H., Karpinski, S., and Mullineaux, P.M. (2004). Evidence for a direct link beween glutathione biosynthesis and stress defence gene expression in *Arabidopsis*. *Plant Cell* 16, 2448-2462.

Bennett, M.H., Gallagher, M.D.S., Bestwick, C.S., Rossiter, J.T., and Mansfield, J.W. (1994). The phytoalexin response of lettuce to challenge by *Botrytis cinerea, Bremia lactucae* and *Pseudomonas syringae* pv *phaseolicol*a. *Physiol. Mol. Plant Pathol*. 44, 321-333.

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.

Chang, M.M., Horovitz, D., Culley, D., and Hadwiger, L.A. (1995). Molecular-cloning and characterization of a pea chitinase gene expressed in response to wounding, fungal infection and the elicitor chitosan. *Plant Mol.Biol*. 28, 105-111.

Cobbett, C.S., May, M.J., Howden, R., and Rolls, B. (1998). The glutathione-deficient, cadmium-sensitive mutant, *cad2-1* of *Arabidopsis thaliana* is deficient in γ-glutamylcysteine synthetase. *Plant J*. 16, 73-78.

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.

Commenil, P., Belingheri, L., and Dehorter, B. (1998). Antilipase antibodies prevent infection of tomato leaves by *Botrytis cinerea*. *Physiol. Mol. Plant Pathol*. 52, 1-14.

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

Denby, K.J., Jason, L.J.M., Murray, S.L., and Last, R.L. (2005) *ups1*, an *Arabidopsis thaliana* camalexin accumulation mutant defective in multiple defence signalling pathways. *Plant J.* 41, 673-684.

Diaz, J., ten Have, A., and van Kan, J.A.L. (2002). The role of ethylene and wound signaling in resistance of tomato to *Botrytis cinerea*. *Plant Physiol*. 129, 1341-1351.

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.

Gil-ad, N.L., Bar-Nun, N., Noy, T., and Mayer, A.M. (2000). Enzymes of *Botrytis cinerea* capable of breaking down hydrogen peroxide. *FEMS Microbiol. Lett*. 190, 121-126.

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

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.

Graham, J.S., Hall, G., Pearce, G., and Ryan, C.A. (1986). Regulation of synthesis of proteinase inhibitor-I and inhibitor-II messenger-RNAs in leaves of wounded tomato plants. Planta 169, 399-405.

Grant, J.J., Yun, B.-W., and Loake, G.J. (2000). Oxidative burst and cognate redox signalling reported by luciferase imaging: identification of a signal network that functions independently of ethylene, SA and Me-JA but is dependent on MAPKK activity. *Plant J.* 24 (5), 569-582.

Greenberg, J.T., Guo, A.L., Klessig, D.F., and Ausubel, F.M. (1994). Programmed cell death in plants – a pathogen-triggered response activated co-ordinately with multiple defence functions. *Cell* 77, 551-563.

Gronover, C.S., Kasulke, D., Tudzynski, P., and Tudzynski, B. (2001). The role of G protein alpha subunits in the infection process of the gray mold fungus *Botrytis cinerea*. *Mol. Plant-Microbe Interact*. 14, 1293-1302.

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

Harms, K., von Ballmoos, P., Brunold, C., Hofgen, R., and Hesse, H. (2000). Expression of a bacterial serine acetyltransferase in transgenic potato plants leads to increased levels of cysteine and glutathione. *Plant J.* 22 (4), 335-343.

Howe, G.A. (2004). Jasmonates as signals in the wound response. *J. Plant Growth Regul*. 23, 223-237.

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

Kishimoto, K., Matsui, K., Ozawa, R., and Takabayashi, J. (2005) Volatile C6-aldehydes and allo-ocimene activates defence genes and induce resistance against *Botrytis cinerea* in *Arabidopsis thaliana*. *Plant Cell Physiol*. 46 (7), 1093-1102.

Kliebenstein, D.J., Rowe, H.C., and Denby, K.J. (2005). Secondary metabolites influence *Arabidopsis/Botrytis* interactions: variation in host production and pathogen sensitivity. *Plant J*. **44,** 25-36.

Klimpel, A., Gronover, C.S., Williamson, B., Stewart, J.A., and Tudzynski, B. (2002). The adenylate cyclase (BAC) in *Botrytis cinerea* is required for full pathogenicity. Mol. *Plant Pathol*. 3, 439-450.

Kuc, J. (2000). Development and future direction of induced systemic resistance in plants. *Crop Prot*. 19, 859-861.

Kuzniak, E., and Sklodowska, M. (1999) The effect of *Botrytis cinerea* infection on ascorbate-glutathione cycle in tomato leaves. *Plant Sci.* 148, 69-76.

Kuzniak, E., and Sklodowska, M. (2001) Ascorbate, glutathione and related enzymes in chloroplasts of tomato leaves infected by *Botrytis cinerea*. *Plant Sci*. 160, 723-731.

Lincoln, J.E., Richael, C., Overduin, B., Smith, K., Bostock, R., and Gilchrist, D.G. (2002). Expression of the antiapoptotic baculovirus p35 gene in tomato blocks programmed cell death and provides broad-spectrum resistance to disease. *Proc. Natl. Acad. Sci. USA* 99, 15217-15221.

Liu, S., Oeljeklaus, S., Gerhardt, B., and Tudzynski, B. (1998). Purification and characterization of glucose oxidase of *Botrytis cinerea*. *Physiol. Mol. Plant Pathol*. 53, 123-132.

Mansfield, J.W., and Hutson, R.A. (1980). Microscopical studies on fungal development and host responses in broad bean and tulip leaves inoculated with 5 species of *Botrytis*. *Physiological Plant Pathology* 17, 131-138.

Marrs, K.A. (1996). The functions and regulation of glutathione-S-transferases in plants. *Ann. Rev. Plant Physiol. Plant Mol. Biol*. **47**, 127-158.

Mauch, F., and Dudler, R. (1993). Differential induction of distinct glutathione-S-transferases of wheat by xenobiotics and by pathogen attack. *Plant Physiol.* 102, 1193-1201.

McLusky, S.R., Bennett, M.H., Beale, M.H., Lewis, M.J., Gaskin, P., and Mansfield, J.W. (1999). Cell wall alterations and localized accumulation of feruloyl-3 '-methoxytyramine in onion epidermis at sites of attempted penetration by *Botrytis allii* are associated with actin polarisation, peroxidase activity and suppression of flavonoid biosynthesis. *Plant J.* 17, 523-534.

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.

Meuwly, P., and Métraux, J.-P. (1993) Ortho-anisic acid as internal standard for the simultaneous quantification of salicylic acid and its putative biosynthetic precursors in cucumber leaves. *Anal. Biochem*. 214, 500-505.

Muckenschnabel, I., Williamson, B., Goodman, B.A., Lyon, G.D., Stewart, D., and Deighton, N. (2001) Markers for oxidative stress associated with soft rots in French beans (*Phaseolus vulgaris*) infected by *Botrytis cinerea*. *Planta* 212, 376-381.

Parashina, E.V., Serdobinskii, L.A., Kalle, E.G., Lavrova, N.V., Avetisov, V.A., Lunin, V.G., and Naroditskii, B.S. (2000). Genetic engineering of oilseed rape and tomato plants expressing a radish defensin gene. Russ. *J. Plant Physiol*. 47, 417-423.

Parisy, V. *et al.,* 2006

Penninckx, I., Thomma, B.P.H.J., 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.M.A., Eggermont, K., Terras, F.R.G., Thomma, B.P.H.J., DeSamblanx, 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.

Pezet, R., Pont, V., and Hoangvan, K. (1991). Evidence for oxidative detoxication of pterostilbene and resveratrol by a laccase-like stilbene oxidase produced by *Botrytis cinerea*. *Physiol. Mol. Plant Pathol*. 39, 441-450.

Pezet, R., Viret, O., and Gindro, K. (2004). Plant-microbe interaction: the *Botrytis* grey mould of grapes. Biology, biochemistry, epidemiology and control management. In *Advances in plant physiology, Volume VI*, Hemantaranjan A., ed (Jodhpur, India: Scientific Publishers), pp. 179-193.

Prins, T.W., Tudzynski, P., Von Tiedermann, A., Tudzynski, B., Ten HAve, A., Hansen, M.E., Tenberge, K., and van Kan, J.A.L. (2000). Infection of *Botrytis cinerea* and related necrotrophic pathogens. In *Fungal pathology*, J.E. Kronstad, ed (Dordrecht: Kluwer), pp. 32-64.

Rebordinos, L., Cantoral, J.M., Prieto, M.V., Hanson, J.R., and Collado, I.G. (1996). The phytotoxic activity of some metabolites of *Botrytis cinerea*. *Phytochem.* 42, 383-387.

Reino, J.L., Hernandez-Galan, R., Duran-Patron, R., and Collado, I.G. (2004). Virulence-toxin production relationship in isolates of the plant pathogenic fungus *Botrytis cinerea*. *J. Phytopathol.* 152, 563-566.

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, 707-719.

Rushton, P. J., Reinstädler, A., Lipka, V., Lippok, B., and Somssich, I.E. (2002). Synthetic plant promoters containing defined regulatory elements provide novel insights into pathogen- and wound-induced signalling. *Plant Cell* 14, 749-762.

Ryan, C.A. (1990). Protease inhibitors in plants: genes for improving defenses against insects and pathogens. *Annu. Rev. Phytopathol*. 28, 425-449.

Schaller, G.E. and Bleecker, A.B. (1995) Ethylene-binding sites generated in yeast expressing the *Arabidopsis* ETR1 gene. *Science* **270**, 1809-1811.

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

Staswick, P.E., Su, W.P. 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., 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.

Stewart, A., and Mansfield, J.W. (1985). The Composition of wall alterations and appositions (reaction material) and their role in the resistance of onion bulb scale epidermis to colonization by *Botrytis allii*. Plant Pathol. 34, 25-37.

ten Have, A., Mulder, W., Visser, J., and van Kan, J.A.L. (1998). The endopolygalacturonase gene *BCPG1* is required for full virulence of *Botrytis cinerea*. *Mol. Plant-Microbe Interact*. 11, 1009-1016.

Terras, F.R.G., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn, R.W., Kester, A., Rees, S.B., Torrekens, S., Vanleuven, F., Vanderleyden, J., Cammue, B.P.A., and Broekaert, W.F. (1995). Small Cysteine-Rich Antifungal Proteins From Radish - Their Role in Host-Defense. *Plant Cell* 7, 573-588.

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

Thomma, B., Eggermont, K., Tierens, K., and Broekaert, W.F. (1999b). Requirement of functional ETHYLENE-INSENSITIVE 2 gene for efficient resistance of *Arabidopsis* to infection by *Botrytis cinerea*. Plant Physiol. 121, 1093-1101.

Thomma, B.P.H.J., Eggermont, K., Penninckx, I.A.M.A., 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.

Tierens, K.F.M.J., Thomma, B.P.H.J., Bari, R.P., Garmier, M., Eggermont, K., Brouwer, M., Penninckx, I.A.M.A., Broekaert, W.F., and Cammue, B.P.A. (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.

Tournas, V.H. (2005). Spoilage of vegetable crops by bacteria and fungi and related health hazards. *Crit. Rev. Microbiol.* 31, 33-44.

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 Baarlen, P., Staats, M., and Van Kan, J.A.L. (2004). Induction of programmed cell death in lily by the fungal pathogen *Botrytis* elliptica. *Mol. Plant Pathol*. 5, 559-574.

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.

Viaud, M., Legeai, F., Pradier, J.M., Brygoo, Y., Bitton, F., Weissenbach, J., Brunet-Simon, A., Duclert, A., Fillinger, S., Fortini, D., Gioti, A., Giraud, C., Halary, S., Lebrun, I., Le Pecheur, P., Samson, D., and Levis, C. (2005). Expressed sequence tags from the phytopathogenic fungus *Botrytis cinerea*. *Eur. J. Plant Pathol*. 111, 139-146.

Vollenweider, S., Weber, H., Stolz, S., Chételat, A., and Farmer, E.E. (2000). Fatty acid ketodienes and fatty acid ketotrienes: Michael addition acceptors that accumulate in wounded and diseased *Arabidopsis* leaves. *Plant J.* 24 (4), 467-476.

VII. General Discussion

The cuticle forms a continuous lipid membrane over the apical epidermal cell walls of essentially all aerial plant organs (Jenks et al., 2002). The cutin polymer, the structural component of the cuticle, is composed of esterified fatty acid derivatives and is embedded in a complex mixture of lipids called waxes. The cuticle plays a major role in the interaction of the plant with its environment. The main functions of the cuticle are to reduce the uncontrolled loss of water and apoplastic solutes, to protect tissue from mechanical damage, to reflect and attenuate radiation, and to form a mechanical barrier against penetration by fungal hyphae and insect mouthparts (Kerstiens, 1996).

The cuticle is often the first contact point with environmental microbes and plays a critical role in the interaction of the plant with microorganisms (Kolattukudy, 2001). Many pathogenic fungi find their way via stomata or other openings, while some penetrate the cuticle directly. In some cases, direct penetration is facilitated by fungal cutinase loosening the cuticular matrix (Kolattukudy, 1985; Köller, 1995). The cuticle was shown to be a source of signals for invading fungal pathogens. Cutin monomers, the breakdown products of fungal cutinases, have been found to stimulate germination and appressorium formation (Gilbert et al., 1996) and to induce expression of the cutinase gene in many fungi (Kolattukudy et al., 1995). It has long been supposed that the plant cuticle functions only as an inert physical barrier against fungal infection. However, there is evidence that cutin monomers may also act as early alarm signals of fungal attack and trigger defence reactions in the host (Namai et al., 1993; Schweizer et al., 1994; Schweizer et al., 1996; Schweizer et al., 1996; Fauth et al., 1998; Kauss et al., 1999).

In this study, the reactions of plants to changes in the structure of their cuticle were investigated, and in particular the potential role of cuticular defects to trigger innate immunity. As model of study, transgenic *Arabidopsis* plants were generated that overexpress a cutinase from *Fusarium solani* f.sp. *pisi* and therefore degrade their own cutin *in situ* (Sieber et al., 2000). These plants that express cutinase constitutively (CUTE plants) show an altered ultrastructure of the cuticle and an enhanced permeability of the cuticle to solutes, in addition to ectopic pollen germination on leaves and strong organ fusions (Sieber et al., 2000). We show that degradation of the cuticular layer in CUTE plants leads to full immunity to *Botrytis cinerea*, an ubiquitous fungal pathogen causing important damages to many crop plants. As one of the main functions of the cuticle is to confer protection to the plant, this counter-intuitive result raised the exciting hypothesis that *Arabidopsis* plants might sense the degradation of their surface layer and induce effective defence responses.

The powerful resistance of CUTE plants to *B. cinerea* was found to be independent of the known defence signalling routes involving salicylic acid (SA), ethylene (ET) or jasmonic acid (JA) and accompanied by changes in gene transcription. After inoculation with *B. cinerea*, the expression of genes coding for lipid transfer proteins (LTP), peroxidases (PER), and protease inhibitors (PI) was strongly enhanced in CUTE plants, in comparison to wild-type (WT) plants.

The involvement of these novel genes in the defence against *B. cinerea* was demonstrated by overexpressing them in WT plants. Such transgenic plants displayed an increased resistance to the fungus, supporting the contribution of these genes in the resistance to *B. cinerea*. The *LTP*, *PER* and *PI* genes are naturally induced by *B. cinerea* in WT plants, albeit to a lower level, indicating that they might be part of an attempted but insufficient defence response against the virulent pathogen.

Several enzymes with cutinolytic activity have been characterised and purified from culture filtrates of *B. cinerea*, e.g. cutinases, esterases and lipases (Salinas et al., 1986; Salinas, 1992; Comménil et al., 1995; Comménil et al., 1999; Gindro and Pezet, 1999). The cutinase gene (*cutA*) of *B. cinerea* was found to be expressed, but not essential for infection (Van der Vlugt-Bergmans et al., 1997; Van Kan et al., 1997), like the lipase gene (*lip1*) (Reis et al., 2005). Overexpression of the *cutA* or *lip1* genes of *B. cinerea* in *Arabidopsis* plants induced, in addition to the typical developmental phenotype of CUTE plants, full resistance to *B. cinerea* similar to that of CUTE plants overexpressing the cutinase of *F. solani*. In WT plants, the rapidly growing fungus can apparently overcome the effect of the cutinolytic enzymes produced during the infection. The quantity and the timing of the production of these enzymes might be insufficient to trigger resistance.

The powerful defence of CUTE plants was found to be accompanied by the diffusion of a strong fungitoxic activity against *B. cinerea*, demonstrated in both *in-vitro* and *in-vivo* assays. The early arrest of fungal growth and the absence of fungal penetration on the surface of inoculated CUTE plants might be related to the secreted fungitoxic compound(s). The fungitoxic activity was shown to diffuse from CUTE leaves or from WT leaves digested with purified cutinase. Fungitoxic activity was also discovered in fractionated diffusates of WT plants, albeit in lower amount, suggesting the existence of a natural fungitoxic substance(s) in the extracellular matrix of *Arabidopsis* plants. The enhanced permeability of the cuticle of CUTE plants might play a role in the diffusion rate of the fungitoxic compound.

Mutants with defects in the cuticle structure have been recently characterised. Poor growth and performance, sensitivity to low humidity, increased sensitivity to chemicals such as pesticides and herbicides, ectopic pollen germination and organ fusions can often be associated with improper cuticle formation in *Arabidopsis* (Yephremov and Schreiber, 2005). The full resistance to *B. cinerea* was not specific to the transgenic CUTE plants, but could also be observed in other plants with cuticular defects, like the mutants *bodyguard* (Kurdyukov et al., 2006) and *lacerata* (Wellesen et al., 2001), the novel mutants *pec1* and *pec9* (*permeable cuticle*), and the overexpressing plants 35S-*SHINE* (Aharoni et al., 2004). The resistance to *B. cinerea* was paralleled with the diffusion of the fungitoxic activity, strengthening the important role of this activity in the defence against the fungus. The increased resistance of *bdg* and *lcr* was correlated with priming of the *LTP*, *PER* and *PI* genes in a way similar to CUTE plants,

whereas the expression of these genes was variable in other mutants tested. This may be taken to suggest that there are other elements involved in the induction of defence responses linked to cuticular defects. In addition, the two mutants *wax2* (Chen et al., 2003) and *adhesion of calyx edges*/*hothead* (Kurdyukov et al., 2006), although they displayed typical phenotypes for cuticular defects, were as susceptible as WT plants to *B. cinerea*.

Arabidopsis plants with cuticular defects induce changes leading to complete resistance to *B. cinerea*. Modification of the cuticle can activate a multi-layered defence syndrome, comprising a diffusible fungitoxic activity and the potentiated induction of genes upon inoculation with the fungus. Thus, the cuticle serves also a source of signals for plants. The perception of cuticular defects might sensitize the plant for further attack and thus induce the priming of defence responses upon pathogen challenge. On the other hand, even though a defective cuticle fails in protecting the plant against pathogen attack, plants with cuticular defects exploit this adverse situation to their benefit. The increased cuticular permeability of CUTE plants and the different mutants might help the diffusion of fungal elicitors towards the epidermal cells and allow the earlier perception of the invader. In addition, permeable cuticle might facilitate the leaking of apoplastic compounds with fungitoxic activity.

Besides degradation of the cuticle by the expression of a fungal cutinase in transgenic *Arabidopsis* plants, disruption of the leaf integrity was also performed by wounding. Wounding of the leaf surface with forceps or with a syringe needle induced full resistance to *B. cinerea* at the wound site. Again, this observation was surprising, since wounding usually provokes entry points for invading microorganisms. Moreover, *B. cinerea* is a necrotrophic fungus that is able to live on dead plant tissues. It was shown that the fungus uses hypersensitive cell death to its advantage for a better colonization of the host (Govrin and Levine, 2000; van Baarlen et al., 2004). However, puncturing the leaf surface with some holes only was sufficient to trigger full immunity against the necrotrophic fungus. The wound-induced resistance of *Arabidopsis* WT plants to *B. cinerea* was demonstrated to be independent of the SA-, ET- and JA-signalling pathways.

Wounding induced a priming of camalexin accumulation; it confirmed the implication of this fungitoxic phytoalexin in the wound-induced resistance to *B. cinerea*, since *Arabidopsis* mutants deficient in camalexin production were less protected by wounding than WT plants. The antioxidant compound glutathione was also found to be required for the resistance, as shown by the susceptibility of mutants deficient in the γ-glutamylcysteine synthetase after wounding and inoculation with *B. cinerea*. WT basal levels of glutathione were shown to be required for full protection induced by wounding. In addition, the wound-induced resistance was accompanied by the primed expression of a gene coding for a glutathione-S-transferase 1. Even although other yet undiscovered elements play a role in this wound-induced protection, these results demonstrate how an abiotic stress can trigger full immunity to the virulent fungus *B. cinerea*.

Wounds are usually caused by insects chewing plants. Activation of wound responses by insect damage is directed to healing of injured tissues, as well as activation of defences to prevent further insect damage. Insect feeding regulates expression of defence genes that contribute to induced resistance against herbivores (Bergey et al., 1996). Resistance against herbivorous insects and some fungal pathogens was found to depend on wound-response signaling via JA and ethylene (Maleck and Dietrich, 1999). Mechanical wounding of first leaves of broad bean was also shown to significantly reduce rust infection caused by *Uromyces fabae* in the wounded first leaf as well as the unwounded second leaf of *Vicia faba* and to follow changes in oxylipins (Walters et al., 2006). Resistance to *B. cinerea* induced by wounding was investigated in one study, showing that green leafy volatiles or isoprenoids produced after mechanical wounding or pathogen/herbivore attack retarded the disease development of the fungus in *Arabidopsis* plants (Kishimoto et al., 2005). The present work reports for the first time a full resistance to *B. cinerea* mounted very rapidly at a wound site. Therefore, the wound-induced responses taking place in plants are also effective against this fungus.

In summary, this study reports two unexpected observations showing that the disruption of the plant surface leads to full immunity to the necrotrophic fungus *B. cinerea*. *Botrytis* diseases are probably the most common and most widely distributed diseases of vegetables, ornamentals, fruits, and even some field crops throughout the world, and in greenhouse-grown crops (Agrios, 2005). Some of the most serious diseases caused by *Botrytis* include grey mold of strawberry, grapes and many vegetables and ornamentals (Agrios, 2005). It can also provoke important damages to foodstuffs during transport and cold storage. Infections are particularly devastating in humid conditions. The resistance syndromes described in this report are rare examples presenting a complete absence of symptoms in *Arabidopsis* plants, usually highly susceptible to this fungus. Whereas defence against *B. cinerea* was previously mostly associated with the JA- and ET-pathway (Penninckx et al., 1996; Penninckx et al., 1998), the immunity conferred by cutinase expression or by wounding is independent of this defence pathway. Recent work on mutants with altered response to *B. cinerea* attribute growing importance of camalexin in plant defence, as observed in the wound-induced resistance (Ferrari et al., 2003; Denby et al., 2004). Thus, the results presented here highlight novel mechanisms that might possibly be exploited to protect plants against this ubiquitous fungal pathogen.

Arabidopsis plants can perceive defects in their cuticular layer and induce a strong resistance to *B. cinerea*, correlated with a priming of the expression of different defence-related genes. These genes were also induced in inoculated susceptible WT plants, but to a lower level. *B. cinerea* is a very virulent pathogen causing extended soft rot symptoms in three days and achieving its life cycle in less than a week under our laboratory conditions. In natural conditions, defence responses induced by cuticular defects might be effective when infections are slower. Under variable environmental conditions, degradation of the cuticle by fungal

cutinolytic enzymes might take some time and the plant defences activated by the perception of the cuticular defects may thus be play a significant role. In addition, the resistance induced by cuticular defects might be involved in the resistance against other pathogens penetrating through the cuticle that were not yet tested on *Arabidopsis* plants with defective cuticles.

The overexpression of *LTP*, *PER* and *PI* genes, the expression of which was primed in CUTE plants upon inoculation with *B. cinerea*, led to increased resistance to the fungus in WT plants. These genes may be potentially interesting candidates for transformation of economically important plants with the aim of improving the resistance to *Botrytis* diseases. For example, transgenic strawberry plants were generated that express the *ch5B* gene coding for a chitinase from *Phaseolus vulgaris*; the transformed plants displayed high levels of resistance to *B. cinerea*, correlating with an increase of chitinolytic activity in leaves (Vellicce et al., 2006). The stilbene synthase gene isolated from grapevine was expressed in tobacco plants and led to increased resistance to infection by *B. cinerea* (Hain et al., 1993). Likewise, transgenic grapevine plants expressing high level of resveratrol under the control of a pathogen-inducible promoter showed an increased tolerance to *B. cinerea* (Coutos-Thevenot et al., 2001). From a biological point of view, the benefits of genetically modified plants for commercial production would be to increase the yield and the resistance against diseases without the need of fungicides. However, the development of such genetically modified plants is not automatically accepted by consumers and farmers in European countries.

Resistance to *B. cinerea* could be induced by external application of purified cutinase from *F. solani* on *Arabidopsis* WT plants, thus mimicking transgenic cutinase-expressing plants. Cutinase can be produced in yeast and purified. As an alternative to the generation of transgenic plants with increased resistance, one could imagine treating plant leaves to induce defence responses against *B. cinerea* diseases, or to apply cutinase on fruits to protect them from infection during storage. Moreover, the powerful fungitoxic activity isolated from leaf diffusates of *Arabidopsis* plants could be used as a natural fungicide against *B. cinerea*. Interestingly, the fungitoxic activity was found to completely inhibit the development of symptoms on *Arabidopsis* and tomato leaves, suggesting a possible use as external treatment on plants. In the future, the active compound(s) should be chemically characterised, and the activity of the purified compound(s) should be confirmed. A method for purification of important amounts of the fungistatic compound(s) or for *in-vitro* synthesis should be developed, in order to allow large scale tests on different crops. The active diffusates were also shown to display fungistatic activity against the fungus *Monilinia*, which provokes brown rot of stone fruits like peaches, cherries, plums, apricots and almonds. Losses result primarily from fruits rotting in the orchard, but serious losses may also appear during transit and marketing of the fruit (Agrios, 2005), strengthening the interest of this natural fungitoxic compound.

References

Agrios, G.N. (2005). Plant Pathology (5th edition). Elsevier Academic Press.

Aharoni, A., Dixit, S., Jetter, R., Thoenes, E., Van Arkel, G., and Pereira, A. (2004). The SHINE clade of AP2 domain transcription factors activates wax biosynthesis, alters cuticle properties, and confers drought tolerance when overexpressed in *Arabidopsis*. Plant Cell **16,** 2463-2480.

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.

Chen, X., Goodwin, M., Boroff, V.L., Liu, X., and Jenks, M.A. (2003). Cloning and characterization of the *WAX2* gene of *Arabidopsis* involved in cuticle membrane and wax production. Plant Cell **15,** 1170-1185.

Comménil, P., Belingheri, L., Sancholle, M., and Dehorter, B. (1995). Purification and properties of an extracellular lipase from the fungus *Botrytis cinerea*. Lipids **30,** 351-356.

Comménil, P., Belingheri, L., Bauw, G., and Dehorter, B. (1999). Molecular characterization of a lipase induced in *Botrytis cinerea* by components of grape berry cuticle. Physiol. Mol. Plant Pathol. **55,** 37-43.

Coutos-Thevenot, P., Poinssot, B., Bonomelli, A., Yean, H., Breda, C., Buffard, D., Esnault, R., Hain, R., and Boulay, M. (2001). *In vitro* tolerance to *Botrytis cinerea* of grapevine 41B rootstock in transgenic plants expressing the stilbene synthase Vst1 gene under the control of a pathogen-inducible PR 10 promoter. J. Exp. Bot. **52,** 901-910.

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

Fauth, M., Shweizer, P., Buchala, A., Markstadter, C., Riederer, M., Kato, T., and Kauss, H. (1998). Cutin monomers and surface wax constituents elicit H_2O_2 in conditioned cucumber hypocotyl segments and enhance the activity of other H_2O_2 elicitors. Plant Physiol. **117,** 1373-1380.

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.

Gilbert, R.D., Johnson, A.M., and Dean, R.A. (1996). Chemical signals responsible for appressorium formation in rice blast fungus *Magnaporte grisea*. Physiol. Mol. Plant Pathol. **48,** 335-346.

Gindro, K., and Pezet, R. (1999). Purification and characterization of a 40.8 kDa cutinase in ungerminated conidia of *Botrytis cinerea*. FEMS Microbiol. Letters **171,** 239-243.

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

Hain, R., Reif, H.J., Krause, E., Langebartels, R., Kindl, H., Vornam, B., Wiese, W., Schmelzer, E., Schreier, P.H., Stocker, R.H., and al., e. (1993). Disease resistance results from foreign phytoalexin expression in a novel plant. Nature **361,** 153-156.

Jenks, M.A., Eigenbrode, S.D., and Lemieux, B. (2002). Cuticular waxes of Arabiodpsis. In: The Arabidopsis Book, Somerville CR, Meyerowitz EM, eds (Rockville, MD: American Society of Plant Biologists).

Kauss, H., Fauth, M., Merten, A., and Jeblick, W. (1999). Cucumber hypocotyls respond to cutin monomers via both an inducible and a constitutive H_2O_2-generating system. Plant Physiol. **120,** 1175-1182.

Kerstiens, G. (1996). Signalling across the divide: a wider perspective of cuticular structure-function relationships. Trends in Plant Science **1,** 123-129.

Kishimoto, K., Matsui, K., Ozawa, R., and Takabayashi, J. (2005). Volatile C6-aldehydes and allo-ocimene activate defense genes and induce resistance against *Botrytis cinerea* in *Arabidopsis thaliana*. Plant Cell Physiol. **46,** 1093-1102.

Kolattukudy, P.E. (1985). Enzymatic penetration of the plant cuticle by fungal pathogens. Annu. Rev. Phytopathol. **23,** 223-250.

Kolattukudy, P.E. (2001). Polyesters in higher plants. In: Advances in Biochemical Engineering Biotechnology: Biopolyesters, W. Babel and A. Steinbüchel, eds (Berlin: Springer-Verlag)**,** 1-49.

Kolattukudy, P.E., Rogers, L.M., Li, D., Hwang, C.S., and Flaishman, M.A. (1995). Surface signaling in pathogenesis. Proc. Natl. Acad. Sci. **92,** 4080-4087.

Köller, W., Yao, C., Trail, F., and Parker, D.M. (1995). Role of cutinase in the invasion of plants. Can. J. Bot. **73,** 1109-1118.

Kurdyukov, S., Faust, A., Tenkamp, S., Bär, S., Franke, B., Efremova, N., Tietjen, K., Schreiber, L., Saedler, H., and Yephremov, A. (2006). Genetic and biochemical evidence for involvement of *HOTHEAD* in the biosynthesis of long chain a-,w-dicarboxylic fatty acids and formation of extracellular matrix. Planta **11,** 1-15.

Kurdyukov, S., Faust, A., Nawrath, C., Bär, S., Voisin, D., Efremova, N., Franke, R., Schreiber, L., Saedler, H., Métraux, J.P., and Yephremov, A. (2006). The epidermis-specific extracellular BODYGUARD controls cuticle development and morphogenesis in *Arabidopsis*. Plant Cell **18,** 321-339.

Maleck, K., and Dietrich, R.A. (1999). Defense on multiple fronts: how do plants cope with diverse enemies? Trends in Plant Science **4,** 215-219.

Namai, T., Kato, T., Yamaguchi, Y., and Hirukawa, T. (1993). Antirice blast activity and resistance induction of C-18 oxygenated fatty acids. Biosci. Biotechnol. Biochem. **57,** 611-613.

Penninckx, I.A., Thomma, B.P., De Samblanx, G.W., Buchala, A., Métraux, 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.M.A., Eggermont, K., Terras, F.R.G., Thomma, G.P.H.J., De Samblanx, G.W., Buchala, A., Métraux, 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.

Reis, H., Pfiffi, S., and Hahn, M. (2005). Molecular and functional characterization of a secreted lipase from *Botrytis cinerea*. Mol. Plant Pathol. **6,** 257-267.

Salinas, J., Warnaar, F., and Verhoeff, K. (1986). Production of cutin hydrolyzing enzymes by *Botrytis cinerea* in vitro. J. Phytopathol. **116,** 299-307.

Salinas, J.C. (1992). Function of cutinolytic enzymes in the infection of gerbera flowers by *Botrytis cinerea.* Ph.D. thesis, University of Utrecht, The Netherlands.

Schweizer, P., Jeanguénat, A., Mösinger, E., and Métraux, J.P. (1994). Plant protection by free cutin monomers in two cereal pathosystems. Adv. Mol. Genet. Plant Microbe Interact. M.J. Daniels, J.A. Downie and A.E. Osbourn, eds. (Kluwer Academic Publishers, Dordrecht, The Netherlands)**,** 371-374.

Schweizer, P., Jeanguénat, Whitacre, D., A., Métraux, J.P., and Mösinger, E. (1996). Induction of resistance in barley against *Erysiphe graminis* f.sp. *hordei* by free cutin monomers. Physiol. Mol. Plant Pathol. **49,** 103-120.

Schweizer, P., Felix, G., Buchala, A., Müller, C., and Métraux, J.P. (1996). Perception of free cutin monomers by plant cells. Plant J. **10,** 331-341.

Sieber, P., Schorderet, M., Ryser, U., Buchala, A., Kolattukudy, P., Métraux, J.P., and Nawrath, C. (2000). Transgenic *Arabidopsis* plants expressing a fungal cutinase show alterations in the structure and properties of the cuticle and postgenital organ fusions. Plant Cell **12,** 721-738.

van Baarlen, P., Legendre, L., and van Kan, J.A.L. (2004). Plant defence compounds against *Botrytis* infection. Y. Elad et al. (eds), *Botrytis*: Biology, Pathology and Control (Kluwer Academic Publishers, Netherlands)**,** 143-161 (Chapter 149).

Van der Vlugt-Bergmans, C.J.B., Wagemakers, C.A.M., and Van Kan, J.A.L. (1997). Cloning and expression of the cutinase A gene of *Botrytis cinerea*. Mol. Plant Microbe Interact. **10,** 21-29.

Van Kan, J.A.L., Van't Klosster, J.W., Wagemakers, C.A.M., Dees, D.C.T., and Van der Vlugt-Bergmans, C.J.B. (1997). Cutinase A of *Botrytis cinerea* is expressed, but not essential, during penetration of gerbera and tomato. Mol. Plant Microbe Interact. **10,** 30-38.

Vellicce, G.R., Ricci, J.C., Hernandez, L., and Castagnaro, A.P. (2006). Enhanced resistance to *Botrytis cinerea* mediated by the transgenic expression of the chitinase gene ch5B in strawberry. Transgenic Res. **15,** 57-68.

Walters, D.R., Cowley, T., and Weber, H. (2006). Rapid accumulation of trihydroxy oxylipins and resistance to the bean rust pathogen *Uromyces fabae* following wounding in *Vicia faba*. Annu. Bot. (Lond.)**,** in press.

Wellesen, K., Durst, F., Pinot, F., Benveniste, I., Nettesheim, K., Wisman, E., Steiner-Lange, S., Saedler, H., and Yephremov, A. (2001). Functional analysis of the *LACERATA* gene of *Arabidopsis* provides evidence for different roles of fatty acid w-hydroxylation in development. Proc. Natl. Acad. Sci. **98,** 9694-9699.

Yephremov, A., and Schreiber, L. (2005). The dark side of the cell wall: molecular genetics of plant cuticle. Plant Biosystems **139,** 74-79.

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IX. Curriculum vitae

Education

PhD in Plant Biology: University of Fribourg (Prof. Jean-Pierre Métraux) 2002 - 2006

- PhD Thesis: study of the defense reactions in *Arabidopsis thaliana* against the fungus *Botrytis cinerea*
  physiological approach: microscopic analysis of fungal growth *in planta*
  molecular approach: analysis of plant and fungal gene expression during infection, generation of transgenic plants
  biochemical approach: isolation of a plant compound with fungitoxic activity

Master of Science in Biology: University of Fribourg 1997 - 2001

- Master Thesis: purification of a protein and characterisation of two systems for inducible gene expression in plants (Dr. Christiane Nawrath)
- Vigener-Price for Master Thesis (2002)

Baccalauréat: Collège St-Michel, Fribourg 1993 - 1997

Experience

University of Fribourg: teaching assistant for practical courses; supervision of Bachelor students and trainees 2002 - 2006

Swiss Center for Scientific Research, Abidjan (Ivory Coast): field work (2 months) 2002

Farchim SA, Bulle (CH): clerical work (2x 2 months) 1999 / 2000

Station Fédérale de Recherches Agronomiques, Changins (CH): field work (2 months) 1998

Technical skills

molecular biology

PCR, cloning, transformation (of bacteria, plants)
protein overexpression in *E. coli*
extraction of DNA, RNA
analysis of gene expression: Northern blot, RT-PCR, real-time PCR, microarrays

biochemistry

protein purification (6 His-tag)
SDS-PAGE, Western bot
dialysis, gel-filtration chromatography
metabolite analysis (extraction of glutathione and camalexin)

microscopy

bright field, fluorescence
different staining methods for infected plant tissues

general

culture and maintenance of plants and different fungal pathogens
plant and microbial bioassays

Languages

French: mother tongue

German: proficient, spoken and written

English: proficient, spoken and written

Publications

Chassot C. and Métraux J.-P. (2005). The cuticle as a source of signals for plant defense. Plant Biosystems, 139: 28-31.

Chassot C., Nawrath C. and Métraux J.-P. Cuticular defects lead to full immunity to a major plant pathogen. *(submitted)*

Chassot C., Lamotte O., Buchala A. and Métraux J.-P. Wound-induced resistance to *Botrytis cinerea*. *(in preparation)*

Bessire M., Chassot C., Borel S., MacDonald-Pététot J., Métraux J.-P. and Nawrath C. The *botrytis-resistant1* mutant of *Arabidopsis* reveals that an increased cuticular permeability leads to a strong resistance to *Botrytis cinerea*. *(submitted)*