Determinants of compatibility between *Arabidopsis* and the hemibiotroph *Colletotrichum higginsianum*

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II Abbreviations

ABA	abscisic acid
ABRC	Arabidopsis Biological Resource Center
AFLP	amplified fragment length polymorphism
A. thaliana	*Arabidopsis thaliana*
Avr	avirulence gene
BAC	bacterial artificial chromosome
BIBAC	binary bacterial artificial chromosome
Bp	base pairs
°C	degree Celsius
CAPS	cleaved amplified polymorphic sequence
CC	coiled-coil
cDNA	complementary DNA
C. higginsianum	*Colletotrichum higginsianum*
cM	centi Morgan
DAB	diaminobenzidine
dCAPs	derived cleaved amplified polymorphic sequence
dmr	downy mildew resistance
DNA	desoxy-ribonucleic acid
dNTP	desoxy-nucleotide-triphosphate
dpi	days post inoculation
E. coli	*Escherichia coli*
ELISA	enyme-linked immunosorbent assay
EMS	ethylmethane sulfonate
ET	ethylene
ETI	effector triggered immunity
ETS	effector triggered susceptibility
F_1	first filial generation
F_2	second filial generation
F_3	third filial generation
g	gram
G. cichoracearum	*Golovinomyces cichoracearum*
G. orontii	*Golovinomyces orontii*
HIF	heterogeneous inbred family
H. parasitica	*Hyaloperonospora parasitica*
HR	hypersensitive response
HSK	homoserine kinase
IL	introgression line
JA	jasmonic acid
kb	kilobase
L	litre
LOD	logarithm of the odds
LRR	leucine-rich repeat
m	milli
M	molar
MAP	mitogen activated protein
Mb	megabase
µ	micro

μm	micrometer
min	minute
MLO	mildew-resistance locus o
NASC	Nottingham Arabidopsis Stock Centre
NBS	nucleotide binding site
NIL	near isogenic line
NO	nitric oxide
OG	oligogalacturonide
PAMP	pathogen associated molecular pattern
PCR	polymerase chain reaction
PGIP	polygalacturonase-inhibiting protein
pmr	powdery mildew resistance
PR	pathogenesis related
PRR	pattern recognition receptor
PTI	PAMP triggered immunity
QTL	quantitative trait locus
R	resistance (gene)
RFLP	restriction fragment length polymorphism
RIL	recombinant inbred line
RLK	receptor like kinase
RLP	receptor like protein
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	rounds per minute
SA	salicylic acid
SAR	systemic acquired resistance
sec	second
SNP	single nucleotide polymorphism
SSLP	short sequence length polymorphism
TAIR	The Arabidopsis Information Resource
T-DNA	transfer DNA
TIR	Toll-interleukine-1 receptor
TLR	Toll-like receptor
TM	transmembrane
TTSS	type III secretion system
UV	ultra violet
V	Volt
wt	wild type
Zn	zinc

III Summary

Plant disease susceptibility is determined by complex interactions between plant and pathogen factors, resulting in co-evolution of a host plant species and its adapted pathogen. Previously, there has been major scientific interest in plant resistance that counteracts pathogen attack. In contrast, mechanisms of plant susceptibility are poorly understood. The aim of this study was to identify genetic determinants of dominant susceptibility of *Arabidopsis thaliana* to the hemibiotrophic ascomycete *Colletotrichum higginsianum*. Two different approaches were used, both based on the hypothesis that if an essential host susceptibility factor is not present or not functional, the plant will not support infection by the fungus. In the first approach, a forward genetics screen was conducted to identify mutants that had lost susceptibility due to chemically induced mutations in essential host susceptibility factors. Screening of 207,000 EMS *Arabidopsis* mutants in highly susceptible genetic backgrounds identified 35 candidates with reduced susceptibility to *C. higginsianum*. However, the reduction was not sufficiently clear-cut to allow identification of the mutated locus through positional cloning. The *C. higginsianum* infection phenotypes of available downy mildew resistant (*dmr*) and powdery mildew resistant (*pmr*) mutants were also analysed. Loss of susceptibility to *C. higginsianum* by specific *dmr* and *pmr* mutant lines indicated that pathogens share some common mechanisms of disease development. In the second approach, analysis of 116 *Arabidopsis* accessions from diverse geographic origins revealed considerable natural variation in response to *C. higginsianum* inoculation. Different modes of inheritance of resistance were identified by crossing resistant accessions to the highly susceptible Ler-0 accession and following segregation, and by quantitative trait loci (QTL) analysis of recombinant inbred line (RIL) populations. It was assumed that accessions lacking an essential dominant susceptibility factor would show monogenic recessively inherited resistance. Alternatively, recessive resistance could be due to the presence of a recessive resistance (*R*) gene. To select for recessive resistance, accessions that had susceptible F_1 progenies and F_2 progenies segregating 3:1 (susceptible : resistant) were characterised further. A single recessive locus was shown to confer resistance in the accessions Ws-0, Gifu-2 and Can-0. The same locus was identified by QTL analysis in the Ler-0 x Kondara RIL population. Positional cloning in a Ler-0 x Ws-0 F_2 mapping population located this recessive resistance locus to the lower arm of chromosome V between the molecular

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markers "236" (18,307,842 bp) and "312" (18,407,860 bp). Twenty candidate genes within the mapping interval, including six TIR (Toll-Interleukin 1 receptor) type NBS-LRR (Nucleotide Binding Site–Leucine Rich Repeats) genes, were analysed to determine whether this locus encodes a dominant susceptibility factor, or alternatively, a recessive *R* gene. Natural variation was also characterised cytologically. This revealed differences between resistant and susceptible accessions at an early stage in the penetration efficiency of the pathogen, or in the establishment of biotrophic primary hyphae, with no indications of involvement of host callose deposition or accumulation of reactive oxygen species in recessive resistance mechanisms.

IV Zusammenfassung

Krankheiten von Pflanzen werden durch komplexe Interaktionen zwischen Kompatibilitätsfaktoren von Pathogenen und deren Wirten bestimmt, wodurch es zur Koevolution zwischen einer Pflanzenart und einem adaptierten Pathogen kommt. In früheren Studien lag der Fokus auf der Erforschung von Abwehrmechanismen gegen Pathogenbefall. Nur wenig ist bisher über Suszeptibilität von Pflanzen bekannt.

Thema dieser Arbeit war die Identifizierung von genetischen Komponenten für dominante Suszeptibilität von *Arabidopsis thaliana* gegenüber dem hemibiotrophen Ascomyceten *Colletotrichum higginsianum*. Dafür wurden zwei verschiedene experimentelle Ansätze durchgeführt, die beide auf der Hypothese basierten, dass die Pflanze gegenüber dem Pilz resistent ist, wenn ein essentieller pflanzlicher Suszeptibilitätsfaktor entweder nicht vorhanden, oder nicht funktionell ist. Der erste Ansatz zielte auf die Identifizierung von *Arabidopsis* Mutanten deren Suszeptibilität durch chemisch induzierte Mutationen in Suszeptibiliätsfaktoren reduziert wurde. Bei einer Musterung von 207.000 Mutanten wurden 35 Kandidatenpflanzen mit verringerter Suszeptibilität gegenüber *C. higginsianum* identifiziert. Diese Reduktion war jedoch nicht eindeutig genug für eine positionelle Klonierung des mutierten Genlokus. Des Weiteren wurden die Phänotypen von „downy mildew resistant" (*pmr*) und „powdery mildew resistant" (*dmr*) Mutanten nach Inokulierung mit *C. higginsianum* analysiert. Die dabei identifizierte Reduktion der Suszeptibilität bestimmter *dmr* und *pmr* Mutantenlinien weisten auf gemeinsame Mechanismen der Pathogenese zwischen *C. higginsianum* und *Hyaloperonospora parasitica* bzw. *Golovinomyces cichoracearum* hin.

In einem zweiten experimentellen Ansatz wurde durch die Analyse von 116 *Arabidopsis* Ökotypen verschiedenen geographischen Ursprungs natürliche Variation in der Resistenz gegenüber *C. higginsianum* identifiziert. Mit Hilfe von Kreuzungen von resistenten Ökotypen mit dem suszeptiblen Ökotyp Ler-0, und der Analyse von Genloci für quantitativ vererbten Merkmalen (quantitative trait loci, QTL) in rekombinanten Inzuchtlinien (RIL) konnte monogene dominante, monogene rezessive und polygene Vererbung der Resistenz ermittelt werden. Dabei wurde von der Arbeitshypothese ausgegangen, dass Ökotypen, denen ein essentieller und dominanter Suszeptibilitätsfaktor durch natürliche Variation fehlt, monogene rezessive Vererbung der Resistenz aufweisen. Alternativ könnte rezessive Resistenz auch durch ein rezessives Resistenzgen (*R*-Gen)

vermittelt werden. In dieser Arbeit wurde ein Genlokus identifiziert, der rezessive Resistenz in den Ökotypen Ws-0, Gifu-2 und Can-0 vermittelt. Dieser Genlokus wurde auch durch QTL-Analyse einer Ler-0 x Kondara RIL-Population ermittelt. Positionelle Klonierung in einer Ler-0 x Ws-0 F_2 Kartierungs-Population lokalisierte die Position dieses rezessiven Resistenz-vermittelnden Genokus auf dem unteren Arm des Chromosoms V zwischen der Position der molekularen Marker „236" (18.307.842 Bp) und „312" (18.407.860 Bp). Zwanzig Kandidatengene, einschließlich sechs TIR (Toll-Interleukin 1) Typ NBS-LRR (Nucleotide Binding Site-Leucine Rich Repeats) Gene, wurden analysiert, um zu bestimmen, ob dieser Genlokus einen dominanten Suszeptibilitätsfaktor, oder alternativ ein rezessives *R*-Gen kodiert. Weiterhin wurde die natürliche Variation in der Resistenz gegenüber *C. higginsianum* zytologisch charakterisiert. Es wurden Unterschiede zwischen resistenten und suszeptiblen Ökotypen beobachtet, die dafür sprechen, dass für Resistenz entweder eine verringerte Invasionsrate, oder ein inhibiertes biotrophes Hyphenwachstum von *C. higginsianum* verantwortlich ist. Dabei gab es keine Hinweise auf eine Beteilung von Kalloseeinlagerung und Ansammlung von Wasserstoffperoxid an rezessiven Resistenzmechanismen.

1.Introduction

Plants have evolved a sophisticated multi-layered immune system in response to the constant challenge by disease-causing pathogens they are exposed to. Evolution of plant resistance mechanisms, in turn resulted in the co-evolution of pathogens that have adapted to infect and reproduce in a narrow range of host species, causing thereby enormous food losses. It is therefore an important challenge to unravel the mechanisms underlying the complex interactions between plants and their pathogens to identify new ways to control plant diseases.

1.1 *Arabidopsis*, the model host for studying plant-microbe interactions

Arabidopsis thaliana, called *Arabidopsis* hereafter, belongs to the mustard family (*Brassicaceae*). It is a widespread annual weed, native to Europe and central Asia and naturalised in North America, Australia and Japan (Mitchell-Olds and Schmitt, 2006). *Arabidopsis* has proven to offer several advantages for laboratory use: a small plant size, a short life-cycle, a high fertility and a large seed production. This makes it possible to grow *Arabidopsis* in a rapid manner in limited space. Because of its relatively small genome size it was the first plant to have its genome fully sequenced. For these reasons, *Arabidopsis* has emerged as a leading model plant, in particular suitable for genetic and molecular research, which has led to the establishment of a large research community with important biological and molecular resources available (Koornneef *et al.*, 2004) such as a multitude of well-characterised mutants and microarray chips. *Arabidopsis* also serves as an invaluable model system in plant pathology. It is host to a large number of microbes belonging to different taxonomic classes, including oomycetes, fungi, viruses and bacteria. Depending on their mode of infection, they are classified as necrotrophs (derive energy from killed cells), obligate biotrophs (derive energy from living cells) or hemibiotrophs (initially biotrophic but later switching to necrotrophy). *Arabidopsis* therefore allows insights into very diverse mechanisms of plant disease and resistance.

1.2 *Colletotrichum higginsianum*

Colletotrichum is a large genus of Ascomycete fungi, containing many species which cause anthracnose on an extremely wide range of temperate and tropical crops and ornamental plants (Bailey *et al.*, 1992). The brassica pathogen *Colletotrichum higginsianum* has a wide host range, attacking many cultivated forms of *Brassica* and *Raphanus* as well as the wild *Brassicaceae Arabidopsis* (Narusaka *et al.*, 2004; O'Connell *et al.*, 2004). Like many other *Colletotrichum* species, *C. higginsianum* invades host plants by a two-step hemibiotrophic infection process, which starts with the germination of spores on the plant surface to form fungal penetration organs, termed appressoria (Fig. 1.1a). Melanisation of the mature appressorium cell wall and accumulation of osmotically active solutes in the cytoplasm leads to the generation of an enormous turgor pressure by osmosis. This mechanical force, in combination with enzymatic activity, enables the fungus to directly penetrate plant epidermal cells. Thin penetrations pegs develop from the base of the appressoria and penetrate the plant cuticle and cell wall and give rise to primary hyphae (Fig. 1.1b). During the initial biotrophic phase, the primary hyphae grow intracellularly between the plant cell wall and the plant plasma membrane and are functionally equivalent to haustoria, the feeding structures of biotrophic pathogens (Shimada *et al.*, 2006). Eventually, *C. higginsianum* switches to a necrotrophic mode of growth, which is associated with a change in fungal morphology; narrow secondary hyphae are generated that kill the plant cells and dissolve cell walls ahead of infection to extensively colonise the host tissue (Fig. 1.1c). At this stage, the fungus feeds on the dead host cells to generate the sporulating structures, the acervuli, in which the spores are produced to complete the fungal asexual life-cycle.
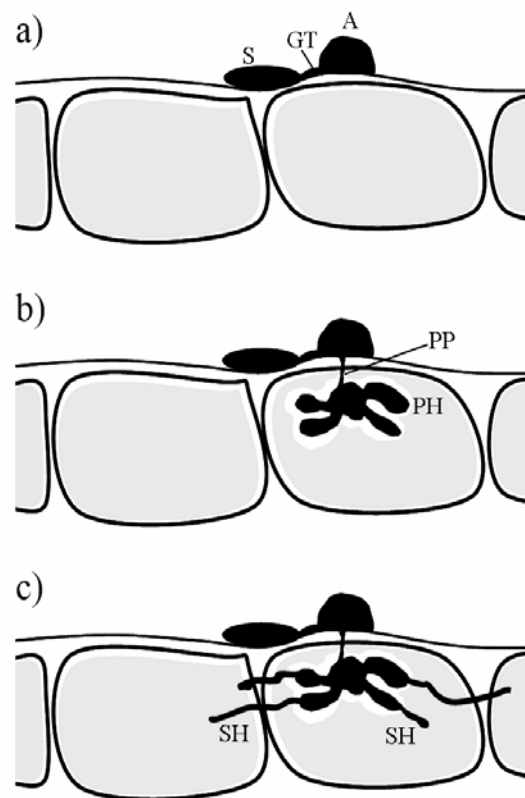
Figure 1.1: The asexual infection cycle of *Colletotrichum higginsianum*

a) Spores (S) adhere to the host cuticle and produce a germtube (GT). The appressorium (A) is formed to penetrate plant epidermal cells directly.

b) A penetration peg (PP) develops from the base of the appressorium and penetrates the host cuticle and cell wall. Primary hyphae develop inside the epidermal cell and invaginate the plant plasma membrane. The host protoplast remains alive during this biotrophic stage of the interaction.

The *Arabidopsis-C. higginsianum* pathosystem is an attractive model system for the study of plant-pathogen interactions as it not only offers the genetic resources available for the host (see 1.1), but also the experimental advantages of the pathogen. *C. higginsianum* is a haploid organism for most of its life cycle. It can be cultured axenically in contrast to obligate biotrophic pathogens and is therefore easy to handle. Furthermore, it can be transformed for mutational analysis and critical assessment of gene function by targeted gene disruption. The complete genome sequence for *Colletotrichum graminicola*, a closely-related maize anthracnose pathogen, will be available in the near future. Due to its hemibiotrophic infection strategy, *C. higginsianum* possesses features of obligate biotrophic pathogens, as well as characteristics of necrotrophic pathogens. Thus, the

Arabidopsis-*C. higginsianum* model system offers insights into general aspects of different fungal life-styles and the switch from a biotrophic to a necrotrophic life-style.

1.3   Plant defense mechanisms

Plants are under attack above- and below-ground by a host of resourceful microbes, including viruses, bacteria, fungi, oomycetes and nematodes (Dangl and Jones, 2001). To combat this plethora of pathogens, plants have evolved a robust innate immune system that exhibits striking similarities as well as significant differences with various metazoan innate immune systems (McDowell and Simon, 2008). The plant immune system consists of both preformed physical and chemical barriers, *e.g.* waxy cuticular layers and anti-microbial compounds, and a barrage of induced defences (Dangl and Jones, 2001). Recent work has shown that the inducible component of the plant immune system can be generally divided into two main branches; the pathogen associated molecular patterns (PAMP)-triggered immunity (PTI) and the effector-triggered immunity (ETI) (Jones and Dangl, 2006).

1.3.1   Pathogen associated molecular patterns (PAMP)-triggered immunity (PTI)

Pathogen associated molecular patterns (PAMPs) are highly conserved molecules and widely distributed among microbial species, where they play an essential role in the microbial lifestyle, but are absent in the potential host (Medzhitov and Janeway, 2002; Nürnberger *et al.*, 2004). Well-characterised examples for PAMPs are bacterial flagellin (Gomez-Gomez and Boller, 2002), the bacterial elongation factor EF-Tu (Zipfel *et al.*, 2006), lipopolysaccharides from bacteria, chitin and ergosterol from true fungi, and heptaglucoside and transglutaminase from oomycetes (Zipfel and Felix, 2005). Once the pathogen has overcome the preformed barriers and has gained access to the plant's interior by either direct penetration or by entering through wounds or natural openings, highly sensitive and specific pattern recognition receptors (PRRs) in the plant can detect PAMPs as "non-self" molecules. These plant plasma membrane-spanning PRRs can be grouped into 2 classes: the receptor-like kinases (RLKs) that carry a serine/threonine kinase domain, and the receptor-like proteins (RLPs) that have a short cytoplasmic tail at the

intracellular side and extracellular domains that can contain leucine-rich repeats (LRRs) or LysM motifs (Göhre and Robatzek, 2008). Recognition of PAMPs by PRRs leads to the activation of PAMP-triggered immunity (PTI) (Chisholm *et al.*, 2006). PTI comprises pre-invasive defence such as stomatal closure, as well as post-invasive defences, *e.g.* mitogen-activated protein kinase (MAPK) signalling, transcriptional activation of pathogen-responsive (PR) genes, production of reactive oxygen species (ROS), deposition of callose to reinforce the cell wall at sites of infection, and ethylene production (Asai *et al.*, 2002; Gomez-Gomez and Boller, 2002). In most cases, PTI is sufficient to avoid microbial growth while ensuring host cell survival (Nürnberger *et al.*, 2004). Through these general defence mechanisms, entire plant species can be resistant to all genetic variants of a specific pathogen and are therefore also referred to as non-host resistance (Thordal-Christensen *et al.*, 2000; Nürnberger *et al.*, 2004). One of the best-studied examples of PAMP recognition by PRRs is the perception of a synthetic 22-amino-acid peptide (flg22) from a conserved flagellin domain. Flagellin is a subunit of flagella, which are indispensable for bacterial motility (Macnab, 1992). A genetic screen using flg22 identified the *Arabidopsis* LRR-receptor kinase FLS2 (Chinchilla *et al.*, 2006), which is internalised upon flagellin perception by receptor-mediated endocytosis (Robatzek *et al.*, 2006). Recently, the bacterial EF-Tu has been shown to be recognised by the *Arabidopsis* LRR-kinase EFR and thereby limiting *Agrobacterium* pathogenicity (Zipfel *et al.*, 2006). The RLK LysM has been identified to recognise fungal chitin (Wan *et al.*, 2008), the RLP LeEix recognises the fungal xylanase EIX (Ron and Avni, 2004) and CEBiP was demonstrated to recognise fungal chitin (Kaku *et al.*, 2006). Treatment with a conserved EF-Tu peptide has been shown to induce expression of a gene set nearly identical to that induced by flg22 and vice versa (Zipfel *et al.*, 2006). This indicates that PAMPs converge on a limited number of signalling pathways and lead to a common set of outputs that require PTI (Jones and Dangl, 2006).

1.3.2 Effector-triggered immunity (ETI)

Plant pathogens can overcome PTI by developing effectors that interfere with PTI mechanisms (da Cunha *et al.*, 2007). For this, pathogens inject a range of effectors during infection that suppress PTI responses at the level of perception, signalling or defense action, which leads to host colonisation (Grant *et al.*, 2006; Göhre and Robatzek) and was therefore recently termed effector triggered susceptibility (ETS) (Chisholm *et al.*, 2006;

Jones and Dangl, 2006). Although it is likely that all pathogenic microbes encode effectors, the best characterised effectors so far come from phytopathogenic bacteria. The bacterial pathogen *Pseudomonas syringae* injects its effectors via the type III secretion system (TTSS) (Espinosa and Alfano, 2004; Galan and Wolf-Watz, 2006; Lindeberg *et al.*, 2006; Brutinel and Yahr, 2008) into the cytosol of plant cells where they contribute to virulence. The *P. syringae* effectors AvrPto and AvrPtoB have been shown to block PTI before the MAPK cascade activation (He *et al.*, 2006; Jones and Dangl, 2006). AvrRpm1 and AvrB bind to RIN4, which might act as an adaptor protein that holds multiple PRR signalling pathways under negative regulation (Kim *et al.*, 2005). Phytopathogenic fungi and oomycetes do not possess a TTSS. However, oomycetes and fungi have also been shown to secrete effector proteins into both the extracellular space and the host cytoplasm where they can play diverse roles in pathogenicity and interactions with host cells (Dean *et al.*, 2005; Kämper *et al.*, 2006). An RxLR amino acid motif that targets the effector proteins for host cells has been shown to be highly conserved among three different oomycete effectors and additional oomycete proteins predicted to be secreted (Kamoun, 2006) and is similar to the RxLx motif of malaria parasites, suggesting a conserved role in pathogenicity (Birch *et al.*, 2006). Enzyme activity has been demonstrated for a few fungal effectors (Jia *et al.*, 2000; Orbach *et al.*, 2000; El Gueddari *et al.*, 2002; van den Burg *et al.*, 2006) and the *CgDN3* secreted protein appears to suppress host-cell death during the initial biotrophic phase of *Colletotrichum gloeosporioides* after infection of *Stylosanthes* (Stephenson *et al.*, 2000).

The evolution of secreted effector proteins by plant pathogens led to the acquisition of plant proteins that specifically recognise these effectors, thereby providing effector-triggered immunity (ETI) (Chisholm *et al.*, 2006). This specific recognition of pathogen effectors, termed avirulence (AVR) factors, by cognate plant resistance (*R*) gene products has been characterised genetically as gene-for-gene resistance (Flor, 1971) and is race-specific. AVR protein recognition initiates a cascade of downstream events, such as an increase in cytosolic calcium depolarisation of the plasma membrane, a localised ROS burst, nitric oxide (NO) production and MAPK cascade activation (Dangl and Jones, 2001). ETI responses therefore show a significant overlap with PTI responses (Nimchuk *et al.*, 2003; Nürnberger *et al.*, 2004; Göhre and Robatzek, 2008). Furthermore, ETI is typically associated with a localised programmed cell death, the hypersensitive response (HR) (Jones and Dangl, 2006), which is correlated with restricting biotrophic pathogens to the infection site.

To date, numerous *R* genes have been cloned from a wide range of plant species and most of them can be classified into two main classes according to their domain organisation: the nucleotide binding leucine-rich repeat (NB-LRR) genes and the extracellular LRR genes (Jones and Dangl, 2006). The NB-LRR genes represent the largest class of *R* genes and can be further subdivided into coiled-coil (CC)-NB-LRR and Toll-interleukin-1 receptor (TIR)-NB-LRR genes according to their N-terminal domain. More than 150 proteins have been predicted to be NB-LRR proteins in *Arabidopsis* alone (Chisholm *et al.*, 2006). The second major class of *R* genes, encoding extracellular LRR proteins, is subdivided into three subclasses. These include RLPs with an extracellular LRR and a transmembrane domain, the RLKs with an extracellular LRR and a transmembrane domain, and the polygalacturonase-inhibiting protein (PGIP) with a cell wall LRR (Chisholm *et al.*, 2006). Although many *R* genes and their corresponding pathogen effectors have been cloned in the past, direct binding between them has rarely been demonstrated. Therefore, in addition to the original model of a direct recognition of the Avr protein and its cognate R protein, several NB-LRR proteins have been identified to recognise effectors indirectly by detecting the products of their action on host targets (Van der Biezen and Jones, 1998). This mode of indirect interaction is formulated in the "guard hypothesis", describing the R protein keeping the host target protein, the guardee, under surveillance. The most extensively studied guardee is *Arabidopsis* RIN4, which constitutively associates with the CC-NB-LRR R protein RPM1. In the presence of the *P. syringae* effectors AvrB or AvrRpm1, RIN4 is hyper-phosphorylated, which has been shown to suppress PTI (Kim *et al.*, 2005). RPM1, in turn, is activated following this phosphorylation (Bisgrove *et al.*, 1994; Mackey *et al.*, 2002) and thereby activating ETI. Thus, as a consequence of the indirect recognition, a limited number of receptors guarding key host targets is sufficient to monitor the presence of multiple effectors having the same target (Jones and Dangl, 2006).

In response to ETI, pathogens have evolved further effectors that specifically interfere with ETI mechanisms in plants, either by shedding or diversifying the recognised effector, or by acquiring additional effectors that suppress ETI (Jones and Dangl, 2006). One example is the *P. syringae* effector AvrRpt2, a cysteine protease, which may have evolved as a mechanism to interfere with the RPM1 disease resistance pathway and which restores pathogen virulence by cleavage of RIN4. However, RPS2 evolved which recognises the activity of AvrRpt2 and triggers ETI again (Axtell and Staskawicz, 2003; Caplan *et al.*, 2008).

Many recent studies have focused on the signal transduction pathway downstream of activated plant immunity receptors. Interestingly, different classes of NB-LRR proteins require different signalling components. The TIR-NB-LRR proteins have been shown to require EDS1 and its interacting partners PAD4 and SAG101 (Glazebrook *et al.*, 1996; Parker *et al.*, 1996; Aarts *et al.*, 1998; Falk *et al.*, 1999; Feys and Parker, 2000; Feys, 2001). In contrast to this, signal transduction by the CC-NB-LRR proteins seems to be generally dependent on NDR1 (Aarts *et al.*, 1998; Coppinger *et al.*, 2004). In the TIR-NB-LRR protein triggered response, EDS1 and its interacting partners are needed for the expression of HR and the accumulation of salicylic acid (SA) (Wiermer *et al.*, 2005). Furthermore, they are involved in the generation of a signal potentiation loop that involves the processing of ROS- and SA-derived signals (Feys, 2001; Rustérucci *et al.*, 2001). SA accumulation primes a mechanism of systemic immunity in which local defences establish a state of heightened resistance throughout the plant against subsequent pathogen attack, known as systemic acquired resistance (SAR) (Durrant and Dong, 2004). The EDS1 pathway is further regulated by LSD1 and ACD11, negative regulators of a cell death pathway that depends on EDS1 and PAD4 activities (Brodersen *et al.*, 2002; Mateo *et al.*, 2004). The MAP kinase MPK4 has been identified to be required for both repression of the SA pathway and activation of the ethylene (ET)/ jasmonic acid (JA) pathway (Petersen *et al.*, 2000; Mateo *et al.*, 2004). SA-dependent defence responses have been shown to be particularly effective against biotrophic and hemibiotrophic pathogens, probably due to the fact that cell death deprives these pathogens of nutrients (Thomma *et al.*, 2001; Glazebrook, 2005). In contrast, necrotrophs commonly take advantage of dead cells and JA and ET seem to be alternative signals in resistance to necrotrophs and generally act synergistically (Glazebrook, 2005).

1.4 Recessive resistance

ETI is conferred by *R* genes that induce an active resistance response after perception of cognate *Avr* genes (see 1.3). As this resistance is typically inherited dominantly (Hammond-Kosack and Jones, 1997), this mode of resistance can also be termed dominant resistance. To date, less attention has been paid to incompatible plant-pathogen interactions controlled by recessive resistance genes. Recessive resistance can, as with dominant resistance, be the result of active resistance mechanisms induced by the plant

recognition of the invading pathogen by an *R* gene that is recessively inherited. Alternatively, recessive resistance might underlie a passive resistance mechanism due to the lack or a mutated version of a specific host factor required by the pathogen to complete its life-cycle. Although knowledge about this aspect of resistance is still limited for bacterial and fungal systems, recessive forms of resistance are fairly common in viral systems, accounting for almost half of all known viral *R* genes (Kang *et al.*, 2005b). Work carried out to characterise loss-of-susceptibility mutants, mainly obtained through chemical mutagenesis of susceptible hosts, together with work on natural recessive resistance in crop species, offers insights into the very diverse mechanisms of recessive resistance.

1.4.1   Recessive resistance in plant-virus interactions

Viruses depend on the host biochemical machinery to complete their biological cycle. The successful infection of a plant by a virus requires a series of compatible interactions between host and viral factors, including the expression and replication of the viral genome, cell-to-cell movement and long distance translocation through the plant vascular system (Carrington *et al.*, 1996; Maule *et al.*, 2002). Recessive resistance seems to be more frequent for potyviruses than for viruses of other families (Diaz-Pendon *et al.*, 2004) and was found to disturb mainly viral replication or movement (Kang *et al.*, 2005b). The recessive resistance genes *TOM1* and *TOM2A* encode integral membrane proteins that are localised in the tonoplast (Ishikawa *et al.*, 1993). The TOM1 protein has been shown to interact with the helicase domain of replication proteins encoded by *Tobacco mosaic virus* (Ishikawa *et al.*, 1991; Ishikawa *et al.*, 1993; Yamanaka *et al.*, 2000; Yamanaka *et al.*, 2002; Hagiwara *et al.*, 2003). It is postulated that the interaction of TOM2A with TOM1 constitutes a component of the tobamoviral replication complex (Tsujimoto *et al.*, 2003) that is essential for successful viral growth. The translation initiation factor *eIF4E* has been identified to be a major determinant of recessive resistance to the family of potyviruses (Robaglia and Caranta, 2006). eIF4E binds to the 5′ cap structure of mRNA and is a key player in the initiation of plant protein synthesis. The binding of the viral protein VPg to eIF4E has been shown to be required for successful infection of the plant. The key role played by *eIF4E* in recessive resistance has been revealed by the discovery that the *lsp1* gene for resistance to Tobacco etch virus (Lellis *et al.*, 2002), the *mo1* gene for lettuce resistance to lettuce mosaic virus (Nicaise *et al.*, 2003), the *sbm1* gene for pea

resistance to *Pea seed-borne mosaic virus* (Gao *et al.*, 2004) and the *pot-1* gene for tomato resistance to *Potato virus Y* and *Tobacco etch virus* (Ruffel *et al.*, 2004) all correspond to mutations in *eIF4E* homologs, affecting virus cell-to-cell movement and viral accumulation. Moreover, recessive resistance to viral infection has also been shown to be caused by mutations in *eIF4G*, a further component of the eukaryotic translation initiation complex (Yoshii *et al.*, 2004).

1.4.2 Recessive resistance in plant-bacteria interactions

Although recessive resistance is well-studied in viral systems, little is understood regarding this phenomenon in plant-bacterial interactions. However, recent research on the *Oryza sativa–Xanthomonas oryzae pv. oryzae* (*Xoo*) pathosystem has shown that nine of the 30 documented *R* genes are recessively inherited (Iyer-Pascuzzi and McCouch, 2007b). Two of them, *xa5* and *xa13*, have recently been cloned. Amino acid substitutions in the γ-subunit of the transcription factor IIA, the xa5 protein, prevent the interaction with bacterial proteins which normally promote disease possibly by the activation of genes that might be involved in nutrient, sugar or iron metabolism (Iyer and McCouch, 2004). *Xa13/Os8N3* encodes a plasma membrane-localised protein which is involved in pollen development of rice (Chu *et al.*, 2006). Mutations in the promoter region of *xa13* seem to eliminate the bacterial induced upregulation of *Xa13* by the *Xoo* race 6, PXO99[A], which leads to resistance, probably by abolishing the interaction of the PthXo1 bacterial type III effector with its target (Chu *et al.*, 2006; Yang *et al.*, 2006). The recessive *RRS1-R* allele has been identified to provide resistance of *Arabidopsis* to the bacterial wilt pathogen *Ralstonia solanacearum* (Deslandes *et al.*, 2002). *RRS1* encodes a new class of TIR-NB-LRR proteins (see 1.3.2) with a nuclear localisation signal and a WRKY domain. Although *RRS1-R* has been identified to confer resistance recessively, it acts as a dominant gene in transgenic plants. Its structure, which is typical for an *R* gene, together with the identification of its corresponding *Avr* gene *PopP2* (Deslandes *et al.*, 2003) strongly suggest that RRS1 acts in a similar way to dominant TIR-NB-LRR proteins.

1.4.3 Recessive resistance in plant-fungal interactions

One of the best-studied examples of recessive resistance of plants to fungal pathogens is recessive resistance to powdery mildew mediated by the loss of the barley *mildew resistance locus o* (*Mlo*). Barley Mlo encodes an integral membrane protein with seven transmembrane domains (Bueschges *et al.*, 1997). *mlo*-based resistance is not restricted to the monocot barley - several mutants with enhanced resistance to powdery mildew were identified in *Arabidopsis* to confer resistance, indicating that this mechanism of resistance may be inducible in any higher plant species (Consonni *et al.*, 2006). Recent work has postulated that MLO proteins function as regulatory components of plant secretory processes involving SNARE domain proteins, and the powdery mildew fungi appear to specifically corrupt MLO for successful pathogenesis (Panstruga, 2005; Humphry *et al.*, 2006). Similarly, the powdery mildew resistance (*pmr*) mutants do not support growth of the pathogen and were suggested to represent true compatibility factors (Vogel and Somerville, 2000). Four of the corresponding *PMR* genes have been cloned. *PMR2* has been shown to be allelic to *AtMLO2* (Consonni *et al.*, 2006). *PMR4* encodes GSL5, a callose synthase that is essential for callose deposition at wound and biotic stress sites (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). The mutation in *pmr4* results in the loss of callose accumulation and the enhanced activation of SA and pathogen-responsive genes. *PMR5* and *PMR6* encode a protein of unknown function and a pectate lyase, respectively (Vogel *et al.*, 2002; Vogel *et al.*, 2004). Both mutants show similar phenotypes, resulting in increased cell wall pectin content, a reduced pectin esterification and a suggested change in the hydrogen-bonding environment of cellulose, leading to reduced powdery mildew growth. Since *pmr5* and *pmr6* confer resistance to *G. orontii* and *G. cichoracearum*, but not to virulent strains of *P. syringae* and *Hyaloperonospora parasitica*, it was proposed that the according proteins are true compatibility factors that are generally specific for single pathogen species.

1.4.4 Recessive resistance in plant-oomycete interactions

Recently, more insights could be gained into recessive resistance to the downy mildew *H. parasitica*. A screen of EMS induced *Arabidopsis* mutants has identified eight downy mildew-resistant (*dmr*) mutants, corresponding to six different loci (Van Damme *et al.*, 2005). The mutants *dmr3*, *dmr4* and *dmr5* are associated with constitutive expressions of the defense-related *PR-1* gene, indicating that these mutants are affected in defence

pathways instead of mechanisms of recessive resistance. The mutants *dmr1* and *dmr6* have been cloned and further characterised. *DMR1* encodes a homoserine kinase (HSK) (Van Damme, 2007) and the mutation in *dmr1* results in elevated homoserine levels. Although homoserine was shown not to have a direct effect on pathogen growth, treatment of *Arabidopsis* with the amino acid results in complete protection from the fungal infection, possibly due to a role in a so far undefined mechanism resulting in plant disease resistance via the modulation of host amino acid metabolism. The mutant *dmr6* carries a mutation in the gene that encodes a 2-oxoglutarate (2OG)-Fe(II) oxygenase of unknown function, resulting in the enhanced expression of a subset of defence-associated genes, including *DMR6* itself (van Damme *et al.*, 2008). It was suggested that the mutation activates either a novel plant defence, or could cause the accumulation of a toxic DMR6 substrate. Alternatively, the DMR6 metabolic product might be required for successful infection by the oomycete.

1.5   Natural variation of *Arabidopsis* accessions

Natural variation between and within species is considered to be the main resource for evolutionary changes. Only a species that has the potential to adapt to changes in the environment by genetic variation within the species can survive and produce successors. Genetic variation is influenced by evolutionary processes that can affect the whole genome, *e.g.* the demographic history and the type of breeding system, or by evolutionary processes that are variable across the genome, *e.g.* the recombination rate, the mutation rate and selection (Schmid *et al.*, 2006). Therefore, at any given locus genetic variation is the result of a combination of genome-wide and locus-specific factors. Analysis of natural variation can provide insights beyond knowledge based on a mutagenised genome and aims to focus on physiological, ecological and evolutionary questions.

The model plant *Arabidopsis* occurs throughout the Northern hemisphere in Europe, Asia and Africa, from the latitude range of 68°N to 0°, and has also been naturalised in North America, Australia and Japan (Koornneef *et al.*, 2004). It has been found from sea level up to 4250 m (Al-Shehbaz and O'Kane Jr, 2002), and occupies very diverse habitats (Shindo *et al.*, 2007). This broad distribution requires a level of genetic variation to adapt to the specific conditions of these diverse environments. The extraordinarily wide phenotypic variation described for *Arabidopsis*, not only in visually obvious phenotypes, but also in

genetic mechanisms, reflects this natural variation. Recent genome-wide studies show that in *Arabidopsis* an average pair of alleles differs at about seven nucleotides per kilobase (Mitchell-Olds and Schmitt, 2006). Hundreds of accessions from natural populations that have been collected from diverse worldwide locations are available from international stock centres, *e.g.* NASC and ABRC (Scholl *et al.*, 2000; Koornneef *et al.*, 2004), offering an immense source of genetic variation. Therefore, natural variation in *Arabidopsis*, a species that offers a large number of genomic tools and resources (see 1.1), allows analysis of three complementary areas: (1) genomic studies of molecular variation and its population structure, (2) identification of genetic polymorphisms underlying natural variation in complex traits, and (3) ecological and evolutionary studies of natural selection and adaptation. In the recent past, analysis of natural variation has proven to be a powerful alternative to mutant-based functional analyses for the identification of single genes and their functions. Natural variation is often more subtle than laboratory generated knock-out mutants and might therefore allow identification of mechanisms of pathway control and cross-pathway linkages that may not be detectable with knock-out mutants, in particular when these are lethal (Tonsor *et al.*, 2005). Furthermore, null or weak wild-type alleles cannot be detected by the mutant-approach, and some phenotypes appear only in certain genetic backgrounds due to epistatic interactions (Koornneef *et al.*, 2004). Furthermore, natural variation can make an important contribution to the understanding of complex traits. Mutagenic knock-outs of single genes cannot elucidate the function of all genes involved in a trait that is determined by several loci. Lastly, natural variation-based analysis allows the elucidation of the mechanisms generating and maintaining the variation. There has been a recent focus on how genetic variation affects phenotypic traits and its ecological aspects, *i.e.* to determine the molecular mechanisms that maintain phenotypic variation in the wild. Genetic polymorphisms might be evolving neutrally, or could be transient variants on their way to being eliminated because they are deleterious, or on their way to fixation because they are beneficial (Mitchell-Olds *et al.*, 2007). Further studies have begun to elucidate the genome-wide evolutionary processes that shape natural variation (Nordborg *et al.*, 2005; Schmid *et al.*, 2006; Clark *et al.*, 2007). In contrast to artificially induced mutations, the mutations underlying natural variation were maintained by natural selection. Hence, genetic variation that exists in high frequency in nature is more likely to be adaptive than artificially induced mutations (Shindo *et al.*, 2007). In conclusion, analysis of genetic variation in natural genomes gives a more complete picture

to understand plant function in an evolutionary context and will therefore be an important complement to mutant analyses in the future.

1.6 Quantitative trait loci (QTL) analysis and Recombinant Inbred Line (RIL) populations

Part of the natural variation is of a qualitative nature, *i.e.* phenotypes in the progeny of crosses exhibit only a limited number of discrete classes, determined by single segregating loci. Positional cloning (mapping), also termed Mendelian genetic analysis, is generally applied to identify the location of the genes responsible for these monogenic traits. This strategy relies on the fact that as physical distance between a gene of interest and a molecular marker decreases, so does the genetic recombination frequency (Jander *et al.*, 2002). In the course of mapping, progressively closer flanking markers on either side of the locus of interest determine recombination events, until a region of 10-20 candidate genes is identified. The genetic basis of the natural variation can then be identified by several approaches, *e.g.* phenotypical analysis of T-DNA insertion lines, sequencing the genetic variation between different accessions, or complementation analysis.

Nevertheless, most variation between accessions is of a quantitative nature due to the effects of allelic variation at several loci. Combined with environmental effects, these quantitative trait loci (QTL) determine a continuous phenotypic distribution of the trait in a segregating population (Koornneef *et al.*, 2004). Therefore, the genotypes at these loci cannot be directly inferred from the phenotype of a plant, which has historically hampered genetic analysis (Alonso-Blanco and Koornneef, 2000). Instead, during a QTL analysis, phenotypic values of the trait are associated with genotypic classes of polymorphic molecular markers to identify the number and the genetic position of loci that control the trait variation and their contribution to the total variance of the trait in that experiment. In principle, QTL detection can be done in the F_2 generation (Alonso-Blanco and Koornneef, 2000), as commonly used for Mendelian genetic analysis. However, for QTL analysis the recombinant inbred line (RIL) populations have proven to be useful. RIL populations are derived by successively selfing single plants from the progeny of individual F_2 plants until homozygousity is achieved at the F_8 generation (Alonso-Blanco and Koornneef, 2000) and therefore RILs represent individual homozygous mosaics of the original parental genomes

(Fig. 1.2a). Despite the time that is required to produce them, they offer major advantages over F_2 populations; once homozygousity has been attained, the lines can be propagated indefinitely without further segregation. Therefore, genotyping of the individual lines only needs to be done once. Moreover, a trait can be measured in the same population that is grown in different environments and it can be analysed on several sister plants per line, which minimises the environmental variation and therefore improves accuracy of QTL mapping. Furthermore, RIL populations, in contrast to F_2 populations, undergo multiple rounds of meiosis before homozygousity is reached. Therefore, linked genes have a greater probability of recombination, which results in the greater chance of detecting recombination events between two linked markers (Burr and Burr, 1991). Currently more than 60 RIL populations are available that have been produced in different laboratories and some of them are publicly available (http://www.inra.fr/internet/Produits/vast/). To allow a proper comparison of the location of genes, it is important to use the same marker framework for their genotyping, and the markers should be preferentially anchored to the physical map of the species. Various molecular marker systems have been used for the genotyping the RIL populations, *e.g*. SSLPs and AFLP markers. In particular the development of a high number of SNP markers has offered a useful tool not only for QTL mapping, but also for Mendelian genetic analysis (Schmid *et al.*, 2003; Schmid *et al.*, 2006), and these are available via TAIR (http://arabidopsis.org), the Cereon database (http://www.arabidopsis.org/Cereon/index.jsp) and (http://walnut.usc.edu/2010/an-arabidopsis-polymorphism-database). By means of QTL mapping, the position of each QTL is assigned to a genetic interval of 5-50 cM, corresponding, on average, to 1.2-12 Mb (Koornneef *et al.*, 2004). Therefore, further fine-mapping and validation of the effects of a QTL requires the generation and analysis of near isogenic lines (NILs) (Fig. 1.2b). In these lines, the allele of interest from one accession has been introduced only in the vicinity of the QTL into the genetic background of the other accession (Alonso-Blanco and Koornneef, 2000; Maloof, 2003).
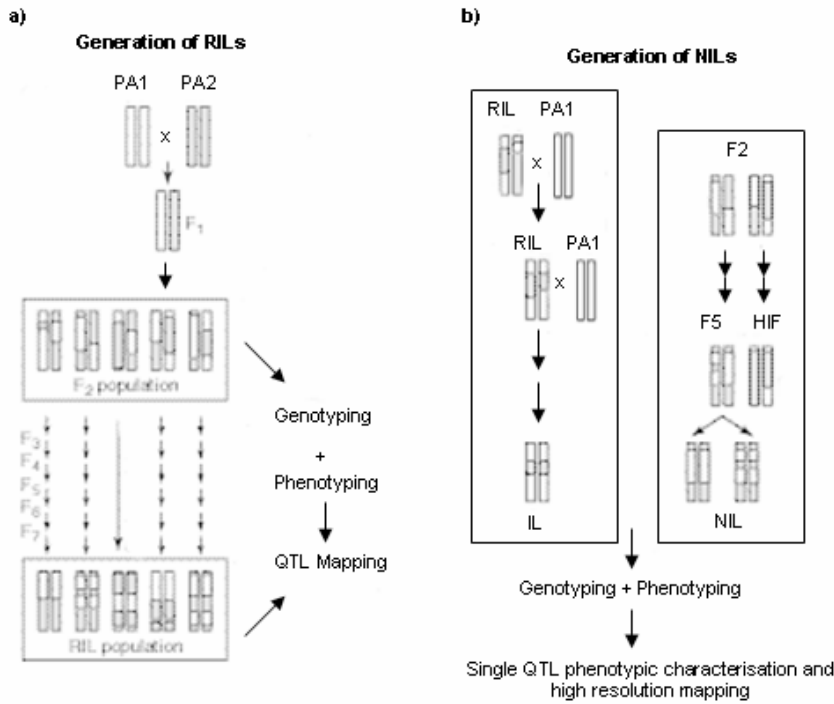
Figure 1.2: The generation of RIL and NIL populations and their application in QTL analysis and validation.

Image modified from: Alonso-Blanco *et al*., (2000). The graphical genotype of individual plants is depicted for a representative single pair of chromosomes.

a) To detect and locate quantitative trait loci (QTL), F_2 and recombinant inbred lines (RIL) populations can be used. In both cases, genotyping and phenotyping of each individual line is necessary for the QTL mapping. RILs are derived by crossing parental accession 1 (PA1) to parental accession 2 (PA2) and successively selfing single plants from the progeny of individual F_2 plants until homozygosity is achieved at the F_8 generation.

b) Near isogenic lines (NILs) differ in the alleles around a single QTL and can be obtained by either the generation of introgression lines (IL) or by the generation of heterogenous inbred families (HIFs). For the generation of ILs, a suitable RIL line is recurrently backcrossed to one of the parental accessions. HIFs can be obtained by continuous selfing of RILs that are not entirely homozygous until the F_5 generation. Genotyping and phenotyping of both types of NILs facilitates the fine mapping of single QTLs and its phenotypic characterisation to validate the effect of the QTL on the variation of a trait.

1.7 Thesis aims

The overall aim of this study was to identify genetic determinants of the interaction between *Arabidopsis* and the hemibiotrophic ascomycete *C. higginsianum*. For this, I used two different approaches, both based on the hypothesis that if an essential susceptibility factor is not present or not functional, the plant will not support infection by the fungus.

In the first approach, I conducted a forward genetic screen for isolation of EMS- and γ-radiation-induced *Arabidopsis* mutants that had lost susceptibility to *C. higginsianum* to identify potential host susceptibility factors. In addition, I analysed the *C. higginsianum* infection phenotypes of available downy mildew resistant (*dmr*) (Van Damme *et al.*, 2005) and powdery mildew resistant (*pmr*) (Vogel and Somerville, 2000) mutants to test whether *C. higginsianum* shares common susceptibility factors with these obligate biotrophs.

The second approach of my study exploited natural variation in susceptibility to *C. higginsianum* between *Arabidopsis* accessions. It was assumed that accessions lacking an essential susceptibility factor would show monogenic recessively inherited resistance. Alternatively, recessive resistance could be due to the presence of a recessive *R* gene. A total of 116 accessions were tested for their infection phenotypes after inoculation with *C. higginsianum* and the mode of inheritance of resistance was analysed by crossing resistant accessions to the highly susceptible Ler-0 accession and following segregation. To identify putative host susceptibility factors, accessions that were recessively resistant, *i.e.* having susceptible F_1 progenies and F_2 progenies segregating 3:1 (susceptible : resistant), were chosen. In parallel, QTL analysis was carried out for two RIL populations originating from crosses of resistant accessions to Ler-0. To identify the genetic basis of the identified natural variation, resistance in these accessions was mapped and candidate genes were analysed according to their possible function in recessive resistance. In addition, the cytological phenotypes of resistant accessions were characterised in detail to obtain clues to the mechanism of resistance.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals and general equipment suppliers

ADGEN (Auchincruive, UK)

Boehringer Mannheim GmbH (Mannheim, Germany)

Carl Roth GmbH + Co. KG (Karlsruhe, Germany)

Invitrogen (Karlsruhe, Germany)

Merck (Darmstadt, Germany)

New England BioLabs (NEB) (Ipswich, MA, USA)

Operon Biotechnologies GmbH (Cologne, Germany)

Oxoid GmbH (Wesel, Germany)

QIAGEN GmbH (Hilden, Germany)

Sigma-Aldrich Chemie GmbH (Deisenhofen, Germany)

2.1.2 Antibiotics

Hygromycin   100 μg/mL
Kanamycin   50 μg/ml

Stock solutions stored at -20°C.

2.1.3 Media

Unless otherwise indicated, all media were sterilised by autoclaving at 121°C for 20 minutes. Heat sensitive solutions were sterilised using filter sterilisation units prior to addition of autoclaved components. For the addition of antibiotics and other heat liable components the solution or media were cooled down to 55°C.

E. coli Media:

LB (Lauria Bertani) Broth

Tryptone peptone	1 %
Yeast extract	0.5 %
NaCl	0.5 %

In H_2O

For selection Kanamycin 50 µg/mL

Agar plates

1.5-2 % agar was added to the LB broth

For selection Kanamycin 50 µg/mL

Colletotrichum media:

Mathur's medium

Glucose	2.8 g
$MgSo_4 \cdot 7H_2O$	1.22 g
KH_2PO_4	2.72 g
Oxoid Mycological peptone	2.8 g

Add to 1L with dH_2O

Agar plates or conical flasks

1.5-2% agar was added to the Mathur's medium

2.1.4 Buffers and solutions

Agarose gel (1 and 4%)

Agarose	1 g/ 4 g
TAE buffer (10x)	100 mL
Ethidium bromide stock (10 mg/ml)	2 µL

Aniline Blue staining solution

Aniline Blue (water soluble)	0.01% (w/v)
K_2HPO_4	0.07 M

In H_2O

DAB (3,3'Diaminobenzidine) staining solution

DAB	1 mg/mL

In H_2O, pH was adjusted to 3.8 with HCL

Edwards buffer

Tris-HCl pH 7.5	200 mM
NaCl	250 mM
EDTA	25 mM
SDS	0.5%

In H_2O

Loading buffer (Orange G Dye 6x)

Sucrose	40%
Orange G (Merck)	0.5%

In TE buffer

PCR buffer

Tris-HCL pH 9	100 mM
KCL	500 mM
$MgCl_2$	15 mM
TritonX-100	1%

In H_2O

TAE (Tris/acetate/EDTA) buffer (10x)

Tris base	24.2 g
Glacial acetic acid	5.71 mL
$Na_2EDTA \cdot 2H_2$	3.72 mL
H_2O	to 1L

TE (Tris/EDTA) buffer

Tris/HCL (pH 8.0)	10 mM
EDTA (pH 8.0)	1 mM
Tris/HCL	1 M
Tris-Base	121 g
H_2O	to 1 L

121 g Tris base was dissolved in 800 mL, adjusted to the desired pH with concentrated HCL, and then adjusted to the volume of 1 L with H_2O.

2.1.5 Organisms

2.1.5.1 Pathogens

C. higginsianum strains (IMI 349061-2, IMI 349061-GFP)

E. coli DH10B (BIBAC-library)

H. parasitica (isolate Cala2)

2.1.5.2 Plant material

Seeds of *Arabidopsis* accessions were obtained from NASC (http://arabidopsis.info/) and SASSC (http://www.brc.riken.jp/lab/epd/SASSC/index.html). *Arabidopsis* accessions used in this study are listed in Table 3.2.

Seeds of the Ler-0 x Kas-2 and the Ler-0 x Kondara RIL populations were kindly provided by Dr. M. Reymond, Cologne, Germany.

Re-mutagenised EMS Ler-0 *rar1*-13 seeds were kindly provided by Dr. P. Muskett and Dr. J. Parker, Cologne, Germany.

EMS mutagenised Ler-0 seeds were obtained by Lehle Seeds, USA

γ-radiation mutagenised Ler-0 seeds were kindly provided by Dr. E. van der Vossen, Wageningen, Netherlands.

Seeds of the *dmr* mutants were kindly provided by Dr. G. Van den Ackerveken, Utrecht, Netherlands.

2.1.6 Oligonucleotides

Primers used in the study were synthesized by Operon. Sequence and length are listed in the Table SD 2, supplementary Data. Primers were stored in 100 µM stock solution in water at -20°C.

2.1.7 Enzymes

2.1.7.1 Restriction endonucleases

Restriction enzymes were purchased from NEB and used following the manufacture's instructions.

2.1.7.2 Nucleic acid modifying enzymes

Standard PCR reactions were performed using homemade *Taq* DNA polymerase.
SuperScript™ II RNase H Reverse Transcriptase (Invitrogen)

2.1.8 Software, databases and other internet resources

Analysis and alignment of sequencing chromatograms

  SeqMan (Lasergene)

Sequence analysis and comparison

  http://www.ncbi.nlm.nih.gov/BLAST/

Databases for genomic sequences of *Arabidopsis*

  http://www.arabidopsis.org

  http://www.tigr.org/tdb/e2k1/ath1/ath1.shtml

Searching for SSLP and CAPs markers

  http://www.arabidopsis.org

  http://msqt.weigelworld.org/

  http://www.inra.fr/internet/Produits/vast/

Primer design

  http://biotools.umassmed.edu/bioapps/primer3_www.cgi

CAPs marker design

  http://helix.wustl.edu/dcaps/dcaps.html

Leica Confocal Imaging Software

Leica Confocal Software, Version 2.61, Leica Microsystems Heidelberg GmbH, Germany

Software for RIL mapping

Van Ooijen, J.W., 2004. MapQTL® 5, Software for the mapping of the quantitative trait loci in experimental populations. Kyazma B.V., Wageningen, Netherlands

2.2 Methods

2.2.1 Growth and culturing of *Arabidopsis*

Plants were sown on soil substrate and stratified for two days at 4°C in darkness to allow an even germination. Germination was induced by transfer of the plants to a light chamber with 21°C during the day, 21°C during the night and a relative humidity of 50%. All plants were grown for three weeks at a day/night cycle of 10 and 14 hours, respectively. For inoculation with *C. higginsianum*, plants were transferred to another light chamber (see 2.2.6.1).

For mapping experiments, F_2 plants of the respective mapping population were grown in 96-well trays on soil together with the respective parents as control and inoculated at an age of three weeks.

2.2.2 Generation of *Arabidopsis* F_1 and F_2 progeny

Fine tweezers and a magnifying-glass were used to emasculate an individual flower. To prevent self-pollination, only flowers that had a well-developed stigma but immature stamen were used for crossing. Fresh pollen from three to four independent donor stamens was dabbed onto each single stigma. Mature siliques containing F_1 seed were harvested and allowed to dry. Approximately five F_1 seeds per cross were grown as described above and allowed to self pollinate. Produced F_2 seeds were collected and stored.

2.2.3 EMS mutagenesis

About 10,000 seeds of Ler *eds1-2* were imbibed in a humid chamber for 2 days at 4°C. The seeds were then transferred to a Falcon tube which was filled with 50 mL deionised water and 0.15 mL of 0.3 % methanesulfonic acid ethyl ester (EMS) solution and incubated for 9 hours on a shaker. Eventually, seeds were extensively washed with 2 L of sterile water. For planting the seeds were transferred to 1 L of 0.08% agarose solution and 5 mL of this solution was pipetted per TEKU tray, filled with soil (approx. 50 seeds per TEKU tray). In about 10% of the M_2 families albino mutants were detected. 773 independent M_2 families were created.

2.2.4 Growth and sub-culturing of pathogens

2.2.4.1 *Colletotrichum higginsianum*

3 mL of spore suspension were dispersed over Mathur's agar medium (Mathur *et al.*, 1950), dispensed in 250 ml Ehrlenmeyer flasks and cultured at 20-25°C (Sherriff *et al.*, 1994). Conidia could be harvested at any time from 6-30 days, but 9-14 days was optimum for sub-culturing. For the harvest of conidia, 5 mL of sterile water was added to each flask and the flasks were vigorously shaken to suspend the conidia.

2.2.4.2 *Escherichia coli*

For sub-culturing of bacterial cultures, colonies were streaked on LB medium, supplemented with Kanamycin for selection and cultured at 37°C.

2.2.4.3 *Hyaloperonospora parasitica*

H. parasitica isolate Noco2 was maintained as mass conidiosporangia cultures on leaves of the susceptible *Arabidopsis* accession Ler-0 for 7 days. Leaf tissue from infected seedlings was harvested into a 50 mL Falcon tube 7 d after inoculation. Conidiospores were collected by vigorously vortexing harvested leaf material in sterile dH_2O for 15 sec and after the leaf material was removed by filtering through miracloth the spore suspension was adjusted to a concentration of $4x10^4$ spores/mL dH_2O using a Neubauer counting cell chamber. Plants to be inoculated had been grown under short day conditions as described above. *H. parasitica* conidiospores were applied onto two-week-old seedlings by spraying until imminent run-off using an aerosol-spray-gun. Inoculated seedlings were kept under a propagator lid to create a high humidity atmosphere and incubated in a growth chamber at 18°C and a 10 h light period.

2.2.5 Glycerol stock preparation

To maintain the properties of a fungal or bacterial culture, a stock of frozen fungal conidia, respectively of frozen bacteria was maintained.

To prepare a glycerol stock, a concentrated aqueous fungal spore, respectively bacterial suspension was mixed with an equal volume of sterile 30% glycerol, aliquoted in 1 mol volumes into cryo-tubes and snap-frozen in liquid nitrogen for storage at -80°C.

2.2.6 Inoculation of *Arabidopsis* with pathogens

2.2.6.1 Inoculation of the plants with *C. higginsianum* isolates

A fungal spore suspension was prepared in 15 mL sterile water by vigorous shaking of the Erlenmeyer flask. Spore concentrations were determined by use of a haemocytometer and spore-suspensions were adjusted to the desired concentrations (5×10^5 spores/mL for microscopical analysis and 2×10^6 spores/ mL for phenotyping of the F_2, F_3 progenies, allelism tests and tests of T-DNA insertion mutant lines) by adding sterile water.

Plants were spray-inoculated by use of an atomiser and sprayed plants were sealed inside a plastic propagator. The lid of the propagator was sprayed with sterile water to provide 100% relative humidity. The propagators were incubated at 25°C in a growth chamber with a 16 h photoperiod and a PPFR of 80 μmol m^{-2} s^{-1} and 81% relative humidity.

2.2.6.2 Inoculation of the plants with *H. parasitica*

Inoculation procedure of *H. parasitica* was identical to procedure of sub-culturing of (see 2.2.4.3)

2.2.7 *Arabidopsis* mutant screen (EMS and γ-radiation mutated M$_2$ seeds)

For screening EMS Ler-0 (Lehle) and Ler *eds1*-2 M$_2$ mutant pools, approx. 20 mg of seeds were grown on soil in a tray (45 x 30 cm). Plants were inoculated after two weeks. For Ler *rar1*-13 EMS mutants and γ-radiation mutated Ler-0 seeds, individual M$_2$ lines were grown separately (approx. 10-20 plants per line). Growth and fungal treatment of plants was carried out as described in section 2.2.1 and section 2.2.6.1.

2.2.8 Determination of infection phenotypes

For determination of phenotypes, a disease score (DS) was applied, based on numerical rating of the extent of pathogen colonisation of the host and the severity of host symptoms. DS 0 describes an intact plant with no symptoms or small pin-point brown flecks. Fungal growth was restricted to the penetration attempt of appressoria or to the production of primary biotrophic hyphae. Plants of the DS 1 were mostly intact with necrotic flecks or limited lesions. Secondary necrotrophic hyphae were either absent or very restricted in extent. DS 2 referred to plants with partially collapsed leaves and with

large brown necrotic lesions and some tissue maceration and water-soaked regions on the leaf surface. Partially, the plants collapsed. In limited regions of the plant secondary mycelium could be observed with few acervuli. Plants of the category DS 3 were completely collapsed and the tissue was extensively macerated and water-soaked. Eventually, the plants disintegrated. The plant material got replaced by fungal secondary hyphae that produced acervuli.

For the screening of *Arabidopsis* accessions and their according F_1 progenies, at least five plants per line were inoculated with *C. higginsianum*. Infection phenotypes of resistant accessions and of RIL lines were confirmed by a second, respectively a third independent test with parental accessions as control. Analysis of RIL populations was carried out without microscopical determination of fungal growth.

2.2.9 Nucleic acid extraction

2.2.9.1 RNA extraction from *Arabidopsis*

Total RNA was prepared from three-week-old plant material. Liquid nitrogen frozen samples (approximately 50 mg) were homogenised 2x15 sec to a fine powder using a Mini-Bead-Beater-8^{TM} (Biospec Products) and 1.2 mm stainless steel beads (Roth) in 2 mL centrifuge tubes. After the first 15 sec of homogenisation, samples were transferred back to liquid nitrogen and the procedure was repeated. 1 mL of TRI$^®$ Reagent (Sigma) was added and samples were homogenised by vortexing for 1 min. For dissociation of nucleoprotein complexes the homogenised sample was incubated for 5 min at room temperature. 0.2 mL of chloroform was added and samples were shaken vigorously for 15 sec. After incubation for 3 min at room temperature samples were centrifuged for 15 min at 12000g and 4°C. 0.5 mL of the upper aqueous, RNA containing phase were transferred to a new microcentrifuge tube and RNA was precipitated by adding 0.5 volumes of isopropanol and incubation for 10 min at room temperature. Subsequently, samples were centrifuged for 10 min at 12000g and 4°C. The supernatant was removed and the pellet was washed by vortexing in 1 mL of 75% ethanol. Samples were again centrifuged for 5 min at 7500g and 4°C, pellets were air dried for 10 min and dissolved in 50 µL DEPC-H_2O. All RNA extracts were adjusted to the same concentration with DEPC-H_2O. Samples were stored at -80°C.

2.2.9.2 DNA extraction from *Arabidopsis*

The extraction of DNA from *Arabidopsis* leaf material was performed after the method of (Edwards *et al.*, 1991), modified.

Arabidopsis leaf material (at least 10x10mm^2 leaf surface) was harvested and the samples were frozen immediately in liquid nitrogen until further processing, or kept cool on ice for direct processing. The samples were ground and immediately 400 µL of Edwards buffer was added and incubated for 10-60 min at room temperature. The samples were then centrifuged for 5 min at 13000rpm and the supernatant was transferred into a new 1.5 mL reaction tube and 300 µL of cold (-20°C) 2-Propanol was added and incubated for 2 min at room temperature. This followed centrifugation for 5 min at 13000rpm and the supernatant was discarded. To wash the pellet 300 µL of 70% Ethanol was added and carefully mixed and the samples were then centrifuged for 5 minutes at 13000rpm. The supernatant was discarded and the pellet was dried and then resuspended in 100 µL sterile water.

2.2.9.3 Plasmid preparation

High quality plasmid DNA was isolated using the QIAGEN Midi prep kit, following the manufacture's manual.

2.2.10 Reverse transcription-polymerase chain reaction (RT-PCR)

RT-PCR was carried out in two steps. SuperScript™ II RNase H$^-$ Reverse Transcriptase (Invitrogen) was used for first strand cDNA synthesis by combining 1-1.5 µg template total RNA, 1 µL oligo dT$_{18}$V (0.5 µg/ µL, V standing for an variable nucleotide), 5 µL dNTP mix (each dNTP 2.5 mM) in a volume of 13.5 µL (deficit made up with DEPC-H$_2$O). The sample was incubated at 65°C for 10 min to destroy secondary structures before cooling on ice. Subsequently the reaction was filled up to a total volume of 20 µL by adding 4 µL of 5x reaction buffer (supplied with the enzyme), 2 µL of 0.1M DTT and 0.5 µL reverse transcriptase. The reaction was incubated at 42°C for 60 min before the enzyme was heat inactivated at 70°C for 10 min. For subsequent normal PCR, 1 µL of the above RT-reaction was used as cDNA template. As template, total RNA for the reverse transcription reaction was not DNase treated, a control reaction for each RNA preparation was performed in which the reverse transcription reaction was incubated without reverse

transcriptase enzyme (enzyme replaced by equal volume of DEPC-H_2O) to check in the following PCR for contamination by genomic DNA.

2.2.11 Polymerase chain reaction (PCR)

Amplification of specific DNA fragments was carried out by PCR. Template DNA from a variety of sources was used. The amount of the template DNA was dependent on the reaction. The reaction was repeated for the appropriate number of cycles using the following conditions.

Reaction mix:

10x PCR buffer	2 µL
2.5 mM dNTPs	2 µL
1 mM primer forward	1 µL
1 mM primer reverse	1 µL
Taq-DNA-Polymerase	0.5 µL
DNA template	1 µL
Added to 20 µL with sterile H_2O	

PCR-cycler conditions:

94 °C 3 min
(94 °C 15 sec, 56 °C 30 sec, 72 °C 30 sec) 10x
(94 °C 30 sec, 53 °C 30 sec, 72 °C 30 sec) 27x
72 °C 10 min
15 °C 5 min

2.2.12 Restriction endonuclease digestion of DNA

Restriction digests were carried out using the manufacture´s recommended conditions. Typically, reactions were carried out in 0.5 mL tubes, using 1 µL of restriction enzyme per 10 µL reaction. All digests were carried out at the appropriate temperature for a minimum of 1 h.

2.2.13 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments according to their size. 12 µL of PCR products were loaded in 6x loading dye on a 4% agarose gel for mapping with PCR markers, otherwise on a 1% agarose gel. Electrophoresis was performed in 1x TAE buffer at 130 V depending on the fragment size and the separation needed. DNA fragments were visualised by staining with Ethidium bromide and could be detected in a transilluminator.

2.2.14 DNA sequencing

DNA sequences were determined by the Automatisches DNA-Isolierung und Sequenzierung (ADIS-Unit) at the MPIZ on Applied Biosystems (Weiterstadt, Germany) Abi Prism 377 and 3700 sequences usig Big Dye-terminator chemistry (Sanger *et al.*, 1977).

2.2.15 Sequence alignment and analysis

Trace files of sequence chromatograms obtained with forward and reverse primers were aligned for each accession and analysed with DNASTAR program of the Lasergene software (see 2.1.8).

2.2.16 Microscopical analysis

2.2.16.1 Determination of penetration efficiency

At least four leaves of three-week-old plants per genotype were inoculated with a 5×10^5 spores/ mL concentrated spore suspension of *C. higginsianum* and harvested at 2 and 3 days after inoculation. Leaves were destained in ethanol:chloroform (3:1) and solution and mounted on slides in lactophenol. Between 200-600 appressoria, equally distributed between the sampled leaves, were counted per genotype and analysed for a successful hyphae production.

2.2.16.2 3-3'Diaminobenzidine (DAB) staining for hydrogen peroxide accumulation

At least four leaves of three-week-old plants per genotype were inoculated with a 5×10^5 spores/ mL concentrated spore suspension of *C. higginsianum* and harvested at 2 and 3

days after inoculation. Leaf-stems were immersed in DAB-staining solution and allowed to take up DAB solution through their petiole under high humidity conditions in darkness overnight. Then the leaves were transferred into glass vials and destained in methanol overnight, followed by an overnight incubation in chloral hydrate. Chloral hydrate was replaced with 70% glycerol and the leaves were mounted in 70% glycerol solution. Between 200-600 appressoria, equally distributed between the sampled leaves, were counted per genotype and analysed for a successful hyphae production and presence of a DAB staining.

2.2.16.3 Aniline Blue staining of callose deposition

At least four leaves of three-week-old plants per line were inoculated with a $5x10^5$ spores/ mL concentrated spore suspension of *C. higginsianum* and harvested at 2 and 3 days after inoculation. After harvest, the leaves were decolourised in ethanol:chloroform (3:1) overnight, followed by lactophenol incubation overnight. Leave samples were re-hydrated by transfer to 75%, then 50% and 25% lactophenol in water, each incubation step for 15 minutes, and was then followed by two changes of pure sterilised water. Samples were stained with Aniline Blue solution for 24-72 h at 4°C and mounted in Aniline Blue solution. The samples were analysed microscopically under UV-light excitation. Between 200-600 appressoria, equally distributed between the sampled leaves, were counted per genotype and analysed for a successful hyphae production and presence of Aniline Blue staining.

2.2.16.4 Quantification of *C. higginsianum* sporulation

To determine sporulation levels, plants were harvested 4 and 5 days after inoculation in a 50 ml Falcon tube and vortexed vigorously in 10 ml water for 15 sec. 10 µl of this spore suspension were removed twice and spores were counted under a light microscope using a Neubauer counting cell chamber. For each tested *Arabidopsis* genotype, three pots, each containing 9 plants, were inoculated per experiment and harvested spores from all plants of each pot were counted with sporulation levels expressed as the number of spores per gram fresh weight.

2.2.17 Quantification of hyphal growth by enzyme-linked immunosorbent analysis (ELISA)

For each tested *Arabidopsis* genotype, 1 g of inoculated plant tissue was harvested for three replicates per genotype per experiment. ELISA was conducted following manufacturer's manual (ADGEN).

2.2.18 Mapping

2.2.18.1 Positional cloning with PCR-based molecular markers

F_2 plants were grown, inoculated and phenotyped as described in section 2.2.1, 2.2.6.1 and 2.2.8. For a first localisation of the target gene in the genome, DNA of 26 resistant and 10 susceptible plants of a Ler-0 x Ws-0 F_2 mapping population was analysed with 27 PCR-based molecular markers, distributed throughout the whole genome, of the marker set published at http://www.inra.fr/internet/Produits/vast/msat.php and the SSLP marker set described by Lukowitz *et al*. (2000). Following a detection of a putative association with markers to one arm of one chromosome, additional F_2 plants were phenotyped, and plants exhibiting a resistant infection phenotype were genotyped with PCR-markers located on the identified arm of the chromosome to identify recombinants. For this, additional molecular markers (SSLP, CAPs and dCAPs) were designed (see 2.1.8), utilised for genotyping with progressively closer markers until no further recombinants were found. The F_3 progeny (15-20 plants per line) originating from F_2 plants that are showing a recombination event for one of the markers flanking the region of interest, were phenotyped to confirm the targeted gene region.

2.2.18.2 QTL mapping and analysis

The RIL populations and their respective parental accessions were grown in 96-well trays as described in section 2.2.1. At an age of three weeks, the plants were inoculated with *C. higginsianum* (see 2.2.6.1) to determine infection phenotypes and to assign them disease scores (DS) (see 2.2.8). Each RIL line was tested in two independent experiments, three plants per experiment. Broad sense heritability (h^2) was estimated for each trait by using the following equation:

$h^2 = VG/(VG + VE)$

where VG is the variance between RILs and VE is the variance within RILs. QTL detections were performed by using the software package MapQTL 5[®] (Kyazma B.V., Wageningen, Netherlands) (see 2.1.8) as described in its reference manual (http://kyazma.nl). In a first step, QTL were detected by interval mapping to determine the putative QTL involved in the variation of the considered trait. Thereafter, the closest marker to each local logarithm-of-odds (LOD) score peak was determined as a cofactor (Van Ooijen and Maliepaard, 1996; Van Ooijen *et al.*, 2000). LOD threshold values applied to declare the presence of QTL were estimated by performing permutation tests implemented in MapQTL version 5.0. On average, the threshold obtained ($\alpha = 5\%$) corresponds to a 2.5 LOD. The additive effects of the detected QTL were estimated from composite interval mapping results. The contribution of each detected QTL to the total variance (R^2) was estimated by variance component analyses.

3 Results

3.1 Analysis of *Arabidopsis* loss-of-susceptibility mutants

3.1.1 Screen of *Arabidopsis* mutant pools for loss of susceptibility

To identify host genes involved in the establishment of a successful *C. higginsianum* infection, *Arabidopsis* mutants were screened for loss of susceptibility to the fungus. I hypothesised that a mutation in a plant susceptibility factor which is essential for the support of pathogenesis of *C. higginsianum* in *Arabidopsis* should result in loss of susceptibility, *i.e.* gain of resistance. Therefore, different lines of a susceptible genetic backgrounds, *i.e.* Ler *rar1*-13, Ler *eds1*-2 mutants and Ler-0 wild type, were chemically mutagenised with EMS treatment. Seeds of Ler-0 wild type mutated by γ-radiation treatment were kindly provided by Dr. E. van der Vossen (Wageningen, NL).

Due to the stringent conditions of the inoculation process, *i.e.* young developmental stage of plants and high inoculum concentration, only plants showing a gain of resistance had survived seven days after spray-inoculation with *C. higginsianum* (Fig. 3.1). The mutant plants with a susceptible infection phenotype were strongly macerated or completely collapsed. This allowed a rapid screen of a large number of plants.

Figure 3.1: Forward genetic screen of EMS mutagenised *Arabidopsis* M$_2$ plants seven days after inoculation with *Colletotrichum higginsianum*.
Arabidopsis Ler-0 was mutagenised by ethylmethane sulfonate (EMS) treatment. Presented are the infection phenotypes of three-week-old M$_2$ plants, seven days after inoculation with *C. higginsianum*.

Approx. 65,500 ethylmethane sulfonate (EMS) -mutagenised Ler-0 M_2 plants obtained commercially from Lehle seeds (USA), were screened by spray-inoculation of two-week-old plants with *C. higginsianum* spore suspension. Forty-five plants were found to exhibit reduced susceptibility at five days after inoculation. However, subsequently these were identified not to be Ler-0 genetic background, probably due to seed contamination while generation. Their intermediate susceptible phenotypes were in accordance with the infection phenotype identified for the Col-0 accession (Fig. 3.7). In addition, 22,000 EMS mutants, generated in a Ler *rar1*-13 mutant background by Dr. P. Muskett (Cologne, Germany), were tested for their infection phenotypes. The *rar1*-13 mutation did not affect susceptibility to *C. higginsianum* (data not shown). I also generated an EMS mutant pool in the Ler *eds1*-2 mutant background. The mutation in the *EDS1* gene rendered the plants more susceptible to *C. higginsianum* than the Ler-0 wild type (Fig. 3.2) (Liu *et al.*, 2007a).

Figure 3.2: Macroscopic infection phenotype of *Arabidopsis* Ler-0 wild-type and Ler *eds1*-2 mutant plants six days after inoculation with *Colletotrichum higginsianum*.
Three-week-old *Arabidopsis* Ler-0 wild-type and Ler *eds1*-2 mutant plants were spray-inoculated with *C. higginsianum*. Presented is the macroscopic infection phenotype at 6 dpi. Bar, 2 cm.

Therefore, this mutant background was chosen for re-mutagenesis in order to facilitate a clear distinction between susceptible and resistant phenotypes in the mutant screen, and 96,000 plants of this mutant pool were screened. A further 24,000 plants of the Ler-0 γ-radiation mutant pool were tested for a loss of susceptibility. Hence, a total number of ~ 207,500 mutagenised M_2 plants were screened (Tab. 3.1) from different genetic backgrounds that were all highly susceptible to *C. higginsianum*. Of these, 309 lines were identified in the primary screen as having reduced susceptibility compared to wild type. These plants were grown on for seed production and their phenotypes were retested in the

M_3 generation. 27% of the candidates were found not to be in the expected Ler-0 genetic background (see above) and were therefore excluded, 29% of the lines had either not produced seeds or the seeds did not germinate so that further analysis was not feasible. The phenotypes of 201 candidate lines were tested in the M_3 generations in two independent infection experiments with *C. higginsianum*. For 52% of the initial candidate lines, reduced susceptibility could not be confirmed in the following generation, as all infected M_3 plants exhibited a wild-type phenotype. Identification of reduced susceptibility in the M_2 generation may have resulted from variation in the inoculation conditions, *e.g.* low humidity or incomplete coverage of the plants with spore-suspension during inoculation. For 38 M_2 lines, a loss of susceptibility could be confirmed in the M_3 generation. Most of the identified candidates showed only a moderate loss of susceptibility, as inoculation with *C. higginsianum* still resulted in the development of necrotic lesions on the leaves.
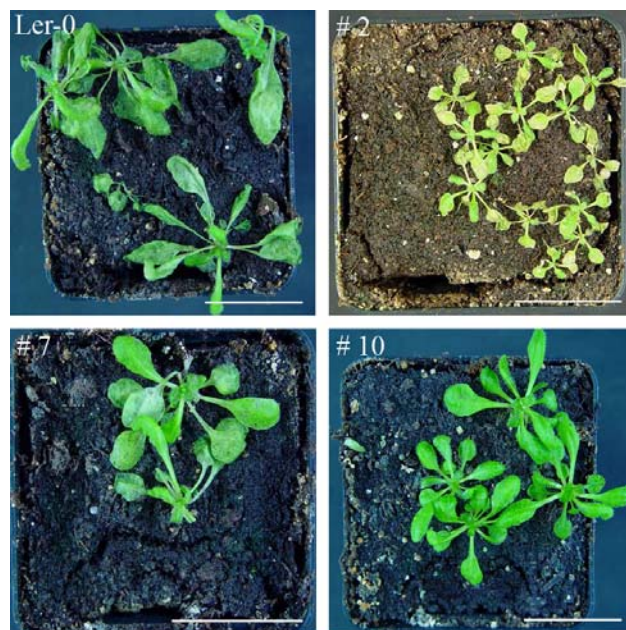
Figure 3.3: *Arabidopsis* Ler-0 wild type and EMS mutant plants five days after inoculation with *Colletotrichum higginsianum*.

Arabidopsis Ler-0 was mutagenised with ethylmethane sulfonate (EMS). Presented are the macroscopic phenotypes of four-week-old *Arabidopsis* Ler-0 wild type and EMS mutant lines (designated as #2, #7 and #10) five days after inoculation with *C. higginsianum*. Bar, 2 cm.
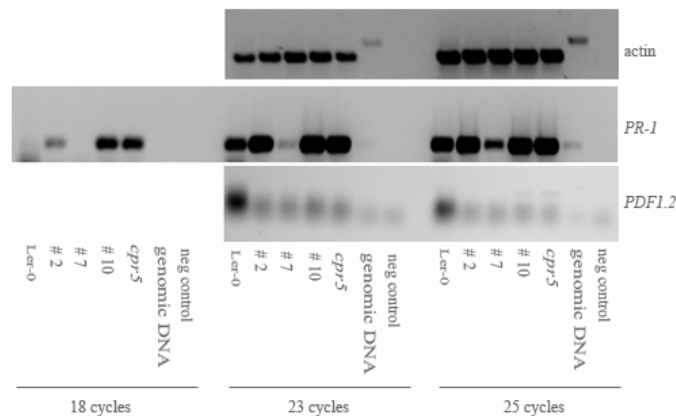
Figure 3.4: Expression analysis of *PR-1* and *PDF1.2* in uninoculated *Arabidopsis* Ler-0 wild-type, EMS mutants and the *cpr5* mutant.

Total RNA was extracted from three-week-old, uninoculated *Arabidopsis* Ler-0 wild-type plants, EMS mutant plants (designated as #2, #7 and #10) and *cpr5* mutant plants and the expression of the marker genes *PR-1* and *PDF1.2* was analysed by semi-quantitative RT-PCR. Equal application of template RNA for the reverse transcription reaction was shown by a control PCR reaction detecting plant *actin*. Numbers of cycles for each PCR reaction are indicated below.

In contrast, plants of the mutant lines designated #2 and #10 in a Ler-0 background exhibited much less severe disease symptoms than the other candidate mutants (Fig. 3.3). The observed loss of susceptibility of the putative mutants was expected to result from either loss of a susceptibility factor or from constitutive or enhanced expression of plant-defence resistance mechanisms, as found for the *cpr*, *edr* and *acd* mutants (Glazebrook, 2001). To test whether plant defence pathways were constitutively activated, the expression levels of marker genes for the SA and the JA/ET defence pathways, namely *PR-1* (Santamaria *et al.*, 2001) and *PDF1.2* (Penninckx *et al.*, 1996), were determined in uninoculated plants of the mutant lines #2 and #10. In addition, mutant #7 was analysed, which showed reduced susceptibility, but to a lesser extent than lines #2 and #10 (Fig. 3.3). The *PR-1* and *PDF1.2* expression levels of uninoculated mutant plants was compared to the expression in uninoculated wild type Ler-0 plants and the *cpr5* mutant, for which a constitutively increased *PR-1* level had been demonstrated (Bowling *et al.*, 1997) (Fig. 3.4). *cpr5* also exhibited an unexpected but faint increase of *PDF1.2* expression. The level of *PR-1* expression in the mutant line #10 was similar to that of the *cpr5* mutant. The increased *PR-1* expression suggests identification of a mutant with a constitutive

activation of defence pathways. In accordance with this finding, mutant #10 also exhibited increased resistance to the oomycete pathogen *H. parasitica* (virulent isolate Cala2), as determined by reduced sporulation of the pathogen and increased plant hypersensitive cell death responses (data not shown). However, these results need to be confirmed due to increased *PDF1.2* expression in *cpr5* that is in contrast to previous studies. An elevated level of *PR-1* expression was also detected for the mutant #2, hinting to effects of the mutation on the SA defence pathway rather than a host susceptibility factor. In contrast, the mutant #7 did not exhibit an increased expression of either *PR-1* or *PDF1.2*, suggesting that the gain of resistance may be the result of a mutation in a *bona fide* susceptibility factor. The expression of *PR-1* and *PDF1.2* was in fact lower than the expression level of these genes in the unchallenged wild type Ler-0 plants. Therefore, repression of one defence pathway due to a mutual antagonism between the SA and JA/ET defence pathways can probably be excluded. The reduction of susceptibility to *C. higginsianum* in mutant #7 (Fig. 3.4), as well as for the additional 35 candidates that have been identified in the initial screen of the M_2 populations, was not sufficiently clear-cut to allow a reliable identification of the mutant phenotype during a mapping process. As this was a prerequisite for the identification of the mutated locus, mapping could not be carried out for any of these initial candidates. Therefore, the screen of EMS and γ-radiation induced mutant lines did not result in the identification of loss-of-susceptibility mutants that allowed a subsequent identification of putative susceptibility factors.

Table 3.1: Screen of *Arabidopsis* plants mutagenised with EMS and γ-radiation treatment for reduced susceptibility to *C. higginsianum*

Total number of tested M_2 plants	207,000
Number of initial candidates in M_2 generation	309
No germination/ no seeds of M_3 generation	90
Wild-type phenotype in M_3 generation	163
Reduced susceptibility in M_3 generation	38
Increased *PR-1* expression	2

M_2 plants of mutagenised pools were sprayed with *C. higginsianum* spore suspension and screened for reduced fungal susceptibility at five days after inoculation. The phenotype of each M_2 line was then tested by two independent infection assays in the M_3 generation.

3.1.2 Phenotypic analysis of *dmr Arabidopsis* mutants

In a forward genetics approach, the *Arabidopsis* downy mildew resistant (*dmr*) mutants were isolated by van Damme *et al.* (2005) (see 1.4.4). As in the present study, the authors aimed to identify plant genes required for disease susceptibility, but to the obligate biotrophic pathogen, the oomycete *H. parasitica*. Similarly to the screen described in section 3.1.1, seeds of the susceptible genotype Ler *eds1*-2 were re-mutagenised to induce mutations in potential susceptibility factors that were expected to result in reduced or complete loss of susceptibility to *H. peronospora*. The mutants *dmr1–dmr6* were found to exhibit a significantly reduced susceptibility to *H. parasitica* (Van Damme *et al.*, 2005) (see 1.4.4). It was of interest to determine whether other pathogen species that exhibit an intracellular biotrophic lifestyle similar to *H. parasitica*, require at least some of the specific plant-pathogen interaction mechanisms identified for *Arabidopsis* and *H. parasitica* (Van Damme *et al.*, 2005; Van Damme, 2007; van Damme *et al.*, 2008). As the infection strategy of the hemibiotrophic ascomycete *C. higginsianum* includes an intracellular biotrophic phase, mutations in the *DMR* genes might cause at least a partial loss of susceptibility to *C. higginsianum*. In order to test this, the infection phenotypes of the mutants *dmr1*-1, *dmr1*-2, *dmr1*-3, *dmr1*-4, *dmr2*, *dmr5* and *dmr6* were analysed macroscopically and microscopically following *C. higginsianum* inoculation (Fig. 3.5). The mutants *dmr1*-1, *dmr1*-2, *dmr1*-3, *dmr2 and dmr5* showed an infection phenotype that was indistinguishable from that of the genetic background line Ler *eds1*-2. For all these mutant lines the plants were strongly affected by the fungal growth, as shown by a partial or complete collapse of the plants at 8 dpi (Fig. 3.5 a). Fungal growth, visible by the constitutive expression of the Green Fluorescence Protein (GFP) in the mycelium of *C. higginsianum*, has a similar extent in these genotypes at three days after inoculation with *C. higginsianum* (Fig. 3.5 b). In contrast, the mutant lines *dmr1*-4 and *dmr6* exhibited a clearly reduced susceptibility to *C. higginsianum* infection. Fungal colonies observed in the leaves of both mutant lines were few and restricted in size and the extent and number of water-soaked lesions produced were similarly reduced compared to Ler *eds1*-2 plants infected by *C. higginsianum*. Therefore, I concluded that the plant factors affected in *dmr1*-4 and *dmr6* are not exclusively required for the infection of *Arabidopsis* by *H. parasitica*, but also for the pathogenesis of *C. higginsianum* in its host. Interestingly, *dmr1*-4 was the only allele of four *dmr1* mutant alleles that showed loss of susceptibility.

a   b

Ler *eds1-2*

dmr1-1

dmr1-2

dmr1-3

dmr1-4

dmr2

dmr6
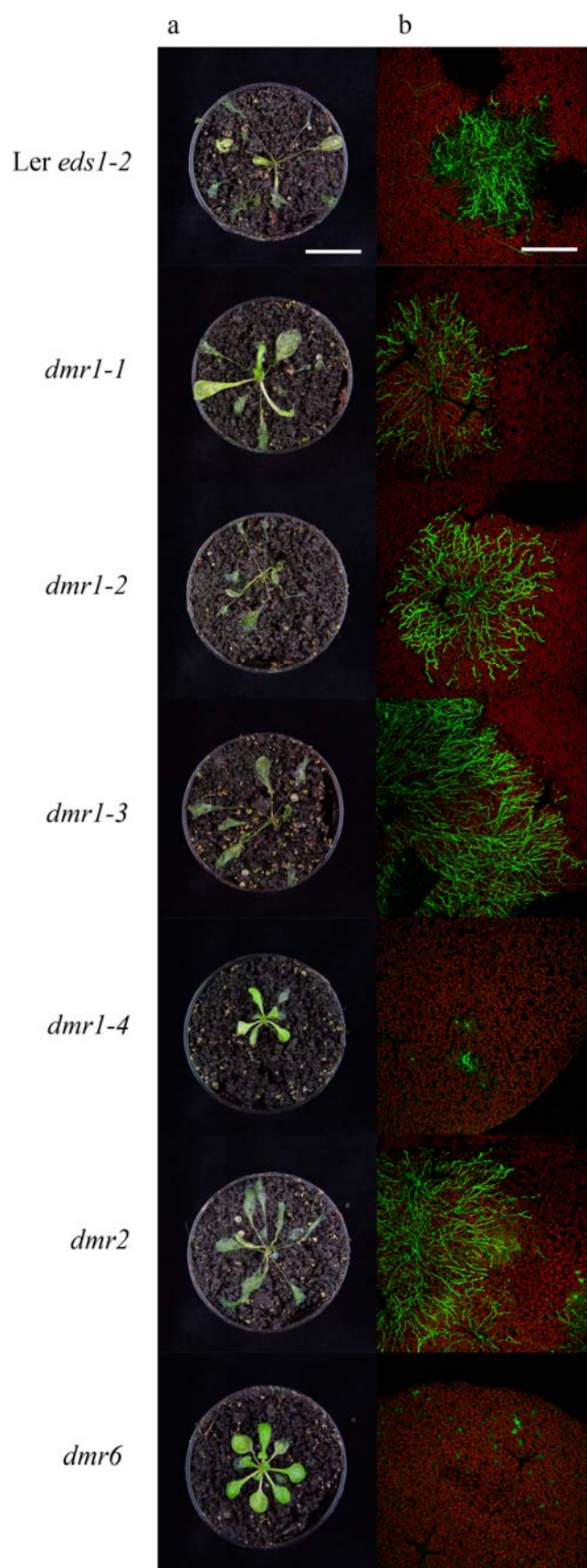
Figure 3.5: Macroscopic and microscopic infection phenotypes of *Arabidopsis* Ler *eds1-2* and *dmr* mutant plants after inoculation with *Colletotrichum higg*insianum.

(a) Column shows four-week-old *Arabidopsis* Ler *eds1-2* and *dmr* mutant plants eight days after inoculation with *C. higginsianum*. Bar, 3 cm.

(b) The images show the overlay of a projection of 15-20 confocal micrographs taken in the green and red fluorescence channel. Leaf samples of three-week-old *Arabidopsis* mutant plants were taken three days after inoculation with a *C. higginsianum* strain that constitutively expresses GFP. Fungal colonies are visible by the green fluorescence of the GFP expressed in the cytoplasm of fungal hyphae. Intact chlorophyll of living plant cells is visible by its red autofluorescence. Bar, 300 μm.

3.1.3 Phenotypic analysis of *pmr* mutants

The *Arabidopsis* powdery mildew resistant (*pmr*) mutants exhibit enhanced resistance to *G. cichoracearum*, the powdery mildew of cruciferous plants that also colonises *Arabidopsis*. Six *pmr* loci (*pmr1-pmr6*) have been identified in an EMS-mutant screen for loss of fungal sporulation (Vogel and Somerville, 2000) (see 1.4.3).

As with the analysis of response of the *dmr* mutants to *C. higginsianum* infection, my aim was to test whether the susceptibility factors affected in the *pmr* mutants are specific for the interaction of *G. cichoracearum* with *Arabidopsis*, or whether susceptibility to powdery mildew shares some features with susceptibility to *C. higginsianum*. Therefore, the mutant lines *pmr2-pmr6* were inoculated with *C. higginsianum* and their macroscopic infection phenotypes were compared with the infection phenotype of wild type Col-0 (Fig. 3.6). Plants of the mutant lines *pmr5* and *pmr6* exhibited a reduced extent of necrotic lesions eight days after inoculation with *C. higginsianum* in comparison to Col-0. Fungal growth in plants of the *pmr4* lines was slightly, reduced, however a precise evaluation of a possible reduction of susceptibility needs further careful analysis. The infection phenotype of *pmr2* mutant plants was not distinguishable from that of Col-0 wild type. Plants of the *pmr3* mutant lines exhibited a slight increase in susceptibility compared to Col-0 plants.

In conclusion, the plant factors affected in the *pmr4*, *pmr5* and *pmr6* lines are not specific for the interaction of *Arabidopsis* with powdery mildews but appear to be similarly important for the interaction with *C. higginsianum*. The mutation in *pmr3* supported stronger *C. higginsianum* growth. The plant factor affected in *pmr2* was shown not to be essential for host infection by *C. higginsianum*.
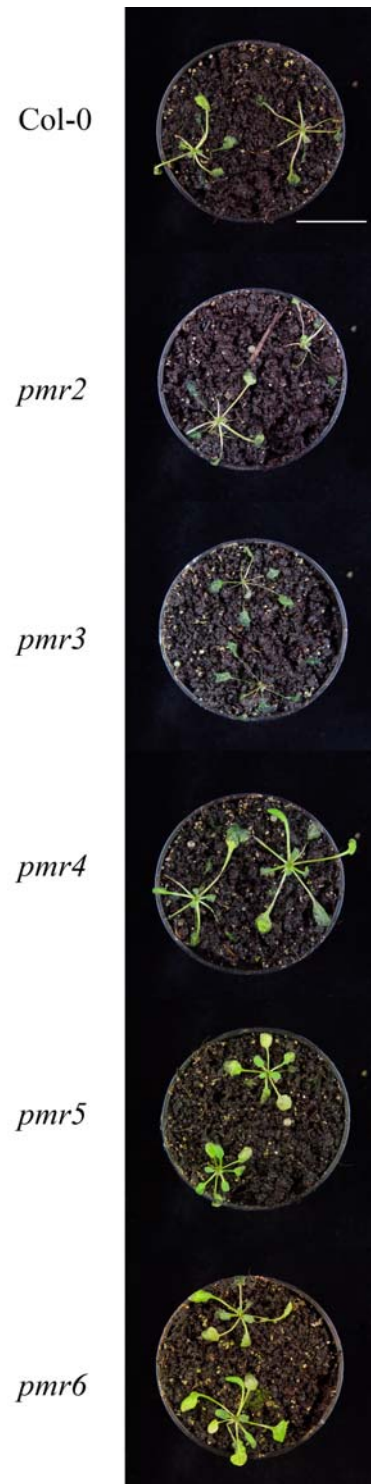
Figure 3.6: Macroscopic infection phenotypes of the *Arabidopsis pmr* mutant plants after inoculation with *Colletotrichum higginsianum*.

Column shows four-week-old Col-0 wild type and *pmr1-pmr6* mutant plants seven days after inoculation with *C. higginsianum.* Bar, 3 cm.

3.2 Natural variation in *Arabidopsis* response to *C. higginsianum*

The initial forward genetic screen of EMS and γ-radiation induced mutant lines did not result in the identification of loss-of-susceptibility mutants with a marked reduction of susceptibility that allowed a subsequent localisation of susceptibility factors. Therefore, the second approach of this study, likewise aiming to identify determinants of compatibility between *Arabidopsis* and *C. higginsianum,* was based on natural variation of *Arabidopsis* accessions in response to *C. higginsianum* inoculation.

3.2.1 Identification of natural variation of *Arabidopsis* accessions in response to *C. higginsianum* infection

C. higginsianum was described to infect and complete its asexual life cycle on *Arabidopsis* (Narusaka *et al.*, 2004; O'Connell *et al.*, 2004). The infection strategy follows the two-stage, hemibiotrophic pattern with a brief initial biotrophic phase associated with primary hyphae, eventually followed by a switch to the necrotrophic stage, associated with the production of secondary hyphae and formation of necrotic lesions. However, within the scope of this study, I observed that *Arabidopsis* accessions showed wide variation in the interaction phenotypes upon *C. higginsianum* infection (Fig. 3.7). While plants of some accessions remained mostly intact, with only a few necrotic flecks or no visible symptoms and almost no production of secondary necrotrophic hyphae, plants of other accessions collapsed totally five to six days after inoculation and the tissue contained extensive secondary mycelium growth. In other accessions, an intermediate infection phenotype could be observed.
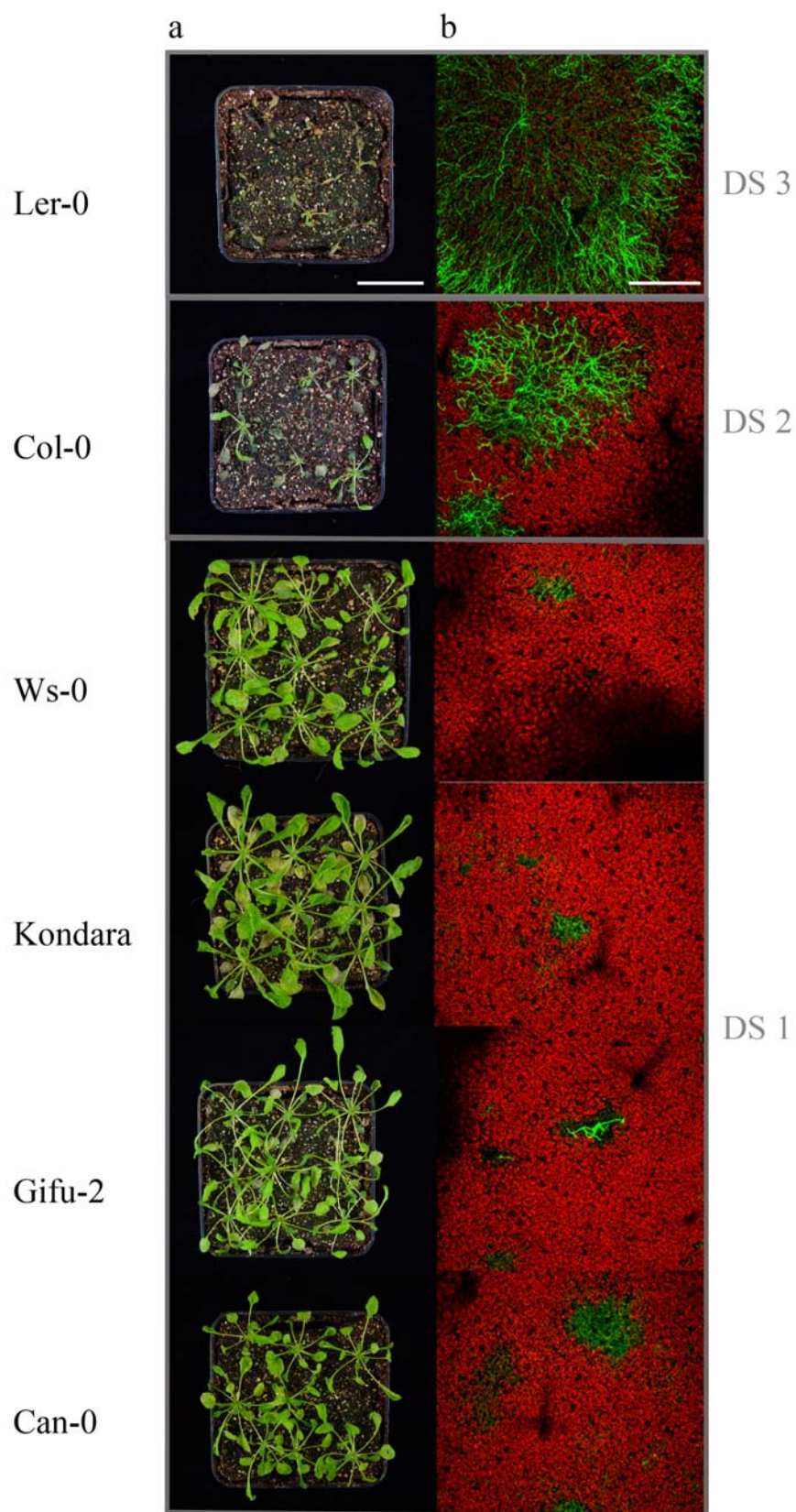
Figure 3.7: Macroscopic and microscopic infection phenotypes of selected *Arabidopsis* accessions after inoculation with *Colletotrichum higginsianum* and their classification into disease scores (DS) 1-3.

(a) Column shows four-week-old *Arabidopsis* plants nine days after inoculation with *C. higginsianum*. Bar, 4.5 cm.

(b) The images show the overlay of a projection of 15-20 confocal micrographs taken in the green and red fluorescence channel. Leaf samples of three-week-old *Arabidopsis* plants were taken three days after infection with a *C. higginsianum* strain that constitutively expresses GFP. Fungal colonies are visible by the green fluorescence of the GFP expressed in the cytoplasm of fungal hyphae. Intact chlorophyll of living plant cells is visible by its red autofluorescence. Bar, 300 μm.

The six presented *Arabidopsis* accessions were allocated a disease score (DS), based on the combined macroscopical and microscopical observations of the plant response to fungal infection as follows:

DS 1, resistant - plants mostly intact with only limited lesions, necrotrophic secondary hyphae are mostly absent or very restricted in extent.

DS 2, intermediate - plants partially collapsed with necrotic lesions and some tissue maceration and water-soaking, extensive necrotrophic mycelium.

DS 3, fully susceptible - plants completely collapsed and tissue extensively macerated and water-soaked, extensive necrotrophic mycelium.

To survey the extent of natural variation among *Arabidopsis* accessions in detail and to eventually identify the molecular components conferring resistance or susceptibility to *C. higginsianum*, the first step was to determine the infection phenotypes of a large number of accessions from different geographical regions and ecological habitats.

A numerical disease score (DS) was applied (see 2.2.8 and Fig. 3.7), based on the extent of pathogen colonisation of the host and the severity of host symptoms in order to analyse 116 *Arabidopsis* accessions (Tab. 3.2). This compilation includes accessions of the Nordborg collection (Nordborg *et al.*, 2005) and the nested core collection (McKhann *et al.*, 2004). The Nordborg collection of accessions was compiled to investigate patterns of polymorphism of *Arabidopsis* accessions and includes frequently used stock centre accessions as well as samples from natural populations. The core collection designed by McKhann *et al*. (2004) was generated based on the results of a polymorphism survey of a worldwide collection of accessions that cover the range of known ecological and geographical habitats. It also contains accessions such as CVI and Shah that have been collected at the edge of the natural distribution of this species and have been shown to be distantly related to other accessions (McKhann *et al.*, 2004). This collection was designed to offer in a minimum of accessions the maximum possible genetic diversity within the

species. The present study furthermore included lines from international stock centres, *i.e.* NASC and SASSC (see 2.1.5.2).

Table 3.2: *Arabidopsis* accessions, their origin and infection phenotypes

Accession	Origin	Disease Score (DS)
Ag-0	Argentat/France	0
An-1	Antwerp/Belgium	1-2
Bay-0	Bayreuth/Germany	3
Bil-5	Billaberget/Sweden	0-1
Bl-1	Bologna/Italy	0-1
Bla-1	Blanes/Spain	2
Bor-1	Borky/Czech Republic	2
Bor-4	Borky/Czech Republic	2
Bur-0	Burren/Eire	3
C24	Coimbra/Portugal	2-3
Can-0	Canary Islands	0-1
Col-0	Columbia/USA	2-3
Ct-1	Catania/Italy	2
CVI	Cape Verdi Islands	3
Eden-1	Eden/Sweden	1
Eden-2	Eden/Sweden	1-2
Edi-0	Edinburgh/UK	2
Ei-2	Eifel/Germany	1-2
Eil-0	Eilenburg/Germany	0-1
En-T	Usmanov Lab/Tajikistan	1-2
Est-1	Estland/Russia	2
Fab-2	Faberget/Sweden	1
Fab-4	Faberget/Sweden	1-2
Fei-0	St. Maria d. Feiria/Portugal	0-1
Ga-0	Gabelstein/Germany	1
Gifu-2	Gifu/Japan	0-1
Gre-0	Greenville, MI/USA	2
Gy-0	La Minière/ France	2
HR-5	Ascot/UK	2
HR-10	Ascot/UK	1
In-0	Innsbruck/Austria	1-2
Jm-0	Jamolice/Czech Republic	2
Kas-2	Kashmir/India	1-2
Kn-0	Kaunas/Lithuania	2
Knox-10	Knox, IN/USA	2
Knox-18	Knox, IN/USA	2
Kondara	Khurmatov/Tajikistan	0-1
Kyoto	Kyoto/Japan	2
Kz-1	Kazakhstan	1
Kz-9	Kazakhstan	1-2
Ler-0	Landsberg, Warthe/Poland	3
Ler-1	Landsberg, Warthe/Poland	3

Table 3.2: continuation

Accession	Accession	Accession
Lip-0	Lipowiec/Poland	2-3
Lov-1	Lovvik/Sweden	2-3
Lov-5	Lovvik/Sweden	1
Lp2-2	Lipovec/Czech Republic	2-3
Lp2-6	Lipovec/Czech Republic	1-2
Lz-0	Lezoux/France	1-2
Mrk-0	Markt/Germany	2-3
Ms-0	Moscow/Russia	2
Mt-0	Martuba//Libya	2-3
Mz-0	Merzhausen/Germany	1
N6 Karelian	Karelian region/Russia	0-1
N7 Pinguba	Pinguba/Russia	0-1
Nd-1	Niederzenz/Germany	1
NFA-8	Ascot/UK	2
NFA-10	Ascot/UK	3
Nok-1	Noordwijk/Netherlands	2
Omo2-1	Ostra Mocklo/Sweden	0-1
Omo2-3	Ostra Mocklo/Sweden	0-1
Oy-0	Oystese/Norway	1
Pa-1	Palermo/Italy	2
Pi-0	Pitztal/Austria	2
Pna-10	Benton Harbor/USA	1-2
Pna-17	Benton Harbor/USA	0
Pro-0	Proaza/Spain	1
Pu2-7	Prudka/Czech Republic	1
Pu2-23	Prudka/Czech Republic	1
PYL-1	Le Pyla/France	1-2
Ra-0	Randan/France	2
RAN	Cale de Mordeuc/France	2-3
Ren-1	Rennes/France	0-1
Ren-11	Rennes/France	1
Ri-0	Richmond B.C./Canada	2-3
Rld-2	Rschew/Russia	2
Rmx-A02	St. Joseph/USA	1
Rmx-A180	St. Joseph/USA	1
RRS-7	North Liberty/USA	0-1
RRS-10	North Liberty/USA	0-1
Rubezhnoe-1	Rubezhnoe/Ukraine	1-2
Sakata	Sakata/Japan	2-3
Sap-0	Sapporo/Japan	2
Se-0	San Eleno/Spain	3
Sendai-1	Sendai/Japan	0-1
Sendai-3	Sendai/Japan	0-1
Sendai-4	Aoba-Ku/Japa	0-1
Shah	Palmiro-Alay/Tajikistan	2
Sorbo	Tadjikistan	1-2
Sp-0	Berlin/Germany	0-1
Spr1-2	Spratteboda/Sweden	2

Table 3.2: continuation

Accession	Accession	Accession
Spr1-6	Spratteboda/Sweden	2
Sq-1	Ascot/UK	1
Sq-8	Ascot/UK	2
St-0	Stockholm/Sweden	0-1
Stw-0	Stobowa/Russia	1
Ta-0	Tabor/Czecg Republic	2
Tamm-2	Tammisari/Finland	1
Tamm-27	Tammisari/Finland	0-1
Te-0	Tenela/Finland	0-1
Ts-1	Tossa der Mar/Spain	0-1
Ts-5	Tossa der Mar/Spain	0-1
Tsu-1	Tsu/Japan	2-3
Ull2-3	Ullstorp, Sweden	1
Ull2-5	Ullstorp, Sweden	2
Uod-1	Ottenhof/Austria	2-3
Van-0	Vancouver/Canada	2
Var2-1	Vancouver/Canada	3
Var2-6	Vancouver/Canada	0-1
Wa-1	Warsaw/Poland	2-3
Ws-0	Wassilewskija/Russia	0-1
Ws-2	Wassilewskija/Russia	1-2
Wt-5	Wietze/Germany	2-3
Yam	Yamagata/Japan	0-1
Yo-0	Yosemite Nat.Park/USA	2
Zdr-1	Zdarec/Czech Republic	0-1
Zdr-6	Zdarec/Czech Republic	2

Plants were inoculated with *C. higginsianum* and analysed microscopically at three days after inoculation and macroscopically at six days after inoculation. The disease score (DS) is based on the combined macroscopic and microscopic observations:

DS 0, fully resistant - plants remain intact with only small necrotic lesions, no hyphae present or only biotrophic hyphae without necrotrophic secondary hyphae

DS 1, intermediate resistant - plants mostly intact with only limited lesions, secondary hyphae are mostly absent or very restricted in extent

DS 2, intermediate susceptible - plants partially collapsed with large necrotic lesions and some tissue maceration and water-soaking, extensive secondary mycelium, sporulation rarely seen

DS 3, fully susceptible - plants completely collapsed and tissue extensively macerated and water-soaked, extensive secondary mycelium with abundant spore production

Of the 116 accessions tested, 41% exhibited a resistant phenotype (DS 0, DS 0-1 and DS 1), 38% an intermediate infection phenotype (DS 1-2 and DS 2) and 20% had a susceptible interaction phenotype (DS 2-3 and DS 3). Only 8% of the accessions tested showed a highly susceptible phenotype (DS 3) after inoculation with *C. higginsianum.*

Due to the missing information about exact geographical origins, *i.e*. coordinates or habitats, for many of the accessions tested, analysis of a correlation between infection phenotypes and geographical origin was based on classification of accessions to countries of origin (Tab. SD.1). However, a more precise analysis for detection of possible correlation remains to be carried out in the future when the missing information will be available. Based on the present data, there was no correlation between infection phenotypes and geographical origins of the accessions identified.

3.2.2 Genetic analysis of the inheritance of *C. higginsianum* resistance

In order to identify the mode of inheritance of resistance in selected *Arabidopsis* accessions, resistant accessions were crossed to the highly susceptible accession Ler-0 (Tab. 3.3). This accession was selected as the susceptible parent, because it has been investigated in genetic analyses and sequencing projects. The focus of this study was on the identification of recessive resistance, conferred by the lack of a functional copy of a host susceptibility factor (see 1.4). I hypothised that the introgression of a dominant susceptibility factor by crossing a susceptible accession to a resistant one, which presumably lacks a functional copy of this factor, should result in gain of full or partial susceptibility in the F_1 generation. The subsequent F_2 generation was expected to segregate 3:1 (susceptible : resistant). Recessive resistance conferred by the presence of a recessive *R* gene (see 1.4), should exhibit the same inheritance features in the F_1 and F_2 generations. In contrast, monogenic, dominant resistance conferred by a dominant *R* gene (see 1.3) would be expected to result in a fully resistant F_1 generation and the subsequent F_2 generation should segregate 1:3 (susceptible : resistant).

Seventeen accessions that had previously been identified to have a DS 0, DS 0-1 or DS 1 were crossed to Ler-0 and the infection phenotypes of their corresponding F_1 generations was determined (Tab. 3.3).

Table 3.3: Disease Score of F_1 generations of *Arabidopsis* derived from crossing resistant accessions with the susceptible Ler-0 accession

Resistant parent	F_1 Disease Score
Eil-0	1
Fei-0	1
St-0	1
Ts-1	1
Sendai-1	1-2
Ws-2	1-2
Sendai-3	2
Sendai-4	2
N6	2
Can-0	2-3
Enheim-T	2-3
Gifu-2	2-3
N7	2-3
Ren-1	2-3
Ws-0	2-3
Oy-0	3
Sp-0	3

At least 10 plants per F_1 line were inoculated with *C. higginsianum* and macroscopic phenotypes were determined at six days after inoculation. Disease score (DS):
DS 0, fully resistant - plants remain intact with only small necrotic lesions
DS 1, intermediate resistant - plants remain mostly intact with only limited lesions
DS 2, intermediate susceptible - plants partially collapsed with necrotic lesions and some tissue maceration and water-soaking
DS 3, fully susceptible - plants completely collapsed and tissue extensively macerated and water-soaked

Six of the F_1 lines originating from crossing a resistant parent to the susceptible parent Ler-0 exhibited a resistant phenotype (DS 1 and DS 1-2). Eleven of the 17 F_1 lines tested exhibited susceptibility, however in varying extents. Three F_1 lines showed an intermediate infection phenotype (DS 2) and were therefore more susceptible than the resistant parent, but more resistant than Ler-0. Six of the F_1 lines showed a phenotype (DS 2-3) that was slightly less susceptible than Ler-0 while the crosses of Oy-0 and Sp-0 to Ler-0 resulted in F_1 phenotypes that were indistinguishable from that of the susceptible parent. Eight F_1 lines were analysed further in the F_2 generation. With this selection it was intended to include examples of different modes of resistance inheritance. Furthermore,

these lines were selected due to the origin of the parental accessions from different geographic regions (Fig. 3.8).
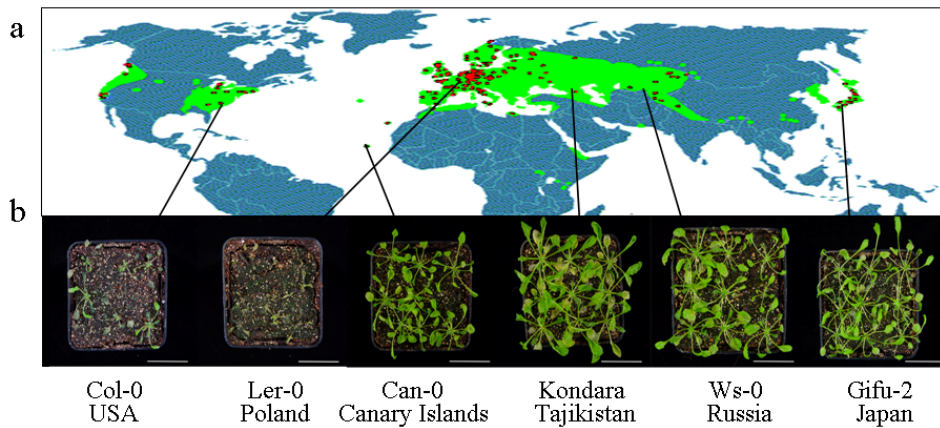
Figure 3.8: Geographical origin of six selected *Arabidopsis* accessions and their macroscopic infection phenotypes after inoculation with *Colletotrichum higginsianum*.

(a) The worldwide natural habitats of *Arabidopsis* are highlighted in green. Red dots indicate the geographical origins of accessions that are publicly available through international stock centres. Black lines point to the geographical origin of the accessions depicted in (b). Image (a) modified from Koornneef *et al*., 2004.

(b) Macroscopic infection phenotypes of four-week-old *Arabidopsis* plants nine days after inoculation with *C. higginsianum* are shown. Bar, 4.5 cm.

The eight selected F$_2$ generations were tested for their segregation in response to *C. higginsianum* inoculation (Tab. 3.4). However, because large numbers of F$_2$ plants had to be screened in a high-throughput manner, intermediate phenotypes could not be reliably distinguished from fully susceptible phenotypes. Therefore, numerical disease scoring was not applied and the F$_2$ plants were simply categorised as either susceptible or resistant (Fig. 3.9).

Figure 3.9: Representative example of *Arabidopsis* F$_2$ population plants inoculated with *Colletotrichum higginsianum* showing segregation of resistance phenotype.

Infection phenotype of four *Arabdidopsis* Ler-0 x Ws-0 F$_2$ generation plants six days after inoculation with *C. higginsianum*. While one F$_2$ plant (left bottom corner) exhibits a resistant phenotype and has clearly survived the infection, the other plants are strongly affected with extensive necrotic lesions and tissue maceration, and are partially or fully collapsed.

Due to the stringent conditions of the infection assay, *i.e.* high inoculum concentrations, resistant plants generally showed some necrotic lesions and water-soaked symptoms, but nonetheless survived infection. Susceptible plants, however, exhibited a strongly affected phenotype at six to seven days after inoculation and eventually collapsed. Intermediary phenotypes could not be easily distinguished with this screen. For each F$_2$ line, at least 90 plants were scored for their infection phenotypes. For many lines it was possible to test a larger number of F$_2$ and analysis was carried out in several independent tests (Tab. 3.4).

						expected for 3 : 1 (S : R) segregation			expected for 1 : 3 (S : R) segregation			
cross of accessions	F_1 Disease Score	No. of tests	Total number of F_2 plants	Susceptible (observed)	Resistant (observed)	Susceptible (expected)	Resistant (expected)	P (χ^2 test)	susceptible (expected)	resistant (expected)	P ($\chi2$ test)	Origin of resistant parent
Ler-0 x Can-0	2-3 (S)	2	186	139	47	139	46,5	0.93	46,5	139,5	2,69E-55	Canary Islands
Ler-0 x Gifu-2	2-3 (S)	4	352	252	100	264	88	0.13	88	264	1,27E-90	Japan
Ler-0 x Ws-0	2-3 (S)	21	1792	1329	463	1344	448	0.41	448	1344	0	Russia
Ler-0 x Eil-0	1-2 (R)	1	100	20	80	75	25	5,79E-23	25	75	0,24	Germany
Ler-0 x Sendai-1	1-2 (R)	1	97	21	76	72,75	24,25	6,92E-34	24,25	72,75	0,44	Japan
Ler-0 x Sendai-3	2 (I)	1	77	23	54	57,75	19,25	5,93E-20	19,25	57,75	0,32	Japan
Ler-0 x Sendai-4	2 (I)	1	86	26	60	64,5	21,5	9,01E-22	21,5	64,5	0,26	Japan
Ler-0 x Ws-2	1-2 (R)	2	115	79	45	86,25	28,75	0	28,75	86,25	3,36E-25	Russia

Table 3.4: Analysis of segregation in response to *Colletotrichum higginsianum* in F_2 generations derived from crosses between eight resistant accessions and the susceptible Ler-0 accession.

Disease scores of F_1 generations were determined as follows. Disease score (DS): 0, fully resistant; 1, intermediate resistant, 2, intermediate susceptible; 3, fully susceptible.

F_2 generations were inoculated with *C. higginsianum* and classified as susceptible or resistant. Expected F_2 segregation ratios refer to either a 3:1 (susceptible : resistant), or 1:3 (susceptible : resistant) F_2 segregation, respectively, and were compared to the observed segregation by a χ^2 test (P = 0,05). S, susceptible infection phenotype, R, resistant phenotype, I, intermediate phenotype.

cross of accessions	F_1 Disease Score	Number of independent tests	Total number	Analysis of F_2 generation expected for 9:7 (S : R) segregation					
				Susceptible (observed)	Resistant (observed)	Susceptible (expected)	Resistant (expected)	P	P (χ^2 test)
Ler-0 x Ws-2	1-2	2	115	79	45	64,68	50,31	0.05	0,05

Table 3.5: Analysis of segregation in response to *Colletotrichum higginsianum* in the F_2 generation derived from crosses between the resistant accession Ws-2 and the susceptible Ler-0 accession.

Disease score of the F_1 generation, originating from a cross of the susceptible accession Ler-0 to resistant accession Ws-2 was determined. Disease score 1-2, intermediate resistant.

According F_2 generation was inoculated with *C. higginsianum* and classified into susceptible and resistant plants. Observed segregation ratios and expected segregation ratios for a 9:7 (susceptible : resistant), was analysed by a χ^2 test (P = 0,05).

For the three F_2 lines derived from crosses with the resistant parents Can-0, Gifu-2 and Ws-0, a 3:1 (susceptible : resistant) segregation could be identified, based on χ^2 analysis with P > 0.05. The F_2 lines derived from crosses with the resistant parents Eil-0, Sendai-1, Sendai-3 and Sendai-4 exhibited a 1:3 (susceptible : resistant) segregation pattern with P > 0.05. The χ^2 analysis of the F_2 lines derived from the resistant parent Ws-2 was not compatible with either of the two segregation patterns, indicating that resistance is not inherited by a monogenic trait. However, the segregation of resistance in the F_2 generation was in agreement with a digenic genetic model. Digenic inheritance of a trait results in F_2 segregation ratios that are variations of the classical 9:3:3:1 ratio for two genetically independent genes, depending on the epistatic relations of the two genes. As the screen of F_2 generation does not facilitate a differentiation between the intermediate susceptible infection phenotype of heterozygotes and a full susceptible infection phenotype of homozygous plants (see above), a 9:7 (susceptible : resistant) segregation of resistance, *i.e.* both genes are dominantly involved in the trait value, was expected and could be confirmed for the Ler-0 x Ws-2 F_2 generation (Tab 3.5).

In summary, the natural variation in response to *C. higginsianum* of various *Arabidopsis* accessions, originating from very different geographical regions, was evaluated. The full spectrum of responses between strong resistance to fungal attack through to full susceptibility to fungal infection could be observed in this collection of 116 accessions. This natural variation can be explained by different scenarios of dominant or recessive monogenic resistance or polygenic resistance.

3.3 Genetic analysis of recessive resistance of *Arabidopsis* to *C. higginsianum*

3.3.1 Mapping populations

To identify the region containing a recessive resistance locus, several resistant accessions had been crossed to the susceptible accession Ler-0 (see 3.2.2). For positional cloning of the responsible loci, the corresponding F_2 generations were selected that exhibited the expected 3:1 (susceptible : resistant) segregation and a susceptible F_1 phenotype, hence accessions Ws-0, Can-0 and Gifu-2. Positional cloning was initially concentrated on the F_2 mapping population derived from the Ler-0 x Ws-0 cross, since this resistant accession

had been the subject of previous genetical analyses and more sequence information was available than for the relatively unexplored accessions Can-0 and Gifu-2. In the course of mapping the resistance loci in the Ler-0 x Gifu-2 and the Ler-0 x Can-0 F_2 populations, I hypothised that the resistance loci might be identical in all three F_2 populations (see 3.3.4). This was subsequently tested by allelism analysis (see 3.3.6).

3.3.2 First-pass mapping

A first-pass mapping strategy (Jander *et al.*, 2002) was used to identify the approximate region containing the locus responsible for recessive resistance. For this "rough mapping" procedure, 26 F_2 plants, exhibiting a resistant phenotype, and 10 susceptible F_2 plants were analysed by PCR with a set of 27 SSLP markers (Lukowitz *et al.*, 2000; Jander *et al.*, 2002; Loudet *et al.*, 2002). These codominant PCR-based molecular markers had been confirmed to exhibit sequence polymorphisms between Ler-0 and Ws-0 and were evenly distributed throughout the five chromosomes. By genotyping this small F_2 mapping population, reduced recombination frequencies were identified with molecular markers on the lower arm of chromosome V (Fig. 3.10). This suggested genetic linkage of these markers to the recessive resistance locus. Since the resistance phenotype is expected to be inherited recessively, the region of interest in resistant plants should genotypically resemble the Ws-0 parent. In accordance with this, resistant plants were either homozygous Ws-0 in this region or less often heterozygous on chromosome V, whereas susceptible plants were heterozygous or resembled the Ler-0 genotype. In contrast, the genotypes identified for marker positions on the other four chromosomes resembled a random distribution.
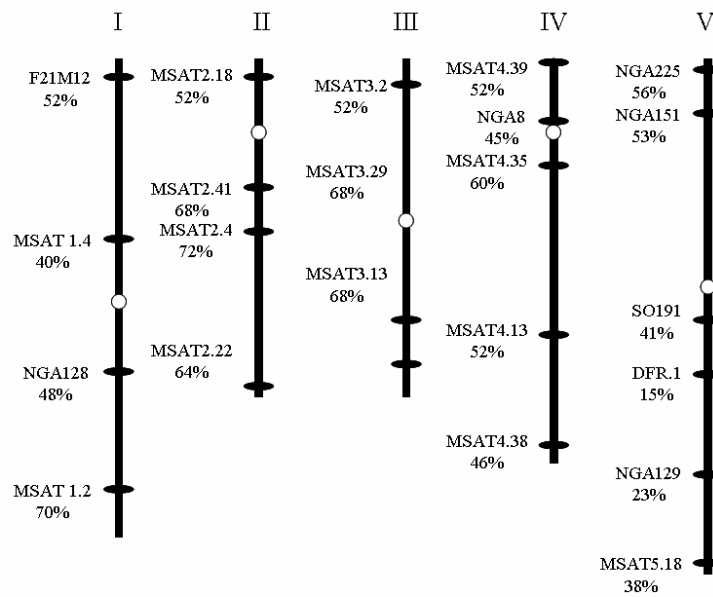
Figure 3.10: First-pass mapping of a locus conferring resistance to *Colletotrichum higginsianum* in an *Arabidopsis* Ler-0 x Ws-0 F$_2$ mapping population.

Schematic representation of SSLP markers and their positions on the five chromosomes of the *Arabidopsis* genome. A Ler-0 x Ws-0 F$_2$ mapping population was screened for resistance after *C. higginsianum* infection and for the resistant plants the recombination frequencies were determined at the indicated marker positions. The reduced recombination frequencies on the lower arm of chromosome V (DFR1 and NGA129) indicated a possible linkage of the resistance locus to this region. Centromers are indicated by white dots.

3.3.3   Fine-mapping of recessive resistance locus Ws-0

In the first-pass mapping, linkage to markers on the lower arm of chromosome V was identified. To define the region of interest more accurately, a fine-scale mapping had to be carried out with a larger number of Ler-0 x Ws-0 F$_2$ progenies. In the following fine-mapping process, a total number of approximately 3,000 plants were phenotyped and 740 of these, exhibiting a resistant phenotype, were then genotyped with various molecular markers.

By the first pass mapping I had identified a recombination frequency of 41% at the molecular marker position SO191, and at the position of marker DFR.1 a recombination frequency of 15%. At the telomeric side, I observed a recombination frequency of 38% at the position of the molecular marker MSAT5.18, and a recombination frequency of 23% at the position of the marker NGA129. F$_2$ plants were identified that showed a recombination event at the positions of one of the molecular markers DFR.1 or NGA129,

and therefore these markers were determined as the initial "flanking" markers of the region of interest. To reduce the size of this region, the mapping population was screened for plants that exhibited further recombination events for one of two additional markers located between the initial flanking markers DFR.1 and NGA129. Following this strategy, the mapping population was genotyped with progressively closer markers until no further recombinants were found (Tab. 3.6). For this approach, suitable SSLP, CAPs and dCAPs markers were identified from available databases (TAIR, Monsanto, see 2.1.8), or were designed on the basis of detected DNA sequence polymorphisms between Ler-0 and Ws-0. Two plants (F_2 lines 51F5 and 54F9) were found that exhibited a recombination event at the position of the centromeric final flanking marker 236 (18,307,842 bp), and two plants (F_2 lines 15D1 and 52H3) with a recombination event at the position of the telomeric final flanking marker 312 (18,407,860 bp) and the target locus was delineated to a region of ~ 100 kb, based on the Col-0 reference sequence information (Tab. 3.6). However, I could not exclude that the target region might be smaller or larger in the parental accessions Ler-0 or Ws-0 than the Col-0 reference genome. To validate the mapped region, I determined the infection phenotypes of F_3 families derived from F_2 lines that exhibited a single recombination event for one of the flanking markers. Given that the identified region contains a locus conferring recessive resistance, it was expected that F_3 plants, originating from resistant F_2 plants, should not segregate in their infection phenotypes, and should all be resistant after *C. higginsianum* inoculation. F_2 plants exhibiting a heterozygous genotype for both flanking markers 236 and 312, however, were expected to result in an F_3 generation that was segregating 3:1 (susceptible : resistant). The infection phenotypes of all F_3 lines were in accordance with these expectations (Tab. 3.6) and therefore confirmed the accuracy of the region of interest.

Table 3.6 — Fine mapping (Chromosome V PCR markers)

PCR - markers and their position on chromosome V (based on Col-0 reference sequence)

F2 line	phenotype of F2 line	DFR.1 (17,181,592 bp)	MBD2-1 (17,222,071 bp)	MSAT5.9 (17,252,309 bp)	17,57Mb (17,570,000 bp)	MRH10-2 (17,719,014 bp)	MSAT5.4 (17,844,441 bp)	78 (17,923,131 bp)	64 (18,039,560 bp)	66 (18,050,701 bp)	68 (18,089,563 bp)	72 (18,262,458 bp)	134 (18,272,105 bp)	146 (18,304,971 bp)	236 (18,307,842 bp)	82 (18,338,008 bp)	RPS4-NT (18,342,772 bp)	332 (18,363,163 bp)	338 (18,379,031 bp)	364 (18,383,701 bp)	312 (18,407,860 bp)	252 (18,410,145 bp)	276 (18,4648,051 bp)	MRA19-1 (18,597,044 bp)	NGA129 (19,007,000 bp)	F3 phenotype
17H2	R	het	n.d.	n.d.	het	Ws	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	n.d.	Ws	n.d.	n.d.	n.d.	Ws	Ws	n.d.	Ws	n.d.	R
15F9	R	het	n.d.	n.d.	het	het	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	n.d.	Ws	n.d.	n.d.	n.d.	n.d.	Ws	n.d.	Ws	Ws	R
31A12	R	n.d.	n.d.	n.d.	n.d.	n.d.	het	het	het	Ws	Ws	Ws	n.d.	n.d.	Ws	n.d.	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	R
30C6	R	n.d.	n.d.	n.d.	n.d.	n.d.	het	het	het	het	het	Ws	n.d.	n.d.	Ws	Ws	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
21G10	R	n.d.	het	n.d.	het	het	het	n.d.	het	het	n.d.	Ws	n.d.	Ws	Ws	Ws	Ws	n.d.	n.d.	n.d.	Ws	Ws	n.d.	Ws	n.d.	R
13E6	R	n.d.	het	n.d.	het	het	het	het	het	het	het	Ws	n.d.	Ws	Ws	Ws	Ws	n.d.	n.d.	n.d.	Ws	Ws	n.d.	Ws	Ws	R
51F5	R	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	het	het	het	het	het	Ws	Ws	n.d.	n.d.	n.d.	Ws	Ws	n.d.	Ws	n.d.	R
54F9	R	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	het	het	het	het	het	Ws	Ws	n.d.	n.d.	n.d.	Ws	Ws	n.d.	Ws	n.d.	R
15B9	R	Ws	n.d.	n.d.	Ws	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	n.d.	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	het	R
15E8	R	Ws	n.d.	n.d.	Ws	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	n.d.	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	het	R
55A2	R	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	Ws	n.d.	n.d.	Ws	Ws	n.d.	Ws	Ws	Ws	Ws	Ws	n.d.	het	n.d.	n.d.	R		R
14D7	R	Ws	n.d.	n.d.	Ws	Ws	Ws	n.d.	Ws	Ws	n.d.	Ws	n.d.	n.d.	Ws	n.d.	Ws	n.d.	n.d.	n.d.	Ws	n.d.	het	het	R
15F1	R	Ws	n.d.	n.d.		Ws	Ws	n.d.	n.d.	n.d.	n.d.	Ws	n.d.	Ws	Ws	Ws	Ws	Ws	Ws	n.d.	het	het	R			R
13B11	R	Ws	Ws	n.d.	Ws	Ws	Ws	Ws	n.d.	Ws	Ws	Ws	n.d.	Ws	n.d.	Ws	Ws	Ws	n.d.	Ws	het	n.d.	het	het	R
14E11	R	Ws	n.d.	n.d.	Ws	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	n.d.	Ws	n.d.	Ws	n.d.	n.d.	n.d.	Ws	het	n.d.	Ws	het	R
52H3	R	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	Ws	n.d.	n.d.	Ws	Ws	Ws	Ws	Ws	Ws	het	het	het	het	n.d.	R
15D1	R	Ws	n.d.	n.d.	Ws	Ws	Ws	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	n.d.	Ws	Ws	Ws	Ws	het	het	n.d.	het	het	R
31D10	S	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	Ws	Ws	Ws	Ws	Ws	Ws	het	het	het	het	het	het	n.d.	het	het	n.d.	het	n.d.	segregating
45D6	S	n.d.	n.d.	n.d.	n.d.	n.d.	Ws	n.d.	Ws	Ws	Ws	het	n.d.	n.d.	het	het	het	n.d.	n.d.	n.d.	n.d.	het	n.d.	het	n.d.	segregating
49E4	R	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	het	het	het	het	het	het	het	het	het	het	het	Ws	n.d.	segregating

Table 3.6: Fine mapping of recessive resistance to *Colletotrichum higginsianum* in *Arabidopsis* Ler-0 x Ws-0 F$_2$ plants.

F$_2$ lines, and their corresponding F$_3$ lines, derived from crosses between the *Arabidopsis* accessions Ler-0 and Ws-0, were phenotyped at seven days after inoculation with *C. higginsianum*. The genotypes of the F$_2$ lines at indicated SSLP marker positions on chromosome V were determined on the basis of the Col-0 reference accession. Flanking markers "236" and "312" delineating the locus are highlighted in red. R, resistant phenotype; S, susceptible phenotype; n.d., not determined; Ws, genotype resembling Ws-0 parent: het, heterozygous genotype.

3.3.4 Targeted mapping of recessive resistance loci in Gifu-2 and Can-0 accessions

It was possible that recessive resistance in the *Arabidopsis* accessions Gifu-2 and Can-0 is conferred by different loci than in Ws-0. As the following comparative cytological analyses (see 3.7) indicated a similar infection phenotype in all three resistant accessions, it was however conceivable that the recessive resistance loci might be identical. In order to test this hypothesis, a small number of plants from F_2 mapping populations originating from crosses between Ler-0 and Gifu-2, and Ler-0 and Can-0 were tested with a limited number of molecular markers (Tab. 3.7 and Tab. 3.8) that had previously been identified as flanking markers for the region of interest in the Ler-0 x Ws-0 F_2 mapping population (Tab 3.6).

Similar to the target region identified in the Ler-0 x Ws-0 F_2 mapping population, Gifu-2 and Can-0 F_2 mapping populations indicated an association of resistance with the lower arm of chromosome V, as shown by recombination frequencies of 7-25%. In accordance with results from the Ler-0 x Ws-0 F_2 mapping population, resistance was associated with a genotype that in most cases corresponded to the homozygous resistant parent, or, less frequently, a heterozygous state. In no case did resistant F_2 plants exhibit a Ler-0 genotype for the tested molecular markers. Susceptible F_2 plants in contrast, corresponded to the homozygous Ler-0 parent genotype or were heterozygous at the tested marker positions. Hence, the observed phenotypes and genotypes were consistent with an expected recessive inheritance of resistance. On the basis of these findings, it was likely that resistance loci in the accessions Ws-0, Gifu-2 and Can-0 might be identical. An allelism test (see 3.3.6) was required to confirm this assumption.

		Recombination frequency				
		22%	20%	18%	25%	22%
		PCR-markers and their position on chromosome V				
F₂ line	phenotype of F₂ line	MRH10-2 (17,719,014 bp)	68 (18,089,563 bp)	72 (18,262,458 bp)	82 (18,338,008 bp)	MRA19-1 (18,597,044 bp)
26A1	R	Can	n.d.	n.d.	Can	Can
26A2	R	Can	Can	Can	Can	Can
26A3	R	Can	Can	Can	Can	Can
26A4	R	Can	Can	Can	Can	Can
26A9	R	Can	Can	Can	Can	Can
26A10	R	Can	Can	Can	Can	Can
26B4	R	Can	Can	Can	Can	Can
26B5	R	Can	Can	Can	Can	Can
26B12	R	Can	Can	Can	Can	Can
26C3	R	het	het	het	het	Ler
26D5	R	Can	Can	Can	Can	Can
26D6	R	Can	Can	Can	Can	Can
26D10	R	Can	Can	Can	Can	Can
26E2	R	Can	Can	Can	Can	Can
26E3	R	Can	Can	Can	Can	het
26E8	R	Can	Can	Can	Can	Can
26E12	R	Can	Can	Can	Can	Can
26G2	R	Can	Can	Can	Can	Can
26G3	R	Can	Can	Can	Can	Can
26G5	R	Can	Can	Can	Can	Can
26H1	R	het	Can	Can	Can	Can
26H10	R	Can	Can	Can	Can	Can
25A5	R	Can	Can	n.d.	n.d.	Can
25A11	R	Can	Can	n.d.	Can	Can
25B1	R	Can	Can	Can	n.d.	Can
25B2	R	Can	Can	Can	Can	Can
25B6	R	Can	Can	Can	Can	Can
25B7	R	Can	Can	Can	Can	Can
25B11	R	Can	Can	Can	Can	Can
25C2	R	Can	Can	Can	Can	Can
25C5	R	het	Can	Can	n.d.	Can
25C9	R	Can	Can	Can	Can	Can
25C11	R	Can	Can	Can	n.d.	Can
25D7	R	Can	Can	Can	Can	Can
25D8	R	Can	Can	Can	Can	Can
25E6	R	Can	Can	Can	Can	Can
25E7	R	Can	Can	Can	n.d.	Can

Table 3.7 Mapping of locus conferring recessive resistance to *Colletotrichum higginsianum* in an *Arabidopsis* Ler-0 x Can-0 F₂ population. Genotypes were identified at the indicated molecular marker positions on chromosome V and infection phenotypes were determined at seven days after *C. higginsianum* inoculation. Can, Can-0-like genotype; Ler, Ler-0-like genotype; het, heterozygous genotype; R, resistant phenotype; S, susceptible phenotype; n.d., not determined.

Table 3.7: Continued

F₂ line	phenotype F₂ line	MRH10-2 (17,719,014 bp)	68 (18,089,563 bp)	72 (18,262,458 bp)	82 (18,338,008 bp)	MRA19-1 (18,597,044 bp)
		PCR-markers and their position on chromosome V				
25F3	R	Can	Can	Can	Can	Can
25F6	R	Can	Can	Can	Can	Can
25G6	R	Can	Can	Can	Can	Can
25G8	R	Can	Can	Can	Can	n.d.
25G10	R	Can	Can	Can	Can	Can
25H4	R	Can	Can	Can	Can	Can
25H6	R	Can	Can	Can	Can	Can
25H8	R	Can	Can	Can	n.d.	Can
25H12	R	Can	Can	Can	Can	Can
26C4	S	Ler	Ler	Ler	Ler	Ler
26D1	S	Ler	Ler	Ler	Ler	Ler
26D8	S	het	het	het	het	het
26D12	S	het	het	het	het	het
26F6	S	het	het	het	het	het
26G8	S	het	het	n.d.	het	het
25C6	S	het	het	het	het	het
25D1	S	Ler	Ler	Ler	Ler	Ler
25D2	S	Ler	Ler	Ler	Ler	Ler
25D3	S	het	het	het	het	Ler
25D5	S	het	het	het	het	Ler
25E3	S	Ler	Ler	Ler	Ler	het
25F9	S	het	het	het	het	het
25G1	S	Ler	Ler	Ler	Ler	het
25H3	S	Ler	Ler	Ler	Ler	Ler
25H10	S	het	het	het	het	het

		Recombination frequency			
		18%	13%	14%	7%
		PCR-markers and their position on chromosome V			
F₂ line	phenotype F₂ line	**MBD2-1** (17,222,071 bp)	**68** (18,089,563 bp)	**72** (18,262,458 bp)	**MRA19-1** (18,597,044 bp)
23A3	R	Gifu	Gifu	Gifu	Gifu
23A4	R	Gifu	Gifu	Gifu	Gifu
23B1	R	Gifu	Gifu	Gifu	Gifu
23B3	R	Gifu	Gifu	Gifu	Gifu
23B4	R	Gifu	Gifu	Gifu	Gifu
23B6	R	n.d	Gifu	Gifu	Gifu
23C2	R	Gifu	Gifu	Gifu	Gifu
23C4	R	Gifu	Gifu	Gifu	Gifu
23C5	R	Gifu	Gifu	Gifu	Gifu
23D1	R	Gifu	Gifu	Gifu	Gifu
23D5	R	Gifu	Gifu	Gifu	Gifu
23D10	R	het	Gifu	Gifu	Gifu
23E2	R	Gifu	Gifu	Gifu	Gifu
23E8	R	Gifu	Gifu	Gifu	Gifu
23E11	R	het	Gifu	Gifu	Gifu
23F7	R	n.d	Gifu	Gifu	Gifu
23H1	R	Gifu	Gifu	Gifu	Gifu
23H6	R	n.d	n.d	Gifu	Gifu
23H8	R	Gifu	Gifu	Gifu	Gifu
23H10	R	Gifu	Gifu	Gifu	Gifu
23H12	R	Gifu	Gifu	Gifu	Gifu
24A8	R	n.d	Gifu	Gifu	Gifu
24A11	R	Gifu	Gifu	Gifu	Gifu
24B1	R	n.d	Gifu	Gifu	Gifu
24B4	R	n.d	Gifu	Gifu	Gifu
24B11	R	n.d	Gifu	Gifu	Gifu
24C3	R	Gifu	Gifu	Gifu	Gifu
24D2	R	Gifu	Gifu	Gifu	Gifu
24E1	R	Gifu	Gifu	Gifu	Gifu
24E2	R	Gifu	Gifu	Gifu	Gifu
24E6	R	Gifu	Gifu	Gifu	Gifu
24E10	R	Gifu	Gifu	Gifu	Gifu
24E11	R	Gifu	Gifu	Gifu	Gifu
24F1	R	Gifu	Gifu	Gifu	Gifu
24F5	R	Gifu	Gifu	Gifu	Gifu
24G6	R	n.d	Gifu	Gifu	Gifu
24G11	R	het	Gifu	Gifu	Gifu

Table 3.8: Mapping of locus conferring recessive resistance to *Colletotrichum higginsianum* resistance in an *Arabidopsis* Ler-0 x Gifu-2 F₂ population. Genotypes were identified at the indicated molecular marker positions on chromosome V and infection phenotypes were determined at seven days after *C.*

higginsianum inoculation. Gifu, Gifu-2-like genotype; Ler, Ler-0-like genotype; het, heterozygous genotype; R, resistant phenotype; S, susceptible phenotype; n.d., not determined.

Table 3.8.: Continued

F₂ line	phenotype F₂ line	PCR-markers and their position on chromosome V			
		MBD2-1 (17,222,071 bp)	**68** (18,089,563 bp)	**72** (18,262,458 bp)	**MRA19-1** (18,597,044 bp)
24G12	R	Gifu	Gifu	Gifu	Gifu
24H1	R	Gifu	Gifu	Gifu	Gifu
24H5	R	Gifu	Gifu	Gifu	Gifu
24H6	R	het	het	het	Gifu
24H7	R	n.d	Gifu	Gifu	Gifu
24H8	R	Gifu	Gifu	Gifu	Gifu
24H9	R	het	het	het	het
24A1	S	Ler	Ler	Ler	Ler
24A4	S	het	het	het	het
24B3	S	het	het	het	het
24B7	S	Ler	Ler	Ler	Ler
24C2	S	Ler	Ler	Ler	Ler
24C12	S	Ler	Ler	Ler	Ler
24D6	S	het	het	het	het
24D8	S	Ler	Ler	Ler	Ler
24F10	S	Ler	Ler	Ler	Ler
24G2	S	Ler	Ler	Ler	Ler
24G8?	S	Ler	Ler	Ler	Ler
24H3	S	Ler	Ler	Ler	Ler
23A1	S	Ler	Ler	Ler	Ler
23A2	S	Ler	het	het	Ler
23D6	S	het	het	het	Ler

3.3.5 Identification of resistance loci by analysis of Quantitative Trait Loci (QTL)

Scoring phenotypes in RIL populations has been proven to be particularly useful for the detection and localisation of quantitative trait loci (QTL), as many traits can be identified that are under control of multiple loci and therefore exhibit a continuous rather than qualitative variation (see 1.6).

Since the accessions Ler-0, Kas-2 and Kondara had been shown to exhibit significantly different resistance responses to *C. higginsianum* (Tab. 3.2), I examined RIL populations derived from the crosses between Ler-0 x Kas-2 and Ler-0 x Kondara for additional sources of potential susceptibility factors. Both RIL populations had already been genotyped with common molecular markers (El-Lithy *et al.*, 2006) to anchor their genetic maps, thereby facilitating comparison of QTL positions between the populations. 164

individual lines of the Ler-0 x Kas-2 RIL population and 131 lines of the Ler-0 x Kondara population were grown in three replicate experiments and three-week old plants were inoculated with *C. higginsianum*. For the Ler-0 x Kas-2 RIL population, disease scores (DS) (Fig. 3.7) of each RIL, determined at four and five days after inoculation, were analysed for QTLs as a combined data set derived from both time points (with the kind help by Dr. M. Reymond, Cologne, Germany). Two major QTLs (at 18.4-23.8 cM on chromosome I and at 12.7-21.1 cM on chromosome V) with LOD scores of 3 and 5, respectively, were mapped (Fig. 3.11, Tab. 3.9). QTL detection with integration of the two cofactors, the molecular markers SNP107 and SNP193, could explain less than 20% of the variance, therefore indicating only small-to-medium effects of these QTLs on the *C. higginsianum* infection phenotype and suggesting the existence of several additional epistatic effects.

Table 3.9: QTL analysis results in Ler-0 x Kas-2 RIL population

chromosome	marker	position (cM)	allelic effect (2a)	LOD score	r^2	heritability (h^2)
1	SNP107	10	0.27	3.5	8.5	0.658
2	SNP193	36.8	0.32	4.8	12.2	

Marker, the closest left marker flanking the LOD score peak.

Position, the position of the QTL is expressed in cM from the first marker of the chromosome.

2a, the mean effect of the replacement of both Kas-2 alleles by Ler-0 alleles at the QTL.

r^2, Percentage of variance explained by the sum of the QTL.

h^2, heritability of the trait (see 2.2.18.2).

For the Ler-0 x Kondara RIL population, disease scores were determined at six days after inoculation. In contrast to the small to medium QTL effects on resistance identified in the Ler-0 x Kas-2 RIL population, QTL mapping for the Ler-0 x Kondara RIL population resulted in the identification of a region on the lower arm of chromosome V (44.8-62.9 cM) with a LOD score > 20, governing a significant major QTL controlling *C. higginsianum* resistance. Interval mapping with integration of cofactors (SNP81, SNP334, M4-9 and SNP97) estimated an r^2 value (phenotypic variances explained by the QTL) of

0.57. The major QTL on chromosome V alone explained more than 4/5 of the phenotypic variance (48%), indicating its major effect on resistance to *C. higginsianum* in contrast to the additional minor QTLs detected on chromosome III ($r^2 = 4.9\%$) and chromosome IV ($r^2 = 7.7\%$ and 4.1%). The allelic effect of 0.77 (Tab. 3.10) indicates that the Ler-0 alleles at marker position of the major QTL on chromosome V increase the susceptibility.
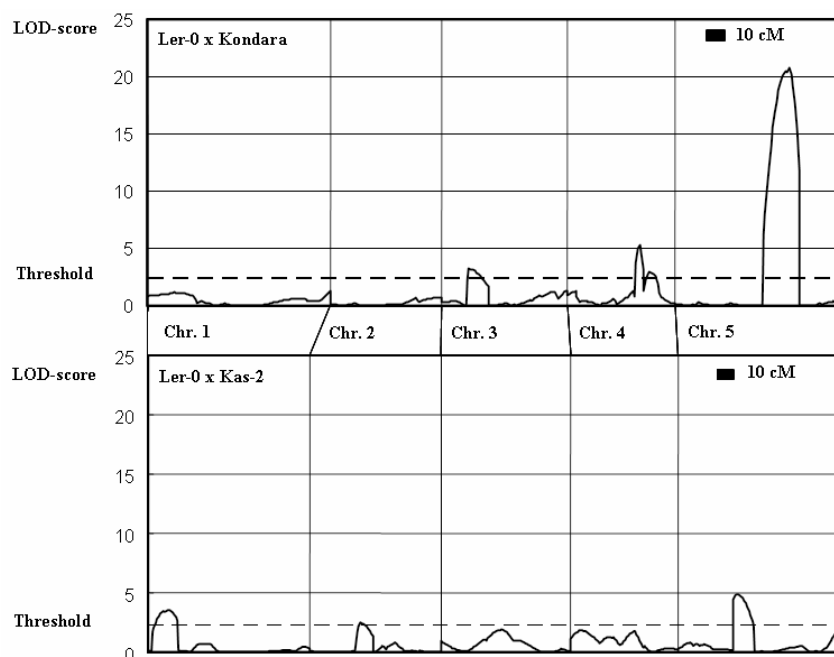
Figure 3.11: Detected QTLs explaining resistance to *Colletotrichum higginsianum* infection in the *Arabidopsis* Ler-0 x Kondara and Ler-0 x Kas-2 recombinant inbred line (RIL) populations.
A total of 131 independent lines of the *Arabidopsis* Ler-0 x Kondara and 164 independent lines of the Ler-0 x Kas-2 RIL populations were inoculated with *C. higginsianum* and infection phenotypes were determined at 6 dpi (Ler-0 x Kondara RIL population), or at 4 and 5 dpi (Ler-0 x Kas-2 RIL population). Interval mapping was performed with MapQTL5. The LOD score was estimated at every marker position and between markers, resulting in the presented LOD profile.

Of particular note was the finding that the map position of the major QTL in the Ler-0 x Kondara RIL population corresponds to that identified by linkage analysis in the Ler-0 x Ws-0 F_2 mapping population (Fig. 3.12), as well as the map positions identified in the Ler-0 x Gifu-2 and Ler-0 x Can-0 F_2 mapping populations. Although it cannot be excluded that different loci in the same region are responsible for resistance in the selected accessions, it seems probable that Mendelian linkage analysis combined with QTL detection in the Ler-0 x Kondara RIL population, have identified a single common locus governing resistance to *C. higginsianum* in multiple accessions.

Table 3.10: QTL analysis results in Ler-0 x Kondara RIL population

chromosome	marker	position (cM)	allelic effect (2a)	LOD score	r^2	heritability (h^2)
3	SNP81	14.14	0.26	3.21	4.9	
4	SNP334	38.38	0.42	4.51	7.7	
4	M4-9	45.8	-0.31	2.47	4.1	0.72
5	SNP97	57.75	0.77	20.39	47.8	

Marker, the closest left marker flanking the LOD score peak.

Position, the position of the QTL is expressed in cM from the first marker of the chromosome.

2a, the mean effect of the replacement of both Kondara alleles by Ler-0 alleles at the QTL.

r^2, Percentage of variance explained by the sum of the QTL.

h^2, heritability of the trait (see 2.2.18.2).

In summary, QTL analysis of the Ler-0 x Kas-2 RIL population detected two QTLs on the upper arms of chromosomes I and V. However, they had only minor to medium effects on the resistance to *C. higginsianum* and additional loci are likely to influence the resistant phenotype. These QTLs were therefore not investigated further in the course of this study. In contrast, a major QTL could be identified in the Ler-0 x Kondara RIL population which had a major effect on resistance to *C. higginsianum*. Strikingly, the map position of the QTL on the lower arm of chromosome V is in accordance with the map positions identified for resistance loci in Ws-0, Gifu-2 and Can-0, and was shown to be allelic as discussed in section 3.3.6. Therefore, fine-mapping of the identified QTL was not continued in RIL lines.
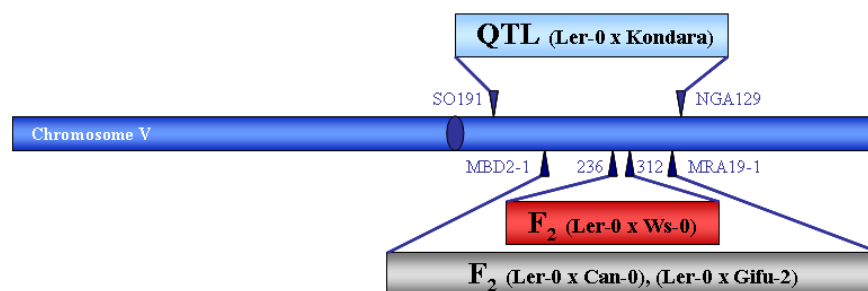
Figure 3.12: Location on *Arabidopsis* chromosome V of loci conferring resistance to *Colletotrichum higginsianum* in the Ler-0 x Kondara recombinant inbred line (RIL) population and Ler-0 x Ws-0, Ler-0 x Can-0 and Ler-0 x Gifu-2 F$_2$ populations.

The major quantitative trait locus (QTL) controlling resistance to *C. higginsianum* in the Ler-0 x Kondara RIL population, and the recessive resistance loci detected in the Ler-0 x Ws-0, Ler-0 x Can-0 and Ler-0 x Gifu-2 F$_2$ mapping populations are represented by the light blue, red and grey bars, respectively. The closest molecular markers on chromosome V (blue bar) of *Arabidopsis* are indicated by arrowheads. The resistance loci of the F$_2$ populations are contained within the QTL region. The centromere of chromosome V is indicated by the blue ellipse.

3.3.6 Allelism Tests

Accessions Ws-0, Gifu-2 and Can-0 had been shown to exhibit a resistant infection phenotype (see 3.2.1) in response to *C. higginsianum* inoculation. All three accessions have a recessive inheritance of the resistance locus (see 3.2.2). For Ws-0, the genomic region harbouring the recessive resistance locus was located in a ~ 100 kb region between the molecular markers 236 and 312 (see 3.3.3) on the lower arm of chromosome V. Likewise, genetic linkage analysis, carried out with small Ler-0 x Gifu-2 and Ler-0 x Can-0 F$_2$ mapping populations (see 3.3.4) indicated potential linkage of resistance to molecular markers located on the lower arm of chromosome V, including the 100 kb region previously identified for the Ler-0 x Ws-0 F$_2$ population. Furthermore, a QTL analysis with the Ler-0 x Kondara RIL population identified a major QTL governing resistance to *C. higginsianum* (see 3.5). The location of this QTL was in accordance with the location of the region of interest determined by Mendelian genetic analysis (Fig 3.12). It cannot be ruled out that resistance in Ws-0, Can-0, Gifu-2 and Kondara is conferred by different loci positioned within the detected region on the lower arm of chromosome V. However

instead, resistance is likely due to the effect of the same locus. In order to test this hypothesis, allelism crosses were generated and the infection phenotypes of the subsequent generations were analysed in response to *C. higginsianum* inoculation. For this, allelism crosses were generated between all four selected resistant accessions and the resulting F_1 and according F_2 generations were tested for their macroscopic infection phenotypes at seven days after inoculation with *C. higginsianum* (Fig. 3.13, Tab. 3.11). If resistance was conferred by different recessive resistance loci in the resistant accessions, the F_1 generations were expected to exhibit a susceptible phenotype and the F_2 lines should segregate 3:1 (susceptible : resistant). However, if resistance was conferred by the identical recessive resistance locus, then F_1 plants were expected to resemble the resistant parental phenotypes and the F_2 progeny should be uniformly resistant.

Figure 3.13 Macroscopic infection phenotypes of *Arabidopsis* accessions and F$_2$ plants of allelism-crosses six days after inoculation with *Colletotrichum higginsianum*.

Presented are infection phenotypes of three-week-old plants of resistant and susceptible *Arabidopsis* accessions (left column) and F$_2$ generations (right column) derived from crosses of Ws-0 (as female or as male parent) to Gifu-2, Can-0 and Kondara, inoculated with *C. higginsianum* at 6 dpi.

Plants of the F$_1$ generations from all allelism crosses exhibited a uniform resistant phenotype at seven days after inoculation with *C. higginsianum* (data not shown), and were therefore not markedly different from the resistant phenotypes of their parents. Likewise, in the F$_2$ generations, no segregation of infection phenotypes could be identified for at least 100 F$_2$ plants per allelism cross (Tab. 3.11). All inoculated F$_2$ plants exhibited resistance resembling that of the parental accessions (Fig. 3.13).

Table 3.11: Infection phenotypes of F$_2$ generations from reciprocal allelism crosses between resistant *Arabidopsis* accessions

Parental accession 1	Parental accession 2		
	Gifu-2	Can-0	Kondara
Ws-0	resistant	resistant	resistant

Parental accession 1 was crossed to parental accession 2 and three-week-old plants of the resulting F$_2$ lines (at least 100 plants per line) were inoculated with *C. higginsianum*. Macroscopic infection phenotypes were determined at seven days after inoculation.

In conclusion, resistance in F$_1$ plants and a uniform resistance in F$_2$ progeny was obtained for all tested allelism crosses in response to *C. higginsianum* inoculation. This observation indicates the presence of a single recessive resistance locus that is identical in the resistant accessions Ws-0, Gifu-2, Can-0 and Kondara.

3.3.7 Summary of genetic analysis of recessive resistance of *Arabidopsis* to *C. higginsianum*

Positional cloning in a Ler-0 x Ws-0 F_2 mapping population was utilised to identify the molecular mechanisms responsible for the observed natural variation in recessive resistance to *C. higginsianum* (see 3.3.3). In addition to identification of recessive resistance loci by classical Mendelian mapping, QTL analysis of a Ler-0 x Kondara RIL populations identified a resistance locus on the lower arm of chromosome V (see 3.3.5). The identified recessive resistance locus could be confirmed to be allelic between Ws-0, Gifu-2, Can-0 and Kondara (see 3.3.6, Fig. 3.12 and Fig. 3.13).

Table 3.12: Inheritance of resistance in F_1 and F_2 generations from crosses of selected *Arabidopsis* accessions and methods used for mapping

Susceptible parent	Resistant parent	DS of F_1 plants	Segregation in F_2 generation (S:R)	Mapping method and position of resistance locus
Ler-0	Eil-0	1 (R)	1:3	positional cloning (*RCH1*) (Narusaka *et al.*, 2004)
Ler-0	Ws-0	2-3 (S)	3:1	positional cloning, chromosome V
Ler-0	Can-0	2-3 (S)	3:1	positional cloning, allelism tests, chromosome V
Ler-0	Gifu-2	2-3 (S)	3:1	positional cloning, allelism tests, chromosome V
Ler-0	Kondara	n.d.	n.d.	QTL-mapping chromosome V

Disease score (DS): 0, fully resistant; 1, intermediate resistant, 2, intermediate susceptible; 3 fully susceptible.

n.d., not determined; S, susceptible phenotype; R, resistant phenotype.

3.4 Candidate genes and expression analysis

The identified ~100 kb region located between the flanking markers 236 (18,307,842 bp), and 312 (18,407,860 bp) in the Ler-0 x Ws-0 F_2 mapping population harbours a locus conferring recessive resistance to *C. higginsianum*. Based on sequence analysis of the Col-0 accession, this region contains 20 candidate genes (Tab. 3.13).

Table 3.13: Candidate genes within the region identified to contain a locus conferring recessive resistance to *C. higginsianum*

Gene ID	Description
At5g45210	Putative disease resistance protein (TIR-NB-LRR)
At5g45220	Putative disease resistance protein (TIR-NB-LRR)
At5g45230	Putative disease resistance protein (TIR-NB-LRR)
At5g45240	Putative disease resistance protein (TIR-NB-LRR)
At5g45250	RPS4, *Pseudomonas syringae* disease resistance protein (TIR-NB-LRR)
At5g45260	RRS1, recessive resistance to *Ralstonia solanecearum* (TIR-NB-LRR with WRKY52 motif)
At5g45275	Similar to nodulin-related gene NFD4, (Nuclear Fusion Defective 4)
At5g45276	Unknown pseudogene
At5g45280	Putative pectinacetylesterase
At5g45290	Zinc finger (C3HC4-type RING finger) family protein
At5g45300	BAM8/BMY2, beta-amylase 8
At5g45307	MicroRNA targeting AGO1
At5g45310	Similar to rice gene Os01g0962100
At5g45320	Unknown protein
At5g45330	Unknown protein
At5g45340	Putative ABA 8'-hydroxylase; involved in ABA catabolism
At5g45350	Non-secreted proline-rich protein
At5g45360	F-box family protein
At5g45370	Nodulin-related integral membrane protein
At5g45380	Sodium symporter protein

Since the resistance locus was identified in the Ler-0 x Ws-0 F_2 mapping population, I cannot rule out that additional genes are present in the region of interest which are absent from Col-0 in this region. Conversely, genes identified in the region of interest in the Col-0 genetic background might not be present in Ler-0 or Ws-0.

The database "Genevestigator" (www.genevestigator.com) is a reference expression database which allows the expression and regulation of genes to be studied by compiling information from hundreds of microarray experiments. For 15 of the 20 candidate genes, expression patterns had been assigned to certain plant organs in uninfected *Arabidopsis* plants of the accession Col-0 by microarray analyses (Fig. 3.14). This organic-specific assignment was analysed in an attempt to exclude genes from the list of candidates, based on their localisation of expression. Plant factors conferring resistance/susceptibility are expected to be localised at sites of interaction with the pathogen and its host, *i.e.* leaves and stems, so that the corresponding gene expression should occur in the appropriate plant organ. Genes whose expression was presumably restricted to root tissue were not expected to be involved in the plant-fungal interaction, and therefore not considered likely candidates for the resistance locus. Enhanced expression in root tissue was identified for genes At5g45210, At5g45230, At5g45240 and At5g45380 (Fig. 3.14). However, At5g45230 and At5gG45240 exhibited expression throughout the plant and therefore cannot be excluded from functioning in aerial tissues interacting with *C. higginsianum*. The exclusive expression of At5g45210 in roots is in accordance with the observations in a comprehensive study by Tan *et al*. (2007). The differential expression of NBS-LRR genes may function in resistance to a variety of pathogens that attack different parts of the plants. The exclusive expression of At5g45210 in the root endodermis and cortex could therefore hint to a function in resistance to root pathogens, *e.g.* nematodes. Expression of At5g45380 has previously been shown to be involved in the uptake of urea in plant roots (Kojima *et al.*, 2007), in accordance with its enhanced expression in root tissue.

Constitutive expression in root tissue was not identified for any other genes in the mapped region, but expression in the parental accessions Ler-0 and Ws-0 might be different from the expression profile in Col-0. Furthermore, inoculation of the plants with the fungus could change the expression of candidate genes in unpredictable ways.

A targeted expression analysis in the Ler-0 genetical background before and after *C. higginsianum* inoculation was not available due to missing information of the Ler-0 sequence in this region (see 3.6). Narusaka *et a.l* (2004) performed a microarray analysis using arrays consisting of approximately 7,000 *Arabidopsis* full-length cDNAs to compare

gene expression of uninoculated Col-0 plants versus plants inoculated with *C. higginsianum*. Their expression profile of 120 *Arabidopsis* genes which were significantly induced by inoculation did not include the candidate genes I identified in the present study.
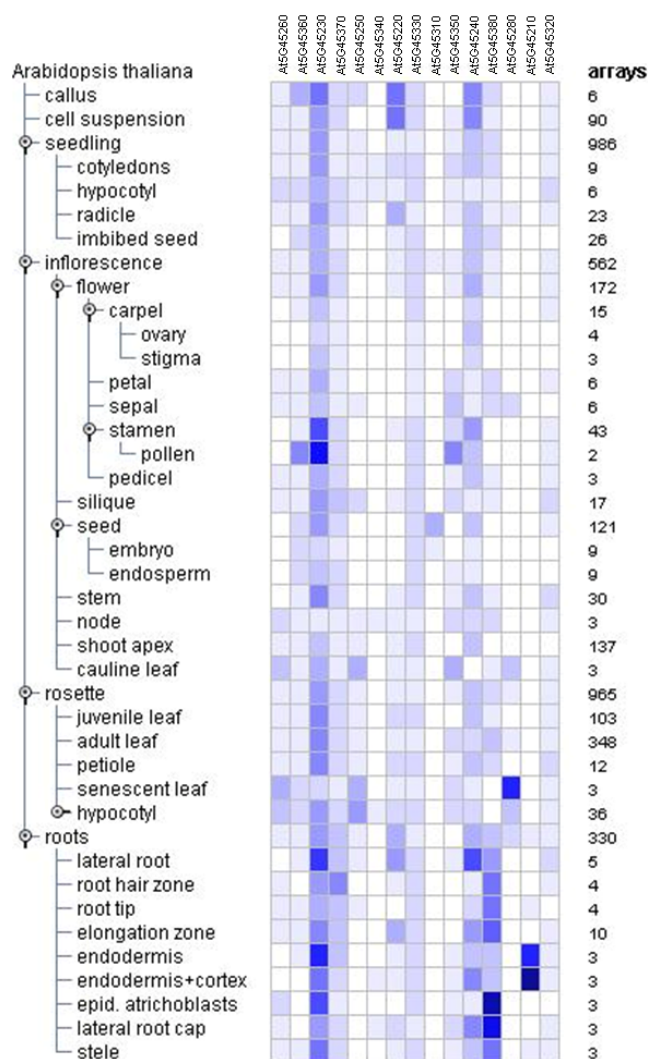
Figure 3.14: Heat map of expression values of 15 *Arabidopsis* genes on chromosome V, according to microarray analysis of plant organs in unchallenged plants.

Varying numbers of microarray analyses (right column) identified the specific expression of *Arabidopsis* (Col-0) genes in individual plant organs and their corresponding cell tissues. The expression potential (EP) of a given gene is defined here as the average of the top 1% signal values of a probe set across all arrays in the database. The darkest colour corresponds to the EP value. Image modified from the genevestigator database (www.genevestigator.com).

3.5   Analysis of *Arabidopsis* T-DNA insertion mutants

A large collection of *Agrobacterium tumefaciens* transferred DNA (T-DNA) insertion transformants of *Arabidopsis* has been generated through gene disruption by the random integration of *Agrobacterium* T-DNA vectors into the plant genome (Alonso *et al.*, 2003). The locations of the insertions were subsequently determined by sequencing of the genomic DNA flanking the T-DNA and are publicly available (http://signal.salk.edu/cgi-bin/tdnaexpress). The generation of mutations due to the gene disruption can result in an altered phenotype compared to the wild type which might reveal the function of the affected gene. Due to the random insertion of the T-DNA, it can be located in promoter regions or exons, but also in an intron and intergenic region, and therefore may not always result in gene disruption.

In order to identify the recessive resistance locus among the 20 identified candidate genes, several *Arabidopsis* mutants with insertions in the candidate genes were analysed for their infection phenotypes after *C. higginsianum* inoculation (Tab. 3.14). For each line, at least 20 individual plants were tested, as most T-DNA insertion mutant lines consist of a mixture of homozygous and heterozygous plants. I expected that only mutants affected in the recessive resistance locus would exhibit a different infection phenotype compared to the corresponding wild type. Several different collections of T-DNA insertion mutants are available (Sundaresan *et al.*, 1995; Tissier *et al.*, 1999; Samson *et al.*, 2002; Alonso *et al.*, 2003). These mutant collections were generated in the genetic backgrounds Col-0, Ws-2 and Ler-0, two of which differ from the parental accessions used for the present mapping analysis. Thus, a screen of the candidate genes in these mutant collections harboured the problem that the gene of interest may not be present in the mutant collection or, due to natural variation between *Arabidopsis* accessions, could have varying effects on the infection phenotype. Furthermore, for many of the selected mutant lines in the Ws-2 and Ler-0 genetic backgrounds, the insertion constructs were predicted not to be located in the coding regions of the genes, based on the Col-0 reference sequence, and are therefore listed according to the gene with the closest proximity.

Table 3.14: List of tested *Arabidopsis* T-DNA insertion mutants and their
response to *Colletotrichum higginsianum* inoculation

Affected gene	Seed-stock identification	Genetic background	Seed-stock centre	Macroscopical phenotype
At5g45200	EOTTV19T3	Ws-2	INRA	wild-type
At5g45210	DYHTV92T3	Ws-2	INRA	wild-type
At5g45210	N564468	Col-0	NASC	wild-type
At5g45220	N140586	Ler-0	NASC	wild-type
At5g45220	N620694	Col-0	NASC	wild-type
At5g45230	N514813	Col-0	NASC	wild-type
At5g45240	ESYTV2T3	Ws-2	INRA	wild-type
At5g45240	N568070	Col-0	NASC	wild-type
At5g45240	N521539	Col-0	NASC	wild-type
At5g45240	N524831	Col-0	NASC	wild-type
At5g45250	N565748	Col-0	NASC	wild-type
At5g45250	GT6567	Ler-0	CSH	wild-type
At5g45260	N561602	Col-0	NASC	wild-type
At5g45260	N599734	Col-0	NASC	wild-type
At5g45275	N663173	Col-0	NASC	wild-type
At5g45275	N607262	Col-0	NASC	wild-type
At5g45275	N662744	Col-0	NASC	wild-type
At5g45276	EYKTV172T3	Ws-2	INRA	wild-type
At5g45276	CVGTV3T3	Ws-2	INRA	wild-type
At5g45280	EXZTV192T3	Ws-2	INRA	wild-type
At5g45280	DXMTV35T3	Ws-2	INRA	wild-type
At5g45280	DYHTV11T3	Ws-2	INRA	wild-type
At5g45280	EXJTV48T3	Ws-2	INRA	wild-type
At5g45280	N524444	Col-0	NASC	wild-type
At5g45290	N604509	Col-0	NASC	wild-type
At5g45290	N625067	Col-0	NASC	wild-type
At5g45290	EAQTV54T3	Ws-2	INRA	wild-type
At5g45290	N660962	Col-0	NASC	wild-type
At5g45300	N661204	Col-0	NASC	wild-type
At5g45307	N594145	Col-0	NASC	wild-type
At5g45310	FBOTV28T3	Ws-2	INRA	wild-type
At5g45330	EEUTV145T3	Ws-2	INRA	wild-type
At5g45340	N601566	Col-0	NASC	wild-type
At5g45340	N578170	Col-0	NASC	wild-type
At5g45340	FCATV57T3	Ws-2	INRA	wild-type
At5g45350	N579478	Col-0	NASC	wild-type
At5g45360	ABWTV8T3	Ws-2	INRA	wild-type
At5g45360	CRCTV7T3	Ws-2	INRA	wild-type
At5g45360	EQMTV47T3	Ws-2	INRA	wild-type
At5g45360	N514089	Col-0	NASC	wild-type
At5g45360	N164485	Col-0	NASC	wild-type
At5g45380	ACCTV4T3	Ws-2	INRA	wild-type

Table 3.14: continuation

Three-week-old plants (at least 20 plants of each mutant line) were inoculated with *C. higginsianum* and at 7 dpi scored for their macroscopic infection phenotypes. The localisation of the insertion constructs in the *Arabidopsis* genome (http://signal.salk.edu/cgi-bin/tdnaexpress) was based on the Col-0 reference sequence. Insertion constructs in the Ws-2 and Ler-0 background might not be localised in the coding region of the indicated genes and are therefore listed according to the gene with the closest proximity.

Wild-type-like, macroscopic phenotype of mutant plants was indistinguishable from that of the wild-type genetic background.

None of the listed 42 mutant lines affected in the coding or intergenic regions of 20 genes (based on the Col-0 reference sequence), showed and altered infection phenotype compared to the corresponding wild-type genetic background. Therefore the phenotypes of the tested T-DNA mutants did not provide any hint to the possible identity of the resistance locus.

3.6 Screening of an *Arabidopsis* Ler-0 binary bacterial artificial chromosome (BIBAC)-library

In the course of developing molecular markers for positional cloning (see 3.3.2), I found that the Col-0 sequence in the region of interest is highly polymorphic to the sequence of the parental accessions Ler-0 and Ws-0. Several of the PCR-based markers that were designed based on the Col-0 sequence were functional in a PCR reaction with Col-0 genomic DNA as template (data not shown). However, use of the same markers in a PCR reaction with template DNA from accessions Ler-0 and Ws-0 did not result in a PCR product. This was especially noticeable for the region ~ 18,300,000-18,330,000 bp, a region containing predicted or verified TIR-NB-LRR genes (see 3.4). This suggests the existence of major sequence divergence between Col-0 and Ler-0/Ws-0 in this region. Sequencing of the entire region of interest in Ler-0 was therefore expected to reveal both major and minor sequence polymorphisms between Col-0 and Ler-0 including possible absence of genes identified in Col-0, or presence of additional genes in this region in Ler-0 that had not been identified for Col-0. Polymorphisms could also be due to gene

duplications, deletions, inversions and truncations (Clark *et al.*, 2007). However, even single nucleotide polymorphisms between the two accessions could result in natural variation in their response to *C. higginsianum* infection (Chu *et al.*, 2006; Clark *et al.*, 2007). To draw conclusions about the nature of polymorphisms responsible for the observed natural variation in resistance to *C. higginsianum*, the Ler-0 sequence of the recessive resistance locus would have to be compared to that of resistant accessions.

A Bacterial Artificial Chromosome (BAC) library, containing large-inserts of Ler-0 genomic DNA, constructed in a plant-transformation-competent binary BAC vector (BIBAC2), was available (Chang *et al.*, 2003). The library contains 11,520 clones with an estimated average insert size of 162 kb and ~ 11.5 coverage of the genome. This Ler-0 BIBAC library (kindly provided by Dr. W. Soppe, Cologne, Germany) was screened (with the kind help of C. Philipp, Cologne, Germany) with the two radioactively labelled probes 176 (18,306,114-18,306,878 bp) and 205 (18,337,817-18,338,557 bp) (Tab. SD2) in a single screening experiment. Hybridisation of at least one of these probes to the insert nucleotide sequences of 10 BACs was identified. Therefore these BACs were expected to contain parts of or the entire region of interest within their inserts. The approximate insert size of the 10 BACs could be identified by digestion with the endonuclease enzyme BamH I to vary between 75,300 bp and 150,000 bp (data not shown) and was therefore in accordance with an expected average insert size of 160 kb (Chang *et al.*, 2003).

BIBAC transformation of Ws-0 plants could test the hypothesis that resistance of Ws-0 is due to natural variation in a true susceptibility factor. Transformation of a functional susceptibility factor into a resistant genetic background, such as Ws-0, would be expected to result in gain of susceptibility. However, previous attempts of BIBAC transformation into *Arabidopsis* plants were not successful, possibly due to the large insert size of the BACs (Prof. B. Weisshaar, Bielefeld, Germany, personal communication), and therefore this strategy was not pursued further in the present study.

To identify the precise insert size and its position within the *Arabidopsis* genome (based to the Col-0 reference sequence), vector end sequencing was carried out by QIAGEN (Hilden) for eight of the 10 BACs. For this purpose, BAC end sequencing reactions were conducted, aiming to determine 800-1200 bp of the insert sequence starting from both ends of the insert. The insert end sequences could then be subjected to homology searches using the Basic Local Alignment Search Tool (BLAST) megablast algorithm (Altschul *et al.*, 1997) against the Col-0 reference sequence to identify putative matches to the sequences. A precise location of the insert sequence (18,308,223-18,377,702 bp) could be

determined for only one BAC clone (designated BAC #8). For the other seven BACs, a genomic location could not be established for at least one insert end due to poor sequence homology, which was consistent with the previously identified major polymorphisms between the Col-0 and Ler-0 sequences in this region.

Assembly of sequencing data of the entire insert of BAC #8 by QIAGEN resulted in the generation of five contigs. Analysis of these contigs identified the presence of the TIR-NB-LRR genes At5g45210, At5g45250 and At5g45260 and presence of the genes At5g45275, At5g45276, At5g45280, At5g45290, At5g45300 and At5g45310 (see 3.4) with varying degrees of polymorphisms to the Col-0 reference sequence. Presence of the genes At5g45220, At5g45230 and At5g45240 as identified in the Col-0 reference accession could not be confirmed. When the sequence of contig #2 was subjected to homology searches using BLASTN, significant alignments to At5g45220 (92% identity) could be identified, indicating the presence of TIR-NB-LRR genes in Ler-0 with polymorphic sequences compared to the TIR-NB-LRR genes identified for Col-0. The high degree of the overall Ler-0 DNA sequence variation to the Col-0 reference sequence in the region containing the cluster of TIR-NB-LRR genes confirms previous findings (see above).

Further analysis of the insert sequence identified the presence of a ~ 5000 bp region located between At5g45290 and At5g45300 in Ler-0 that could not be identified at this position for the Col-0 reference sequence. Homology analysis for this sequence identified the presence of a sequence of a 600 bp-length with a 99% homology to the reverse transcriptase *Ta24* gene, which could indicate an insertion of a non-LTR retrotransposon into the Ler-0 sequence (Wright *et al.*, 1996).

Analysis of the genes At5g45320 – At5g45380 was not possible as their DNA sequence was not included in the insert of BAC #8.

3.7 Cytological analysis of fungal pathogenesis and host responses in resistant and susceptible *Arabidopsis* accessions

Natural variation in responses to *C. higginsianum* inoculation between the resistant *Arabidopsis* accessions Ws-0, Gifu-2, Can-0, Kondara and the susceptible accession Ler-0 had been identified to be caused by a single recessive resistance locus. It was therefore of interest to characterise the cytological basis of recessive resistance. The comparative

cytological analysis aimed to determine whether all resistant accessions show resistance at the same stage of fungal pathogenesis as expected for resistance due to the effect of a single recessive resistance locus. I also attempted to quantify the extent of resistance in the different accessions.

Two additional accessions, Eil-0 and Col-0, were included in this cytological analysis. Eil-0 has been described previously by Narusaka *et al.* (2004) as a resistant accession in which resistance to C. *higginsianum* was conferred by the single dominant resistance locus *RCH1*. The accession was included in several comparative tests to identify whether the dominant resistance in Eil-0 is associated with a different infection phenotype or defence responses compared to the recessive resistance identified in Ws-0, Gifu-2 and Can-0 and Kondara. The accession Col-0 had been shown to exhibit an intermediate susceptible phenotype (DS 2-3) (Tab. 3.2) and the analysis of symptoms and microscopical tests indicated that fungal pathogenesis is more restricted compared to fully susceptible Ler-0 (Fig. 3.7). Therefore, Col-0 was an interesting accession which may offer additional natural variation in response to *C. higginsianum* infection.

Resistance could conceivably occur at different stages of fungal pathogenesis. It could manifest at early stages of infection, *e.g.* with appressorium formation and penetration of epidermal cells. Therefore, the presence of biotrophic primary hyphae beneath appressoria was quantified (see 3.7.1) in order to estimate the efficiency of appressorial penetration. Resistance could also occur at later stages of pathogenesis by restriction of the development of biotrophic primary hyphae and of necrotrophic secondary hyphae. Therefore, the extent of hyphal colonisation was quantified by ELISA (enzyme-linked immunosorbent assay) (see 3.7.2). In addition, restriction of fungal spread within the plant might limit fungal asexual reproduction. Fungal spore-production *in planta* was therefore also quantified (see 3.7.3). Moreover, typical active plant resistance mechanisms, *e.g.* production of reactive oxygen species (ROS) and callose deposition at pathogen entry sites, were analysed histochemically and quantified to assess their possible contribution to the observed resistance of selected accessions (see 3.7.4).

3.7.1 Analysis of appressorial penetration efficiency

Following the germination of spores on the plant surface, *C. higginsianum* invades the host tissue by the development of a series of specialised infection structures, including germ-tubes, appressoria, penetration pegs, biotrophic primary hyphae and necrotrophic

secondary hyphae (Perfect *et al.*, 1999) (see 1.2). Initial microscopical analyses focused on the early penetration stages to identify whether resistance of the selected accessions was associated with termination or delay of fungal penetration attempts. The percentage of appressoria producing biotrophic primary hyphae inside host epidermal cells was quantified at three different time points for all selected accessions to estimate the efficiency of fungal penetration and therefore the extent of plant resistance at this fungal developmental stage (Fig. 3.15 and see 2.2.16.1). For this purpose, leaf samples were examined microscopically at one, two and three days after inoculation. The data represent mean values from three independent experiments.
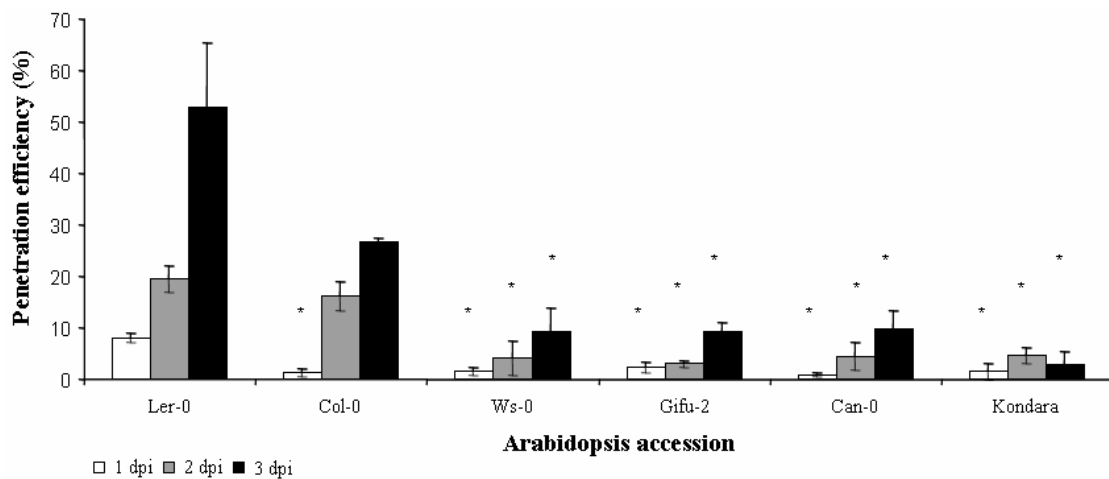
Figure 3.15: Penetration efficiency of appressoria formed by *Colletotrichum higginsianum* on selected *Arabidopsis* accessions at one, two and three days post inoculation (dpi).
The frequency with which appressoria of *C. higginsianum* formed biotrophic primary hyphae in selected *Arabidopsis* accessions was analysed at one, two and three days post inoculation of three-week-old plants. Percentages of appressoria are presented as mean ± standard error (n=3) of three independent experiments. For each experiment, at least 200 interaction sites were analysed by light microscopy for the presence of biotrophic hyphae beneath appressoria. Significant differences from Ler-0 (Student`s test, $P < 0.05$) are indicated by asterisks.

I found that appressoria were produced with the same frequency on leaves of all accessions tested. However, further development of the fungus varied between resistant and susceptible accessions. Almost 10% of the appressoria examined had penetrated host epidermal cells and produced fully developed biotrophic hyphae at 1 dpi in the susceptible accession Ler-0 (Fig. 3.15 and Fig. 3.16 c). The percentage of appressoria that had

successfully penetrated, increased at 2 dpi to 20%, and at 3 dpi, more than 50% of the appressoria examined had penetrated host epidermal cells.

In accordance with previous observations, Col-0 presented an intermediate susceptible phenotype (DS 2). Absolute fungal penetration efficiency on this accession was only slightly reduced in comparison to Ler-0, but the timing of penetration was delayed. Hence, at 1 dpi the percentage of appressoria producing biotrophic hyphae was significantly lower than for Ler-0, but percent penetration had increased to almost 30% at 3 dpi (Fig. 3.15). Therefore, penetration was not significantly different to Ler-0 at later time points.
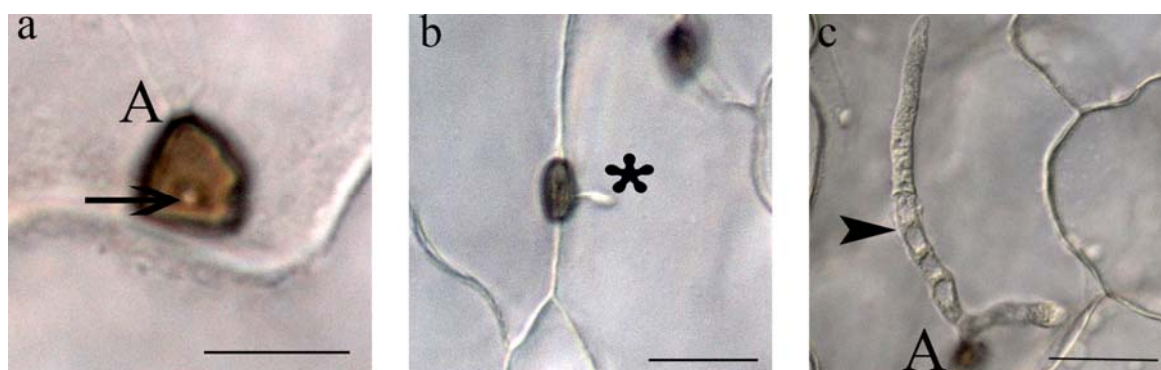
Figure 3.16: Differing outcomes of attempted penetration by *Colletotrichum higginsianum* appressoria into *Arabidopsis* leaf epidermal cells.

Arabidopsis leaves were harvested three days after inoculation with *C. higginsianum,* cleared and analysed by light microscopy without staining. Upon germination, *C. higginsianum* spores produced a specialised infection structure, the melanised appressorium (A), which generates enormous turgor pressure to penetrate host epidermal cells.

(a) Penetration attempt of *C. higginsianum* appressorium (A) into an epidermal cell of the resistant *Arabidopsis* accession Ws-0. Note basal penetration pore (arrow). Bar, 8 µm.

(b) Penetration by an appressorium into an epidermal cell of resistant *Arabidopsis* accession Gifu-2 resulted in the production of a small penetration peg (asterisk), but fungal development did not progress further. Bar, 15 µm.

(c) Penetration by an appressorium into an epidermal cell of susceptible *Arabidopsis* accession Ler-0 resulted in the production of biotrophic primary hyphae (arrowhead). Bar, 15 µm.

In contrast to these findings, penetration efficiency of the fungus in all the resistant accessions (Ws-0, Gifu-2, Can-0 and Kondara) was significantly lower than in Ler-0 and Col-0. Fully developed melanised appressoria of normal appearance could be observed in these resistant accessions and the detection of appressorial penetration pores and short penetration pegs (Fig. 3.16a and b) suggests that these appressoria attempted to penetrate

the underlying plant epidermal cells. However, successful penetration occurred with a significantly lower frequency than in Ler-0. Less than 2% of appressoria formed in the resistant accessions had produced fully developed biotrophic hyphae at 1 dpi, and this did not increase beyond 10% at 3 dpi. Thus, penetration efficiency was significantly lower than on Ler-0 at all three time points, and overall penetration efficiency on all accessions was less than 20% of the efficiency on Ler-0 at 3 dpi (Fig. 3.15).

Although the level of biotrophic hyphae production in resistant accessions at 3 dpi was similar to that in Ler-0 at 1 dpi, it could be demonstrated that the reduced penetration efficiency was not simply due to a delay in fungal penetration. Quantification of appressoria forming biotrophic hyphae at the later time point of 7 dpi (Fig. 3.17) when Ler-0 plants had already fully collapsed, showed that in resistant accessions only ~3.6% of appressoria had successfully penetrated. Therefore, even four days later, penetration efficiency was markedly reduced compared to that on susceptible Ler-0 plants at 3 dpi. This indicates that fungal development is terminated at the penetration stage in all the resistant accessions examined. This result was in accordance with the macroscopic phenotypes at 9 dpi (Fig. 3.7), where the resistant accessions showed few symptoms, whereas Ler-0 plants died as early as 5-6 dpi.

Recapitulating the findings, I could establish that in all *Arabidopsis* accessions tested the fungus produced normal fully developed appressoria at a high frequency. Furthermore, penetration attempts could be observed in all accessions by the formation of penetration pores and penetration pegs at the base of appressoria. However, the production of biotrophic hyphae reflecting the efficiency of penetration was significantly reduced in the resistant accessions Ws-0, Gifu-2, Can-0 and Kondara compared to the susceptible accession Ler-0. Col-0 exhibited an intermediate efficiency of penetration. On the susceptible Ler-0 accession, appressorial penetration efficiency was not only higher than on resistant accessions, but also occurred more rapidly, with an earlier development of biotrophic hyphae. It can be concluded that active or passive resistance mechanisms responsible for the reduced fungal growth during penetration of the plant cuticle or cell wall must act at a very early stage of fungal development seen in tested resistant accessions.
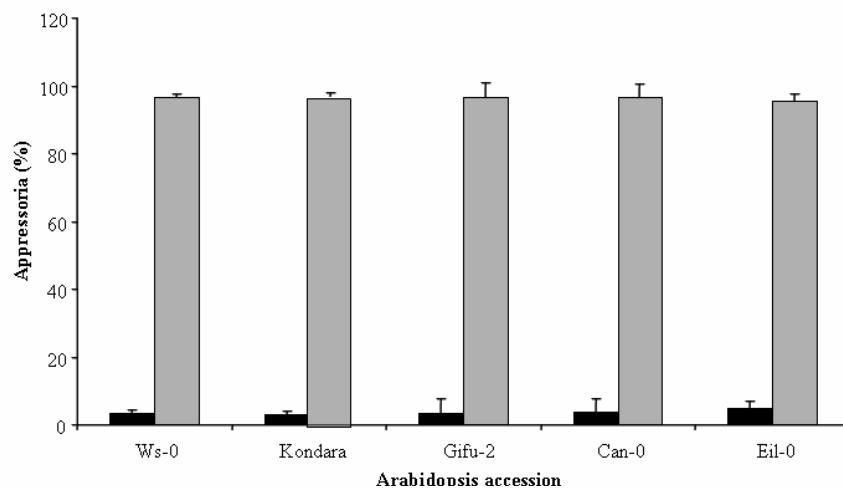
Figure 3.17: Penetration efficiency of *Colletotrichum higginsianum* appressoria on five resistant *Arabidopsis* accessions at seven days post inoculation (dpi).

The proportions of appressoria which had penetrated (visible biotrophic hyphae) or not penetrated (no visible hyphae) epidermal cells of five resistant *Arabidopsis* accessions were analysed by light microscopy after inoculation of three-week-old plants with *C. higginsianum*. Percentages of appressoria are presented as means from three independent experiments and error bars show the standard deviation. For each experiment, at least 300 interaction sites were analysed for the presence of biotrophic hyphae beneath appressoria.

3.7.2   Quantification of hyphal growth

In a susceptible plant, after successful penetration by the appressorium, the fungus invades a single epidermal cell by the production of biotrophic primary hyphae (see 1.2). This initial stage, in which the plant cell remains alive, is eventually followed by a switch to a necrotrophic mode of infection. It is plausible to expect that in resistant accessions the extent of fungal colonisation of host tissue would be limited, as I had previously shown that a low percentage of appressoria successfully penetrate the epidermis (Fig. 3.15). Thus, it was expected that a reduced number of biotrophic hyphae would result in a reduced number of necrotrophic hyphae, and therefore in a reduced size and number of fungal colonies. In accordance with this, following inoculation of the resistant accessions Ws-0, Gifu-2, Can-0 and Kondara with a transgenic *C. higginsianum* strain that constitutively expresses GFP in the cytoplasm (Fig. 3.7b), fungal colonies were indeed reduced in size and number. The red autofluorescence of plant chlorophyll indicated that the plant tissue adjacent to fungal colonies remained intact at this time point in the resistant accessions (3 dpi). This observation was in a clear contrast to the much larger

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size and number of fungal colonies identified after inoculation of Ler-0 plants. Presumably, the higher proportion of appressoria that had successfully penetrated resulted in more extensive fungal growth and colonisation. Furthermore, I could identify by the lack of red autofluorescence of the invaded plant cells that these were no longer intact and destroyed by fungal growth. This effect was also visible in directly adjacent cells not yet invaded by fungal hyphae, demonstrating that the fungus seems to be able to disrupt cells ahead of infection, possibly by the secretion of diffusible cell wall degrading enzymes or fungal toxins. However, it remains unclear whether the reduced size and number of fungal colonies on the resistant accessions is solely due to the reduced penetration efficiency of the appressoria, or whether growth of the hyphae themselves is also inhibited. Consistently, in the intermediate infection phenotype of Col-0, the size and number of fungal colonies was less than in susceptible Ler-0 plants, but more than in any of the resistant accessions.

To quantify hyphal growth in different accessions, an enzyme-linked immunosorbent analysis (ELISA) was conducted (Fig 3.18 and see 2.2.17). Fungal mycelium could be detected and therefore quantified by the binding of a *Colletotrichum* genus specific antibody MAFF27 (Cook *et al.*, 1995). Using immunofluorescence microscopy, this commercially available monoclonal antibody MAFF27 was shown to bind specifically to the biotrophic primary hyphae and secondary, necrotrophic hyphae of *C. higginsianum*, but not to spores or appressoria (Fig. 3.19). For use in ELISA, a secondary antibody conjugated to alkaline phosphatase was used together with the substrate P-nitrophenyl phosphate. The colour of the reaction product was quantified in a plate reader by measuring the specific absorbance of the samples at 405 nm (A_{405}). Three samples of each accession, each consisting of 1g plant material, were estimated.
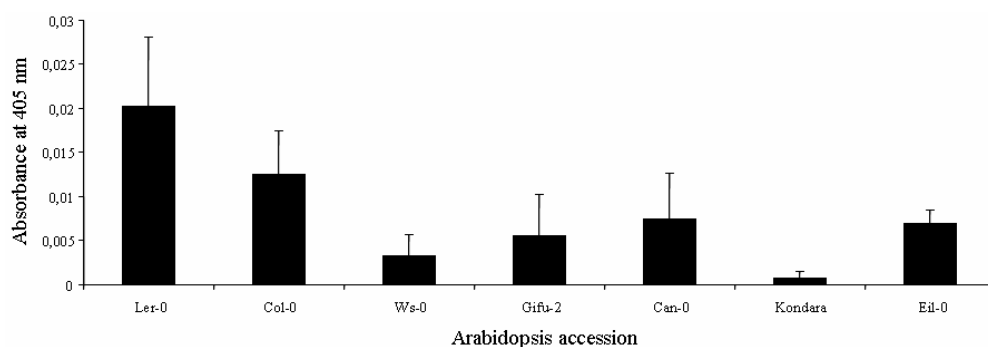
Figure 3.18: Quantification of *Colletotrichum higginsianum* hyphal growth in selected *Arabidopsis* accessions four days post inoculation (dpi).

The extent of hyphal growth of *C. higginsianum* in leaf tissue of selected *Arabidopsis* accessions was quantified four days after spray-inoculation of three-week-old plants by ELISA using a genus-specific monoclonal antibody. Results are presented as mean absorption units in 1g of plant fresh-weight and error bars show the standard deviation of three replicate samples. Independent repetitions of the experiment showed similar results (data not shown).

The ELISA confirmed that more extensive hyphal growth occurred in Ler-0 plants compared to all other accessions tested (Fig. 3.18). In accordance with the microscopical analysis, the extent of hyphal growth in Col-0 was intermediate and lower in all of the resistant accessions. Thus, the detected A_{405} for the resistant accessions were at least 50% lower than the absorbance value for Ler-0. However, these findings must be interpreted with care since the ELISA experiment exhibited several technical problems that prevented an exact quantification of fungal growth to be made *in planta*. Samples from plants were harvested at four days after inoculation which was the earliest time point a clear difference in fungal hyphal growth could be detected by ELISA. At this time point, the integrity of the plant tissue is already affected by fungal infection. The susceptible accessions Ler-0 and Col-0 exhibited water soaked lesions which affected the overall fresh weight of the plants. However, fresh weight was the most suitable measure to standardise the amount of plant material analysed in these experiments. Therefore, the influence of infection on tissue fresh weight is likely to bias the amount of fungal growth. Moreover, due to variation between the three independent samples of each accession originating from the same batch of infected plants, the standard deviations obtained were high. I concluded that a precise quantification of hyphal growth in different accessions is not possible by ELISA. In order to develop a more accurate estimation of hyphal growth *in planta*, an analysis by semi-quantitative PCR was attempted. The aim was to quantify hyphal growth by PCR-based detection of the expression of the transgene *hygromycin B phosphotransferase* (*hph*) in plant samples after inoculation with a *C. higginsianum* strain which expresses *hph* under control of a strong constitutive promoter from the *Apergillus nidulans GPDA* gene (O'Connell *et al.*, 2004). However, it was not possible to compare hyphal growth *in planta* reliably between different plant genotypes due to problems with normalising the amount of sample analysed. As with the ELISA test (see above), significant differences in hyphal growth could only be detected when plants already exhibited strong symptoms of fungal infection. Therefore, both the fresh-weight and size (area) of plant tissue samples were

strongly affected by presence of the water-soaked and necrotic lesions, leading to inaccurate equalisation between the samples and to a misleading estimation of pathogen growth. Degradation of plant genomic DNA at this stage of fungal infection prevented a normalisation by plant internal standards, such as *Rubisco* (data not shown). Furthermore, the same number of plants or leaves could not be analysed because the tested genotypes had significantly different sizes of plants and leaves. Hence, aberrant PCR kinetics associated with sampling heavily necrotised tissues cannot easily be avoided.

Nevertheless, the microscopical observations of GFP-tagged mycelia, in combination with the quantification of hyphal growth by ELISA, even though inaccurate, convincingly demonstrated a strong reduction in hyphal growth in the resistant accessions Ws-0, Gifu-2, Can-0 and Kondara compared to the intermediate susceptible Col-0 and the susceptible accession Ler-0. This is consistent with the previous findings of a reduced appressorial penetration efficiency which resulted in reduced production of biotrophic and necrotrophic hyphae. However, it remains unclear whether this difference in hyphal growth also results from post-penetration defence responses or deficiency in a plant factor required for post-penetration development of the fungus.
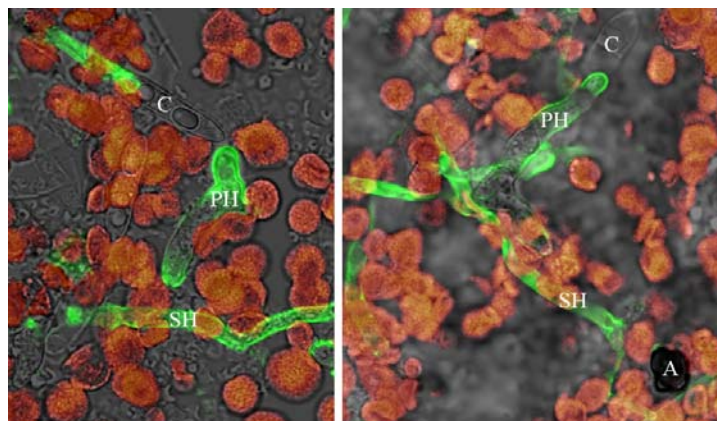
Figure 3.19: Immunofluorescencelabelling of *Colletotrichum higginsianum* biotrophic and necrotrophic hyphae isolated from infected leaves of *Arabidopsis*.

A method for isolating *C. higginsianum* fungal structures from infected leaves of *Arabidopsis* was adapted from (Pain *et al.*, 1994). Inoculated *Arabidopsis* leaves were homogenised filtered to remove plant debris and the fungal structures collected by isopycnic centrifugation on Percoll. After drying onto microscope slides, the fungal structures were then incubated in undiluted monoclonal antibody, raised in rats, which is highly specific for *Colletotrichum* (Cook *et al.*, 1995), followed by incubation with a secondary goat monoclonal anti-rat-antibody, which was fluorescein (FITC) conjugated. Specific binding of the monoclonal antibody to primary, biotrophic hyphae (PH) and secondary, necrotrophic hyphae (SH) was identified by confocal microscopy. No binding of the monoclonal antibody to fungal appressoria (A) and conidia (C) or plant chloroplasts could be detected.

3.7.3 Quantification of spore production

Sporulation of the fungus indicates completion of the pathogenic life-cycle. Only if the fungus passes through all developmental stages can the necrotrophic hyphae produce sporangiophores with spore acervuli. This, in turn enables the fungus to disperse its propagules to other areas of the same leaf, further leaves of the same plant or adjacent plants. Hence, this final stage of the fungal infection cycle can also be considered an indicator of host resistance or susceptibility.

As spores can be produced only after successful penetration of the plant and production of necrotrophic hyphae, it was expected that the selected resistant accessions should exhibit a reduced production of spores compared to the susceptible accessions. Therefore, the number of fungal spores washed from 1g fresh weight of plant material and was quantified at four and five days after inoculation (Fig. 3.20 and see 2.2.16.4). Three replicate samples (nine plants per sample), prepared from the same inoculation experiment, were evaluated and the mean and standard deviation was calculated. Independent repetitions of this experiment showed similar results (data not shown). At 4 dpi, the sporulation of *C. higginsianum* on Ler-0 was significantly greater than in Col-0 and the resistant accessions. This is in accordance with microscopical observations, suggesting earlier penetration and colonisation of Ler-0 than other accessions. The lower sporulation in all other investigated accessions was highly significant and in agreement with the more limited production of necrotrophic hyphae in these accessions as shown by ELISA and confocal microscopy. Differences in sporulation became more evident at 5 dpi. In the resistant accessions sporulation remained at a very low level comparable to that at 4 dpi, while in Col-0 a clear increase in spore production was detectable at 5 dpi. However, this increase is significantly different ($P < 0.02$) from the enormous increase in sporulation detected in Ler-0 at 5 dpi. This confirms microscopical observations of abundant acervuli on Ler-0 leaves at 5 dpi.

In conclusion, quantitative analysis of fungal spore production *in planta* revealed a highly significant difference in sporulation at 4, and particularly at 5 dpi, between susceptible, intermediate susceptible and resistant accessions. This seems likely to be a consequence of fungal development being affected at the early stage of penetration and biotrophic hyphae production. Reduced production of biotrophic and necrotrophic hyphae would be expected to result in fewer specialised fruiting structures essential for the generation of spores and completion of its life cycle. The results are consistent with an early expression of

penetration resistance in the resistant accessions, but do not exclude that the recessively resistant plants lack additional factors required by the fungus for generation of spores.
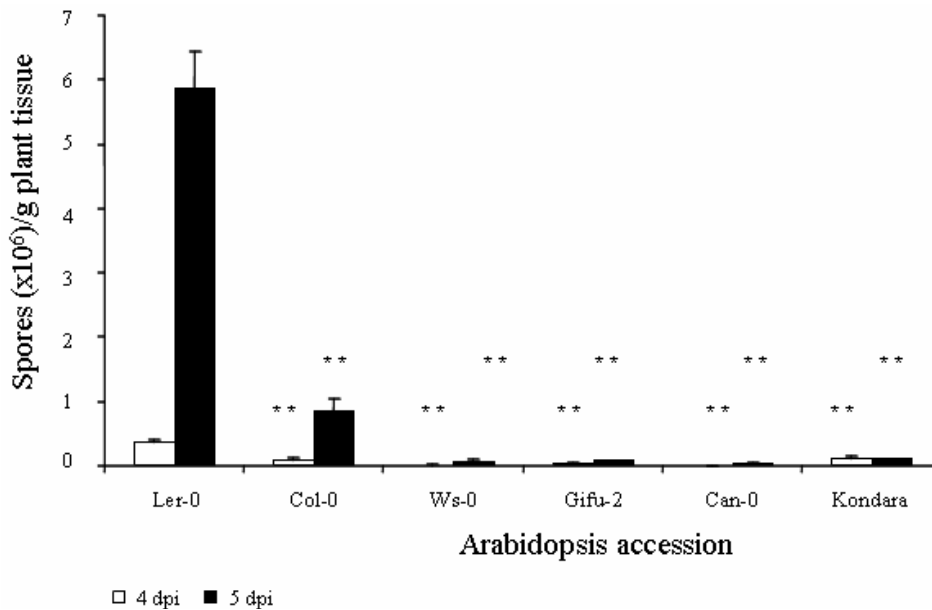
Figure 3.20: Quantitative analysis of *Colletotrichum higginsianum* sporulation on selected *Arabidopsis* accessions at four and five days post inoculation (dpi).

Sporulation of *C. higginsianum* on selected *Arabidopsis* accessions was quantified at four and five days after spray-inoculation of three-week-old plants. For each accession, sporulation was analysed in plant material from three pots (nine plants per pot), each pot was analysed individually. The average number of spores per gram plant tissue is presented. Error bars show standard deviations. Highly significant differences from Ler-0 (Student's test, $P < 0.02$) are indicated by two asterisks. Independent repetitions of the experiment showed similar results (data not shown).

3.7.4 Histochemical characterisation of host defence responses to *C. higginsianum* infection

Active plant resistance is correlated with the activation of defence responses. The perception of the pathogenic invader by the plant induces a series of defence reactions such as a hypersensitive response (HR), defence gene expression and cell wall strengthening (see 1.3).

Local generation of ROS is a frequent plant response to attack by many microbial pathogens (Apel and Hirt, 2004; O'Connell and Panstruga, 2006) and is often linked to the HR and to cell wall-based defence responses such as papilla deposition. However, recent reports suggest that successful pathogenesis by some necrotrophic or hemibiotrophic

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fungal pathogens relies on, or is at least supported by, a high concentration of H_2O_2 (Hückelhoven and Kogel, 2003).

During fungal infection, callose has been shown to be deposited in localised cell wall appositions, termed papillae, that form beneath infection sites and are thought to function as both physical and chemical barriers to pathogen penetration (Zeyen *et al.*, 2002; Hückelhoven, 2007) (see 4.4.3). Therefore, papilla production was commonly assumed to be an active resistance response to invading pathogens. Surprisingly, in contrast to this assumption, the *pmr4* mutant which lacks pathogen-induced callose synthase had reduced susceptibility to virulent powdery mildew fungi and to *H. parasitica* (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). It has therefore been postulated that PMR4 is important for successful infection by these pathogens.

Recessive resistance mediated by the lack of a functional host susceptibility factor is expected to not induce active defence responses (O'Connell and Panstruga, 2006; Iyer-Pascuzzi and McCouch, 2007a). Thus, the mechanisms mediating dominant resistance, *e.g.* resistance conferred by the dominant *RCH1* gene in Eil-0, may differ from those mediating recessive resistance in Ws-0, Gifu-2, Can-0 and Kondara. On the other hand, if recessive resistance in these accessions is conferred by the presence of a recessive *R* gene, active defence responses, possibly similar in nature to those triggered by the *RCH1* gene, can be expected.

As ROS production and callose deposition are common active plant responses that can be induced early after pathogen challenge, I studied their involvement in resistance to *C. higginsianum*.

3.7.4.1 Accumulation of hydrogen peroxide

The accumulation of H_2O_2 as a predominant ROS in plant cells after infection with *C. higginsianum* was determined by staining with 3'-3'-diaminobenzidine (DAB) (Thordal-Christensen *et al.*, 1997) (see 2.2.16.2). DAB polymerises locally at sites of H_2O_2 activity into a reddish-brown polymer which is stable in most solvents and therefore microscopically detectable.

Plants were stained with DAB at two and three days after *C. higginsianum* inoculation. To test whether ROS production is associated with resistance responses to *C. higginsianum*, the presence of H_2O_2 in epidermal cells beneath fungal appressoria was quantified (Fig. 3.21). Thus, for at least 600 appressoria per tested accession I determined (i) whether the

appressoria had penetrated, microscopically visible by the production of biotrophic hyphae, and (ii) if the penetration attempt was associated with DAB staining of the penetrated plant cell; *i.e.* each appressorium was classified into one of four possible categories as shown in Fig. 3.21a. Average values from three independent experiments are presented (Fig. 3.21b), Tab. SD4 and Tab. SD5). Consistent with previous microscopical analyses, ~ 20% of the appressoria had successfully penetrated Ler-0 at two days after inoculation with *C. higginsianum*. Penetration was observed for < 10% of appressoria in the intermediate susceptible accession Col-0, and for < 5% of appressoria in the resistant accessions. It was therefore possible that the plants had already perceived and responded to the fungal attack at this early time point. However, for all tested accessions < 1% of the penetrated appressoria had induced H_2O_2 accumulation as shown by DAB staining of the penetrated cell. Likewise, < 1% of the appressoria which had not penetrated were associated with H_2O_2 accumulation in the underlying plant epidermal cell.

At three days after inoculation with *C. higginsianum*, 60% of the appressoria examined in Ler-0 had penetrated to produce primary hyphae (Fig. 3.21 and 3.22). 14% of penetrated appressoria were associated with DAB staining in the penetrated plant cell and 10% of appressoria which had not penetrated were associated with a DAB staining in the underlying plant cell. Similarly, for appressoria in the Col-0 accession, 10% of the penetrated appressoria were associated with DAB staining of the penetrated cell. However, the DAB staining observed in Ler-0 (Fig. 3.22, right panel) and Col-0 plants at 3 dpi was mostly diffuse and not confined to a single epidermal cell, but was rather distributed in patches of multiple epidermal and mesophyll cells. Since penetrated cells stained in this way generally contained necrotrophic secondary hyphae, the staining may reflect plant cell death caused by the activities of the fungus rather than a localised defence response to pathogen attack. Consistent with previous findings, in all the selected resistant accessions, including Eil-0, < 10% of the penetration attempts by appressoria resulted in the development of biotrophic hyphae by 3 dpi (Fig. 3.21 and Fig. 3.22). However, in clear contrast to the susceptible Ler-0 accessions and intermediate susceptible Col-0 accessions, a negligible percentage (~ 1%) of both penetrated and non-penetrated appressoria were associated with DAB staining of the underlying plant epidermal cell. This finding differs from observations by Narusaka *et al*. (2004), who reported a high frequency of H_2O_2 production in Eil-0 plants between two and three days after infection by *C. higginsianum*. Furthermore, the frequency of penetration attempts associated with DAB staining did not increase at 5 dpi (data not shown). Therefore, no detection of H_2O_2

production in Eil-0 plants, in contrast to observations by Narusaka *et al*. (2004), is not just due to a delayed plant response.

(a)

Scoring categories for host cell responses

I	II	III	IV
penetrated, stained	penetrated, unstained	not penetrated, stained	not penetrated, unstained

(b)

penetrated, stained (I)

penetrated, unstained (II)

not penetrated, stained (III)

not penetrated, unstained (IV)
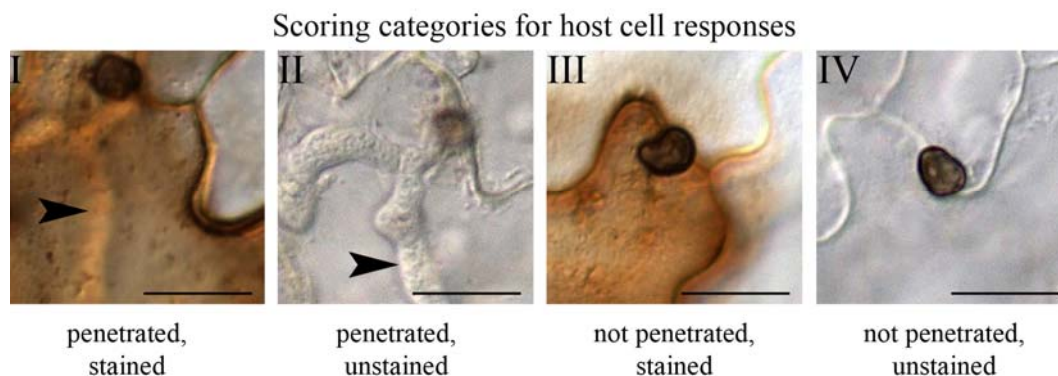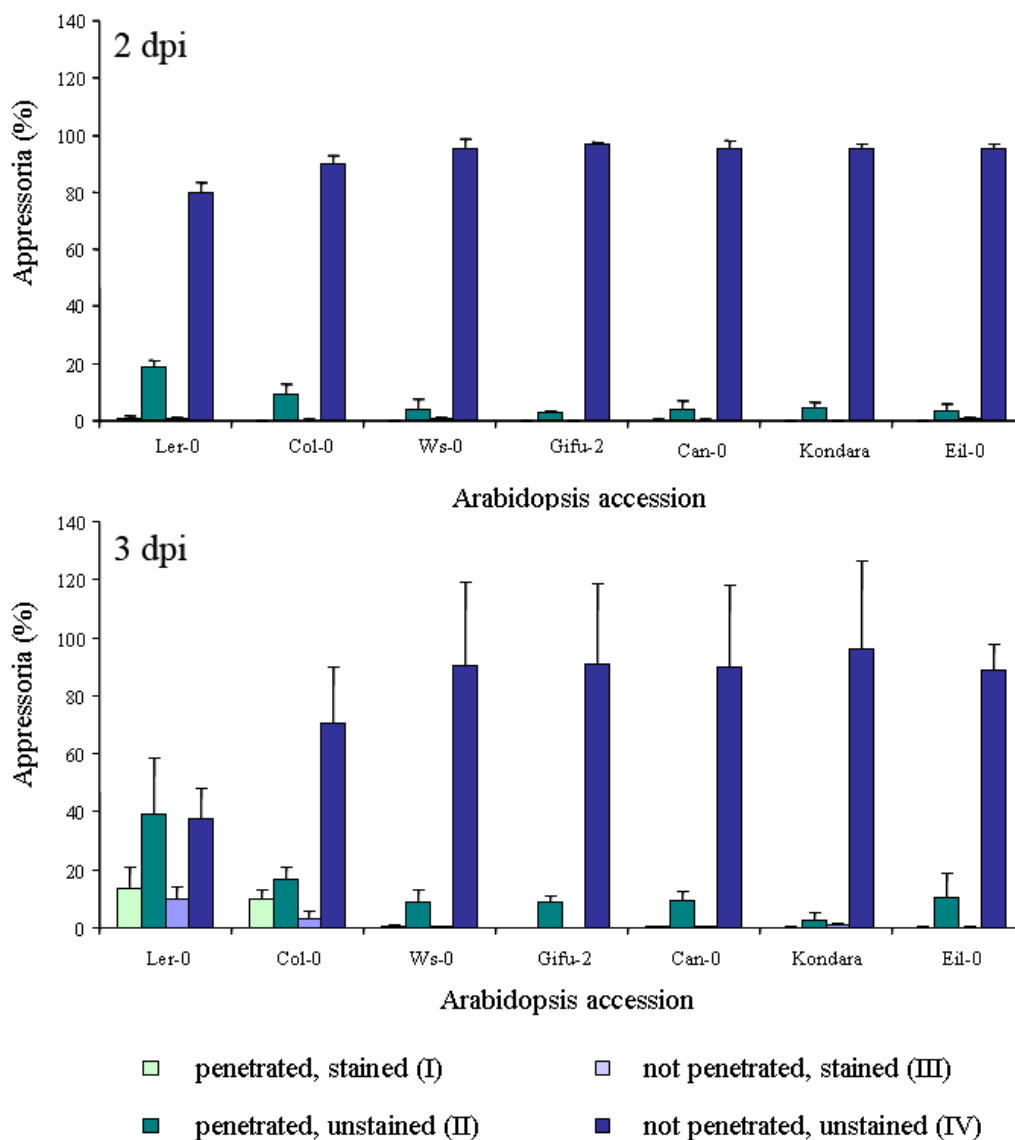
Figure 3.21: Quantitative analysis of the accumulation of reactive oxygen species (ROS) in epidermal cells of selected *Arabidopsis* accessions in response to *Colletotrichum higginsianum* appressoria.

Arabidopsis leaves were harvested two and three days after inoculation with *C. higginsianum* and stained with 3,3'-diaminobenzidine (DAB) for visualisation of H_2O_2 accumulation in plant epidermal cells. The DAB staining was viewed by light microscopy and host cells were scored according to whether they were penetrated (visible biotrophic hyphae) or not penetrated (no visible hyphae).

(a) Examples of categories defined for quantitative analysis of DAB staining. Bar, 15 μm. Biotrophic hyphae are indicated by arrowheads.

Category I: "penetrated, stained". Successful penetration of the appressorium resulted in the production of biotrophic hyphae. The epidermal cell attacked by the appressorium is DAB stained.

Category II "penetrated, unstained". Successful penetration of the appressorium, visible by the presence of biotrophic hyphae. The penetrated host cell shows no DAB staining.

Category III: "not penetrated, stained". Appressorium did not produce visible biotrophic hyphae, but the underlying epidermal cell is DAB stained.

Category IV: "not penetrated, unstained". The appressorium did not produce biotrophic hyphae. No DAB staining of the underlying epidermal cell.

(b) DAB staining in relation to penetration of host cells by biotrophic hyphae was scored according to the categories defined in (a). The percentages of appressoria represent the mean and standard error (n=3) of at least 600 interaction sites distributed over 12 leaves, determined in 3 independent experiments (4 leaves per experiment).
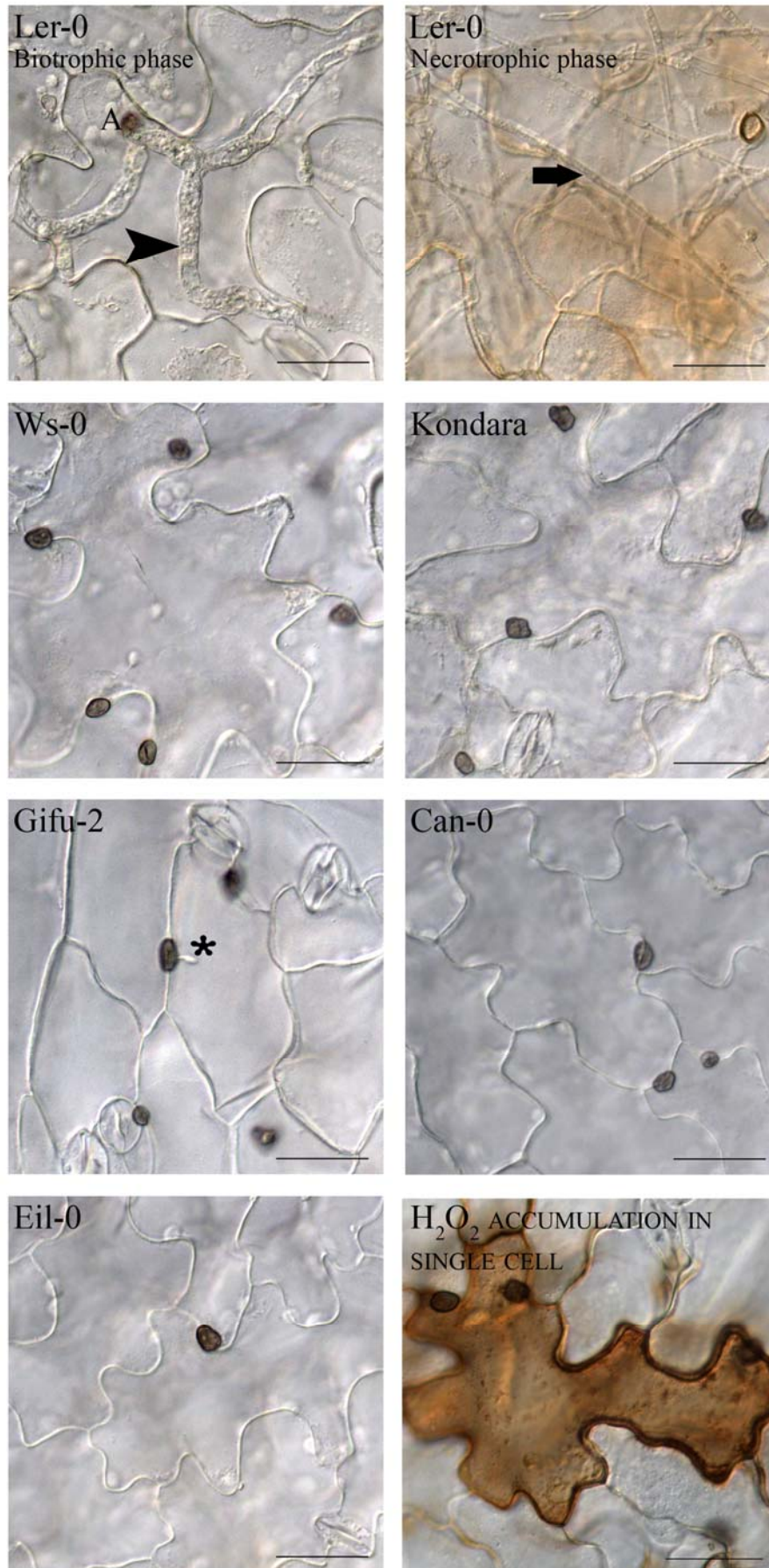
Figure 3.22: Localisation of reactive oxygen species (ROS) in epidermal cells of selected *Arabidopsis* accessions in response to *Colletotrichum higginsianum* inoculation.

Leaves of three-week-old *Arabidopsis* plants were harvested three days after inoculation with *C. higginsianum,* stained with 3-3' diaminobenzidine (DAB) to visualise H_2O_2 accumulation and analysed by light microscopy. Bar, 2.5 μm. A, appressoria; arrowhead, biotrophic hyphae; bold arrow, necrotrophic hyphae; asterisk, penetration peg. Representative micrographs are shown.

(Ler-0, left panel) The appressorium successfully penetrated Ler-0 plants and large biotrophic primary hyphae developed in the epidermal cell. At this biotrophic stage of fungal development no DAB staining was detectable in penetrated epidermal cells.

(Ler-0, right panel) Thin necrotrophic secondary hyphae of *C. higginsianum* colonising Ler-0 plants. At the necrotrophic stage, H_2O_2 accumulation was visible by DAB staining. This diffuse staining was not confined to a single epidermal cell.

(Ws-0), (Kondara), (Gifu-2), (Can-0) and (Eil-0) Appressoria were formed and penetration attempts were clearly visible by the production of penetration pores and penetraton pegs, but no generation of biotrophic hyphae were formed and no H_2O_2 accumulation was detected.

(H_2O_2 accumulation in single cell) H_2O_2 accumulation in a single epidermal cell beneath a successfully penetrated appressorium in Ler-0. This response was rarely seen in Ler-0 and contrasts with the diffuse staining observed in the necrotrophic stage (Ler-0, right panel)

In summary, ROS production was not associated with a rapid localised response to attempted fungal invasion in either resistant or susceptible interactions. Instead, ROS production occurred during the necrotrophic phase of susceptible interactions, affecting large conglomerations of host cells and may therefore be a consequence of host cell death caused by fungal activities, which also affects the adjacent cells not yet been penetrated by the fungus. Furthermore, the results suggest that ROS production is not a significant component of the resistance conferred by either a dominant *R* gene in Eil-0, or putative recessive resistance genes in Ws-0, Gifu-2, Can-0 and Kondara.

3.7.4.2 Callose production

To address the question whether callose production is a plant response associated with resistance to *C. higginsianum*, Aniline Blue staining was performed (Dietrich *et al.*, 1994) (see 2.2.16.3) on inoculated plants to visualise the callose, which produces an intense UV light-induced fluorescence with the Aniline Blue fluorochrome Siroflour. To test whether callose production was associated with attempted penetration by fungal appressoria (Fig. 3.23) at least 600 appressoria per accession were classified into one of four possible

categories, shown in Fig. 3.23a and average values from three independent repetitions are presented (Fig. 3.23b), Tab. SD6 and Tab. SD7).

As expected, at two days after inoculation with *C. higginsianum* a higher percentage of appressoria had successfully penetrated plant cells in Ler-0 (16%) and Col-0 (13%) than in all the resistant accessions (~ 4%). In Ler-0, 7% of the penetrated appressoria were associated with callose deposition in the cell beneath the appressorium while in Col-0 10% of the penetrated appressoria were associated with callose deposits. For all tested accessions at 2 dpi, > 80% of the appressoria had not penetrated and were not associated with callose production. In contrast, only 0-4% of appressoria on resistant plants had successfully penetrated, but all of these exhibited a callose production in the penetrated plant cell.

At 3 dpi, fungal penetration had increased to 53.7% in Ler-0 plants but only reached 14.6% of appressoria in Col-0 plants. In Ler-0 plants only a small proportion of the successfully penetrated appressoria was associated with callose deposits (16%) (Fig. 3.23 and Fig. 3.24). However, in Col-0, 10% of the total 14.6% of successfully penetrated appressoria were associated with callose production beneath the appressorium. Frequently, biotrophic hyphae were seen to penetrate through the callose deposit both in Ler-0 and Col-0. In the resistant accessions Ws-0, Gifu-2 and Kondara, an increase of callose production was detectable from 2 to 3 dpi (Fig. 3.23). In Kondara and Gifu-2, the percentage of unpenetrated appressoria associated with callose deposits (74%) was even higher than the percentage of unpenetrated appressoria not associated with callose production (50%). In contrast, no clear increase of unpenetrated appressoria associated with callose production occurred at 3 dpi in Can-0 and Eil-0. Furthermore, in all susceptible and resistant accessions, specific staining of callose at plant-pathogen interaction sites sometimes occurred along the entire cell wall at 3 dpi (Fig. 3.24), which may indicate an active response to pathogen attack by adjacent living plant cells.

(a)

Scoring categories for host cell responses

I	II	III	IV
penetrated, stained	penetrated, unstained	not penetrated, stained	not penetrated, unstained
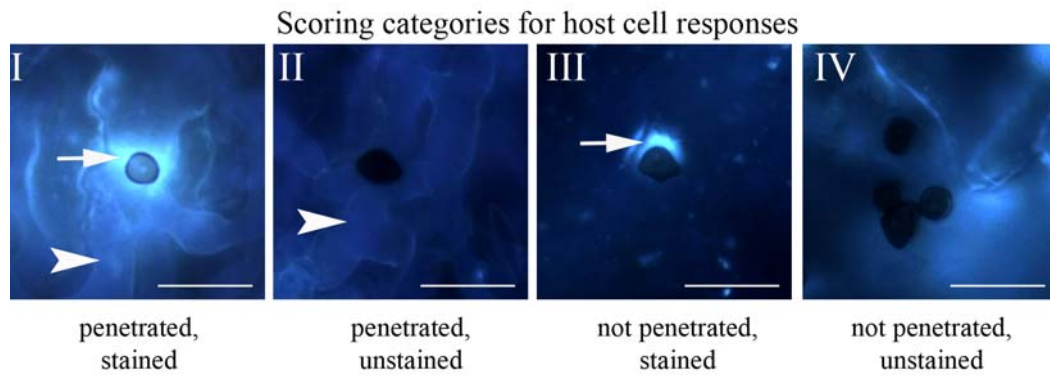
(b)
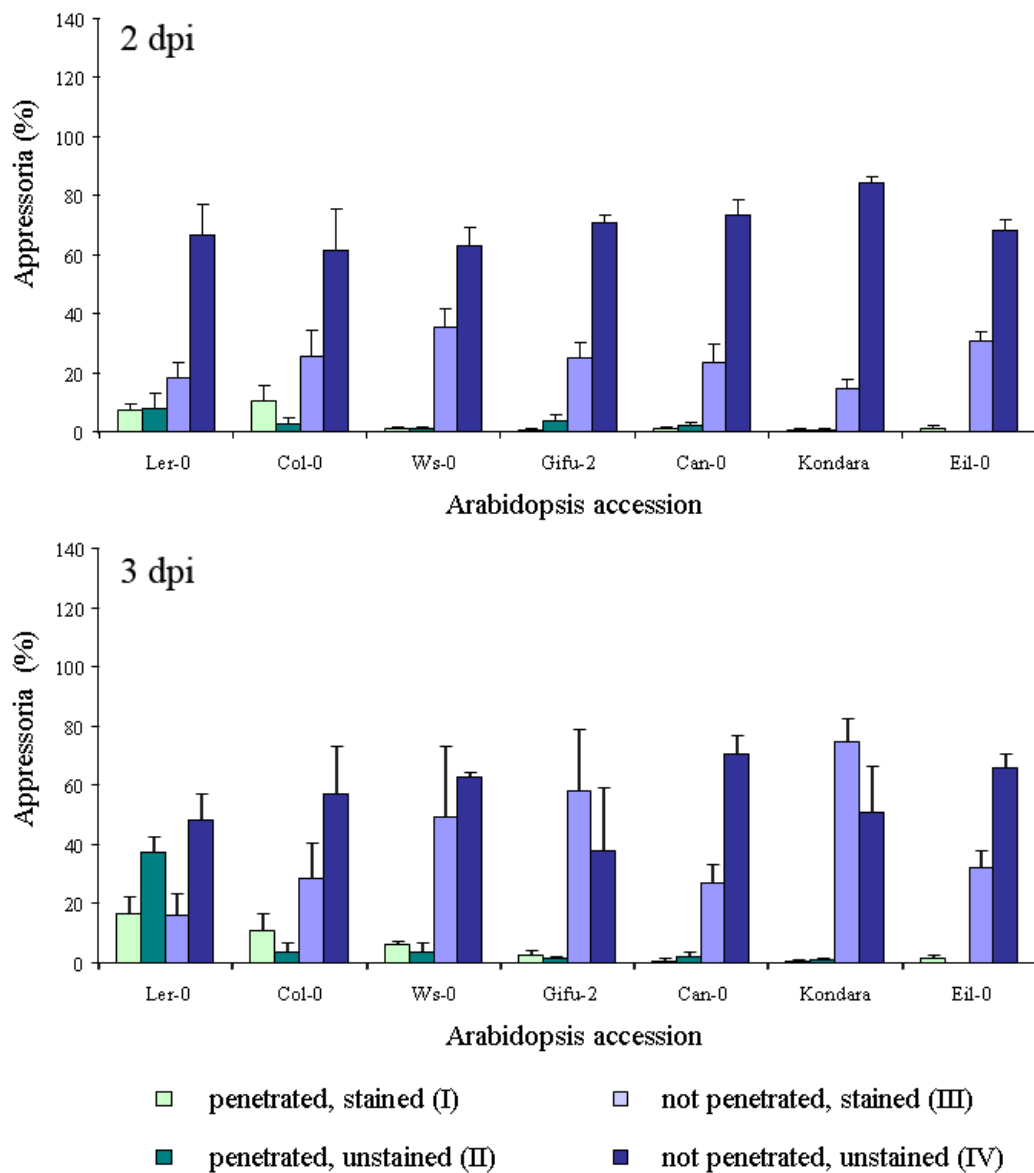
Figure 3.23: Quantitative analysis of callose production in epidermal cells of selected *Arabidopsis* accessions in response to *Colletotrichum higginsianum* appressoria.

Arabidopsis leaves were harvested two and three days after inoculation with *C. higginsianum* and stained with Aniline Blue to visualise callose deposition by fluorescence microscopy in relation to whether the cells were penetrated (visible biotrophic hyphae) or not penetrated (no visible hyphae).

(a) Examples of categories defined for quantitative analysis of callose production. Bar, 15 μm. Callose depositions are indicated by arrows, biotrophic hyphae by arrowheads. The fungal cell wall was weakly stained by Aniline Blue due to the presence of β1,3 glucans.

Category I: "penetrated, stained". Successful penetration by the appressorium resulted in the production of biotrophic hyphae. A callose papilla has been deposited beneath the appressorium, but has been penetrated by the hypha.

Category II: "penetrated, unstained". Successful penetration by the appressorium has resulted in the production of biotrophic hyphae. There is no detectable callose deposited beneath the appressorium.

Category III: "not penetrated, stained". Appressorium has not produced visible biotrophic hyphae. A callose papilla is present beneath the appressorium.

Category IV: "not penetrated, unstained". No biotrophic hyphae and no papillae are visible beneath the appressorium.

(b) Callose production in relation to penetration of host cells by biotrophic hyphae was scored according to the categories defined in (a). Percentages of appressoria represent the mean and standard error (n=3) of at least 600 interaction sites distributed over 12 leaves, determined in three independent experiments (four leaves per experiment).
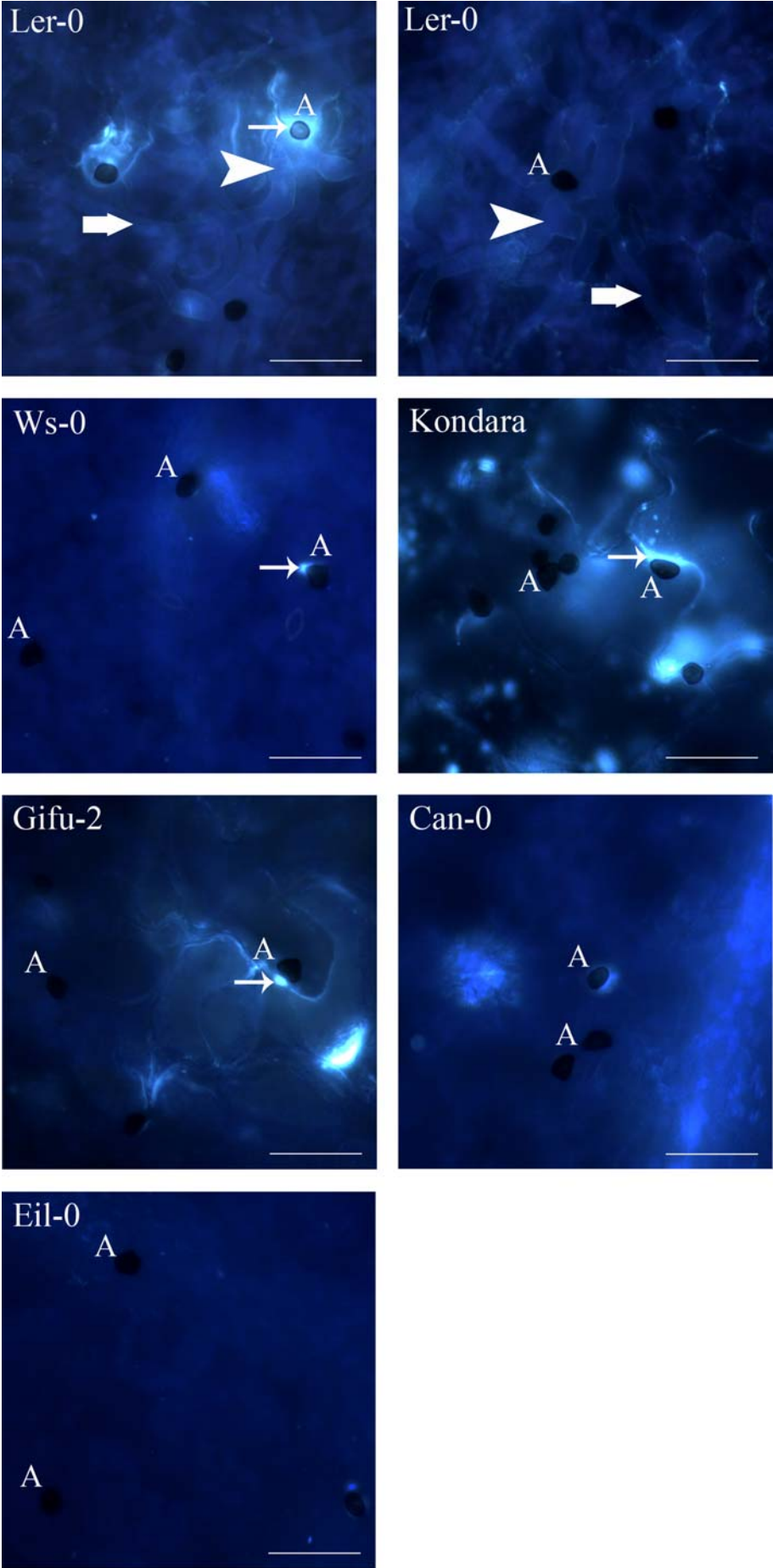
Figure 3.24: Detection of callose production in epidermal cells of selected *Arabidopsis* accessions after *Colletotrichum higginsianum* inoculation.

Leaves from three-week-old plants of selected *Arabidopsis* accessions were harvested three days after inoculation with *C. higginsianum* and stained with Aniline Blue to visualise callose deposition. The Aniline Blue staining was viewed by fluorescence microscopy. Bar, 2.5 μm. A, appressoria; arrowheads, biotrophic hyphae; bold arrows, necrotrophic hyphae; arrows, papillae.

(Ler-0, left panel) Callose production could be detected beneath two of the appressoria. Biotrophic hyphae penetrated through the callose deposit to give rise to necrotrophic hyphae.

(Ler-0, right panel) Callose deposition is not visible beneath any appressoria. Biotrophic and necrotrophic hyphae developed from the appressoria.

(Ws-0) For one of four appressoria, callose production could be detected by Aniline Blue staining. No biotrophic developed from any of the appressoria.

(Kondara) For two appressoria papillae were visible in association with a callose thickening of the cell walls of affected epidermal cells. No biotrophic hyphae emerged from any of the appressoria.

(Gifu-2) No biotrophic hyphae developed from any of the appressoria. A callose deposition was present beneath one appressorium.

(Can-0) For one of three appressoria weak Aniline Blue fluorescence was visible, indicating callose production. No biotrophic hyphae emerged from any of the appressoria.

(Eil-0) No biotrophic hyphae or callose production were visible beneath any of the appressoria.

In summary, callose production was found to be a response to attempted fungal entry in susceptible as well as resistant accessions and occurred as early as 2 dpi. While there was a clear difference in penetration efficiency between susceptible (Ler-0 and Col-0) and resistant accessions, the percentage of unpenetrated appressoria associated with callose deposits and unpenetrated appressoria without callose deposits were similar for all tested accessions. This suggests that failure to penetrate is not directly related to callose deposition. At 3 dpi, biotrophic hypha production had increased in susceptible Ler-0 plants and these hyphae successfully penetrated through callose deposits in most cases where callose was present. In contrast, Ws-0, Gifu-2 and Kondara showed a large increase in the proportion of unpenetrated appressoria associated with callose staining which was not observed for the accessions Col-0, Can-0 and Eil-0. Notably, the dominant resistance of Eil-0 plants was not associated with abundant callose deposition.

3.7.5 Summary of comparative analysis of fungal pathogenesis and host defence responses in selected *Arabidopsis* accessions

A detailed cytological analysis of selected susceptible, intermediate susceptible and resistant *Arabidopsis* accessions was carried out to determine at what stage of fungal development resistance is expressed and which defence mechanisms might underlie this resistance. Also, the cytology of resistance in a dominant resistant accession was directly compared with that of accessions exhibiting recessive resistance.

The stage of fungal development reached by *C. higginsianum* in different host accessions was studied and the extent of fungal colonisation quantified. I showed that the early fungal developmental stage of appressorial penetration and biotrophic hyphae formation was arrested in resistant accessions. Progress to subsequent developmental stages, analysed by ELISA (biotrophic and necrotrophic hyphae production) and spore quantification (asexual reproduction) clearly differed in resistant accessions compared to the susceptible ones. To identify possible plant defence mechanisms that might contribute to resistance, H_2O_2 accumulation and callose deposition were evaluated histochemically. This identified differences in plant defence responses between the resistant accessions and susceptible Ler-0. However, neither H_2O_2, nor callose deposition were clearly linked to the resistance phenotype, *i.e.* failure to penetrate. Furthermore, no significant differences in defence responses were found between Eil-0 in which resistance is conferred by the dominant *RCH1* gene, and accessions exhibiting recessively inherited resistance.

4   Discussion

In the present study, genetic determinants of the interaction between *Arabidopsis* and the hemibiotrophic ascomycete *C. higginsianum* were analysed, with the emphasis on the identification of plant susceptibility factors. This was expected to provide important new insights into the plant contribution to the interaction between the fungus and its host.

Two different approaches were used to identify potential *Arabidopsis* susceptibility factors in the interaction with *C. higginsianum*. The first approach was a forward genetic screen based on the identification of ethylmethane sulfonate (EMS) and γ-radiation-induced mutants (in Ler-0 wild type, Ler *eds1*-2 and Ler *rar1*-13 genetic backgrounds) that had lost susceptibility to *C. higginsianum*. A screen of 207,000 mutant lines identified 38 mutants that exhibited a loss-of-susceptibility phenotype, but since the reduction of susceptibility was not sufficiently clear, positional cloning of these loci was not possible. Analysis of available induced mutants exhibiting reduced susceptibility to *H. parasitica* (*dmr1*-4 and *dmr6*) (Van Damme *et al.*, 2005), and to *G. cichoracearum* (*pmr4-pmr6*) (Vogel and Somerville, 2000), revealed that these loci also play a role in the compatible interaction with *C. higginsianum*.

The second approach was based on natural variation between accessions of *Arabidopsis* in response to challenge by *C. higginsianum*. Recessive resistance factors were sought by crossing resistant accessions to the susceptible accession Ler-0 and following segregation in the F_1 and F_2 progeny. Furthermore, QTL analysis was utilised for the identification of molecular determinants of resistance to *C. higginsianum*. A single recessive resistance locus, conferring recessive resistance in at least four resistant *Arabidopsis* accessions originating from different geographic regions, was identified by positional cloning and QTL mapping on the lower arm of chromosome V. The cellular basis of this recessive resistance and its effects on fungal pathogenesis were investigated microscopically.

4.1   *Arabidopsis* accessions vary in their resistance to *C. higginsianum*

In the present study, I identified natural variation of *Arabidopsis* accessions in resistance to *C. higginsianum* inoculation (see 3.2.1). A total of 116 *Arabidopsis* accessions, originating from various geographic origins and reflecting a maximum possible genetic diversity of the species in a minimum of accessions (McKhann *et al.*, 2004), were

classified according to their disease scores after *C. higginsianum* inoculation. The accessions showed a complete spectrum of responses from extreme resistance to extreme susceptibility. The assignment of disease scores to accessions was in overall accordance with a similar study by Narusaka *et al.* (2004), except for one accession. While in the present study Oy-0 showed a resistant phenotype with only a limited number of lesions and restricted fungal growth, Narusaka *et al.* (2004) identified Oy-0 as a highly susceptible accession. Nevertheless, the overall good agreement between the results of these two independent infection studies indicates that *C. higginsianum* infection phenotypes are robust and reproducible, being relatively independent of laboratorial conditions, fungal isolates and infection procedures.

The broad range of infection phenotypes indicated the existence of genetic variation in response to *C. higginsianum* inoculation within and between populations. Natural variation within populations was exemplified by accessions originating from the same geographical region, which showed varying responses to *C. higginsianum* infection. Among these, in particular noticeable are the infection phenotypes of Ws-0 and Ws-2. Although originating from the same geographic region in Russia, they displayed differing infection phenotypes in response to *C. higginsianum*. A different F_1 generation infection phenotype and contrasting segregation patterns in the F_2 generation after crossing to the susceptible accession Ler-0 (Tab. 3.3, Tab. 3.4 and Tab 3.5) suggest that different mechanisms are responsible for resistance in Ws-0 and Ws-2 as discussed in section 4.3.

Identification of a range of resistance responses, indicating allelic variation between and within populations, was in accordance with studies that aimed to determine the relationship between genome-wide genetic diversity and biogeography. Positive correlation between genetic variation and the geographical origin of accessions, *i.e.* isolation by distance, has been reported, suggesting the existence of population structures at a global geographical scale (Nordborg *et al.*, 2005; Shindo *et al.*, 2007). Population structures were further analysed by the use of a large number of accessions that were collected not to represent a well-designed hierarchical sampling scheme (Schmid *et al.*, 2006). Accessions from central Asia were shown to have a low level of genome-wide polymorphisms relative to accessions from the Iberian Peninsula and Central Europe. Accessions from the Iberian Peninsula and from Central Asia constitute distinct populations, whereas Central and Eastern European accessions represent admixed populations in which genomes had been reshuffled by historical recombination events. These patterns were suggested by Schmid *et al.* (2006) to result from the rapid post-glacial

recolonisation of Eurasia from glacial refuge populations. It was shown that individuals from the same local populations can be genetically different, indicating that they originated from multiple source populations, whereas geographically distant accessions can be highly similar. These findings were in agreement with the observations that, despite the occurrence of local inbreeding, a large proportion of the global variation reported was segregating within and between local populations within major geographical regions (Bakker *et al.*, 2006a). A more recent study confirmed a clear east-west genetic structure both with proposed Pleistocene refugia and post-Pleistocene colonised regions (Beck *et al.*, 2008). The refugia themselves are genetically differentiated from one another and displayed elevated levels of within-population genetic diversity relative to recolonised areas.

A high-density array resequencing 20 diverse accessions identified many SNPs between the tested accessions with large effects on gene integrity, including premature stop codons, alteration of methionine residues and removal of annotated stop codons (Clark *et al.*, 2007) indicating the possible effects of polymorphisms within and between accessions.

4.2 *Arabidopsis* infection phenotypes do not correlate with geographic origins

In the present study, analysis of natural variation did not identify a correlation between infection phenotype and distinct geographic origin of the accessions (see 3.2.1, Tab. SD1), although this requires confirmation by more precise analyses, *e.g.* analysis according to habitats or analysis according to coordinates of accession origins (see 3.2.1). The lack of a correlation of infection phenotypes with geographic origins raises the question whether resistance or susceptibility to *C. higginsianum* is a trait that is under any evolutionary pressure to maintain genetic diversity.

If resistance to *C. higginsianum* offers a fitness advantage, it was reasonable to expect a correlation between the occurrence of resistant accessions and the geographical distribution of *C. higginsianum*, caused by adaptive evolution. In this way, variation in traits such as timing of flowering and germination, reflect adaptations to specific environments and bear ecological significance (Koornneef *et al.*, 2004). However, few

studies have documented the distribution of *C. higginsianum* (Tab. 4.1) (O'Connell *et al.*, 2004).

Table 4.1: Documented distribution of *Colletotrichum higginsianum*

continent	countries
Asia	Brunei, China (South), Honk Kong, Japan, New Guinea, Sabah, Sarawak, South Korea, Taiwan
Africa	South Africa
Europe	Italy, Netherlands
North-America	USA
South America	Argentina, French Antilles, Jamaica, Trinidad

Countries (ordered according to their continents) for that distribution of *C. higginsianum* was documented (personal communication, Dr. R. O'Connell, Cologne, Germany)

C. higginsianum has been shown to favour high temperatures with a high humidity. Although *Arabidopsis* has a worldwide distribution and can be found in diverse habitats, it was shown for *Arabidopsis* that high temperature above 22°C in summer is one factor limiting its distribution (Hoffmann, 2002). Therefore, as *Arabidopsis* prefers lower temperatures than the fungus, environmental conditions favourable to both *C. higginsianum* and *Arabidopsis* might not be widespread under natural conditions. Hence, it remains unclear whether *C. higginsianum* is a natural pathogen of *Arabidopsis*. Due to the lack of selection pressure represented by *C. higginsianum*, alleles conferring resistance to this pathogen might not have been selected in distinct geographic regions, explaining the lack of correlation between resistant accessions and geographic origins. This is further supported by the finding that the dominant *R* gene *RCH1*, the first resistance locus identified in *Arabidopsis* to *C. higginsianum*, was only present in Eil-0 among 37 accessions collected from around the world (Narusaka *et al.*, 2004). Natural variation in response to *C. higginsianum* might therefore represent a neutral distribution of polymorphisms resulting from the lack of selection pressure applied by the fungus.

However, a striking observation was that alleles conferring full susceptibility to C. *higginsianum* were relatively rare in the collection of accessions. I identified that just 7% of the accessions showed a highly susceptible phenotype (DS 3), whereas 41% exhibited resistant phenotypes (DS 0-DS 1). Therefore, alternatively to a neutral distribution of

resistance, *C. higginsianum* might represent a selection pressure driving the evolution of resistance among populations. Intensive studies on selection mechanisms in adaptive evolution have revealed that *R* genes show stronger patterns of selection than sets of non-*R* genes or random sequences in the genome (Bakker *et al.*, 2006b; Clark *et al.*, 2007; Salvaudon *et al.*, 2008). Although all *R* genes do not show the same clear evidence of selection, balancing selection to maintain polymorphism has been suggested for a fair number of *R* genes (Salvaudon *et al.*, 2008), resulting in the existence of both resistance and susceptibility alleles that frequently occur together within natural populations and are thus common across the *Arabidopsis* range. For the *R* genes *RPM1* (Stahl *et al.*, 1999), *RPS2* (Caicedo *et al.*, 1999) and *RPS5* (Tian *et al.*, 2002) balancing selection has been suggested, explaining a trade-off between the benefits and costs of resistance, *e.g.* lower reproductive efficiency, which thereby contributes to the maintenance of polymorphisms in *R* genes (Tian *et al.*, 2003; Korves and Bergelson, 2004; Clark *et al.*, 2007) and the maintenance of susceptibility, respectively. Therefore, although *C. higginsianum* might be a driving force of adaptation, balancing selection could have maintained susceptibility alleles.

The low frequency of susceptible alleles of *Arabidopsis* to *C. higginsianum* raises the question whether selection pressures other than the fungus might have maintained allelic variation despite the ostensible fitness disadvantage of susceptibility to *C. higginsianum*. If population differentiation in a trait is adaptive, the degree of quantitative genetic differentiation in the trait among populations is expected to be greater than the genetic differentiation among populations in neutral molecular markers (Mitchell-Olds and Schmitt, 2006). Understanding this aspect of natural variation requires the complex determination of genetic and physiological trade-offs between different traits in a distinct environment; thus, any positive trait might involve a cost for the individual to maintain the trait under specific environmental conditions. Therefore, to understand why susceptible alleles might have been maintained by balanced polymorphism in some *Arabidopsis* accessions, it is necessary to understand whether the susceptibility alleles impact up on other traits that might be under the selection pressure of distinct environmental factors. For example, it is possible that the susceptible alleles might encode proteins with fundamental functions for the plant only under specific environmental conditions, thereby conferring a fitness advantage to the plant. Therefore, selection pressure might be higher on this trait than on resistance to *C. higginsianum*.

It is furthermore possible that the absence of correlation between phenotypes and geographic regions was due to the small sample size of accessions analysed in this study and a strong bias towards accessions from Central Europe. Therefore, regions where *C. higginsianum* is mainly expected to occur might not be well represented. However, recent studies have demonstrated that sampling bias due to over-representation of accessions from Central Europe relative to other geographical regions of similar size does not confound estimates of genetic variation (Schmid *et al.*, 2006).

Admixture of local populations, especially in Central Europe, resulting from human disturbance, might further complicate our ability to understand the evolutionary forces shaping genetic variation within *Arabdidopsis* populations (Mitchell-Olds and Schmitt, 2006). It therefore cannot be excluded that the distribution of susceptible alleles in the susceptible accessions, originating from Central Europe, Cape Verde Islands and North America, may have been affected by human-induced migration and disturbances, so that occurrence of resistance and susceptibility to *C. higginsianum* might not have been shaped exclusively by environmental selection pressures.

A further consideration is that the standard growth conditions in growth chambers and greenhouses used in the present study might fall outside the natural range of the tested accessions. The identification of natural variation in resistance to *C. higginsianum* in this study must therefore be interpreted with care, as the laboratory conditions might influence the infection phenotypes. The artificial growth conditions may provide unfavourable environments for some accessions which might skew the results of this study of evolutionary relatedness.

4.3 Resistance of *Arabidopsis* to *C. higginsianum* is conferred by dominant, recessive and polygenic resistance

In the present study I aimed to exploit natural variation in *Arabidopsis* to identify determinants of the interaction with *C. higginsianum*. To determine the genetic basis of resistance and susceptibility to *C. higginsianum*, I crossed resistant accessions to the susceptible Ler-0 accession and the inheritance of resistance was analysed in F_2 generations.

Extensive work in recent decades on race-specific resistance has elucidated the role of R genes in the sophisticated plant defence mechanisms against potential pathogens. Most R genes in bacterial and fungal systems have been identified to show dominant or semi-dominant inheritance (Hammond-Kosack and Jones, 1996; Iyer-Pascuzzi and McCouch, 2007a). Also for the *Arabidopsis*–*C. higginsianum* interaction, a dominant resistance locus (*RCH1*) was shown to confer the highly resistant phenotype of Eil-0 (Narusaka *et al.*, 2004). Although *RCH1* is so far the only identified dominant resistance locus to *C. higginsianum*, and was not detected in 36 other accessions, it is reasonable to expect that R genes other than *RCH1* may confer resistance in some of the *Arabidopsis* accessions tested in the present study. In contrast to Narusaka *et al.* (2004), who used Col-0 as susceptible parent in dominance tests, Ler-0 was chosen as susceptible parent in this present study. In addition, most of the resistant accessions I tested were not examined by Narusaka *et al.* (2004). The dominant effect of the resistance locus *RCH1* was confirmed in this study by identification of a fully resistant F_1 generation resulting from crossing Eil-0 to Ler-0. Furthermore, the segregation of the corresponding F_2 generation (Tab. 3.4) was in accordance with the expected 1:3 (susceptible : resistant) segregation for dominant R genes. Similarly, the F_1 generations I generated from crosses with the resistant accessions Fei-0, St-0 and Ts-1 also exhibited highly resistant infection phenotypes, hinting to the presence of a dominant R gene. Since Fei-0 and Ts-1 were not included in the study by Narusaka *et al.* (2004), it is possible that the resistance of these two accessions is conferred by *RCH1*. However, as the focus of the present study was on the identification of susceptibility factors, and therefore on the identification of recessive resistance (see 4.3), I did not study further corresponding F_2 generations. The F_1 generation derived from crossing accessions Sendai-1, Sendai-3 and Sendai-4 to Ler-0 were assigned intermediate disease scores (DS) 1-2 and 2, which was therefore not in accordance with the effect of a dominant R gene. Nevertheless, analysis of the corresponding F_2 generations revealed statistically significant 1:3 (susceptible : resistant) segregation patterns for all three Sendai accessions. Therefore, although this was not consistent with observations of the F_1 generations, these data suggest the action of a dominant R gene. Since these three accessions were also not included in the previous study by Narusaka *et al.* (2004), their resistance might possibly be conferred by *RCH1*. It is noticeable that Sendai-1, Sendai-3 and Sendai-4 originate from the same geographic region in Japan. As accessions from Asia had been shown to exhibit a low level of genome-wide polymorphisms (see 4.1), it is possible that resistance of these three accessions might be due to the same R gene. A more

detailed study of the molecular components conferring resistance in these accessions might be interesting from an evolutionary point of view, because *C. higginsianum* occurs naturally in Japan (Tab. 4.1) (O'Connell *et al.*, 2004). It remains to be elucidated whether this Japanese population of *Arabidopsis* accessions has adapted to selection pressure from *C. higginsianum*.

The molecular basis of recessive resistance, in contrast to the resistance conferred by dominant *R* genes, has not attracted much attention until recently (O'Connell and Panstruga, 2006) and was the main focus of the present study. Recessive resistance was expected to result in a susceptible F_1 generation after crossing a resistant accession to the susceptible accession Ler-0. Furthermore, the subsequent F_2 generation was expected to exhibit a 3:1 (susceptible : resistant) segregation. It was shown that 47% of the generated F_1 lines exhibited fully susceptible phenotypes after *C. higginsianum* inoculation (Tab. 3.3). Three of the corresponding F_2 lines were tested and their infection phenotypes were in accordance with the segregation pattern expected for recessive resistance. Further five F_1 generations also hinted to a possible recessive inheritance of resistance in the parental accession, but it remains to be confirmed by analysis of infection phenotypes in the corresponding F_2 generations.

Crossing the moderately resistant accession Ws-2 (DS 1-2) to Ler-0 did not result in a susceptible infection phenotype in the F_1 generation, and the segregation of the F_2 generation did not point to the effect of either a dominant or recessive resistance locus (Tab 3.4). However, the segregation analysis indicated a digenic inheritance of the trait, because I detected a statistically significant variation from the expected classical segregation of 9:3:3:1 (Tab. 3.5). This finding is particularly interesting, as it suggests that a different mechanism for resistance operates in the accession Ws-0 (see 4.1).

In addition to the digenic/polygenic inheritance of resistance in Ws-2, QTL analysis of the Ler-0 x Kas-2 RIL population also pointed to a quantitative resistance in the accession Kas-2 resulting from the effects of allelic variation of several loci. Two major QTLs on chromosome I and chromosome V, having small-to-medium phenotypic effects on resistance, and several minor QTLs, were shown to control the resistance trait (see 3.3.5). Only a few studies have focused on the genetic basis of quantitative variation in *Arabidopsis* resistance to pathogens (Buell and Somerville, 1997; Wilson *et al.*, 2001; Kliebenstein *et al.*, 2002; Godiard *et al.*, 2003; Denby *et al.*, 2004; Kover *et al.*, 2005; Perchepied *et al.*, 2006). However, the polygenic resistance of accessions Ws-2 and Kas-2 is in agreement with recent observations that most physiological or morphological traits

exhibit a continuous phenotypic distribution within or between populations and are thus quantitative (Shindo *et al.*, 2007). In fact, the presence of QTLs for many different traits appears to be the norm rather than the exception, as shown by many loci mapped in RIL populations (Loudet *et al.*, 2002; Koornneef *et al.*, 2004). It was therefore interesting that QTL analysis of the Ler-0 x Kondara RIL population (see 3.3.5) detected a single major QTL exerting a major effect on resistance, with negligible minor effect QTLs, indicating a monogenic inheritance of resistance to *C. higginsianum* in Kondara.

In summary, the analysis of resistance responses of 116 *Arabidopsis* accessions identified a high level of natural variation in response to *C. higginsianum* as shown by the broad spectrum of resistance. I could not identify obvious correlation between the infection phenotypes and geographic origins. Strong susceptibility to *C. higginsianum* was found to be the exception in *Arabidopsis*. Crosses of resistant accessions to the fully susceptible accession Ler-0, and the analysis of the resulting F_1 and F_2 generations identified different modes of inheritance of resistance among the tested accessions. Besides the identification of monogenic recessive resistance, monogenic dominant resistance could also be found, confirming the results of a previous study. Furthermore, polygenic resistance, conferred by two or more loci, respectively, was also identified.

4.4 Cytological analyses of recessive resistance

4.4.1 Recessive resistance affects fungal penetration of epidermal cells

Successful pathogenesis of *C. higginsianum* in *Arabidopsis* includes three distinct steps each of which require close interaction of the fungus with its host:

- Initial penetration of host cells by fungal appressoria
- Production of specialised feeding structures - the biotrophic primary hyphae in living host cells
- Host-cell killing and cell wall dissolution by necrotrophic secondary hyphae

At all infection stages, modulation of host cell processes by the fungus is probably critical for successful pathogenesis. Only if the fungus successfully interacts with its host at all three steps of fungal pathogenesis it can complete its life-cycle by asexual reproduction, *i.e.* the sporulation. Therefore, in the present study, cytological analyses aimed to determine which stage of fungal development is affected during recessive resistance. This

was expected to give indications about the functions and effects of recessive resistance loci. Furthermore, it aimed to determine whether the mechanisms of recessive resistance varied between the accessions, and if the mechanisms of recessive resistance differed from those of dominant resistance.

Since I found appressoria of normal appearance on all accessions tested (see 3.7.1), it can be concluded that host resistance mechanisms did not affect events up to this stage of fungal development, including initial spore adhesion and germ-tube formation. A normal morphogenesis and maturation of appressoria, including the formation of a penetration pore and melanin deposition in the appressorial cell wall was observed on all *Arabidopsis* accessions tested. Also I could identify penetration attempts by appressoria by the formation of penetration pegs on all accessions tested (Fig. 3.16). However, the successful penetration of the plant cell wall, a process which requires a combination of mechanical force and enzymatic degradation (Perfect *et al.*, 1999), differed markedly between Ler-0 and the resistant accessions. In the susceptible accession Ler-0, > 50% of the penetration attempts resulted in the formation of biotrophic primary hyphae. In contrast, in the resistant accessions only ~ 10% of penetration attempts resulted in the formation of biotrophic primary hyphae. Besides this difference in penetration frequency, the timing of penetration was also different in Ler-0 compared to the resistant accessions, as appressoria penetrated Ler-0 cell walls more rapidly than cell walls of resistant accessions. Furthermore, analysis of penetration efficiency at a later time point confirmed that pathogenesis was not just delayed, but effectively reduced in all resistant accessions tested. These observations pinpoint the effects of recessive resistance to the stage of penetration of the cuticle and cell wall, or alternatively, to the initial establishment of biotrophic hyphae in host epidermal cells.

The plant cuticle and cell wall are important barriers that shield the plant cell membrane and cytoplasm from invasion by potential pathogens. It is therefore plausible that minor differences in the composition or structure of the cell wall could result in major effects on disease resistance. Natural variation for cell wall composition between and within species, shaped by environmental factors, has been documented (Hazen *et al.*, 2003). For initial penetration of the cell wall rupture of the cuticle is required. The cuticle covers the epidermal cell wall layer of aerial plant tissues and although the primary role of the cuticle is probably to reduce water loss, it has also been shown to be involved in the generation and distribution of signals in development and in plant-pathogen interactions (Nawrath, 2006). Evidence from various experimental approaches has indicated a possible sensing of

cuticular degradation products by plants which probably activates defence responses (Chassot and Métraux, 2005). Furthermore, the *Arabidopsis att1* mutant was isolated in an attempt to identify host factors involved in type III gene induction in *P. syringae* pv. *phaseolicola* (Xiao *et al.*, 2004). *Att1* encodes CYP86A2, a cytochrome P450 monooxygenase which was shown to be involved in cuticle formation because the cutin content was reduced to 30% in the *att1* mutant. Interestingly, the mutation also greatly enhanced the expression of the bacterial type III genes *avrPt* and *hrpL*, suggesting a role of CYP86A2 in disease resistance. Closer analysis of the mutant indicated that alteration of the cuticle membrane structure by itself activated the type III gene expression. It was suggested that the cuticle membrane inside the substomatal chamber may repress bacterial type III gene expression, either by blocking access to a positive host factor or by active repression of bacterial gene expression. The less compact structure of the substomatal chamber cuticle membrane in mutant plants may lead to leakage of this host factor, or facilitates the attachment of bacteria to the host cell within intercellular spaces. Alternatively, lipids synthesised by CYP86A2 may repress type III gene expression. The *LACS2* gene is also involved in cuticle formation in *Arabidopsis* (Schnurr and Shockey, 2004). It was suggested that LACS2 is essential for activation of cutin monomers for their assembly into cutin domains that are then transported across the membrane to the outermost layer of the cell wall for final polymerisation (Bessire *et al.*, 2007). It was therefore surprising that the *lac2-3* mutant, exhibiting increased cuticle permeability, was strongly resistant to *Botrytis cinerea*. However, it was found that the increase of cuticle permeability directly correlates with the amount of antifungal compounds released to the plant surface, resulting in resistance to *Botrytis*. Thus, the increased resistance to *Botrytis* was explained by an increased diffusion of fungal signals, *e.g.* the effector molecules, across the more permeable cuticle of the mutant resulting in the release of antifungal compounds that arrest fungal invasion.

Impairment of pathogen attachment can be caused by alterations in the composition of not only the cutin layer, but also the polysaccharide component of the cell wall. The *Arabidopsis rat4* mutation, which confers resistance to *Agrobacterium tumefaciens* (Zhu *et al.*, 2003b), corresponds to the *CSLA9* gene, a putative processing glycosyltransferase that is believed to participate in the synthesis of an unknown cell wall polysaccharide (Zhu *et al.*, 2003a). The *rat4* mutants had a decreased number of lateral roots and the lateral roots also grew more slowly. The mutant was shown to be blocked in an early step in *Agrobacterium*-mediated transformation, possibly due to an altered ability to secrete

particular cell wall polysaccharides that are essential for the *Agrobacterium tumefaciens* recognition and subsequent attachment. The *Arabidopsis cev1* mutant was recovered by screening for constitutive activation of JA (Ellis and Turner, 2001) and was found to have enhanced resistance to powdery mildew. This mutation in *CESA3*, which encodes a cellulose synthase involved in primary cell wall synthesis, leads to a decrease in the amount of cellulose, which was found to trigger JA and ET production (Ellis *et al.*, 2002b). Although it remains to be determined whether the cell wall alteration causes pathogen defence directly or indirectly through activation of the JA pathway, it was shown to confer resistance to *G. cichoracearum*, *P. syringae* and the aphid *Myzus persicae* (Ellis *et al.*, 2002a). The *Arabidopsis pmr5* and *pmr6* mutants were found to be highly resistant to powdery mildew (Vogel *et al.*, 2002; Vogel *et al.*, 2004). This resistance was not dependent on constitutively activated SA or ET and JA signal transduction pathways. *PMR5* and *PMR6* encode a protein of unknown function and a pectate lyase, respectively. For both mutants, alterations in cell wall composition were detected, indicated by increased pectin content, reduced pectin esterification and a suggested change in the hydrogen-bonding environment of cellulose. Although the powdery mildew pathogen is still able to penetrate, at 2 dpi the fungal colonies consist only of shrivelled hyphae that are loosely attached to the leaf surface. It cannot be excluded that the mutations activate a novel defence pathway. Alternatively, it was suggested that the changes in the cell wall architecture are associated with powdery mildew resistance. Hypothetically, the pathogen may have a limited ability to digest the outer epidermal cell wall of the mutants or the mutated cell wall might carry latent signalling molecules to activate novel defences (Vorwerk *et al.*, 2004). Alternatively, the mutants are less hospitable hosts, offering only a decreased nutrient availability to the pathogen.

In the light of the above findings, it is possible that in the interaction of *Arabidopsis* with *C. higginsianum,* alterations in cuticular or polysaccharide components of the cell wall, or in pathways leading to their biosynthesis, might result in major effects on fungal pathogenesis. An aberrant cell wall composition could affect the structure of the plant surface, which in turn could impair attachment of fungal appressoria. Such appressoria would be unable to exert the mechanical pressure required for successful penetration of the cuticle cell wall. Likewise, a cell wall containing increased amounts of cutin or epicuticular waxes or a less permeable polysaccharide composition may be more resistant to mechanical penetration by appressoria. Alternatively, the altered composition of a putative target for a fungal enzyme, such as a cutinase or pectinase, could impair

enzymatic penetration of the cell wall. Furthermore, aberrant permeability of the cell wall might affect the transport diffusion of fungal elicitors or, plant antimicrobial compounds across the cell wall.

Due to the intimate and complex interaction between *C. higginsianum* and living host cells during the biotrophic stage of intracellular hyphal growth, variation in a plant factor could significantly affect fungal development after penetration. A plant signal that might be required for the induction of hyphal growth after penetration may be affected in resistant accessions, resulting in failure of fungal differentiation of specialised biotrophic hyphae. Alternatively, in accordance with the genetic evidence from the *pmr5* and *pmr6* mutants (see above), variations in plant cell wall composition might result in a physiological state of the apoplast that is less favourable for fungal growth. The switch from penetration of the plant cell wall to intracellular hyphal growth is also associated with the modulation of host cell processes by the fungus for fungal nutrition. Therefore, aberrations in plant factors might affect nutrient, sugar or iron metabolism or transporters. Alternatively, plant factors that are required for generation of the interfacial matrix, which separates the fungal cell wall from the invaginated plant plasma membrane, may compromise nutrient availability for the fungus.

Plant factors exploited for pathogen growth have been identified in other pathosystems. For example the *xa5* and *xa13* genes were shown to be involved in recessive resistance of rice to the bacterial pathogen *Xanthomonas oryzae* pv. *oryzae* (Iyer and McCouch, 2004). *Xa5* encodes a small subunit of the transcription factor IIA, one of several general transcription factors that work with RNA polymerase II to transcribe DNA (Iyer and McCouch, 2004). It was suggested that a single amino acid substitution might prevent the interaction of *xa5* with a bacterial protein, possibly a transcriptional activator of eukaryotic genes, and this might prevent disease. *Xa13* encodes a unique plasma membrane-localised protein with a predicted role in pollen development (Chu *et al.*, 2006). Mutations in the promoter region of *Xa13* blocked induction of *Xa13* during pathogen inoculation, which seems to be the key to resistance in this case (Iyer-Pascuzzi and McCouch, 2007a). It was suggested that the promoter of the *Xa13* gene is targeted by the bacterial type III effector PthXo1 (Yang *et al.*, 2006). However, it remains unclear how the developmental and disease-resistance pathways are related.

Resistance to several species of the family of *Potyviridae* was shown to result from mutations in the translation initiation factors *eIF4E* and *eIF(iso)4E* (Diaz-Pendon *et al.*,

2004; Kang *et al.*, 2005b). The eIF4E protein is involved in the expression of eukaryotic mRNA. Although the exact mechanisms by which *eIF4E* mutations control resistance remain to be elucidated, it could be shown that a small number of amino acid changes in the protein prevent interaction with a small viral protein known as VPg (viral protein genome-linked) during the virus infection (Ruffel *et al.*, 2002; Gao *et al.*, 2004; Kang *et al.*, 2005a; Yeam *et al.*, 2007). As a result the virus is unable to complete its life cycle and successfully infect the plant.

Host-specific toxins (HSTs) constitute a special class of pathogen effectors. HSTs are secondary metabolites that are toxic only to the specific host of the pathogen and are innocuous to the great majority of other plants (Friesen *et al.*, 2008). HSTs are required for the virulence of necrotrophic fungi by reducing or eliminating disease responses in the host (Toyoda *et al.*, 2002). However, only specific genotypes of the host are sensitive to the toxin and sensitivity could be shown to be a dominant trait, *i.e*. absence of the toxin target results in recessive resistance. This is implying that perception of the toxin is conferred by the direct or indirect interaction with a toxin receptor and this perception can be expected to be affected by alterations in the structure of the receptor, resulting in a decreased growth ability of the fungus.

Natural variation in plant factors exploited by pathogens for host susceptibility could result in reduced fungal penetration or development of biotrophic primary hyphae growth as discussed above. Alternatively, reduced fungal growth could be caused by active resistance mechanisms that are either not present or not induced in susceptible accessions. Thus, the resistance of accessions exhibiting reduced fungal growth could be due to the presence of *R* genes that are activated by the perception of fungal penetration attempts. Their activation, in turn, induces a complex signalling cascade leading to expression of plant defence reactions (Dangl and Jones, 2001). Narusaka *et al*. (2004) previously identified the presence of a dominant, monogenic *R* gene in the *Arabidopsis* accession Eil-0, conferring resistance to *C. higginsianum*. Although resistance in the other resistant accessions tested in this study was inherited in a recessive manner, the presence of active, but recessive *R* genes cannot be excluded and is discussed in section 4.3. Interestingly, cytological analysis did not identify cellular defence responses to *C. higginsianum* such as H_2O_2 accumulation (see 4.4.2) or callose deposition (see 4.4.3) in any of the resistant accessions. This is consistent with the finding that recessive resistance in the four accessions Ws-0, Gifu-2, Can-0 and Kondara is conferred by the same resistance locus

(see 3.3.6). However, it also raises the question why recessively resistant accessions are indistinguishable from the dominant resistance identified in Eil-0. This could indicate that the mechanisms underlying recessive resistance and dominant resistance are similar as discussed in section 4.3, although those resistance mechanisms remain elusive.

A further possibility is that plant antimicrobial compounds could differ in composition between resistant and susceptible accessions so that the compounds in susceptible accessions such as Ler-0 are less effective in restricting *C. higginsianum,* allowing more extensive colonisation by biotrophic primary hyphae.

4.4.2   Accumulation of hydrogen peroxide does not confer resistance to *C. higginsianum*

Recessive resistance, due to alterations in susceptibility factors, can be considered to be a passive response, defined more by the lack of susceptibility than the activation of defence signalling pathways (O'Connell and Panstruga, 2006; Iyer-Pascuzzi and McCouch, 2007a). Alternatively, recessive resistance might be conferred by the presence of a recessive *R* gene, which would be expected to activate a cascade of defence reactions leading to containment of the pathogen. Accumulation of ROS such as H_2O_2 is closely associated with plant defence reactions against many pathogens, *e.g.* the hypersensitive reaction (HR), expression of defence genes and cell wall strengthening by oxidative cross-linking reactions (Levine *et al.*, 1994; Lamb and Dixon, 1997; Thordal-Christensen *et al.*, 1997; Grant and Loake, 2000; Hückelhoven and Kogel, 2003; Apel and Hirt, 2004). Additionally, H_2O_2 might be fungitoxic and hence provide a direct penetration resistance mechanism (Hückelhoven, 2005). In the interaction of the barley powdery mildew fungus *Blumeria graminis* f.sp. *hordei* (*Bgh*) with its host, H_2O_2 accumulation has been shown to occur in three phases: while in the first phase the germ tube tip appearance is linked to H_2O_2 accumulation, in the second phase H_2O_2 accumulation is subcellularly confined to the cytoplasm close to the site of attack. In the third phase, H_2O_2 accumulation spreads over the whole cell, meaning that it is not restricted to subcellular sites (Hückelhoven and Kogel, 2003). H_2O_2 starts to accumulate either at the mesophyll-epidermis interface or near penetration sites depending on the type of *R* gene that mediates the defence response (Hückelhoven and Kogel, 2003). In any case, phase three was shown to be closely linked to subsequent cell death and arrest of the pathogen (Thordal-Christensen *et al.*, 1997; Vanacker *et al.*, 2000; Hückelhoven and Kogel, 2003). Thus, the detection of H_2O_2

accumulation might indicate active resistance responses of the plant. In the present study, I analysed H_2O_2 accumulation to determine (i) whether recessive resistance to *C. higginsianum* was based on active or passive resistance mechanisms and (ii) if resistance mechanisms differed between recessive and dominant resistant accessions.

ROS production occurred predominantly during the necrotrophic phase of susceptible interactions in Ler-0 and Col-0 plants, affecting large conglomerations of host cells instead of single epidermal cells. At this late stage of infection, H_2O_2 production was considered to be a consequence of extensive host cell death caused by fungal activities rather than specific defence responses of a single cell to confine pathogen growth. Occurrence of highly localised ROS production at penetration sites, suggesting *R* gene-mediated defence responses, could not be detected for resistant accessions. This suggests that cellular processes other than ROS production are varying between resistant and susceptible accessions. However, *H. parasitica* growth was inhibited in the *dmr* mutants without accumulation of ROS or visible cell death (Van Damme *et al.*, 2005), but most of the mutants could be identified not to be true loss-of-susceptibility mutants (see 4.8). Therefore, recessive resistance could involve active defence responses other than ROS production. Furthermore, although ROS production has been proposed to play a central role in the process of host cell death during HR, recent studies have supported earlier suspicions that ROS may not be sufficient for the complete host cell death response during HR (Grant and Loake, 2000). Mechanisms other than ROS production might contribute to active cell death responses and therefore active resistance responses cannot definitely be ruled out in response to *C. higginsianum* inoculation.

Interestingly, observations in the present study are not in accordance with the findings of Narusaka *et al*. (2004). Although both studies confirmed that ROS production was not a rapid response to fungal invasion, Narusaka *et al*. (2004) had observed an accumulation of H_2O_2 exclusively in plants of the Eil-0 accession between two to three days after inoculation with *C. higginsianum*. The H_2O_2 accumulation was reported to be in agreement with the detection of infection hyphae within epidermal cells, which were, however, collapsed and highly vacuolated and did not affect adjacent mesophyll cells. It was therefore suggested that resistance in Eil-0 may be mediated through a process of hypersensitive cell death in the epidermal cells which limit subsequent invasion of mesophyll cells by *C. higginsianum*. DAB staining in the present study however confirmed that there was no detectable H_2O_2 generation in any resistant accessions even at 5 dpi, which excluded the possibility that ROS accumulation was delayed in comparison

to studies by Narusaka *et al.* (2004). However, infection assays conducted by Narusaka *et al.* (2004), utilised a different fungal culture (MAFF305635) and different plant growth and inoculation conditions than in the present study. It is possible that these variables explain the contrasting observations of ROS production in Eil-0 plants.

4.4.3 Reduced penetration efficiency is not directly related to callose deposition

Newly synthesised depositions of callose between the plasma membrane and the cell wall in the paramural space typically occur in plants in response to various abiotic and biotic stresses (Stone and Clarke, 1992). These cell wall appositions, termed papillae, are composed of altered cell wall material and, among other constituents, contain callose (β-1,3-glucans) and phenolics. Other constituents of papillae can be lignin, cellulose, pectin, suberin, chitin, lipids, silicon and diverse cell wall proteins (Schmelzer, 2002). During fungal infections, papilla formation occurs in a localised area beneath infection sites and is thought to hamper hydrolytic and osmotic pressure from fungal appressoria (Aist, 1976; Hückelhoven, 2005). The formation of papillae is typically rapid and occurs before the integrity of host cells has been damaged. It was observed that papillae are deposited regardless of a plant's eventual resistance or susceptibility to penetration (Zeyen *et al.*, 2002). Papillae are sites of induced protein cross-linking for structural reinforcement of the plant cell wall, thereby presenting a physical barrier to the invading pathogen. They are furthermore thought to be sites of localised accumulation of induced antimicrobial or microbial-static compounds, such as phenolics, reactive oxygen species, thionins or peroxidases (Aist, 1976; Thordal-Christensen *et al.*, 1997; Zeyen *et al.*, 2002), and are providing an additional chemical barrier to pathogens.

Although it has long been believed that callose papillae impede pathogen entry into plant cells, recent work contradicts these observations. Analysis of plants lacking PMR4/GSL5, a callose synthase which is required for wound and papillary callose formation, showed reduced susceptibility to virulent powdery mildew fungi and to the oomycete *H. parasitica* (Jacobs *et al.*, 2003; Nishimura *et al.*, 2003). However, although papillary callose was not detectable in *pmr4/gsl5* plants, typical papillae formed beneath fungal appressoria that were microscopically indistinguishable from those in wild-type plants (Jacobs *et al.*, 2003). It therefore seems unlikely that callose serves as an essential component in papillae. Mutations in the SA pathway in the *pmr4/gsl5* mutant restored susceptibility of

pmr4 (Nishimura *et al.*, 2003), suggesting that PMR4/GSL5 or its product negatively regulate the SA signalling pathway. It was therefore postulated that the SA pathway acts as a mechanism of *pmr4/gsl5*-based resistance (Nishimura *et al.*, 2003). Alternatively, it is possible, because the papilla response is so widespread in plants, that some fungi might have evolved to depend on the presence of a papilla. Pathogens may have exploited components of this wound response for successful pathogenesis, *e.g.* as a structural scaffold to accommodate fungal complexes, or callose might serve as a pathogen-induced protection barrier that prevents recognition of pathogen-derived molecules by the host (Jacobs *et al.*, 2003).

To test whether callose deposition is an *Arabidopsis* response associated with resistance or susceptibility to *C. higginsianum*, I quantified its association with attempted penetration by appressoria at two and three days after inoculation (see 3.7.4.2). I found that callose deposition is an active plant response to attempted fungal entry in susceptible as well as resistant accessions, and there was no significant correlation between the presence of callose and failure of appressoria to penetrate. Callose deposition did not occur earlier in resistant than in susceptible accessions and callose was deposited before the majority of appressoria had penetrated susceptible Ler-0 and Col-0 plants. Hence, natural variation in resistance to *C. higginsianum* did not appear to be related to the timing of the callose deposition.

In Ler-0 and Col-0 plants primary hyphae had successfully penetrated through the callose deposits at 3 dpi, suggesting that callose does not effectively terminate hyphal growth. However, as callose is just one component of the papilla, components other than callose might be responsible for the reduced penetration efficiency in resistant accessions. They may generate a localised microenvironment at the penetration site with physical or chemical conditions unfavourable for appressorial penetration or initial growth of *C. higginsianum* primary hyphae in resistant accessions. Nevertheless, this cannot be the only mechanism of resistance to the fungus, because a large number of unsuccessful appressorial penetration attempts were not associated with callose papilla production. This clearly suggests that factors other than callose deposition and papilla production must account for natural variation in resistance to *C. higginsianum*. This is supported by the observation that increased callose deposition was shown for Ws-0, Gifu-2 and Kondara, but not for the accessions Can-0 and Eil-0, even though all of them are resistant to *C. higginsianum*. It therefore seems likely that callose deposition is an active plant response to challenge by *C. higginsianum* in both susceptible and resistant accessions, and that the

occurrence of callose deposits does not correlate with the resistance of distinct accessions to fungal infection. In agreement with previous studies which had observed that the fungus penetrated through the papilla into the host epidermal cell (Wharton and Julian, 1996; Mims and Vaillancourt, 2002; Narusaka *et al.*, 2004), and the observation that plants harbouring the *pmr4/gsl5* mutation do not show strong resistance to *C. higginsianum* (see 3.1.3 and 4.8), callose deposition does not appear to be the primary mechanism for resistance. This probably explains why callose deposition in recessively resistant accessions does not markedly differ from callose deposition in Eil-0 harbouring a dominant resistance locus.

4.5   Recessive resistance –recessive R gene or true susceptibility factor?

Positional cloning of a recessive resistance locus responsible for the resistance of *Arabidopsis* to *C. higginsianum* in the Ler-0 x Ws-0 F_2 generation (see 3.3.3) identified a ~ 100 kb region on the lower arm of chromosome V. However, it was hindered by a high frequency of polymorphisms between the Col-0 reference sequence and the Ler-0 accession. Positional cloning is dependent on the use of molecular markers and attempts to design these markers were based on the Col-0 reference sequence. Although I could identify these markers to be functional in PCR reactions with Col-0 genomic DNA as template, PCR reactions with template DNA from the Ler-0 and Ws-0 accessions did not result in PCR products. This strongly suggests that sequences are highly divergent, in particular in the region of 18,300,000-18,330,00 bp. This problem impeded further reduction of the region of interest by genetic linkage analysis. Furthermore, the missing sequence information for the two parental accessions did not facilitate the design of primers required for expression analysis of candidate genes before and after inoculation with *C. higginsianum*, which could help to detect possible differential expression between resistant and susceptible accessions. Previous microarray analysis (Narusaka *et al.*, 2004), comparing gene expression of uninoculated Col-0 plants to Col-0 plants inoculated with *C. higginsianum*, did not identify significantly increased expression of any of the candidate genes of the present study. This could indicate that the intermediate susceptibility of the Col-0 expression is determined by loci other than those identified by the analysis of natural variation between Ler-0 and Ws-0. Alternatively, although essential

for successful pathogenesis or resistance, the expression of particular candidate genes might not be significantly induced upon inoculation with *C. higginsianum.*

Analysis of T-DNA insertion mutants (see 3.5) did not identify mutant lines with aberrant infection phenotypes after *C. higginsianum* inoculation. However, most of the available T-DNA insertion mutant lines had a different genetic background to the accessions for that the natural variation in response to *C. higginsianum* was identified. Therefore it cannot be excluded that, despite no effect on the infection phenotype could be identified, one of the genes affected in the T-DNA insertion mutant lines is nevertheless the recessive resistance locus.

The identified recombination frequency was in accordance with an expected average of 1% per 250 kb (Lukowitz *et al.*, 2000; Jander *et al.*, 2002; Peters *et al.*, 2003). This suggests that genotyping of further F_2 plants might indicate additional recombinants to further reduce the region of interest to allow the identification of the recessive resistance locus.

4.5.1 Potential role of candidate genes in *Arabidopsis- C. higginsianum* interactions

There are two possible scenarios to explain the mechanisms of recessive resistance (Fraser, 1999). The first scenario proposes that resistance is the result of an active mechanism, in which the resistant plant interferes with some stage of the pathogen; susceptibility would therefore be due to the lack, or a non-functional copy, of such a factor involved in resistance. According to the second scenario, resistance might be the result of a passive mechanism whereby a plant is resistant due to the lack of a functional host factor required by the pathogen to complete is life-cycle. In the present study, neither a detailed cytological analysis (see 3.7 and 4.4) did provide convincing evidence for either of the possible scenarios, nor the positional cloning, the T-DNA insertion mutants and expression analysis allowed the identification of the recessive resistance mechanism yet. Therefore, the possible involvement of candidate genes and their corresponding gene products was considered on the basis of their predicted biological function in recessive resistance. The region identified to contain the recessive resistance locus comprises 20 genes, based on the Col-0 reference sequence, although it cannot be excluded that Ler-0 or the resistant accessions tested in the present study lack genes identified for Col-0, or contain additional genes not present in Col-0.

4.5.1.1 Candidate *R* genes

The region of interest contains six genes with a TIR-NB-LRR sequence; therefore they are predicted or confirmed to encode R proteins responsible for active disease resistance. One of them, *RPS4* (At5g45250) is a TIR-NB-LRR gene that confers resistance to the bacterial pathogen *P. syringae* pv. *tomato* strain DC3000 expressing *avrRPS4* (Hinsch, 1996; Gassmann *et al.*, 1999). It has been shown in earlier studies that this gene confers dominant resistance. As T-DNA insertion mutant lines in a Ler-0 genetic background did not alter the susceptible phenotype of the wild type in response to *C. higginsianum* (see 3.5), presumably *RPS4* is not responsible for the recessive resistance observed in the present study. At5g45210-At5g45240 have not been characterised yet and the function of their gene products as disease resistance proteins is only predicted based on their DNA sequence. However, the constitutive expression of At5g45210 exclusively in roots (see 3.4) (Tan *et al.*, 2007) hints to a function in resistance to root pathogens, suggesting that At5g45210 is probably not involved in interactions of *Arabidopsis* with *C. higginsianum.* Nevertheless, I cannot rule out that inoculation with the fungus induces gene expression in aerial tissues of the plant.

Although most TIR-NB-LRR genes in bacterial and fungal systems show dominant inheritance, the *RRS1* gene has been shown to confer recessive resistance of *Arabidopsis* to *Ralstonia solanecearum* (Deslandes *et al.*, 2002). Interestingly, RRS1-R was shown to be a new member of the TIR-NB-LRR protein family with a C-terminal extension including a putative nuclear localisation signal and a WRKY domain. Although genetically defined as a recessive allele, *RRS1-R* acts as a dominant gene when delivered as a transgene into *RRS1-S* genotype plants (Lahaye, 2002). Furthermore, transgenic plants of the resistant accession Nd-1 carrying the *RRS1-S* gene failed to develop wilt disease, strongly suggesting that RRS1-S is not a host susceptibility protein required by the pathogen for disease development. In addition, structural similarities between *RRS1* genes and other cloned *R* genes, and a requirement for SA and NDR1, make the hypothesis that RRS1 functions as host susceptibility protein rather unlikely. Indeed, more recent studies have shown that RRS1-R recognises the avirulence protein PopP2, an *R. solanecearum* type III effector. The two genes, *RRS1-R* and *RRS1-S* differ in the position of a stop codon that leads in RRS1-S to the formation of a protein truncated by 90 amino acids. However, an interaction between PopP2 and both RRS1-R and RRS1-S was identified in resistant and susceptible accessions (Deslandes *et al.*, 2003). It was therefore

proposed that RRS-R and RRS1-S might compete for bacterial or plant components essential for pathogen perception and/or signalling. Since the RRS1 proteins differ mainly in their C-terminal transcription factor, they may give distinct transcriptional read-outs, resulting in plant resistance only in the case of RRS1-R. Hence, similar to the identification of *RRS1*, it cannot be excluded that natural variation in the genes At5g45210-At5G45240 and At5g45260 leads to the identification of active, but recessive resistance and that the resistance mechanism may differ from that of most cloned *R* genes. Interestingly, RRS1-R mediated resistance does not involve hypersensitive responses upon inoculation which typifies many *R* genes. This would be consistent with the absence of active defence responses in the cytological analysis.

Although most of the identified TIR-NB-LRR *R* genes have been identified to confer dominant resistance, a semi-dominant mode of inheritance has been observed for some *R* genes (Xiao *et al.*, 2005; Göllner *et al.*, 2008). Although resistance in Ws-0, Gifu-2 and Can-0 was consistent with a recessive inheritance mode, intermediate phenotypes could not be reliably distinguished from fully susceptible phenotypes due to the constraints of high-throughput screening of F_2 plants (see 3.2.2). Therefore, it was not feasible to identify possible semi-dominant effects of a resistance locus. Although the infection phenotypes of the F_1 generations derived from crossing these resistant accessions to Ler-0 were strongly susceptible (DS 2-3), therefore suggesting a recessive inheritance of resistance, the F_1 generations were not as fully susceptible as Ler-0 plants (DS 3). I therefore cannot exclude that resistance is due to an *R* gene that is acting in a gene-dosage dependent manner, or alternatively, the different genetic backgrounds used in the present analysis affect the penetrance of a possible *R* gene.

Recent studies have identified natural variation in the response of *Arabidopsis* to Victoria blight, caused by the fungus *Cochliobolus victoriae* (Lorang *et al.*, 2007). Pathogenicity of *C. victoriae* depends on the production of a toxin called victorin, and mapping of an F_2 population resulting from crossing the victorin-sensitive accession Cl-0 to the victorin-insensitive accession Col-4, identified the locus *LOV1* as conferring susceptibility to the fungus. Interestingly, *LOV1* encodes a CC-NB-LRR protein and is a member of the *RPP8* disease resistance gene family. Lorang *et al.* (2007) could show that *LOV1* mediates typical disease resistance responses, *e.g.* SA-dependent induction of *PR-1*, production of the phytoalexin camalexin and HR-like cell death. These findings strongly suggest that LOV1 functions in a manner analogous to resistance proteins. It was therefore proposed that *LOV1* might be required as a resistance gene to a naturally-occurring pathogen of

Arabidopsis, and this mechanism is exploited for susceptibility by *C. victoriae*. Since LOV1 function requires ATTRX5, a cytosolic thioredoxin that may play a role in protecting plant cells from oxidative stress (Reichheld *et al.*, 2002; Laloi *et al.*, 2004), it was suggested that ATTRX5 could be a target of pathogen virulence effectors and be guarded by a NB-LRR protein (Sweat *et al.*, 2008). In this case, it is possible that ATTRX5 is targeted both by victorin and by an avirulence effector of the putative pathogen for which *LOV1* may act as a resistance gene. It is conceivable that similar mechanisms could operate resistance to *C. higginsianum* involving one of the identified TIR-NB-LRR genes. Therefore, the function of a gene product in disease resistance to one pathogen might not rule out an alternative function of the same gene product in susceptibility to *C. higginsianum*. However, the cytological analysis by DAB staining did suggest that resistance was associated with typical disease resistance responses such as ROS accumulation.

4.5.1.2 Plant susceptibility factors

In addition to six candidate genes with a TIR-NB-LRR structure, and therefore with a putative function in disease resistance, 14 further genes are included in the region of interest identified by positional cloning. At5g45310-At5g45330 are genes encoding proteins with unknown function and therefore their possible involvement in interactions with *C. higginsianum* cannot be concluded. At5g45380, a sodium symporter protein, is known from previous microarrays studies to be constitutively expressed in root tissue (see 3.4). In agreement with this differential expression in roots, previous studies have revealed an involvement in the uptake of urea in plant roots (Kojima *et al.*, 2007). It is therefore reasonable to exclude At5g45380 from the list of candidate genes. Furthermore, At5g45275 and At5g45370, encoding nodulin-related integral membrane proteins, are likely involved in the establishment of symbiotic interactions of *Arabidopsis* with rhizobia for nitrogen fixation. Therefore, it is reasonable to assume that these genes are not essential for the interaction of a pathogen with a hemibiotrophic lifestyle with its host. Nevertheless, different ways of endosymbioses, both mutualistic and parasitic, probably have evolved by exploiting some common core components (Parniske, 2000). Hence, an involvement of these two genes cannot completely be ruled out.

Microarray expression analysis did not give any reasons to exclude the remaining seven candidate genes. The putative or confirmed functions of the corresponding gene products

suggest a function in metabolism or development and may impact pathogen virulence invasion ways in similar ways as discussed in section 4.4.1.

Particularly interesting is At5g45280, coding for a putative pectin acetylesterase. Pectins are one of the major components of the middle lamella of plant cells and are composed of D-galacturonic acid polymers with interspersed methylgalacturonic acid residues which can be esterified by acetyl or methyl groups. The presence of acetyl esterification is known to modify the properties of the pectin molecules and contributes to the structural complexity of the pectin network (Vercauteren *et al.*, 2002). Pectin acetylesterases catalyse the deacetylation of esterified pectin. The removal of acetyl esters from the pectin backbone makes the polysaccharide more accessible to pectin-degrading enzymes, such as polygalacturonase and pectate lyases. A study has identified that *Erwinia* sp. cleaves only galacturonic acid residues that are not acetyl–esterified (Davis *et al.*, 1984). It is conceivable that natural variation in this gene might provide cell wall microenvironments in susceptible accessions that are more accessible to fungal penetration or for nutrition than in resistant accessions. In a recent differential proteomic analysis expression of At5g45280 was shown to be induced by oligogalacturonides (OGs) in Col-0 *Arabidopsis* seedlings (Casasoli *et al.*, 2008). OGs are elicitors of plant defence responses released from the homogalaturonan of the plant cell wall during the attack by pathogenic microorganisms. Therefore, this finding suggests a possible role of the pectin acetylesterase in the perception of pathogens.

At5g45290 encodes a zinc finger (C3HC4-type RING finger) family protein. The zinc finger is a structural feature shared by various proteins that bind to DNA and act as transcriptional regulators. Zinc finger proteins are among the most abundant proteins in eukaryotes and have been shown to play important roles in various cellular functions including transcriptional activation, regulation of apoptosis and protein folding and assembly (Yang *et al.*, 2008). The RING finger protein is a specialised type of zinc finger and consists of 40-60 residues that bind two atoms of zinc. The specific function of RING fingers, however, is still poorly understood. Studies have shown that many RING finger proteins have E3 ubiquitin ligase activities and can specifically interact with E2 ubiquitin-conjugating enzymes and can thereby promote ubiquitination. COP1 is one of the best characterised proteins with a RING-finger domain and has been shown to be a repressor of photomorphogenesis development and a light-regulated developmental molecular switch (McNellis *et al.*, 1994; McNellis *et al.*, 1996). HOS1 functions in low temperature signal transduction (Lee *et al.*, 2001). HUB2, also a C_3HC_4 Zn RING finger, is implicated in

chromatin remodelling during seed dormancy (Liu *et al.*, 2007b). RING-finger proteins have been identified to function in plant defense responses (Hong *et al.*, 2007). A recent study identified that the overexpression of the pepper *CaRFP1* encoding the C_3HC_4 type RING finger protein that physically interacts with the basic PR-1 protein CABPR1 to confer disease susceptibility to *P. syringae* pv *tomato* (Hong *et al.*, 2007). It was suggested that CaRPF1 acts as an E3 ligase for polyubiquitination of target PR proteins. It is therefore conceivable that C_3HC_4 Zn RING finger are involved in the interaction of *C. higginsianum* with its host.

Starch is synthesized in many organs of *Arabidopsis*, including leaves, flowers, developing seeds and root caps. During starch breakdown, β-amylase hydrolyses α-1,4 glycosidic linkages of polyglucan chains at the non-reducing end to produce maltose, which is thought to be metabolised in the cytosol following hydrolytic cleavage. β-amylase was suggested to regulate maltose accumulation and thereby contributes to protection of proteins and membranes during temperature shocks (Kaplan and Guy, 2005). Although cytological analysis of fungal pathogenesis hinted to an effect at the initial penetration stage, it cannot be excluded that a β-amylase encoded by At5g45300, might be involved in nutrition of the fungus at a very early stage of hyphal growth. Natural variation in activity of the gene activity could therefore result in decreased biotrophic growth of primary hyphae.

The gene product of At5g45307 is a microRNA (miRNA) that targets AGO. miRNAs constitute a class of endogenous single-stranded small RNAs (21-23 nt) that exists in animals and plants. miRNAs derive from long ssRNAs that fold and form imperfect hairpin dsRNAs. The Dicer-Like1 (DCL1) cuts the fold-back stem loop of the miRNA precursors, resulting in a miRNA duplex. The 3´ends of miRNA duplex are methylated and loaded onto AGO1, where the mature single-stranded miRNA guides the RNA slicing activity of AGO1 to partially complementary mRNAs. The miRNA-guided cleavage of mature mRNAs occurs in the cytoplasm and is thought to be the predominant mechanism of miRNA-guided regulation in plants (Mallory and Vaucheret, 2006). The negative regulation of gene expression by mRNA cleavage controls many fundamental biological processes, *e.g.* induction of cell differentiation in response to an endogenous stimulus and activation of adaptive responses to particular exogenous stresses. Natural variation in At5g45307 could therefore cause differences in susceptibility by regulating RNA interference (gene silencing).

The phytohormone abscisic acid (ABA) is involved in stress tolerance, stomata closure, flowering, seed dormancy and further physiological events (Nambara and Marion-Poll, 2005). ABA is also involved in regulation of shoot elongation and root growth. Endogenous levels of ABA in plants are properly and cooperatively controlled by synthesis, transport, and catabolic inactivation in response to environmental changes (Ueno *et al.*, 2007). Catabolic inactivation of ABA is mainly mediated by ABA 8´-hydroxylases (CYP707A1-4), which catalyse the 8´hydroxylation of ABA into 8´-hydroxy-ABA and its more stable tautomer phaseic acid. The transcript levels of all four CYP707As have been shown to be induced by abiotic stress and dehydration. At5g45340, a putative ABA 8´hydroxylase, might be similarly involved in ABA catabolism. A direct role of ABA in resistance has been observed for various plant-pathogen interactions, including virus and fungal infections (Dunn *et al.*, 1990). It was observed that the severity of symptoms which developed on hypocotyls of *Phaseolus vulgaris* after inoculation with *Colletotrichum lindemuthanium* was inversely related to the ABA content in the hypocotyls. Since ABA had no significant effect on the growth of *C. lindemuthanium in vitro*, it was proposed that resistance might be due to ABA-induced changes in the host tissues, such as mechanical properties of the tissue, or alterations in the host plasmalemma which could disturb the biotrophic interphase (Dunn *et al.*, 1990) and therefore result in susceptibility. It is plausible to expect that natural variation in regulation of ABA can affect susceptibility to *C. higginsianum*.

At5g45350 belongs to one of the largest plant superfamilies encoding F-box proteins (Kuroda *et al.*, 2002). They are part of SCF complexes that function as ubiquitin E3 ligases and are responsible for substrate recognition and substrate recruitment to the SCF complex. F-box proteins of *Arabidopsis* have been shown to be essential for auxin, methyl jasmonate, gibberellin and ethylene signalling (van den Burg *et al.*, 2008). Furthermore, they regulate the circadian clock, senescence photomorphogenesis, floral development, self-incompatibility and responses to biotic and abiotic stresses. An important role in the interaction of *C. higginsianum* with its host is therefore possible. A polymorphism in an F-box gene having a major impact on the interaction with *C. higginsianum* would be in accordance with earlier observations that F-box genes harbour the second-highest occurrence of major-effect changes (Clark *et al.*, 2007).

In summary, the region I identified to harbour the recessive resistance locus comprises 20 candidate genes. Further restriction of the region of interest was not feasible by genetic linkage analysis. Microarray expression analysis suggests that At5g45210 and At5g45380

are not likely to be involved in the interaction between *C. higginsianum* and its host. Furthermore, the genes At5g45275 and At5g45370 can possibly be excluded due to their predicted function in symbiotic interaction with rhizobial bacteria. Gene products of At5g45220-At5g45260 are putative disease resistance proteins. Therefore they constitute candidate genes for a resistance locus that functions in an active defence response to *C. higginsianum* in a recessive or possibly semidominant manner, although cytological analysis did not identify typical features of an active resistance response. Alternatively, disease resistance responses conferred by an *R* gene might be exploited by the fungus to mediate susceptibility to *C. higginsianum*. Gene products of At5g45275-At5g45370 are expected to be involved in fundamental plant processes that might be essential for the plant-pathogen interaction mechanisms and are therefore possible candidates for dominant susceptibility factors to *C. higginsianum*, resulting in recessive resistance when aberrant in their structure and function.

4.6 Why was only a single recessive resistance locus identified in this study?

Four resistant accessions (Ws-0, Gifu-2, Can-0 and Kondara) and one susceptible accession Ler-0 were selected to identify the molecular basis of their natural variation in response to *C. higginsianum* inoculation (see 3.2). Due to their origin from very different geographic regions (Tab. 3.2), it was expected that analysis of the natural variation would reveal more than one recessive resistance locus conferring resistance by different molecular mechanisms. However, interestingly, the present analysis identified only a single recessive resistance locus in all four resistant accessions (see 3.3.6). This raises the question why no evidence was found for the contribution of further recessive resistance loci.

A striking finding of the present study was that only a minority of accessions showed strong susceptibility to fungal infection. This observation that susceptibility is a rare trait among *Arabidopsis* accessions (see 4.1) suggests that the identification of only one recessive resistance locus varying between the resistant accessions and Ler-0, is not due to the small number of resistant accessions tested, but rather might reflect the natural frequency of susceptibility to *C. higginsianum*. It seems unlikely that this finding of a

limited number of recessive resistance loci present in accessions from very different geographic regions may indicate that the identified resistance is conferred by a recessive *R* gene. As it is expected that there is a limited overlap of natural habitats of *Arabidopsis* and *C. higginsianum* (see 4.2), it is unlikely that selection pressure applied by the fungus would have resulted in many *Arabidopsis* accessions carrying the same resistance allele. It seems more plausible to argue that the identified recessive resistance is determined by natural variation in a dominant host susceptibility factor. This factor might have fundamental functions for the plant, *e.g.* in metabolism or development. As discussed in section 4.1, occurrence of a polymorphism in this gene could be beneficial only under distinct environmental conditions in only some habitats, as a result of a trade-off between fundamental functions and selection advantages under these conditions. This allele might therefore have been established in only a rare number of accessions, such as Ler-0. It is possible that the variation in this plant factor coincidentally confers susceptibility to *C. higginsianum* and functions therefore as a host susceptibility factor. In conclusion, due to the expected limited overlap of host and pathogen distribution, the fungus might not have evolved different mechanisms of interactions independently in different geographic regions, leading therefore to only a limited number of recessive resistance factors as observed in the present study. Nevertheless, including more susceptible *Arabidopsis* accessions in further studies of natural variation to *C. higginsianum* inoculation might possibly reveal recessive resistance loci other than the single one identified in this study.

4.7 Why did the mutant screen not identify true loss-of-susceptibility mutants?

In the course of the present study, I screened 207,000 EMS and γ-radiation mutated *Arabidopsis* M_2 generation plants in several susceptible genetic backgrounds (Ler-0, Ler *eds1*-2 and Ler *rar1*-13), aiming to identify mutations affecting susceptibility factors and therefore resulting in loss of susceptibility. Two mutants, designated #2 and #10 (see 3.1.1) were shown to be affected in disease resistance, identified by constitutive expression of the defence associated genes *PR-1* and *PDF1.2*, instead of loss of susceptibility (see 3.1.1). A total of 309 M_2 mutant lines exhibited a reduced susceptibility to *C. higginsianum* in a primary screen. However, for 163 of the corresponding M_3 lines,

the loss of susceptibility was not confirmed since the plants showed a wild-type infection phenotype in two independent infection experiments. For further 90 M_3 lines, either seed production was not successful, or the M_3 seeds did not germinate, which might be a result of the inoculation process with the fungus as the plant development was heavily affected by the infection, and therefore confirmation of the infection phenotype was not feasible. For 38 M_3 lines, reduced susceptibility to *C. higginsianum* could be confirmed. However, this reduced susceptibility was not sufficiently clear to allow a reliable identification of the mutant phenotype, which is essential for mapping the affected locus. It is possible that genes mediating susceptibility belong to a gene family. Hence, the effects of mutations in one member of this family would be masked by the functional redundancy or compensatory effects of other family members, resulting in only a moderate loss of susceptibility. Furthermore, disease susceptibility might be determined by multiple genes, each one making only a partial contribution to the trait. As with redundancy, the effects of mutations in only one of these genes might not lead to a sufficiently large phenotypic change in disease susceptibility. To enable the identification of mutants with less strong phenotypes, the mutant screen would have to be performed in less stringent inoculation conditions, *i.e.* lower inoculum concentrations, or a less susceptible genetic background, such as the accession Col-0 (DS 2-3). However, Col-0 already exhibits a certain degree of resistance. Therefore, this would lead to the identification of a large number of false positives, thereby reducing the efficiency of the mutant screen.

In addition, mutations in genes required for disease susceptibility might be essential for growth and development of the plant. Hence, mutations in these genes may result in lethality in an embryonic or juvenile stage of plant development and therefore such mutants would not have been identified in the screen.

It was surprising that the mutant screen did not identify alleles of the downy mildew resistant *dmr1*-4 and *dmr6* mutants. These EMS mutants, identified in a similar screen for loss of susceptibility to *H. parasitica* (Van Damme *et al.*, 2005) were found to be strongly resistant to *C. higginsianum* (see 3.1.2). The fact that mutations in these genes were not identified in the present analysis suggests that the mutant screen might have been not fully saturated and screening a larger number of mutants may be required to detect true loss-of-susceptibility mutants.

4.8 The role of *dmr* and *pmr* mutations in *Arabidopsis* susceptibility to *C. higginsianum*

To gain insight into the molecular basis of susceptibility of *Arabidopsis* to *H. parasitica*, van Damme *et al*. (2005) generated EMS mutants in the highly susceptible Ler *eds1-2* background. In a forward genetic approach, these mutants were screened for loss of susceptibility to *H. parasitica*. Twenty *dmr* mutants were identified; eight of these correspond to six different loci (*dmr1-dmr6*) (Van Damme *et al.*, 2005). Resistance of *dmr3*, *dmr4* and *dmr5* was associated with constitutive expression of *PR-1*, indicating that these mutants are probably not affected in susceptibility factors, but in resistance. In contrast *dmr1*, *dmr2* and *dmr6* mutants were initially suggested to be affected in true host susceptibility factors (Van Damme *et al.*, 2005) although recently a possible involvement in novel defence pathways was proposed (Van Damme, 2007; van Damme *et al.*, 2008). Although host susceptibility factors are generally expected to be involved in highly specific plant-pathogen interactions (O'Connell and Panstruga, 2006), it was of interest to determine whether *H. parasitica* and *C. higginsianum*, both exhibiting an intracellular biotrophic lifestyle, share requirement for at least some of these plant susceptibility factors. Therefore, I tested mutants harbouring four alleles of *dmr1, dmr2* and *dmr6* for their response to *C. higginsianum* inoculation (see 3.1.2). The infection phenotypes of *dmr1*-1, *dmr1*-2, *dmr1*-3 and *dmr2* were microscopically and macroscopically indistinguishable from that of the genetic background line Ler *eds1*-2, indicating that mutations in these loci do not play a role in the interaction of *C. higginsianum* with its host. Interestingly, *dmr1*-4 and *dmr6* exhibited a highly resistant infection in response to *C. higginsianum* inoculation.

The four different alleles of *dmr1* mutants were shown to carry different amino acid substitutions in a plant homoserine kinase (HSK) (At2g17265) (Van Damme, 2007). HSK is a key enzyme in the aspartate metabolic pathway for the biosynthesis of the essential amino acids methionine, threonine and isoleucine in plants. It could be shown that although the *dmr1* mutants have a reduced HSK activity, and therefore the substrate homoserine accumulates, the levels of methionine, threonine and isoleucine were increased rather than decreased in the *dmr1* mutants. This was suggested to be caused by a possible feedback mechanism that shuttles sufficient aspartate into the pathway so that homoserine accumulates to high levels and residual HSK activity, in the presence of high substrate concentrations, is sufficient to produce equal amounts of methionine, threonine

and isoleucine. Alternatively, the three amino acids might be synthesised by an alternative pathway independent of homoserine. Exogenous application of homoserine did not indicate effects on spore germination, suggesting that homoserine itself is not toxic for *H. parasitica*. However, exogenous application of L-homoserine mimicked the *dmr1* mutant phenotype, *i.e.* the haustoria appeared to be encased by papillae and growth of *H. parasitica* was arrested after formation of the first haustorium. Callose deposition was shown to be a secondary response which is not responsible for the arrest of pathogen growth (Van Damme, 2007). Therefore it was postulated that homoserine itself plays a role in a novel mechanism of plant disease resistance via modulation of the host amino acid metabolism and is therefore not a true susceptibility factor (Van Damme, 2007). However, it remains unclear why only *dmr1*-4, and not the other *dmr1* alleles, results in loss of susceptibility to *C. higginsianum*. Levels of homoserine in *Arabidopsis* seedlings were shown to be higher in *dmr1*-4 than in *dmr1*-1 and *dmr1*-3, but slightly lower than in *dmr1*-2, indicating that the loss of susceptibility in *dmr1*-4 is not due to a higher homoserine concentration in this mutant line. It might be possible that the *dmr1*-4 line, although having been back-crossed twice, contains additional EMS-induced mutations, that are responsible for the resistant infection phenotype to *C. higginsianum* rather than the mutation in *DMR1*. This possibility could be an interesting focus for future studies.

DMR6 (At5g24530) encodes a 2OG-Fe(II) oxygenase, a member of the superfamily of oxygenases that catalyses different hydroxylation and desaturation steps in plants (van Damme *et al.*, 2008). Transcription of *DMR6* was shown to be strongly induced during the interaction with both compatible and incompatible isolates of *H. parasitica*. Moreover, the activation of *DMR6* was locally induced within cells that are in direct contact with the pathogen, suggesting a role during plant defense. As the mutation in *DMR6* was shown to result in constitutive activation of defence-associated genes, *e.g. ACD6, PR-1, PR-2, PR-4* and *PR-5*, it was postulated that *DMR6* might negatively affect the expression of these genes. Possibly, in the *dmr6* mutants the substrate of the *DMR6* encoded 2OG-Fe(II) oxygenase is accumulated, which could have a direct toxic effect on the pathogen, or indirectly stimulate the expression of defence-associated genes. Alternatively, the product of *DMR6* is either negatively regulating defense-associated genes or positively affecting susceptibility to *H. parasitica*. If *dmr6* affects, directly or indirectly, the activation of defence-associated genes, it would raise the question why this does not result in a broader range of resistance, since the *dmr6*-1 mutant is resistant to *H. parasitica* and *C. higginsianum*, but remains susceptible to *P. syringae* and the obligate biotrophs *G. orontii*

and *Albugo candida*. Specific loss of susceptibility to only *H. parasitica* and *C. higginsianum* might therefore suggest that DMR6 functions as a plant susceptibility factor and is essential for the interaction of both these pathogens with *Arabidopsis*. This however, raises the question why the plant maintained *DMR6* despite the apparent fitness disadvantage. It is possible that negative regulation of defence-associated genes is required for the control of defence responses by negative feedback loops, because constitutive activation of these responses would be too metabolically expensive for the plant. Alternatively, susceptibility to *H. parasitica* and *C. higginsianum* does not represent a selection pressure driving the loss of *DMR6* in a trade-off between the advantageous effects of *DMR6* and susceptibility to these pathogens, as similarly discussed in section 4.1. A further possibility is that *DMR6* is involved in fundamental metabolic or development plant processes. The intracellular lifestyle of *H. parasitica* and *C. higginsianum* might be dependent on these processes during a specific step of their pathogenesis (see 4.2). Nevertheless, it is an interesting observation that pathogens with an obligate biotrophic life-style can differ more in their requirement for susceptibility mechanisms than two pathogens with different life-styles, *i.e.* an obligate biotroph and a hemibiotroph.

In a similar forward genetic approach, *Arabidopsis* mutants in a Col-0 background were identified that did not support normal growth of the powdery mildew pathogen, *G. cichoracearum*, without constitutive accumulation of *PR1* and *PDF1.2*. Four *pmr* loci, *pmr2*, *pmr4*, *pmr5* and *pmr6* have been cloned and characterised (Vogel and Somerville, 2000; Vogel *et al.*, 2002; Jacobs *et al.*, 2003; Nishimura *et al.*, 2003; Vogel *et al.*, 2004; Consonni *et al.*, 2006). As with the *dmr* mutants, the *pmr* mutants were tested for reduced susceptibility to *C. higginsianum* (see 3.1.3), aiming to identify common mechanisms of host-pathogen interactions between pathogens with an intracellular biotrophic lifestyle. While *pmr2*, *pmr3* and *pmr4* did not show a marked difference in their infection phenotype after inoculation with *C. higginsianum*, *pmr5* and *pmr6* plants exhibited a significant reduction in their susceptibility in comparison to Col-0 wild-type plants.

PMR2 has been shown to be allelic to *AtMLO2* (Consonni *et al.*, 2006) and *mlo*-mediated resistance was identified to be effective against the adapted powdery mildew species *G. orontii* and *G. cichoracearum* without requirement of SA-, JA- or ET- mediated signalling pathways (Panstruga, 2005). In contrast, *Atmlo2* plants were fully susceptible to *P. syringae* and to *H. parasitica*. MLO is an integral plasma membrane-localised protein,

possessing seven hydrophobic membrane-spanning helices with a C-terminus that harbours an amphiphilic α-helix that serves as a calmodulin binding domain. *AtMLO2* belongs to a phylogenetic clade of three *Arabidopsis* genes (*MLO2*, *MLO6* and *MLO12*) which represent co-orthologs of the barley Mlo (Consonni *et al.*, 2006). Recent studies have shown that host cell entry, but not conidiophore formation (asexual sporulation) is restored in *Atmlo2 pen1* double mutants. *Atmlo2 pen2* and *Atmlo2 pen3* double mutants did not only exhibit restored pathogen entry rates, but also wild-type-like conidiation. This was an intriguing observation, since *PEN1*, *PEN2* and *PEN3* are required for powdery mildew non-host resistance (Collins *et al.*, 2003; Lipka *et al.*, 2005; Stein *et al.*, 2006). Furthermore, the SNARE domain protein HvSNAP34 was shown to be a contributor of mlo-based and non-host resistance (Collins *et al.*, 2003; Douchkov *et al.*, 2005). The fact that both *mlo*-resistance and powdery mildew non-host resistance are largely independent of common defence signalling pathways such as SA and JA/ET, and that both require PEN1/PEN2/PEN3 and further factors, strongly suggests that these two modes of resistance are mechanistically identical (Humphry *et al.*, 2006). It is currently thought that MLO proteins function as regulatory components of plant secretory processes involving SNARE domain proteins such as PEN1 (Panstruga, 2005). Therefore, it has been postulated that adapted powdery mildew species might have evolved to circumvent these defence mechanisms by specifically corrupting MLO activity for successful pathogenesis. In contrast to adapted powdery mildew species, AtMLO2/PMR2 seems not to be required for successful entry of *C. higginsianum*, as shown by the comparable susceptibility of *pmr2* and wild-type plants.

Interestingly, a mutation in *PMR3* did not cause increased resistance to *C. higginsianum*, as observed in response to inoculation with *G. cichoracearum* and *H. parasitica,* but *pmr3* plants were even more susceptible to *C. higginsianum* than Col-0 wild-type plants (see 3.1.3). Therefore, cloning of the *pmr3* locus in the future might reveal a mechanism that confers susceptibility to *G. cichoracearum*, but inhibits growth of *C. higginsianum*.

For *pmr4* plants, I observed a slight reduction of susceptibility to *C. higginsianum* (see 3.1.3). However, this needs more detailed analysis of the level of reduced of susceptibility since the Col-0 genetic background already confers a certain degree of resistance to *C. higginsianum*. The reduced susceptibility of *pmr4* plants would be in agreement with a mutation in *GSL5*, a callose synthase isoform, which is suggested to negatively regulate the SA pathway, as discussed in section 4.4.3 (Nishimura *et al.*, 2003). Reduced susceptibility to *C. higginsianum,* possibly due to the loss of negative regulation of the SA

pathway, supports previous findings that the SA signalling pathway contributes to resistance of Col-0 to *C. higginsianum* (O'Connell *et al.*, 2004; Liu *et al.*, 2007a). Alternatively, it has been suggested that biotrophic pathogens such as *H. parasitica* and *G. cichoracearum* might have evolved to exploit components of the plant wound response for successful pathogenesis, whereby GSL5 callose synthase may facilitate nutrient uptake or serve as a pathogen-induced protection barrier preventing recognition by the plant (Jacobs *et al.*, 2003). In the present study, I found callose deposition not to affect growth of *C. higginsianum* in the tested *Arabidopsis* accessions (see 4.4.3) and therefore callose deposition does not seem to be a component required to support successful pathogenesis. This raises the question of how the loss of *pmr4*, and therewith loss of callose deposits, might result in slightly reduced *C. higginsianum* growth, if *pmr4*-based resistance mechanisms should not involve effects by the regulation of the SA signalling pathway as proposed by Nishimura *et al.* (2003).

The *PMR5* and *PMR6* loci encode a protein of unknown function and a pectate lyase, respectively, as discussed in detail in section 4.4.1. Both mutants exhibit alterations in cell wall composition, notably an increased pectin content, reduced pectin esterification and a change in the hydrogen-bonding environment of cellulose (Vogel *et al.*, 2002; Vogel *et al.*, 2004), suggesting that changes in the cell wall architecture are associated with powdery mildew resistance. Although a conclusive model for susceptibility to powdery mildews remains elusive, it was hypothesised that effects on the cell wall might alter nutrient availability for the fungus, or result in reduced penetration efficiency. Susceptibility to *C. higginsianum* was also decreased in *pmr5* and *pmr6* compared to Col-0 (Fig. 3.6), strongly suggesting that the mutations affect plant-pathogen interaction mechanisms that are common between two different species of powdery mildew, *i.e. G. cichoracearum* and *G. orontii*, and also *C. higginsianum*. This is particularly interesting, as plants challenged with the oomycete *H. parasitica* were equally susceptible as Col-0 wild-type plants. As with the observations on *dmr6*, this suggests that pathogens with an obligate biotrophic life-style can differ more in their requirement for susceptibility mechanisms than an obligate biotroph and a hemibiotroph.

4.9 Why have *dmr1*, *dmr6*, *pmr4-6* not been identified in the analysis of natural variation?

The present study identified *dmr* and *pmr* loci contributing to susceptibility to *C. higginsianum* that had previously been found in mutant screens. However, none of these loci were detected by analysis of natural variation between Ler-0 and the resistant accessions Ws-0, Gifu-2, Can-0 and Kondara. It is possible that the *dmr* and *pmr* mutations, induced by chemical treatment, have not occurred in nature. This is most likely for loci that are involved in fundamental plant processes, *e.g.* growth or development. Naturally occurring mutations in these genes would result in high fitness costs or even lethality which would therefore not drive the fixation of these alleles in the population. Furthermore, many of these chemically induced mutations have been shown to have pleiotropic effects, resulting in microlesions, altered leaf morphology and decreased plant size (Vogel and Somerville, 2000; Vogel *et al.*, 2002; Vogel *et al.*, 2004; Van Damme *et al.*, 2005), indicating additional effects on the plant that may reduce fitness. Likewise, the mutation in barley MLO is known to be associated with the spontaneous deposition of callose and premature onset of leaf senescence (Panstruga, 2005). This impaired leaf physiology has been shown to result in reduced grain yield of *mlo* mutants compared with wild-type plants. Moreover, as previously discussed, it is likely that susceptibility to *C. higginsianum* might not be strongly disadvantageous under natural conditions. Therefore, *C. higginsianum* might not represent a selection pressure driving the fixation of any of the identified *dmr* or *pmr* alleles. Similarly, *mlo* resistance does not seem to be required in wild populations to keep the spread of powdery mildew pathogens in check (Panstruga, 2005).

The *pmr* mutants were generated in a Col-0 genetic background and therefore differ from the genetic background analysed in the present study of natural variation in response to *C. higginsianum*. The genetic background might have effects on the loci identified, and thereby on the extent of susceptibility. It is therefore possible that naturally occurring *pmr5* and *pmr6* mutations would not be detectable in the resistant accessions of this study. Furthermore, the analysis was based on natural variation between Ler-0 and the tested resistant accessions. It therefore cannot be excluded that fixation of the *pmr5* and *pmr6* alleles has occurred, but in all accessions tested, and therefore identification of the loci was not feasible by analysis of natural variation.

4.10 Summary

The focus of the present study was on the identification of determinants of the interaction between *Arabidopsis* and the ascomycete pathogen *C. higginsianum,* based on a forward genetics screen for mutants that exhibit a loss of susceptibility to *C. higginsianum,* and on the analysis of natural variation in resistance.

A high-throughput screen of 207,000 chemically or γ-radiation induced *Arabidopsis* mutants of susceptible genetic backgrounds did not lead to the identification of mutant lines with a reduction of susceptibility to *C. higginsianum* that was significant enough for the localisation of the responsible susceptibility loci. However, infection assays of previously identified mutants with loss-of-susceptibility infection phenotypes to *H. parasitica* (*dmr* mutants) and *G. cichoracearum* (*pmr* mutants) revealed some mutant lines that also showed a loss of susceptibility to *C. higginsianum*, suggesting the existence of mechanisms of plant-pathogen interactions that are common between the hemibiotrophic pathogen *C. higginsianum* and two obligate biotrophic pathogens.

In the second approach I identified a single recessive resistance locus to *C. higginsianum* by crossing the resistant accessions Ws-0, Gifu-2 and Can-0 to the susceptible Ler-0 accession and following segregation in the F_1 and F_2 progeny, and parallely by QTL analysis of the Ler-0 x Kondara RIL population. By positional cloning in a Ler-0 x Ws-0 F_2 population, this recessive resistance locus could be located on the lower arm of chromosome V between the molecular markers 236 (18,307,842 bp) and 312 (18,407,860 bp).

A cytological analysis demonstrated that in the resistant accessions, *C. higginsianum* growth was arrested either at the stage of appressorial penetration or at the initial stage of biotrophic primary hyphal growth. Recessive resistance mechanisms were shown not to involve either reactive oxygen species accumulation or callose deposition.

4.11 Outlook

A chromosomal region harbouring a recessive resistance locus, involved in the interaction of *Arabidopsis* with *C. higginsianum*, was identified in this study. The next challenge will be to isolate the plant gene conferring recessive resistance and to determine the nature of

the sequence polymorphism responsible for the natural variation between Ler-0 and resistant accessions.

Positional cloning of an enlarged Ler-0 x Ws-0 F_2 mapping population might reduce further the size of the region harbouring the recessive resistance factor. Targeted expression analysis of candidate genes that has become feasible for some genes by the DNA sequence analysis of the Ler-0 BIBAC insert might indicate a function of one of these genes in recessive resistance. Eventually, a comparative DNA sequence analysis of the identified recessive resistance locus in Ler-0 and in resistant accessions will elucidate the character of the natural variation. Analysis of the gene structure, biological function, and eventually the transformation of the recessive resistance locus of the susceptible Ler-0 accession into one of the four resistant accessions and vice versa will help to identify whether the recessive resistance locus acts as either a dominant susceptibility factor or as a recessive *R* gene.

A detailed study of polymorphisms of the recessive resistance locus in additional accessions, including in particular highly susceptible and distantly related accessions, *e.g.* CVI, will shed light on intraspecific variability and might indicate mechanisms of evolutionary adaptation.

The *Arabidopsis-Colletotrichum* interaction might be model pathosystem that is not of relevance in nature. It will therefore be important to analyse homologues of the identified recessive resistance factor in natural hosts, *e.g. Brassica campestris* and *Raphanus*.

The involvement of the recessive resistance locus in interactions with other hemibiotrophic, obligate biotrophic and necrotrophic pathogens should also be examined to learn us about the general relevance of this determinant of the interaction between *Arabidopsis* and *C. higginsianum* to elucidate whether it is uniquely required for susceptibility to *C. higginsianum*.

4.12 General perspectives

Plants are continuously the target of attacks by disease-causing organisms, including bacteria, fungi, viruses and nematodes. The economic and social impact of food losses due to plant diseases is enormous for a constantly growing world population with decreasing areas of cultivable land. Therefore, understanding the molecular mechanisms of resistance and susceptibility of plants to pathogens will help to identify new ways to control through

plant breeding diseases providing environmentally friendly ways to improve food production. Unravelling the mechanisms of susceptibility and resistance of *Arabidopsis* to *C. higginsianum* should increase our understanding of the molecular basis of plant susceptibility to other *Colletotrichum* species which cause devastating diseases, an important pathogen on numerous crop and ornamental plants around the world. Ultimately this will improve our understanding of general mechanisms of plant-pathogen interactions to create durable resistance in the field, similar to the successful deployment of *mlo* alleles in European agriculture in elite barley lines.

V Supplementary Data

Table SD1: Geographic origin of *Arabidopsis* accessions, and infection phenotypes

Origin	Accession	Disease Score (DS)
Austria	In-0	1-2
Austria	Pi-0	2
Austria	Uod-1	2-3
Belgium	An-1	1-2
Canada	Ri-0	2-3
Canada	Van-0	2
Canada	Var2-1	3
Canada	Var2-6	2-3
Canary Islands	Can-0	0-1
Cape Verdi Islands	CVI	3
Czecg Republic	Ta-0	2
Czech Republic	Bor-1	2
Czech Republic	Bor-4	2
Czech Republic	Jm-0	2
Czech Republic	Lp2-2	2-3
Czech Republic	Lp2-6	1-2
Czech Republic	Pu2-23	1
Czech Republic	Pu2-7	1
Czech Republic	Zdr-1	0-1
Czech Republic	Zdr-6	2
Eire	Bur-0	3
Finland	Tamm-2	1
Finland	Tamm-27	0-1
Finland	Te-0	0-1
France	Ag-0	0
France	Gy-0	2
France	Lz-0	1-2
France	PYL-1	1-2
France	Ra-0	2
France	RAN	2-3
France	Ren-1	0-1
France	Ren-11	1
Germany	Bay-0	3
Germany	Ei-2	1-2
Germany	Eil-0	0-1
Germany	Ga-0	1
Germany	Mrk-0	2-3
Germany	Mz-0	1
Germany	Nd-1	1
Germany	Sp-0	0-1
Germany	Wt-5	2-3
India	Kas-2	1-2
Italy	Bl-1	0-1
Italy	Ct-1	2
Italy	Pa-1	2
Japan	Gifu-2	0-1
Japan	Kyoto	2
Japan	Sakata	2-3
Japan	Sap-0	2
Japan	Sendai-1	0-1
Japan	Sendai-3	0-1
Japan	Sendai-4	0-1

Table SD1: continuation

Origin	Accession	Disease Score (DS)
Japan	Tsu-1	2-3
Japan	Yam	0-1
Kazakhstan	Kz-1	1
Kazakhstan	Kz-9	1-2
Libya	Mt-0	2-3
Lithuania	Kn-0	2
Netherlands	Nok-1	2
Norway	Oy-0	1
Poland	Ler-0	3
Poland	Ler-1	3
Poland	Lip-0	2-3
Poland	Wa-1	2-3
Portugal	C24	2-3
Portugal	Fei-0	0-1
Russia	Est-1	2
Russia	Ms-0	2
Russia	N6 Karelian	0-1
Russia	N7 Pinguba	0-1
Russia	Rld-2	2
Russia	Stw-0	1
Russia	Ws-0	0-1
Russia	Ws-2	1-2
Spain	Bla-1	2
Spain	Pro-0	1
Spain	Se-0	3
Spain	Ts-1	0-1
Spain	Ts-5	0-1
Sweden	Bil-5	0-1
Sweden	Eden-1	1
Sweden	Eden-2	1-2
Sweden	Fab-2	1
Sweden	Fab-4	1-2
Sweden	Lov-1	2-3
Sweden	Lov-5	1
Sweden	Omo2-1	0-1
Sweden	Omo2-3	0-1
Sweden	Spr1-2	2
Sweden	Spr1-6	2
Sweden	St-0	0-1
Sweden	Ull2-3	1
Sweden	Ull2-5	2
Tadjikistan	Sorbo	1-2
Tajikistan	En-T	1-2
Tajikistan	Kondara	0-1
Tajikistan	Shah	2
UK	Edi-0	2
UK	HR-5	1
UK	HR-10	2
UK	NFA-8	2
UK	NFA-10	3
UK	Sq-1	1
UK	Sq-8	2
Ukraine	Rubezhnoe-1	1-2
USA	Col-0	2-3
USA	Gre-0	2
USA	Knox-10	2
USA	Knox-18	2
USA	Pna-10	1-2

Table SD1: continuation

Origin	Accession	Disease Score (DS)
USA	Pna-17	0
USA	Rmx-A02	1
USA	Rmx-A180	1
USA	RRS-7	0-1
USA	RRS-10	0-1
USA	Yo-0	2

Plants were inoculated with *Colletotrichum higginsianum* and analysed microscopically at three days after inoculation and macroscopically at six days after inoculation. The disease score (DS) is based on the combined macroscopic and microscopic observations:

DS 0, fully resistant - plants remain intact with only small necrotic lesions, no hyphae present or only biotrophic hyphae without necrotrophic secondary hyphae

DS 1, intermediate resistant - plants mostly intact with only limited lesions, secondary hyphae are mostly absent or very restricted in extent

DS 2, intermediate susceptible - plants partially collapsed with large necrotic lesions and some tissue maceration and water-soaking, extensive secondary mycelium, sporulation rarely seen

DS 3, fully susceptible - plants completely collapsed and tissue extensively macerated and water-soaked, extensive secondary mycelium with abundant spore production

Table SD2: Geographic origin of *Arabidopsis* accessions, and infection phenotypes

Marker	Primer	Sequence 5`-3`	Position	Descrip.	Ch
NGA225	NGA225F	GAAATCCAAATCCCAGAGAGG	1507104	SSLP	5
	NGA225R	TCTCCCCACTAGTTTTGTGTCC	1507224		5
NGA151	NGA151F	GTTTTGGGAAGTTTTGCTGG	4669932	SSLP	5
	NGA151R	CAGTCTAAAAGCGAGAGTATGATG	4670082		5
SO191	SO191F	CTCCACCAATCATGCAAATG	15021915	SSLP	5
	SO191R	TGATGTTGATGGAGATGGTCA	15022062		5
DFR.1	DFR.1F	TGTTACATGGCTTCATACCA	17181581	CAP	5
	DFR.1R	AGATCCTGAGGTGAGTTTTTC	17182723	(Bsa AI)	5
MBD2-1	MBD2-1F	ACAATTCGTTGACAAAAAGC	17222001	SSLP	5
	MBD2-1R	TCAACCTCCATAGTTTGAGC	17222608		5
MSAT5.9	17,57Mb-F	CGTCATTTTTCGCCGCTCT	17252309	SSLP	5
	17,57Mb-R	CATGGTGGCGCGTAGCTTA	17253907		5
17,57Mb	17,57Mb-F	GGTCTCTTCTCCACTGTTTG	17569614	SSLP	5
	17,57Mb-R	GAACAAGAAGTCCTTGGAGA	17570096		5
MRH10-2	MRH10-2F	TTTTGTTGTGAATGAATTGG	17719014	SSLP	5
	MRH10-2R	AGCCGTTAGAACCAAAATTA	17719212		5
MSAT5.4	MSAT5.4F	TCAACCCTAGATGGTGTCGAT	17844441	SSLP	5
	MSAT5.4R	TTCAATCATTTTTGCCGTGA	17844702		5
78	MFC16-2F	AATCTGCCACTGTGCTTAAT	17923050	SSLP	5
	MFC16-2R	ACTAATGCTTGGGCAATCTA	17923280		5
64	K23L20-F	TTTTAAAACGACTCATGCTTT	18039450	SSLP	5
	K23L20-R	ACCGTTTGCTATGCTTCTAA	18039640		5
66	K23L20-2F	AAACATCGCTCTTCTCAGTC	18050610	SSLP	5
	K23L20-2R	GGGAATTATGACACCAACAC	18050801		5
68	K23L20-3F	GGTGAGATCGTCTTAGTCGT	18089460	SSLP	5
	K23L20-3R	TCTCAATCCAATGTTCAGGT	18089670		5
72	K17O22-1F	GCGACTGTGAAGTTTGAGAT	18262301	SSLP	5
	K17O22-1R	GAGCCTTAAACTGCCACTAA	18262501		5
134	18272105F	CAGCACCGAAATGACAAAAA	18272105	CAPs	5
	18272105R	AGCCGAAAATGTTTTGAAATAA	18273001	(HphI)	5
146	18304971F	ATGCAGAAAATTTTACGTACC	18304971	CAPs	5
	18304971R	TTGGGTTAACTTTTTGTTAA	18305240	(HpaI)	5
236	18307842F	TAACTCCAACACGTCACTCA	18307842	CAPs	5
	18307842R	TCATCGGGAGTGTTGCTAA	18308089	(MseI)	5
82	K9E15-1F	AGCCCAAAACTGAAAAACTC	18337817	SSLP	5
	K9E15-1R	ATCTGTGGGTCAGAAATCCT	18338088		5
RPS4-NT	RPS4-NTF	TCATCAATTTCCGTGGGGCA	18342772	CAPs	5
	RPS4-NTR	GGTACCTTTTCTTGTCAATG	18343194	(XhoI)	5
332	18363163F	GTCATATAATCGTGATGGAAGA	18363163	CAPs	5
	18363163R	AAAATGTGAGTGTATCCGAAA	18363459	(EcoRV)	5
338	18379031F	CATTTTGATCAATGAATTACACATTG	18379031	CAPs	5
	18379031R	GAGGGAATGAATGAAATTGA	18379231	(RsaI)	5
364	18383701F	GACGGAGAAGGTACAGATGA	18383701	CAPs	5
	18383701R	AAGAAGAACATGGGCCTAAT	18384001	(XhoI)	5
312	18407860F	ATGTTACATACTTACATGTCAGTCTGA	18407860	CAPs	5
	18407860R	TTCCACTTGGACAATGATG	18408204	(DdeI)	5
252	MFC19F	CTTTGGCTGGAGGACTTAAA	18410145	SSLP	5
	MFC19R	TAGAGCCACGTGAACTGAAG	18410422		5
276	18464805F	AGTTTGAAGCCCTTGTCAGA	18464805	CAPs	5
	18464805R	TCTCAAATGATCCCTCAATC	18465025	(SacI)	5
MRA19-1	MRA19-1F	CGGTACTTTTTATTTTTCTTTTG	18597044	SSLP	5
	MRA19-1R	TCAATTATCCGAATCACTAAAA	18597265		5
NGA129	NGA129-F	CACACTGAAGATGGTCTTGAGG	19007000	SSLP	5
	NGA129-R	TCAGGAGGAACTAAAGTGAGGG	19008000		5
MSAT5.18	MSAT5.18F	GATTATAGGTTATTTTCGTT	26321176	SSLP	5
	MSAT5.18R	ACAGAAGAACCGATTC	26321467		5
MSAT1.4	MSAT1.4F	CTAAACTAGAACCAGGGGTAA	14160180	SSLP	1
	MSAT1.4R	CTAAACTAGAACCAGGGGTAA	14160420		1
NGA128	NGA128-F	ATCTTGAAACCTTTAGGGAGGG	20225000	SSLP	1
	NGA128-R	GGTCTGTTGATGTCGTAAGTCG	20226000		1

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Table SD2: continuation

Marker	Primer	Sequence 5`-3`	Position	Descrip.	Ch.
MSAT1.2	MSAT1.2F	TTGAGTGGTGCCGCTTG	28894896	SSLP	1
	MSAT1.2R	ATATCTCCATCGCTGCAACC	28895060		1
MSAT2.18	MSAT2.18F	TAGTCTCTTTTGGTGCGCATA	2799644	SSLP	2
	MSAT2.18R	AGCCTCTCCAAGCTTAGGTCT	2799849		2
MSAT2.41	MSAT2.41F	GACTGTTTCATCGGATCCAT	11095452	SSLP	2
	MSAT2.41R	ACAAACCATTGTTGGTCGTG	11095596		2
MSAT2.4	MSAT2.4F	TGGGTTTTTGTGGGTC	13831870	SSLP	2
	MSAT2.4R	GTATTATTGTGCTGCCTTTT	13832158		2
MSAT2.22	MSAT2.22F	CGATCCAATCGGTCTCTCT	19632943	SSLP	2
	MSAT2.22R	TGGTAACATCCCGAACTTC	19633191		2
MSAT3.2	MSAT3.2F	AAGGTACGGCGGTGGATATTG	9055511	SSLP	3
	MSAT3.2R	CGGGGATTTCTTCTTCCTGTG	9055722		3
MSAT3.29	MSAT3.29F	CGGATGAGATCCAA	20486867	SSLP	3
	MSAT3.29R	GACAGAGGTTTACTAATGT	20487105		3
MSAT3.13	MSAT3.19F	TTGTGTGTTTGCGATC	21377089	SSLP	3
	MSAT3.19R	CATATCCGTTTTTATGTTTT	21377370		3
MSAT4.39	MSAT4.39F	GTTATCACATTAAAATCACC	89498	SSLP	4
	MSAT4.39R	CCAATTGTAATATATGAACA	89659		4
NGA8	NGA8F	GAGGGCAAATCTTTATTTCGG	5628810	SSLP	4
	NGA8R	TGGCTTTCGTTTATAAACATCC	5628967		4
MSAT4.35	MSAT4.35F	CCCATGTCTCCGATGA	7549254	SSLP	4
	MSAT4.35R	GGCGTTTAATTTGCATTCT	7549471		4
MSAT4.13	MSAT4.13F	GGAACAAGAACACAGTGAA	15297044	SSLP	4
	MSAT4.13R	ATAAATCTAGGCAGGACAAG	15297270		4
MSAT4.38	MSAT4.38F	GCCTTATAGTACACCCAAA	18412248	SSLP	4
	MSAT4.38R	CCACTCCACTCTCGAA	18412437		4

Table SD3: Quantitative analysis of penetration efficiency of appressoria formed by *C. higginsianum* on selected *Arabidopsis* accessions at 1, 2 and 3 dpi

	1 dpi		2 dpi		3 dpi	
	penetrated		penetrated		penetrated	
accession	mean (%)	st. error	mean (%)	st. error	mean (%)	st. error
Ler-0	8,003048	0,947387	19,49267	2,676852	52,82208	12,45068
Col-0	1,229047	0,84419	9,729622	2,87824	26,57381	0,895933
Ws-0	1,443968	0,751967	4,087382	3,375533	9,232545	4,648783
Gifu-2	2,328511	1,08488	2,982167	0,665575	9,186286	1,931711
Can-0	0,822644	0,434752	4,478539	2,748108	9,775386	3,477102
Kondara	1,587302	1,587302	4,605132	1,63019	2,801478	2,641318

Results are presented as mean values
st. error, standard error (n=1,73)

Table SD4: Quantitative analysis of DAB production in epidermal cells of selected *Arabidopsis* accessions in response to *C. higginsianum* appressoria at 2 dpi

	penetrated				unpenetrated			
	stained		unstained		stained		unstained	
accession	mean (%)	st. error	mean (%)	st. error	mean (%)	st. error	mean (%)	st. error
Ler-0	0,740741	0,740741	18,75193	2,011531	0,493827	0,493827	80,0135	3,139223
Col-0	0,124378	0,124378	9,605244	2,960968	0,248756	0,248756	90,02162	2,722306
Ws-0	0	0	4,087382	3,375533	0,68522	0,343703	95,2274	3,497983
Gifu-2	0	0	2,982167	0,665575	0,104822	0,104822	96,91301	0,610017
Can-0	0,254453	0,254453	4,224087	2,847623	0,254453	0,254453	95,26701	2,669219
Kondara	0	0	4,605132	1,63019	0,11554	0,11554	95,27933	1,56178
Eil-0	0,093458	0,093458	3,774612	1,832865	0,859269	0,111605	95,27266	1,814717

Results are presented as mean values
st. error, standard error (n=1,73)

Table SD5: Quantitative analysis of DAB production in epidermal cells of selected *Arabidopsis* accessions in response to *C. higginsianum* appressoria at 3 dpi

	penetrated				unpenetrated			
	stained		unstained		stained		unstained	
accession	mean (%)	st. error	mean (%)	st. error	mean (%)	st. error	mean (%)	st. error
Ler-0	13,59944	7,236545	39,22264	19,07147	9,688254	4,424604	37,48967	10,73623
Col-0	9,830549	3,34798	16,74327	4,239602	3,081642	2,451681	70,34454	19,71863
Ws-0	0,458716	0,458716	8,773829	4,299023	0,45942	0,264841	90,30803	28,6653
Gifu-2	0,104167	0,104167	9,08212	2,020092	0,104167	0,104167	90,70955	28,0727
Can-0	0,477193	0,271166	9,298193	3,206357	0,328407	0,328407	89,89621	28,15771
Kondara	0,16835	0,16835	2,633127	2,47308	0,869407	0,470994	96,32912	30,29149
Eil-0	0,218341	0,218341	10,52871	8,248581	0,218341	0,218341	89,03461	8,685262

Results are presented as mean values
st. error, standard error (n=1,73)

Table SD6: Quantitative analysis of Aniline Blue staining in epidermal cells of selected *Arabidopsis* accessions in response to *C. higginsianum* appressoria at 2 dpi

| | penetrated | | | | unpenetrated | | | |
| | stained | | unstained | | stained | | unstained | |
accession	mean (%)	st. error	mean (%)	st. error	mean (%)	st. error	mean (%)	st. error
Ler-0	7,274786	2,22665	7,851656	5,38974	18,34639	4,982756	66,52717	10,42815
Col-0	10,17515	5,236578	2,857201	1,812342	25,61929	8,674553	61,34836	13,94272
Ws-0	0,865801	0,865801	1,064812	0,559607	35,2113	6,519941	62,85809	6,492815
Gifu-2	0,735083	0,385553	3,629717	2,276893	24,85119	5,209125	70,78401	2,658556
Can-0	1,116519	0,535917	1,910793	1,207463	23,44178	6,339483	73,53091	4,939302
Kondara	0,510204	0,510204	0,621035	0,314727	14,64302	2,87685	84,22575	2,359469
Eil-0	1,060071	0,865544	0	0	30,85813	2,790371	68,0818	3,655915

Results are presented as mean values
st. error, standard error (n=1,73)

Table SD7: Quantitative analysis of Aniline Blue staining in epidermal cells of selected *Arabidopsis* accessions in response to *C. higginsianum* appressoria at 3 dpi

| | penetrated | | | | unpenetrated | | | |
| | stained | | unstained | | stained | | unstained | |
accession	mean (%)	st. error	mean (%)	st. error	mean (%)	st. error	mean (%)	st. error
Ler-0	16,71604	5,468463	37,51628	4,910676	16,25404	7,305748	47,99785	9,244826
Col-0	10,86843	5,598676	3,846771	2,89036	28,44883	12,11563	56,83597	16,34168
Ws-0	6,263065	0,967	3,59999	3,170849	49,15812	23,78832	62,97604	1,432375
Gifu-2	2,38774	1,533766	1,512849	0,783761	58,12444	20,43512	37,97497	21,33765
Can-0	0,751614	0,55863	2,101231	1,526143	26,84135	6,584865	70,30581	6,552888
Kondara	0,459559	0,375228	0,919118	0,750456	74,85666	7,837798	50,98344	15,51192
Eil-0	1,642906	0,931128	0,125628	0,102575	32,29542	5,429131	65,93604	4,600578

Results are presented as mean values
st. error, standard error (n=1,73)

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VII Acknowledgements

VIII Erkärung

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