Genetic analysis of disease susceptibility in the Arabidopsis-*Hyaloperonospora parasitica* interaction

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(met een samenvatting in het Nederlands)

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Mireille van Damme:
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Contents

Mireille van Damme:
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Abbreviations

AVR	avirulence
ATR	*Arabidopsis thaliana* recognized
BTH	benzothiadiazole
CC	coiled coil
DAB	3'-3'-di-amino-benzidine
2-DDG	2-deoxy-D-glucose
dpi	days post inoculation
EBI	European Bioinformatics Institute
EMS	ethane methyl sulfonate
EST	expressed sequencing tags
ETI	effector-triggered immunity
GHMP	galactokinase, homoserine kinase, mevalonate kinase, phospho-mevalonate kinase
HS	homoserine
HSK	homoserine kinase
HR	hypersensitive response
Ile	isoleucine
LRR	leucine rich repeats
MAP	mitogen-activated protein
Met	methionine
NBS	nucleotide binding site
NCBI	National Center for Biotechnology Information
PAMPs	pathogen associated molecular patterns
PCD	programmed cell death
PTI	PAMP-triggered immunity
PR	pathogen-related
R	resistance
ROIs	reactive oxygen intermediates
RPP	recognition of *Peronospora parasitica*
SA	salicylic acid
Thr	threonine
TIR	Toll interleukin 1 receptor
TTSS	type III secretion system

Mireille van Damme:
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Chapter 1

General Introduction and Outline

Genetics of Plant Disease Susceptibility

Plants are immobile and constantly exposed to microbes. Nevertheless plants are able to deal with most attacks and are therefore resistant to the majority of potential pathogens. However, a limited number of microbes has evolved the ability to infect a given host plant resulting in susceptibility and the development of disease. On a global scale the impact and costs of plant diseases on agriculture is enormous, highlighting the importance of the research on this topic. At the molecular level, however, very little is known about the processes that underlie disease susceptibility. In this introductory chapter different aspects of plant disease susceptibility will be discussed. To place the concept of susceptibility in a framework I will first provide a brief overview of the current knowledge on resistance to pathogens, in particularly on the dominant forms of resistance that involve specific pathogen recognition as the initial trigger. The focus will then change to basic compatibility and the infection of susceptible plants by biotrophic pathogens, with special emphasis on the Arabidopsis-*Hyaloperonospora parasitica* interaction. Approaches to identify susceptibility genes will be discussed, starting with the analysis of recessive forms of resistance. Recessive forms of resistance can occur due to mutations in susceptibility genes that are required for a successful infection or basic compatibility. In the last part of this chapter the approaches to identify susceptibility genes by mutagenesis will be discussed. Finally, the approach I have taken to identify Arabidopsis genes required for susceptibility to downy mildew is outlined.

Resistance triggered by pathogen recognition

Plants resist most infection attempts of pathogenic microbes by activating a successful defence response that is triggered by early pathogen recognition. The triggers can be (i) pathogen-associated molecular patterns (PAMPs) that belong to the general elicitors of defence or they can be (ii) isolate-specific effectors or compounds that are recognized by cognate resistance proteins in

the host. In this chapter the nomenclature by Chisholm et al. (2006) is used for these two types of resistance being (i) PAMP-triggered immunity (PTI) and (ii) effector-triggered immunity (ETI). These responses can be activated once the pathogen has gained access to the plant's interior. This can occur by direct penetration through the surface or indirect by entering through wounds or natural openings, e.g. stomata. Several lines of defence have to be crossed before the pathogen can propagate on a plant. The first line of plant defence is actually a passive one; the pathogen has to pass the cuticle and cell wall which function as physical barriers. The second line of defence is at the plant cell membrane where surface receptors monitor the environment for potential PAMPs. Recognition of PAMPs by these receptors leads to the activation of PTI (Chisholm et al., 2006). PTI comprises mitogen-activated protein (MAP) kinase signalling, transcriptional activation of pathogenesis-related (PR) genes (Zipfel et al., 2004), reinforcement of cell wall components, and production of reactive oxygen intermediates (ROIs) (Kotchoni and Gachomo, 2006). The perception of flagellin as a bacterial PAMP is one of the best studied examples of PTI in plants. Flagellin is a subunit of flagella, which are required for bacterial motility (Macnab, 1992). Upon recognition of flagellin by FLS2, the Arabidopsis flagellin receptor like kinase, a MAP kinase cascade is initiated (Asai et al., 2002) that leads to the activation of host defence responses (Yang et al., 2001).

To overcome this host defence pathogens have evolved specific virulence factors that suppress PTI. Over the past years great progress has been made in the identification of plant defence suppressors in bacterial-plant interactions. Bacterial virulence factors, so called effector proteins, are introduced via the type III secretion system (TTSS) into the host cell where they are postulated to have their activity contributing to a successful infection (Chisholm et al., 2006). Several *P. syringae* type III effectors, e.g., AvrPto and AvrPtoB have been shown to act as virulence factors and promote pathogen growth (Abramovitch et al., 2003). In Arabidopsis, AvrPto suppresses cell wall based defences (Hauck et al., 2003). The activation of the MAP kinase cascade as a result of flagellin perception by FLS2 is suppressed by AvrPto and AvrPtoB (Zipfel et al., 2006). In addition, AvrPtoB suppresses HR- based programmed cell death (PCD) (de Torres et al., 2006). However, in a protoplast transient

assay it was shown that AvrPto and AvrPtoB could not suppress PCD mediated by *R* gene perception of the type III effectors AvrRpm1, AvrB and AvrRpt2 (He et al., 2006). Also in plant-oomycete interactions suppression of defence has been observed, e.g. by the AVR3a protein of *Phytophthora infestans. AVR3a* was found to suppress the HR triggered in *Nicotiana benthamiana* by INF1, a host-specific elicitor protein of *P. infestans* (Bos et al., 2006).

Effector proteins primarily contribute to pathogen fitness but the nomenclature of many of these virulence factors reveals that they were earlier identified as 'avirulence' (AVR) factors. The recognition of AVR factors can be observed as a third line of defence which has evolved in plants in response to effector proteins. The resistance initiated by recognition of such AVR factors is also referred to as effector-triggered immunity (ETI) (Chisholm et al., 2006). ETI involves the direct or indirect interaction of AVR factors from the pathogen with specific resistance proteins from the plant. Many resistance genes have been cloned in the last two decades. The majority of these dominant resistance genes are involved in race specific resistance. Six main classes of resistance genes have been described so far; (1) extracellular LRR with a kinase domain (e.g. *Xa21*), (2) extracellular LRRs with a membrane anchor (e.g. *Cf-9*), (3) serine/threonine kinase (*Pto*), (4) Toll interleukin1 receptor nucleotide binding site(TIR:NBS):LRR (e.g. *N*), (5) coiled coil (CC):NBS:LRR (e.g. *RPS2*) and (6) signal anchor with a CC domain (*RPW8*) (Jones, 2001). The direct or indirect perception of the pathogen AVR protein by a specific plant resistance (R) protein activates a hypersensitive response (HR). The HR is a fundamental element of plant resistance which includes local PCD and activation of biochemical defence responses. PCD is confined to a few cells that are sacrificed and which could aid the arrest of pathogen development. Biochemical responses include the production of reactive oxygen intermediates (ROIs), structural changes in the cell wall, accumulation of defence-related proteins, and biosynthesis of anti-microbial compounds (Greenberg and Yao, 2004). In plant-bacterium interaction, no ETI is triggered by AVR factors in the absence of a plant *R*-gene or in the absence of the evolutionarily conserved bacterial TTSS. This system can deliver type III effector proteins, e.g., AVR proteins, into host cells (Hueck, 1998). AVR factors that trigger ETI have been identified

from different pathogens, including oomycetes. Four oomycete AVR genes have been cloned so far, *Avr1b-1* from *Phytophthora sojae* (Shan et al., 2004), *ATR13* (Allen et al., 2004) and *ATR1*^{NdWsB} (Rehmany et al., 2005) from *H. parasitica* and *Avr3a* from *Phytophthora infestans* (Armstrong et al., 2005). The detection of ATR (*Arabidopsis thaliana Recognized*) proteins is mediated by cognate plant resistance proteins, the RPPs (*Recognition of Peronospora parasitica*). AVR1b-1 triggers necrosis in leaves of soybean containing the *Rps1b* resistance gene (Shan et al., 2004). The *P. infestans* AVR3a protein is detected by the potato R3a resistance protein (Armstrong et al., 2005). Sequence alignment of oomycete AVR proteins, revealed a conserved motif (RxLR) positioned C-terminal of the signal peptide sequence in these proteins. The RxLR motif shows resemblance in sequence to the host-cell targeting signal, RxLx(E/Q), identified in the malaria parasite *Plasmodium falciparum.* The RxLx(E/Q) motif is required for translocation of *Plasmodium* proteins into the host erythrocytes cytoplasma (Hiller et al., 2004; Marti et al., 2004). A similar function is suggested for translocation of oomycetes secreted proteins from the apoplast into the plant cell cytoplasm where recognition by the cognate R-protein occurs (Rehmany et al., 2005). If no AVR proteins from the pathogen are detected by cognate plant R-proteins the pathogen can infect the plant successfully. The plant is a host for the pathogen resulting in a compatible interaction.

Basic compatibility

Basic compatibility can be defined as an evolved state of symbiosis that exists between taxa of a plant and a pathogen (Ellingboe, 1976). In fact, the evolution of compatibility can be seen as the combined evolution of virulence factors of the pathogen with recognition and defence factors of the host. A host can be infected by diverse plant pathogens such as viruses, bacteria, fungi or oomycetes. A compatible pathogen is adapted to penetrate, survive and proliferate on its host, and the plant is providing the required growth factors to the pathogen (Holub, 2006). In a recent review Holub (2006) indicated that exploring the interaction between oomycetes and Arabidopsis could contribute to fundamental discoveries in plant-pathogen compatibility.

The oomycetes include some major plant pathogens, causing various diseases such as seedling damping-off and mold root rotting (*Pythium* and *Phytophthora*), white rust (*Albugo*) and downy mildew (e.g., *Bremia, Plasmopara, Peronospora, Pseudoperonospora,* and *Hyaloperonospora*). Oomycetes lack taxonomic affinity with the so-called true fungi (e.g., ascomycetes and basidiomycetes). Instead, oomycetes are eukaryotes that are more closely related to brown algae and that belong to the kingdom Stramenopila.

The oomycete *H. parasitica* causes downy mildew on Arabidopsis and other members of the Brassicae family. *H. parasitica* is an obligate biotroph and depends solely on living plant tissue for growth and reproduction. Isolates from Arabidopsis are host specific and do not infect Brassica plants. Koch and Slusarenko (1990) described and illustrated the infection of Arabidopsis by *H. parasitica,* previously known as *Peronospora parasitica*. *H. parasitica* spores germinate on the leaf surface and form appressoria from which the penetrating hyphae extend and penetrate between the anticlinal walls of adjoining epidermal cells. The mycelium grows intercellularly and haustoria are formed in the epidermal and mesophyll cells (Koch and Slusarenko, 1990). Haustoria are specialized infection structures that enlarge the plant-pathogen contact area and absorb plant factors needed for pathogen growth. *H. parasitica* has a sexual and asexual infection life cycle. The asexual life cycle of *H. parasitica* is depicted in Figure 1.

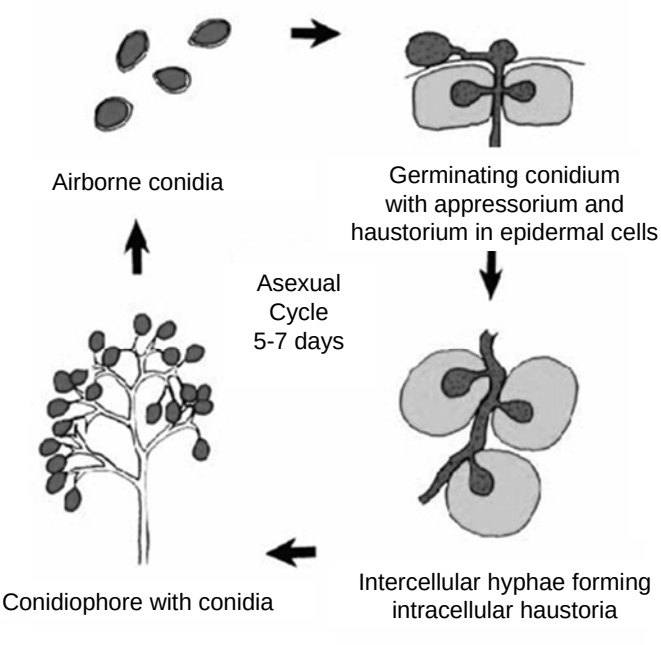

Figure 1. The asexual infection cycle of *Hyaloperonospora parasitica*. The airborne conidia (spores) land on the leaf surface and germinate. Appressoria are formed to penetrate the leaf. Hyphae are formed and grow intercellularly between the epidermal and mesophyll cells. Hyphae penetrate the cell walls and haustoria are formed in the adjacent cells. After 5 -7 days the pathogen produces conidiophores that grow

out of the stomata. The conidia formed on the conidiophores can start a new infection cycle.

Microscopic responses during infection and defence are visualized in Figure 2 for a compatible and incompatible interaction. In the incompatible interaction shown, pathogen recognition is mediated by the *R*-gene *RPP2* (Sinapidou et al., 2004) that detects the presence of the *ATR2* gene product of *H. parasitica* present in isolate Cala2. The infection can be visualized by use of three different stainings. Trypan blue staining visualizes the *H. parasitica* cells (Figure 2A) and the death plant cells (Figure 2D). Detection of ROIs is shown in the incompatible interaction by the brown precipitate in Figure 2E. Callose is detectable surrounding the haustorial neck (Figure 2C) in the compatible interaction. In the incompatible interaction callose accumulates around the haustoria and in the infected plant cell (Figure 2F).

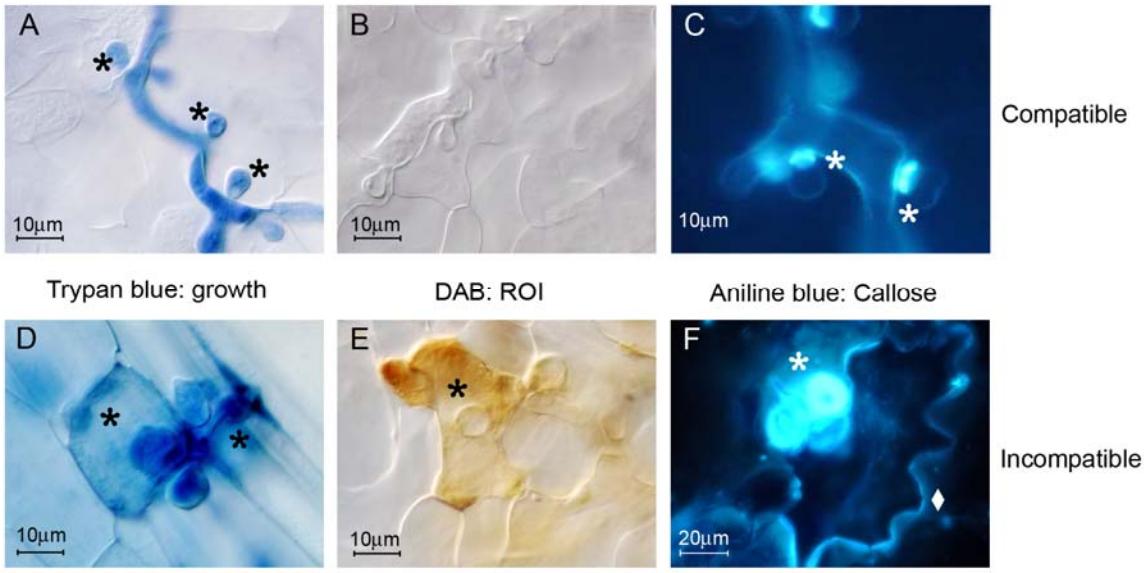

Figure 2. Microscopic images of compatible and incompatible *H. parasitica* interactions in Arabidopsis visualized by three different methods.
A. Trypan blue staining of a compatible interaction. The *H. parasitica* hyphae grow between the cells and haustoria invaginate the cells (indicated by *). (**D**) During the incompatible interaction the haustoria-containing Arabidopsis cells were stained (indicated by *) demonstrating cell death which results in arrest of *H. parasitica* growth. Reactive oxygen intermediates (ROIs) can be detected by di-amino-benzidine (DAB) as a brown precipitate inside the cells. **B**. No ROIs are detectable in the compatible interaction. **E**. In the incompatible interaction ROIs are visualized, as brown

precipitate within the Arabidopsis cells (indicated by *) that have encountered *H. parasitica*. **C**. By aniline blue staining the callose formation surrounding the haustorial neck (indicated by *) is visualized in the compatible interaction. **F**. It is observed that callose can completely surround the haustoria (*) that have invaded the plant cells and at the plasma membrane of infected Arabidopsis cells (♦).

Haustoria have been linked to nutrient uptake ever since their discovery (Voegele and Mendgen, 2003). Investigation of nutrient uptake in obligate biotrophs is complicated since they cannot be cultured on artificial media. In the biotrophic rust fungus *Uromyces fabae*, a pathogen of *Vicia faba*, it was shown that the flow of sugars to the rust seems to occur largely through the haustorial complex (Voegele et al., 2001). A hexose transporter gene, *HXT1* was isolated from an *U. fabae* haustorial cDNA library. The HXT1 protein is localized in the haustorial membrane where it is thought to mediate the uptake of D-glucose and D-fructose (Voegele et al., 2001). The haustoria of the fungal biotroph *Erysiphe cichoracearum*, causing powdery mildew, are thought to play an important role in sugar metabolism and transport. The Arabidopsis sugar transporter protein 4 (AtSTP4) and the cell-wall invertase, Atβfruct1, transcript levels were increased in Arabidopsis during infection with *E. cichoracearum* (Fotopoulos et al., 2003). Furthermore, the putative plasma membrane H^+-ATPase gene (*EcPMA1*) from *E. cichoracearum* showed an elevated expression during infection, correlating with the growth of the pathogen (Fotopoulos et al., 2006).

The establishment of haustoria in plant cells, as well as the transport of plant nutrients towards the haustoria are examples which illustrate that the plant has an active role in supporting pathogen growth and development. To determine this role of the plant in the infection process, host genes need to be identified that contribute to disease susceptibility. Presumably, a mutation in a plant gene that contributes to the infection process would result in reduced susceptibility. This form of resistance is expected to be recessive in contrast to the 'classic' resistance genes, which are dominant.

Identification of susceptibility genes

Recessive forms of resistance

Recessive resistance genes can be viewed as deleted, mutated or otherwise defective host genes that are essential for pathogen growth but do not pose a problem for the normal functioning of the host. In contrast to the resistance conferred by dominant resistance genes, knowledge is limited on recessive resistance genes. Recessive forms of resistance have been identified in many plant-pathogen interactions, including plant-virus, -bacteria, -fungus or -oomycete interactions.

Plant-Virus interaction

Successful infection of a plant by a virus requires host factors and a limited number of viral gene products. In infected plant cells a virus is dependent on its host for the production of viral proteins, for replication and systemic spread. Recessive resistance, also referred to as "passive resistance", could occur at any stage of the viral life cycle (Kang et al., 2005b). The absence of host factors or the mutation of host factors has long been postulated as recessive inherited disease resistance in plants. Many recessive viral resistance loci have been identified in host plants. Most appear to disturb viral replication or movement as reviewed by Kang et al., (2005b). If viral replication can not take place the resistance occurs at the cellular level. For example, the Arabidopsis *tom1* and *tom2A* mutants (Tobamovirus multiplication) do not support tobacco mosaic virus accumulation in single cells. *TOM1* encodes a multipass transmembrane protein that interacts with the tobamovirus encoded replicase protein (Yamanaka et al., 2000). *TOM2A* encodes a transmembrane protein and interacts with TOM1; both proteins appear to be essential constituents of the tobamovirus replication complex (Tsujimoto et al., 2003). Another example of disturbed replication is found in the Arabidopsis *loss-of-susceptibility to potyviruses 1* (*lsp1*) mutant, that is resistant to Tobacco etch virus (Lellis et al., 2002). *LSP1* encodes for the eukaryotic translation initiation factor eIF(iso)4E, an isoform of eIF4E, that has cap binding activity. In the Arabidopsis *cum1* and *cum2* mutants the viral cell-to-cell movement of the cucumber mosaic virus was inhibited. The *CUM1* and *CUM2* genes encode for eukaryotic translation initiation factors 4E and 4G, respectively (Yoshii et al.,

2004). In addition, the pepper *PVR1* (Kang et al., 2005a) and the pea *SMB1* (Gao et al., 2004) genes also encode for eIF4E. The plant eIF4E protein appears to participate in virus cell-to-cell movement, besides its involvement in virus replication, (reviewed by Kang 2005b).

Plant-bacteria interaction

The bacterial blight pathogen of rice, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*), can be refrained from growth by several resistance genes. Most resistance genes in rice, found so far, are dominant except *xa5*, which is partially dominant, and *xa13* that is recessive (Li et al., 2001). The *xa5* gene encodes for the gamma subunit of transcription factor IIA (TFIIAγ5) (Iyer and McCouch, 2004), a factor involved in transcription by RNA polymerase II. In rice, an additional *TFIIAγ1* gene, on chromosome 1, is identified and both are expressed in all rice organs, except in the panicle were only *TFIIAγ5* is expressed (Jiang et al., 2006). For the *xa13* rice plants Chu and colleges suggest that resistance is not mediated in a similar manner as with the dominant *R* genes *Xa4*, *Xa10* and *Xa26* (Chu et al., 2004). The rice *Xa13/Os8N3* gene is a homolog of the gene *MtN3*, a member of the nodulin family (Chu et al., 2006; Yang et al., 2006). In Medicago, *MtN3* gene expression is induced during root nodule development by *Rhizobium* (Gamas et al., 1996). Expression of the *Xa13/Os8N3* product is required for *Xoo* race 6 (strain PX099) susceptibility (Chu et al., 2006; Yang et al., 2006). PX099 resistant rice lines contain an insertion, deletion or substitution in the -69 to -86 promoter region of the *Xa13* gene. Mutations in this promoter region abolish the activation of *xa13* expression required for *Xoo* race 6 (strain PX099) susceptibility (Chu et al., 2006).

In Arabidopsis, the recessive *RRS1-R* allele is responsible for resistance against bacterial wilt caused by *Ralstonia solanacearum*. The *RRS1-R* resistance gene was identified in a cross between the resistant Nd-1 and the susceptible Col-5 accessions of Arabidopsis. *RRS-1R* encodes for a TIR-NBS-LRR protein with a carboxy-terminal WRKY-domain (Deslandes et al., 2003). The gene was also identified in a mutant screen for sensitively to low humidity (Noutoshi et al., 2005). The WRKY domain is suggested to suppress the activation of defence

genes. This *RRS-1R* gene could be regarded as an *R*-gene containing its own intragenic negative regulator.

Plant-fungus interaction

In barley plants homozygous for the recessive *mlo* gene, the entry of powdery mildew (*Blumeria graminis* f.sp. *hordei*) into epidermal cells is blocked. *mlo* plants show a defence mimic phenotype in the absence of the pathogen. Barley *Mlo* encodes a plasma membrane protein with seven transmembrane domains (Buschges et al., 1997). The MLO protein is required for successful penetration of the host cell wall (Panstruga, 2005). The cytoplasmic C-terminus contains an amphiphilic α-helix that serves as a calmodulin binding domain. Calcium-dependent calmodulin binding contributes to Mlo dependent susceptibility (Kim et al., 2002). In Arabidopsis, 15 *Mlo* orthologs were identified. To test if the *Mlo* orthologs confer resistance when mutated, homozygous T-DNA lines were generated and challenged with the powdery mildew fungus, *Golovinomyces orontii*. Only the homozygous *Atmlo2* line did not show any macroscopic disease symptoms nor condiophore formation, but microscopic analysis indicated some residual pathogen growth (Consonni et al., 2006). *AtMLO2* belongs to a clade of three *Mlo* orthologs, including *AtMLO6* and *AtMLO12.* The single and double mutants *Atmlo6* and *Atmlo12* did not result in *G. orontii* resistance, but *Atmlo2/Atmlo6* and *Atmlo2/Atmlo12* double mutants supported lower levels of fungal growth that the single *Atmlo2* mutant. The *Atmlo2/Atmlo6/Atmlo12* triple mutant was fully resistant, similar to Barley *mlo* plants (Consonni et al., 2006).

Genetic analysis of susceptibility

Recessive resistance genes can be considered as natural variants of dominant susceptibility genes. Therefore another approach to obtain resistant plants and to identify susceptibility genes is by mutagenesis. In a screen for loss of susceptibility to the powdery mildew pathogen *Erysiphe cichoracearum*, 26 powdery mildew resistant (*pmr*) mutants were identified (Vogel and Somerville, 2000). Four of the corresponding *PMR* genes have been cloned so far. *PMR4* (*GLS5=CalS12*) encodes for a callose synthase, resulting in a loss of callose accumulation. Gene expression profiling of *pmr4-1* plants showed an

enhanced activation of salicylic acid (SA) and pathogen-responsive genes. The up-regulation of these genes in *pmr4-1* mutants increased even further in response to pathogen infection. *pmr4*-based resistance probably acts through both *NPR1*-dependent and *NPR1*-independent signalling pathways since the *pmr4*/*npr1* double mutant still supported an intermediate level of fungal growth (Nishimura et al., 2003). *PMR6* encodes a pectate lyase-like gene, and *PMR5* is a gene of unknown function belonging to a large family of plant-specific genes. The phenotypes of *pmr5* and *pmr6* plants are very similar; a smaller rosette, and shorter, rounder and cupped leaves when compared to the wild-type plant. The cell-wall composition of *pmr6* is altered and analysis of *pmr5* indicates similar changes, i.e. increased levels of pectin (Vogel et al., 2002; Vogel et al., 2004). *PMR2* was found to be identical to *AtMLO2* which is described above as an ortholog of the Barley *Mlo* gene encoding a plasma membrane protein with seven transmembrane domains (Consonni et al., 2006). The PMR proteins are suggested to participate at diverse locations near the haustorial interaction site. PMR5, PMR6 and PMR2 might be participating near the extrahaustorial membrane and PMR4 at the haustorial neck (Figure 3).

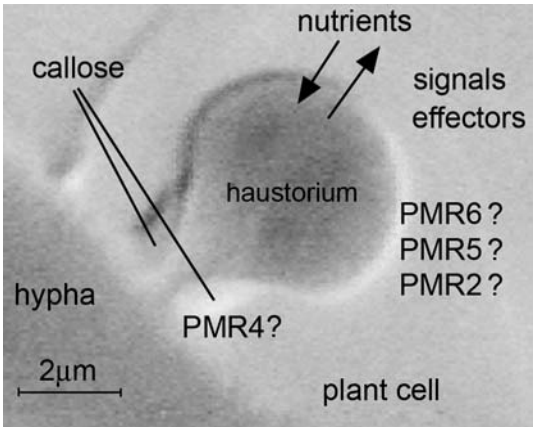

Figure 3. Schematic representation of a haustorium and proposed sites of action of PMR proteins. PMR4 known to be responsible for callose formation is placed near the haustorial neck were callose is located. PMR5 and PMR6 are involved in the cell wall composition that could interfere with haustorial development. And the *pmr2* mutants show an enhanced callose response at the haustorium. This figure is adapted from Panstruga (2003).

Besides the screen for *pmr* mutants another screen in Arabidopsis for loss of susceptibility to the fungal hemibiotroph *Colletotrichum higginsianum* has been

performed. So far, three candidate *chr (C. higginsianum* resistant) mutants have been isolated (O'Connell and Panstruga, 2006).

These genetic studies to identify plant susceptibility genes, that can also be considered recessive resistance genes, provide a first glance of the molecular processes during plant-pathogen compatibility. In contrast to recessive viral-, bacterial-, and fungal- resistance genes, knowledge is limited on recessive plant-oomycete resistance genes. Genetic studies on susceptibility of plants to highly specialized obligate biotrophic oomycete pathogens could provide us with new insights into the genes required for establishing basic compatibility.

Outline of thesis

The aim of this study was to identify Arabidopsis genes required for disease susceptibility to the oomycete *Hyaloperonospora parasitica*. A forward genetics approach was carried out to isolate *H. parasitica* resistant mutants. Mutants were generated in the highly susceptible L*er eds1-2* (*enhanced disease susceptibility1-2*) mutant by ethane methyl sulfonate (EMS) treatment. Mutants that lost susceptibility to *H. parasitica* were selected and mapping populations were generated to clone the corresponding genes responsible for the downy mildew resistant phenotype.

The identification and characterization of the downy mildew resistant (*dmr*) Arabidopsis mutants is described in **chapter two**. Twenty *dmr* mutants were identified of which eight were further characterized. Three *dmr* mutants, *dmr1*, *dmr2* and *dmr6*, showed no enhanced defence responses, e.g. no induced expression of the defence-associated gene *PR-1*, and absence of PCD and ROIs. In contrast, *PR-1* expression was elevated in the *dmr3*, *dmr4*, and *dmr5* mutants indicating that these mutants have an enhanced defence-response. The *dmr1*, *dmr2*, *dmr5*, and *dmr6* mutants were still susceptible to other pathogens, such as the bacterium *Pseudomonas syringae* pv. *tomato* and the fungus *Golovinomyces orontii*. The *dmr1* locus was mapped on chromosome 2, the d*mr5* locus on chromosome 3 and *dmr2*, *dmr3*, and *dm6* were mapped on chromosome 5.

In the **third chapter** the cloning of the *DMR1* gene and its functional analysis is described. The *DMR1* gene, At2g17265, encodes for homoserine kinase. Homoserine kinase phosphorylates homoserine to phospho-homoserine. Amino

acid analysis of the *dmr1-1*, *dmr1-2*, *dmr1-3*, and *dmr1-4* mutants revealed high levels of homoserine and sarcosine that are absent in the parental line, L*er eds1-2*. Infiltration of homoserine into the L*er eds1-2* seedlings resulted in *H. parasitica* resistance. High levels of homoserine cause *H. parasitica* resistance in the *dmr1* mutants via an as yet unknown mechanism.

Chapter four describes the cloning of *DMR6*, At5g24530, encoding an oxido reductase. Oxido reductases catalyze the transfer of electrons from one molecule, the oxidant, to another, the reductant. For the *DMR6* encoded oxido reductase no biological function has been demonstrated nor do we know the substrate and product of the predicted enzyme. *DMR6* is locally up regulated by biotic stress caused by *H. parasitica* infection, during compatible and incompatible interactions. Biotic stress (pathogens) and abiotic stress (hormones, e.g., SA) result in an up regulation of the *DMR6* transcript which indicates that *DMR6* expression is defence-associated. However, lack of *DMR6*, in the *dmr6* mutants, results in resistance that is accompanied by the enhanced expression of a set of defence-associated transcripts, including *DMR6*. These results suggest a dual role of *DMR6* during pathogen infection.

Finally, in **chapter 5,** a general discussion of the thesis is provided, in which the obtained results are summarized and discussed, and suggestions for future research are given.

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Chapter 2

Identification of Arabidopsis loci required for susceptibility to the downy mildew pathogen *Hyaloperonospora parasitica*

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Abstract

Plants are susceptible to a limited number of pathogens. Most infections fail due to active defence, or absence of compatibility. Many components of the plant's surveillance system and defence arsenal have been identified in the last decades. However, knowledge is limited on compatibility; in particular the role of plant factors in the infection process. To gain insight into these processes we have initiated an Arabidopsis mutant screen for reduced susceptibility to the downy mildew pathogen *Hyaloperonospora parasitica.* EMS-mutants were generated in the highly susceptible Arabidopsis line L*er eds1-2*. Eight downy mildew resistant (*dmr*) mutants were analyzed in detail, corresponding to 6 different loci. Microscopic analysis showed that in all mutants *H. parasitica* growth was severely reduced. Resistance of *dmr3*, *dmr4* and *dmr5* was associated with constitutive expression of *PR-1*. Furthermore, *dmr3* and *dmr4*, but not *dmr5*, were also resistant to *Pseudomonas syringae* and *Golovinomyces orontii*, respectively. However, enhanced activation of plant defence was not observed in *dmr1*, *dmr2*, and *dmr6*. We postulate that in these susceptibility mutants, cellular processes are disrupted which are required for *H. parasitica* infection. This interesting new set of mutants provides a basis to elucidate the molecular processes underlying susceptibility to downy mildew in Arabidopsis.

Additional keywords: compatibility, disease resistance, disease susceptibility, downy mildew resistance, obligate biotroph, oomycete pathogen.

Introduction

Susceptibility to plant diseases can be considered more exception than rule. Only a limited number of microbes have the capacity to retrieve nutrients and survive on living plant tissue; also referred to as compatibility. In most cases,

plants resist infection due to active defence mechanisms or the absence of compatibility towards a given pathogen. Knowledge on disease resistance has increased tremendously over the last two decades. Numerous resistance genes have been cloned and many defence-associated and signal transduction genes have been identified (Glazebrook, 2001; Rathjen and Moffett, 2003). However, little is known about the molecular basis of disease susceptibility and the role of the plant in the infection process. We consider three distinct steps during infection of plants by biotrophic pathogens that involve an array of different host cell processes.

The first step towards compatibility is the formation of specialized penetration and feeding structures at the correct time and location. Their development requires coordinated expression of infection-related genes of the pathogen, triggered by the environment and signals from the host plant. To come into close contact with the host some pathogens can actively move to the plant, e.g. zoospores of *Phytophthora* and *Pythium* species that are attracted by root exudates (Tyler, 2002). To access the host, spores attach to the plant surface, germinate, and often develop specialized structures, called appressoria, to penetrate the plant cuticle and cell wall (Tucker and Talbot, 2001). Although for some pathogens surface hydrophobicity and hardness are sufficient to stimulate appressorium development (Carzaniga et al., 2001; Tucker and Talbot, 2001) others require specific signals to develop infection structures, e.g. the sensing of cutin by *Colletotrichum* species (Dickman et al., 2003), or the height of the guard cell lip by *Uromyces appendiculatus* (Allen et al., 1991). Also during later stages of infection by fungal and oomycete biotrophs, signals or characteristics of the cellular environment in the host play an important role in the regulated development of haustoria and sporulation structures (Mendgen and Hahn, 2002). It is striking to see that an obligate biotroph, like the downy mildew pathogen *Hyaloperonospora parasitica*, develops a single haustorium in each plant cell it encounters (Koch and Slusarenko, 1990).

Once within the host tissue, we consider a second step when pathogens are exposed to a wide range of activated plant defence responses. Early pathogen

detection can occur through recognition of specific elicitors, mediated by plant resistance genes (Nimchuk et al., 2003), or through perception of general elicitors e.g. pathogen associated molecular patterns (PAMPs) i.e. fungal chitin, oomycete glucans, and bacterial flagellin (Nurnberger et al., 2004).The circumvention of recognition and suppression of plant defence responses is therefore an important step for pathogens to take. It will be clear that for successful infection pathogens need to reduce or modify their plant-exposed PAMPs to circumvent their recognition. This “stealth” strategy, however, does not seem to offer complete protection. Recent literature shows that pathogens have evolved advanced mechanisms to actively suppress plant defence responses. The effector protein AvrRpt2 of *Pseudomonas syringae* targets the Arabidopsis RIN4 protein, which is required for RPM1-mediated resistance (Mackey et al., 2002), resulting in RIN4 elimination (Axtell and Staskawicz, 2003; Mackey et al., 2003). Another *Pseudomonas* effector, AvrPtoB, suppresses programmed cell death thereby preventing the hypersensitive response (Abramovitch et al., 2003). Also in fungi and oomycetes evidence is emerging that pathogen effectors exert their function on host proteins, e.g. the inhibition of the tomato pathogenesis related protease P69B by a *Phytophthora infestans* Kazal-type protease inhibitor (Tian et al., 2004). Strikingly, like many bacterial effectors, also fungal and oomycete effectors appear to act inside the host cell, e.g. AvrL567 of the rust fungus *Melampsora lini* (Dodds et al., 2004) and ATR13 of *H. parasitica* (Allen et al., 2004).

The modulation of host cell processes is an important third step in the infection process. To create a favourable environment for growth and for the acquisition of nutrients, pathogens are thought to actively influence host gene expression and metabolism. Although this seems obvious, there is, so far, little knowledge about this phenomenon. A direct link between a pathogen effector protein and host gene expression was provided by Marois *et al.* (2002), who showed that a set of pepper genes is specifically induced by the bacterial effector AvrBs3. In plant-bacteria interactions, the role of induced host gene expression in nutrient acquisition by the bacteria is unclear. In plant-fungus interactions, the proposed process of nutrient transport over the host cell membrane towards the pathogen is supported by the fact that sugar transporters and amino acid

permeases are highly expressed in rust haustoria reviewed in Mendgen and Hahn (2002). Also in the host plant *Vicia faba*, the rust fungus *Uromyces fabae* induces the expression of genes encoding an ATPase, amino acid transporter, asparagine synthetase, and sucrose synthase (Wirsel et al., 2001). Their contribution in providing nutrients to the intruding pathogen awaits functional analysis. In fact, no host proteins specific for the extrahaustorial membrane have been identified, so far.

The role of the host in disease susceptibility has been addressed in several recent studies. In a search for Arabidopsis loci required for susceptibility to *Erysiphe cichoracearum*, Vogel and Somerville (2000) screened EMS and T-DNA insertion mutants for loss of susceptibility. Twenty six powdery mildew resistant (*pmr*) mutants were isolated, corresponding to 6 loci that displayed strongly reduced sporulation, of which 3 have been cloned. *PMR6* encodes a pectate lyase–like protein (Vogel et al., 2002), *PMR4* a callose synthase (Nishimura et al., 2003), and *PMR5* a protein of unknown function (Vogel et al., 2004). The PMR proteins all appear to act at the cell wall to provide resistance to powdery mildew. Natural variation in disease susceptibility of Arabidopsis to the fungal pathogen *Botrytis cinerea* was explored by Denby *et al.* (2004) who analyzed recombinant inbred lines of a L*er* x Col-0 cross. They identified several small to medium effect quantitative trait loci that govern disease susceptibility to *Botrytis*.

To gain more insight into disease susceptibility we study the interaction between the oomycete downy mildew pathogen *H. parasitica* and its natural host Arabidopsis. This pathosystem has been extensively studied over the past years and has revealed fascinating aspects of pathogen recognition, defence signal transduction, and resistance gene evolution (Holub, 2001; Slusarenko and Schlaich, 2003). The infection process starts with the germination of a spore and subsequent penetration of the leaf surface through the anticlinal wall of the epidermal cell layer (Koch and Slusarenko, 1990). Once inside the leaf *H. parasitica* makes intercellular hyphae from which haustoria develop in almost each adjacent plant cell. Although the pathogen breaches through the plant cell wall it remains separated from the host cell cytoplasm by the

extrahaustorial membrane. To identify host genes involved in the establishment of a successful infection we have undertaken a genetic approach. Here we describe the isolation and characterization of 8 downy mildew resistant (*dmr*) mutants corresponding to 6 different loci. In three *dmr* mutants resistance is associated with constitutive activation of plant defence responses. Three other mutants, *dmr1*, *dmr2*, and *dmr6*, show resistance in the absence of enhanced defence responses, suggesting that the corresponding genes are required for susceptibility to downy mildew.

Results

Isolation of downy mildew resistant mutants

Mutants were created by EMS in the Landsberg *erecta* (L*er*) mutant *eds1-2* (Parker et al., 1996). EMS was chosen to find not only knock-out mutants but also missense mutations generating more subtle phenotypes. An additional advantage was that we could perform the mutagenesis in the genetic background of the *eds1-2* mutant. *EDS1* (for enhanced disease susceptibility) is a lipase-like protein (Falk et al., 1999) and is an important component in the signal transduction pathway of several resistance genes of the TIR-NBS-LRR class and in basal resistance to a number of pathogens (Aarts et al., 1998) L*er* *eds1-2* plants are highly susceptible to the downy mildew pathogen *H. parasitica*; they support more abundant sporulation of *H. parasitica* than the susceptible parental L*er* line. The high level of *H. parasitica* infection of *eds1-2* facilitates the convenient distinction between phenotypes in our mutant screen for loss of susceptibility.

Downy mildew resistant (*dmr*) mutants were identified among the susceptible M_2 plants by visual screening of cotyledons and primary leaves for lack of *H. parasitica* sporulation at 7-10 days post inoculation with isolate Cala2 (see Figure 1 for the screening approach). From our screening of 3600 M_1 families (approximately 100.000 M_2 plants), we identified approximately 300 putative mutants. Of these, less than 10% could be confirmed in the next generation (M_3) as being truly resistant; the remaining 90% apparently were false positives. To rule out the possibility of pollen- or seed-contamination, mutants were genotyped using *EDS1* primers for the parental *eds1-2* deletion. A total of

20 confirmed *dmr* mutants were identified. The detailed analysis of eight *dmr* mutants is described below.

Genetic analysis

Complementation crosses between the mutants revealed 6 independent loci, denoted downy mildew resistance 1 to 6 (*dmr1* to *dmr6*). For *dmr1* three independent alleles were identified (*dmr1-1* to *-3*). To determine the chromosomal map position of the *DMR* genes the mutants were crossed to the *FN2* Col-0 mutant. This mutant is susceptible to the *H. parasitica* isolate Cala2, due to a fast neutron mutation in the *RPP2A* gene (Sinapidou et al., 2004). All eight *dmr* mutations appeared to be recessive as the F_1 plants were susceptible to Cala2, and approximately a quarter of the F_2 plants displayed *H. parasitica* resistance. From each cross the resistant F_2 plants were selected, genotyped and re-screened for resistance in the F_3 generation. The resulting map positions for the different *dmr* mutations are depicted in Figure 2. The map position of *dmr1* was determined to be below the centromere on chromosome 2 in a 1-Mb region, between BAC T24I21 and T17A5. *dmr2* and *dmr6* mapped between BAC MOP9 and T11H3 on chromosome 5. Although they map to the same region, *dmr2* and *dmr6* are not allelic, as F_1 plants resulting from complementation crosses were susceptible to *H. parasitica*. *dmr3* is also located on chromosome 5, in a region flanked by marker nga151 and BAC MVA3. The *dmr5* mutation mapped on chromosome 3 to a 4-Mb region, between marker T17B22 and BAC MAG2. The *dmr4* mutant confers a very strong and reproducible resistance to *H. parasitica*, but could not be linked to a distinct position on the chromosomal map in a cross with Col-0 *FN2*, as the F_2 and F_3 progeny did not give a reproducible resistance phenotype.

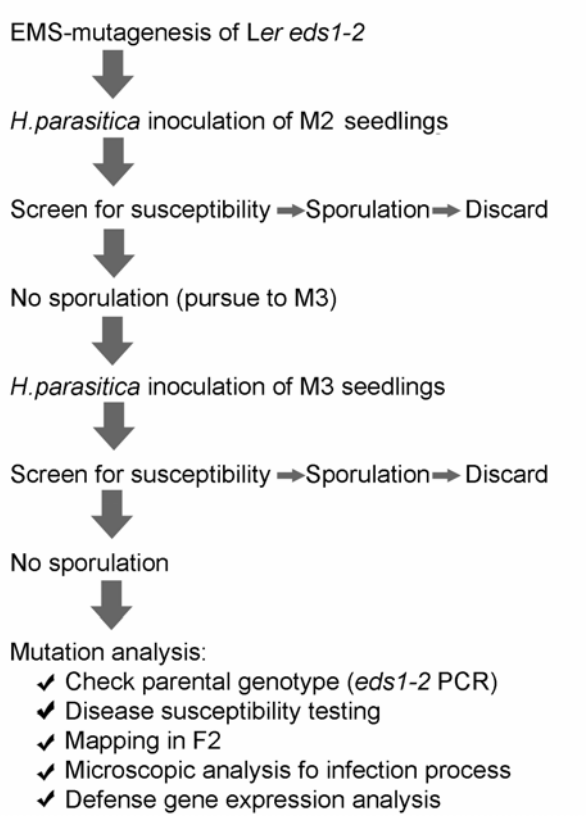

Figure 1. Screening approach for the isolation of downy mildew resistant mutants.

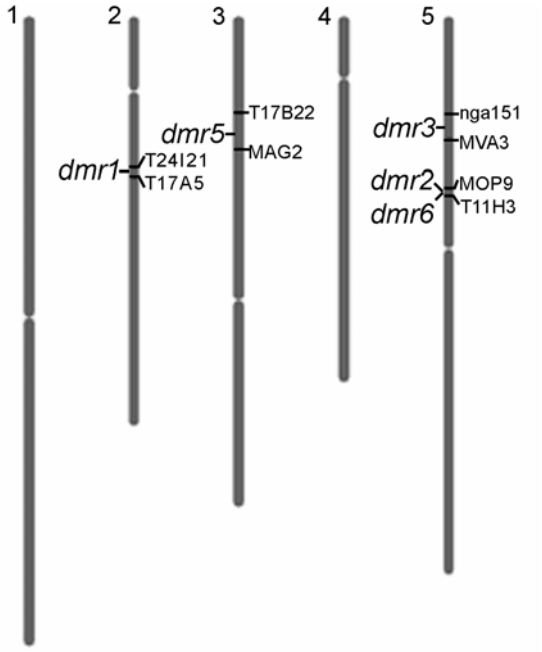

Figure 2. The position of five downy mildew-resistant (*dmr*) loci on the *Arabidopsis* chromosome map. The location of each *dmr* locus is placed on the physical map using the program Chromosome Map Tool.

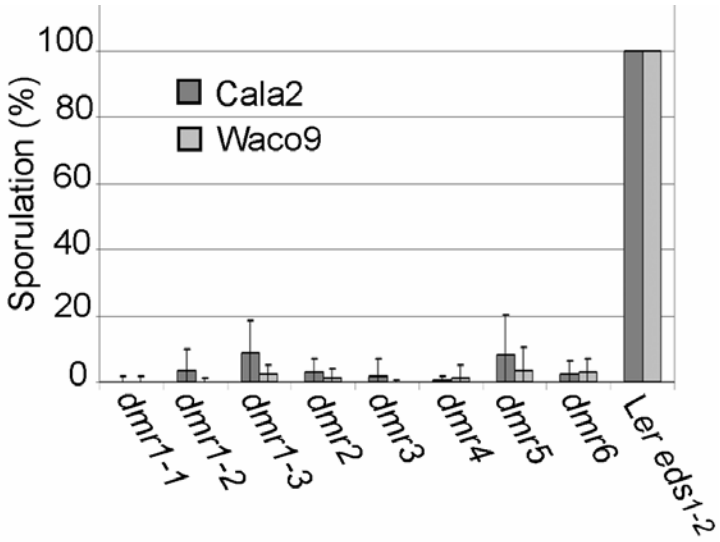

Figure 3. Quantification of *Hyaloperonopora parasitica* sporu-lation on the downy-mildew-resistant (*dmr)* mutants compared to the parental L*er eds1-2* line (set to 100%) at 7 days postinoculation with the isolates Cala2 and Waco9.

Disease susceptibility of dmr mutants

The *dmr* mutants vary in degree of resistance to *H. parasitica*. To quantify the level of resistance we counted the number of sporangiophores on mutant seedlings (Figure 3). Compared to the parental line, L*er eds1-2*, all mutants showed strongly reduced sporulation after infection with the isolates Cala2 and Waco9. In particular mutants *dmr1-1*, *dmr3*, and *dmr4* showed strong to complete resistance. Mutant *dmr5* still supported some pathogen growth resulting in sporulation levels that are 10% of that of the parental line. Interestingly, the different alleles of *dmr1* showed different levels of resistance, with *dmr1-1* giving higher levels of resistance than *dmr1-2* and *dmr1-3*. Besides their resistance to *H. parasitica*, some of the *dmr* mutants have additional phenotypes. *dmr3* (Figure 4F) and *dmr4* (Figure 4G) show dwarfism, and *dmr1-1* shows chlorosis and reduced growth (Figure 4B). Except for the dwarfism in *dmr3* that appears to be a pleiotropic effect, the additional phenotypes did not co-segregate with resistance to *H. parasitica* in F_2 mapping populations, indicating that they are caused by additional point mutations resulting from the EMS mutagenesis.

The infection process in the different *dmr* mutants was studied microscopically to determine when and where *H. parasitica* growth and development was arrested. Infected leaves were stained with trypan blue 3 days post inoculation with Cala2, and analyzed by differential interference contrast microscopy (Figure 4). Colonization of cotyledons and

leaves of *dmr1-1*, *dmr2*, *dmr3* and *dmr4* by *H. parasitica* was strongly reduced as compared to the parental L*er eds1-2* line. Hyphal growth could still be detected in the *dmr1-2*, *dmr1-3*, *dmr2*, *dmr5* and *dmr6* mutants, although to a lesser extent as in the parental line L*er eds1-2* (Figure 4A). In the three *dmr1* mutants cell wall appositions were often observed around arrested haustoria (Figure 4B-D). These papillae, containing callose (as detected by aniline blue staining, data not shown), are possibly a secondary effect on the invading haustoria. In case of the *dmr2* mutant no papillae formation was detected but hyphal growth was often arrested after the first haustoria had formed (Figure 4E). In many cases haustoria had aberrant shapes with globular extension on the otherwise spherical haustoria, in particular in mutants *dmr3* (Figure 4F) and *dmr6* (data not shown). On *dmr4* leaves spores did not seem to be able to penetrate efficiently and to grow intercellular hyphae (Figure 4G). Although there was significant *H. parasitica* colonization in *dmr5*, haustoria were not formed in every adjacent cell and hyphal growth was irregular (Figure 4H). In the *dmr6* mutants the growth of *H. parasitica* was constrained after the first haustoria had formed (Figure 4I). Inspection of *H. parasitica* infections in the *dmr* mutants at 5 days post inoculation (data not shown) were very similar to those at 3 days post inoculation indicating that further colonization remained disturbed. Overall, the growth and development of *H. parasitica*, as observed by microscopic analysis of trypan blue stained plants, is in good agreement with the level of sporulation that we have observed (as shown in Figure 3).

The production of reactive oxygen intermediates (ROIs) is often linked to the hypersensitive response and to cell wall based defence responses (Beers and McDowell, 2001). Microscopic detection of ROIs by DAB (3'-3'-diaminobenzidine) staining showed that in none of the *dmr* mutants ROIs are produced in response to *H. parasitica* infection (data shown for *dmr1-3* in Figure 4L), as is the case in the L*er eds1-2* parental line (Figure 4K). As a positive control for the DAB staining Col-0 was inoculated with the incompatible isolate Cala2. ROIs could be clearly visualized by the brown DAB precipitate that was formed in close vicinity to the invading pathogen (Figure 4J).

To analyze if the *dmr* mutants display a spectrum of resistance broader than to *H. parasitica* alone, susceptibility to the Gram-negative bacterial pathogen *Pseudomonas syringae* pathovar *tomato* (*Pst*) DC3000 and the biotrophic powdery mildew fungus *Golovinomyces orontii* was tested. As shown in Figure 5, the parental line supported growth of *Pst* (a 100-fold increase in 3 days), whereas seedlings treated with BTH, a chemical inducer of systemic acquired resistance, showed 20-fold less bacterial growth. Mutants *dmr1*, *dmr2*, *dmr5*, and *dmr6* did not seem to be significantly affected in their susceptibility to *Pst*. In contrast, *dmr3* and *dmr4* displayed a strong resistance to *Pst* that is comparable to BTH treated control plants. In addition the *dmr3* and *dmr4* mutants also showed resistance to the powdery mildew pathogen, *G. orontii*. This fungal pathogen, formerly referred to as *Erysiphe orontii*, was previously reported to represent one out of three powdery species that are able to successfully colonize Arabidopsis (Plotnikova et al., 1998). The *dmr* mutants were screened for resistance to *G. orontii* (Table 1). Two additional accessions, Col-0 and Shakdara (Sha), were included as susceptible and resistant controls, respectively. Like the susceptible control (Col-0, average disease resistance score 2.5) our parental line L*er-eds1-2* was susceptible (scored 2.8) to powdery mildew, whereas Sha proved resistant (scored 0.5). The level of susceptibility of the *dmr1* mutants to *G. orontii* was not univocal. Whereas *dmr1-1* was resistant (score 0.5), *dmr1-2* and *dmr1-3* were equally susceptible (~2.8) as the parental line. A fourth *dmr1* mutant, *dmr1-4*, that we recently isolated was also susceptible (data not shown), suggesting that *dmr1* does not mediate resistance to *G. orontii*. Resistance to powdery mildew in the *dmr1-1* mutant could be caused by another independent mutation, e.g. the mutation that gives rise to the chlorotic growth phenotype of *dmr1-1*. The *dmr3* and *dmr4* mutants displayed a strong reduction in susceptibility with *dmr4* showing resistance comparable to the Sha control. Also, the *dmr5* and *dmr6* mutants were more resistant to powdery mildew than the parental control, although the reduction in disease symptoms was not as strong as in the resistant control and the *dmr3* and *dmr4* plants. Interestingly, *dmr2* was reproducibly more susceptible to *G. orontii* than the parental line. Based on the different infection assays we conclude that *dmr3* and *dmr4* show broad spectrum disease resistance against oomycete, bacterial and fungal pathogens. The other

mutants appear to be more specifically affected in their interaction with *H. parasitica*.

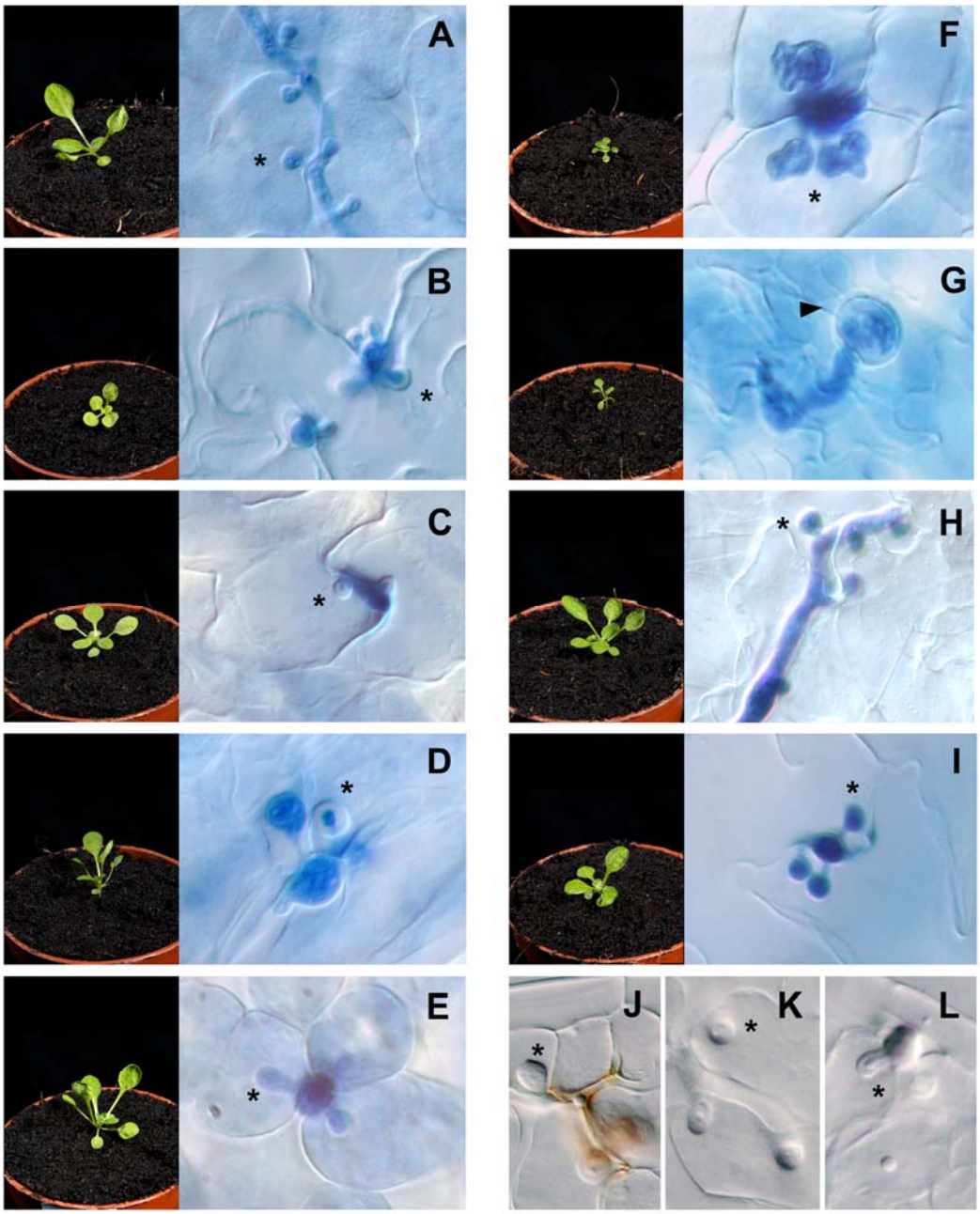

Figure 4. Phenotypes of downy mildew resistant (*dmr*) mutants. For each mutant, a 3 week-old plant is shown, as well as a microscopic image of *Hyaloperonospora parasitica*-infected tissue of 14-day-old seedlings. For each mutant, representative images of trypan blue-stained seedlings 3 days postinoculation with isolate Cala2 (5 x 10^4 spores·ml^{-1}) were taken. For clarity, a single haustorium is marked with an asterisk (*) in every microscopic image. **A**. In the parental L*er eds1-2* line *H. parasitica* hyphal growth was abundant and haustoria were formed in adjacent plant cells. **B.** Mutant *dmr1-1* is slightly smaller and chlorotic, whereas **C**. *dmr1-2* and **D**. *dmr1-3* appear

phenotypically similar to the parental line (A). *H. parasitica* growth was arrested in all three *dmr1* mutants and underdeveloped haustoria were often surrounded by cell wall appositions containing callose (B, C, and D). **E.** Infection of *dmr2* also showed arrested haustorial development and no additional macroscopic phenotype. **F**. A dwarf mutant, *dmr3,* showed aberrantly-shaped haustoria and strongly arrested hyphal growth. **G**. In the *dmr4* mutant, which also has a dwarf phenotype, *H. parasitica* penetration of the epidermal cell layer was hampered resulting in strongly reduced intercellular growth (note the failed penetration of the germinated spore, marked with the black arrowhead). **H**. The *dmr5* mutant shows normal plant development, but *H. parasitica* growth was reduced. There was significant hyphal growth, but haustoria were not formed in all adjacent plant cells as in the parental line (A). **I**. The *dmr6* mutant shows slightly rounded leaves. In *dmr6*, some *H. parasitica* growth occurred and haustoria were formed, although they often had aberrant shapes (data not shown) or stayed immature (I). **J**, **K**, and **L.** Detection of reactive oxygen intermediates (ROIs) by 3'-3'-diaminobenzidine staining in seedlings inoculated with *H. parasitica* isolate Cala2. **J**. Col-0 plants that recognize Cala2 through *RPP2* accumulated ROIs at the infection site (visible as the red-brown precipitate), whereas **K**. L*er eds1-2* and the *dmr* mutants, such as **L**. *dmr1-3,* did not show ROIs production nor hypersensitive cell death.

Defence responses

In order to distinguish between activated defence and susceptibility mutants, the expression level of the defence-related *PR-1* gene was determined in healthy and *H. parasitica*-inoculated seedlings of the different *dmr* mutants. As shown by Northern blot analysis (Figure 6) the parental line L*er-eds1-2* does not show any *PR-1* expression, whereas BTH-treated seedlings, as expected, showed a strong induction of *PR-1*. In *dmr3*, *dmr4*, and *dmr5* elevated levels of *PR-1* expression were observed. The defence gene activation in *dmr3* and *dmr4* was not unexpected as these mutants display resistance to *Pst* and *G. orontii*. The *dmr5* mutant, however, remained susceptible to *Pst* despite elevated expression of *PR-1.* Like the parental line, mutants *dmr1*, *dmr2*, and *dmr6* do not show elevated *PR-1* expression (Figure 6). In addition, *PR-1* expression was not induced 3 days after inoculation with the compatible *H. parasitica* isolate Cala2 (data not shown). We conclude that the mutants *dmr1*, *dmr2*, and *dmr6* are susceptibility mutants as the do not show broad spectrum resistance nor do they show enhanced defence gene expression.

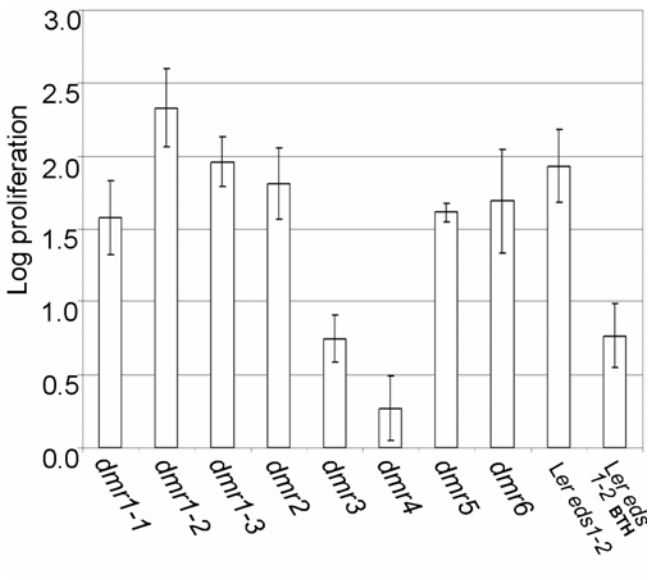

Figure 5. Quantification of bacterial (*Pseudomonas syringae* pv *tomato*) proliferation in the downy mildew-resistant mutants (*dmr*) and control plants over 3 days. The *dmr1*, *dmr2*, *dmr5*, and *dmr6* mutants showed about a 100-fold increase in leaf tissue that was comparable to that of the parental L*er eds1-2* line. In contrast, mutants *dmr3* and *dmr4* show a strongly reduced growth that is comparable to that of benzothiadiazole (BTH)-treated plants (3 days prior to *P. syringae* pv. *tomato* inoculation) in which systemic acquired resistance is induced.

Table 1. Susceptibility of the downy mildew-resistant mutants to *Golovinomyces orontii*.

Genotype	Score of susceptibility Average (n=6) [a]
dmr1-1	0.5
dmr1-2	2.8
dmr1-3	2.7
dmr2	3.0
dmr3	1.2
dmr4	0.9
dmr5	2.0
dmr6	1.5
L*er eds1-2*	2.8
Col-0	2.5 (susceptible)
Sha	0.5 (resistant)

[a] The level of sporulation was scored on a disease resistance scale of 0-3 at 10 days post inoculation. The total number of plants tested per accesion or mutant was 6. The assay was repeated once with comparable results.

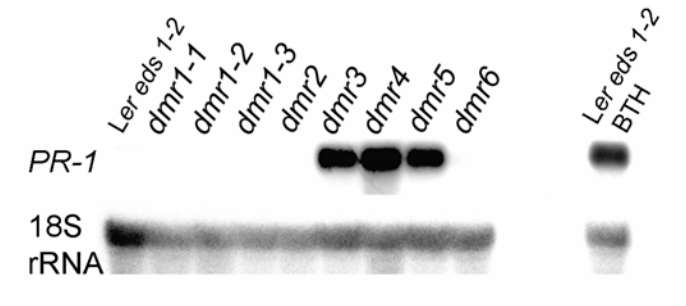

Figure 6. Northern blot analysis of *PR-1* expression in non-inoculated seedlings of different downy mildew-resistant (*dmr*) mutants compared to the parental L*er eds1-2* line. L*er eds1-2* treated with benzothiazole (BTH) was used as a positive control for *PR-1* expression. The same membrane was also probed with 18S rRNA as a loading control.

Is dmr3 mutated in the DND1 gene?

The *dmr3* mutant resembles the previously described *dnd1* mutant in many aspects. Shared features include its map position on the upper arm of chromosome 5, resistance to both *H. parasitica* and *Pst*, constitutive expression of *PR-1*, and a dwarf phenotype. *DND1* (Defense No Death 1) encodes a cyclic-nucleotide gated anion channel that, when mutated, leads to enhanced plant defence and constitutive pathogen resistance (Clough et al., 2000; Yu et al., 1998). To test if *dmr3* is mutated in the *DND1* gene we sequenced the *DND1* coding region of both the parental L*er eds1-2* line and the *dmr3* mutant. No polymorphisms were detected between the mutant and parental line. This was confirmed by complementation crosses between *dmr3* and the Col-0 *dnd1* mutant. The resulting F_2 and F_3 generation segregated for resistance to *H. parasitica* indicating that *dmr3* and *dnd1* are not allelic.

Discussion

In this paper we describe the isolation and identification of a set of eight Arabidopsis mutants that are disturbed in the interaction with the downy mildew pathogen, *H. parasitica*. The mutant screening was based on the assumption that mutations leading to reduced pathogen growth would also result in low numbers of sporangiophores on the leaves, allowing the identification of mutants by visual screening of sporulation on M_2 seedlings. Our approach is very similar to the one used by Vogel and Somerville (2000) for the isolation of *pmr* mutants conferring resistance to powdery mildew. We

expected to find two classes of downy mildew resistant mutants, the first resulting from enhanced defence responses, and the second due to impairment of the infection process (lack of susceptibility mutants). From more than 100,000 M_2 seedlings from 3600 M_1 families we have isolated 20 independent *dmr* mutants of which 8 were analyzed in more detail. Complementation crosses showed that the 8 *dmr* mutants correspond to 6 loci which were subsequently mapped in segregating F_2 populations. Two of the loci map to regions with known defence genes. Although *dmr3* maps near *dnd1*, complementation crosses and *DND1* sequencing of the *dmr3* mutant showed that these mutants are not affected in the same gene. On chromosome 2, *dmr1* maps near *son1*(Kim and Delaney, 2002), which is a suppressor of *nim1* (also known as *npr1*). Like *dmr1*, *son1* is a recessive mutation conferring resistance to *H. parasitica*. However, *son1* mutants show constitutive activation of *PR-1* and resistance to *Pseudomonas* bacteria, whereas the three different *dmr1* loci do not show these phenotypes. Comparison of the *dmr* map positions to those of the powdery mildew resistance (*pmr*) loci (Vogel and Somerville, 2000), that provide resistance to *Erysiphe cichoracearum*, indicated that they are not linked. The *pmr4* locus, however, not only confers resistance to powdery mildew, but also to *H. parasitica* (Vogel and Somerville, 2000). *PMR4* encodes a callose or glucan synthase (Jacobs et al., 2003; Nimchuk et al., 2003). *NahG* transgenics, which do not accumulate SA, appeared to be fully susceptible to *E. cichoracearum* (Nimchuk et al., 2003). We have not identified *pmr4* in our screening, either because its resistance phenotype could require *EDS1*, or because we did not yet reach saturation in our genetic screen.

Enhanced defence response mutants

Many mutants are already known that show constitutive or activated plant defence responses, e.g. the *cpr*, *cim*, *acd*, *dnd*, and *edr* mutants reviewed in (Glazebrook, 2001). In these mutants, negative regulators of plant defence are mutated (e.g. in *edr1*, a MAPKKK mutant, (Frye et al., 2001) or constitutive defence activation is occurring as a result of mutated or truncated signalling proteins (e.g. *acd6-1*, an ankyrin repeat membrane protein; (Lu et al., 2003)). Multiple mutants with constitutive defence responses form spontaneous lesions caused by cell death; these include *acd1*, *acd2*, *acd6*, *cpr5, lsd1 to 7*, and *ssi1*

(Bowling et al., 1997; Dietrich et al., 1994; Greenberg et al., 1994; Rate et al., 1999; Shah et al., 1999; Tanaka et al., 2003). In our *dmr* mutants no lesions or cell death was detected after *H. parasitica* inoculation. As the mutant screening was performed in the *eds1-2* background we counter-selected most of these mutations. Indeed several enhanced defence mutations, i.e. *cpr1* and *cpr6,* require a functional *EDS1* gene to mediate disease resistance (Clarke et al., 2001). However, *eds1* mutants are still able to mount a hypersensitive response and produce ROIs (Rusterucci et al., 2001). EDS1 is an important component in the signal transduction pathway following pathogen recognition by a subgroup of resistance proteins, the TIR-NBS-LRR class (Falk et al., 1999). A second role of EDS1 is in basal pathogen resistance, explaining why *eds1* mutants are more susceptible to pathogen infection than susceptible wild-type plants (Parker et al., 1996).

Three of the *dmr* mutants, *dmr3*, *dmr4* and *dmr5,* can be considered enhanced defence mutants. Resistance of *dmr3* is tightly linked to dwarfism and *PR-1* expression. This was confirmed by the analysis progeny of a backcross of *dmr3* to the parental line (data not shown). We have excluded the possibility that *dmr3* is allelic to *dnd1*, although they map to the same chromosomal region. The map-based cloning of *DMR3* will reveal its function in enhanced activation of plant defence. The dwarfism of *dmr4* is not linked to resistance to *H. parasitica*, as shown in segregating F_2 populations, and is therefore probably caused by other EMS mutations. The resistance of *dmr4* is particularly interesting as it appears to act at the level of penetration. Penetration resistance is a rare phenomenon but well known from powdery mildew resistant barley *mlo* mutants (Panstruga and Schulze-Lefert, 2002). Unlike these, however, the *dmr4* mutant also shows elevated levels of *PR-1* gene expression and resistance to a bacterial pathogen (*Pseudomonas syringae*). We are currently testing if NPR1 (for no PR1 expression) is required for resistance in the *dmr4* mutant. Although the resistance of *dmr4* to *H. parasitica* is strong and reproducible, it appeared not to be genetically tractable in crosses with the *A. thaliana* accession Col-0. About one quarter of the F_2 plants showed a clear resistance phenotype. However, F_3 plants obtained from resistant F_2 plants often proved to be susceptible. Also, several backcrosses of F_2 plants to the

Col-0 mutant *FN2* did not result in a reproducible phenotype. Crosses to other Arabidopsis accessions and mutants are now in progress to assess if the *dmr4* phenotype is more stable in genetic backgrounds other than Col-0.

The *dmr5* mutant constitutively expresses the *PR-1* gene and is resistant to *H. parasitica* but not to *Pseudomonas* or *G. orontii.* We do not know of any mutant that displays this combination of phenotypes, suggesting that *dmr5* is affected in a yet unknown component in defense signal transduction. Uncoupling of oomycete and bacterial resistance is also seen in the *cpr5 npr1* and *cpr5 nahG* double mutants where constitutive expression of *PR-1* and resistance to *P. syringae* maculicola ES4326 is abolished but not resistance to *H. parasitica* Noco2 (Bowling et al., 1997). The dominant disease resistant mutant *cpr6* constitutively expresses *PR-1*, *BGL-2* and *PR-5* and mediates resistance to *P. syringae* pv. maculicola and *H. parasitica* Noco2. Constitutive expression of *PR* genes in the *cpr6* mutant is SA but not NPR1 dependent. Resistance to *P. syringae* but not to *H. parasitica* is suppressed in the *cpr6 npr1* double mutant, despite *PR*-gene expression (Clarke et al., 1998). However,
besides constitutive *PR* gene expression, both *cpr5* and *cpr6* exhibit elevated levels of PDF1.2 indicating that different defence pathways are involved (Bowling et al., 1997; Clarke et al., 1998). Moreover a functional *EDS1* is fully required for resistance mediated by *cpr6* and at least partially required for *cpr5*-mediated resistance (Clarke et al., 2001). In contrast, resistance mediated by *dmr5* is *EDS1* independent. To unravel the role of *DMR5* in plant defence crosses to known defence signalling mutants are currently being made and defence gene expression will be monitored by DNA micro arrays.

Lack-of-susceptibility mutants

The resistance of *dmr* mutants to *H. parasitica* can also be due to lesions in host genes that play an important role in the infection process. These, so called, susceptibility mutations could well affect processes like signalling, nutrient transport, or membrane biogenesis. So far, very little is known about such host compatibility factors. Several recessive resistance loci have been described that might have arisen as a result of mutation of important

"susceptibility genes" (Panstruga, 2003), e.g. barley *mlo* and Arabidopsis *pmr* (Vogel and Somerville, 2000). MLO, a plant-specific seven transmembrane protein from barley, is required for compatibility to powdery mildew and modulates cell death and senescence (Buschges et al., 1997). Whether MLO is a true compatibility factor or a suppressor of plant defence awaits further molecular characterization of *Mlo*-mediated processes in the plant. The isolation of three *PMR* genes, that are required for compatibility to powdery mildew in Arabidopsis, provided an indication to their possible role in the infection process. In *pmr6*, a pectate lyase-like gene is mutated resulting in an altered composition of the plant cell wall (Vogel et al., 2002), which did not block penetration by the fungus. The mechanism of *pmr6*-mediated resistance could not be pinpointed to a defined stage in fungal development. The powdery mildew resistance in the *pmr5* mutant is also correlated to an altered cell wall composition (Vogel et al., 2004). However, currently it is not known how the change in cell wall architecture in *pmr5* and *pmr6* mutants translates into the post-haustorial growth cessation of the fungal pathogen. Resistance of the *pmr4* mutant is associated with an enhanced pathogen-induced salicylic acid-dependent defence response that is caused by the loss of a specific callose synthase isoform (Nishimura et al., 2003).

In our screen we have identified 3 candidate loci for susceptibility, *dmr1*, *dmr2*, and *dmr6*. For *dmr1*, three different alleles were obtained from M2 mutants of independent families and confirmed by complementation crosses. The *dmr1*, *dmr2*, and *dmr6* mutants showed resistance to *H. parasitica*, but not to *Pseudomonas* or *G. orontii*. *H. parasitica* growth was inhibited in these *dmr* mutants without visible cell death or accumulation of reactive oxygen intermediates. In addition, expression of the defence-related gene *PR-1* was not constitutive, nor more strongly expressed after inoculation with *H. parasitica*. Taken together, these data strongly suggest that cellular processes, other than defence, are disturbed in the *dmr1*, *dmr2*, and *dmr6* mutants leading to a reduced level of *H. parasitica* infection. The *dmr1* locus was mapped on chromosome 2 to a region covering 109 genes. *dmr2* and *dmr6* are both located near marker nga139 on chromosome 5. Genetic analysis of complementation crosses showed that *dmr2* and *dmr6* are not allelic. The

dmr2/*dmr6* region still encompasses 74 genes. Fine mapping is in progress to clone the *DMR1*, *DMR2* and *DMR6* genes.

In conclusion, our genetic approach to identify Arabidopsis genes required for infection by *H. parasitica* has yielded six different mutant loci. Three of these display enhanced defence responses, which could explain their resistance phenotype. More interestingly, we have identified 3 new *dmr* loci that appear to play an important role in the *H. parasitica* infection process. The isolation of the corresponding *DMR* genes and their functional analysis will provide us with the tools to start exploring the molecular basis of susceptibility to disease, in particular to downy mildew.

Materials and Methods

Plant lines and growth conditions

Arabidopsis thaliana lines used in this study are Landsberg-*erecta* (L*er*), Shakdara (Sha), Columbia (Col-0), L*er eds1-2* (Parker et al., 1996) and the Col-0 mutant *FN2* (Sinapidou et al., 2004). Plants were grown on potting soil in a growth chamber (Snijders, The Netherlands) at 22ºC with 16 h of light (100 µE/m2/sec) and a relative humidity of 75%.

Growth and infection of downy mildew

Hyaloperonospora parasitica isolate Cala2 was kindly provided by Dr. E. Holub (HRI, Wellesbourne) and maintained on Arabidopsis L*er* by weekly transfer to healthy 10-14 day-old seedlings (Holub et al., 1994). To obtain large amounts of sporangiospores for bioassays, inoculum was collected from L*er eds1-2* seedlings that support abundant Cala2 growth and sporulation (Parker et al., 1996). Inoculum ($5x10^4$ spores·ml^{-1}) was applied on 14 day-old seedlings using a spray gun. After inoculation, plants were allowed to dry for 15-60 minutes and subsequently incubated under a sealed lid (100% relative humidity) in a growth chamber at 16ºC with 9 h light/day (100µ E/m^2/sec). The amount of sporulation was quantified at 7 days post inoculation by counting the number of sporangiophores on the cotyledons and leaves. In Figure 3 the number of

sporangiophores was determined per leaf for both isolates for at least 40 to 100 seedlings per mutant. The amount of sporangiophores on L*er-eds1-2* was set as 100%.

EMS mutagenesis

Seeds of L*er eds1-2* backcrossed twice to L*er* were kindly provided by Dr. Jane Parker (MPI, Cologne). About 10,000 seeds were imbibed in water for ~2 days at 4°C, then treated for 18.5 h in 0.2125% EMS (21.25 µl in 10 ml water), and extensively washed with 2 L of sterile water. M_1 plants were grown on autoclaved soil (7 parts of potting compost and 5 parts of sand) and 4 week-old plants were treated with BTH (100 mg/L active compound/ml H_2O) to protect them from disease. From more than 3600 individual M_1 plants seeds were collected as M_2 families. In about 10% of the M_2 families albino mutants were detected.

Mutant screening

30-40 M_2 plants/family were sown in plug trays (22x13 wells) with fine potting soil. After 10-14 days the seedlings were inoculated with *H. parasitica* Cala2. Seven days later susceptible plants were removed with tweezers. The remaining seedlings were rescreened 3 days later and resistant plants were transferred and grown for M_3 seed production. About 30 M_3 plants per putative M_2 mutant were re-screened for resistance. True resistant lines were then genotyped for the parental *eds1-2* mutation by PCR to exclude seed contamination.

Microscopy

Infections in the leaves were visualized by trypan blue staining of *H. parasitica*. For this, infected leaves were collected in a 1.5 ml centrifuge tube. An adequate volume of lactophenol-trypan blue (1:1:1:1 volume of lactic acid: glycerol: phenol: H_2O) was added. The tubes were placed in a boiling water bath for 1 minute. Leaves were destained in chloral hydrate. The tubes were placed in a speed-vacuum infiltrator for 1 minute to remove air bubbles from the leaves. *H. parasitica* growth was detected by differential interference contrast microscopy.

Reactive oxygen intermediates (ROIs) were detected by staining for H_2O_2 accumulation. 3'-3'-diaminobenzidine (DAB) staining visualizes H_2O_2 as described by (Thordal-Christensen et al., 1997). DAB staining was performed for 8 hours on 10 day-old *A. thaliana* seedlings at 3 days post inoculation with *H. parasitica*, isolate Cala2. Seedlings dissected from the root were placed in 96-well plates containing 100 µl DAB solution a plastic box under high humidity (5-6 h). Afterwards the leaves were cleared of chlorophyll in alcoholic lactophenol (1 volume of phenol: glycerol: lactic acid: water (1:1:1:1) and 2 volumes of ethanol) for 15-30 min at 65°C, followed for 2 hours at room temperature in fresh solution. Prior to microscopy the samples were placed in chloral hydrate in a speed-vacuum infiltrator for 1 minute to remove air bubbles. Arabidopsis leaves were visualized by differential interference contrast microscopy.

Bacterial growth curve

The bacterial growth of *Pseudomonas syringae* pathovar tomato strain DC3000 was essentially performed as described by Tornero and Dangl (2001). Ten day old seedlings were inoculated by surface dipping. At time points 0 (1 hour) and 3 days post inoculation samples were taken. For this, the aerial parts of 5 seedlings were weighed, treated as described to release bacteria, and plated on Kings B agar plates to determine bacterial numbers per mg fresh weight.

Golovinomyces orontii inoculation

To assess powdery mildew infection phenotypes, rosette leaves of six week old *dmr* mutants, the L*er eds1* parental line and two control plants (including the fully susceptible Col-0 and the resistant Sha accessions) were inoculated with a high density of *Golovinomyces orontii* conidiospores. Disease symptoms were evaluated 8-10 days post inoculation by macroscopic and microscopic inspection and classified according to the disease resistance (DR) score described by Adam *et al*.(Adam et al., 1999).

Northern blot analysis

Total RNA was isolated from frozen tissue samples using the RNeasy Plant Mini Kit (QIAGEN) according to the manufacturer's protocol. RNA samples were

denatured for 1 hour at 50 °C in glyoxal (10 mM sodium phosphate; 1.0 M glyoxal: 0.5x DMSO). 8 μg RNA samples were separated on agarose (1.5%) gel, in 10 mM sodium phosphate (pH 7.0) running buffer. After transfer of the RNA to nylon membranes (Hybound N; Amersham Biosciences) the RNA was cross-linked to the membrane by UV-cross linking (175 mJ/cm^2). The membrane was hybridized with ^{32}P-labeled probes using the Random DNA Labeling Kit (Fermentas). First with *PR-1* and after stripping (15 minutes in boiling, 0.1xSSC; 0.5%SDS) with 18S rRNA, at 64°C in a hybridization buffer (7% SDS, 0.5 M sodium phosphate pH 7.0, 10 mM EDTA, 1% BSA) , for both probes. The *PR-1* probe was generated by PCR amplification using the primers 5'-gtaggtgctcttgttcttcc-3' and 5'- ttcacataattcccacgagg-3'. The 18S probe was derived from an Arabidopsis cDNA clone (Pruitt and Meyerowitz, 1986). Filters were rinsed twice with pre-warmed (65°C) buffer (0.5% SDS, 2x SSC) and washed twice 15 min in 0.5% SDS, 2x SSC at 65°C.

Genetic mapping

The *dmr* mutants were crossed to the *FN2* mutant (Sinapidou *et al*., 2004) in the Col-0 background to generate a mapping population. F_1 plants were checked for heterozygosity by genotyping with the *eds1-2* PCR based marker (forward EDS6 primer 5'-gtggaaaccaaatttgacattag-3', forward EDS4 primer 5'-ggcttgtattcatcttctatcc-3'and reverse EDS2B primer 5'-acacatcggtgatgcgagaca-3'). The F_1 seedlings were drop inoculated to test for susceptibility. F_2 seedlings were screened for disease resistance as described above, resistant F_2 plants were genotyped. For fine-mapping of the *dmr* mutants PCR-based markers were used. The markers were based on insertion or deletion polymorphisms between Colombia and Landsberg depicted at http://www.arabidopsis.org/Cereon/index.html. The markers T24I21 (forward primer, 5'-aatccaaatttcttgcgagaacaca-3' and reverse primer, 5'-aaacgaagagtgacaatggttggag-3') based on 12bp (CER459636) deletion in the Landsberg accession. The T17A5 marker (forward primer 5'-cgatgtctcaccggtgtaaccttta-3' and reverse primer, 5'-ttgcagagaacttcatgactggcta-3') results in a 32bp (CER458639) deletion in Landsberg. The MOP9 marker based on CER456575, a 31 bp deletion, in the Landsberg accession (forward primer 5'-tttgggaacagaaaaagttggaggt-3' and reverse primer 5'-

catattcaaaagggaaaatcccaga-3'). A 24bp insertion (CER458037) in the Landsberg accession was used for the T11H3 marker (forward primer 5'-ccaattgggttatttacttcgatt-3'and reverse primer 5'-cggcttttaacaacatattttcca -3'). The nga151 marker is depicted at http://www.arabidopsis.org and the MVA3 marker resulting in a 16bp insertion (CER457398) in the Landsberg accession (forward primer 5'-cttatcgaaacccccatttgtaag-3'and reverse primer 5'-aagaaagaggtcagagtcggagaa-3'). The T17B22 marker resulting in a 13 bp insertion (AC012328) in the Landsberg accession (forward primer 5'-gagattgtcttatactcggaatgtg-3' and reverse primer 5'-ataaaatcataaccccaccaaaag-3') and MAG2 marker, based on a 37bp insertion (CER464868) in the Landsberg accession (forward primer 5'- ttctattattcggtggaagatcaag-3'and reverse primer 5'-tagatttctgcgaagatttct-3').

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Author-recommended internet resource

The Arabidopsis Information Resource (TAIR) website: www.arabidopsis.org

TAIR Chromosome Map Tool:

www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp

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Chapter 3

Plant disease resistance through modulation of host amino acid metabolism

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Abstract

Plant disease resistance is commonly triggered by early recognition of pathogens and subsequent activation of immunity. We report on an alternative form of resistance in Arabidopsis mediated by recessive alleles of *DOWNY MILDEW RESISTANT 1*. Map-based cloning revealed that *DMR1* encodes for homoserine kinase (HSK). Five independent Arabidopsis *dmr1* mutants carrying different amino acid substitutions in HSK all accumulate homoserine, which is not detected in wild-type plants. Although homoserine does not have a direct effect on pathogen growth, treatment of Arabidopsis with the amino acid results in complete protection. Thus, homoserine accumulation *in vivo* provides resistance to downy mildew infection.

Keywords: Arabidopsis, downy mildew resistance, compatibility, homoserine kinase

Introduction

Many major plant diseases are caused by obligate biotrophic fungi and oomycetes (fungal-like members of the kingdom *Stramenopila*). Pathogens causing powdery mildew, rust, and downy mildew disease, are in most cases highly specialized on particular host species on which they fully depend for growth and reproduction. They do not cause disease on other plant species as the infection is obstructed by the plant-controlled process of non-host resistance that involves both pre- and post-invasion defences (Wiermer et al., 2005). In host plants, pathogens circumvent or actively suppress this non-host resistance (Chisholm et al., 2006). A second layer of defence has evolved in host plants that is mediated by single dominant resistance (*R*) genes. The R proteins allow the direct or indirect detection of pathogen determinants followed by the activation of plant immunity (Jones, 2001). Despite all these

effective intrinsic defence mechanisms plants are still vulnerable to pathogen attack. The molecular mechanisms underlying disease susceptibility, and more specifically the role of host-specific processes that sustain pathogen development and growth, are still largely unknown. In recent years genetic studies on the model plant *Arabidopsis thaliana* have resulted in the identification of a number of genes that mediate susceptibility to biotrophic pathogens (O'Connell and Panstruga, 2006; Vogel and Somerville, 2000). Mutants in the *PMR5* and *PMR6* genes, encoding for a pectate-lyase and protein of unknown function, respectively, show alterations in the plant cell wall, caused by defects in pectin structure or composition, that lead to their *powdery mildew resistant* (*pmr*) phenotype (Vogel et al., 2002; Vogel et al., 2004). In the *pmr4* mutant a callose synthase gene is mutated which leads to enhanced defence responses during pathogen infection (Nishimura et al., 2003). In barley a recessive gene, named *mlo*, has provided durable resistance to powdery mildew already for more than 30 years. MLO is thought to modulate powdery mildew immunity through modulation of vesicle traffic (Panstruga, 2005). Recently, it was shown that loss-of-function of *AtMLO2*, an Arabidopsis co-ortholog of the barley *Mlo* gene, confers resistance to powdery mildew by activation of a defence mechanism that is present in wild-type Arabidopsis (Consonni et al., 2006). The Arabidopsis *pmr5, pmr6,* and *mlo* mutations are highly specific as they reduce the susceptibility to powdery mildew but not to the bacterial pathogen *Pseudomonas syringae* and the downy mildew pathogen *Hyaloperonospora parasitica*. A similar specificity was found for the *downy mildew resistant* (*dmr*) mutants *dmr1*, *dmr2*, and *dmr6* that are still normally susceptible to powdery mildew and *Pseudomonas* (Van Damme et al., 2005). The mode of *H. parasitica* resistance of these mutants could be due to the absence of a specific host protein required for infection or by a so far unknown defence mechanism.

Here we describe the cloning and characterization of the *DOWNY MILDEW RESISTANT 1* (*DMR1*) gene. The *DMR1* gene was identified by map-based cloning and encodes for homoserine kinase (At2g17265). In five independent *dmr1* mutants nucleotide changes were identified, leading to amino acid substitutions in the HSK protein. Amino acid analysis of four *dmr1* mutants compared to the parental line L*er eds1-2* shows an accumulation of

homoserine in these mutants. Resistance was also obtained by external application of homoserine. These results have uncovered modulation of host amino acid metabolism as a novel mechanism for resistance against downy mildew.

Results

dmr1-mediated resistance to Hyaloperonospora

The *dmr1* mutants were back-crossed twice (BC_2), to reduce the number of unlinked EMS-induced mutations, to the parental line L*er eds1-2* and to L*er* containing the wild-type *EDS1* gene. In the L*er* background the *dmr1* mutations provide full resistance indicating that *dmr1* is independent of *EDS1* (data not shown). Resistance to *H. parasitica* of the BC_2 mutants (in L*er eds1-2*), containing the *dmr1-1*, *dmr1-2*, *dmr1-3*, and *dmr1-4* mutation, respectively, was tested. Except for *dmr1-3* all mutants showed complete resistance to *H. parasitica*. The *dmr1-3* mutant supported a very minor level of sporulation as depicted in figure 1. At the cellular level, the growth of *H. parasitica* hyphae was arrested after formation of the first haustoria in the *dmr1* mutants. The haustoria in the *dmr1* mutants appeared to be encased by papillae that are visible as translucent structures surrounding the haustoria and that are absent in the parental control line (Figure 1C). To detect if the papillae surrounding the haustoria in the *dmr1* mutants (Figure 1C) contain callose, the infected seedlings were stained with aniline blue. In Figure 2A an aniline staining of three different *dmr1* mutants and L*er eds1-2* seedlings is depicted, three days post infection with *H. parasitica*, Cala2. The incompatible interaction with Cala2 on Col-0 indicated high levels of callose deposition in those cells that contain haustoria. Callose deposition in the susceptible L*er eds1-2* line is only localized around the haustorial neckband. The haustoria in the *dmr1* mutants are surrounded by callose, indicating that the papillae formed contain callose (Figure 2A, *dmr1-1*, *dmr1-2* and *dmr1-3*). Another indication of an activated plant defence response is the presence of reactive oxygen intermediates (ROIs). ROIs can be visualized by 3'-3'-diaminobenzidine (DAB) as a brown precipitate. No ROIs could be detected in the *dmr1* mutants and the L*er eds1-2* parental line three days post infection of *H. parasitica*, Cala2. *eds1* mutants are still capable of mounting a hypersensitive response and

produce ROIs (Rusterucci et al., 2001). In control plants, Col-0 infected with the incompatible isolate Cala2, ROIs are detected as a brown precipitate in cells containing haustoria and that are undergoing hypersensitive cell death (Figure 2B). If activated defence is the cause of the resistant phenotype in the *dmr1* mutants it could be related to formation of callose and not to the activation of ROIs.

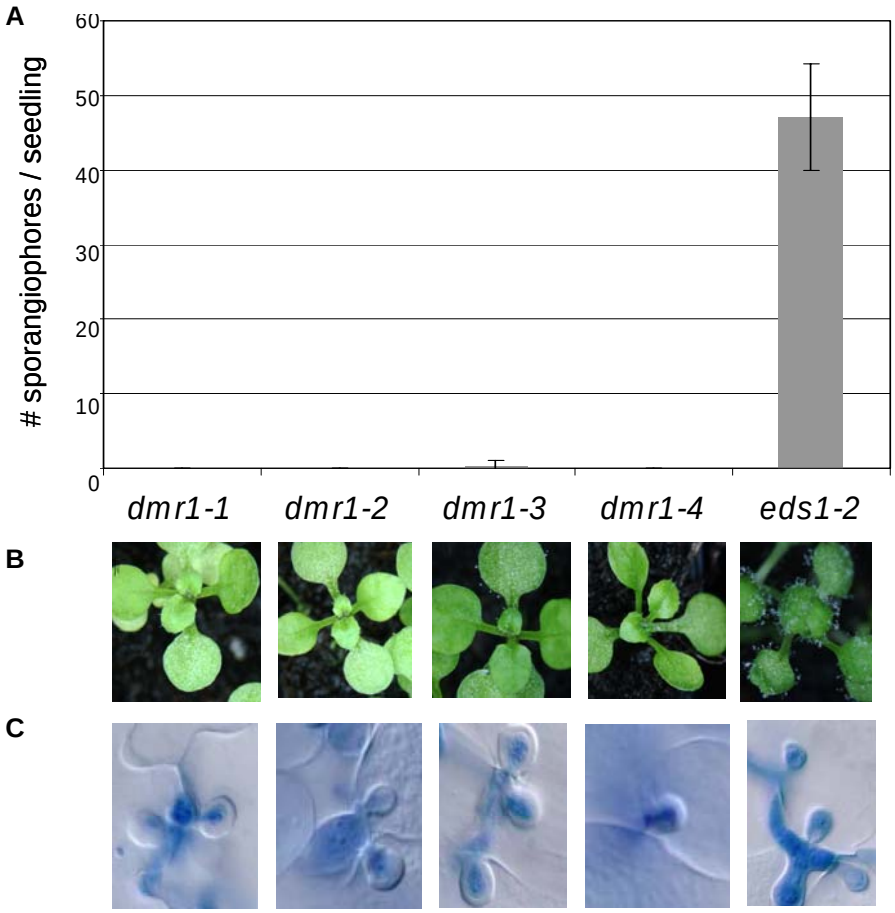

Figure 1. Quantification and visualization of *Hyaloperonospora parasitica* growth on the *dmr1* BC_2 mutants. The *dmr1* mutants confer nearly complete resistance to the downy mildew pathogen *H. parasitica.* **A**. Sporangiophore formation 5 days post inoculation (dpi) on 10 day old Arabidopsis seedlings. **B**. Macroscopic images of sporangiophores on Arabidopsis seedlings 8 dpi with *H. parasitica*. **C**. Microscopic examination of trypan blue-stained hyphae and haustoria in leaves of Arabidopsis seedling 5 dpi with *H. parasitica*.

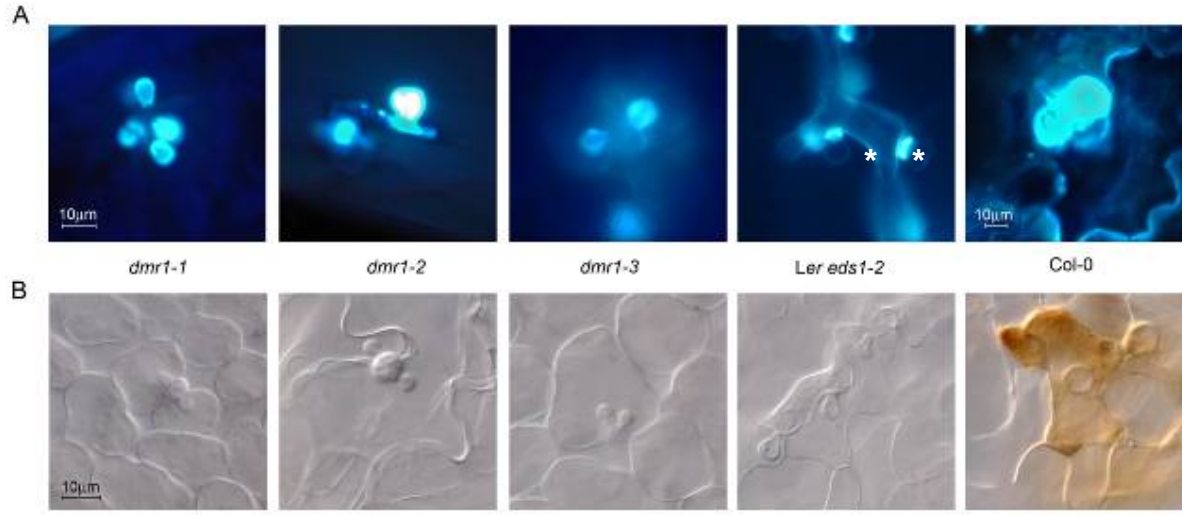

Figure 2. **A**. Microscopic analysis of callose formation in Arabidopsis leaves during the interaction with *H. parasitica* isolate, Cala2 visualized by aniline blue staining. In the *dmr1* mutants callose can be detected surrounding the *H. parasitica* haustoria, in L*er eds1-2* the callose is only surrounding the haustoria neckband (∗) and in an incompatible interaction with Col-0 callose can be detected around haustoria and at the cell wall of the haustoria-containing cells. **B**. Microscopic visualization of reactive oxygen intermediates (ROIs) by DAB staining. The brown precipitate can only be visualized in the incompatible interaction, with Col-0. In none of the *dmr1* mutants ROIs can be detected during the *H. parasitica* interaction.

Cloning of DMR1

The recessive *dmr1* mutation was mapped on the long arm of chromosome 2 (Van Damme et al., 2005d). Further analysis mapped it to a chromosomal region of approximately 130 kb covered by three BACs; F6P23, T23A1, and F5J6. Analysis of 650 F_2's resulted in the isolation of 5 recombinants between markers on BACs F6P23 to F5J6. Analysis of these recombinants allowed the fine mapping of *DMR1* to a region of eight candidate genes; At2g17230 to At2g17290 (Figure 3A). DNA sequencing of the eight genes in the *dmr1-1*, *dmr1-2*, *dmr1-3*, *dmr1-4*, and the recently isolated *dmr1-5* mutant, revealed single point mutations in the At2g17265 gene (Figure 3A), encoding for homoserine kinase (HSK). All five mutations result in amino acid substitutions (Figure 3A) in the At2g17265 encoded protein at positions that are conserved in plant HSK proteins (Figure 4).

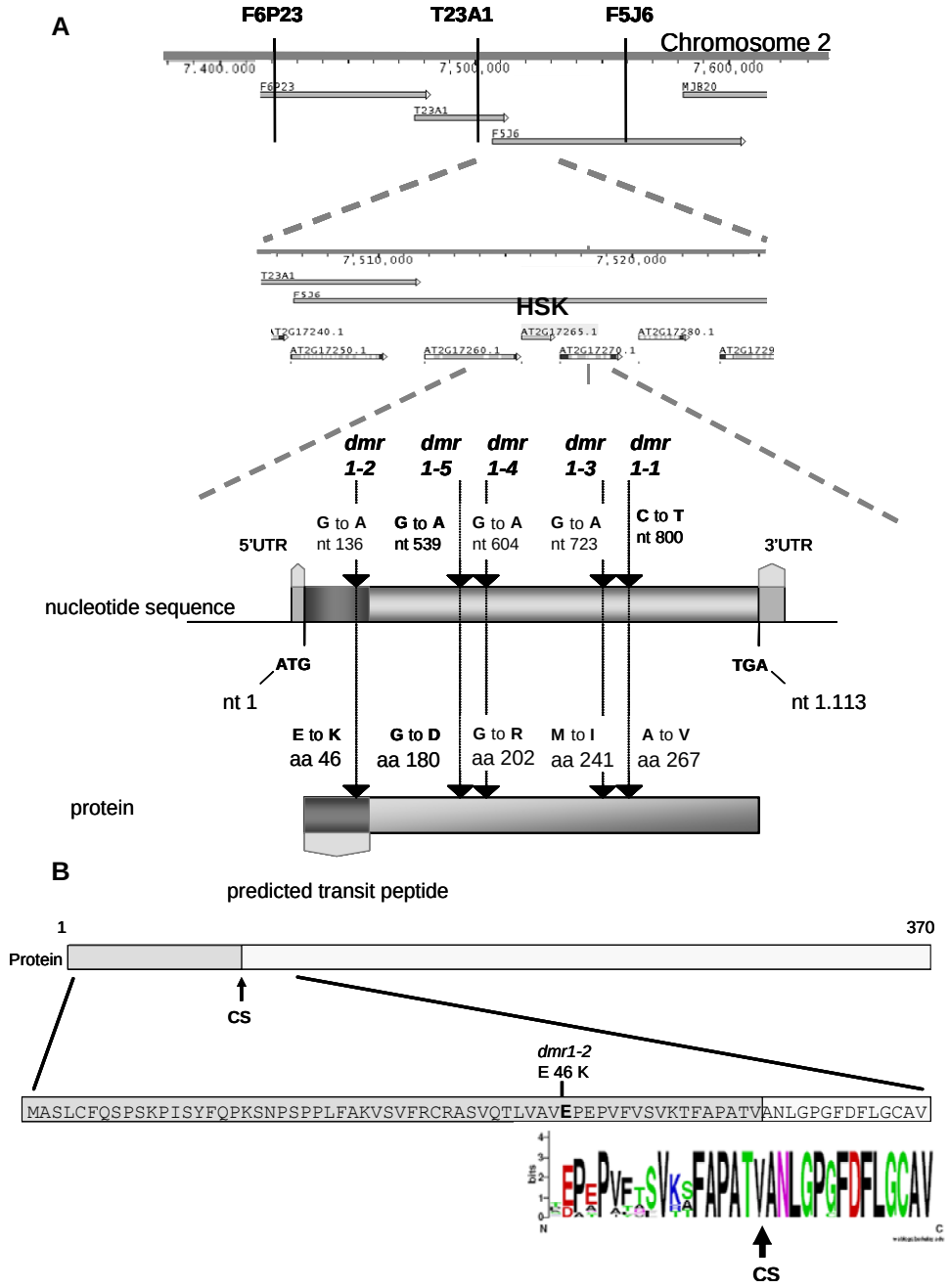

Figure 3 **A**. Map-based cloning and structure of the *DMR1* gene. The *dmr1* locus was fine-mapped from a region containing three BACs to a region of eight genes. Sequencing of these genes resulted in the finding of mutations in the At2g17265 gene encoding for homoserine kinase. The position of the nucleotide changes in the HSK coding sequence and amino acid changes in the HSK protein are indicated for the *dmr1-1*, *dmr1-2*, *dmr1-3*, *dmr1-4* and *dmr1-5* mutants. Note that all mutations are GC to AT transitions typical of EMS-induced mutations. **B**. The *dmr1-2* mutation is located in the putative chloroplast transit peptide which is predicted to be cleaved between valine 62 and alinine 63 (as indicated by the arrow). A strech of 17 amino

acids preceding the cleavage site (CS) is conserved in 14 plant HSK proteins (Figure 4) as depicted in the logo (weblogo.berkeley.edu).

HSK is a single copy gene in Arabidopsis and rice, based on the annotated genome sequences. In many other species ESTs were identified that belong to single orthologous *HSK* genes. However, in poplar and potato, two orthologous genes appear to be present. A multiple sequence alignment of the predicted orthologous HSK protein sequences is depicted in Figure 4. The nuclear-encoded HSK protein carries a predicted N-terminal transit sequence for chloroplast targeting.

The three indicated motifs (Figure 4) are highly conserved in all members of the superfamily of kinases; galactokinase, homoserine kinase, mevalonate kinase and phosphomevalonate kinase (GHMP) (Zhou et al., 2000). Although the *dmr1* mutations affect amino acid residues that are conserved in plant HSK proteins, they are not the residues that are likely to be involved in substrate- and Mg-ATP-binding (motifs 1 to 3 in Figure 4) as predicted by comparison with the *Methanococcus jannashii* HSK protein, of which the crystal structure has been described (Zhou et al., 2000).

```
Arabidopsis_thaliana      ---MASLCFQSPS---KPISYFQPKS-----NPSPPLFAKVSVFRCRASVQTLVA----- 44
Citrus_sinensis           ----MAICFSSAV---KPANHFTVFF-----NPAP----KKPIFKCSCSLPTVTT----- 39
Populus_trichocarpa_1     ---MAICCFPSPL---KPMTPATPLT-----NLKPK---RPDILRCNFSLPTITT----- 41
Populus_trichocarpa_2     ---MAIC-FLSPL---KPITPTTSLTT----NLNPK---KPNILRCNFSLPIITT----- 41
Solanum_tuberosum_2       ----MAVLCQSP---LNLKLITSSSSS-----SRNRTANP----SFRLNLSAHSR----- 39
Solanum_tuberosum_1       ----MAITYQSP---MKLNFITSNGFS-----NPPSLYPINTHFSFGFNLSSVSSKTQT- 47
Solanum_lycopersicum      ----MAITFQSP---MKLSFITSNGFS-----NPPSLYPINTHFSFGFNLSSVSSKTQT- 47
Nicotiana_benthamiana     ---MAAICYQSP---VKLNFTTSNAFSNPIPNNPPPLYPIKTRFSSGFNLSAVPSKTQTT 54
Ipomoea_nil               ------------------ASISSTRHP-----NPPLCLPALNISRCGPLFSAVTSSTLA- 36
Glycine_max               ----MATSTCFLC--PSTASLKGRARF------RIR---IRCSSS----VSVNIR----- 36
Phaseolus_vulgaris        ----MATAMSFLC--PSPATFKGTEMP------IAR---FRCCSSNTNSVSLNTR----- 40
Pinus_taeda               MESVFAQTKNHCFYLEPDLGLINSCFGLSRFRTKFSRGHLPHVFNVRCNAQQVSLKP--- 57
Zea_mays                  ----MAPAATSTAS--APSSFHSTGRHR----ARVGARPSLVSLRVRAANPNVT------ 46
Oryza_sativa              ---MAAAAAAAAAP--SPAPCFPSTRH-----TLPGLVSVRVSRRVKVA--VAI------ 44

                               K(dmr1-2)        Motif1
                                  ▼
Arabidopsis_thaliana      ----VEPEPVFVSVKTFAPATVANLGPGFDFLGCAVDG----LGDHVTLRVDPSVRAGEV 96
Citrus_sinensis           ----TEPEPVFTSVKTFAPATVANLGPCFDFLGCAVDG----LGDYVSLKVDPSVHPGEV 91
Populus_trichocarpa_1     ----TEPEPVFTSVRSFAPATVANLGPGFDFLGCAVDG----LGDFVSLRVDPSVHPGEL 93
Populus_trichocarpa_2     ----TEPEPVFTSVRSFAPATVANLGPGFDFLGCAVDG----LGDFVSLRVDPSVHPGEL 93
Solanum_tuberosum_2       ----SEPSPVFTSVKSFAPATVANLGPGFDFLGCAVDG----IGDFVTLRLDPNVHPGEV 91
Solanum_tuberosum_1       HITIPEPEPVFTSVKSFAPATVANLGPGFDFLGCAVDG----IGDFVTLRVDPNVKAGEV 103
Solanum_lycopersicum      HITIPEPEPVFTSVKSFAPATVANLGPGFDFLGCAVDG----VGDFVTLRVDPNVKAGEV 103
Nicotiana_benthamiana     HITIPEPEPVFASVKSFAPATVANLGPGFDFLGCAVDG----IGDFITLRVDSKVKPGEV 110
Ipomoea_nil               ---VSDPEPVYASVKSFAPATVANLGPGFDFLGCAVDG----IGDFVTVRVDPDVPPGQV 89
Glycine_max               ----REPEPVTTLVKAFAPATVANLGPGFDFLGCAVDG----LGDIVSVKVDPQVHPGEI 88
Phaseolus_vulgaris        ----TEPQPVTTFVKAFAPATVANLGPGFDFLGCAVDG----IGDIVSVRVDPEVRPGEI 92
Pinus_taeda               -VIQFEATPILQSVKAFAPATIANLGPGFDFLGCAVEG----LGDHVTVEVNEDVEPGKI 112
Zea_mays                  ----ADPAPAFQSVTTFAPATVANLGPGFDFLGCAVADASLSLGDTVTATLDPSLPPATV 100
Oryza_sativa              ----ADPAPAFNSVTAFAPATVANLGPGFDFLGCAVADASLSLGDTVTATLDPSLPPGTV 98
                               .  *    * :*****:***** ******** .    :** ::  :: .: .. :
                                                                        Motif2

Arabidopsis_thaliana      SISEITGTTT-----KLSTNPLRNCAGIAAIATMKMLGIRSVGLSLDLHKGLPLGSGLGS 151
Citrus_sinensis           SISEVIGPS------KLSKNPLWNCAGIAAISAMKMLGVRSVGLSLSLEKGLPLGSGLGS 145
Populus_trichocarpa_1     SISDISGPK------KLSKNPLYNCAGIAAIATMKMLNIRSVGLSLSLEKGLPLGSGLGS 147
Populus_trichocarpa_2     SISDISGTK------NLSKNPLNNCAGIAAIATMKMLNIRSVGLSLSLEKGLPLGSGLGS 147
Solanum_tuberosum_2       SISDISGAG-----KKLRRNPRWNCAGIAAISVMKMLNIRSVGLTLSLHKGLPLGSCLGS 146
Solanum_tuberosum_1       SISDISGAG-----NRLSKDPLSNCAGIAAISVMKMLNIQSVGLSISLEKGLPLGSGLGS 158
Solanum_lycopersicum      SISDISGAG-----NRLSKDPLSNCAGIAAISVMKMLNIQSVGLSISLEKGLPLGSGLGS 158
Nicotiana_benthamiana     SISDISGAG-----GKLSKDPLSNCAGIAAISVMKMLNIQSVGLSISLEKGLPLGSGLGS 165
Ipomoea_nil               SISEISGAG-----NKLSKNPLWNCAGIAAIAVMKMLRIQSVGLSLSLEKGLPLGSGLGS 144
Glycine_max               CISDISGHAP----NKLSKNPLWNCAGIAAIEVMKMLSIRSVGLSLSLEKGLPLGSGLGS 144
Phaseolus_vulgaris        RISDITGHAP----NKLSTNPLWNCAGIAAIEVMKMLAIRSVGLSLSLQKGLPLGSGLGS 148
Pinus_taeda               VISFIDGDN-----NRLSLNPMKNCAGIAAKATMELLGVRSVGLSLGLHKGLPLGSGLGS 167
Zea_mays                  SIASVTSPSRPNLAERLSRDPLRNCAGVAAIAALRALGVRSHAVSIHLTKGLPLGSGLGS 160
Oryza_sativa              AIASVTSPSRPTLADRLSRDPLRNCAGVAAIAALRALDVKSHAVSIHLTKGLPLGSGLGS 158
                           *: : .        .*  :*  ****:**  .:. * ::* .::: * ******* ***

                          Motif2                          D(dmr1-5)              R(dmr1-4)
                                                             ▼                      ▼
Arabidopsis_thaliana      SAASAAAAAVAVNEIFGRKLGSDQLVLAGLESEAKVSGYHADNIAPAIMGGFVLIRNYEP 211
Citrus_sinensis           SAASAAAAAVAVNEMFGNKLLPDELVLAGLESEAKVSGYHADNIAPAIMGGFVLIRSYEP 205
Populus_trichocarpa_1     SAASAAAAAVAVNELFGRKLEVKDLVLAGLESEAKVSGYHADNIAPAIMGGFVLIRSYDP 207
Populus_trichocarpa_2     SAASAAAAAVAVNEMFGRKLEVKDLVLAGLESEAKVSGYHADNIAPAIMGGFVLIRSYDP 207
Solanum_tuberosum_2       SAASAAAAAVAVNELFGRPLTLTDLVLAGLDSESKVSGYHADNVAPAIMGGFVLIRSYHP 206
Solanum_tuberosum_1       SAASAAAAAVAVNEIFGRKLSVDDLVLAGLESETKVSGYHADNIAPSIMGGFVLVRSYDP 218
Solanum_lycopersicum      SAASAAAAAVAVNEIFGRKLSVDDLVLAGLESETKVSGYHADNIAPSIMGGFVLIRSYDP 218
Nicotiana_benthamiana     SAASAAAAAVAVNELFGGKLSVSDLVLAGLESETKVSGYHADNIAPAIMGGFVLIRSYDP 225
Ipomoea_nil               SAASAAAAAVAVNELFGSRLSVSDLVFAGLESESKVSGYHADNVAPSIMGGFVLIRSYDP 204
Glycine_max               SAASAAAAAVAVNELFGKKLSVEELVLASLKSEEKVSGYHADNVAPSIMGGFVLIGSYSP 204
Phaseolus_vulgaris        SAASAAAAAVAVNEMFGKRLSVEDLVVASLKSEEKVSGYHADNVAPAIMGGFVLIQSYEP 208
Pinus_taeda               SAASAAAAAVAVNGLFGNKLTKSDLVLAGLESEAAVSGYHADNVAPSLMGGFVLVRSYSP 227
Zea_mays                  SAASAAAAAKAVDALFGSRLGRDDLVLAGLESEKAVSGFHADNIAPAILGGFVLVRSYDP 220
Oryza_sativa              SAASAAAAAKAVDALFGSLLHQDDLVLAGLESEKAVSGFHADNIAPAILGGFVLVRSYDP 218
                          ********* **: :**  *   :**.*.*.**  ***:****:**:::*****: .* *
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                                                    I(dmr1-3)                 V(dmr1-1)
                                                    ▼                         ▼
Arabidopsis_thaliana        LDLKPLRFPSDKDLFFVLVSPDFEAPTKKMRAALPTEIPMVHHVWNSSQAAALVAAVLEG 271
Citrus_sinensis             LDLMRLNFPEKKQLLFVLVTPEFEAPTKKMRAALPAEVGMPHHIWNCSQAGALVAAVLNG 265
Populus_trichocarpa_1       LELMSLQFPVEKDLIFVLVSPDFEAPTKKMRAALPAEIGMPHHVWNCSQAGAFVASVLQG 267
Populus_trichocarpa_2       LELMSLQFPVEKDLIFVLVSPDFEAPTKKMRAALPAEIGMSHHVWNCSQAGALVASVLQG 267
Solanum_tuberosum_2         LELIQLNFPHEKDLFFVLANPEFEAPTKKMREALPQEITMSHHIWNCSQAGALVASVLLG 266
Solanum_tuberosum_1         LELISLKFPFEKDLFFVLVNPEFEAPTKKMRAVLPSEVTMSHHIWNCSQAGALVAAILQG 278
Solanum_lycopersicum        LELIPLKFPFEKDLFFVLVNPEFEAPTKKMRAVLPSEVTMSHHIWNCSQAGALVAAILQG 278
Nicotiana_benthamiana       LELIELKFPLEKDLFFVLVNPEFEAPTKKMRAALPNEVTMSHHIWNSSQAGALVAAILQG 285
Ipomoea_nil                 LELIQLKFPQEKSLFFVLVNPEFEAPTKKMRAALPAEITMSSHVWNCSQAGALVASVLQG 264
Glycine_max                 LELMPLKFPAEKELYFVLVTPEIEAPTKKMRAALPTEIGMPHHVWNCSQAGALVASVLQG 264
Phaseolus_vulgaris          LRLIELKFPAEKELYFVLVSPEFEAPTKKMRAALPGEIAMAHHVWNCSQAGALVAAVLQG 268
Pinus_taeda                 LDLIHLPFPSEKELFFVLVTPAFEAPTKEMRAVLPKNITMKDHIQNCSQAAALVAAILQG 287
Zea_mays                    FHLVPLSFPPALRLHFVLVTPDFEAPTSKMRAALPRQVDVQQHVRNSSQAAALVAAVLQG 280
Oryza_sativa                FHLIPLSSPPALRLHFVLVTPDFEAPTSKMRAALPKQVAVHQHVRNSSQAAALVAAVLQG 278
                            : *  *  *    * ***..* :****.:** .** :: :  *: *.***.*:**::* *
                                                                 Motif3
                                                                 ‾‾‾‾‾‾

Arabidopsis_thaliana        DAVMLGKALSSDKIVEPTRAPLIPGMEAVKKAALEAGAFGCTISGAGPTAVAVIDSEEKG 331
Citrus_sinensis             DPVGLGKALSSDKIVEPNRAPLIPGMEAVKKVAVEAGAYGCTISGAGPTAVAVVDNEEKG 325
Populus_trichocarpa_1       DLVGLGKALSSDKIVEPKRAPLIPGMEGVKKAALEAGAFGCTISGAGPTAVAVVDSEERG 327
Populus_trichocarpa_2       DLVGLGKALSSDKIVEPKRAPLIPGMVGVKKAALEAGAFGCTISGAGPTAVAVVGSEDRG 327
Solanum_tuberosum_2         DVSGFGKALSSDKIVEPRRTPLIPGMEGVKKAAMEAGAFGCTIRGAGPTVVAVTDNEETG 326
Solanum_tuberosum_1         DSRGLGKALSSDKIVEPRRGPLIPGMEGVKKAALKAGAFGCTISGAGPTLVAVTDDEERG 338
Solanum_lycopersicum        DSRGLGKALSSDKIVEPRRGPLIPGMEGVKKAALKAGAFGCTISGAGPTLVAVTDDEERG 338
Nicotiana_benthamiana       DSRGLGKALSSDKIVEPKRGPLIPGMEGVKKAALEAGAFGCTISGAGPTLVAVTDGEERG 345
Ipomoea_nil                 DLPGLGKALSSDKIVEPRRAPLIPGMEAVKKAAIQAGAFGCTISGAGPTAVAVTDDEEKG 324
Glycine_max                 DVVGLGKALSSDKIVEPRRAPLIPGMEAVKRAAIQAGAFGCTISGAGPTAVAVIDDEQTG 324
Phaseolus_vulgaris          DVVGLGKALSSDKIVEPRRAPLIPGMEAVKKAALQAGAFGCTISGAGPTAVAVIDDELAG 328
Pinus_taeda                 DPCLLGAALSSDSIVEPKRGPFIPGMMAVKAAALETGAFGCTISGAGPTAVAITDTAEKG 347
Zea_mays                    DAGLIGSAMSSDGIVEPTRAPLIPGMAAVKAAALQAGALGCTISGAGPTVVAVIQGEERG 340
Oryza_sativa                DATLIGSAMSSDGIVEPTRAPLIPGMAAVKAAALEAGALGCTISGAGPTAVAVIDGEEKG 338
                            *   :* *:*** **** * *:**** .** .*:::** **** ***** **:      *

Arabidopsis_thaliana        QVIGEKMVEAFWKVGHLKSVASVKKLDNVGARLVNSVSR---- 370
Citrus_sinensis             KVIGEKMVEAFWKEGNLKAVSMVKRLDRVGARLVGSVRAPR-- 366
Populus_trichocarpa_1       VEIGERMVETFWKEGKLKAVASVKRLDRVGARLVGSVPR---- 366
Populus_trichocarpa_2       MEVGERMVEAFWKEGNLKAVAMVKRLDRVGARLVGSVPR---- 366
Solanum_tuberosum_2         REIGQRMVEVFLEHGKLKALAMVKKLDRIGARLVSSQPI---- 365
Solanum_tuberosum_1         REIGERMVDAFMKEGNLKALAMVKKLDRVGARLVSSNS----- 376
Solanum_lycopersicum        REIGERMVEAFMKEGNLKALAMVKKLDRVGARLVSSNS----- 376
Nicotiana_benthamiana       REIGERMVEAFMKEGKLKALAMVKQLDRVGARLVSSNPR---- 384
Ipomoea_nil                 MEIGKRMVEAFIQEGNLKALAMVKRLDRVGARLVSKNGSICN- 366
Glycine_max                 HLIAKHMIDAFLHVGNLKASANVKQLDRLGARRIPN------- 360
Phaseolus_vulgaris          NAIAEHMIHAFLHHGNLKASAKVLQLDRLGARRILD------- 364
Pinus_taeda                 KAIAVAMVDMFQKKGQLEATASVQKLDRIGARVV--------- 381
Zea_mays                    EEVARKMVDAFWSAGKLKATATVAQLDTLGARVIATSSLN--- 380
Oryza_sativa                EEVGRRMVEAFANAGNLKATATVAQLDRVGARVISTSTLE--- 378
                              :.  *:. *   *:*:: : * :** :*** :
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Figure 4. Multiple Sequence Alignment of plant homoserine kinase amino acid sequences generated using the CLUSTAL W programme from the European Bioinformatics Institute (EBI). HSK sequences are derived from mRNA, genomic or assembled EST sequences which are available from GenBank on the National Center for Biotechnology Information (NCBI) website. Below the sequence the conserved amino acids are indicated by the dots, and identical amino acids by the asterisks. The black triangles indicate the amino acids that are substituted in the five Arabidopsis *dmr1* mutants. The three motifs indicate the conserved sequences that are involved in substrate and co-factor binding.

To test if a functional At2g17265 gene could complement the *dmr1* phenotype, the *dmr1-1*, *dmr1-2*, *dmr1-3* and *dmr1-4* mutants were transformed with the wild-type Arabidopsis Col-0 HSK coding sequence under control of a 35S promoter. Transgenic lines showed restoration of susceptibility to *H. parasitica* (data not shown). The complementation and the identification of 5 independent alleles, confirm that the mutations in the *HSK* gene are responsible for resistance to *H. parasitica*.

The dmr1 mutants accumulate homoserine

HSK is a key enzyme in the aspartate metabolic pathway for the biosynthesis of the essential amino acids methionine, threonine and isoleucine in plants (Figure 5A). To test if the HSK mutations affect amino acid biosynthesis we quantified soluble amino acids in seedlings of the *dmr1* mutants and the parental line L*er eds1-2*. High levels of homoserine were detected in the *dmr1* mutants, but not in the parental line, L*er eds1-2* (Figure 5B). The levels of homoserine in the *dmr1* mutants are in between 500 and 7000 nmol/mg tissue (fresh weight) and undetectable in the parental line. The accumulation of homoserine indicates that the dmr1 mutants have a reduced HSK activity. Remarkably, the levels of threonine, methionine, and isoleucine (further downstream in the aspartate pathway) were not reduced, but increased in some of the *dmr1* mutants (Figure 5C-E). The methionine level was 2 to 5 times higher in all four *dmr1* mutants and isoleucine and threonine levels were 2 to 3 times higher in *dmr1-2* and *dmr1-4*, when compared to the parental line. This is unexpected as the accumulation of homoserine points to a reduced HSK activity which would lead to lower levels of the products further down in the pathway. As the *dmr1* mutants only carry missense mutations they may have retained some HSK activity that is sufficient in the presence of high levels of homoserine to synthesize phospho-homoserine. We hypothesize that in the *dmr1* mutants a feedback mechanism channels more aspartate into the pathway to compensate for the reduced HSK activity resulting in even higher levels of homoserine. The levels of aspartate were equal in *dmr1-1* and *dmr1-3* and 2 times lower in *dmr1-2* and *dmr1-4* compared to the parental line (data not shown). Both *dmr1-2* and *dmr1-4* have the highest homoserine levels

(approximately 7000 nmol/mg). The aspartate levels in *dmr1-1* and *dmr1-3* are equal to the levels in the parental line but the homoserine levels are respectively approximately 1000 nmol/mg and 500 nmol/mg. Another explanation could be that methionine, threonine and isoleucine might be synthesized by an alternative pathway independent of homoserine.

Homoserine provides resistance to H. parasitica in Arabidopsis

To test whether resistance to downy mildew was directly caused by high homoserine levels the inhibitory effect of the compound was tested in growth assays. As *H. parasitica* is an obligate biotroph and cannot be cultivated *in vitro*, the effect of homoserine could only be tested on spore germination. No inhibitory effect of homoserine on spore germination was found up to concentrations of 100 mM (data not shown). The insensitivity to homoserine could be confirmed using the related non-obligate oomycete pathogen *Phytophthora brassicae*. Homoserine levels, up to 10 mM, did not affect radial growth of *P. brassicae in vitro* (data not shown). These data indicate that homoserine itself, at the concentrations used, is not toxic for the pathogen but instead suggests an active role of the plant in mediating homoserine-induced resistance.

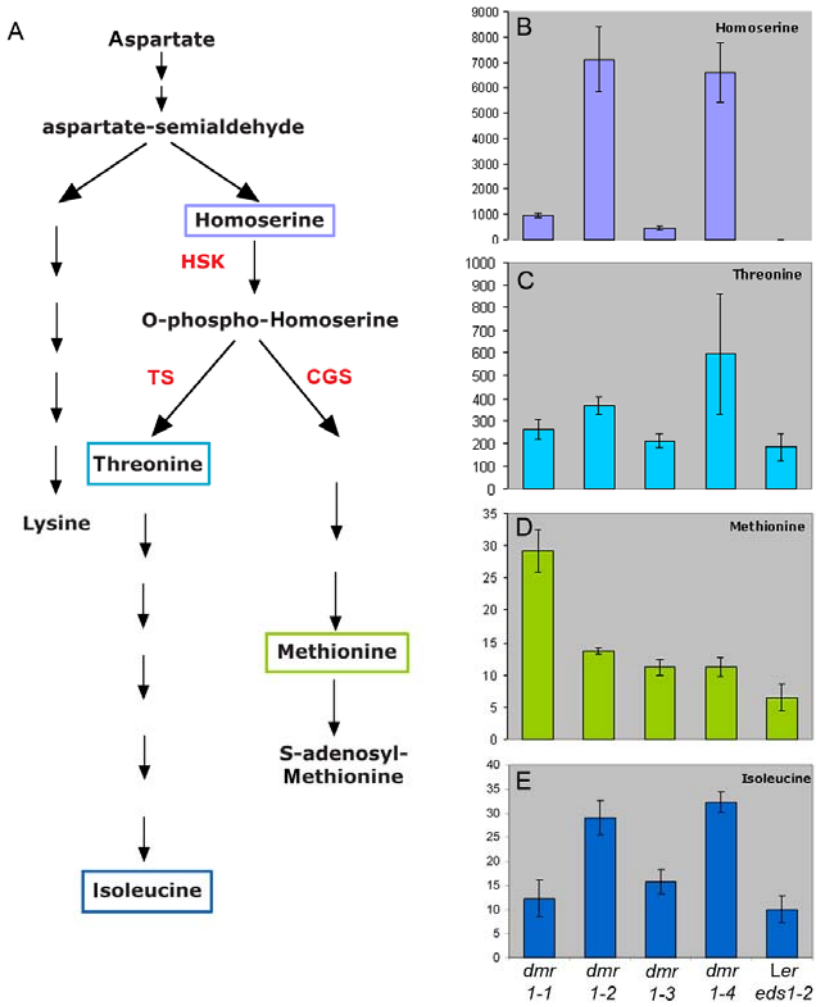

Figure 5. Mutation of the HSK gene in the *dmr1* mutants results in homoserine accumulation and altered levels of the amino acids threonine, methionine and isoleucine. **A**. The role of HSK in the aspartate metabolic pathway in Arabidopsis (adapted from www.aracyc.org). Homoserine is the common precursor for the essential amino acids threonine, isoleucine, and methionine, but not for lysine. In the chloroplast stroma HSK produces homoserine-phosphate that is the direct substrate for threonine synthase (TS) or cystathionine γ-synthase (CGS). The levels (in nmol/mg fresh weight) of homoserine (**B**) threonine (**C**) methionine (**D**) and isoleucine (**E**) in seedlings of four *dmr1* mutants compared to the parental line, L*er eds1-2* is shown.

The exogenous application of homoserine during downy mildew infection of wild-type seedlings was tested. For this, solutions of L-homoserine and the control compound D-homoserine were infiltrated into the leaf extracellular space, such that all leaf cells were directly exposed to the compound. Treatment with L-homoserine at a concentration of 5 mM resulted in complete resistance to downy mildew, i.e. the absence of sporangiophore formation (Figure 6A). Microscopic analysis of trypan blue stained infections showed arrested *H. parasitica* growth (Figure 6B and C) and encased haustoria in the L-homoserine-treated leaves (Figure 6C and D). Similar effects were observed in the *dmr1* mutants suggesting that they are indeed the result of the

presence of homoserine (Figure 1C and 2A). The *dmr1-1* mutant also contain elevated levels of methionine and the *dmr1-2* and *dmr1-4* mutants have elevated levels of threonine and isoleucine. However, resistance was not obtained after infiltration with methionine, threonine, isoleucine or the stereo-isomer D-homoserine indicating that the effect is L-homoserine-specific (Figure 6A-C). No sporangiophore formation could be detected (Figure 6A) or visualized on L-homoserine treated seedlings (Figure 6B and C). Trypan blue staining of methionine, threonine and isoleucine infiltrated seedlings infected with *H. parasitica* did not show any altered haustoria (Figure 6D). Although the level of several amino acids is altered in the *dmr1* mutants only exogenous application of L-homoserine mimicked the *dmr1* mutant phenotype (Figure 6E). Callose deposition was only detectable in L-homoserine treated seedlings, besides the presence of a callose around the haustorial neckband (Figure 6E).

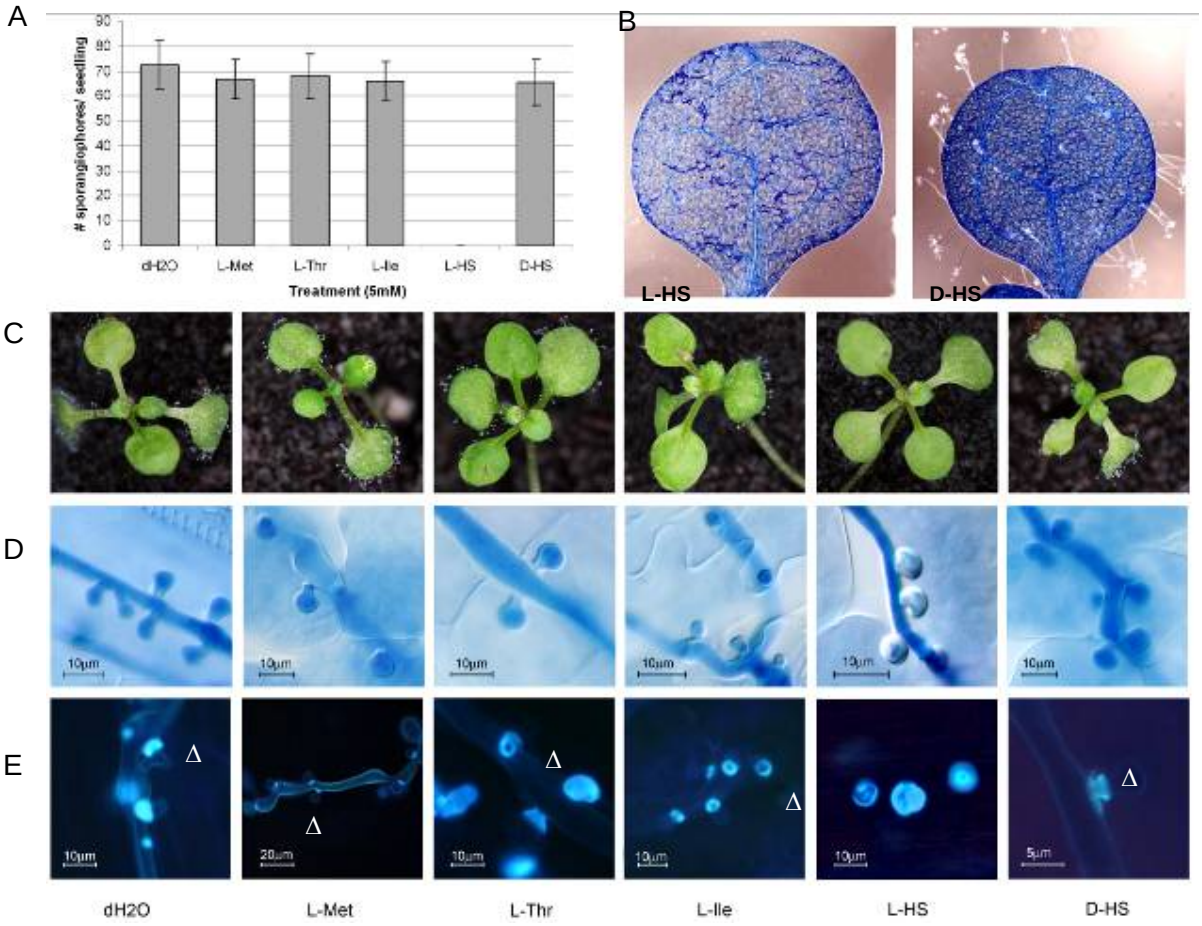

Figure 6. Exogenous application of L-homoserine leads to H. parasitica resistance in Arabidopsis. **A**. Quantification of sporangiophore formation in L*er eds1-2* seedlings, 5 days past inoculation with *H. parasitica* and 2 days after infiltration with the different described amino acids (5mM), **B.** Whole seedling leaf microscopic analysis of *H. parasitica* growth visualized 5 days past inoculation by trypan blue staining

(4x magnification). The seedlings were infiltrated with 5 mM L-HS (left panel) and 5 mM D-HS (right panel) 2 days prior staining. Sporangiophores are only emerging from the leaf infiltrated with D-HS. **C**. Images of whole L*er eds1-2* seedlings 5 dpi with Cala2 and 2 days post amino acid infiltration. No emerging sporangiophores can be detected in L-HS treated seedlings, in the fifth panel. **D.** Microscopic analysis of *H. parasitica* haustoria by trypan blue staining (40x magnification). The haustoria of the L-HS treated seedlings show papillae formation surrounding the haustoria. **E.** Callose formation visualized by aniline blue staining in seedlings (40x magnification). Callose detectable in the haustorial neckbands is indicated with a Δ, the last panel in of **E** shows a side view of the haustorial neckband after infiltration with D-homoserine, similar to the L-Methionine and dH_2O infiltration. The L-Threonine and L-Isoleucine depict a top view, represent as a circle. Callose deposition surrounding the formed haustoria is detectable in the L-HS treated seedlings.

Preliminary data indicate that besides the elevated homoserine levels in the *dmr1* mutants also sarcosine (67-365 nmol/mg), homocitrulline (2-6 nmol/mg), and cystathionine (5-6 nmol/mg) accumulate. The levels of sarcosine in the *dmr1* mutants correlate with the detected levels of homoserine with a correlation coefficient of 0.99, suggesting that they are metabolically linked. As the level of sarcosine is particularly high, the amino acid (5 mM) was tested for its effect on the infection process by infiltration of L*er eds1-2* seedlings. Sarcosine treatment did not result in *H. parasitica* resistance (data not shown).

Callose inhibition

Encasement of haustoria by papillae containing callose is a well known defence response. Inhibition of callose synthase by 2-deoxy-D-glucose (2-DDG) (Jaffe and Leopold, 1984) was used to assess whether callose was required for the homoserine-induced resistance. Callose was quantified by measuring fluorescence levels in aniline blue stained samples of *H. parasitica* infected seedlings treated with homoserine and 2-DDG. The fluorescence intensity levels in leaf tissue infiltrated with water or L-HS and 2-DDG were approximately 100 (arbitrary value), whereas the levels in L-HS infiltrated tissues was 150 (Figure 7). This indicates that callose formation was completely inhibited by the 2-DDG treatment. However, 2-DDG treatment did not affect L-homoserine induced-resistance to *H. parasitica* as co-infiltration of L-homoserine and 2-DDG still resulted in the arrest of *H. parasitica* growth. This strongly suggests that homoserine-induced callose formation is a secondary response that is not responsible for the arrest of pathogen growth.

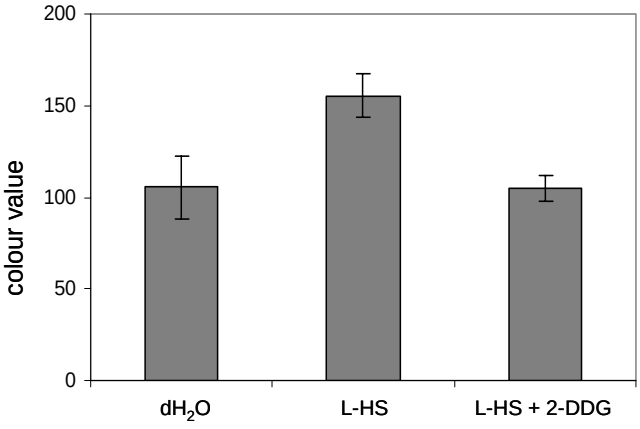

Figure 7. Quantification of callose deposition in *H. parasitica* infected seedlings treated with L-homoserine and 2-DDG. Callose was quantified from microscopic images, 20x magnification, of Arabidopsis seedlings 2 days past dH_2O, L-HS (5 mM) and L-HS (5 mM) + 2-deoxy-D-Glucose (2 mM) infiltration and 5 days past *H. parasitica* infection. The levels of callose are depicted as the mean blue fluorescence colour value per image.

Discussion

Downy mildew resistant 1 (*dmr1*) mutants were previously identified in a genetic screen for Arabidopsis genes required for *H. parasitica* susceptibility (Van Damme et al., 2005). Five alleles of *dmr1* were isolated and the *dmr1-1* mutant was used to map-base-clone the *DMR1* gene. The *DMR1* gene was identified as At2g17265 encoding homoserine kinase (HSK). The five *dmr1* alleles all carried GC to AT transitions, typical of EMS induced mutations. All five mutations resulted in amino acid substitutions in the HSK protein. The fact that no null mutations were identified suggests that HSK is an essential plant protein. In *Escherichia coli* and *Saccharomyces cerevisae* (Arevalo-Rodriguez et al., 1999) deletion of the *HSK* gene leads to threonine auxotrophy. Furthermore, in the Arabidopsis genome sequence a single *HSK* gene is predicted. A second gene (At4g35295) is annotated as "putative *HSK*", but the gene has many stop codons and the predicted protein is only 111 amino acids long, whereas HSK is 370 amino acids. The At4g35295 is probably a pseudogene that has degenerated from a duplicated *HSK* gene. The HSK region on chromosome 2 and the At4g35295 region on chromosome 4 are syntenic and probably originated from an ancient segmental duplication (Blanc et al., 2000; Terryn et al., 1999). A single *HSK* gene was also identified in rice, but in poplar two predicted *HSK* genes have been identified, that could result from a more recent genome duplication (Tuskan et al., 2006). Analysis

of ESTs and generated contigs, identified from ten other plant species for which the complete *HSK* coding sequence was obtained, showed in most cases a single *HSK* gene except in potato which appears to contain two *HSK* orthologs. The HSK amino acid alignment shows an overall of 40% identity among the different *HSK*s orthologs.
The fact that the predicted chloroplast transit peptide cleavage site falls into the conserved motif 1, raises doubt about the accuracy of this prediction. Also the mutation in *dmr1-2* (E46K) is in the predicted transit peptide. Interestingly, a stretch of 17 amino acids that precedes the predicted transit peptide cleavage site, including an acidic residue at position 46, is conserved in all plant HSK proteins as shown by the alignment in figure 4. However, as no proteomics data on the N-terminal amino acid sequence of the processed HSK protein is available one cannot draw any conclusions about the correct cleavage site. Biochemical fractioning of barley resulted in 83% of the homoserine kinase activity that was recovered from chloroplasts (90%) (Wallsgrove et al., 1983). And Wallsgrove et al. (1993) showed that the HSK protein from the chloroplast was active.

HSK phosphorylates homoserine to phospho-homoserine, the precursor for methionine, threonine and via threonine for isoleucine. The *dmr1* mutants contain high levels of homoserine, whereas this amino acid was not detectable in wild-type plants and the parental line L*er eds1-2*. This suggests that the HSK activity is limiting in the *dmr1* mutants leading to the accumulation of the substrate homoserine. Low HSK activity was expected to lead to lower levels of downstream products. However, the levels of methionine, threonine and isoleucine were increased rather than decreased in the *dmr1* mutants. We postulate that a feedback mechanism shuttles sufficient aspartate into the pathway so that homoserine accumulates to high levels. The residual HSK activity in the presence of high substrate concentrations is probably sufficient to produce equal amounts or even more of methionine, threonine and isoleucine. Resistance to *H. parasitica* in Arabidopsis was only obtained by exogenous application of L-homoserine and not by any of the other amino acids that were increased in levels in the *dmr1* mutants. The fact that *H. parasitica* spore germination or *P. brassicae* mycelium growth was not

inhibited by direct treatment with L-homoserine implies that the plant plays an active role in mediating homoserine-induced resistance. The resistance is independent of mutation of *DMR1* since exogenous application of L-homoserine to wild-type plants also results in *H. parasitica* resistance.

Resistance of the *dmr1* mutants is specific to *H. parasitica*, as they are still susceptible to other pathogens. Previously we reported that the *dmr1* mutants are susceptible to *P. syringae* pv. *tomato* and *Golovinomyces orontii* (Van Damme et al., 2005). Preliminary data indicate that *dmr1* plants are also susceptible to the anthracnose fungus *Colletotrichum higginsianum* (R. O'Connell, personal communication) and the white rust pathogen, *Albugo candida* (E. Holub, personal communication). The specific resistance is directly linked to high homoserine levels and could be caused by (i) the activation of a highly specific and so far unknown defence response, or (ii) the sensitivity of *H. parasitica* to high homoserine levels *in planta*. Gene expression profiling of the *dmr1* mutant is in progress and a suppressor screen in the *dmr1* background has been initiated to identify genes required for homoserine-induced resistance.

The phosphorylation of homoserine by HSK, as well as the enzymatic steps in the first part of the aspartate pathway leading to the synthesis of homoserine, are assumed to take place in the chloroplast (Azevedo et al., 1997). However, *H. parasitica* is not in direct contact to the chloroplasts as it is separated from it by the plant cell membrane and cytoplasm. It is unknown whether homoserine can be transported (active or passive) across the chloroplast membrane. However, the fact that exogenous (i.e. extracellular) application of homoserine leads to resistance implies that homoserine can have its effect outside of the chloroplast, or that the amino acid is transported over the plasma membrane and chloroplast membranes to have its activity in the chloroplast stroma. We are currently testing these ideas in transgenic *dmr1* plants expressing a HSK enzyme targeted to the cell cytoplasm so that homoserine located outside of the chloroplast is effectively removed.
Callose was detected around the *H. parasitica* haustoria after L-homoserine infiltration of the parental lines and in the *dmr1* mutants. Wound callose and

papillary callose formation is a widely recognized early response of host plants to microbial attack. Callose formation could result in reduction of nutrient transport and signals over the haustorial membrane, which could arrest the developing hyphae. But the similar detectable callose levels in the water infiltrated seedlings that remained *H. parasitica* susceptible, to the *H. parasitica* resistant seedlings co-infiltrated with L-HS and 2-DDG suggests another so far unknown response of defence caused by the high levels of homoserine.

Homoserine was not detectable in wild type Arabidopsis seedlings. However, in other plant species such as pea and other members of the legume subfamily Vicieae, high levels of homoserine are present. The high endogenous homoserine level in pea plants was found to act as an inducer of the fungal pathogen *Nectria haematococca*. It was hypothesized that *N. haematococca* has evolved the ability to sense homoserine, but also asparagine, as a signal to induce expression of virulence genes *in planta* (Yang et al., 2005).

We conclude that with the isolation of the *DMR1* gene encoding homoserine kinase we identified a new mechanism resulting in plant disease resistance via the modulation of host amino acid metabolism. The results provide the first evidence that the accumulation of a single amino acid, L-homoserine, can lead to plant disease resistance.

Materials and Methods

Plant lines and growth conditions

Arabidopsis thaliana accessions used in this study are Landsberg *erecta* (L*er*), L*er eds1-2* (Parker et al., 1996) and the Col-0 mutant *FN2* (Sinapidou et al., 2004). Plants were grown on potting soil in a growth chamber (Snijders, The Netherlands) at 22ºC with 16 h of light (100 µE/m2/sec) and a relative humidity of 75%.

Growth and infection of downy mildew

H. parasitica isolate Cala2 was kindly provided by Dr. E. Holub (HRI, Wellesbourne) and maintained on Arabidopsis L*er* by weekly transfer to healthy 10-14 day-old seedlings (Holub et al., 1994). To obtain large amounts

of sporangiospores for bioassays, inoculum was collected from L*er eds1-2* seedlings that support abundant Cala2 growth and sporulation (Parker et al., 1996). Inoculum ($5x10^4$ spores·ml^{-1}) was applied on 14 day-old seedlings using a spray gun. After inoculation, plants were allowed to dry for 15-60 minutes and subsequently incubated under a sealed lid (100% relative humidity) in a growth chamber at 16ºC with 9 h light/day (100µ E/m^2/sec). The amount of sporulation was quantified at 5 days post inoculation by counting the number of sporangiophores on the cotyledons and leaves.

Microscopy

Infections of *H. parasitica* in Arabidopsis leaves were visualized by trypan blue staining. Infected seedlings or leaves were stained in lactophenol (1:1:1:1 volume of lactic acid/glycerol/phenol/H_2O) containing 1 mg/mL trypan blue, by boiling for 1-2 minutes and destaining overnight in choral hydrate. Trapped air bubbles were removed by 1 minute speed vacuum infiltration. *H. parasitica* growth was visualized by interference contrast microscopy. Reactive oxygen intermediates (ROIs) were detected by staining with 3'-3'-diaminobenzidine (DAB) which visualizes H_2O_2 (Thordal-Christensen et al., 1997). DAB staining was performed for 8 hours on 10 day-old Arabidopsis seedlings at 3 days post inoculation with *H. parasitica*, isolate Cala2. Seedlings dissected from the root were placed in 96-well plates containing 100 µl DAB solution a plastic box under high humidity. Afterwards the leaves were cleared of chlorophyll in alcoholic lactophenol (1 volume of phenol: glycerol: lactic acid: water (1:1:1:1) and 2 volumes of ethanol) for 15-30 min at 65°C, followed for 2 hours at room temperature in fresh solution. Prior to microscopy the samples were placed in chloral hydrate. Arabidopsis leaves were visualized by differential interference contrast microscopy. Prior to aniline blue staining, seedlings (10-14 days) were cleared in lacto-phenol alcohol twice for 5 minutes at 65°C, followed by two 50% ethanol rinses and one rinse with 0.07 M NaH2PO4 (pH = 9.0), followed by a 30 minutes incubation in 0.07 M phosphate buffer (pH = 9.0) at room temperature. Aniline staining was preformed in freshly prepared 0.05 % aniline blue (w/v) in 0.07 M NaH2PO4 (pH = 9.0) and incubate for 1 hour at room temperature. The slides were prepared in a 0.05 % aniline blue staining solution and callose was visualized

on an Olympus AX with DAPI & WU filters (excitation between 330-385 nm and 420 nm barrier).

Image analysis

Digital images of aniline blue stained seedlings were taken with a Nikon DXM1200 camera. All images were photographed under standard conditions. Image analysis was carried out with a PC-based system equipped with the KS400 version 3.0 software (Carl Zeiss Vision, Oberkochen, Germany). A program was developed in KS400 to quantify the mean blue fluorescence of representative images of aniline blue stained tissues. Images were converted into grey and all tissue was selected on the basis of grey level threshold. Inversion of this binary image results in the selection of the background necessary to determine the mean fluorescence in the background area. To exclude areas displaying glare of the fluorescent structures the background image was eroded 15 times. The mean blue fluorescence is determined in the blue component of the colour image. All blue fluorescing structures were selected in the color image based on the Hue Lightness Saturation model. Objects below 50 pixels in size were excluded. Artefacts were excluded interactively. The total blue fluorescence and total area is measured in the blue component of the colour image. The blue fluorescence in the equivalent background area is subtracted. The resulting value divided by the total fluorescent area presents the mean blue fluorescence.

Cloning DMR1

The isolation of the *dmr1* mutant together with the generation of the EMS mutant and the screening has been previously described (Van Damme et al., 2005). From each cross the resistant F_2 plants were selected, genotyped and rescreened for resistance in the F_3. For the initial mapping 48 resistant F_2 plants were selected and codominant SSLP markers, http://carnegiedpb.stanford.edu/
publications/methods/ppsuppl.html evenly distributed over the 5 Arabidopsis chromosomes were checked. The *dmr1* phenotype is cosegregating with an area on chromosome 2, close to the ciw3 and nga1126 marker. The Cereon database was used to design new PCR markers based on insertion, deletion

(IND) and single nucleotide polymorphisms (SNP) between the Colombia and Landsberg isolates. All designed markers were designed on the Arabidopsis chromosome 2, by blasting in SeqViewer from TAIR (www.arabidopsis.org). An additional screen for new recombinants was initiated for 650 F_2 plants resulting in ~90 F_2 recombinant plants between two IND based markers 6.25Mb and 7.3Mb, leaving a contig of 5 BAC. The F_2 plants were genotyped and the F_3 generation was phenotyped, giving a resistant, segregating or susceptible phenotype. The *dmr1* mutation was fine mapped to a ~130 kb region including 3 BACs between 2 IND based markers, located on BAC F6P23, at 7.43 Mb and F5J6, at 7.56 Mb. This resulted in an area of 30 candidate genes for the *dmr1* locus, between At2g17060 and At2g17380. Additional cleaved amplified polymorphic markers (CAPS) were designed based on SNP linked to 6 specific At2g17190, At2g17200, At2g17270, At2g17300, At2g17310 and At2g17360 coding genes. Analysing the remaining recombinants left 8 candidate genes At2g17230 till At2g17290 followed by sequencing analysis of these eight genes. In the At2g17265 gene point mutations were identified.

Amino Acid extraction

Amino acids were extracted from 14 day old seedlings that were grinded in the liquid nitrogen. Samples were dissolved in final a concentration of 80% methanol, vortexed for 1 minute and incubated 10 minutes at room temperature (twice) followed by 10 minutes centrifugation at 8000 rpm. The supernatant was collected in a new fresh tube, and 20% methanol was added to the pellet followed by a 1 minute vortex and 10 minutes incubation at room temperature step (twice). The extract was centrifuged for 10 minutes (at 8000 rpm) and the supernatant was added to the previously collected 80% methanol extracted supernatant. Sample volumes were reduced by speed vacuum centrifugation and freeze drying. Dried samples were dissolved in 150 µl dH_2O. An equal volume (150 µl) of internal standard, S-amino-ethyl-cysteine (SAEC), 750 µmol/L, was added. Prior to amino acid detection, remaining proteins were removed by treatment with 10% sulfosalicylic acid (SSA). Plant amino acids were detected and quantified by automated ion-

exchange chromatography with post column ninhydrin derivatization on a JOEL AminoTac JLC-500/V (Tokyo, Japan).

cDNA synthesis

RNA was isolated with RNaesy kit (Qiagen, Venlo, The Netherlands) and treated with the RNase-free DNase set (Qiagen) from approximately 100 mg tissue from 10 day old seedlings. The total RNA yield was quantified by using an UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo(dT)$_{15}$ (Promega, Madison, WI, USA), according to the manufactures instructions.

HSK constructs

Complementation lines were generated by transforming *dmr1* plants by the floral dipping method with *Agrobacterium tumefaciens* strain containing the At2g17265 gene from Col-0 behind the 35S promoter. The construct was generated by PCR amplification of the full length At2g17265 gene from Col-0 cDNA with primers which included restriction sites that were used for directional cloning. At2g17265 was directionally cloned after digesting pBn 35S-Tn with BamHI and EcoRI. A forward primer (For.HSKclonAT._BamHI: 5'-CTCATTACTGGATCCTCAATGGCAAGTCT-3') containing a BamHI restriction site near the start, ATG codon, amplified the 5'-end of the *DMR1* and at the 3'-end at stop codon site an EcoRI site was generated with a reverse primer (Rev.HSKclonAT._EcoRI: 5'-GTTCCAATCTTAACGAATTCAAACAGCACAC-3') and the insert was cloned into the pGreenII0229 (Hellens et al., 2000). Transformed seeds with the 35S::HSK construct expressed the BASTA resistance gene. 300 µM DL-Phosphinothricin (BASTA) was sprayed on 10 day old seedlings and repeated 3 times, resistant seedlings (T_1) were transferred and the next generation, the T_2 was analyzed for *H. parasitica* susceptibility. HSK expression levels were quantified by Q-PCR according manufactures guidelines (Applied Biosystems, Foster City, CA, USA). cDNA was generated according the previous described isolation method. The reporter dye SYBR GreenI was detected with the ABI PRISM 7700 sequence detection system from Applied

Biosystems to determine the (HSK_QPCRF: 5'- ATTTGGGACCAGGGTTTGA -3' by Q-PCR and HSK_QPCRR: 5'- GAGACCTCACCGGCTCGTA -3').

Over expressing lines of DMR1

DMR1 over expressing lines were made by transforming Col-0 and L*er eds1-2* plants by the floral dipping method with *Agrobacterium tumefacien* strains containing the construct generated for the complementation of *dmr1*.

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Chapter 4

The Arabidopsis defence-associated *DMR6* gene is required for downy mildew susceptibility.

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Abstract

The Arabidopsis mutant *downy mildew resistant 6* (*dmr6*) carries a recessive mutation that leads to loss of susceptibility to *Hyaloperonospora parasitica*. Here we describe the map-based cloning of the *DMR6* gene encoding for an oxidoreductase (At5g24530) with unknown function. In the *dmr6-1* mutant a single base pair change was uncovered that results in a premature stop codon. The requirement of *DMR6* in susceptibility was confirmed in lines homozygous for a second loss-of-function allele, *dmr6-2*, which was identified in a T-DNA insertion line. Complementation of the *dmr6-1* mutant by transformation with the At5g24530 gene resulted in restoration of susceptibility to *H. parasitica*, confirming that At5g24530 is indeed *DMR6*. Expression analyses revealed a dual role of *DMR6* during pathogen infection. In wild type plants, *DMR6* expression is induced in response to pathogen infection and treatment with salicylic acid, suggesting a role in plant defence. However, in *dmr6* mutants, the absence of a functional DMR6 protein results in downy mildew resistance that is associated with the enhanced expression of a set of defence-related transcripts, including *DMR6*. The potential role of DMR6 in determining susceptibility or resistance to *H. parasitica* is discussed.

Keywords: Arabidopsis, downy mildew resistance, compatibility, disease susceptibility

Introduction

Plants are challenged with a large variety of biotic and abiotic stresses. Despite their immobile nature they are able to cope with most forms of stress, including the attack of pathogens. However, plants are susceptible to a limited number of often specialized pathogen species. In such compatible plant-pathogen interactions one can envisage that the plant is actively involved in

supporting the growth and development of the pathogen. The host proteins involved in establishing this basic compatibility can be considered susceptibility factors. Absence of such a host susceptibility protein, e.g. due to a mutation, could lead to reduced susceptibility or resistance. The dependence on the host is particularly important for obligate biotrophic pathogens that require living plant tissue for their growth and reproduction. Most biotrophic fungi and oomycetes form specialized feeding structures, haustoria, within the host cells they infect. Haustoria are believed to be important for nutrient uptake. In the rust pathogen *Uromyces fabae*, e.g., sugar and amino acid transporters, i.e. the D-glucose and D-fructose transporter, HXT1 (Voegele et al., 2001) and the amino acid transporters, AAT1 and AAT2 (Struck et al., 2002) are specifically localized to the pathogen's haustorial membrane. It remains unclear how the plant contributes to the transport of nutrients over the plant cell membrane (the extra-haustorial membrane). Besides the obvious feeding relation, hardly anything is known about other aspects of disease susceptibility to fungal and oomycete biotrophs, i.e. the production of signals for pathogen development, the accommodation of infection structures, and the vulnerability to suppression of plant defence responses. Genetic studies on Arabidopsis have great potential to identify genes that are important for compatibility to biotrophic pathogens, i.e. the powdery mildew fungus *Erysiphe cichoracearum* and the downy mildew oomycete *Hyaloperonospora parasitica*. *E. cichoracearum* grows epicuticular forming haustoria from the outside in the epidermal cells. To identify compatibility genes required for powdery mildew susceptibility, Vogel and colleges have isolated twenty-six recessive Arabidopsis *powdery mildew resistance* (*pmr)* mutants (Vogel and Somerville, 2000). Four of the corresponding powdery mildew resistance (*PMR)* genes have been cloned and characterized. *PMR4* (*GLS5=CalS12*) encodes a callose synthase (Nishimura et al., 2003). The *pmr4* mutant could no longer induce a callose response and showed an enhanced activation of salicylic acid-dependent defence genes. *PMR6* encodes a pectate lyase-like gene, and *PMR5* is a gene of unknown function belonging to a large family of plant-specific genes. The cell-wall composition of both *pmr5* and *pmr6* is altered, in particular the levels of pectin

are increased (Vogel et al., 2002; Vogel et al., 2004). Recently, *PMR2* was identified as *AtMlo2*, which is an Arabidopsis ortholog of the Barley *mlo* gene (Consonni et al., 2006). Barley *mlo* encodes a plasma membrane protein with seven transmembrane domains (Buschges et al., 1997). The MLO protein is required for successful entry of the host cell by the powdery mildew pathogen *Blumeria graminis* f.sp. *hordei* (Panstruga, 2005).

In contrast to the powdery mildews, downy mildews do penetrate into the host cell tissue and grow intercellularly, forming haustoria in mesophyll and epidermal cells (Koch and Slusarenko, 1990). A loss-of-susceptibility mutant screen in Arabidopsis resulted in the identification of twenty downy mildew resistant (*dmr*) mutants, of which eight were studied in more detail that corresponded to six independent loci, *dmr1* to *dmr6* (Van Damme et al., 2005). The *dmr3*, *dmr4* and *dmr5* mutants showed elevated levels of *PR-1* gene expression in the absence of pathogen infection indicating that these are enhanced defence-response mutants. The *dmr1*, *dmr2* and *dmr6* mutants are postulated as mutants in which cellular processes, required for downy mildew infection are disturbed. The corresponding Arabidopsis *DMR* gene products could contribute to the *H. parasitica* infection process. Recently, we have cloned the *DMR1* gene which was found to encode for homoserine kinase (HSK). In *dmr1* plants homoserine accumulates as a result of reduced homoserine kinase activity (Chapter 3). Infiltration of homoserine to susceptible Arabidopsis lines was found to results in resistance to *H. parasitica* similar to that observed in the *dmr1* mutants.

Here we describe the cloning and characterization of *DMR6* which encodes for a predicted oxidoreductase. *DMR6* expression is locally induced by *H. parasitica*, both in compatible and incompatible interactions. Transcript profiling of the *dmr6-1* mutant showed induced expression of a pronounced set of defence-related genes including *DMR6.* The dual activity of *DMR6* in both susceptibility and defence will be discussed.

Results

Cloning of DMR6

Multiple *dmr6-1* back-cross 2 (BC_2) lines were generated in the parental L*er eds1-2* and in the L*er* background. The reduced susceptibility of the BC_2 lines was determined by quantification of *H. parasitica* sporulation (Figure 1A). The *dmr6-1* BC_2 line number E37 showed a ten fold reduction in sporulation when compared to the parental line, L*er eds1-2*. Similar results were obtained for other independent *dmr6-1* BC_2 lines. Previously, we mapped the recessive *dmr6-1* mutation near the nga139 marker on chromosome 5 to a region encompassing 74 genes. Fine mapping linked the *H. parasitica* resistance in the *dmr6-1* interval to the chromosomal region that is covered by the BACs T13K7 and K18P6 in between two markers located in the genes At5g24420 and At5g24590. This allowed the *dmr6-1* interval to be confined to 18 candidate genes. Comparative sequence analysis of the 18 genes in *dmr6-1* and the parental line, L*er eds1-2*, revealed a point mutation in the second exon of the At5g24530 gene. This single base change of G to A, typical for an EMS mutation, changes TGG (trp codon) to a TGA (premature stop codon) at nucleotide position 691 of the genomic sequence (Figure 1C). The At5g24530 gene is predicted to encode for an oxidoreductase protein with a mass of 39.4 kDa. The premature stop codon truncates the predicted oxidoreductase enzyme of 342 aa at position 141 before the conserved catalytic domain suggesting that *dmr6-1* is a null-allele. Oxidoreductases are enzymes that catalyze the transfer of electrons from one molecule, the oxidant, to another, the reductant. No biological function has so far been described for At5g24530.

A second allele, *dmr6-2*, was identified in a T-DNA insertion line (FLAG_445D09) from the mutant collection from INRA, Versailles. The presence and location of the T-DNA insert in the second intron of At5g24530 (Figure 1C) was confirmed by PCR and sequence analysis (data not shown). Progeny of the FLAG_445D09 line homozygous for the T-DNA insertion was resistant to *H. parasitica* isolate Waco9, whereas the parental line (Ws-4) was susceptible (Figure 1A). The At5g24530 transcript could be amplified by RT-

PCR using primers in exon 2 and 3 in Ws-4, but not in the homozygous *dmr6-2* line (data not shown), indicating that *dmr6-2* can be considered a second null-allele.

To corroborate the idea that At5g24530 is required for susceptibility to *H. parasitica* the *dmr6-1* mutant was transformed with the cDNA from At5g24530 cloned under control of the 35S promoter. In five independent *dmr6-1* T_2 seedlings the overexpression of At5g24530 was confirmed by RT-PCR (data not shown). All T_3 lines showed restoration of susceptibility to *H. parasitica* isolate Cala2 (Figure 1B), confirming that At5g24530 is *DMR6*. The complementation, together with the identification of two independent *dmr6* mutants clearly indicates that a functional At5g24530 gene is required for susceptibility to *H. parasitica*.

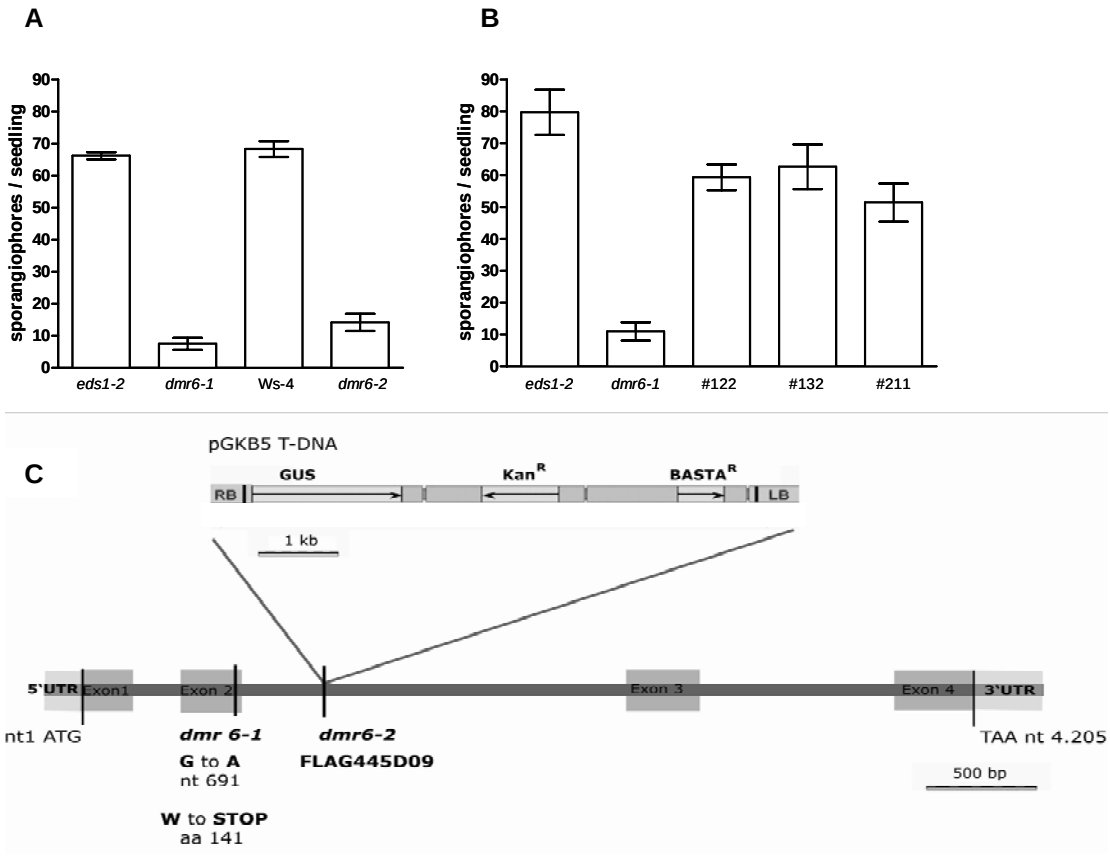

Figure 1. Downy mildew resistance of the *dmr6* mutants. **A**. Quantification of sporangiophores of *H. parasitica* isolate Waco9, 7 days post inoculation, on the *dmr6-*

1 mutant (BC_2, line E37) compared to its parental line L*er eds1-2* and on the *dmr6-2* mutant (FLAG_445D09 T-DNA line) compared to its parental line Ws-4. **B**. Restoration of susceptibility by complementation with the At5g24530 gene in the *dmr6-1* mutant. *H. parasitica* sporangiophores per seedling were quantified on L*er eds1-2*, *dmr6-1* and 3 complementation lines, #122, #132 and #211. **C**. The *DMR6* gene contains four exons and a coding sequence of 1026 bases. The two alleles are indicated; *dmr6-1* with a base change in exon 2, and *dmr6-2* with a T-DNA insertion in intron 2.

DMR6 is transcriptionally activated during H. parasitica infection

To study the expression of *DMR6* during infection with *H. parasitica* relative transcript levels were measured by quantitative PCR at six different time points from 0 days post inoculation (dpi) to 5 dpi (Figure 2). RNA was extracted from ten day old L*er* seedlings that were sprayed with water (mock), a compatible, or an incompatible *H. parasitica* isolate. At 0 dpi (2 hours pi) no significant difference in *DMR6* transcripts could be detected between the different treatments. At 1 dpi the level of *DMR6* transcript was significantly increased in both the compatible and incompatible interaction compared to mock-treated seedlings. The *DMR6* transcript level was slightly but significantly higher at 1 dpi in the incompatible (ΔC_T of 3.5, approximately 11 fold induction) than in the compatible interaction (ΔC_T of 3.0, approximately 8 fold induction). Expression levels increased in time until 4-5 dpi. At these time points the *DMR6* transcript levels were higher in the compatible than in the incompatible interaction. The elevated *DMR6* transcript levels during compatible and incompatible *H. parasitica* interactions suggest a role of *DMR6* in plant defence. The defence-associated expression of *DMR6* was also found in three of our enhanced-defence mutants, *dmr3*, *dmr4*, and *dmr5* (Van den Ackerveken et al., unpublished). Furthermore, *in silico* analysis of *DMR6* levels in the Genevestigator Mutant Surveyor (Zimmermann et al., 2005) showed that the gene is also strongly induced in the pathogen resistant mutants *mpk4* and c*pr5*. In the *cpr5/npr1* double mutant the *DMR6* transcript level remained high indicating that the induction of *DMR6* expression is mostly *NPR1* independent. Salicylic acid appears to be an important signal in the induction of *DMR6* expression during senescence as *nahG* transgenic plants

(expressing the bacterial salicylate hydroxylase gene) showed only low levels of *DMR6* transcript.

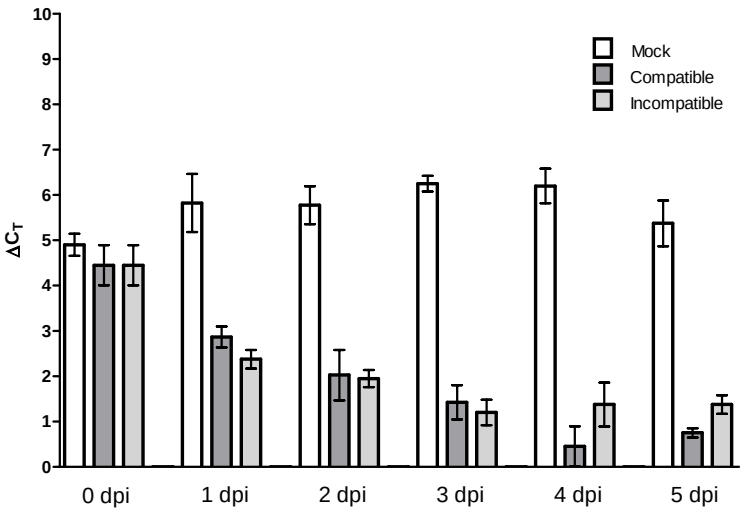

Figure 2. Relative transcript levels of *DMR6* in L*er* plants either mock treated or inoculated with a compatible or incompatible *H. parasitica* isolate. Transcript levels were determined at different days post inoculation. The difference in cycle threshold (ΔC_T) values reflect the number of additional PCR amplification cycles required to reach an arbitrary threshold product concentration as compared to *ACTIN2*. A lower ΔC_T value indicates a higher transcript level.

Responses to biotic and abiotic stress were further analysed *in silico* using the Genevestigator Response Viewer (Zimmermann et al., 2004). *DMR6* was found to be induced after treatment by salicylic acid (SA) (Log-ratio 3.67) and abscisic acid (ABA) (Log-ratio 1.2). Furthermore, *DMR6* was also found to be induced during senescence (Log-ratio 3.47), after exposure to high levels of ozone (Log-ratio 1.96) or low levels of nitrate (Log ratio 1.36), and during osmotic (Log-ratio 1.13) and cold stress (Log ratio 1.22). In response to pathogens, the *DMR6* gene was induced after infection with *Phytophthora infestans* (Log-ratio 1.9), and *Erysiphe cichoracearum* (Log-ratio 1.71). Overall, these data show that *DMR6* is induced by a large variety of abiotic and biotic stress responses, of which many can be linked to plant defence.

To investigate in more detail how the expression of *DMR6* is activated during biotic and abiotic stress, *DMR6* reporter lines were generated. The localisation of *DMR6* expression was studied in transgenic Col-0 and L*er eds1-2* plants containing the *DMR6* promoter linked to the *uidA* (β-glucuronidase, GUS) reporter gene (p*DMR6*::GUS). To visualise both *H. parasitica* hyphal growth,

by staining with trypan blue, as well as GUS activity, magenta-Xgluc was used as a β-glucuronidase substrate yielding a magenta precipitate. In uninfected plants no GUS expression could be detected in the different plant organelles; roots, meristem, flower, pollen and seed. The expression of *DMR6* was induced in the compatible interactions, L*er eds1-2* infected with Cala2 (Figure 3A), and Col-0 infected with isolate Waco9 (Figure 3B). GUS expression was also induced in the incompatible interaction L*er eds1-2* inoculated with isolate Emoy2 (Figure 3C). As shown in figure 3A and 3B, *DMR6* expression was confined to the cells in which *H. parasitica* had formed haustoria. Plant cells containing the most recently formed haustoria did not show detectable levels of GUS activity (Figure 3A, indicated by asterisk). During the incompatible interaction (Figure 3C) activity of the *DMR6* promoter could only be detected in the cells that were in contact with the initial invading hyphae and haustoria. In death cells, resulting from the hypersensitive response (HR, visualized by trypan blue staining indicated in Figure 3C by asterisk) no GUS activity could be detected, possibly due to protein degradation in these cells. To test if the *DMR6* expression in haustoria-containing cells is caused by a wound-like response, seedlings were wound by incision with scissors and stained for GUS activity 3 days later. No detectable GUS expression was seen, indicating that the expression of *DMR6* is not induced by wounding (Figure 3D). Furthermore the local induction of *DMR6* expression was tested in response to treatment with benzothiadiazole (BTH), a functional analogue of salicylic acid (SA). At 3 days post BTH treatment GUS activity was mainly localized in the newly formed, but not in the mature leaves (Figure 3E). Analysis of p*DMR6*::GUS lines confirm the Q-PCR expression data described above and highlights the strictly localized induction of *DMR6* in response to *H. parasitica* infection.

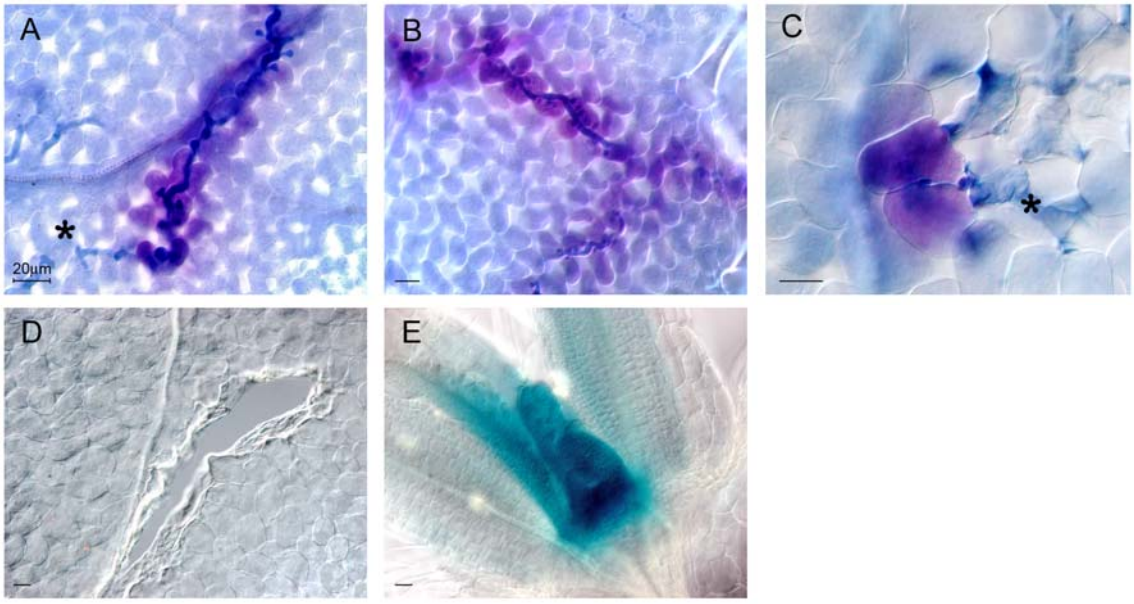

Figure 3. p*DMR6*::GUS expression studies of transgenic Arabidopsis lines, visualized with only X-gluc as substrate(Figure D and E) or Magenta-Xgluc (Figure A-C) and trypan blue staining of *H. parasitica* growth (Scale bar size is 20 µm). **A**. L*er eds1-2* (p*DMR6*::GUS) 3 dpi with *H. parasitica* isolate Cala2. **B**. Col-0 (p*DMR6*::GUS) 3 dpi with *H. parasitica* isolate Waco9. **C**. L*er eds1-2* (p*DMR6*::GUS) 3 dpi with *H. parasitica* isolate Emoy2. **D**. Col-0 (p*DMR6*::GUS) 3 days after wounding. **E**. Col-0 (p*DMR6*::GUS) 3 days after BTH application.

The dmr6-1 mutant constitutively expresses defence associated transcripts

To elucidate how the lack of *DMR6* results in *H. parasitica* resistance, the transcriptome of the *dmr6-1* mutant compared to the L*er eds1-2* parental line was analysed. Probes derived from mRNA of the above-ground parts of 14 day old *dmr6-1* and L*er eds1-2* seedlings were each hybridised to four 25k CATMA arrays (Allemeersch et al., 2005; Hilson et al., 2004). A total of 58 genes were found to be significantly differentially expressed in *dmr6-1* (Table 3A and 3B of the supplementary data). The differentially expressed genes found were predominantly up regulated (51 genes) and associated with activated plant defence responses, e.g., *ACD6*, *PR-5*, *PR-4/HEL* and *PAD4*. These data indicate that loss of *DMR6* results in the activation of a specific set of defence-associated transcripts. The finding that *DMR6* is among the *dmr6-1*-induced

genes corroborates the idea that *DMR6* is defence-associated. To test if the induced expression of the defence-associated genes was due to the loss of *DMR6* and not due to additional EMS mutations remaining in the backcrossed *dmr6-1* mutant the transcript level of a selection of genes (At4g14365, At1g14880, *ACD6*, *PR-1*, *PR-2* and *PR-5*) was verified by quantitative PCR in both the *dmr6-1* and *dmr6-2* mutant. *PR-1* (not on the CATMA array) and *PR-2* were included because these genes are generally used as markers for defence gene expression (Figure 4). *DMR6* transcript level was only tested in the *dmr6-1* mutant (Figure 4A) because the *dmr6-2* mutant (Figure 4B) has a T_DNA insertion disrupting the *DMR6* transcript. The induction of *DMR6* as observed in the micro array analysis was confirmed by Q-PCR in *dmr6-1* compared to L*er eds1-2* (Figure 4A). Figure 4A and B show that all six defence genes were indeed elevated in both *dmr6* mutants as compared to the parental lines and this could be the primary cause of *H. parasitica* resistance. Figure 4A also includes one of the three complementation lines of *dmr6-1* that were verified by Q-PCR for the expression of the seven tested genes. All three complementation lines, #122, #132 and #211, were susceptible for *H. parasitica* (Figure 1B). The restoration of defence gene expression to wild-type levels in the complementation line #132 (Figure 4A) demonstrates that the mutation in the At5g24530 gene in *dmr6-1* is responsible for the induced expression levels of the six defence associated genes tested. The up regulated expression levels of the six defence associated genes in the *dmr6-1* mutant are confirmed in the *dmr6-2* mutant in Figure 4B. The relative transcript levels of the six tested genes in the *dmr6-2* (Figure 4B) are higher then in the *dmr6-1* mutant. This could be due to the difference in genetic background, as *dmr6-2* is in the Ws-4 and *dmr6-1* in the L*er eds1-2* background.

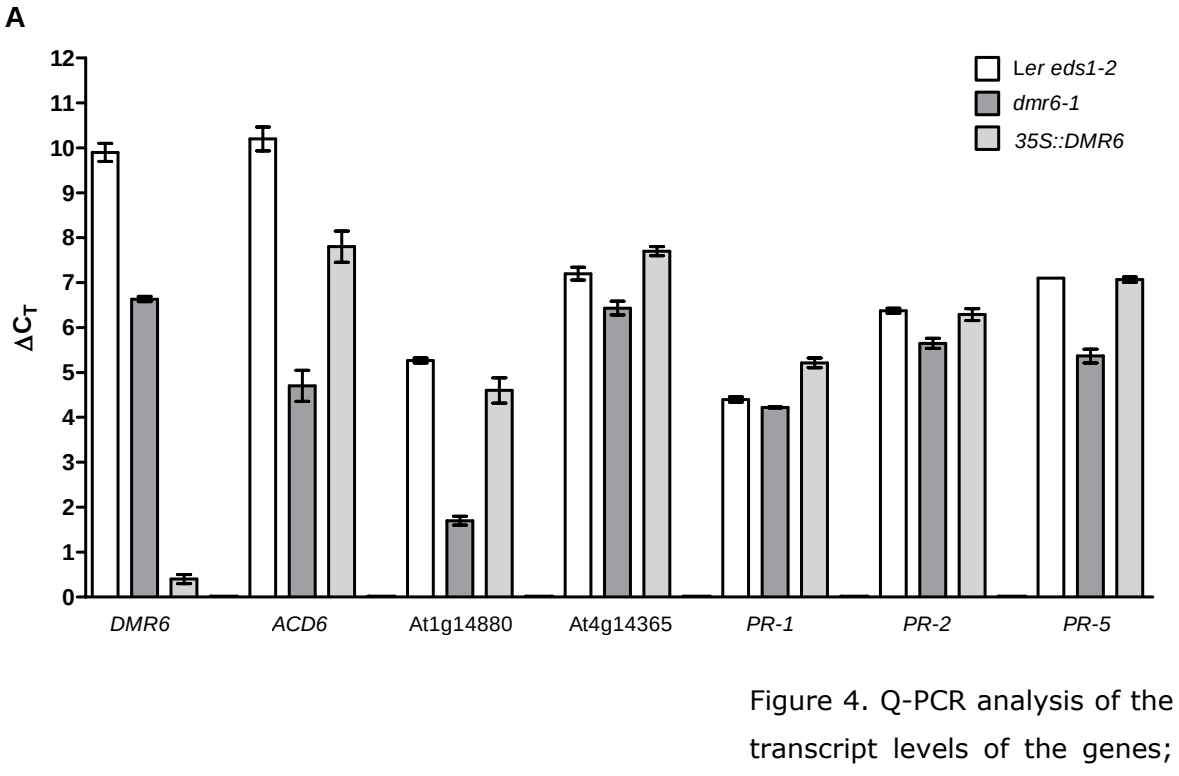

Figure 4. Q-PCR analysis of the transcript levels of the genes; At4g14365, At1g14880, *ACD6*, *PR-1*, *PR-2* and *PR-5*, selected as up regulated in the *dmr6-1* micro array analysis. **A**. Transcription levels of the six genes and additionally the *DMR6* transcript in L*er eds1-2* compared to *dmr6-1* and in the complementation line #132. **B**. Elevated transcripts of six defence-associated genes in *dmr6-2* versus Ws-4. ΔC_T reflects the number of additional PCR amplification cycles required to reach the level of *ACTIN2* transcripts. A lower ΔC_T value indicates a higher transcript level.

To analyse if higher levels of *DMR6* transcript result in increased or reduced *H. parasitica* susceptibility, the 35S::*DMR6* construct was transformed into L*er*

eds1-2 and Col-0 plants. All the transgenic *DMR6* over expression lines were verified for elevated *DMR6* expression by RT-PCR. Over expression of *DMR6* did not result in higher levels of *H. parasitica* susceptibility, compared to the parental lines (data not shown).

Discussion

The *dmr6* locus was identified in a genetic screen for loss of susceptibility to the downy mildew pathogen *H. parasitica* (Van Damme et al., 2005). *DMR6* was map-based-cloned and identified as gene At5g24530, encoding for an oxidoreductase. An additional allele, *dmr6-2*, was identified in line FLAG_445D09 that has a T-DNA insertion in the At5g24530 gene. Both the *dmr6-1* and *dmr6-2* mutants show a strongly reduced susceptibility to *H. parasitica.* Overexpression of the oxidoreductase gene At5g24530 restored susceptibility to *H. parasitica* in the *dmr6-1* mutant thereby confirming that the At5g24530 gene is required for susceptibility to *H. parasitica* and that it is indeed *DMR6*. Overexpression of the *DMR6* gene also restored the expression of the six defence associated genes to the wild type levels tested in the *H. parasitica* susceptible complementation lines. This demonstrates that the *dmr6* mutation induces both defence gene expression and *H. parasitica* resistance, suggesting that there is a causal relationship between the two. The DMR6 protein is a member of the 2OG-Fe(II) oxygenase family of oxidoreductases that are characterized by the presence of the conserved Pfam PF03171 2OG-Fe(II) oxygenase superfamily domain, which is identified in 242 so far known and predicted Arabidopsis proteins. Although At5g24530 is annotated as "similar to flavanone 3-hydroxylase (F3H)" the identity to F3H (At3g51240) is less than 35% at the amino acid level. Ten other proteins annotated as oxidoreductases in Arabidopsis have a higher homology to DMR6 than F3H, but for none of these proteins the biological function has been demonstrated. As the *dmr6* mutants do not show an altered seed colour or other anthocyanin-related phenotypes it is unlikely that the *DMR6* gene plays an important role in the flavanoid and anthocyanin metabolic pathways. Metabolic profiling of the *dmr6* mutants will be pursued to reveal the possible

substrate(s) and product(s) of the DMR6 oxidoreductase enzyme. In Arabidopsis 283 genes are associated with the gene ontology (GO) term "oxidoreductase" at 'The Arabidopsis Information Resource' (TAIR) in the category "Molecular Function". Of these, 88 belong to the category of oxidoreductases that use 2-oxoglutarate as one of the two donors. Known members of the 2-oxyglutarate and Fe(II) dependent oxygenase superfamily include flavanone 3-hydroxylase (F3H), flavanol synthase, gibberellin oxidase and dioxigenase, 1-aminocyclopropane-1-carboxylate oxidase, and leucoanthocyanidin dioxygenase.

Enzymes that act in a metabolic pathway often show co-regulation at the transcriptional level. A good example is the co-regulation of genes in the flavanoid biosynthesis i.e. 4-coumarate-ligase, chalcone synthase, chalcone isomerase and flavanone-3 hydroxylase (Claire et al., 2005). Preliminary data derived from expression analysis using the expression angler tool (Toufighi et al., 2005) indicate that no other genes encoding metabolic enzymes show a clear co-regulation with *DMR6*. Although this does not mean that DMR6 is not part of a metabolic pathway it may suggest that DMR6 acts on its own on a particular substrate and that the derived product is not further metabolized to other compounds.

DMR6 expression analysis by quantitative PCR and promoter GUS lines revealed that the gene is strongly induced in the interaction with compatible and incompatible *H. parasitica* isolates, suggesting that it is infection-associated. However, lack of the DMR6 protein, in the *dmr6* mutants, does not result in reduced defence but provides resistance, suggesting a dual role of DMR6 during pathogen infection. A set of defence-associated genes that are constitutively upregulated in the *dmr6* mutants could be responsible for the observed resistance. However, the question remains why the *dmr6-1* mutant is only resistant to *H. parasitica* and *C. higginsianum* (O'Connell and Panstruga, 2006), and not to *Pseudomonas syringae*, *Golovinomyces orontii* (Van Damme et al., 2005) and the white rust oomycete, *Albugo candida* (E.

Holub, personal communication). If the enhanced defence is causing the resistance one would expect that the *dmr6* mutants would show broad resistance. Possibly, the subset of defence genes that is induced in the *dmr6* mutants only provides protection against *H. parasitica* and *C. higginsianum*. The mechanism of regulation of this subset of genes through mutation of *DMR6* is unknown. One could envisage that due to loss-of-function of DMR6 a substrate accumulates that induces a subset of defence genes. This phenomenon was observed in the *dmr1* mutant, where the accumulation of homoserine, the substrate of the *DMR1* encoded HSK enzyme, leads to loss of susceptibility. Alternatively, the product of the *DMR6* encoded oxidoreductase could be an important metabolite that is required for disease development in the Arabidopsis-*H. parasitica* interaction. Such a compound might act as a trigger for pathogen development or be important on the plant side where it aids accommodation of the pathogen. In this case the activation of defence gene expression would be a secondary effect. Genetic studies using defence regulatory mutants will reveal whether the enhanced defence gene expression is an essential component of *dmr6*-mediated resistance. Future studies aimed at identifying the DMR6 substrate and product, as well as genetic suppressor studies, will increase our understanding on the role of the *DMR6*-encoded oxidoreductase in both defence and susceptibility to downy mildew.

Supplementary data

Table 1. IND markers used for fine mapping of *DMR6*.

Name primer	Gene	Forward primer	Reverse primer
IND_MOP9	At5G24210	tttgggaacagaaaaagttggaggt	catattcaaaagggaaaatcccaga
IND_K16H17	At5g24420	tggggttgtggtttattctgttgac	tggccaatagtagttgatacgcaaga
IND_T4C12	At5g24820	tctcgggtaagacacaagtcgagat	tattccaacttgcgacgtagagcat
IND_T11H3	At5g24950-60	ccaattgggttatttacttcgatt	cggcttttaacaacatattttcca
IND_F21J6	At5g25270	aacacatcaccaagatgaatccaga	cctctgccccaagaaatattgagat

Table 2. Additional IND and CAPS markers used for final fine mapping of *DMR6*.

Name primer/ Gene	INDEL/ enzyme	Forward primer	Reverse primer
AT5G24450	18	agctttgtatggtagtgccaatga	gcggtatacgggggttaaaatcta
At5g24490	TaqI	atggccaaccactctttgttac	acaagcaagaagaacagcgaag
At5g24520-30	TaqI	gaaatttggttgttggcatttatc	tcaagatcttcatattctcattcca
At5G24540/50	41	cagctgaagtatgtttcatcccttt	cttgcaattgttgggactaggtaa
At5G24550/60	14	tcactaaccagtgaaaaaggttgc	tatacagcgaatagcaaagccaag
At5g24470	HphI	ccgcgagtgtaatatatctctcct	cagtttaacgcatgaagtgctagt
At5g24590	PdmI	gcatcatttgtaccgtactgagtc	tagtggatactctgtccctgaggt

Table 3**A**. Arabidopsis transcripts that are more than 2 fold induced in the *dmr6-1* mutant compared to L*er eds1-2* (Pearson correlation coefficient < 0.05)

AGI code	CATMA ID	Log ratio	P Value	Gene	Annotation
At1g14880	1a13910	4.044	0.001		unknown protein
At1g31580	1a29810	3.652	0.001	ECS1	Encodes cell wall protein. ECS1
At5g10760*	5a09500	3.132	0.001		aspartyl protease family protein
At3g48640	3a41600	2.910	0.003		hypothetical protein
At2g25510	2a23850	2.850	0.001		expressed protein
At5g45380	5a41330	2.696	0.008		sodium:solute symporter family protein
At4g14400	4a14770	2.686	0.002	ACD6	ACD6, ankyrin repeat family protein
At1g35710	1a33890	2.681	0.003		leucine-rich repeat transmembrane protein kinase
At5g10760*	4a06910	2.384	0.004		aspartyl protease family protein
At5g54610	5a50460	2.123	0.004		Induced in response to salicylic acid,belongs to the ankyrin repeat protein family.
At2g41090	2a39435	2.120	0.001		calmodulin-like calcium-binding protein, 22 kDa (CaBP-22)
At5g24530	5a22130	1.910	0.003	DMR6	oxidoreductase, 2OG-Fe(II) oxygenase family protein
At5g03350	5a02460	1.782	0.003		legume lectin family protein
At3g01290	3a00270	1.778	0.003		band 7 family protein, similar to hypersensitive-induced response protein
At3g23120	3a23080	1.603	0.004		leucine-rich repeat family protein, contains leucine rich-repeat (LRR) domains
At1g32750	1a31080	1.597	0.006	HAC13	HAC13 protein (HAC13), identical to HAC13 (Arabidopsis thaliana)
At1g75040	1a64376	1.557	0.006	PR-5	pathogenesis-related protein 5 (PR-5),Thaumatin family
At2g31880	2a30140	1.505	0.004		leucine-rich repeat transmembrane protein kinase, putative
At4g14365	4a14700	1.504	0.024		zinc finger (C3HC4-type RING finger) family protein/ankyrin repeat family protein
At1g73800	1a63120	1.494	0.005		calmodulin-binding protein, similar to calmodulin-binding protein
At4g02520	4a02835	1.489	0.005		Encodes glutathione transferase belonging to the phi class of GSTs.
At3g56710	3a49680	1.485	0.013		Sig1 binding protein; interacts with Sig1R4.
At3g04720	3a03743	1.473	0.005	PR-4	Encodes a protein similar to the antifungal chitin-binding protein hevein latex
At3g04210	3a03160	1.452	0.005		disease resistance protein (TIR-NBS class)
At1g69730	1a59040	1.446	0.007	WLK	WAK-like kinase (WLK)
At1g03850	1a02710	1.401	0.030		disease resistance protein (TIR-NBS class)
At1g27570	1a25800	1.336	0.008		phosphatidylinositol 3- and 4-kinase family protein
At1g72930	1a62155	1.322	0.028		disease resistance protein (TIR class), putative
At1g66970	1a56260	1.292	0.023		glycerophosphoryl diester phosphodiesterase family protein
At2g42320	2a40710	1.269	0.006		nucleolar protein gar2-related
At1g19960	1a18950	1.244	0.025		expressed protein
	2a05820	1.235	0.019		unknown protein
At1g72020	1a61280	1.227	0.029		DNAJ heat shock N-terminal domain-containing protein
At5g42520	5a38290	1.225	0.008		expressed protein
At5g11780	5a10570	1.211	0.013		expressed protein
At5g60900	5a56585	1.190	0.031		S-receptor kinase homolog 2 precursor
At4g23170	4a24930	1.180	0.027		Induced in response to Salicylic acid.Similar to receptor-like kinase 4 and 5.
At1g45145	1a38000	1.160	0.029		encodes a cytosolic thioredoxin
At1g21270	1a20325	1.152	0.014		wall-associated kinase 2
At4g25940	4a27612	1.149	0.015		epsin N-terminal homology (ENTH) domain-containing protein
At1g49750	1a40830	1.145	0.032		leucine-rich repeat family protein
At1g23840	1a22710	1.125	0.020		expressed protein
At5g50200	5a46160	1.122	0.045		High-affinity nitrate transporter. Up-regulated by nitrate.
At5g62630	5a58220	1.108	0.016		expressed protein
At4g13820	4a14000	1.096	0.030		disease resistance family protein / LRR family protein
At3g52430	3a45345	1.061	0.043	PAD4	PAD4 Encodes a lipase-like gene
At1g70620	1a59900	1.055	0.029		cyclin-related
At2g26440	2a24760	1.053	0.017		putative pectinesterase
At5g20230	5a18670	1.045	0.016		blue copper binding protein
At2g24160	2a22500	1.033	0.020		leucine rich repeat protein family
	1a23020	1.027	0.018		unknown protein
At1g52240	1a43280	1.025	0.045		Encodes a member of KPP-like gene family

Table 3**B**. Arabidopsis transcripts that are more than 2 fold repressed in the *dmr6-1* mutant compared to L*er eds1-2* (Pearson correlation coefficient <0.05)

AGI code	CATMA ID	Log ratio	P Value	Gene	Annotation
At2g35200	2a33340	-1.374	0.020		hypothetical protein
At5g15260	5a13540	-1.301	0.007		genomic putative protein
At5g10430	5a09183	-1.301	0.015	AGP4	arabinogalactan-protein (AGP4)
At3g25190	3a25040	-1.247	0.020		integral membrane protein, putative, similar to nodulin 21 (N-21)
At5g58390	5a54150	-1.201	0.022		peroxidase, putative, similar to peroxidase (Nicotiana tabacum)
At5g14920	5a13190	-1.095	0.038		gibberellin-regulated family protein,similar to proline-rich family protein
At4g22480	4a24200	-1.010	0.025		putative glycine-rich protein

Materials and Methods

Hyaloperonospora parasitica growth and infection

H. parasitica isolates Waco9, kindly provided by Dr. M. Aarts (WUR, Wageningen, N.L.) and Cala2 kindly provided by Dr. E. Holub (Warwick HRI, Wellsbourne, U.K.) were maintained on Arabidopsis Ws-0 and L*er* respectively. Inocula (4 x 10^5 spores ml^{-1}) were weekly transferred to 10 day old healthy seedlings (Holub et al., 1994) by use of a spray gun. Seedlings were air-dried for approximately 45 minutes and incubated under a sealed lid at 100% relative humidity in a growth chamber at 16°C with 9 hours of light per day (100μE/m2/s). The sporulation levels were quantified 7 days post inoculation (dpi) by counting the number of sporangiophores per seedling, for at least 40 seedlings per tested line (Figure 1B).

Generation of backcrossed dmr6-1 lines

The *dmr6-1* mutants were back crossed twice (BC_2) to the parental line L*er eds1-2* as well as to L*er*. The BC_2 lines generated with L*er* were selected for the presence of the wild type *EDS1* gene by PCR analysis.

Cloning DMR6

Fine mapping of the *DMR6* gene was done with PCR markers designed using the Cereon database to identify insertion and deletion (IND) differences between Col-0 and L*er*. The markers: IND_MOP9 in gene At5G24210; IND_K16H17 in gene At5G24420; IND_T4C12 in gene At5G24820; IND_T11H3 in between genes At5G24950_60 and IND_F21J6 in gene At5G25270 were used for mapping (Table 1). An additional screen for new recombinants was initiated on 300 F_2 plants resulting in eight F_2 recombinant plants between the two IND based markers IND_MOP9 and IND_T4C12, which flanked a region of 61 genes. Six additional IND markers (Table 2) reduced the region to eighteen candidate genes for the *dmr6* locus, between

At5g24420 and At5g24590. Sequence analysis of At5g24530 indicated a point mutation leading to a stop codon in exon 2 result in the *dmr6-1* mutant.

Identification of T-DNA insertion line

A second *dmr6* allele was identified, 445D09, a FLAG T-DNA insertion line generated by INRA Versailles in the Ws-4 accession background (Samson et al., 2002). The T-DNA insertion was confirmed by PCR using a primer designed in the At5g24530 gene, LP primer (5'-CAGGTTTATGGCATATCTCACGTC-3'), in combination with the T-DNA right border primer, Tag3' (5'-CTGATACCAGACGTTGCCCGCATAA-3') or RB4 (5'-TCACGGGTTGGGGTTTCTACAGGAC-3'). The exact T-DNA insertion in the second intron of At5g24530 was confirmed by sequencing of amplicons generated with the T-DNA primers from both the left and right border in combination with the gene specific primers LP or RP (5'-ATGTCCAAGTCCAATAGCCACAAG-3').

cDNA synthesis

RNA was isolated (from approximately 100 mg leaf tissue from 10 day old seedlings) with the RNeasy kit (Qiagen, Venlo, The Netherlands) and treated with the RNase-free DNase set (Qiagen). Total RNA was quantified using an UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan). cDNA was synthesized with Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and oligo$(dT)_{15}$ (Promega, Madison, WI, USA), according manufactures instructions.

Complementation of the dmr6-1 mutant

A construct was generated by PCR amplification of the full length At5g24530 gene from Col-0 cDNA with primers which included restriction sites that were used for directional cloning. A forward primer (5'-TTCTGGGATCCAATGGCGGCAAAGCTGATATC-3') containing a BamHI restriction site near the start codon (ATG), amplified the 5'-end of *DMR6* and at the 3'-end after the stop codon an EcoRI site was generated with a reverse primer (5'-GATATATGAATTCTTAGTTGTTTAGAAAATTCTCGAGGC-3'). The 35S-*DMR6*-Tn was

cloned into the pGreenII0229 (Hellens et al., 2000). Complementation lines were generated by transforming *dmr6-1* plants by the floral dip method with *Agrobacterium tumefaciens* (Clough and Bent, 1998) containing the At5g24530 gene from Col-0 behind the 35S promoter. DL-Phosphinothricin (BASTA, 300 µM) resistant seedlings were isolated and analyzed for *H. parasitica* susceptibility and for *DMR6* expression levels by RT-PCR.

Construction of DMR6 over expressing lines

DMR6 over expression lines were made by transforming Col-0 and L*er eds1-2* plants with the construct generated for the complementation of *dmr6-1*.

Promoter DMR6::GUS transgenic seedlings

A 2.486 bp fragment of the *DMR6* promoter sequence was amplified using p*DMR6*F (5'-GACTCTGTCTGAGTCTGCAGTCCCAAACCATG-3') and p*DMR6*R (5'-GCCGCCATTGGATCCCAGAA
AATTGAAGAAG-3'), generating a *PstI* and a *BamHI* restriction sites. The two restriction sites allowed cloning of the fragment into the pGREENII-0229G plasmid (http://www.pgreen.ac.uk) (Hellens et al., 2000) in front of the GUS gene. p*DMR6*::GUS transgenic lines were generated by transforming L*er eds1-2* and Col-0 plants by the floral dip method with *Agrobacterium tumefaciens* strain (Clough and Bent, 1998). p*DMR6*::GUS transgenic (T_3) seedlings were infected with *H. parasitica* and 3 dpi the seedlings were vacuum infiltrated with Magenta-Xgluc solution (50 mM $NaPO_4$ (pH 7.0), 0.5 mM $K_3FE(CN)_6$, 0.5 mM $K_4FE(CN)_6$, 0.1% TritonX-100, 0.5 mg/ml Magenta-GlcA (Duchefa)) and stained overnight at 37 °C. Seedlings were cleared with 70% EtOH followed by trypan blue staining to stain *H. parasitica* inside the seedlings.

Gene expression profiling of the dmr6 mutant

Total RNA was isolated as described above. mRNA was amplified with the MessageAmp aRNA kit (Ambion). CATMA array version 2 (Crowe et al., 2003) slides containing approximately 25.000 gene specific tags were hybridized according to standard conditions (de Jong et al., 2006). For quantitative PCR,

cDNA templates were generated as described previously. Cycle thresholds were determined per transcript in triplicate using the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I (Applied Biosystems, Foster City, CA, USA) as reporter dye. Primer sets for the transcripts are *DMR6* (QDMR6F:5'-TGTCATCAACATAGGTGACCAG-3' and QDMR6R: 5'-CGATAGTCACGGATTTTCTG TG-3'), At1g14880 (QAt1g14880F:5'-CTCAAGGAGAATGGTCCACA-3' and QAt1g14880R: 5'-CGACTTGGCCAAATGTGATA-3'), At4g14365 (QAt4g14365F: 5'-TGGTTTTCTGAGGCATGTAAA-3' and QAt4g14365R:5'-AGTGCAGGAACATTGG TTGT-3'), *ACD6* (QACD6F:5'-TGGACAGTTCTGGAGCAGAT-3' and QACD6R: 5'-CAACTCCTCCGCTGTGAG-3'), *PR-5* (QPR-5F:5'-GGCAAATATCTCCAGTATTCACA -3' and QPR-5R: 5'-GGTAGGGCAATTGTTCCTTAGA-3'), *PR-2* (QPR-2 F:5'-AAGGAGCTTAGCCTCACCAC-3' and QPR-2R: 5'- GAGGGAAGCAAGAATGGAAC -3'), PR-1 (QPR-1F:5'-GAACACGTGCAATGGAGTTT-3'and QPR-1R: 5'-GGTTCCA CCATTGTTACACCT-3') and *ACT-2* (QACT2 F:5'- AATCACAGCACTTGCACCA-3' and QACT2R: 5'- GAGGGAAGCAAGAATGGAAC-3') generating 100 base pair fragments.

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Chapter 5

General Discussion

Compatibility between plants and adapted pathogens is the basis of susceptibility, which eventually leads to disease. To establish compatibility, pathogens have to overcome the plant's defence repertoire and to gain access to the host's interior and metabolites. This is a complex task and therefore plants are resistant to the vast majority of potential pathogens. In exceptional cases, microbes have adapted to the host and have established compatibility. Our understanding of the molecular mechanisms underlying compatibility or disease susceptibility is limited. In chapter 1 the current knowledge on plant genes required for susceptibility to biotrophic pathogens was discussed. In this chapter the work on the *dmr* mutants, described in this thesis, is placed in a broader perspective and compared to work on other loss-of-susceptibility mutants identified in genetic screens. Based on the underlying molecular mechanisms these mutants can be placed in one of four main classes; (i) broad range enhanced defence mutants, (ii) pathogen-specific enhanced defence mutants, (iii) cell wall affected mutants and (iv) lack of susceptibility mutants.

i. Broad range enhanced defence mutants

A diverse range of mutants, derived from different genetic screens, display broad resistance to pathogens that is based on the enhanced or constitutive activation of plant defence responses. In most cases, resistance is associated with the high expression of defence genes, such as the pathogenesis-related (*PR*) genes (Durrant and Dong, 2004). Some PR proteins are known to possess antibacterial or antifungal/oomycete properties and are directly effective against the invading organisms (Van Loon et al., 2006). The broad resistance mutants often accumulate high amounts of salicylic acid, leading to the activation of a subset of defence genes, such as *PR-1*. Examples of mutants or mutant groups that constitutively express *PR-1* are *acd6-1*(*accelerated cell death6-1*) (Lu et al., 2003), *dnd1* (*defence no death1*) (Yu et al., 1998), *agd2* (*aberrant growth and death2*) (Rate and Greenberg, 2001) and the *cpr* (*constitutive expressor of PR genes*) mutants (Bowling et al., 1997). The enhanced or constitutive activation of plant defence can be caused by loss of suppressors, or by disturbed defence signalling. A subclass of

mutants displays spontaneous cell death, often visible as micro lesions that are associated to activated defence gene expression (Dietrich et al., 1994). Several constitutive plant defence mutants, e.g. *cpr1* and *cpr6* require a functional *ENHANCED DISEASE SUSCEPTIBILITY1* (*EDS1)* gene for resistance (Clarke et al., 2001). Our screen for downy mildew resistance was performed in the *eds1-2* background so that we counter-selected most of these mutations. Three of the *dmr* mutants, *dmr3*, *dmr4* and *dmr5* are considered as enhanced defence mutants (chapter 2) and are independent of *EDS1*. All three mutants express the *PR-1* gene constitutively. However, only *dmr3* and *dmr4* display broad resistance, as they are resistant to *H. parasitica*, *Pseudomonas syringae*, and partially to *Golovinomyces orontii* and *Albugo candida* (E. Holub, personal communication). In addition, the *dmr3* and *dmr4* mutants have a dwarf phenotype that is linked to resistance, a phenomenon that is observed in many enhanced defence mutants. Expression analysis of the *dmr3* and *dmr4* mutants revealed a pronounced set of up regulated genes that are known to be associated with biotic stress, including *PR*-genes (Van den Ackerveken et al, unpublished).

ii. Pathogen-specific enhanced defence mutants

Several Arabidopsis mutants have been identified that show resistance to only one or two pathogen species and do not show the broad range resistance as described for the previous class of mutants. Due to their specificity they were originally identified as lack of susceptibility mutants. However, as they show elevated expression of defence-associated genes, they can be grouped in the enhanced defence class of mutants. Whereas the broad resistance mutants generally have a high constitutive *PR-1* expression, the mutants, *pmr2*, *pmr4* (*powdery mildew resistance*), *dmr1* and *dmr6* (*downy mildew resistance*) do not show a high *PR-1* expression in non infected plants. However, they do have activated plant defence responses that are apparently not broad, but effective against a limited number of pathogens. The effectiveness of resistance against only a limited number of pathogens could be due to the expression of only a subset of defence responsive genes and/or due to the level of expression of the defence-associated genes.

The *pmr2* (*Atmlo2*) mutant does not support *G. cichoracearum* (Vogel and Somerville, 2000) and *G. orontii* sporulation but still supported pathogen growth inside the leaves. This growth was fully arrested in the triple mutant *Atmlo2*/*Atmlo6*/*Atmlo12* (three members from the same phylogenetic clade) (Consonni et al., 2006). The *Atmlo* mutant lines, including the double and triple were fully susceptible for *H. parasitica* and *P. syringae*. Resistance to the non-adapted powdery mildew pathogens *Erysiphe pisi* and *Blumeria graminis* f.sp. *hordei* was similar as to the adapted powdery mildew pathogens *G. cichoracearum* and *G. orontii*. *Alternaria alternata*, *A. brassicicola* and *Phytophthora infestans* gave enhanced disease symptoms and cell death in the double and triple *Atmlo* mutants. In the *Atmlo* triple mutant, the level of free SA is related to the developmental stage of the plant; higher SA levels were detected in older mutants (Consonni et al., 2006). Unchallenged barley and Arabidopsis *mlo* mutants show developmentally controlled callose deposition coinciding with the production of ROIs. The *pmr2* mutant also showed leaf chlorosis and necrosis (Consonni et al., 2006). Callose deposition and other cell wall alterations which are linked to chlorosis and necrosis (Swarbrick et al., 2006) could be the primary cause of *pmr2* based resistance.

The *pmr4* mutant confers resistance to powdery mildew and *H. parasitica* (Vogel and Somerville, 2000). Transcription profiling indicated that in infected *pmr4* plants the expression of SA-dependent defence genes was elevated. *PMR4* encodes for the callose synthase (glucan synthase-like; GSL5) protein. In the *pmr4* mutants the of lack callose is suggested to trigger the defence pathway (Nishimura et al., 2003). Powdery mildew resistance in the *gsl5-1* mutant (a homozygous T-DNA insertion line) to *S. fusca* and *G. orontii* was similar to that found in the *pmr4* mutant (Jacobs et al., 2003). In contrast, the non-host pathogen *B. graminis* could better penetrate the mutant probably due to the depletion of callose. Callose, which is synthesized as a response to the invading pathogen by GSL5, could be required for pathogen growth. Jacobs (2003) and coworkers suggested that the callose might protect the fungus during pathogenesis. The double mutants *pmr4-1*/*NahG* and *pmr4-1*/*pad4-1* supported wild-type levels of pathogen growth, implicating that the *pmr4*-based resistance is dependent on the SA pathway.

The *dmr5* mutant confers only resistance to the downy mildew pathogen, *H. parasitica* (chapter 2). But expression analysis of the *dmr5* mutant revealed a set of up regulated defence related transcripts (Huibers et al., in preparation). Presumably, the level of elevated defence related transcript is only sufficient to confer resistance to *H. parasitica* and not to other pathogens.

The *dmr1* mutant confers specific resistance only to *H. parasitica* (chapter 2 and 3). *DMR1* encodes for homoserine kinase and point mutations in the *DMR1* gene result in high levels of homoserine. In the *dmr1* mutants, callose deposition was elevated around the *H. parasitica* haustoria. Deposition of callose was also detectable around the haustoria after L-homoserine infiltration of *H. parasitica* infected wild-type seedlings. The callose deposition was presumably not directly responsible for the resistant phenotype. Co-infiltration of L-homoserine with the callose inhibitor 2-deoxy-D-Glucose still resulted in a resistant phenotype (chapter 3). The resistance in the *dmr1* mutants is induced by L-homoserine but the mechanism remains to be discovered. Callose deposition could slow down or arrest invasion of *H. parasitica*, giving the plant more time to respond with antimicrobial compounds. For example the callose deposition could block nutrient transport from the plant to the haustorium required for *H. parasitica* growth.

The *dmr6* mutant confers resistance to *H. parasitica* (chapter 2) and *C. higginsianum* (O'Connell and Panstruga, 2006) and not to *A. candida*, *G. orontii* and *P. syringae* (chapter 2 and 4). Micro array analysis of the *dmr6-1* mutant versus the parental line showed enhanced expression of a set of defence-associated genes (Table 3A, chapter 4). The elevated transcript set and level of defence genes activated in the *dmr6-1* mutant is suggested to be only effective against *H. parasitica* and *C. higginsianum*. Absence of the DMR6 protein in the *dmr6* mutant was known to be responsible for the enhanced defence gene activation. The loss of function of the oxidoreductase encoded by *DMR6* could result in substrate accumulation or lack of the product resulting in *H. parasitica* resistance. Eventhough no clear activation of defence related transcripts was identified in the *dmr1* or *pmr2* mutants, both mutants show an enhanced level of callose deposition in response to pathogen invasion. Since callose is known to be involved in defence these two mutants are classified with the pathogen-specific enhanced defence mutants.

iii. Cell wall affected mutants

The cell wall is an important barrier that shields the plant cell membrane and cytoplasm from potential pathogens. To cross that barrier, pathogens deploy hydrolytic enzymes, physical pressure, or a combination of both to penetrate the cell wall. The plant cell wall composition varies greatly between cell types, plant species and accessions within species (Hazen et al., 2003). Differences or alterations in composition or make-up of the plant cell wall could arrest pathogen growth and development. Genetic studies have provided evidence for a role of cell wall polysaccharides in disease resistance (Vorwerk et al., 2004). *Resistance to Agrobacterium tumefaciens* (*rat*) mutants, e.g. *rat1*, *rat3* and *rat4,* were identified as cell wall-affected mutants (Zhu et al., 2003b). *RAT1* was found to encode for AGP17, an arabinogalactan-protein (Gaspar et al., 2004) and *RAT4* for GSLA9, a cellulose synthase-like protein (Zhu et al., 2003a). In the *rat1* mutant AGP17 no longer reduces the SAR response which appears to be required for the *A. tumefaciens* infection process. *A. tumefaciens* was unable to down regulate the expression of *PR1* and *PR5* (Gaspar et al., 2004). The *rat4* mutant has a decreased number and reduced growth rate of lateral roots. The roots of the *rat4* mutant have a reduced ability to bind *A. tumefaciens*, indicating that GSLA9 is involved in an early step in the *A. tumefaciens*-mediated plant transformation process. The mutation in a cellulose synthase like gene suggests that an alternation of the plant cell wall architecture is responsible for a reduction of *A. tumefaciens* attachment. Although the *rat4* mutant was identified as a cell-wall affected mutant, cell-wall analysis did not indicate any major alterations in cell wall linkage or composition. However, *A. tumefaciens* is suggested to bind to a compound in the cell wall that is absent in the *rat4* mutant (Zhu et al., 2003a). The mutants, *rat1*, *rat3* and *rat4,* were originally identified as cell wall affected mutants but further functional studies on the mutants and identified genes suggest that other factors besides cell-wall composition could also be involved. It would be interesting to test the response of the *rat* mutants, in particular of *rat1* in the interaction with other pathogens, since the SAR pathway is know to mediate broad spectrum resistance.

Three powdery mildew resistant mutants could be classified as cell wall-affected mutants, *cev1, pmr5* and *pmr6.* The *cev1* mutant was identified in a screen for mutants with a constitutive activation of the jasmonate (JA) pathway (Ellis and Turner, 2001). *CEV1* encodes for a cellulose synthase, which is implicated in primary cell wall synthesis (Ellis et al., 2002b). No null mutant was identified since loss of the cellulose synthase is lethal. The mutated enzyme has a reduced cellulose synthase activity. The decreased amount of cellulose and corresponding defects in cell wall structure was found to trigger JA and ethylene production (Ellis et al., 2002b). For this mutant further research should indicate if the cell wall alteration is directly responsible for resistance, or indirectly by triggering of the JA pathway and subsequent activation of defence. The constitutive activation of JA in the *cev1* mutant is correlated to the resistance of the powdery mildew pathogen *Erysiphe cichoracearum*, and additionally to the bacterium *Pseudomonas syringae* and the aphid *Myzus persicae* (Ellis et al., 2002a). Due to the broader pathogen resistance that correlates to the levels of JA, this cell-wall affected *cev1* mutant could also be placed in the above mentioned class of broad range enhanced defence resistance mutants. In the powdery mildew resistant mutants *pmr5* and *pmr6*, analysis of the cell-wall composition by Fourier transform infrared (FTIR) spectroscopy on isolated cell walls revealed increased levels of pectin (Vogel et al., 2002; Vogel et al., 2004). The *PMR6* gene encodes for a pectate lyase-like gene. *PMR5* is a gene of unknown function belonging to a large family of plant-specific genes. Although *pmr5* and *pmr6* have an altered cell-wall composition the powdery mildew pathogen is still able to penetrate and grow at the initial stage of infection. However, at 2 days post infection most of the fungal colonies consisted of shrivelled hyphae, attached loosely to the leaf surface. In the *pmr5* and *pmr6* mutants the production of asexual conidiophores per colony 6 days post infection was approximately eight times less than in the parental line, Colombia. It is unclear how the change in cell wall architecture in the *pmr5* and *pmr6* mutants translates into the post-haustorial growth cessation of the fungal pathogen. Hypothetically, nutrient transport to the haustoria is altered, or pathogenic effectors can no longer cross the cell wall due to the changed composition of the cell wall. The *dmr* mutants described in this thesis do not

appear to be linked to cell wall changes. However, as we have not analyzed potential alterations of the cell wall in our *dmr* mutants we cannot exclude that they are cell wall affected.

iv. Lack of susceptibility mutants

Mutants that are resistant to a given pathogen but that do not show any form of enhanced defence or altered cell wall composition could be classified as susceptibility mutants. The number of, what could be considered "true", susceptibility genes identified so far is very limited. Two recessive rice genes, *xa5* and *xa13*, mediate resistance to the bacterial blight pathogen of rice, *Xanthomonas oryzae* pv. *oryzae* (*Xoo*). The dominant *Xa5* and *Xa13* genes could be considered susceptibility genes. The *Xa5* gene encodes for TFIAγ5 that is involved in RNA transcription by polymerase II (Iyer and McCouch, 2004). The *Xa13* or *OsN3* gene in rice is a homolog of the *Medicago* nodulin gene, *MtN3* (Chu et al., 2004; Yang et al., 2006). The promoter of the *Xa13* gene is a target site for the *Xoo* type III effectors PthXo1 and AvrXa7 (Yang et al., 2006). *xa13* plants are resistant because the bacterial effector protein is unable to bind the *xa13* promoter due to a 243-bp insertion. The *Xa13* gene is a good example of a susceptibility gene because its expression is induced and required for *Xoo* growth (Chu et al., 2006; Yang et al., 2006).

Mutagenesis in Arabidopsis has resulted in the isolation of mutants disturbed in plant virus interaction. These mutants lack a protein necessary for viral replication and transport. For example *TOM1* and *TOM2*, that encode multipass transmembrane proteins, are required for virus replication (Tsujimoto et al., 2003; Yamanaka et al., 2000). Another protein identified as a virus susceptibility factor is the eIF4E protein, a translation initiation factor. Different plants mutated in the *eIF4E* gene showed reduced viral replication and movement (Gao et al., 2004; Kang et al., 2005; Lellis et al., 2002; Yoshii et al., 2004). The eIF4E protein is involved in the expression of eukaryotic mRNA and its activity is known to be regulated by transcription, phosphorylation, inhibitory proteins, and proteolytic cleavage. Because a virus depends on the host for transcription and translation this factor could be required for new virus particle formation. It is striking to note that of the cloned powdery mildew resistance (*PMR*) and downy mildew resistance (*DMR*)

genes none can be classified as a true susceptibility gene. Only the *dmr2* mutant, for which the corresponding gene is not yet cloned, can be classified as a susceptibility mutant. Expression profiling of the *dmr2* mutant, compared to the L*er eds1-2* parental line, did not show an induced expression of known defence genes (Van den Ackerveken et al, unpublished). The cloning of *DMR2* will reveal the role of the DMR2 protein in disease susceptibility.

Evaluation of genetic approaches to study susceptibility

We have undertaken a forward genetics approach to study susceptibility to downy mildew. Seeds of a susceptible Arabidopsis L*er eds1-2* line were mutagenised with ethyl methyl sulfonate (EMS) and *H. parasitica* resistant seedlings were isolated and further characterized. For two candidate susceptibility mutants, *dmr1* and *dmr6*, the corresponding genes have been cloned (chapters 3 and 4). Both identified genes were placed in the pathogen-specific enhanced defence class (ii) mutants but we should not exclude them completely from the lack of susceptibility class (iv). To clarify the exact resistance mechanism in the *dmr1* mutant, including the function of homoserine, further studies are necessary. The mechanism by which homoserine in the *dmr1* mutants leads to *H. parasitica* resistance could be via a novel defence mechanism. The *dmr6* mutant hints strongly to fit into the pathogen-specific enhanced defence mutants class, as a set of defence-associated genes are activated in *dmr6*. However, we have not proven that resistance depends on the expression of this set of all of the up regulated defence associated genes. To test if defence depends on the upregulation of defence associated genes, double mutants need to be generated by crosses of *dmr6* to e.g., the Arabidopsis *nonexpressor of PR genes 1* (*npr1*) mutant (Cao et al., 1994; Delaney et al., 1995; Glazebrook et al., 1996) that is disturbed in SA-induced *PR-1* gene expression, as well as to other mutants that are impaired in defence related gene expression. One could envisage that susceptibility depends on a functional DMR6 enzyme. The DMR6 enzyme activity could lead to a product needed for pathogen development or plant processes related to susceptibility.

For five of the six *dmr* mutants only a single allele has been identified so far. Only for the *dmr1* mutant, five alleles have been isolated. This indicates that the mutagenesis screen is far from saturated. Additional screening of a larger number of mutants will not only lead to the isolation of more alleles for the known *dmr* loci, but will probably also lead to the identification of new unknown *dmr* loci. The genetic background of the parental line used for mutagenesis is also of influence on the loci that are identified. As the L*er* *eds1-2* background is highly susceptible to *H. parasitica* the resistant mutants have a strong phenotypic change resulting from the mutation of an important gene. The mutant screen was initiated in the *eds1-2* mutant background in order to prevent the identification of a large number of enhanced defence mutants. In addition, the *eds1-2* mutation also facilitated the screen because it reduced the identification of false positives. Performing a similar genetic screen in a less susceptible background could allow the identification of *dmr* loci with less strong phenotypes. However, the efficiency of such a screen would be hampered by the large number of false positives as the parental line already has a certain degree of resistance.

Out of 100,000 M_2 EMS mutants, twenty *dmr* mutants were isolated, and ten were studied in more detail, corresponding to the loci *dmr1* to *dmr6*. Of these *dmr* mutants only *dmr2* could be classified in the lack of susceptibility class of mutants. However, the identification of the corresponding *DMR2* gene is required. Still the main question that remains is; why have we not identified more true susceptibility mutants? One explanation for the lack of identification of real susceptibility genes is that certain Arabidopsis genes required for disease susceptibility are also indispensable for growth and development of Arabidopsis. Mutations in such genes would be lethal to the plant and therefore such mutants can not be identified. Furthermore, disease susceptibility in plants is most probably largely determined by multiple genes with small effects. Mutations in single genes will therefore not lead to a detectable phenotypic change in disease susceptibility. In addition, a susceptibility gene can be a member of a gene family, of which the individual members have the same function (redundancy). The above mentioned effects of lethality, small effects or redundancy make the identification of susceptibility genes difficult by a forward genetic approach.

An alternative approach to identify susceptibility genes is by reverse genetics. Genes that are differentially regulated during *H. parasitica* compatible versus incompatible interactions can be identified via transcriptional profiling. Genes suppressed or induced specifically during a compatible interaction could be putative susceptibility gene candidates. To test if putative susceptibility genes are required for disease, knock out mutant lines in the corresponding genes can be tested for loss of susceptibility or for alterations in the infection process. Large collections of Arabidopsis T-DNA insertion lines containing insertions in most of the genes in the Arabidopsis genome are available to the public (e.g. Alonso et al., 2003). Loss of susceptibility due to the effect of multiple genes, as was discussed previously, will also not be detected in a single gene knock out line. The potentially positive effect on susceptibility of genes induced during a compatible plant pathogen interaction could be tested by generating transgenic Arabidopsis lines over-expressing these genes. In single gene knock out lines the reduced susceptible phenotypic change can be too subtle, but the over expression of this specific gene could result in a detectable more susceptible phenotype. The *WRKY7* gene for example which is induced by *P. syringae* infection and is a negative regulator of plant defence against the bacterial pathogen. *P. syringae* growth in the *wrky7-1*, T-DNA insertion, and *W7*-RNAi mutant plants was only 6- to 7 fold lower than that in wild-type plants. In contrast, the *WRKY7*-overexpressing plants supported more growth of *P. syringae* and developed more severe disease symptoms than wild-type plants (Kim et al., 2006). Both phenotypes, the reduced *P. syringae* growth in the *wrky7-1* and *W7* line, and the induced *P. syringae* growth in the *WRKY7*-overexpressor, were subtle but significant. So the actual role of *WRKY7* as a susceptibility gene is confirmed through the higher susceptibility of the *WRKY7*-overexpressor lines.

Although being powerful genetic approaches, both the forward and the reverse approach have some disadvantages in the identification of susceptibility genes. The disadvantage of the forward genetics approach is that it is a labour and time-consuming method. The advantage is that a resistant phenotype is present in the mutant and in general the mutated gene involved can be identified genetically and cloned by map-based approaches. Forward genetics

results in the identification of only one gene per mutant. Via the reverse genetics approach multiple genes that could be involved in compatibility can be identified directly. The disadvantage is that if multiple genes with small effects are required they can not be detected nor confirmed in T-DNA knock out lines. The same holds true for redundant genes, which remain undetectable because they can complement each other. The forward and reverse genetic approaches can supplement each other to clarify the molecular mechanism of compatibility. For example, the *DMR6* gene, identified by the forward genetic approach, is found to be up regulated during *H. parasitica* infection. Therefore, the *DMR6* gene was also identified by a reverse genetic approach and confirmed by an available knock out T-DNA insertion line in the Ws-4 Arabidopsis accession in the *DMR6* gene, resulting in a resistant phenotype. The undertaken forward genetics approach to study susceptibility to downy mildew has proven to be an excellent method to identify new forms of resistance and to provide a better understanding of the molecular basis of disease susceptibility. The *DMR1* gene, for example, would not have been identified in a reverse genetic approach, because (i) the expression of the *HSK* gene is not altered by *H. parasitica* infection, (ii) no T-DNA insertion lines in the *HSK* gene are available, and (iii) homozygous insertion mutants (or null mutants) are expected to be lethal.

Conclusion

Several genes involved in pathogen compatibility have been identified and their molecular insights analysed, showing completely new aspects of susceptibility. Despite the progress made on mutants and genes related to disease susceptibility many aspects of biotrophic plant-pathogen interactions remain unexplained. A limiting aspect in obligate biotrophic plant pathogen studies is that one can not use the available molecular tools on the pathogens, as no *in vitro* cultivation, transformation or gene disruption is possible yet. Therefore, the aspects involved in a susceptible interaction were mainly studied on the plant side. The haustoria of *H. parasitica* invaginate single Arabidopsis host cells to extract the nutrients needed for growth. New techniques suitable to extract molecules from single infected host cells will give new insights in the compatible interaction. By comparing an infected

versus and uninfected host cell new molecules could be identified that are involved in e.g., accommodation of haustoria, nutrients uptake, and suppression of host cell defence. These host molecules, or potential targets for pathogen effectors can be considered as susceptibility factors.

The knowledge on the *DMR* genes can be used to explore the generation of resistant crop plants. Many crops are susceptible to downy mildew causing pathogens resulting in large yield losses. Application of fungicides is enormous and economically and environmentally costly. A durable downy mildew resistance trait is a very important aspect in crop breeding. Orthologs of both *DMR1* and *DMR6* have been identified in a large number of crop plants. Downy mildew resistant crops mediated by *dmr1* or *dmr6* may one day become reality. To achieve this, mutations in the *DMR1* and *DMR6* orthologs have to be identified in mutagenised or natural variant plants by TILLING (Till et al., 2003) or direct sequencing. Alternatively, gene silencing or knock down lines can be generated via RNAi. These techniques will make the application of *dmr1* and *dmr6* based downy mildew resistance in crops feasible. The cloning of *DMR1* as a homoserine kinase and *DMR6* as an oxidoreductase enzyme has lead to the discovery of novel mechanisms of resistance to *H. parasitica*. The exact molecular mechanism of resistance in both mutants needs to be studied in more detail. Currently suppressor screens are initiated in the *dmr1* and *dmr6* mutant backgrounds to identify genes required for *dmr1*- and *dmr6*-based resistance. The mechanism of *dmr6* resistance is suggested to be based on elevated defence gene activation. Since *DMR6* encodes for an oxidoreductase enzyme either the accumulation of the substrate or the lack of the product could cause the resistant phenotype. The identification of two *DMR* genes has provided new insights in downy mildew resistance based on novel mechanism in *H. parasitica* resistance. Further functional studies on the *dmr* mutants and corresponding genes will learn us more about the molecular mechanism determining resistance or susceptibility, and feature will reveal whether resistance and susceptibility are just two side of the same coin.

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Summary

On a global scale the impact and costs of plant diseases on agriculture is enormous, highlighting the importance of the research on this topic. Plant disease is the result of a compatible interaction between plants and adapted pathogens. The knowledge on the molecular mechanisms underlying compatibility or disease susceptibility is limited. The aim of this study was to identify Arabidopsis genes required for disease susceptibility to the oomycete *Hyaloperonospora parasitica*. We have undertaken a forward genetics approach to study susceptibility to downy mildew. Seeds of the susceptible Arabidopsis L*er eds1-2* line were mutagenised and twenty independent downy mildew resistant (*dmr*) Arabidopsis mutants were isolated of which eight were further characterized (described in **chapter 2**). Three *dmr* mutants, *dmr1*, *dmr2* and *dmr6*, showed no induced expression of the defence-associated gene *PR-1*, and absence of programmed cell death and reactive oxidative intermediates, suggesting they are susceptibility mutants. In contrast, *PR-1* expression was elevated in the *dmr3*, *dmr4*, and *dmr5* mutants indicating that these mutants have an enhanced defence-response. The *dmr1*, *dmr2, dmr5*, and *dmr6* mutants were still susceptible to other pathogens, such as the bacterium *Pseudomonas syringae* pv. *tomato* and the fungus *Golovinomyces orontii*. For two candidate susceptibility mutants, *dmr1* and *dmr6*, the corresponding genes have been cloned (**chapters 3 and 4**).

The *DMR1* gene, At2g17265, was map-based cloned and found to encode for homoserine kinase (described in **chapter 3**). Homoserine kinase phosphorylates homoserine to phospho-homoserine. Amino acid analysis of the *dmr1* mutants revealed high levels of homoserine that are absent in the parental line, L*er eds1-2*. Infiltration of homoserine into L*er eds1-2* seedlings resulted in *H. parasitica* resistance. High levels of homoserine cause *H. parasitica* resistance in the *dmr1* mutants via an as yet unknown mechanism.

The *DMR6* gene was found to encode for an oxido reductase (described in **chapter 4**). Oxido reductases catalyze the transfer of electrons from one molecule, the oxidant, to another, the reductant. For the *DMR6* encoded oxido reductase no biological function has been demonstrated nor do we know the substrate and product of the predicted enzyme. *DMR6* is locally up regulated

during *H. parasitica* infection, in compatible and incompatible interactions. Other forms of biotic stress and abiotic stress result in an up regulation of *DMR6*. However, lack of *DMR6*, in the *dmr6* mutants, results in resistance that is accompanied by the enhanced expression of a set of defence-associated transcripts, including *DMR6*. These results suggest a dual role of *DMR6* during pathogen infection.

The cloning of the *DMR1* and *DMR6* genes, which are required for *H. parasitica* susceptibility, has revealed novel ways to obtain disease resistance in plants, that have the potential to be applied in breeding for disease resistance. Future studies will reveal the molecular mechanisms that lead to loss of susceptibility to *H. parasitica* in the Arabidopsis *dmr1* and *dmr6* mutants.

Samenvatting

Wereldwijd wordt er veel geld besteed aan de bestrijding van plantenziekten. De gebruikte fungiciden hebben naast de hoge kosten ook een schadelijke invloed op het milieu. Veel fungiciden zijn daarom inmiddels op de lijst van verboden bestrijdingsmiddelen terecht gekomen. Alternatieve methoden van resistentie tegen ziekteverwekkers zijn daardoor zeer gewenst. Het onderzoek aan plant-pathogeen interacties levert niet alleen fundamentele kennis over de moleculaire processen die plaatsvinden tussen plant en pathogeen, maar biedt ook de mogelijkheid voor toepassing in de praktijk. Mijn onderzoek binnen de leerstoelgroep Moleculaire Genetica in Utrecht richtte zich op de vraag waarom planten vatbaar zijn voor infectieziekten, met name valse meeldauw (downy mildew) en op welke manier planten meewerken aan het infectieproces. De kennis van het moleculaire mechanisme dat de vatbaarheid voor valse meeldauw bij planten bepaalt, is tot op heden zeer beperkt. Als moleculair biologe ben ik vooral geïnteresseerd in de plantengenen die betrokken zijn bij vatbaarheid.

Voor mijn onderzoek heb ik gebruik gemaakt van de modelplant *Arabidopsis thaliana*, of in het nederlands, de Zandraket. De veroorzaker van valse meeldauw op Arabidopsis is de biotrofe oomyceet *Hyaloperonospora parasitica*. Biotroof wil zeggen dat dit pathogeen voor zijn gehele levenscyclus afhankelijk is van de levende gastheer. Mijn onderzoeksvraag was welke genen in de plant belangrijk zijn voor vatbaarheid voor *H. parasitica*. In de inleiding van mijn proefschift, **hoofdstuk 1**, geef ik in het kort weer wat er tot nu toe bekend is over verschillende plantengenen die een rol spelen tijdens de vatbaarheid voor verscheidene ziekteverwekkers.

Om de plantengenen die een rol spelen bij de *H. parasitica*-infectie te identificeren, heb ik gebruik gemaakt van een klassieke genetische aanpak (forward genetics). Meeldauw gevoelige Arabidopsis planten van de L*er eds1-2* plant zijn gemutageniseerd en vervolgens getest voor *H. parasitica* resistentie. Twintig onafhankelijke meeldauw resistente, *downy mildew resistant* (*dmr*) mutanten zijn hieruit geïsoleerd. De identificatie en beschrijving van acht van deze mutanten, die corresponderen met zes verschillende loci of genen, is beschreven in **hoofdstuk 2**. Drie *dmr* mutanten, *dmr1*, *dmr2* and *dmr6*, vertoonden geen verhoogde afweerrespons van de plant. Deze actieve

afweerrespons was wel aanwezig in de *dmr3*, *dmr4*, and *dmr5* mutanten. De reden waarom de *dmr1*, *dmr2* en *dmr6* mutanten ongevoelig zijn voor meeldauw is mogelijk de afwezigheid of mutatie van een vatbaarheids gen voor *H. parasitica* (**hoofdstuk 2**). De identificatie van het gen *DOWNY MILDEW RESISTANT 1* (*DMR1*) staat beschreven in **hoofdstuk 3**. *DMR1* codeert voor het enzym homoserine kinase. Homoserine kinase fosforyleert het aminozuur homoserine tot fosfo-homoserine. Aminozuuranalyse van de *dmr1* mutanten vertoonde hoge niveau's van homoserine die afwezig waren in de ouderlijn en wild-type Arabidopsis. *H. parasitica* resistentie werd ook verkregen door infiltratie van homoserine in de gevoelige L*er eds1-2* planten. Deze bevindingen bewijzen dat de accumulatie van homoserine in de *dmr1* mutanten resulteeert in resistentie tegen *H. parasitica*. Het exacte mechanisme van resistentie door homoserine accumulatie in Arabidopsis wordt verder onderzocht.

De identificatie van het *DMR6* gen staat beschreven in **hoofdstuk 4**. *DMR6* codeert voor een oxidoreductase enzym, waarvoor nog geen biologische functie is aangetoond. De expressie van het *DMR6* gen wordt lokaal geïnduceerd door infectie met *H. parasitica*. Naast de biotische stress veroorzaakt door het pathogeen, induceert ook abiotische stress het *DMR6* gen. In de *dmr6* mutant worden ook andere genen opgereguleerd, met name genen die geassocieerd zijn met afweer tegen pathogenen. Nader onderzoek is nodig om het exacte mechanisme van de resistentie in de *dmr6* mutant te begrijpen. Daarbij moet met name onderzocht worden of de *H. parasitica* resistentie wordt veroorzaakt door de opregulatie van de geassocieerde afweergenen of door een andere oorzaak, zoals de afwezigheid van het produkt dat gemaakt wordt door het DMR6 enzym.

In de discussie (**hoofdstuk 5**) wordt een indeling gegeven voor de in de hoofdstukken 1-4 beschreven mutanten. Daarnaast wordt de algemene aanpak en eventuele alternatieven van dit onderzoek besproken. Door de identificatie van het *DMR1* en *DMR6* gen, die nodig zijn voor *H. parasitica* gevoeligheid, hebben we een nieuwe vorm van ziekte resistentie in planten gevonden. De kennis uit dit onderzoek kan in de nabije toekomst toepasbaar zijn, omdat de geïdentificeerde genen ook aanwezig zijn in gewassen die gevoelig zijn voor valse meeldauw. Nader onderzoek aan de *dmr1* en *dmr6* mutanten zal het moleculaire mechanisme dat tot deze nieuwe vorm van valse meeldauw resistentie resulteert, verklaren.

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