# Genetic analysis of susceptibility of Arabidopsis to downy mildew infection

**Robin P. Huibers**

**Printing**

Ipskamp, Enschede

**ISBN**

9789039348802

The studies described in this thesis were performed in the sections Plant-Microbe Interactions and Molecular Genetics, Department of Biology, Science Faculty, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands, and were funded by grants from ALW (810.36.004) of the Netherlands Organization for Scientific Research (NOW), and from the Center for Biosystems Genomics (CBSG), which is part of the Netherlands Genomics Initiative.

# Genetic analysis of susceptibility of Arabidopsis to downy mildew infection

Genetische analyse van vatbaarheid van Arabidopsis voor valse meeldauw infectie

(met een samenvatting in het Nederlands)

**Proefschrift**

Ter verkrijging van de graad van doctor aan de Universiteit Utrecht

op gezag van rector magnificus, prof.dr. J.C. Stoof,

ingevolge het besluit van het college voor promoties

in het openbaar te verdedigen op maandag 8 september 2008 des middags te 2.30 uur

door

**Robin Paul Huibers**

geboren op 27 februari 1980 te Alphen aan den Rijn

**Promotor:** Prof.dr. P.J. Weisbeek

**Co-promotor:** Dr. G. van den Ackerveken

De natuur is te bespieden, niet te ontraadselen.

Pythagoras

## Table of Contents

## Abbreviations

| | |
|---|---|
| AVR | avirulence |
| ATR | Arabidopsis thaliana recognized |
| BTH | benzothiadiazole |
| CC | coiled coil |
| DAB | 3’-3’-di-amino-benzidine |
| dpi | days post inoculation |
| EMS | ethane methyl sulfonate |
| HR | hypersensitive response |
| LRR | leucine rich repeats |
| MAMPs | microbe-associated molecular patterns |
| MAP | mitogen activated protein |
| NBS | nucleotide binding site |
| PAMPs | pathogen-associated molecular patterns |
| PR | pathogen-related |
| R | resistance |
| ROS | reactive oxygen species |
| RPP | recognition of *Peronospora parasitica* |
| SA | salicylic acid |
| TIR | Toll interleukin 1 receptor |
| NO | nitric oxide |

# Chapter 1

## General introduction and outline

In their natural environment plants are exposed to a wide variety of microorganisms. Most microbes are harmless to the plant and do not cause disease. In fact many examples exist of mutually beneficial symbioses. For example *Rhizobium* bacteria interact with legumes where they reduce atmospheric nitrogen into ammonia. Ammonia is a source of nitrogen that can be used by the plant. In exchange the *Rhizobium* bacteria receive nutrients from the plant, leading to a mutual beneficial symbiosis. Disease is caused by only a relatively low number of, often specialized, microbes that are capable to grow and reproduce on a particular host plant. Plant diseases cause major losses in crop yield and quality, making research in this field relevant and important for agriculture. This introductory chapter highlights different aspects of how plants perceive and defend themselves against (pathogenic) microbes and what is known about disease susceptibility, in particular to biotrophic and hemibiotrophic fungal and oomycete pathogens.

**Plant innate immunity**

The ability of multicellular organisms to defend themselves against infection by pathogens (bacteria, fungi, oomycetes, etc.) and viruses depends on the ability to mount immune responses. Both animals and plants have inborn defence mechanisms that constitute innate immunity. Vertebrates, such as humans, have, in addition to innate immunity, a defence mechanism that is called adaptive immunity that involves the synthesis of antigen-specific antibodies. The adaptive immune response provides the vertebrate immune system with a 'learning' ability to recognize and remember specific pathogen epitopes, and to mount a stronger attack each new time the pathogen is encountered. This adaptiveness is not part of the innate immune responses. Plants lack an adaptive immune system, however, their innate immune system is capable of defending the plant against most microbial attacks.

Plant innate immune responses become effective in defence when microbes are not deterred by the chemical and physical barriers of the plant. The plant innate immune system is thought to rely on two branches. The first branch consists of (transmembrane) receptors that recognise molecules that are generally associated with microbes and/or pathogens (microbe- or pathogen-associated molecular patterns MAMPs or PAMPs). These molecules are derived from structural components that are conserved among and vital for whole classes of microbes. The second branch consists of receptors that largely act inside the cell

and are encoded by often polymorphic *R* genes that recognise molecules or activities of certain pathogen strains or isolates.

*MAMP/PAMP perception and the activation of basal defence*

Perception of MAMPs/PAMPs generally results in the activation of a basal defence response that includes the production of reactive oxygen species (ROS), activation of mitogen-activated protein (MAP) kinases, the biosynthesis of stress hormones, production of antimicrobial compounds and the enhanced expression of defence genes (Kunze et al., 2004; Felix and Boller, 2003; Brunner et al., 2002; Nuhse et al., 2000). It is thought that MAMP/PAMP perception and the consequent activation of basal plant defences considerably limits the number of microbes that can be pathogenic. Only specialised microbes are able to grow and reproduce on a given host plant, thereby being pathogenic, despite activation of MAMP/PAMP triggered defences (Jones and Dangl, 2006; Bent and Mackey, 2007).

The best studied example of MAMP/PAMP perception is that by Arabidopsis FLS2, a transmembrane protein that binds and recognises a specific part of flagellin, the protein that is the major component of the flagellum of many bacteria (Chinchilla et al., 2006). Arabidopsis plants lacking a functional *FLS2* gene have enhanced sensitivity to spray application of *Pseudomonas syringae* DC3000 but not to syringe infiltration into the leaf apoplast (Zipfel et al., 2004), indicating that flagellin perception limits bacterial growth at an early stage of infection. Moreover, silencing of the *FLS2* ortholog from *Nicotiana benthamiana* (*NbFLS2*) led to increased growth of compatible and interestingly, also non-pathogenic strains of *P. syringae* (Hann and Rathjen, 2007). Flagellin perception apparently limits growth of non-pathogenic microbes as well. Similar findings were made with Arabidopsis plants lacking *EFR1*, the MAMP/PAMP receptor of elongation factor Tu (EF-Tu) (Zipfel et al., 2006). Absence of *EFR1* in Arabidopsis resulted in more efficient delivery of T-DNA by *Agrobacterium*. It is clear that pathogen growth is inhibited by MAMP/PAMP-triggered defences. However, to address which potential pathogens are actually not pathogenic due to MAMP/PAMP-triggered basal immunity is difficult. Microbes usually express multiple MAMPs/PAMPs perceived by different MAMP/PAMP receptors. Therefore, knocking out a single MAMP/PAMP receptor will only have a small effect on immunity (Bent and Mackey, 2007).

*R-gene mediated resistance*

Successful pathogens are not arrested by MAMP/PAMP-triggered basal defence responses. However, plants can still mount effective defence responses. This depends on the perception of molecules (proteins) that are not considered MAMPs or PAMPs as they are often isolate or strain specific. This form of immunity or resistance was hypothesised to be mediated by the products of specific plant resistance (*R*) and pathogen a-virulence (*Avr*) genes (Flor, 1971) and was termed gene-for-gene resistance or *R*-gene mediated resistance. Because the specific R and avr proteins are not present in all individuals of each species, interactions can be either compatible or incompatible. The interaction is incompatible when corresponding *Avr* and *R* gene products are present and trigger the activation of defence responses and disease resistance. In absence of corresponding *Avr* and *R* gene products pathogen growth is not halted and the interaction is termed compatible.

Many *Avr* and *R* genes have now been cloned from different pathogen and plant species, respectively. Avr proteins were first identified on the basis of their avirulence activity. However, it has become clear that they provide important virulence functions for the pathogen (in absence of recognition by R proteins) and are termed effector proteins. Pathogen effector proteins often act inside the host cell. Gram-negative bacterial pathogens use their type III secretion system (TTSS) to deliver effector proteins into the host cell. Fungal and oomycete pathogens secrete effector proteins into the extracellular spaces, after which some become translocated to the host cell. For several effector proteins it has been shown that they interfere with cellular processes of the host and suppress defences (for several excellent reviews concerning bacterial, fungal and oomycete effector proteins, see Kamoun, 2007; Grant et al., 2006; Jones and Dangl, 2006 and Chisholm et al., 2006). However, despite their virulence function during compatible interactions, their presence in plants containing a cognate *R* gene triggers a rapid defence response resulting in pathogen resistance in incompatible interactions.

Recognition of Avr proteins by R proteins can be direct in the sense that both proteins physically interact as was shown for *RRS1-R* and *PopP2* (Deslandes et al., 2003), AvrL567 proteins and L5 L6 and L7 (Dodds et al., 2006), Avr-Pita and Pi-ta (Jia et al., 2000) and AvrPto and Pto (Scofield et al., 1996; Tang et al., 1996). In other cases direct interactions between R and Avr proteins could not be detected. An alternative model was put forward, the guard hypothesis (Van der Biezen and Jones, 1998), stating that R proteins guard

effector/Avr protein targets. In this scenario R proteins respond to perturbations of a host protein by an Avr protein. A clear example is the recognition of AvrRpm1 and AvrB by RPM1. Both effector proteins phosphorylate RIN4 (and possibly other proteins) and this phosphorylation activates RPM1 (Mackey et al., 2002). Interestingly, RIN4 is also targeted by AvrRpt2 which cleaves the protein. Cleavage of RIN4 is not recognised by RPM1 but by RPS2 (Axtell and Staskawicz, 2003; Mackey et al., 2003). RIN4 as may other proteins that become phosphorylated by AvrRpm1 and AvrB, is thought to act as a negative regulator of defence. Phosphorylation of RIN4 is thought to stabilize its negative regulatory state. How cleavage of RIN4 can maintain its negative regulatory function is unclear. Several other effectors are suspected to be recognised indirectly, e.g. Avr2 interacting with the protease Rcr3 followed by recognition by Cf-2 (Rooney et al., 2005) and AvrPphB cleaving PBS1 a protein kinase that is then recognized by RPS5 (Shao et al., 2003; Ade et al., 2007).

Despite the wide variation in pathogen encoded Avr proteins, a limited number of R protein classes are known, divided on the basis of known functional domains (Bent, 1996; Dangl and Jones, 2001). This includes (1) proteins with an extracellular leucine rich repeat (LRR) motif and a cytoplasmic kinase domain e.g. Xa21 of rice (Song et al., 1995), (2) proteins that resemble Xa21 in having an extracellular LRR domain but lack the cytoplasmic kinase domain e.g. Cf-2, 4, 5 and 9 of tomato (de Wit and Joosten, 1999), (3) Pto a serine/threonine kinase of tomato, (4) RPW8 of Arabidopsis containing a signal anchor with a cytoplasmic coiled coil (CC) domain (Jones, 2001) and (5) cytoplasmic proteins having a conserved nucleotide binding site (NBS) and a LRR domain. The latter class is by far the most common type of R protein and is present in a wide range of both monocot and dicot plant species (Pan et al., 2000; DeYoung and Innes, 2006). This class can be subdivided in those having an N-terminal CC , Toll/Interleukin1 (TIR) or other domain (Aarts et al., 1998).

Several studies have shown that the LRR domain of the R-proteins, L, Pi-ta, Rx, Cf-4 and Cf-9 confers Avr-specific recognition (Dodds et al., 2006; Ellis et al., 1999; Jia et al., 2000; Rairdan and Moffett, 2006; Van der Hoorn et al., 2001; Wulff et al., 2001). Similar observations were made for the N-terminal domain of L (Ellis et al., 1999; Luck et al., 2000), indicating that both domains can confer recognition specificity. In addition to a role in recognition, the N-terminal domain is thought to be involved in downstream signalling, as suggested by a different requirement of downstream signalling components of TIR- or CC-NBS-LRR proteins (Aarts et al., 1998). The central NBS domain is thought to act as a molecular

switch controlling the activation state of the protein, depending on the nucleotide bound, ADP (off state) or ATP (on state) (Tameling et al., 2006; Takken et al., 2006). According to current models of NBS-LRR protein function and activation (Figure 1), binding of an Avr protein or the perturbation of a guarded host protein to the LRR or N-terminal domain induces a conformational change of the R-protein, followed by the release of ADP and binding of ATP. The ATP bound state is then the active state of the R-protein. The activated R-protein then directly recruits downstream signalling components or requires oligomerization first as has been shown for the activation of the tobacco N protein (Mestre and Baulcombe, 2006).

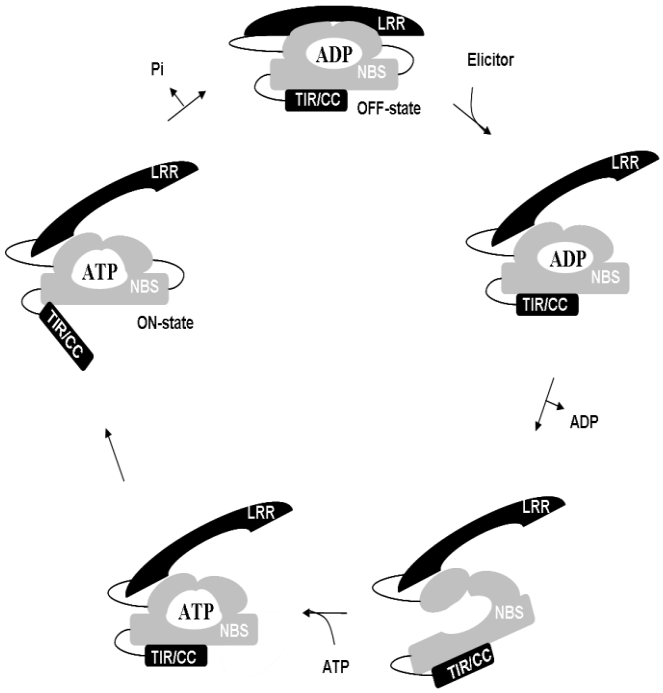


**Figure 1**. Model of NBS-LRR protein function and activation (adapted from Takken et al., 2006). In the absence of an elicitor, the NBS-LRR protein is in the OFF-state. The LRR exerts a negative role by stabilizing the ADP-bound state. The presence of an elicitor (Avr-protein) induces a conformational change of the protein that allows the release of ADP. The subsequent binding of ATP triggers a second conformational change in the N-terminal TIR or CC domain, releasing its signalling potential, the ON-state of the protein. The ATPase activity of the protein attenuates the signalling response and returns the protein to the OFF-state.

R-protein activation usually results in a hypersensitive response (HR), a localised cell death at the site of attempted pathogen entry. The HR is a fundamental element of plant resistance which includes local programmed cell death (PCD) and the activation of biochemical defence responses. PCD is in general confined to a few cells and could aid the arrest of pathogen development. Biochemical defence responses include the production of nitric oxide (NO) and reactive oxygen species, enhanced expression and accumulation of

defence-related genes and proteins, structural changes in the cell wall and the biosynthesis of antimicrobial compounds (Greenberg and Yao, 2004). Many of these responses are remarkably similar to those activated by MAMPs or PAMPs. Moreover, gene expression profiles from plants inoculated with virulent or avirulent pathogen isolates are remarkably similar. Differences are predominantly seen in the timing and level of gene expression (Tao et al., 2003; Li et al., 2006). These reports support the view that *R*-gene mediated responses accelerate and amplify MAMP/PAMP-triggered responses, resulting in the transcriptional activation of predominantly similar defence-associated genes (Jones and Dangl, 2006).

**Compatible plant pathogen interactions**

In the absence of *R*-gene mediated resistance, pathogens are able to grow and reproduce on their host plants and the interaction is said to be compatible. Infection strategies differ considerably among different plant pathogens. Here the focus will be on hemibiotrophic and biotrophic fungal and oomycete pathogens. In contrast to necrotrophic pathogens that kill host tissue before colonising it, biotrophic pathogens colonise living tissue. Hemibiotrophs start off as biotrophs before shifting to a necrotrophic phase. Many hemibiotrophic and biotrophic pathogens form specialised infection structures called haustoria that invaginate host cells after having breached the plant cell wall (Figure 2). Contact between host and pathogen is most intimate at these sites. The extrahaustorial membrane (EHM) surrounds the haustorium and is continuous with the plant plasma membrane. Exchange of signals as well as the flow of water and nutrients from host to pathogen is thought to occur predominantly over this membrane (Voegele and Mendgen, 2003).

Haustoria are thought to be the sites from which pathogen effector proteins become secreted and translocated to the host. This was shown for Avr3a of *Phytophthora infestans*. *P. infestans* transformants expressing an Avr3a-GFP fusion protein showed haustorium-specific secretion of the protein into the extra-haustorial matrix during infection of potato leaves (Whisson et al., 2007). The Avr3a protein contains a conserved RxLR sequence motif (Win et al., 2007). This sequence motif was shown to be equivalent to the *Plasmodium* host-targeting motif in its ability to translocate proteins from *Plasmodium* to human erythrocytes (Bhattacharjee et al., 2006). Moreover, the RxLR motif was found to be required for translocation of the protein into the host cell (Whisson et al., 2007).

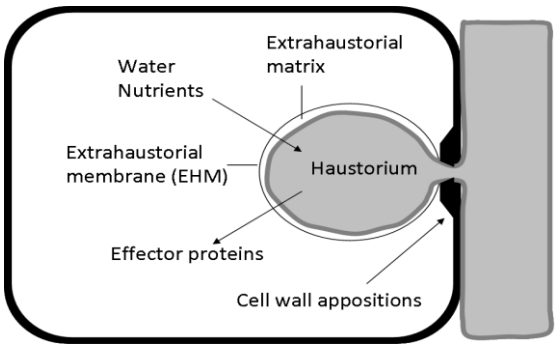


**Figure 2.** Schematic representation of a haustorium. The pathogen has breached through the cell wall and invaginated the host cell. The extrahaustorial membrane (EHM) is continuous with the plant cell membrane and is the site where exchange of signals and nutrients occurs. Plants often respond to invading haustoria by forming cell wall appositions around the haustorial neck.

The role of haustoria in nutrient uptake, such as that of sugars, has been studied in *Uromyces fabae*. Measurements of $H^+$-ATPase activity of microsomal vesicles obtained from different stages of rust development showed that vesicles obtained from haustoria had by far the highest activity (Struck et al., 1996). These results suggested that there is an electrochemical gradient, generated by the H+-ATPase, over the haustorial membrane that could facilitate nutrient uptake from host cells (Struck et al., 1996). In addition it was found that the *HXT1* gene of *U. fabae* is abundantly and predominantly expressed in haustoria and that the protein locates in the haustorial plasma membrane (Voegele et al., 2001). Expression of *HXT1* in yeast showed that it encodes a functional hexose transporter which activity can be blocked by addition of protonophores, indicating that it transports sugars by a proton symport mechanism. Nutrient uptake by haustoria is poorly studied especially in plant-oomycete interactions. Nevertheless, the data regarding *U. fabae* indicate that haustoria function in active uptake of nutrients.

**Genetic analysis of basic compatibility**

Basic compatibility can be considered as an evolved state of symbiosis that occurs at the species level between plant and pathogen. Pathogens have adapted to penetrate, survive and proliferate on their specific hosts and encode specific effector proteins that aid the infection process e.g. by suppressing MAMP/PAMP-triggered defences (Chisholm et al., 2006; Jones and Dangl, 2006). Despite the existence of basic compatibility between a pathogen and host species, compatible interactions only occur in the absence of *R*-gene mediated resistance that could be considered a defence layer of the plant that is superimposed on basic compatibility (Holub, 2006). Knowledge about host components that contribute to basic compatibility and the successful colonisation of fungal and oomycete

pathogens is very limited. Host components could serve as cues for pathogen development, or nutrients that are synthesized and available to the pathogen. Host proteins could be targets for manipulation by pathogen effector proteins, or could be activated as negative regulators of defence, making the plant vulnerable to infection. All host genes that encode components that in one way or another contribute to successful pathogen colonisation could be considered to constitute the genetic basis of basic compatibility of the host plant. Plants in which such genes are absent, mutated or otherwise defective can exhibit reduced susceptibility or disease resistance. This form of resistance is expected to be recessive as it results from loss of gene function, in contrast to the 'classic' *R*-genes that are dominant. The identification and understanding of these compatibility genes will provide a major contribution to our understanding of compatible plant pathogen interactions.

*Broad range disease resistant mutants*

A diverse range of Arabidopsis mutants derived from different mutagenesis screens have been identified that show resistance to a broad range of pathogens, including oomycetes. These mutants were not selected for their loss of compatibility but to show activated defence response phenotypes, such as (1) constitutive expression of *PR* genes (*cpr*, mutants, Bowling et al., 1994), (2) aberrant growth and death phenotypes (*agd*, mutants, Rate and Greenberg, 2001), (3) accelerated cell death (*acd*, mutants Greenberg and Ausubel, 1993), (4) lesions simulating disease (*lsd* mutants, Dietrich et al., 1994), or (5) resistance in absence of an HR (defence no death mutants, Yu et al., 1998). In general, these mutants show resistance to a broad spectrum of different pathogens, have high SA and *PR*-gene expression levels and have an altered plant form, showing reduced stature, altered leaf shape and/or spontaneous cell death. Although these mutants provide valuable insights into the regulation of plant defence mechanisms, they are not particularly helpful in understanding the genetic basis of disease susceptibility.

*mlo mutants are resistant to powdery mildew*

Barley *mlo* plants are resistant to the powdery mildew fungus *Blumeria graminis* f.sp. *hordei*. Resistance results from the failure of the fungus to penetrate the epidermal cell wall. Besides resistance to powdery mildew, *mlo* mutants also exhibit several pleiotropic features, including the spontaneous deposition of callose-containing cell wall appositions (papillae) in

absence of pathogen infections and the premature onset of leaf senescence (Piffanelli et al., 2002; Peterhansel et al., 1997; Wolter et al., 1993), indicating that MLO negatively affects these responses. *MLO* encodes an integral plasma membrane-localized protein, possessing 7 hydrophobic membrane-spanning helices (Devoto et al., 1999). The cytoplasmic C-terminus contains an amphiphilic α-helix that serves in calcium-dependent calmodulin binding. Calmodulin binding is important for *MLO* function, as loss of this domain halved the ability of MLO to negatively regulate defence against powdery mildew *in vivo* (Kim et al., 2002). A mutant screen for suppressors of *mlo* resistance identified *ror2* (*required for mlo resistance 2*) that encodes a plasma membrane-resident syntaxin (Collins et al., 2003). Syntaxins belong to the superfamily of SNARE (soluble N-ethylmaleimide-sensitive fusion protein attachment protein receptor) proteins that contain some key mediators of membrane fusion events in vesicle trafficking (Bock et al., 2001). Requirement of *ROR2* for *mlo* resistance indicates that syntaxin activity is required for *mlo* resistance. Studies using fluorophore-tagged MLO, calmodulin and ROR2 protein variants showed that MLO and ROR2 focally accumulate at sites of attempted fungal cell-wall penetration and physically interact (Bhat et al., 2005). Moreover, interactions between MLO and calmodulin transiently increased at these sites during successful pathogen entry (Bhat et al., 2005). The data suggest that MLO negatively modulates vesicle transport to sites of pathogen entry. Likely, delivered cargo contains antimicrobial components to block pathogen entry.

The Arabidopsis genome encodes 15 *MLO* orthologs and only loss of *AtMLO2* function showed reduced susceptibility to *Golovinomyces orontii* (Consonni et al., 2006). Sequence analysis of Arabidopsis MLO proteins showed that AtMLO2 is most homologous with AtMLO6 and AtMLO12. Double mutants of *Atmlo2* with either *Atmlo6* or *Atmlo12* showed even less susceptibility then *Atmlo2* alone. The triple mutant showed complete resistance comparable with the barley *mlo* mutant (Consonni et al., 2006). Silencing the *SlMlo1* gene of tomato resulted in reduced susceptibility to the powdery mildew *Oidium neolycopersici* (Bai et al., 2008). Moreover, tomato plants homozygous for the *ol-2* allele show broad-spectrum powdery mildew resistance that can be complemented with a wild-type copy of *SlMlo1*, suggesting that *SlMlo1* is mutated in *ol-2* plants. A 19bp deletion in the cDNA of SlMlo1 was detected in resistant *ol-2* plants. Resistance co-segregated with the 19 bp deletion in progeny of crosses between *ol-2* plants and wild-type plants, further substantiating that *SlMlo1* is *Ol-2* (Bai et al., 2008). Disruption of *MLO* genes in barley,

Arabidopsis and tomato results in powdery mildew resistance, indicating that MLO function is conserved throughout many plant species.

*Arabidopsis pmr mutants are resistant to Golovinomyces cichoracearum*

The first mutagenesis approach aimed to identify host genes that contribute to basic compatibility was performed by Vogel and Somerville (2000). They identified 26 *powdery mildew resistant* (*pmr*) mutants in Arabidopsis that had lost susceptibility to *Erysiphe cichoracearum*, also known as *Golovinomyces cichoracearum*. Four of the corresponding genes have been identified so far. *PMR2* was found to be identical to *AtMLO2*, an ortholog of the barley *MLO* gene, as described above. *PMR4* encodes a callose synthase (*GLS5*/*CalS12*) that is required for callose accumulation during pathogen infection (Nishimura et al., 2003). Although *pmr4* mutants lack pathogen-induced callose accumulation they exhibit higher SA and defence gene expression levels. High SA levels are important for *pmr4*-mediated resistance as mutations in genes encoding proteins that functions in the SA pathway are capable of restoring susceptibility to *pmr4-1* (Nishimura et al., 2003). *PMR5* belongs to a large family of plant specific proteins of unknown function and *PMR6* encodes a pectate lyase-like protein. Both mutants show an altered cell wall composition, including increased pectin levels (Vogel et al., 2004; Vogel et al., 2002), indicating that cell wall composition is important in basic compatibility to powdery mildew.

*Arabidopsis is susceptible to the downy mildew pathogen Hyaloperonospora parasitica*

Forward genetic screens in Arabidopsis are a powerful tool to study biological phenomena. The study regarding basic compatibility to *G. cichoracearum* as described above has resulted in the identification of several interesting host genes involved in powdery mildew pathogenesis. In addition, Arabidopsis could be used to study basic compatibility to other groups of plant pathogens. Among other cruciferous plants, Arabidopsis is a host to *H. parasitica* the causal agent of downy mildew disease. *H. parasitica* belongs to the phylum oomycota within the kingdom Stramenopila, which also contains the photosynthetic golden-brown algae and diatoms. The filamentous growth of oomycetes and fungi is very similar but both groups are evolutionary very distantly related. Oomycetes include some of the major plant pathogens e.g. *Pythium* and *Phytophthora* species causing seedling damping off and root rot, white rusts e.g. *Albugo candida* and downy mildews, e.g. *Bremia*, *Plasmopara*,

*Peronospora*, *Pseudoperonospora* and *Hyaloperonospora* species. *H. parasitica* is an obligate biotroph, completely depending on living plant tissue for growth and reproduction. The *H. parasitica* lifecycle (Figure 3) starts when conidia germinate on the plant's leaf surface. Appressoria are formed from which a hyphae extends that penetrates between the anticlinal walls of adjoining epidermal cells. The mycelium grows intercellular and haustoria are regularly formed in epidermal and mesophyl cells (Koch and Slusarenko, 1990). After 5-7 days conidiophores grow out of the stomata bearing new conidia that can infect new plant tissue. In addition to this asexual life-cycle *H. parasitica* can reproduce sexually resulting in the formation of oospores. Hyphae differentiate in oogonia and antheridia; these two structures fuse and form oospores, which are thick-walled spores able to survive harsh conditions.

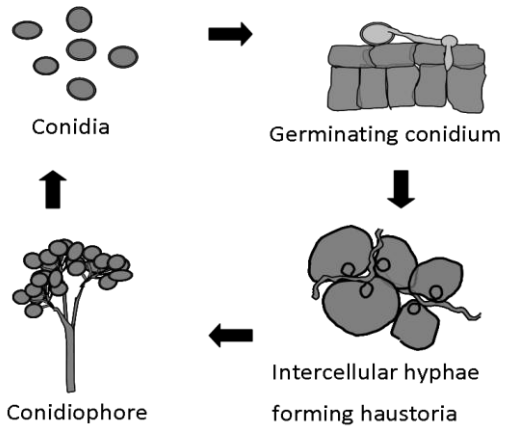


**Figure 3.** The asexual life cycle of *H. parasitica.* Conidia germinate on the surface of a leaf. The pathogen penetrates between the anticlinal walls of adjoining epidermal cells. The mycelium grows intercellular and haustoria are regularly formed in epidermal and mesophyl cells. After 5-7 days conidiophores grows out of the stomata bearing new conidia that can infect new plant tissue.

The interaction between Arabidopsis and *H. parasitica* has been a suitable model to study plant-microbe interactions. Many aspects of *R* gene function and defence signalling, among other subjects, have been elucidated by making use of this patho-system. The research described in this thesis has used this patho-system as a model to study basic compatibility to biotrophic pathogens.

**Outline of thesis**

The aim of this study was to identify Arabidopsis genes controlling cellular processes that influence basic compatibility to *H. parasitica*. **Chapter 2** describes a forward genetic approach to identify Arabidopsis mutants that confer pathogen resistance in particular to *H. parasitica*. Twenty *downy mildew resistant* (*dmr*) mutants were identified of which 8 were further characterised. In **chapter 3** the *dmr5* mutant is characterised in more detail. The *dmr5* mutant shows constitutively enhanced expression of defence-associated genes. The expression of defence-associated genes, as well as *H. parasitica* resistance is largely dependent on *NPR1* function, indicating that resistance is caused by enhanced defence activation. *dmr5* is mutated in *RPM1*, encoding a R-protein of the NBS-LRR class that confers resistance to *P. syringae* strains carrying the *avrB* or *avrRPM1* genes. *RPM1* function is largely lost in *dmr5* plants, although not completely. The obtained data indicates that *dmr5* encodes a mild constitutive active form of RPM1 that mediates *H. parasitica* resistance. **Chapter 4** describes the map based cloning of *DMR6* that encodes a putative 2OG-Fe(II) oxygenase. *DMR6* is strongly induced during compatible and incompatible *H. parasitica* infections and constitutively high expressed in broad range disease resistance mutants. In addition the gene can be induced by application of the SA analog BTH. These data strongly indicate that *DMR6* plays a role in plant defence. *dmr6*-mediated *H. parasitica* resistance is accompanied with the enhanced expression of defence-associated genes, suggesting that *DMR6* negatively affects plant defence responses. **Chapter 5** describes a transcriptomics approach to identify genes that influence susceptibility to *H. parasitica*. Using whole genome CATMA microarrays expression profiles were generated from compatible interactions and compared to profiles from incompatible interactions. Most differentially expressed genes during compatible interactions are similarly expressed in incompatible interactions and are likely involved in MAMP/PAMP and *R*-gene triggered defence responses. A small set of genes was specifically induced in the compatible interaction and could have a different role in infection and disease development. The identification of these compatible specific genes could provide a good starting point to study non-defence-related processes during compatible *H. parasitica* interactions and their role in susceptibility. **Chapter 6** provides a general discussion about the work described in this thesis.

## References

**Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E.** (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signallingpathways in Arabidopsis. Proc Natl Acad Sci U S A. 95, 10306-11.

**Ade, J., DeYoung, B.J., Golstein, C. and Innes, R.W.** (2007). Indirect activation of a plant nucleotide binding site-leucine-rich repeat protein by a bacterial protease. Proc Natl Acad Sci U S A. 104, 2531-6.

**Axtell, M.J. and Staskawicz, B.J.** (2003). Initiation of RPS2-specified disease resistance in Arabidopsis is coupled to the AvrRpt2-directed elimination of RIN4. Cell. 112, 369-77.

**Bai, Y., Pavan, S., Zheng, Z., Zappel, N.F., Reinstädler, A., Lotti, C., De Giovanni, C., Ricciardi, L., Lindhout, P., Visser, R., Theres, K. and Panstruga, R.** (2008). Naturally occurring broad-spectrum powdery mildew resistance in a Central American tomato accession is caused by loss of mlo function. Mol Plant Microbe Interact. 21, 30-9.

**Bent, A.F.** (1996). Plant Disease Resistance Genes: Function Meets Structure. Plant Cell. 8, 1757-71.

**Bent, A.F. and Mackey, D.** (2007). Elicitors, effectors, and R genes: the new paradigm and a lifetime supply of questions. Annu Rev Phytopathol. 45, 399-436.

**Bhat, R.A., Miklis, M., Schmelzer, E., Schulze-Lefert, P. and Panstruga, R.** (2005). Recruitment and interaction dynamics of plant penetration resistance components in a plasma membrane microdomain. Proc Natl Acad Sci U S A. 102, 3135-40.

**Bhattacharjee, S., Hiller, N.L., Liolios, K., Win, J., Kanneganti, T.D., Young, C., Kamoun, S. and Haldar, K.** (2006). The malarial host-targeting signal is conserved in the Irish potato famine pathogen. PLoS Pathog. 2, e50.

**Bock, J.B., Matern, H.T., Peden, A.A. and Scheller, R.H.** (2001). A genomic perspective on membrane compartment organization. Nature. 409, 839-41.

**Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F. and Dong, X.** (1994). A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. Plant Cell. 6, 1845-57.

**Brunner, F., Rosahl, S., Lee, J., Rudd, J.J., Geiler, C., Kauppinen, S., Rasmussen, G., Scheel, D. and Nürnberger, T.** (2002). Pep-13, a plant defence-inducing pathogen-associated pattern from Phytophthora transglutaminases. EMBO J. 21, 6681-8.

**Chinchilla, D., Bauer, Z., Regenass, M., Boller, T. and Felix, G.** (2006). The Arabidopsis receptor kinase FLS2 binds flg22 and determines the specificity of flagellin perception. Plant Cell. 18, 465-76.

**Chisholm, S.T., Coaker, G., Day, B. and Staskawicz, B.J.** (2006). Host-microbe interactions: shaping the evolution of the plant immune response. Cell. 124, 803-14.

**Collins, N.C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J.L., Hückelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S.C. and Schulze-Lefert, P.** (2003). SNARE-protein-mediated disease resistance at the plant cell wall. Nature. 425, 973-7.

**Consonni, C., Humphry, M.E., Hartmann, H.A., Livaja, M., Durner, J., Westphal, L., Vogel, J., Lipka, V., Kemmerling, B., Schulze-Lefert, P., Somerville, S.C. and Panstruga, R.** (2006). Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. Nat Genet. 38, 716-20.

**Dangl, J.L. and Jones, J.D.** (2001). Plant pathogens and integrated defence responses to infection. Nature. 411, 826-33.

**Deslandes, L., Olivier, J., Peeters, N., Feng, D.X., Khounlotham, M., Boucher, C., Somssich, I., Genin, S. and Marco, Y.** (2003). Physical interaction between RRS1-R, a protein conferring resistance to bacterial wilt, and PopP2, a type III effector targeted to the plant nucleus. Proc Natl Acad Sci U S A. 100, 8024-9.

**De Wit, P.J. and Joosten, M.H.** (1999). Avirulence and resistance genes in the Cladosporium fulvum-tomato interaction. Curr Opin Microbiol. 2, 368-73.

**DeYoung, B.J. and Innes, R.W.** (2006). Plant NBS-LRR proteins in pathogen sensing and host defence. Nat Immunol. 7, 1243-9.

**Devoto, A., Piffanelli, P., Nilsson, I., Wallin, E., Panstruga, R., von Heijne, G. and Schulze-Lefert, P.** (1999). Topology, subcellular localization, and sequence diversity of the Mlo family in plants. J Biol Chem. 274, 34993-5004.

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

**Dodds, P.N., Lawrence, G.J., Catanzariti, A.M., Teh, T., Wang, C.I., Ayliffe, M.A., Kobe, B. and Ellis, J.G.** (2006). Direct protein interaction underlies gene-for-gene specificity and coevolution of the flax resistance genes and flax rust avirulence genes. Proc Natl Acad Sci U S A. 103, 8888-93.

**Ellis, J.G., Lawrence, G.J., Luck, J.E. and Dodds, P.N.** (1999). Identification of regions in alleles of the flax rust resistance gene L that determine differences in gene-for-gene specificity. Plant Cell. 11, 495-506.

**Felix, G. and Boller, T.** (2003). Molecular sensing of bacteria in plants. The highly conserved RNA-binding motif RNP-1 of bacterial cold shock proteins is recognized as an elicitor signal in tobacco. J Biol Chem. 278, 6201-8.

**Flor, H.H.** (1971). Current status of the gene-for-gene concept. Annu Rev Phytopathol. 9, 275-96.

**Grant, S.R., Fisher, E.J., Chang, J.H., Mole, B.M. and Dangl, J.L.** (2006). Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. Annu Rev Microbiol. 60, 425-49.
**Greenberg, J.T. and Ausubel, F.M.** (1993). Arabidopsis mutants compromised for the control of cellular damage during pathogenesis and aging. Plant J. 4, 327-41.
**Greenberg, J.T. and Yao, N.** (2004). The role and regulation of programmed cell death in plant-pathogen interactions. Cell Microbiol. 6, 201-11.
**Hann, D.R. and Rathjen, J.P.** (2007). Early events in the pathogenicity of Pseudomonas syringae on Nicotiana benthamiana. Plant J. 49, 607-18.
**Holub, E.B.** (2006). Evolution of parasitic symbioses between plants and filamentous microorganisms. Curr Opin Plant Biol. 9, 397-405.
**Jia, Y., McAdams, S.A., Bryan, G.T., Hershey, H.P. and Valent, B.** (2000). Direct interaction of resistance gene and avirulence gene products confers rice blast resistance. EMBO J. 19, 4004-14.
**Jones, J.D.** (2001). Putting knowledge of plant disease resistance genes to work. Curr Opin Plant Biol. 4, 281-7.
**Jones, J.D. and Dangl, J.L.** (2006). The plant immune system. Nature. 444, 323-9.
**Kamoun, S.** (2007). Groovy times: filamentous pathogen effectors revealed. Curr Opin Plant Biol. 10, 358-65.
**Kim, M.C., Panstruga, R., Elliott, C., Müller, J., Devoto, A., Yoon, H.W., Park, H.C., Cho, M.J. and Schulze-Lefert, P.** (2002). Calmodulin interacts with MLO protein to regulate defence against mildew in barley. Nature. 416, 447-51.
**Koch, E. and Slusarenko, A.** (1990). Arabidopsis is susceptible to infection by a downy mildew fungus. Plant Cell. 2, 437-45.
**Kunze, G., Zipfel, C., Robatzek, S., Niehaus, K., Boller, T. and Felix, G.** (2004). The N terminus of bacterial elongation factor Tu elicits innate immunity in Arabidopsis plants. Plant Cell. 16, 3496-507.
**Luck, J.E., Lawrence, G.J., Dodds, P.N., Shepherd, K.W. and Ellis, J.G.** (2000). Regions outside of the leucine-rich repeats of flax rust resistance proteins play a role in specificity determination. Plant Cell. 12, 1367-77.
**Mackey, D., Holt, B.F. 3rd, Wiig, A. and Dangl, J.L.** (2002). RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell. 108, 743-54.
**Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R. and Dangl, J.L.** (2003). Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. Cell. 112, 379-89.
**Mestre, P. and Baulcombe, D.C.** (2006). Elicitor-mediated oligomerization of the tobacco N disease resistance protein. Plant Cell. 18, 491-501.
**Nishimura, M.T., Stein, M., Hou, B.H., Vogel, J.P., Edwards, H. and Somerville, S.C.** (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science. 301, 969-72.
**Nühse, T.S., Peck, S.C., Hirt, H. and Boller, T.** (2000). Microbial elicitors induce activation and dual phosphorylation of the Arabidopsis thaliana MAPK 6. J Biol Chem. 275, 7521-6.
**Pan, Q., Wendel, J. and Fluhr, R.** (2000). Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. J Mol Evol. 50, 203-13.
**Peterhansel, C., Freialdenhoven, A., Kurth, J., Kolsch, R. and Schulze-Lefert, P.** (1997). Interaction Analyses of Genes Required for Resistance Responses to Powdery Mildew in Barley Reveal Distinct Pathways Leading to Leaf Cell Death. Plant Cell. 9, 1397-409.
**Piffanelli, P., Zhou, F., Casais, C., Orme, J., Jarosch, B., Schaffrath, U., Collins, N.C., Panstruga, R. and Schulze-Lefert, P.** (2002). The barley MLO modulator of defence and cell death is responsive to biotic and abiotic stress stimuli. Plant Physiol. 129, 1076-85.
**Rairdan, G.J. and Moffett, P.** (2006). Distinct domains in the ARC region of the potato resistance protein Rx mediate LRR binding and inhibition of activation. Plant Cell. 18, 2082-93.
**Rate, D.N. and Greenberg, J.T.** (2001). The Arabidopsis aberrant growth and death2 mutant shows resistance to Pseudomonas syringae and reveals a role for NPR1 in suppressing hypersensitive cell death. Plant J. 27, 203-11.
**Rooney, H.C., Van't Klooster, J.W., van der Hoorn, R.A., Joosten, M.H., Jones, J.D. and de Wit, P.J.** (2005). Cladosporium Avr2 inhibits tomato Rcr3 protease required for Cf-2-dependent disease resistance. Science. 308, 1783-6.
**Scofield, S.R., Tobias, C.M., Rathjen, J.P., Chang, J.H., Lavelle, D.T., Michelmore, R.W. and Staskawicz, B.J.** (1996). Molecular Basis of Gene-for-Gene Specificity in Bacterial Speck Disease of Tomato. Science. 274, 2063-5.
**Shao, F., Golstein, C., Ade, J., Stoutemyer, M., Dixon, J.E. and Innes, R.W.** (2003). Cleavage of Arabidopsis PBS1 by a bacterial type III effector. Science. 301, 1230-3.
**Song, W.Y., Wang, G.L., Chen, L.L., Kim, H.S., Pi, L.Y., Holsten, T., Gardner, J., Wang, B., Zhai, W.X., Zhu, L.H., Fauquet, C. and Ronald, P.** (1995). A receptor kinase-like protein encoded by the rice disease resistance gene, Xa21. Science. 270, 1804-6.
**Struck, C., Hahn, M. and Mendgen, K.** (1996). Plasma Membrane H+-ATPase Activity in Spores, Germ Tubes, and Haustoria of the Rust Fungus Uromyces viciae-fabae. Fungal Genet Biol. 20, 30-5.
**Takken, F.L., Albrecht, M. and Tameling, W.I.** (2006). Resistance proteins: molecular switches of plant defence. Curr Opin Plant Biol. 9, 383-90.

**Tameling, W.I., Vossen, J.H., Albrecht, M., Lengauer, T., Berden, J.A., Haring, M.A., Cornelissen, B.J. and Takken, F.L.** (2006). Mutations in the NB-ARC domain of I-2 that impair ATP hydrolysis cause autoactivation. Plant Physiol. 140, 1233-45.

**Tang, X., Frederick, R.D., Zhou, J., Halterman, D.A., Jia, Y. and Martin, G.B.** (1996). Initiation of Plant Disease Resistance by Physical Interaction of AvrPto and Pto Kinase. Science. 274, 2060-3.

**Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G. and Katagiri, F.** (2003). Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen Pseudomonas syringae. Plant Cell. 15, 317-30.

**Van der Biezen, E.A. and Jones, J.D.** (1998). Plant disease-resistance proteins and the gene-for-gene concept. Trends Biochem Sci. 23, 454-6.

**Van der Hoorn, R.A., Roth, R. and De Wit, P.J.** (2001). Identification of distinct specificity determinants in resistance protein Cf-4 allows construction of a Cf-9 mutant that confers recognition of avirulence protein Avr4. Plant Cell. 13, 273-85.

**Voegele, R.T., Struck, C., Hahn, M. and Mendgen, K.** (2001). The role of haustoria in sugar supply during infection of broad bean by the rust fungus Uromyces fabae. Proc Natl Acad Sci U S A. 98, 8133-8.

**Voegele, R.T. and Mendgen, K.** (2003). Rust haustoria: nutrient uptake and beyond. New Phytol. 159, 93-100.

**Vogel, J. and Somerville, S.** (2000). Isolation and characterization of powdery mildew-resistant Arabidopsis mutants. Proc Natl Acad Sci U S A. 97, 1897-902.

**Vogel, J.P., Raab, T.K., Schiff, C. and Somerville, S.C.** (2002). PMR6, a pectate lyase-like gene required for powdery mildew susceptibility in Arabidopsis. Plant Cell. 14, 2095-106.

**Vogel, J.P., Raab, T.K., Somerville, C.R. and Somerville, S.C.** (2004). Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. Plant J. 40, 968-78.

**Whisson, S.C., Boevink, P.C., Moleleki, L., Avrova, A.O., Morales, J.G., Gilroy, E.M., Armstrong, M.R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I.K., Pritchard, L. and Birch, P.R.** (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature. 450, 115-8.

**Win, J., Morgan, W., Bos, J., Krasileva, K.V., Cano, L.M., Chaparro-Garcia, A., Ammar, R., Staskawicz, B.J. and Kamoun, S.** (2007). Adaptive evolution has targeted the C-terminal domain of the RXLR effectors of plant pathogenic oomycetes. Plant Cell. 19, 2349-69.

**Wolter, M., Hollricher, K., Salamini, F. and Schulze-Lefert, P.** (1993). The mlo resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defence mimic phenotype. Mol Gen Genet. 239, 122-8.

**Wulff, B.B., Thomas, C.M., Smoker, M., Grant, M. and Jones, J.D.** (2001). Domain swapping and gene shuffling identify sequences required for induction of an Avr-dependent hypersensitive response by the tomato Cf-4 and Cf-9 proteins. Plant Cell. 13, 255-72.

**Yu, I.C., Parker, J. and Bent, A.F.** (1998). Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis dnd1 mutant. Proc Natl Acad Sci U S A. 95, 7819-24.

**Zipfel, C., Robatzek, S., Navarro, L., Oakeley, E.J., Jones, J.D., Felix, G. and Boller, T.** (2004). Bacterial disease resistance in Arabidopsis through flagellin perception. Nature. 428, 764-7.

**Zipfel, C., Kunze, G., Chinchilla, D., Caniard, A., Jones, J.D., Boller T. and Felix, G.** (2006). Perception of the bacterial PAMP EF-Tu by the receptor EFR restricts Agrobacterium-mediated transformation. Cell. 125, 749-60.

# Chapter 2

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

**Mireille Van Damme[1], Annemiek Andel[1], Robin P. Huibers[1], Ralph Panstruga[2], Peter J. Weisbeek[1], and Guido Van den Ackerveken[1]**

[1] Plant-Microbe Interactions, Department of Biology, Science Faculty, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands.

[2]Max-Planck Institute for Plant Breeding Research, Department of Plant-Microbe Interactions, 50829 Köln, Germany.

Adapted from Molecular Plant-Microbe Interactions. 2005. 18, 583-92.

**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 thaliana* mutant screen for reduced susceptibility to the downy mildew pathogen *Hyaloperonospora parasitica.* Ethyl methane sulfonate (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 six 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*, also were 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:* disease resistance, disease susceptibility, downy mildew resistance, obligate biotroph, oomycete.

**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; this capacity also is referred to as compatibility. In most cases, plants resist infection due to active defence mechanisms or the absence of compatibility toward a given pathogen. Knowledge of disease resistance has increased tremendously over the last two decades. Numerous resistance genes have been cloned and many defenceassociated 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 considered three distinct steps during infection of plants by biotrophic pathogens that involve an array of different host cell processes.

The first step toward 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 spp. 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 surface hydrophobicity and hardness are sufficient to stimulate appressorium development in some pathogens (Carzaniga et al. 2001; Tucker and Talbot 2001), others require specific signals to develop infection structures (e.g., the sensing of cutin by Colletotrichum spp. [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, such as the downy mildew pathogen Hyaloperonospora parasitica, develops a single haustorium in each plant cell it encounters (Koch and Slusarenko 1990).

The second step occurs when pathogens are within the host tissue and 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] such as fungal chitin, oomycete glucans, and bacterial flagellin [Nürnberger et al. 2004]). Therefore, the circumvention of recognition and suppression of plant-defence responses is an important step for pathogens to take. 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, evidence is emerging in fungi and oomycetes 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, fungal and oomycete effectors also 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 favorable 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 and associates (2002), who showed that a set of pepper genes is induced specifically 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 toward the pathogen is supported by the fact that sugar transporters and amino acid permeases are highly expressed in rust haustoria (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 ethyl methane sulfonate (EMS) and T-DNA insertion mutants for loss of susceptibility. Twenty-six powdery mildew-resistant (*pmr*) mutants were isolated, corresponding to six loci that displayed strongly reduced sporulation, of which three 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 and associates (2004), who analyzed recombinant inbred lines of a L*er* × Col-0 cross. They identified several small- to medium-effect quantitative trait loci that govern disease susceptibility to *Botrytis* spp.

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 eight downy mildew-resistant (*dmr*) mutants corresponding to six different loci. In three *dmr* mutants, resistance was associated with constitutive activation of plant-defence responses. Three other mutants, *dmr1, dmr2,* and *dmr6,* showed resistance in the absence of enhanced defence responses, suggesting that the corresponding genes are required for susceptibility to downy mildew.

**Results**

*Isolation of dmr 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 (enhanced disease susceptibility 1) 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 toll interleukin 1 receptor nucleotide binding site leucine-rich repeat (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* facilitated the convenient distinction between phenotypes in our mutant screen for loss of susceptibility.

The *dmr* mutants were identified among the susceptible M2 plants by visual screening of cotyledons and primary leaves for lack of *H. parasitica* sporulation at 7 to 10 days postinoculation with isolate Cala2 (Figure 1 illustrates the screening approach). From our screening of 3,600 M1 families (approximately 100,000 M2 plants), we identified approximately 300 putative mutants. Of these, less than 10% could be confirmed in the next generation (M3) 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. In all, 20 confirmed *dmr* mutants were identified. The detailed analysis of eight *dmr* mutants is described below.

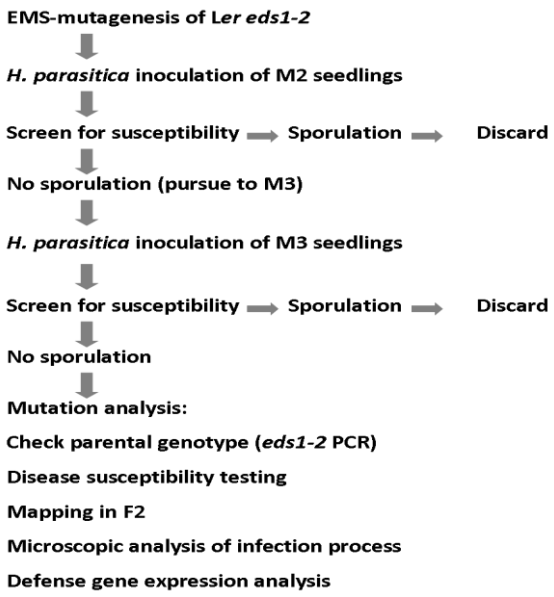


**Figure 1**. Screening approach for the isolation of downy mildew-resistant

*Genetic analysis.*

Complementation crosses between the mutants revealed six independent loci, denoted *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 because the F1 plants were susceptible to Cala2, and approximately a quarter of the F2 plants displayed *H. parasitica* resistance. From each cross, the resistant F2 plants were selected, genotyped, and rescreened for resistance in the F3 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. Loci *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, because F1 plants resulting from complementation crosses were susceptible to *H. parasitica*. Locus *dmr3* also is 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 conferred 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*, because the F2 and F3 progeny did not give a reproducible resistance phenotype.

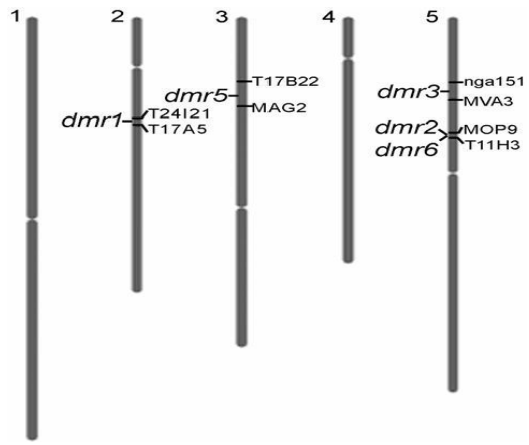


**Figure 2.** Position of five downy mildew-resistant (*dmr*) loci on the *Arabidopsis* chromosome map. The location of each *dmr* locus is placed on the physical map using the program Chromosome Map Tool.

*Disease susceptibility of dmr mutants.*

The *dmr* mutants varied in degree of resistance to *H. parasitica*. To quantify the level of resistance, we counted the number of conidioiophores on mutant seedlings (Figure 3). Compared with 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 were 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*. In addition to their resistance to *H. parasitica*, some of the *dmr* mutants have additional phenotypes. For example, *dmr3* (Figure 4F) and *dmr4* (Figure 4G) showed dwarfism, and *dmr1-1* showed 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 F2 mapping populations, indicating that they were caused by additional point mutations resulting from the EMS mutagenesis.

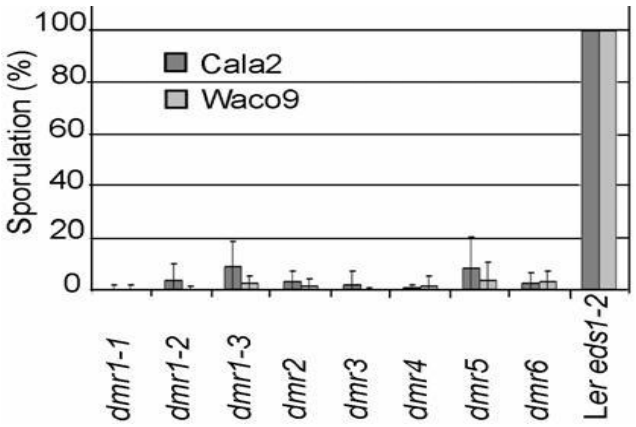


**Figure 3.** Quantification of *H. parasitica* sporulation on the downy mildew-resistant (*dmr*) mutants compared with the parental L*er eds1-2* line (set to 100%) at 7 days postinoculation with the isolates Cala2 and Waco9.

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 postinoculation 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 compared with the parental L*er eds1-2* line. Hyphal growth still could be detected in the *dmr1-2*, *dmr1-3*, *dmr2*, *dmr5,* and *dmr6* mutants, although to a lesser extent than in the parental line L*er eds1-2* (Figure 4A). In the three *dmr1* mutants, cell wall appositions often were observed around arrested haustoria (Figure 4B through D). These papillae, containing callose (as

detected by aniline blue staining; data not shown), possibly were a secondary effect on the invading haustoria. In the case of the *dmr2* mutant, no papillae formation was detected; however, hyphal growth often was 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). *H. parasitica* infections in the *dmr* mutants at 5 days postinoculation (data not shown) were very similar to those at 3 days postinoculation, indicating that further colonization remained disturbed. Overall, the growth and development of *H. parasitica*, as observed by microscopic analysis of trypan blue-stained plants, was in good agreement with the level of sporulation that was observed in figure 3.

The production of reactive oxygen intermediates (ROI) often is linked to the hypersensitive response and to cell wall-based defence responses (Beers and McDowell 2001). Microscopic detection of ROI by 3′-3′-diaminobenzidine (DAB) staining showed that ROI were produced in none of the *dmr* mutants in response to *H. parasitica* infection (Figure 4L; data shown for *dmr1-3*), 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. ROI could be visualized by the brown DAB precipitate that was formed in close vicinity to the invading pathogen (Figure 4J).

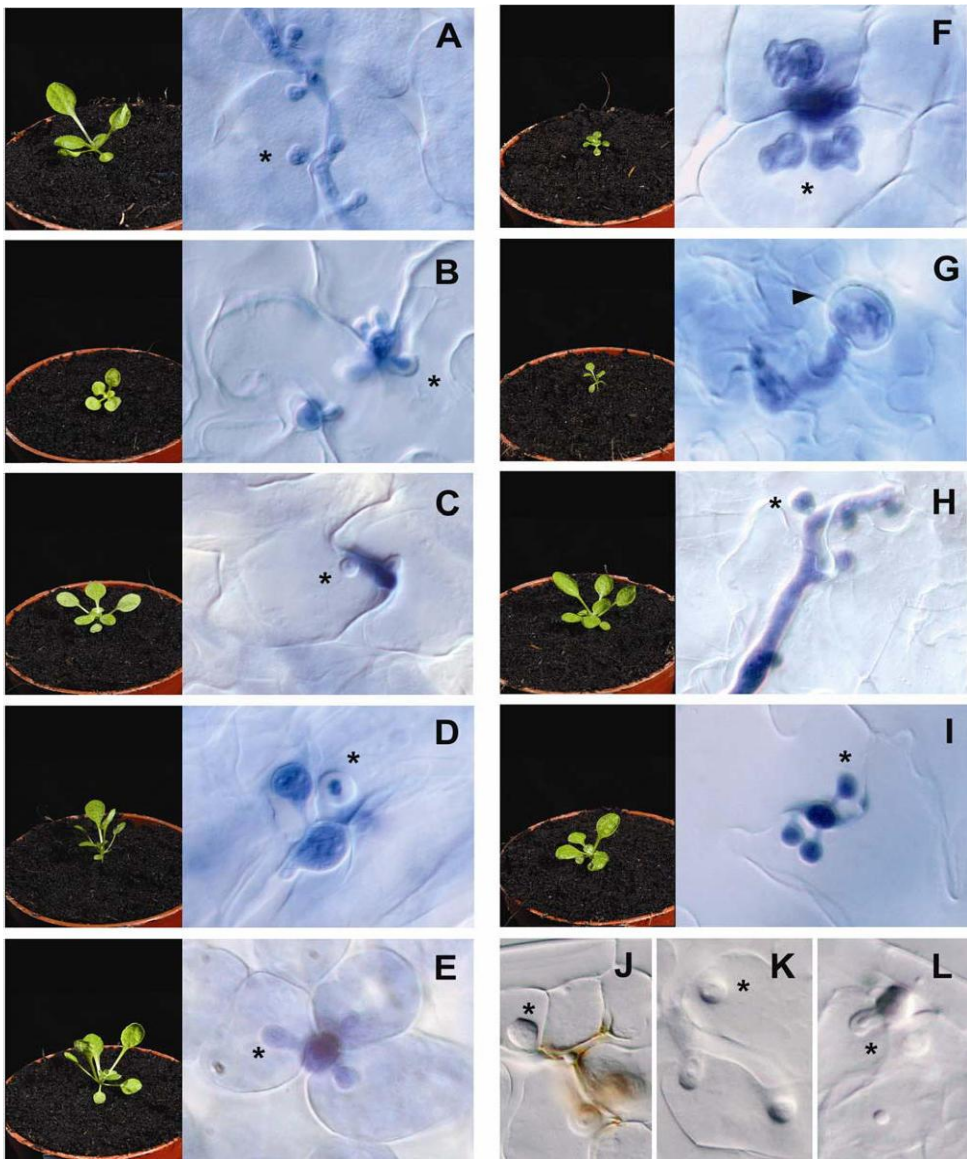


**Figure 4.** Phenotypes of downy mildew-resistant (*dmr*) mutants. For each mutant, a 3-week-old plant is shown, as well as a microscopic image of *Hyaloperonospora parasitica*-infected tissue of 14-day-old seedlings. For each mutant, representative images of trypan blue-stained seedlings 3 days post inoculation with isolate Cala2 ($5 \times 10^4$ 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 often were 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 a 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 (ROI) by 3′-3′-diaminobenzidine staining in seedlings inoculated with *H. parasitica* isolate Cala2. (**J**), Col-0 plants that recognize Cala2 through *RPP2* accumulated ROI 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 ROI production or hypersensitive cell death.

To analyze whether the *dmr* mutants displayed a spectrum of resistance broader than to *H. parasitica* alone, susceptibility to the gram-negative bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 and the biotrophic powdery mildew fungus *Golovinomyces orontii* was tested. The parental line supported growth of *P. syringae* pv. *tomato* (a 100-fold increase in 3 days), whereas seedlings treated with benzothiadiazole (BTH), a chemical inducer of systemic acquired resistance, showed 20-fold less bacterial growth (Figure 5). Mutants *dmr1*, *dmr2*, *dmr5*, and *dmr6* did not seem to be significantly affected in their susceptibility to *P. syringae* pv. *tomato*. In contrast, *dmr3* and *dmr4* displayed a strong resistance to *P. syringae* pv. *tomato* that was comparable with BTH-treated control plants. In addition, the *dmr3* and *dmr4* mutants also showed resistance to

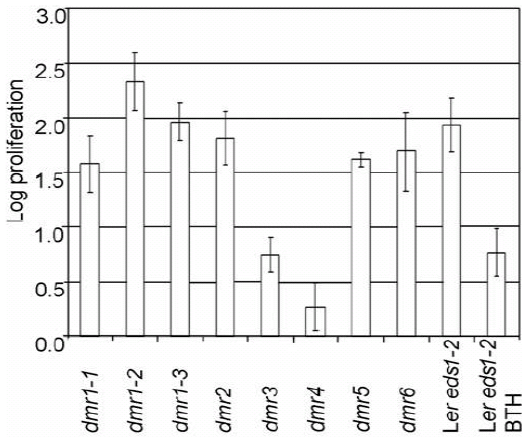


**Figure 5.** Quantification of bacterial (*Pseudomonas syringae* pv. *tomato*) proliferation in the downy mildew-resistant (*dmr*) mutants and control plants over 3 days. The *dmr1*, *dmr2*, *dmr5*, and *dmr6* mutants showed an approximately 100-fold increase in leaf tissue comparable with that of the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

the powdery mildew pathogen, *G. orontii*. This fungal pathogen, formerly referred to as *E. orontii*, previously was reported to represent one of three powdery species that are able to successfully colonize *Arabidopsis thaliana* (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 (score 2.8) to powdery mildew, whereas Sha proved resistant (score 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 (approximately 2.8) with 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 with 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*.

*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* did 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 because these mutants displayed resistance to *P. syringae* pv. *tomato* and *G. orontii*. The *dmr5* mutant, however, remained susceptible to *P. syringae* pv. *tomato* despite elevated expression of *PR-1.* Like the parental line, mutants *dmr1*, *dmr2*, and

*dmr6* did 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 because they do not show broad-spectrum resistance, nor do they show enhanced defence gene expression.

**Table 1.** Susceptibility of the downy mildew-resistant mutants to *Golovinomyces orontii.* [a] Level of sporulation was scored on a disease resistance scale of 0 to 3 at 10 days post inoculation. The total number of plants tested per accession or mutant was six. The assay was repeated once with comparable results.

| Genotype | Average disease resistance score[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*ereds1-2* | 2.8 |
| Col-0 | 2.5 (susceptible) |
| Sha | 0.5 (resistant) |

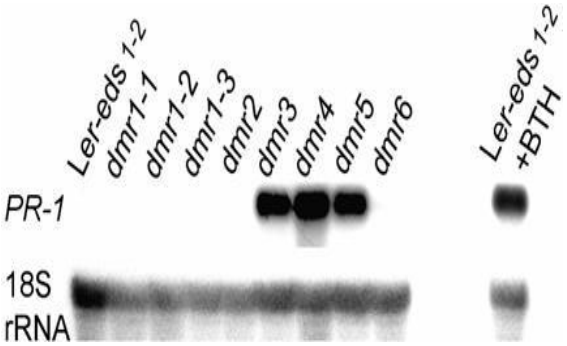


**Figure 6.** Northern blot analysis of *PR-1* expression in non inoculated seedlings of different downy mildew-resistant (*dmr*) mutants compared with the parental L*er eds1-2* line. L*er eds1-2* treated with benzothiadiazole (BTH) was used as a positive control for *PR-1* expression. The same membrane also was 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 *P. syringae* pv. *tomato*, constitutive expression of *PR-1*, and a dwarf phenotype. *DND1* (defence 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 whether *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 F2 and F3 generation segregated for resistance to *H. parasitica,* indicating that *dmr3* and *dnd1* are not allelic.

**Discussion**

In this article, 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 also would result in low numbers of conidiophores on the leaves, allowing the identification of mutants by visual screening of sporulation on M2 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 M2 seedlings from 3,600 M1 families, we isolated 20 independent *dmr* mutants, of which eight were analyzed in more detail. Complementation crosses showed that the eight *dmr* mutants corresponded to six loci which subsequently were mapped in segregating F2 populations. Two of the loci mapped to regions with known defence genes. Although *dmr3* mapped near *dnd1*, complementation crosses and *DND1* sequencing of the *dmr3* mutant showed that these mutants were not affected in the same gene. On chromosome 2, *dmr1* mapped 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 showed constitutive activation of *PR-1* and resistance to

*Pseudomonas* bacteria, whereas the three different *dmr1* loci did not show these phenotypes. Comparison of the *dmr* map positions to those of the *pmr* loci (Vogel and Somerville 2000), that provide resistance to *E. cichoracearum*, indicated that they are not linked. The *pmr4* locus, however, confers resistance not only to powdery mildew but also to *H. parasitica* (Vogel and Somerville 2000). *PMR4* encodes a callose or glucan synthase (Jacobs et al. 2003, Nishimura et al. 2003). *NahG* transgenics, which do not accumulate salicylic acid (SA), appeared to be fully susceptible to *E. cichoracearum* (Nishimura 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 already are known that show constitutive or activated plant-defence responses (e.g., the *cpr*, *cim*, *acd*, *dnd*, and *edr* mutants) (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 signaling 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. The mutant screening was performed in the *eds1-2* background; therefore, we counterselected most of these mutations. Indeed several enhanced-defence mutations (i.e., *cpr1* and *cpr6*) required a functional *EDS1* gene to mediate disease resistance (Clarke et al. 2001). However, *eds1* mutants still were able to mount a hypersensitive response and produce ROI (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 F2 populations, and therefore probably is caused by other EMS mutations. The resistance of *dmr4* is particularly interesting because 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 showed elevated levels of *PR-1* gene expression and resistance to a bacterial pathogen (*P. syringae*). We currently are testing whether *NPR1* (no *PR-1* 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 *Arabidopsis* accession Col-0. Approximately one-quarter of the F2 plants showed a clear resistance phenotype. However, F3 plants obtained from resistant F2 plants often proved to be susceptible. Also, several backcrosses of F2 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 whether 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 syringae* pv. *tomato* or *G. orontii.* We do not know of any mutant that displays this combination of phenotypes, suggesting that *dmr5* is affected in an as-yet-unknown component in defence signal transduction. Uncoupling of oomycete and bacterial resistance also is seen in the *cpr5 npr1* and *cpr5 nahG* double mutants, where constitutive expression of *PR-1* and resistance to *P. syringae* pv. *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, in addition to 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 signaling mutants currently are being made and defence gene expression will be monitored by DNA microarrays.

*Lack-of-susceptibility mutants.*

The resistance of *dmr* mutants to *H. parasitica* also can 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 signaling, 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 (Büschges 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 also is 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 posthaustorial growth cessation of the fungal pathogen. Resistance of the *pmr4* mutant is associated with an enhanced pathogeninduced SA-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 three 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* spp. or *G. orontii*. *H. parasitica* growth was inhibited in these *dmr* mutants without visible cell death or accumulation of ROI.

In addition, expression of the defencerelated 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. Both *dmr2* and *dmr6* are 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 three 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 downy mildew.

**Experimental procedures**

*Plant lines and growth conditions.*

*A. thaliana* lines used in this study were 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 Scientific, Tilburg, The Netherlands) at 22°C with 16 h of light (100 µE/m2/s) and a relative humidity of 75%.

*Growth and infection of downy mildew.*

*H. parasitica* isolate Cala2 was kindly provided by Dr. E. Holub (Warwick HRI, Wellesbourne, U.K.) and maintained on *Arabidopsis* L*er* by weekly transfer to healthy 10- to 14-dayold seedlings (Holub et al. 1994). To obtain large amounts of sporangiospores for bioassays, inoculum was collected from L*er eds1-2 s*eedlings that supported abundant Cala2 growth and sporulation (Parker et al. 1996). Inoculum (5 × 104 spores ml–1) was applied on 14-day-old seedlings using a spray gun. After inoculation, plants were allowed to dry for 15 to 60 min and subsequently incubated under a sealed lid (100% relative humidity) in a growth chamber at 16°C with 9 h of light/day (100 µE/m2/s). The amount of sporulation was quantified at 7

days postinoculation by counting the number of conidiophores on the cotyledons and leaves. The number of conidiophores was determined per leaf for both isolates for at least 40 to 100 seedlings per mutant (Figure 3). The amount of conidiophores on L*er-eds1-2* was set as 100%.

*EMS mutagenesis.*

Seed of L*er eds1-2* backcrossed twice to L*er* were kindly provided by J. Parker (MPIZ, Köln, Germany). Approximately 10,000 seed were imbibed in water for approximately 2 days at 4°C, then treated for 18.5 h in 0.2125% EMS (21.25 µl in 10 ml of water), and extensively washed with 2 liters of sterile water. M1 plants were grown on autoclaved soil (seven parts of potting compost and five parts of sand) and 4-week-old plants were treated with BTH (active compound at 100 mg/liter/ml of H2O) to protect them from disease. Seeds were collected as M2 families from more than 3,600 individual M1 plants. Albino mutants were detected in approximately 10% of the M2 families.

*Mutant screening.*

In all, 30 to 40 M2 plants/family were sown in plug trays (22 by 13 wells) with fine potting soil. After 10 to 14 days, the seedlings were inoculated with *H. parasitica* Cala2. Susceptible plants were removed with tweezers 7 days later. The remaining seedlings were rescreened 3 days later and resistant plants were transferred and grown for M3 seed production. Approximately 30 M3 plants per putative M2 mutant were rescreened for resistance. True resistant lines then were genotyped for the parental *eds1-2* mutation by polymerase chain reaction (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 (1:1:1:1 volume of lactic acid/glycerol/phenol/H2O) with trypan blue (1 mg/ml) was added. The tubes were placed in a boiling water bath for 1 min. Leaves were destained in chloral hydrate. The tubes were placed in a speed-vacuum infiltrator for 1 min to remove air bubbles from the leaves. *H. parasitica* growth was detected by differential interference contrast microscopy. ROI were detected by staining for H2O2 accumulation. As described by

Thordal-Christensen and associates (1997), DAB staining visualized H2O2. DAB staining was performed for 8 h on 10-day-old *A. thaliana* seedlings at 3 days postinoculation with *H. parasitica* isolate Cala2. Seedlings dissected from the root were placed in 96-well plates containing 100 µl of DAB solution a plastic box under high humidity (5 to 6 h). Afterward, the leaves were cleared of chlorophyll in alcoholic lactophenol (one volume of phenol/glycerol/lactic acid/water [1:1:1:1] and two volumes of ethanol) for 15 to 30 min at 65°C, followed for 2 h at room temperature in fresh solution. Prior to microscopy, the samples were placed in chloral hydrate in a speed-vacuum infiltrator for 1 min to remove air bubbles. *Arabidopsis* leaves were visualized by differential interference contrast microscopy.

*Bacterial growth curve.*

The bacterial growth of *P. syringae* pv. *tomato* strain DC3000 was performed essentially as described by Tornero and Dangl (2001). Ten-day-old seedlings were inoculated by surface dipping. Samples were taken at time points 0 (1 h) and 3 days postinoculation. For this, the aerial parts of five seedlings were weighed, treated as described to release bacteria, and plated on King's B agar plates to determine bacterial numbers per milligram of fresh weight.

*G. orontii inoculation.*

To assess powdery mildew infection phenotypes, rosette leaves of 6-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 *G. orontii* conidiospores. Disease symptoms were evaluated 8 to 10 days postinoculation by macroscopic and microscopic inspection and classified according to the disease resistance score described by Adam and associates (1999).

*Northern blot analysis.*

Total RNA was isolated from frozen tissue samples using the RNeasy plant mini kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA samples were denatured for 1 h at 50°C in glyoxal (10 mM sodium phosphate, 1.0 M glyoxal, and 0.5× dimethyl sulfoxide). RNA samples (8 µg) 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 (Hybond N; Amersham Biosciences, Buckinghamshire, U.K.), the RNA was crosslinked to the

membrane by UV-cross linking (175 mJ/cm2). The membrane was hybridized with 32P-labeled probes using the random DNA labeling kit (MBI Fermentas, Vilnius, Lithuania), first with *PR-1* and after stripping (15 min in boiling, 0.1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate] and 0.5% sodium dodecyl sulfate [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% bovine serum albumen), 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 prewarmed (65°C) buffer (0.5% SDS, 2× SSC) and washed twice 15 min in 0.5% SDS, 2× 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. F1 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′-acacatcg gtgatgcgagaca-3′). The F1 seedlings were drop inoculated to test for susceptibility. F2 seedlings were screened for disease resistance as described above, and resistant F2 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 on The Arabidopsis Information Resource (TAIR) website. The markers T24I21 (forward primer, 5′-aatccaaatttcttgcgagaacaca-3′ and reverse primer, 5′-aaacgaagagtgacaatggttggag-3′) based on a 12-bp (CER459636) deletion in the Landsberg accession. The T17A5 marker (forward primer, 5′-cgatgtctcaccggtgtaaccttta-3′ and reverse primer, 5′-ttgcagagaacttcatgactggcta-3′) resulted in a 32-bp (CER458639) deletion in Landsberg. The MOP9 marker was based on CER456575, a 31-bp deletion, in the Landsberg accession (forward primer, 5′-tttgggaacagaaaaagttggaggt-3′ and reverse primer, 5′-catattcaaaagggaaaatcccaga-3′). A 24-bp 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 on the TAIR website and the MVA3 marker resulted in a 16-bp insertion (CER457398) in the Landsberg accession (forward primer, 5′-cttatcgaaacccccatttgtaag-3′ and reverse primer, 5′-

aagaaagaggtcagagtcggagaa-3′). The T17B22 marker resulted 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 37-bp insertion (CER464868) in the Landsberg accession (forward primer, 5′- ttctattattcggtggaagatcaag-3′ and reverse primer, 5′-tagatttctgcgaagatttct-3′).

**Author-recommended internet resource**

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

TAIR Chromosome Map Tool: www.arabidopsis.org/jsp/ChromosomeMap/tool.jsp.

**Acknowledgements**

The research of G. van den Ackerveken was made possible by a fellowship of the Royal Netherlands Academy of Arts and Sciences. We are grateful to J. Parker for providing us with BC2 L*er eds1-2* seed for EMS mutagenesis, E. Holub for the *H. parasitica* isolates and his instructions on how to work with them, J. Beynon for the *FN2* seed, and A. Bent for the *dnd1* mutant. We thank P. Schoondermark and B. Beusink for their efforts at the start of the mutagenesis and screening part of the project.

## References

**Aarts, N., Metz, M., Holub, E., Staskawicz, B.J., Daniels, M.J. and Parker, J.E.** (1998). Different requirements for EDS1 and NDR1 by disease resistance genes define at least two R gene-mediated signaling pathways in Arabidopsis. Proc. Natl. Acad. Sci. U.S.A. 95, 10306-11.

**Abramovitch, R.B., Kim, Y.J., Chen, S., Dickman, M.B. and Martin, G.B.** (2003). Pseudomonas type III effector AvrPtoB induces plant disease susceptibility by inhibition of host programmed cell death. EMBO (Eur. Mol. Biol. Organ.) J. 22, 60-69.

**Adam, L., Ellwood, S., Wilson, I., Saenz, G., Xiao, S., Oliver, R.P., Turner, J.G. and Somerville, S.** (1999). Comparison of Erysiphe cichoracearum and E. cruciferarum and a survey of 360 Arabidopsis thaliana accessions for resistance to these two powdery mildew pathogens. Mol. Plant-Microbe Interact. 12, 1031-43.

**Allen, E.A., Hazen, B.E., Hoch, H.C., Kwon, Y., Leinhos, G.M.E., Staples, R.C., Stumpf, M.A. and Terhune, B.T.** (1991). Appressorium formation in response to topographical signals by 27 rust species. Phytopathology. 81, 323-31.

**Allen, R.L., Bittner-Eddy, P.D., Grenville-Briggs, L.J., Meitz, J.C., Rehmany, A.P., Rose, L.E. and Beynon, J.L.** (2004). Host-parasite coevolutionary conflict between Arabidopsis and downy mildew. Science. 306, 1957-60.

**Axtell, M.J. and Staskawicz, B.J.** (2003). Initiation of RPS2-specified disease resistance in Arabidopsis is coupled to the AvrRpt2-directed elimination of RIN4. Cell. 112, 369-77.

**Beers, E.P. and McDowell, J.M.** (2001). Regulation and execution of programmed cell death in response to pathogens, stress and developmental cues. Curr. Opin. Plant Biol. 4, 561-7.

**Bowling, S.A., Clarke, J.D., Liu, Y., Klessig, D.F. and Dong, X.** (1997). The cpr5 mutant of Arabidopsis expresses both NPR1-dependent and NPR1 independent resistance. Plant Cell. 9, 1573-84.

**Büschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Töpsch, S., Vos, P., Salamini, F. and Schulze-Lefert, P.** (1997). The barley Mlo gene: A novel control element of plant pathogen resistance. Cell. 88, 695-705.

**Carzaniga, R., Bowyer, P. and O'Connell, R.J.** (2001). Production of extracellular matrices during development of infection structures by the downy mildew Peronospora parasitica. New Phytol. 149, 83-93.

**Clarke, J.D., Liu, Y., Klessig, D.F. and Dong, X.** (1998). Uncoupling PR gene expression from NPR1 and bacterial resistance: Characterization of the dominant Arabidopsis cpr6-1 mutant. Plant Cell. 10, 557-69.

**Clarke, J.D., Aarts, N., Feys, B.J., Dong, X. and Parker, J.E.** (2001). Constitutive disease resistance requires EDS1 in the Arabidopsis mutants cpr1 and cpr6 and is partially EDS1-dependent in cpr5. Plant J. 26, 409-20.

**Clough, S.J., Fengler, K.A., Yu, I., Lippok, B., Smith, R.K. and Bent, A.F.** (2000). The Arabidopsis dnd1 "defence, no death" gene encodes a mutated cyclic nucleotide-gated ion channel. Proc. Natl. Acad. Sci. U.S.A. 97, 9323-8.

**Denby, K.J., Kumar, P. and Kliebenstein, D.J.** (2004). Identification of Botrytis cinerea susceptibility loci in Arabidopsis thaliana. Plant J. 38, 473-86.

**Dickman, M.B., Ha, Y.S., Yang, Z., Adams, B. and Huang, C.** (2003). A protein kinase from Colletotrichum trifolii is induced by plant cutin and is required for appressorium formation. Mol. Plant-Microbe Interact. 16, 411-21.

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

**Dodds. P.N., Lawrence, G.J., Catanzariti, A.M., Ayliffe, M.A. and Ellis, J.G.** (2004). The Melampsora lini AvrL567 avirulence genes are expressed in haustoria and their products are recognized inside plant cells. Plant Cell. 16, 755-68.

**Falk, A., Feys, B.J., Frost, L.N., Jones, J.D., Daniels, M.J. and Parker, J.E.** (1999). EDS1, an essential component of R gene-mediated disease resistance in Arabidopsis has homology to eukaryotic lipases. Proc. Natl. Acad. Sci. U.S.A. 96, 3292-7.

**Frye, C.A., Tang, D. and Innes, R.W.** (2001). Negative regulation of defence responses in plants by a conserved MAPKK kinase. Proc. Natl. Acad. Sci. U.S.A. 98, 373-8.

**Glazebrook, J.** (2001). Genes controlling expression of defence responses in Arabidopsis—2001 status. Curr. Opin. Plant Biol. 4, 301-8.

**Greenberg, J.T., Guo, A., Klessig, D.F. and Ausubel, F.M.** (1994). Programmed cell death in plants: A pathogen-triggered response activated coordinately with multiple defence functions. Cell. 77, 551-63.

**Holub, E.B.** (2001). The arms race is ancient history in Arabidopsis, the wildflower. Nat. Rev. Genet. 2, 516-27.

**Holub, E.B., Beynon, J.L. and Crute, I.R.** (1994). Phenotypic and genotypic characterization of interactions between isolates of Peronospora parasitica and accessions of Arabidopsis thaliana. Mol. Plant-Microbe Interact. 7, 223-39.

**Jacobs, A.K., Lipka, V., Burton, R.A., Panstruga, R., Strizhov, N., Schulze-Lefert, P. and Fincher, G.B.** (2003). An Arabidopsis callose synthase, GSL5, is required for wound and papillary formation. Plant Cell. 15, 2503-13.

**Kim, H.S. and Delaney, T.P.** (2002). Arabidopsis SON1 is an F-box protein that regulates a novel induced defence response independent of both salicylic acid and systemic acquired resistance. Plant Cell. 14, 1469-82.

**Koch, E. and Slusarenko, A.** (1990). Arabidopsis is susceptible to infection by a downy mildew fungus. Plant Cell. 2, 437-45.

**Lu, H., Rate, D.N. Song, J.T. and Greenberg, J.T.** (2003). ACD6, a novel ankyrin protein, is a regulator and an effector of salicylic acid signaling in the Arabidopsis defence response. Plant Cell. 15, 2408-20.
**Mackey, D., Holt, B.F., Wiig, A. and Dangl, J.L.** (2002). RIN4 interacts with Pseudomonas syringae type III effector molecules and is required for RPM1-mediated resistance in Arabidopsis. Cell. 108, 743-54.
**Mackey, D., Belkhadir, Y., Alonso, J.M., Ecker, J.R. and Dangl, J.L.** (2003). Arabidopsis RIN4 is a target of the type III virulence effector AvrRpt2 and modulates RPS2-mediated resistance. Cell. 112, 379-89.
**Marois, E., Van den Ackerveken, G. and Bonas, U.** (2002). The Xanthomonas type III effector protein AvrBs3 modulates plant gene expression and induces cell hypertrophy in the susceptible host. Mol. Plant- Microbe Interact. 15, 637-46.
**Mendgen, K. and Hahn, M.** (2002). Plant infection and the establishment of fungal biotrophy. Trends Plant Sci. 7, 352-6.
**Nimchuk, Z., Eulgem, T., Holt, B.F., 3rd and Dangl, J.L.** (2003). Recognition and response in the plant immune system. Annu. Rev. Genet. 37, 579-609.
**Nishimura, M.T., Stein, M., Hou, B.H., Vogel, J.P., Edwards, H. and Somerville, S.C.** (2003). Loss of callose synthase results in salicylic acid-dependent disease resistance. Science. 301, 969-72.
**Nürnberger, T., Brunner, F., Kemmerling, B. and Piater, L.** (2004). Innate immunity in plants and animals: Striking similarities and obvious differences. Immunol. Rev. 198, 249-66.
**Panstruga, R.** (2003). Establishing compatibility between plants and obligate biotrophic pathogens. Curr. Opin. Plant Biol. 6, 320-6.
**Panstruga, R. and Schulze-Lefert, P.** (2002). Live and let live: Insights into powdery mildew disease and resistance. Mol. Plant Pathol. 3, 495-502.
**Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D. and Daniels, M.J.** (1996). Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora parasitica specified by several different RPP genes. Plant Cell. 8, 2033-46.
**Plotnikova, J.M., Reuber, T.L., Ausubel, F.M. and Pfister, D.H.** (1998). Powdery mildew pathogenesis of Arabidopsis thaliana. Mycologia. 90, 1009-16.
**Pruitt, R.E. and Meyerowitz, E.M.** (1986). Characterization of the genome of Arabidopsis thaliana. J. Mol. Biol. 187, 169-83.
**Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S. and Greenberg, J.T.** (1999). The gain-of-function Arabidopsis acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defences, and cell growth. Plant Cell. 11, 1695-708.
**Rathjen, J.P. and Moffett, P.** (2003). Early signal transduction events in specific plant disease resistance. Curr. Opin. Plant Biol. 6, 300-6.
**Rusterucci, C., Aviv, D.H., Holt, B.F., 3rd, Dangl, J.L. and Parker, J.E.** (2001). The disease resistance signaling components EDS1 and PAD4 are essential regulators of the cell death pathway controlled by LSD1 in Arabidopsis. Plant Cell. 13, 2211-24.
**Shah, J., Kachroo, P. and Klessig, D.F.** (1999). The Arabidopsis ssi1 mutation restores pathogenesis-related gene expression in npr1 plants and renders defensin gene expression salicylic acid dependent. Plant Cell. 11, 191-206.
**Sinapidou, E., Williams, K., Nott, L., Bahkt, S., Tör, M., Crute, I., Bittner- Eddy, P. and Beynon, J.** (2004). Two TIR:NB:LRR genes are required to specify resistance to Peronospora parasitica isolate Cala2 in Arabidopsis. Plant J. 38, 898-909.
**Slusarenko, A.J. and Schlaich, N.L.** (2003). Downy mildew of Arabidopsis thaliana caused by Hyaloperonospora parasitica (formerly Peronospora parasitica). Mol. Plant Pathol. 4, 159-70.
**Tanaka, R., Hirashima, M., Satoh, S. and Tanaka, A.** (2003). The Arabidopsis accelerated cell death gene ACD1 is involved in oxygenation of pheophorbide a: Inhibition of the pheophorbide a oxygenase activity does not lead to the "stay-green" phenotype in Arabidopsis. Plant Cell Physiol. 44, 1266-74.
**Thordal-Christensen, H., Zhang, Z., Wei, Y. and Collinge, D.B.** (1997). Subcellular localization of H2O2 in plants. H2O2 accumulation in papillae and hypersensitive response during the barley-powdery mildew interaction. Plant J. 11, 1187-94.
**Tian, M., Huitema, E., Da Cunha, L., Torto-Alalibo, T. and Kamoun, S.** (2004). A Kazal-like extracellular serine protease inhibitor from Phytophthora infestans targets the tomato pathogenesis-related protease P69B. J. Biol. Chem. 279, 26370-7.
**Tornero, P. and Dangl, J.L.** (2001). A high-throughput method for quantifying growth of phytopathogenic bacteria in Arabidopsis thaliana. Plant J. 28, 475-81.
**Tucker, S.L. and Talbot, N.J.** (2001). Surface attachment and pre-penetration stage development by plant pathogenic fungi. Annu. Rev. Phytopathol. 39, 385-417.
**Tyler, B. M.** (2002). Molecular basis of recognition between Phytophthora pathogens and their hosts. Annu. Rev. Phytopathol. 40, 137-67.
**Vogel, J. and Somerville, S.** (2000). Isolation and characterization of powdery mildew-resistant Arabidopsis mutants. Proc. Natl. Acad. Sci. U.S.A. 97, 1897-902.

**Vogel, J.P., Raab, T.K., Schiff, C. and Somerville, S.C.** (2002). PMR6, a pectate lyase like gene required for powdery mildew susceptibility in Arabidopsis. Plant Cell. 14, 2095-106.
**Vogel, J.P., Raab, T.K., Somerville, C.R. and Somerville, S.C.** (2004). Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. Plant J. 40, 968-78.
**Wirsel, S.G.R., Voegele, R.T. and Mendgen, K.W.** (2001). Differential regulation of gene expression in the obligate biotrophic interaction of Uromyces fabae with its host Vicia faba. Mol. Plant-Microbe Interact. 14, 1319-26.
**Yu, I.C., Parker, J. and Bent, A.F.** (1998). Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis dnd1 mutant. Proc. Natl. Acad. Sci. U.S.A. 95, 7819-24.

# Chapter 3

# Arabidopsis *Downy mildew resistant 5* is a gain of function allele of *RPM1*

**Robin P. Huibers and Guido Van den Ackerveken**

Plant-Microbe Interactions, Department of Biology, Science Faculty, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands.

**Abstract**

**The recessive Arabidopsis mutant *downy mildew resistant 5* mediates resistance to *Hyaloperonospora parasitica* but not to *Pseudomonas syringae*. *dmr5*-mediated resistance is accompanied by constitutive high expression of the defence-related gene *PR-1*. Here we show that *H. parasitica* resistance and constitutive high expression of defence-related genes largely depend on a functional *NPR1* gene, suggesting that plant defence responses cause *H. parasitica* resistance. *dmr5* is genetically linked to a base pair substitution in *RPM1*, resulting in loss of resistance to *P. syringae* strains carrying *avrRpm1* or *avrB*. *RPM1* function is largely lost in *dmr5*. However, *rpm1* null plants are not resistant to *H. parasitica*, indicating that loss of *RPM1* function does not cause *dmr5*-mediated *H. parasitica* resistance. Over-expression of the *RPM1* gene containing the *dmr5* mutation resulted in the enhanced expression of defence-related genes and *H. parasitica* resistance, whereas over-expression of wild-type *RPM1* did not. We conclude that *dmr5* is a gain of function allele of *RPM1* that mediates *H. parasitica* resistance.**

*Additional keywords: downy mildew resistance, R proteins.*

**Introduction**

Plants have constitutive and inducible defence mechanisms to resist invading microbes. Microbes that are not deterred by the plant's constitutive first layer of defence become recognized by the innate immune system of the host through perception of microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively). Successful pathogens, however, are able to suppress or circumvent MAMP/PAMP-triggered basal defence responses enabling them to cause disease. Suppression of basal defence can be mediated by pathogen effector proteins. An example is the suppression of RIN4-regulated basal defence in Arabidopsis by the *Pseudomonas syringae* effector proteins *avrRpm1* and *avrB* (Kim et al., 2005; Belkhadir et al., 2004). Although pathogen effector proteins can increase virulence, they can also have a disadvantage to the pathogen. Specific resistance (R) proteins are encoded in plant genomes that are able to recognize the presence or activity of pathogen effector proteins. Upon recognition an elaborate defence response is activated that includes changes in membrane ion fluxes, the production of reactive oxygen species and

changes in gene expression and metabolite production (Greenberg and Yao, 2004). This set of responses normally includes a localized cell death at sites of pathogen entry, termed the hypersensitive response (HR). Functional *R* genes have been identified that mediate resistance against viruses, bacteria, fungal and oomycete pathogens and even against nematodes and insects (Dangl and Jones, 2001).

The most common class of R proteins has a central nucleotide binding site (NBS) and a leucine rich repeat (LRR) domain at the carboxyl terminus (Dangl and Jones, 2001). The amino-terminus of most R proteins contains either a coiled-coil (CC) motif in CC-NB-LRR proteins or a domain that has homology to the *Drosophila* Toll and mammalian interleukin-1 receptor in TIR-NB-LRR proteins (Pan et al., 2000). In Arabidopsis, R proteins containing a CC motif usually depend on *NDR1* for downstream defence activation whereas R proteins having TIR domains usually depend on *EDS1* (Feys and Parker, 2000), indicating that the amino-terminal part of these R proteins is involved in downstream signaling. The carboxy-terminal LRR domain is found in many other proteins and often functions as a region for protein-protein interactions (Kobe and Kajava, 2001; Bell et al., 2003), and could directly or indirectly interact with pathogen effectors.

The NBS consists of 3 conserved motifs found in many ATP/GTP binding proteins (Traut, 1994). The first is the kinase 1a motif, also known as P-loop or Walker A motif that forms a flexible Gly-rich loop containing an invariant Lys residue involved in binding the phosphates of the nucleotide (Walker et al., 1982; Saraste et al., 1990; Traut, 1994). The second motif, the kinase-2 or Walker B motif, is less conserved and contains an invariant Asp residue involved in the coordination of the divalent metal ion required for the phosphotransfer reactions. The third motif, the kinase 3a motif, is involved in binding the purine base or the pentose of the nucleotide (Traut, 1994). The NBS domains of plant R proteins are part of a larger NB-ARC (nucleotide binding adaptor shared by APAF-1, R proteins, and CED-4) domain (van der Biezen and Jones, 1998; Aravind et al., 2004; Leipe et al., 2004) that shares additional homology to the animal ATPases APAF-1 and CED-4, various bacterial transcription factors and many uncharacterized proteins (Leipe et al., 2004).

Mutational analyses of the *R* genes *N*, *RPS2* and *RPM1* have shown that the NB-ARC domain is indispensable for the biological function of R proteins (Dinesh-Kumar et al., 2000; Tao et al., 2000; Tornero et al., 2002). That R protein NB-ARC domains form a functional nucleotide binding pocket was shown for the tomato R proteins I-2 and Mi-1 (Tameling et al.,

2002). Moreover, mutational analyses of several conserved residues within the NBS of I-2 that block ATP hydrolysis but not binding resulted in autoactive forms of I-2 indicating that the ATP-bound state of R proteins is the active form (Tameling et al., 2006). A similar finding was reported for RPS5 activation. Mutations that are known to block ATP binding resulted in a protein that was impaired in activation, in the presence of the elicitor AvrPphB, whereas mutations known to block ATP hydrolysis led to autoactivation of RPS5 (Ade et al., 2007). Recently it was shown that the tobacco R protein N oligomerizes in the presence of its elicitor, the helicase domain of tobacco mosaic virus (TMV) replicase protein. Interestingly, oligomerization was abolished by a mutation in the P-loop motif of the NBS domain, revealing an additional function for this domain (Mestre and Baulcombe, 2006).

Amino acid substitutions in the NB-ARC domain of *R* proteins that disrupt their function could provide further insight into how these proteins function in disease resistance. Here we show that *dmr5* carries a mutation that leads to an amino acid residue substitution in the NBS domain of RPM1. *dmr5* was previously identified in an EMS mutant screen initiated to identify Arabidopsis genes involved in basic compatibility towards the oomycete pathogen *H. parasitica* (Van Damme et al, 2005). *dmr5* provides resistance to *H. parasitica* and has high constitutive expression of the defence-associated gene *PR-1*. Here we show that *H. parasitica* resistance and the constitutive high expression of defence-associated genes largely depend on the presence of a functional *NPR1* gene, suggesting that defence activation is the cause of *H. parasitica* resistance. Over-expression of the *RPM1* gene containing the *dmr5* mutation resulted in *H. parasitica* resistance and high expression of defence-associated genes. The obtained data indicate that *dmr5* encodes for a weak autoactive form of RPM1 that provides resistance to *H. parasitica* but not to *P. syringae*.

**Results**

*Defence-associated gene expression in dmr5.*

The Arabidopsis *dmr5* mutant is resistant to *H. parasitica* and shows enhanced expression of the defence-associated gene *PR-1* suggesting that enhanced defence activation is the cause of resistance (Chapter 2; Van Damme et al., 2005). To determine if more defence-associated genes have higher basal expression levels in *dmr5*, the transcriptome of the *dmr5* mutant was compared to that of the parental line L*er eds1-2* using CATMA DNA microarrays. Transcripts were identified as differentially expressed when they had $Log_2$ ratio of *dmr5*/L*er*

*eds1-2* > 1 or < -1 and in addition a p-value < 0.05. A total of 64 genes were found to be differentially expressed. 44 genes were higher en 20 genes were lower expressed in *dmr5* plants compared to L*er eds1-2* (supplementary table S1). Many genes that were higher expressed have been associated with plant defence responses before, e.g. *DMR6* (Chapter 4; Van Damme et al., 2008), *PR-5* (Uknes et al., 1992), *PAD4* (Glazebrook et al., 1996), *ACD6* (Rate et al., 1999; Lu et al., 2003) and *AIG1* (Reuber et al., 1996). Because the obtained data was derived from a single biological replicate, enhanced expression of 6 genes (*PR-1*, *DMR6*, *At5g03350*, *At5g10760*, *At1g14880* and *At2g25510*) in *dmr5* was verified by Q-PCR in multiple independent biological replicates. These 6 genes are associated with defence as they are constitutively high expressed in *dmr6* mutants (Chapter 4; Van Damme et al., 2008) and become induced after compatible and incompatible *H. parasitica* infections (Chapter 5). All 6 genes were higher expressed in *dmr5* than in the parental line L*er eds1-2* (data not shown).

Enhanced expression of many defence-associated genes including *PR-1* depends on the presence of a functional *NPR1* gene (Dong, 2004). If *dmr5*-mediated *H. parasitica* resistance is caused by the enhanced expression of defence-associated genes, resistance could be largely lost in defence signaling mutants e.g. *npr1*. To test this, *dmr5* was crossed with the *npr1-1* mutant. Because the *dmr5* (L*er-eds1-2*) and *npr1-1* (Col-0) mutants are in different genetic backgrounds, progeny segregated for 4 different loci, namely *RPP2*, *EDS1*, *NPR1* and *DMR5*. Lines were selected based on their genotype in the F3 that had the desired combination of homozygous alleles for the 4 loci and were tested for *H. parasitica* resistance and enhanced expression of the 6 defence-associated genes *PR-1*, *DMR6*, *At5g03350*, *At5g10760*, *At1g14880* and *At2g25510* (Figure 1). The first of the 5 F3 lines was homozygous for *RPP2* that mediates resistance to *H. parasitica* isolate Cala2 used in this experiment (Figure 1G). The other F3 combinations were homozygous for *rpp2* and used to study the role of *NPR1* as they are compatible with the Cala2 isolate. All 6 defence-associated genes are highly expressed in plants carrying only the *dmr5* mutation (Figure 1A-F). Whereas the original mutant is in the *eds1-2* background, these lines carry the wild-type *EDS1* gene showing that the enhanced expression of defence-associated genes in *dmr5* mutants is independent of *eds1-2*. The high expression of *PR-1* is completely lost in *dmr5*/*npr1-1* plants (Figure 1A) and strongly reduced for the other defence-associated genes (Figure 1B-F), showing that *NPR1* is important for the constitutive high expression of these genes in the

*dmr5* mutant. Furthermore, *dmr5*-mediated *H. parasitica* resistance also largely depends on a functional *NPR1* gene (Figure 1G). *H. parasitica* forms around 2.5 x $10^6$ conidia per mg of leaf tissue in *DMR5*/*NPR1* plants at 6 dpi. Significantly more conidia are formed in *DMR5*/*npr1-1* plants, showing that *npr1-1* mutants are enhanced disease susceptible. *dmr5*/*NPR1* plants are resistant as expected and this resistance is largely lost in *dmr5*/*npr1-1* plants. However, *dmr5*-mediated *H. parasitica* resistance is not completely dependent on *NPR1* as *DMR5*/*npr1-1* plants are still more susceptible than *dmr5*/*npr1-1* plants.

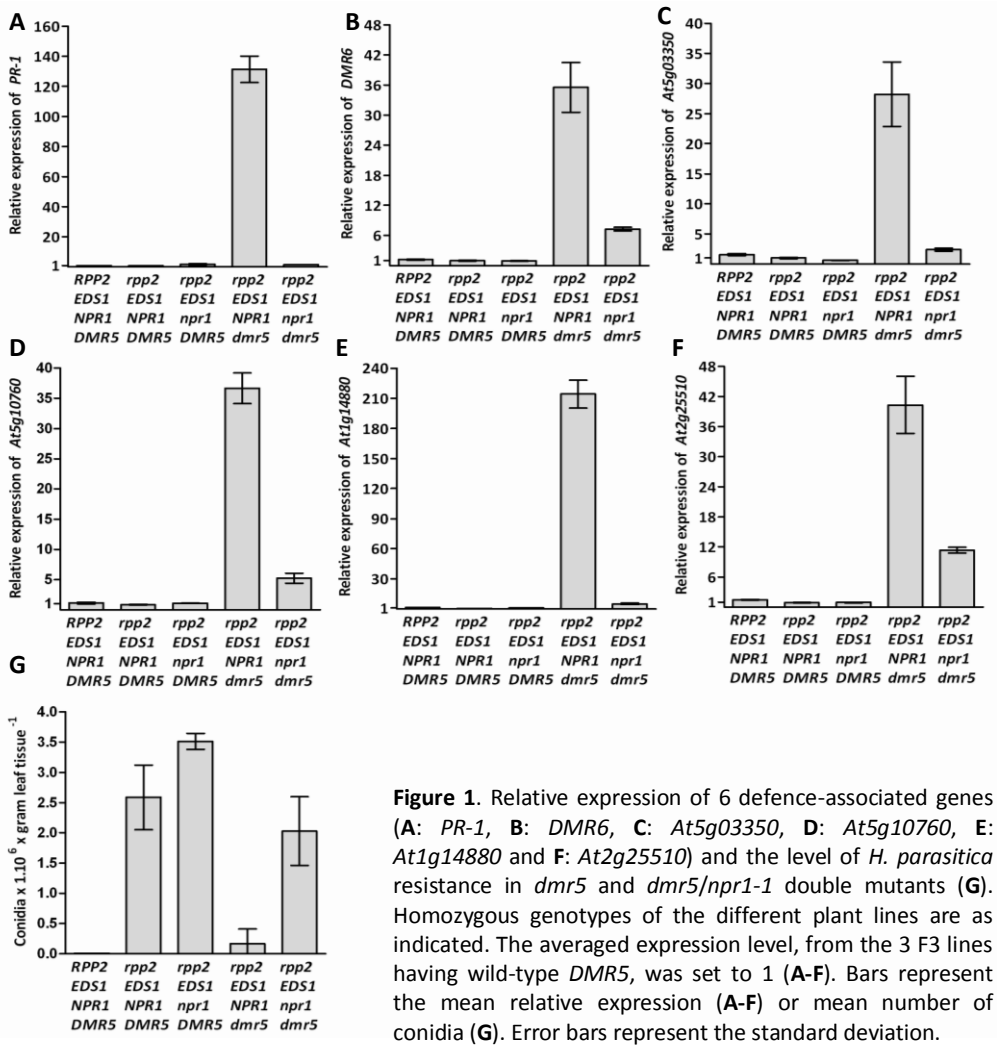


**Figure 1**. Relative expression of 6 defence-associated genes (**A**: *PR-1*, **B**: *DMR6*, **C**: *At5g03350*, **D**: *At5g10760*, **E**: *At1g14880* and **F**: *At2g25510*) and the level of *H. parasitica* resistance in *dmr5* and *dmr5*/*npr1-1* double mutants (**G**). Homozygous genotypes of the different plant lines are as indicated. The averaged expression level, from the 3 F3 lines having wild-type *DMR5*, was set to 1 (**A-F**). Bars represent the mean relative expression (**A-F**) or mean number of conidia (**G**). Error bars represent the standard deviation.

*dmr5 has an amino acid substitution in RPM1*

Previously it was shown that *dmr5*-mediated *H. parasitica* resistance is a recessive trait that was mapped on the upper arm of chromosome III (Chapter 2; Van Damme et al., 2005). Fine mapping allowed us to link *H. parasitica* resistance to an interval located on the 2 BAC clones, F17A9 and T1B9, between the INDEL markers INDEL-F17A9 and INDEL-7080 (Figure 2A). This interval is predicted to contain 7 genes (according to the Arabidopsis genome annotation TAIR 6). Comparative sequence analysis of all 7 candidate genes in the *dmr5* mutant and the

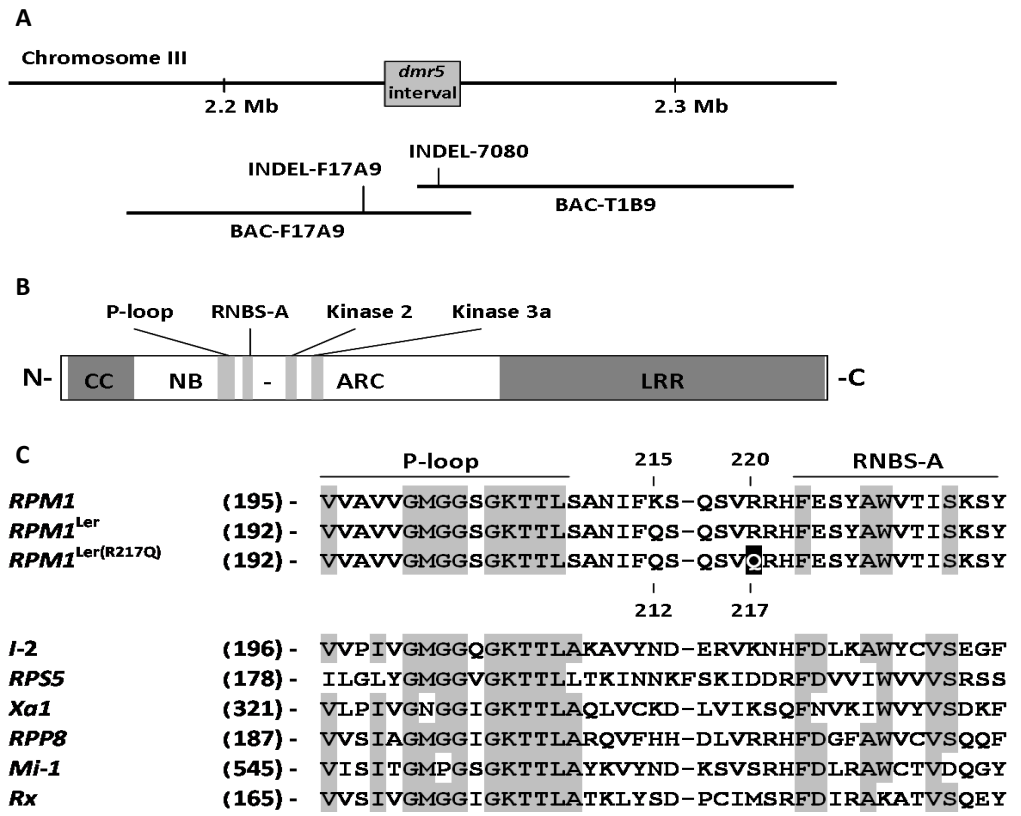


**Figure 2.** (**A**), Mapping interval of *dmr5* after extended recombinant analysis. *H. parasitica* resistance is linked to the genomic interval on chromosome III between 2.2 and 2.3 Mb between the INDEL markers INDEL-F17A9 and INDEL-7080 located on BAC clone F17A9 and T1B9, respectively. (**B**), Schematic representation of RPM1. RPM1 contains 3 domains, an N-terminal coiled coil domain (CC), a NB-ARC domain (nucleotide binding adaptor shared by APAF-1, R proteins and CED4) and a C-terminal Leucine-rich repeat (LRR). Several conserved motifs within the NB-ARC domain are indicated (P-loop, RNBS-A, kinase2 and kinase3a motifs). (**C**), Alignment of P-loop and RBBS-A amino acid sequence motifs and the non-conserved region in between, of Arabidopsis RPM1 (Col-0

sequence, GenBank accession number X87851), RPM1$^{Ler}$ and RPM1$^{Ler(R217Q)}$ (Supplementary table 2), tomato I-2 (AF004879), Arabidopsis RPS5 (AF074916), rice Xa1 (AB002266), Arabidopsis RPP8 (AF089710), tomato Mi-1 (CS025317) and potato Rx (CAB50786). Alignments were made with the publicly available tool ClustalW (http://www.ebi.ac.uk/Tools/clustalw/) using default settings. Numbers in parentheses indicate the positions of the first residue depicted in the full-length sequence of the proteins. Conserved residues are depicted in grey boxes. In the *dmr5* mutant, R217 is changed to Q (black box).

parental line L*er eds1-2* revealed a single point mutation in the coding sequence of *RPM1* (At3g07040). This single base change, from G to A, is typical for EMS mutations and changes a CGG codon (R217) into a CAG (Q) codon within the NB-ARC domain of RPM1 (Figure 2B and C). R217 is a non-conserved residue of the NB-ARC domain of plant R proteins. The *RPM1* nucleotide sequence of L*er* differs at 4 positions from the Col-0 sequence which encodes the characterized RPM1 protein (Grant et al., 1995). In L*er* basepairs 445-453 of the coding sequence (based on Col-0) are absent making the L*er* encoded protein 3 amino acids shorter. In addition, the L*er* sequence has 3 basepair substitutions compared to Col-0, resulting in 2 amino acid substitutions in the encoded protein (DNA and amino acid sequences are shown in Supplementary table S2). Basepair and amino acid substitutions with respect to the Col-0 coding sequences are: A643C giving K212Q, A1986T giving E262D, and C2097A, being a silent mutation. The L*er* and *dmr5* alleles of *RPM1* will hereafter be referred to as *RPM1*$^{Ler}$ and *RPM1*$^{Ler(R217Q)}$ respectively.

*RPM1*$^{Ler(R217Q)}$ *is a partial loss of function allele of RPM1*

In the accession Col-0, *RPM1* encodes a NBS-LRR protein that confers resistance to *P. syringae* strains that express either *avrRpm1* or *avrB*. To test whether L*er* (*RPM1*$^{Ler}$) and *dmr5* (*RPM1*$^{Ler(R217Q)}$) encode a functional RPM1 protein, *dmr5* and its parental line L*er eds1-2* were inoculated with *P. syringae* DC3000 carrying *avrRpm1*, *avrB* or an empty vector (pVSP61). In addition, plants from the Arabidopsis accession Cvi that lack a functional *RPM1* gene were inoculated with all 3 strains to test if *avrRPM1*- and *avrB*- containing strains are as virulent as the control strain with the empty vector. All 3 strains proliferated equally (Figure 3A). Figure 3B shows that L*er eds1-2* is susceptible for *P. syringae* carrying the empty vector control. The proliferation of bacteria is around 2.5 log scales in 4 days. A decrease in bacterial number is observed for bacteria expressing either *avrRpm1* or *avrB* showing that L*er eds1-2* carries a functional *RPM1* gene (*RPM1*$^{Ler}$). *dmr5* shows proliferation of *P. syringae*

carrying the empty vector control that equals that of L*er eds1-2*, which is around 2.5 log scales (Figure 3C). Interestingly, *dmr5* mediates reduced susceptibility to *H. parasitica* (Figure 1G and Chapter2) that depends largely on the constitutive expression of defence-related genes (Figure 1). However, this does not effect the proliferation of *P. syringae* containing the empty vector control (compare Figure 3B and C). This suggests that *H. parasitica* is particularly more sensitive to enhanced expression of defence-related genes then *P. syringae*. *dmr5* has clearly lost the ability to arrest bacterial growth for strains carrying *avrRpm1* or *avrB* (Figure 3C). However, in *dmr5* plants there is still some residual RPM1 activity. Strains carrying *avrRpm1* or *avrB* do not proliferate to the same degree as the empty vector control strain (Figure 3C), showing that *RPM1*$^{Ler(R217Q)}$ is a partial loss of function allele of *RPM1.*

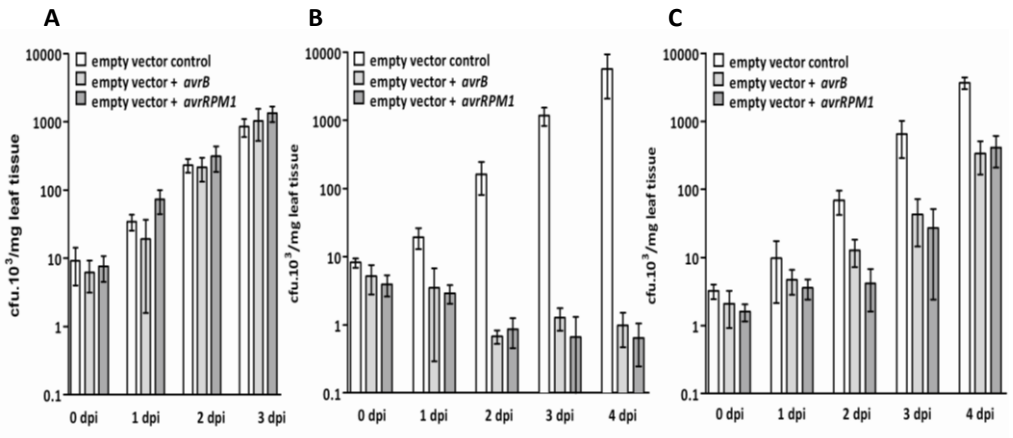


**Figure 3.** Proliferation of *P. syringae* strains carrying an empty vector (pVSP61) or the empty vector containing *avrRpm1* or *avrB* on Cvi (**A**), L*er eds1-2* (**B**) and *dmr5* (**C**). Bars represent the average cfu/mg of leaf tissue, with error bars representing the standard deviation.

*Loss of RPM1 function does not result in H. parasitica resistance*

*dmr5*-mediated resistance inherits as a recessive trait in the mapping population. The recessive nature of *dmr5* suggests that *RPM1* negatively regulates defences effective against *H. parasitica*. To test this, an *rpm1* null mutant, *rpm1-3* (Grant et al., 1995) and the Col-0 wild-type were tested for susceptibility to the *H. parasitica* isolate Waco9. Both the wild-type and *rpm1-3* mutant showed equal levels of susceptibility to *H. parasitica* at 6 dpi (data

not shown), indicating that *RPM1* does not negatively modulate defences effective against *H. parasitica*. To corroborate this, L*er eds1-2* (*RPM1*$^{Ler}$) was crossed with Nd-0 (a natural *rpm1* null accession). If *RPM1*$^{Ler}$ would have a positive effect on susceptibility one would expect that introducing *RPM1*$^{Ler}$ in the Nd-0 genetic background would result in enhanced susceptibility to *H. parasitica*. Figure 4A shows that there is no significant difference in susceptibility between Nd-0 and progeny of Nd-0 crossed with L*er eds1-2*, indicating that *RPM1*$^{Ler}$ does not affect susceptibility to *H. parasitica*. Interestingly, progeny of *dmr5* crossed with Nd-0 showed a slight but significant reduction in susceptibility compared to Nd-0 and progeny of Nd-0 crossed with L*er eds1-2* (Figure 4A). This suggests that *dmr5* is not a true recessive trait, but instead a semi-dominant one, suggesting it is a gain of function allele. However, this semi-dominant behaviour of *dmr5* was not seen in progeny of *dmr5* crossed with L*er eds1-2* (Figure 4B) or in the mapping population obtained from a cross between *dmr5* and Col-0.

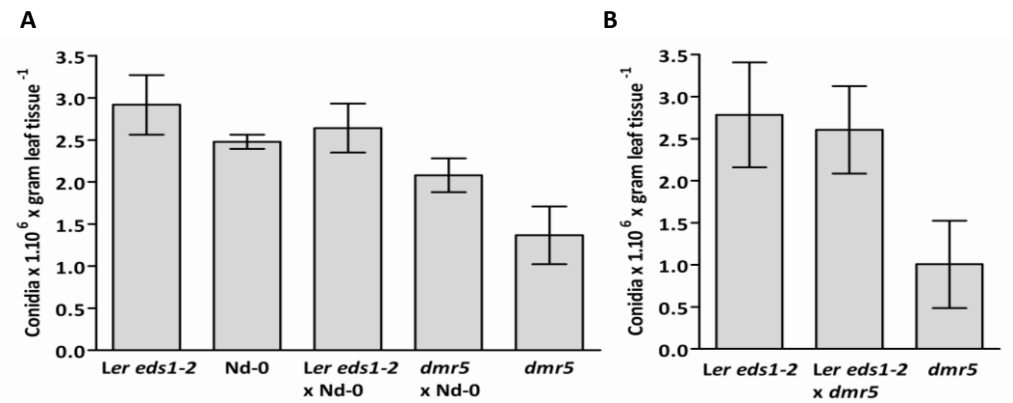


**Figure 5.** *H. parasitica* resistance (number of conidia per mg of leaf tissue) at 6 dpi on different plant lines. (**A**), *H. parasitica* growth on L*er eds1-2*, Nd-0, *dmr5* and progeny of crosses between L*er eds1-2* and *dmr5* with Nd-0. (**B**), *H. parasitica* growth on L*er eds1-2*, *dmr5* and progeny of L*er eds1-2* and *dmr5*. Bars represent the average number of conidia per mg of leaf tissue, with error bars representing the standard deviation.

### *RPM1*$^{Ler(R217Q)}$ is a gain of function allele of *RPM1* capable of mediating *H. parasitica* resistance and the enhanced expression of defence-related genes

The data described above show that *dmr5*-mediated resistance is not caused by loss of *RPM1* function. Therefore, it was tested whether *RPM1*$^{Ler(R217Q)}$ is a gain of function allele

that mediates *H. parasitica* resistance. L*er eds1-2* plants were transformed with a construct that expresses either *RPM1*$^{Ler(R217Q)}$ or *RPM1*$^{Ler}$ under control of the 35S promoter. Plants containing the construct were selected (T1 plants) and expression of *RPM1*$^{Ler(R217Q)}$ and *RPM1*$^{Ler}$ was confirmed in 5 plants of each line (data not shown). The majority of *35S::RPM1*$^{Ler(R217Q)}$ plants showed mild or severe growth phenotypes. Leafs were stunted and often showed necrotic patches, suggestive of uncontrolled defence activation. No growth defects were observed in *35S::RPM1*$^{Ler}$ plants. The level of susceptibility to *H. parasitica* was tested on three week-old plants of both lines. All *35S::RPM1*$^{Ler}$ plants were susceptible, with more then 50 conidiophores per seedling (Figure 5A). In contrast, 70 % of the *35S::RPM1*$^{Ler(R217Q)}$ plants showed significantly less, or no sporulation at all (Figure 5A). Moreover, the level of *H. parasitica* resistance correlated with the severity of growth defects, indicating that both result from expression of *RPM1*$^{Ler(R217Q)}$. The relative expression of both *RPM1* alleles was measured in 5 different plants of each line, with all five *35S::RPM1*$^{Ler(R217Q)}$ plants having mild growth phenotypes. Overall *35S::RPM1*$^{Ler(R217Q)}$ plants show expression levels that are similar to *35S::RPM1*$^{La}$ plants (Figure 5B). This shows that the phenotypic differences between *35S::RPM1*$^{Ler(R217Q)}$ and *35S::RPM1*$^{Ler}$ plants is not due to a difference in expression of *RPM1*$^{Ler(R217Q)}$ and *RPM1*$^{Ler}$ respectively. Figure 1 showed that *dmr5*-mediated resistance largely depends on the constitutive high expression of defence-realated genes. To test whether expression of *RPM1*$^{Ler(R217Q)}$ results in higher expression levels of defence-related genes, relative expression levels of 4 genes were measured in the same 5 plants of each line. All five *35S::RPM1*$^{Ler(R217Q)}$ plants show higher expression levels of the defence-associated genes compared to the *35S::RPM1*$^{Ler}$ plants, showing that in the absence of AvrRpm1 or AvrB RPM1$^{Ler(R217Q)}$ activates the expression of defence-related genes.

**Discussion**

*dmr5* was previously identified in a genetic screen initiated to identify genes that positively affect basic compatibility to the downy mildew pathogen *H. parasitica* (Chapter 2; Van Damme et al., 2005). *dmr5* was shown to mediate resistance to *H. parasitica* but not to *P. syringae* despite enhanced expression of *PR-1*. Here we demonstrate that *H. parasitica* resistance and enhanced expression of defence-associated genes in *dmr5* largely depends on a functional *NPR1* gene, suggesting that resistance is caused by plant defence responses.

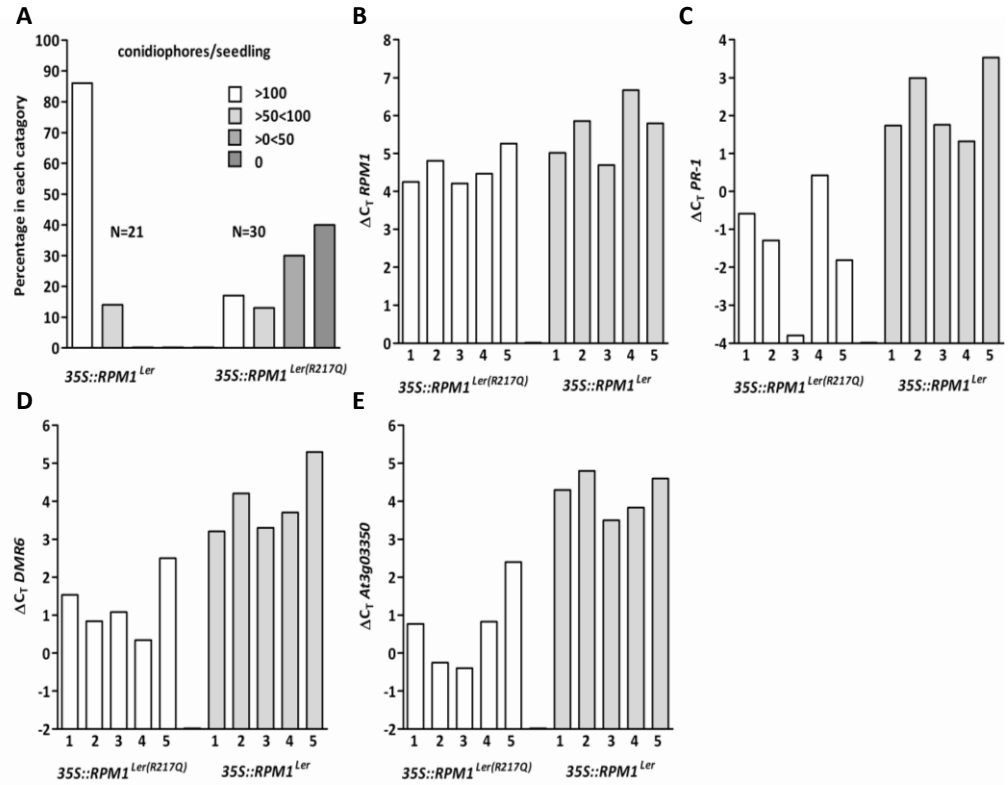


**Figure 5.** Analyses of transgenic L*er eds1-2* plants containing either a *35S::RPM1*$^{Ler}$ or *35S::RPM1*$^{Ler(R217Q)}$ contruct. (**A**), susceptibility to downy mildew was quantified by counting the number of conidiophores per seedling and displayed in four classes of infection severity. (**B**), (**C**), (**D**) and (**E**), expression of *RPM1, PR-1, DMR6* and *At3g03350* respectively relative to *Actin2* in five different *35S::RPM1*$^{Ler(R217Q)}$ or *35S::RPM1*$^{Ler}$ transgenic plants. $\Delta C_T$ values reflect the number of additional PCR-cycles that was required to reach an arbitrary product concentration compared to *Actin2*. Note that a lower bar indicates higher transcript abundance.

Enhanced expression of defence-associated genes and broad range pathogen resistance is often accompanied by dwarfism, such as seen in *dmr3* and *dmr4* (Chapter 2; Van Damme et al., 2005). The fact that the *dmr5* mutant does not show dwarfism and broad range pathogen resistance suggests that the level of defence activation is lower in *dmr5* compared to broad range disease resistant mutants. This is supported by the fact that far more genes are constitutively higher expressed and to a higher degree in *dmr3* and *dmr4* compared to *dmr5* (Van den Ackerveken, unpublished data).

*dmr5* was mapped and a single mutation was found in *RPM1*, which encodes a CC-NBS-LRR protein that confers resistance to *P. syringae* strains carrying *avrRpm1* or *avrB*. The mutation in *dmr5* changes a CGG codon (R217) into a CAG (Q) codon (*RPM1*$^{Ler(R217Q)}$). This

mutation changes a positive charged arginine for a neutral glutamine within the NBS domain of RPM1 between the conserved P-loop and RNBS-A motif. The conserved P-loop in the NBS domain contains an invariant Lys residue that is involved in binding the phosphate of the nucleotide (Walker et al., 1982; Saraste et al., 1990; Traut, 1994). Changing this Lys residue into an Arg in the tomato I-2 (I-$2^{K207R}$) protein greatly reduces its ability to bind ATP (Tameling et al., 2002). The RNBS-A motif is conserved among CC-NBS-LRR proteins but its function is unknown (Meyers et al., 1999; Pan et al., 2000). However, changing the conserved Ser residue in I-2 (I-$2^{S233F}$) resulted in an autoactive form of the protein (Tameling et al., 2006), suggesting that this motif is important for the normal function of CC-NBS-LRR proteins.

The mutation found in *dmr5* does not alter a conserved amino acid residue in the P-loop or RNBS-A motifs. However it certainly affects normal protein function as resistance against *P. syringae* strains carrying *avrRpm1* or *avrB* is largely lost. The partial loss of *RPM1* function together with the recessive nature of *dmr5* raised the possibility that the wild-type RPM1 protein would positively affects *H. parasitica* growth. However, no evidence was found to support this, as the null mutant *rpm1-3* did not show reduced susceptibility to *H. parasitica*. A more plausible explanation is that *dmr5* is a gain-of-function allele of *RPM1*. Gain-of-function mutations usually inherit as dominant traits e.g. as was shown for *acd6* (Rate et al., 1999). Interestingly, F1 progeny of *dmr5* crossed with Nd-0 (lacking a *RPM1* gene) showed less growth of *H. parasitica* compared with F1 progeny of L*er eds1-2* crossed with Nd-0, suggesting that *dmr5* is a semi-dominant mutation. Over-expression of *RPM1*$^{Ler(R217Q)}$ resulted in the enhanced expression of defence-related genes, growth defects and *H. parasitica* resistance, whereas over-expression of *RPM1*$^{Ler}$ did not, showing that *RPM1*$^{Ler(R217Q)}$ is a gain-of-function allele of *RPM1* which encodes a weak autoactive form. It is unlikely that *RPM1*$^{Ler(R217Q)}$ encodes a fully autoactive form as (I), the enhanced defence capacity of *dmr5* does not result in necrosis as was shown for mutant autoactive forms of tomato I-2, transiently expressed in Nicotiana (Tameling et al., 2006) and (II), *dmr5* does not mediate resistance towards *P. syringae* DC3000.

According to current models of R protein function (Takken et al., 2006), R proteins are in 'OFF-state' in the absence of elicitor. In this state the NBS domain has bound an ADP nucleotide. The presence of an elicitor provokes a conformational change leading to exchange of the ADP nucleotide for an ATP nucleotide. The ATP-bound R protein subsequently activates downstream signalling (ON-state). The ATP-ase activity of the NBS

domain attenuates the signalling response and returns the R protein in the OFF-state. Several mutations, within the region that encodes the NBS domain of plant R proteins, have been described that cause autoactivity (Tameling et al., 2006; Takken et al., 2006; Ade et al., 2007). It has been shown that ATP hydrolysis is impaired in these R proteins (Tameling et al., 2006; Ade et al., 2007), subsequently increasing the number of R proteins in the ON-state. In analogy with these models, one could imagine that *RPM1*$^{Ler(R217Q)}$ has a higher affinity for ATP or is impaired in ATP hydrolysis, resulting in weak autoactivity. Alternatively, it may be that the signalling potential of the R protein is partly released regardless of the nucleotide bound. Resistance to *P. syringae* carrying *avrRpm1* or *avrB* is largely abolished. This suggests that the ATP-bound state is not stabilised or that the signalling potential can not be fully released in the presence of elicitor.

The data regarding crosses between L*er eds1-2* and *dmr5* with Nd-0 (*rpm1* null), suggested that *dmr5* is a semi-dominant allele of *RPM1*. This was corroborated by over-expression of *RPM1*$^{Ler(R217Q)}$. However, this semi-dominant behaviour was not observed in progeny of *dmr5* crossed with L*er eds1-2* (*RPM1*$^{Ler}$) or in crosses with Col-0 (*RPM1*) (mapping population). This could mean that *RPM1*$^{Ler(R217Q)}$ is suppressed by a functional *RPM1* gene. However, if suppression exists it should be dosage-dependent as *RPM1*$^{Ler(R217Q)}$ is capable to induce *H. parasitica* resistance in the presence of *RPM1*$^{Ler}$ (over-expression lines). Suppression may be explained by a model were RPM1 activation requires oligomerization first. Plant NBS-LRR proteins belong to the wider class of STAND (signal transduction ATPases with numerous domains) proteins. Several STAND members have been shown to oligomerize upon activation (Inohara et al., 2000; Jaroszewski et al., 2000; Leipe et al., 2004). That plant R proteins can oligomerize was shown for the TIR-NBS-LRR N protein of tobacco that mediates resistance against Tobacco mosaic virus (Mestre and Baulcombe, 2006). Elicitor induced oligomerization was abolished by a mutation in the P-loop motif, indicating that a functional NBS is required for oligomerization. In addition, a positive correlation was found between N oligomerization and the elicitation of resistance, indicating that oligomerization is required for elicitation of defence. Oligomerization was not affected by silencing of *EDS1* a presumed downstream signalling component of N, indicating that oligomerization is an early event in the elicitor activation of N (Mestre and Baulcombe, 2006). It could be that oligomerization can be blocked by incorporation of non-activated monomers. This would set a threshold for defence activation as it would occur only in the presence of multiple activated monomers. If

*RPM1*$^{Ler(R217Q)}$ requires oligomerization, this could explain suppression of *RPM1*$^{Ler(R217Q)}$ by a wild-type gene. However, future studies will demonstrate whether *RPM1*$^{Ler(R217Q)}$ can or can not be suppressed by expression of *RPM1*$^{Ler}$ under control of its native and 35S promoter in the *dmr5* mutant. *RPM1*$^{Ler(R217Q)}$ is the first mutation in a *R* gene described that combines partial loss of function with weak autoactivity of the encoded protein. Further analysis of *RPM1*$^{Ler(R217Q)}$ could provide new and interesting aspects of R protein function.

**Experimental procedures**

*Plant growth conditions and H. parasitica inoculations*

Plants were grown on potting soil in a growth chamber (Snijders, The Netherlands) at 22˚C with 16h of light (100µE/m2/sec) and a relative humidity of 75% before inoculation. *H. parasitica* isolate Cala2 was maintained on the Arabidopsis accession L*er*. 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). Sporulation levels were quantified 6-7 days post inoculation by counting the number of conidiophores per seedling (N> 25).

*Pseudomonas inoculations and quantification of growth*

*Pseudomonas syringae* pv tomato DC3000 derivatives containing pVSP61 (empty vector, no *avr* gene), *avrRPM1*, or *avrB* were kindly provided by Dr. J. Dangl. Plant inoculations and counting of the bacteria were performed as described (Tornero and Dangl, 2001) with a few alterations; bacterial suspensions with an $OD_{600}$ of 0.04 were applied to 14 day old seedlings and all above ground parts were harvested at 0 (1 hour), 1, 2, 3 and 4 days post inoculation.

*dmr5 mutant background*

*dmr5* was identified previously and crossed to the Col-0 mutant *FN2* (Sinapidou et al., 2004) to create a mapping population (Chapter 2; Van Damme et al., 2005). For all other experiments *dmr5* mutants were used that were back crossed twice to the parental line L*er eds1-2* (Parker et al., 1996).

*Map based cloning*

INDEL markers were used for fine mapping. Primers were designed around known insertion and deletion polymorphisms between Col-0 and L*er* as listed in the Cereon database (http://www.arabidopsis.org/Cereon/index.html). INDEL-F17A9 was based on the AC016827 polymorfism, a 24 bp insertion in the Landsberg ecotype and amplified with the forward primer CCTCCACCATATCCGTCATC and the reverse primer TGGATATCAGGGTGGCAGA. INDEL-7080 was based on the AC016827 polymorphism, a 12 bp insertion in the Col-0 ecotype and amplified with the forward primer TCTCTTGTTTACTGCTCTTTTCCTC and reverse primer TCCAGAATCTTGAACGATTTCAT.

*CATMA arrays*

Microarray analysis was performed with CATMA version 2 arrays (complete Arabidopsis transcriptome microarray; Allemeersch et al., 2005; Hilson et al., 2004). Arrays were spotted with 24411 gene-specific tags (GSTs). The GSTs are between 150 and 500 bp in length and show less then 70% identity with any other sequence in the genome. GSTs were spotted on GAPSII glass slides (Corning Incorporated, Acton, MA, USA) using a BioRobotics Microgrid II TAS spotter (Genomics Solutions, Ann Arbor, MI, USA) and cross-linked for 4 hours at 80˚C. Detailed information about CATMA and database access can be found at http://www.catma.org/ (Crowe et al., 2003) and http://genomics.bio.uu.nl/.

*Labelling, hybridization and scanning*

mRNA from total RNA extracts from L*er eds1-2* and *dmr5* were amplified with the MessageAmp aRNA kit (Ambion). Amplified RNA (5 µg) was used as template to synthesize modified cDNA, by incorporation of 5-(3-aminoallyl)-dUTP (Ambion; ratio dUTP/dTPP of 7/3) with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random nonamers (Gene Link, Westchester County, NY, USA) for 2 hours at 42˚C. RNA template was removed by hydrolysis using 3 µl 2.5 M NaOH per 30 µl reaction volume for 15 min at 37˚C. Hydrolysis was stopped by adding 15 µl 2 M MOPS buffer per 30 µl reaction volume. Modified cDNA was purified using the MineLute PCR purification kit (Qiagen) and labeled with Cy3 or Cy5 mono-reactive dye (Amersham, Buckinghamshire, UK). Modified cDNA derived from L*er eds1-2* and *dmr5* RNA was labeled twice with Cy3 and twice with Cy5. The reaction was quenched after 60 min using 4.5 µl 4 M hydroxylamine (Sigma-Aldrich) and

incubated in the dark for 15 min. Labeled cDNA was purified using the MineLute PCR purification kit (Qiagen) and incorporation of Cy3 or Cy5 was determined using a UVmini-1240 spectrophotometer at 550 or 650 nm, respectively.

CATMA arrays were denatured in boiling demineralized water for 3 min and dipped in ethanol afterwards. Slides were spun dry for 3.5 min at 300 g in 50 ml tubes, covered with a LifterSlip (Erie Scientific Company, Portsmouth, NH, USA) and subsequently prehybridized with 100 µl filtered prehybridization solution containing 25% formamide (Merck, Whitehouse Station, NJ, USA), 5x SSC, 0.1% SDS and 1% BSA, fraction V, minimum 96% (Sigma-Aldrich) for 15 min at 55˚C. Microarrays were dipped 5 times in demineralized water and once in isopropanol and spun dry for 3.5 min at 300 g in 50 ml tubes and subsequently covered with a LifterSlip. For each hybridization, 50 µl filtered (0.2 lm) 2x hybridization mix was made, containing 50% deionized formamide (Sigma-Aldrich), 10x SSC and 0.2% SDS. Herring-sperm DNA (1 µl of 11 mg/ml stock; Sigma-Aldrich) was added, and the mix was heated to 42 ˚C to prevent precipitation of SDS. A fixed amount of 1450 ng cDNA was used for all hybridizations, corresponding to an incorporation of Cy3 or Cy5 between 259 and 590 pmol. Each pair of labeled cDNA (2 times L*er eds1-2* labelled with Cy3 and *dmr5* labelled with Cy5 and 2 times vica versa) was concentrated in a Speed Vac (type SC100; Savant Instruments, Hollbrook, NY, USA) to a volume of 50 µl and added to 50 µl 2x hybridization mix. The probes were denatured for 5 min at 95˚C and centrifuged for 2 min at 15000g in a standard tabletop centrifuge and immediately applied to the arrays. The arrays were put in hybridization chambers (Corning Incorporated) containing one drop of 20 µl water on each side, covered by foil, and placed for 16–20 h at 42_C in a water bath. After the hybridization, the arrays were washed twice in a lowstringency wash solution containing 1x SSC, 0.2% SDS and 0.1 mM DTT for 4 min at 55_C. The arrays were subsequently washed in a high-stringency wash solution containing 0.1x SSC, 0.2% SDS and 0.1 mM DTT for 4 min at 55_C and in a final wash solution containing 0.1x SSC and 0.1 mM DTT each for 4 min at room temperature. The slides were dipped five times in demineralized water and immediately submerged in isopropanol. The slides were spun dry as described above and scans of the arrays were made using a ScanArray Express HT (PerkinElmer, Wellesley, MA, USA). Spot intensities of the scans were determined by ImaGene software version 6.5.1 (BioDiscovery, El Segundo, CA, USA).

*Statistics (CATMA arrays)*

Spot intensities from the 4 CATMA arrays were analysed by LIMMA (Smyth, 2004) version 1.7.2 and limmaGUI version 1.2.5 (Wettenhall and Smyth, 2004) software packages from Bioconductor (Gentleman et al., 2004) running in R version 1.9.1 (CR Foundation, Vienna, Austria; Ihaka and Gentleman, 1996). The intensities were normalized by the print tip LOESS to correct for possible within array, dye and print-tip effects. Subsequently, all arrays were normalized between arrays by scaling to obtain the same median absolute deviation for each array, thereby enhancing the comparison between them (Smyth and Speed, 2003). A linear statistical model was created to estimate the log2(*dmr5*/L*er eds1-2*) effect and a moderated t-test (empirical Bayes) was performed to determine differential transcripts between L*er eds1-2* and *dmr5*.

*Selection of F3 progeny of dmr5 crossed with npr1-1*

*dmr5* was crossed with *npr1-1* and F2 progeny was genotyped with INDEL markers genetically linked to the corresponding loci. *dmr5* was linked to the INDEL-F17A9 marker (see previous section). *RPP2* was linked to the F24J7-12 marker, with forward primer TCCGGAAAAGGAACAGAATG and reverse primer CCTCCTGTTTTCGTGGTTTC amplifying a 123 bp fragment in L*er* and a 111 bp fragment in Col-0. *NPR1* was linked to the F22C12+18 marker, with forward primer TCCACGAGTCAGAAGGATGA and reverse primer CCTGCTCAAATGCGTTACAA amplifying a 191 bp fragment in Col-0 and a 173 bp fragment in L*er*. *EDS1* was genotyped with a combinantion of 3 primers, EDS2A: ACACAAGGGTGATGCGAGACA, EDS4: GGCTTGTATTCATCTTCTATCC and EDS6: GTGGAAACCAAATTTGACATT amplifying a 750 bp fragment for *EDS1* or a 600 bp fragment for *eds1-2*.

*Quantitative PCR*

Total RNA was extracted with an RNeasy kit (Qiagen, http://www.qiagen.com) and treated with the RNase-free DNase set (Qiagen). RNA was quantified using an UVmini-1240 spectrophotometer (Shimadzu, http://www.shimadzu.com). cDNA was synthesized with SuperScript-III reverse transcriptase (Invitrogen, http://www.invitrogen.com) and oligo(dT)15 (Promega, http://www.promega.com) from total RNA. Cycle thresholds were determined per transcript in triplicate in multiple biological replicates using the ABI PRISM

7700 sequence detection system (Applied Biosystems, http://www.appliedbiosystems.com) using SYBR Green I (Applied Biosystems) as the reporter dye. The data were normalized using Arabidopsis *ACT2* levels (*At3g18780*). Primer sets for the transcripts are *DMR6* (QDMR6F: 5'-TGTCATCAACATAGGTGACCAG-3' and QDMR6R: 5'-CGATAGTCACGGATTTTCTGTG-3'), *At1g14880* (QAt1g14880F: 5'-CTCAAGGAGAATGGTCCACA-3' and QAt1g14880R: 5'-CGACTTGGCCAAATGTGATA-3'), *At5g10760* (QAt5g10760F: 5'-GGAGCAAAACCCGACTCGT-3' and QAt5g10760R: 5'-TGCAGGGAATGATGACCTT-3'), *PR-1* (QPR-1F: 5'-GAACACGTGCAATGGAGTTT-3'and QPR-1R: 5'-GGTTCCACCATTGTTACACCT-3'), *At5g03350* (QAt5g03350F: 5'-TGGCACTGTTCTGGAAGGT-3' and QAt5g03350R: 5'- AGGTTTTTCTTCAAAATATGTACCC), *At2g25510* (QAt5g25510F: 5'-CAAAGGCACAAACGAACCT and QAt5g25510R: 5'- GACAGAAGCCACAAACCAAA), *RPM1* (QRPM1F: TCTGAAGCTCAAACATTGCAC and QRPM1R: TTTTGCACAAGGATTTCAGAAT) and *ACT-2* (QACT2F: 5'-AATCACAGCACTTGCACCA-3' and QACT2R: 5'-GAGGGAAGCAAGAATGGAAC-3') generating 99-101 base pair fragments.

*Construction of $RPM1^{Ler}$ and $RPM1^{Ler(R217Q)}$ over expression lines*

*$RPM1^{Ler}$ and $RPM1^{Ler(R217Q)}$* were amplified from L*er eds1-2* and *dmr5* respectively, using forward primer aaaaagcaggcttcATGGCTTCGGCTACTGTTGA and reverse primer agaaagctgggtaCTAAGATGAGAGGCTCACAT. Products were diluted and a second PCR amplification step was performed with forward primer GGGGACAAGTTTGTACAAAAAAGCAGGCT and reverse primer GGGGACCACTTTGTACAAGAAAGCTGGGT. PCR products now contained attB1 and attB2 recombination sites attached to the full *RPM1* sequences. The products containing the attB sites were recombined in pDON221 (Gateway™, Invitrogen) using the Gateway system. All procedures were performed according to the manufactures protocols. pDON221 clones containing the *RPM1* sequences were subsequently recombined with the pB7WG2 vector (Karimi et al., 2002). pB7WG2 clones containing the *RPM1* sequences were sequenced and used for transformation of L*er eds1-2* plants with the floral dip method (Clough and Bent, 1998). DL-Phosphinothricin (BASTA, 300 µM) resistant seedlings were selected and analysed as described in the result section. Expression of *$RPM1^{Ler}$ and $RPM1^{Ler(R217Q)}$* was confirmed in five T1 plants of each line. Therefore cDNA was synthesized from the ten individual T1 plants and a specific 185 bp fragment of both *RPM1* sequences amplified using forward

primer CAAGTTTTTCCACCAACTCTCTGTAG and reverse primer GAATATCTTCAAGTCTCAAAGTCTGC. Due to the single base difference between *RPM1*$^{Ler}$ *and* *RPM1*$^{Ler(R217Q)}$ sequences, the product amplified from *RPM1*$^{Ler(R217Q)}$ could be digested with Pst I resulting in a 22 bp smaller fragment.

**Acknowledgements**

This research was funded by a research grant from The Netherlands Organization for Scientific Research (NWO) and Centre for BioSystems Genomics (CBSG), which is part of the Netherlands Genome Initiative. We are grateful to J. Dangl for providing us the *P. syringae* strains.

**Supplementary material**

**Table S1.** Genes that are differentially expressed in *dmr5* compared to L*er eds1-2*. For every transcript CATMA-ID and their corresponding AGI-ID are given. M-values reflect the log2 ratio of *dmr5*/L*er eds1-2* transcript levels. Genes were selected as differentially expressed when they had an M-value < -1.0 or > 1.0 and in addition a P-value < 0.05.

| M | P | AGI-ID | Description |
|---|---|---|---|
| 3.306428257 | 0.00088859 | AT5G03350 | legume lectin family protein, contains Pfam domain, PF00139: Legume lectins beta domain |
| 2.809246675 | 0.00088859 | AT5G10760 | aspartyl protease family protein, contains Pfam domain, PF00026: eukaryotic aspartyl protease |
| 2.305083922 | 0.00088859 | AT1G14880 | expressed protein, similar to PGPS/D12 (Petunia x hybrida) GI:4105794; contains Pfam profile PF04749: Protein of unknown function, DUF614 |
| 2.192027062 | 0.00103662 | AT1G31580 | expressed protein, identical to ORF1 (Arabidopsis thaliana) gi:457716:emb:CAA50905 |
| 2.174710697 | 0.00088859 | AT2G25510 | expressed protein |
| 2.104750699 | 0.00088859 | AT2G28190 | superoxide dismutase (Cu-Zn), chloroplast (SODCP) / copper/zinc superoxide dismutase (CSD2), identical to GP:3273753:AF061519 |
| 2.052824775 | 0.00088859 | AT3G48640 | expressed protein |
| 1.885265037 | 0.0009562 | AT5G24530 | oxidoreductase, 2OG-Fe(II) oxygenase family protein, similar to flavanone 3-hydroxylase (Persea americana)(GI:727410); contains PF03171 2OG-Fe(II) oxygenase superfamily domain |
| 1.839542425 | 0.00757813 | AT3G47480 | calcium-binding EF hand family protein, contains INTERPRO:IPR002048 calcium-binding EF-hand domain |
| 1.659116455 | 0.00200588 | AT3G20340 | expressed protein |
| 1.610320284 | 0.00183529 | AT1G73800 | calmodulin-binding protein, similar to calmodulin-binding protein TCB60 GI:1698548 from (Nicotiana tabacum) |
| 1.600560421 | 0.00183774 | AT1G73870 | zinc finger (B-box type) family protein |
| 1.581850762 | 0.01601428 | AT5G45380 | sodium:solute symporter family protein, contains Pfam profile: PF00474 sodium:solute symporter family |
| 1.566289833 | 0.00115746 | AT1G35710 | leucine-rich repeat transmembrane protein kinase, putative, similar to many predicted protein kinases |
| 1.525557566 | 0.00137891 | AT4G02520 | glutathione S-transferase, putative |
| 1.505407629 | 0.0009562 | AT4G11900 | similar to S-locus lectin protein kinase family protein [Arabidopsis thaliana] (TAIR:At2g19130.1); similar to putative serine/threonine kinase [Oryza sativa (japonica cultivar-group)] (GB:NP_916406.1); |
| 1.504299067 | 0.00461122 | AT3G28540 | AAA-type ATPase family protein, contains Pfam profile: ATPase family PF00004 |
| 1.476419521 | 0.00295858 | AT1G69730 | protein kinase family protein, contains Pfam profile: PF00069 Eukaryotic protein kinase domain |
| 1.472937412 | 0.00445342 | AT3G61280 | similar to expressed protein [Arabidopsis thaliana] (TAIR:At2g45840.1); similar to OSJNBb0039L24.21 [Oryza sativa (japonica cultivar-group)] (GB:XP_473304.1); contains InterPro domain Arabidopsis thaliana protein of unknown function DUF821 (InterPro:IPR008539); contains InterPro domain Lipopolysaccharide-modifying protein |
| 1.450951173 | 0.00201843 | AT1G75040 | pathogenesis-related protein 5 (PR-5), identical to SP:P28493 Pathogenesis-related protein 5 precursor (PR-5) {Arabidopsis thaliana}; contains Pfam profile: PF00314 Thaumatin family |

| M | P | AGI-ID | Description |
|---|---|---|---|
| 1.449974497 | 0.00094156 | AT4G18280 | glycine-rich cell wall protein-related, glycine-rich protein 1.0 precursor, Phaseolus vulgaris, PIR1:S01821 |
| 1.385091916 | 0.0028754 | AT5G45820 | CBL-interacting protein kinase 20 (CIPK20), identical to CBL-interacting protein kinase 20 (Arabidopsis thaliana) gi:14486384:gb:AAK61493 |
| 1.30185958 | 0.00653624 | AT5G39670 | [AT5G39680, pentatricopeptide (PPR) repeat-containing protein, contains INTERPRO:IPR002885 PPR repeats ];[AT5G39670, calcium-binding EF hand family protein, contains INTERPRO:IPR002048 calcium-binding EF-hand domain ] |
| 1.30185958 | 0.00653624 | AT5G39680 | [AT5G39680, pentatricopeptide (PPR) repeat-containing protein, contains INTERPRO:IPR002885 PPR repeats ];[AT5G39670, calcium-binding EF hand family protein, contains INTERPRO:IPR002048 calcium-binding EF-hand domain ] |
| 1.267426478 | 0.00122529 | AT2G46700 | calcium-dependent protein kinase, putative / CDPK, putative, similar to calcium/calmodulin-dependent protein kinase homolog MCK1 (Zea mays) gi:1839597:gb:AAB47181 |
| 1.252006478 | 0.00505587 | AT3G52430 | phytoalexin-deficient 4 protein (PAD4), identical to phytoalexin-deficient 4 protein (Arabidopsis thaliana) GI:6457331; contains Pfam profile PF01764: Lipase |
| 1.204798221 | 0.00119477 | AT4G14365 | zinc finger (C3HC4-type RING finger) family protein / ankyrin repeat family protein, contains Pfam profile: PF00097 zinc finger, C3HC4 type (RING finger) and Pfam profile: PF00023 ankyrin repeat |
| 1.183927875 | 0.00152715 | AT3G25882 | NPR1/NIM1-interacting protein 2 (NIMIN-2), identical to cDNA NIMIN-2 protein (nimin-2 gene)GI:12057155 |
| 1.167391146 | 0.00206589 | AT5G20230 | plastocyanin-like domain-containing protein |
| 1.135651578 | 0.00194043 | AT3G04210 | disease resistance protein (TIR-NBS class), putative, domain signature TIR-NBS exists, suggestive of a disease resistance protein. |
| 1.129245827 | 0.00475493 | AT4G14400 | ankyrin repeat family protein, contains ankyrin repeats, Pfam domain PF00023 |
| 1.125688308 | 0.00733253 | AT4G01460 | basic helix-loop-helix (bHLH) family protein, contains Pfam profile: PF00010 helix-loop-helix DNA-binding domain |
| 1.097054106 | 0.00665685 | AT1G21270 | wall-associated kinase 2 (WAK2), identical to wall-associated kinase 2 (Arabidopsis thaliana) GI:4826399; induced by salicylic acid or INA (PMID:10380805) |
| 1.095887557 | 0.00168994 | AT2G26440 | pectinesterase family protein, contains Pfam profile: PF01095 pectinesterase |
| 1.056011753 | 0.00811001 | AT3G01290 | band 7 family protein, similar to hypersensitive-induced response protein (Zea mays) GI:7716470; contains Pfam profile PF01145: SPFH domain / Band 7 family |
| 1.053782098 | 0.00314222 | AT2G31865 | poly (ADP-ribose) glycohydrolase (PARG) family protein, contains Pfam profile: PF05028 poly (ADP-ribose) glycohydrolase (PARG) |
| 1.043433856 | 0.0020785 | AT5G03210 | expressed protein |
| 1.042856167 | 0.00175081 | AT1G69530 | expansin, putative (EXP1), identical to expansin (At-EXP1) (Arabidopsis thaliana) GI:1041702; alpha-expansin gene family, PMID:11641069 |
| 1.037305777 | 0.01057796 | AT1G33960 | avirulence-responsive protein / avirulence induced gene (AIG1), identical to AIG1 (exhibits RPS2- and avrRpt2-dependent |

| M | P | AGI-ID | Description |
|---|---|---|---|
| | | | induction early after infection with Pseudomonas) SP:U40856 (Arabidopsis thaliana) (Plant Cell 8 (2), 241-249 (1996)) |
| 1.026606147 | 0.0028754 | AT5G06980 | expressed protein |
| 1.026435271 | 0.01126422 | AT1G10340 | ankyrin repeat family protein, contains ankyrin repeat domains, Pfam:PF00023 |
| 1.026186584 | 0.00294702 | AT2G41080 | pentatricopeptide (PPR) repeat-containing protein, contains INTERPRO:IPR002885 PPR repeats |
| 1.022628474 | 0.00673186 | AT4G23260 | protein kinase family protein, contains Pfam domain PF00069: Protein kinase domain |
| 1.01452464 | 0.00257004 | AT3G44450 | expressed protein |
| -1.01253892 | 0.025153 | AT2G42530 | cold-responsive protein / cold-regulated protein (cor15b), nearly identical to cold-regulated gene cor15b (Arabidopsis thaliana) GI:456016; contains Pfam profile PF02987: Late embryogenesis abundant protein |
| -1.11092511 | 0.002544 | ATMG00830 | [AT2G07783, pseudogene, similar to Ccl1, blastp match of 86% identity and 6.4e-177 P-value to GP:1694629:dbj:BAA12329.1::D84373 Ccl1 {Brassica napus} ];[ATMG00830, cytochrome c biogenesis orf382 ] |
| -1.11092511 | 0.002544 | AT2G07783 | [AT2G07783, pseudogene, similar to Ccl1, blastp match of 86% identity and 6.4e-177 P-value to GP:1694629:dbj:BAA12329.1::D84373 Ccl1 {Brassica napus} ];[ATMG00830, cytochrome c biogenesis orf382 ] |
| -1.13053391 | 0.00183987 | AT5G37260 | myb family transcription factor, contains Pfam profile: PF00249 myb-like DNA-binding domain |
| -1.14361708 | 0.00988805 | AT2G05910 | expressed protein, contains Pfam profile PF04525: Protein of unknown function (DUF567) |
| -1.14782041 | 0.03703633 | AT2G01050 | expressed protein |
| -1.5235981 | 0.00102283 | AT3G28270 | expressed protein, similar to At14a protein (GI:11994571 and GI:11994573) (Arabidopsis thaliana) |
| -1.55781715 | 0.00391352 | AT2G47880 | glutaredoxin family protein, contains INTERPRO Domain IPR002109, Glutaredoxin (thioltransferase) |
| -1.6102724 | 0.00157459 | AT1G09080 | luminal binding protein 3 (BiP-3) (BP3), Similar to Arabidopsis luminal binding protein (gb:D89342); contains Pfam domain PF00012: dnaK protein |
| -1.64305787 | 0.01155437 | AT5G54780 | similar to RabGAP/TBC domain-containing protein [Arabidopsis thaliana] (TAIR:At4g27100.1); similar to putative GTPase activating protein [Oryza sativa (japonica cultivar-group)] (GB:XP_468334.1); contains InterPro domain RabGAP/TBC domain (InterPro:IPR000195) |
| -1.83155022 | 0.00889487 | AT2G04020 | GDSL-motif lipase/hydrolase family protein, similar to family II lipase EXL6 (GI:15054390), EXL1 (GI:15054382) (Arabidopsis thaliana); contains Pfam profile PF00657: Lipase/Acylhydrolase with GDSL-like motif |
| -2.10851399 | 0.00088859 | AT4G35830 | aconitate hydratase, cytoplasmic / citrate hydro-lyase / aconitase (ACO), identical to SP:Q42560 Aconitate hydratase, cytoplasmic (EC 4.2.1.3) (Citrate hydro-lyase) (Aconitase) {Arabidopsis thaliana}; contains Pfam profiles PF00330: Aconitase family (aconitate hydratase), PF00694: Aconitase C-terminal domain |

| M | P | AGI-ID | Description |
|---|---|---|---|
| -2.39673621 | 0.00095135 | AT4G14170 | pentatricopeptide (PPR) repeat-containing protein, contains Pfam profile PF01535: PPR repeat |
| -2.47934628 | 0.00239988 | AT2G03970 | pseudogene, similar to SAE1-S9-protein, blastp match of 33% identity and 1.2e-17 P-value to GP:4760708:dbj:BAA77394.1::AB012866 SAE1-S9-protein {Brassica rapa} |
| -1.18000977 | 0.00154234 | AT3G33000 | pseudogene, ATP synthase A subunit, blastp match of 68% identity and 1.3e-82 P-value to SP:P06288:ATPI_TOBAC ATP synthase A chain precursor (EC 3.6.3.14) (Subunit IV). (Belladonna, Deadly nightshade) {Atropa belladonna} |
| -1.18114719 | 0.00181602 | AT3G33002 | pseudogene, ribosomal protein S2p family, blastp match of 84% identity and 7.7e-92 P-value to SP:Q9BBS6:RR2_LOTJA Chloroplast 30S ribosomal protein S2. {Lotus japonicus} |
| -1.21025705 | 0.00135087 | AT2G28820 | similar to alanine aminotransferase, putative [Arabidopsis thaliana] (TAIR:At1g72330.1); similar to ribosomal protein L16 [Vigna angularis] (GB:AAN04889.1); contains InterPro domain Ribosomal protein L16 (InterPro:IPR000114) |
| -1.24139181 | 0.0013061 | AT3G33004 | pseudogene, DNA-directed RNA polymerase beta-chain |
| -1.30262917 | 0.02794093 | AT2G01280 | transcription factor IIB (TFIIB) family protein, contains Pfam domain, PF00382: Transcription factor TFIIB repeat |
| -1.47092096 | 0.00626292 | AT3G51550 | protein kinase family protein, contains protein kinase domain, Pfam:PF00069 |

**Table S2.** Alignment of DNA and amino acid sequences of *RPM1* in *dmr5*, L*er eds1-2* and *Col-0* plants. The numbers depict the position of the first base-pair or amino acid of each line of the coding or protein sequence, respectively. Differences between the Col-0 and L*er* sequences are highlighted in grey. Difference between L*er* and *dmr5* are highlighted in black.

### DNA sequences

```
dmr5          1    ATGGCTTCGGCTACTGTTGATTTTGGGATCGGACGGATTCTTAGTGTCCTGGAAAACGAG
Ler eds1-2    1    ATGGCTTCGGCTACTGTTGATTTTGGGATCGGACGGATTCTTAGTGTCCTGGAAAACGAG
Col-0         1    ATGGCTTCGGCTACTGTTGATTTTGGGATCGGACGGATTCTTAGTGTCCTGGAAAACGAG

dmr5          61   ACCTTGTTATTGTCAGGAGTCCATGGTGAGATTGATAAAATGAAGAAGGAGTTGCTGATC
Ler eds1-2    61   ACCTTGTTATTGTCAGGAGTCCATGGTGAGATTGATAAAATGAAGAAGGAGTTGCTGATC
Col-0         61   ACCTTGTTATTGTCAGGAGTCCATGGTGAGATTGATAAAATGAAGAAGGAGTTGCTGATC

dmr5          121  ATGAAGTCCTTTCTTGAGGATACTCATAAACATGGTGGAAATGGATCCACAACAACAACA
Ler eds1-2    121  ATGAAGTCCTTTCTTGAGGATACTCATAAACATGGTGGAAATGGATCCACAACAACAACA
Col-0         121  ATGAAGTCCTTTCTTGAGGATACTCATAAACATGGTGGAAATGGATCCACAACAACAACA

dmr5          181  ACTCAACTCTTCCAAACTTTTGTGGCAAACACGAGAGATTTGGCTTACCAAATCGAAGAC
Ler eds1-2    181  ACTCAACTCTTCCAAACTTTTGTGGCAAACACGAGAGATTTGGCTTACCAAATCGAAGAC
Col-0         181  ACTCAACTCTTCCAAACTTTTGTGGCAAACACGAGAGATTTGGCTTACCAAATCGAAGAC

dmr5          241  ATTCTCGACGAGTTTGGCTATCACATCCACGGTTATCGTAGCTGCGCCAAAATTTGGCGT
Ler eds1-2    241  ATTCTCGACGAGTTTGGCTATCACATCCACGGTTATCGTAGCTGCGCCAAAATTTGGCGT
Col-0         241  ATTCTCGACGAGTTTGGCTATCACATCCACGGTTATCGTAGCTGCGCCAAAATTTGGCGT

dmr5          301  GCGTTTCATTTCCCGAGGTATATGTGGGCGAGGCACTCGATAGCTCAAAAGCTAGGAATG
Ler eds1-2    301  GCGTTTCATTTCCCGAGGTATATGTGGGCGAGGCACTCGATAGCTCAAAAGCTAGGAATG
Col-0         301  GCGTTTCATTTCCCGAGGTATATGTGGGCGAGGCACTCGATAGCTCAAAAGCTAGGAATG

dmr5          361  GTTAATGTCATGATTCAATCCATTTCTGATTCCATGAAAAGGTACTATCATTCAGAAAAC
Ler eds1-2    361  GTTAATGTCATGATTCAATCCATTTCTGATTCCATGAAAAGGTACTATCATTCAGAAAAC
Col-0         361  GTTAATGTCATGATTCAATCCATTTCTGATTCCATGAAAAGGTACTATCATTCAGAAAAC

dmr5          421  TACCAAGCAGCATTATTACCGCCT---------GGCGATGCAAAGTGGGTGAACAACATC
Ler eds1-2    421  TACCAAGCAGCATTATTACCGCCT---------GGCGATGCAAAGTGGGTGAACAACATC
Col-0         421  TACCAAGCAGCATTATTACCGCCTATTGATGATGGCGATGCAAAGTGGGTGAACAACATC

dmr5          472  AGTGAGTCATCTCTTTTCTTTAGTGAAAATAGTCTTGTAGGGATTGATGCACCCAAGGGA
Ler eds1-2    472  AGTGAGTCATCTCTTTTCTTTAGTGAAAATAGTCTTGTAGGGATTGATGCACCCAAGGGA
Col-0         481  AGTGAGTCATCTCTTTTCTTTAGTGAAAATAGTCTTGTAGGGATTGATGCACCCAAGGGA

dmr5          532  AAGCTCATCGGACGGCTTCTAAGTCCCGAACCTCAGCGGATTGTTGTCGCGGTGGTTGGG
Ler eds1-2    532  AAGCTCATCGGACGGCTTCTAAGTCCCGAACCTCAGCGGATTGTTGTCGCGGTGGTTGGG
Col-0         541  AAGCTCATCGGACGGCTTCTAAGTCCCGAACCTCAGCGGATTGTTGTCGCGGTGGTTGGG

dmr5          592  ATGGGCGGTTCAGGGAAAACTACACTCTCAGCGAATATCTTCCAGTCTCAAAGTGTGCAG
Ler eds1-2    592  ATGGGCGGTTCAGGGAAAACTACACTCTCAGCGAATATCTTCCAGTCTCAAAGTGTGCGG
Col-0         601  ATGGGCGGTTCAGGGAAAACTACACTCTCAGCGAATATCTTCAAGTCTCAAAGTGTGCGG

dmr5          652  AGGCATTTTGAGTCTTACGCTTGGGTTACAATCTCGAAATCTTATGTGATTGAAGATGTG
Ler eds1-2    652  AGGCATTTTGAGTCTTACGCTTGGGTTACAATCTCGAAATCTTATGTGATTGAAGATGTG
Col-0         661  AGGCATTTTGAGTCTTACGCTTGGGTTACAATCTCGAAATCTTATGTGATTGAAGATGTG

dmr5          712  TTTAGGACCATGATCAAGGAGTTTTACAAAGAAGCAGATACACAGATTCCAGCTGAATTA
Ler eds1-2    712  TTTAGGACCATGATCAAGGAGTTTTACAAAGAAGCAGATACACAGATTCCAGCTGAATTA
Col-0         721  TTTAGGACCATGATCAAGGAGTTTTACAAAGAAGCAGATACACAGATTCCAGCTGAATTA

dmr5          772  TACTCCTTAGGCTACAGAGAGTTGGTGGAAAAACTTGTGGAGTATTTGCAGTCGAAGAGG
Ler eds1-2    772  TACTCCTTAGGCTACAGAGAGTTGGTGGAAAAACTTGTGGAGTATTTGCAGTCGAAGAGG
Col-0         781  TACTCCTTAGGCTACAGAGAGTTGGTGGAAAAACTTGTGGAGTATTTGCAGTCGAAGAGG

dmr5          832  TACATTGTTGTGCTTGATGATGTATGGACCACTGGTCTATGGAGGGAGATAAGTATTGCT
Ler eds1-2    832  TACATTGTTGTGCTTGATGATGTATGGACCACTGGTCTATGGAGGGAGATAAGTATTGCT
Col-0         841  TACATTGTTGTGCTTGATGATGTATGGACCACTGGTCTATGGAGGGAGATAAGTATTGCT

dmr5          892  TTACCAGATGGTATTTATGGAAGTCGTGTTATGATGACTACTCGGGACATGAACGTGGCT
Ler eds1-2    892  TTACCAGATGGTATTTATGGAAGTCGTGTTATGATGACTACTCGGGACATGAACGTGGCT
Col-0         901  TTACCAGATGGTATTTATGGAAGTCGTGTTATGATGACTACTCGGGACATGAACGTGGCT
```

```
dmr5          952  TCTTTCCCATATGGAATTGGGAGCACAAAGCACGAGATTGAGCTCTTAAAAGAAGACGAA
Ler eds1-2    952  TCTTTCCCATATGGAATTGGGAGCACAAAGCACGAGATTGAGCTCTTAAAAGAAGACGAA
Col-0         961  TCTTTCCCATATGGAATTGGGAGCACAAAGCACGAGATTGAGCTCTTAAAAGAAGACGAA

dmr5         1012 GCTTGGGTGCTTTTCTCCAACAAAGCTTTTCCGGCAAGTCTTGAGCAATGCAGAACGCAG
Ler eds1-2   1012 GCTTGGGTGCTTTTCTCCAACAAAGCTTTTCCGGCAAGTCTTGAGCAATGCAGAACGCAG
Col-0        1021 GCTTGGGTGCTTTTCTCCAACAAAGCTTTTCCGGCAAGTCTTGAGCAATGCAGAACGCAG

dmr5         1072 AATTTGGAGCCGATAGCACGAAAATTAGTCGAGAGATGTCAGGGTTTGCCATTAGCTATT
Ler eds1-2   1072 AATTTGGAGCCGATAGCACGAAAATTAGTCGAGAGATGTCAGGGTTTGCCATTAGCTATT
Col-0        1081 AATTTGGAGCCGATAGCACGAAAATTAGTCGAGAGATGTCAGGGTTTGCCATTAGCTATT

dmr5         1132 GCTTCTTTGGGAAGCATGATGTCAACCAAGAAGTTTGAATCAGAATGGAAGAAAGTCTAC
Ler eds1-2   1132 GCTTCTTTGGGAAGCATGATGTCAACCAAGAAGTTTGAATCAGAATGGAAGAAAGTCTAC
Col-0        1141 GCTTCTTTGGGAAGCATGATGTCAACCAAGAAGTTTGAATCAGAATGGAAGAAAGTCTAC

dmr5         1192 AGTACTTTAAACTGGGAACTGAACAACAATCATGAACTCAAGATTGTTCGAAGCATTATG
Ler eds1-2   1192 AGTACTTTAAACTGGGAACTGAACAACAATCATGAACTCAAGATTGTTCGAAGCATTATG
Col-0        1201 AGTACTTTAAACTGGGAACTGAACAACAATCATGAACTCAAGATTGTTCGAAGCATTATG

dmr5         1252 TTCCTTAGCTTCAATGATTTGCCATACCCGCTTAAACGTTGTTTCTTATATTGTTCTCTT
Ler eds1-2   1252 TTCCTTAGCTTCAATGATTTGCCATACCCGCTTAAACGTTGTTTCTTATATTGTTCTCTT
Col-0        1261 TTCCTTAGCTTCAATGATTTGCCATACCCGCTTAAACGTTGTTTCTTATATTGTTCTCTT

dmr5         1312 TTCCCTGTGAACTATCGGATGAAAAGAAAGAGGCTCATTAGGATGTGGATGGCACAAAGG
Ler eds1-2   1312 TTCCCTGTGAACTATCGGATGAAAAGAAAGAGGCTCATTAGGATGTGGATGGCACAAAGG
Col-0        1321 TTCCCTGTGAACTATCGGATGAAAAGAAAGAGGCTCATTAGGATGTGGATGGCACAAAGG

dmr5         1372 TTTGTGGAACCGATCAGAGGAGTGAAAGCAGAAGAAGTGGCTGATAGTTACCTCAATGAA
Ler eds1-2   1372 TTTGTGGAACCGATCAGAGGAGTGAAAGCAGAAGAAGTGGCTGATAGTTACCTCAATGAA
Col-0        1381 TTTGTGGAACCGATCAGAGGAGTGAAAGCAGAAGAAGTGGCTGATAGTTACCTCAATGAA

dmr5         1432 CTTGTTTACAGAAATATGCTTCAAGTTATCTTGTGGAATCCTTTTGGACGACCCAAGGCG
Ler eds1-2   1432 CTTGTTTACAGAAATATGCTTCAAGTTATCTTGTGGAATCCTTTTGGACGACCCAAGGCG
Col-0        1441 CTTGTTTACAGAAATATGCTTCAAGTTATCTTGTGGAATCCTTTTGGACGACCCAAGGCG

dmr5         1492 TTCAAGATGCATGACGTGATATGGGAGATTGCTCTATCTGTATCCAAACTTGAGAGGTTC
Ler eds1-2   1492 TTCAAGATGCATGACGTGATATGGGAGATTGCTCTATCTGTATCCAAACTTGAGAGGTTC
Col-0        1501 TTCAAGATGCATGACGTGATATGGGAGATTGCTCTATCTGTATCCAAACTTGAGAGGTTC

dmr5         1552 TGTGATGTATATAATGATGACAGTGATGGTGATGATGCTGCAGAAACAATGGAGAATTAC
Ler eds1-2   1552 TGTGATGTATATAATGATGACAGTGATGGTGATGATGCTGCAGAAACAATGGAGAATTAC
Col-0        1561 TGTGATGTATATAATGATGACAGTGATGGTGATGATGCTGCAGAAACAATGGAGAATTAC

dmr5         1612 GGTAGTCGCCATTTATGCATTCAGAAAGAGATGACACCTGATAGTATACGTGCAACAAAC
Ler eds1-2   1612 GGTAGTCGCCATTTATGCATTCAGAAAGAGATGACACCTGATAGTATACGTGCAACAAAC
Col-0        1621 GGTAGTCGCCATTTATGCATTCAGAAAGAGATGACACCTGATAGTATACGTGCAACAAAC

dmr5         1672 CTTCACTCTCTTTTGGTTTGCTCTTCTGCTAAACACAAGATGGAATTACTTCCAAGTCTG
Ler eds1-2   1672 CTTCACTCTCTTTTGGTTTGCTCTTCTGCTAAACACAAGATGGAATTACTTCCAAGTCTG
Col-0        1681 CTTCACTCTCTTTTGGTTTGCTCTTCTGCTAAACACAAGATGGAATTACTTCCAAGTCTG

dmr5         1732 AACCTTCTGAGAGCCTTAGACCTTGAAGACTCTTCCATCAGCAAGTTACCAGATTGTTTA
Ler eds1-2   1732 AACCTTCTGAGAGCCTTAGACCTTGAAGACTCTTCCATCAGCAAGTTACCAGATTGTTTA
Col-0        1741 AACCTTCTGAGAGCCTTAGACCTTGAAGACTCTTCCATCAGCAAGTTACCAGATTGTTTA

dmr5         1792 GTAACTATGTTCAACTTAAAGTACTTGAATTTGTCCAAGACACAGGTGAAAGAACTGCCA
Ler eds1-2   1792 GTAACTATGTTCAACTTAAAGTACTTGAATTTGTCCAAGACACAGGTGAAAGAACTGCCA
Col-0        1801 GTAACTATGTTCAACTTAAAGTACTTGAATTTGTCCAAGACACAGGTGAAAGAACTGCCA

dmr5         1852 AAAAACTTCCATAAACTCGTCAATTTGGAGACGTTGAACACAAAGCACTCCAAGATTGAG
Ler eds1-2   1852 AAAAACTTCCATAAACTCGTCAATTTGGAGACGTTGAACACAAAGCACTCCAAGATTGAG
Col-0        1861 AAAAACTTCCATAAACTCGTCAATTTGGAGACGTTGAACACAAAGCACTCCAAGATTGAG

dmr5         1912 GAACTTCCTCTAGGGATGTGGAAATTGAAGAAGTTGCGATATCTCATTACTTTTCGCCGC
Ler eds1-2   1912 GAACTTCCTCTAGGGATGTGGAAATTGAAGAAGTTGCGATATCTCATTACTTTTCGCCGC
Col-0        1921 GAACTTCCTCTAGGGATGTGGAAATTGAAGAAGTTGCGATATCTCATTACTTTTCGCCGC

dmr5         1972 AATGATGGACATGATTCCAATTGGAATTATGTTTTAGGTACAAGGGTTGTTCCTAAAATC
Ler eds1-2   1972 AATGATTGGACATGATTCCAATTGGAATTATGTTTTAGGTACAAGGGTTGTTCCTAAAATC
Col-0        1981 AATGAAGGACATGATTCCAATTGGAATTATGTTTTAGGTACAAGGGTTGTTCCTAAAATC
```

```
dmr5          2032 TGGCAGTTGAAGGATTTGCAAGTCATGGACTGCTTCAACGCAGAAGATGAGCTTATAAAA
Ler eds1-2    2032 TGGCAGTTGAAGGATTTGCAAGTCATGGACTGCTTCAACGCAGAAGATGAGCTTATAAAA
Col-0         2041 TGGCAGTTGAAGGATTTGCAAGTCATGGACTGCTTCAACGCAGAAGATGAGCTTATCAAA

dmr5          2092 AATCTCGGGTGTATGACTCAGCTCACAAGAATAAGTCTTGTCATGGTTAGGAGAGAACAT
Ler eds1-2    2092 AATCTCGGGTGTATGACTCAGCTCACAAGAATAAGTCTTGTCATGGTTAGGAGAGAACAT
Col-0         2101 AATCTCGGGTGTATGACTCAGCTCACAAGAATAAGTCTTGTCATGGTTAGGAGAGAACAT

dmr5          2152 GGAAGAGACTTGTGCGATTCACTGAATAAGATTAAAAGAATTCGATTCTTATCTTTAACG
Ler eds1-2    2152 GGAAGAGACTTGTGCGATTCACTGAATAAGATTAAAAGAATTCGATTCTTATCTTTAACG
Col-0         2161 GGAAGAGACTTGTGCGATTCACTGAATAAGATTAAAAGAATTCGATTCTTATCTTTAACG

dmr5          2212 TCGATTGATGAAGAGGAACCGTTAGAGATAGATGACCTGATCGCAACTGCAAGCATTGAG
Ler eds1-2    2212 TCGATTGATGAAGAGGAACCGTTAGAGATAGATGACCTGATCGCAACTGCAAGCATTGAG
Col-0         2221 TCGATTGATGAAGAGGAACCGTTAGAGATAGATGACCTGATCGCAACTGCAAGCATTGAG

dmr5          2272 AAGCTCTTTCTTGCTGGGAAACTGGAGAGAGTACCAAGTTGGTTCAACACTCTTCAAAAC
Ler eds1-2    2272 AAGCTCTTTCTTGCTGGGAAACTGGAGAGAGTACCAAGTTGGTTCAACACTCTTCAAAAC
Col-0         2281 AAGCTCTTTCTTGCTGGGAAACTGGAGAGAGTACCAAGTTGGTTCAACACTCTTCAAAAC

dmr5          2332 CTTACCTATTTGGGTTTGCGTGGATCTCAGCTACAGGAAAACGCCATTCTCTCTATCCAA
Ler eds1-2    2332 CTTACCTATTTGGGTTTGCGTGGATCTCAGCTACAGGAAAACGCCATTCTCTCTATCCAA
Col-0         2341 CTTACCTATTTGGGTTTGCGTGGATCTCAGCTACAGGAAAACGCCATTCTCTCTATCCAA

dmr5          2392 ACGCTTCCTAGATTGGTATGGCTTTCATTTTACAATGCATACATGGGACCTAGGTTGCGT
Ler eds1-2    2392 ACGCTTCCTAGATTGGTATGGCTTTCATTTTACAATGCATACATGGGACCTAGGTTGCGT
Col-0         2401 ACGCTTCCTAGATTGGTATGGCTTTCATTTTACAATGCATACATGGGACCTAGGTTGCGT

dmr5          2452 TTTGCACAAGGATTTCAGAATCTGAAGATTCTAGAGATAGTTCAGATGAAACATCTGACA
Ler eds1-2    2452 TTTGCACAAGGATTTCAGAATCTGAAGATTCTAGAGATAGTTCAGATGAAACATCTGACA
Col-0         2461 TTTGCACAAGGATTTCAGAATCTGAAGATTCTAGAGATAGTTCAGATGAAACATCTGACA

dmr5          2512 GAAGTTGTTATAGAAGACGGTGCAATGTTTGAGCTTCAGAAGCTTTATGTCAGGGCTTGT
Ler eds1-2    2512 GAAGTTGTTATAGAAGACGGTGCAATGTTTGAGCTTCAGAAGCTTTATGTCAGGGCTTGT
Col-0         2521 GAAGTTGTTATAGAAGACGGTGCAATGTTTGAGCTTCAGAAGCTTTATGTCAGGGCTTGT
```

**Amino acid sequences**

```
dmr5             1    MASATVDFGIGRILSVLENETLLLSGVHGEIDKMKKELLIMKSFLEDTHKHGGNGSTTTT
Ler eds1-2       1    MASATVDFGIGRILSVLENETLLLSGVHGEIDKMKKELLIMKSFLEDTHKHGGNGSTTTT
Col-0            1    MASATVDFGIGRILSVLENETLLLSGVHGEIDKMKKELLIMKSFLEDTHKHGGNGSTTTT

dmr5            61    TQLFQTFVANTRDLAYQIEDILDEFGYHIHGYRSCAKIWRAFHFPRYMWARHSIAQKLGM
Ler eds1-2      61    TQLFQTFVANTRDLAYQIEDILDEFGYHIHGYRSCAKIWRAFHFPRYMWARHSIAQKLGM
Col-0           61    TQLFQTFVANTRDLAYQIEDILDEFGYHIHGYRSCAKIWRAFHFPRYMWARHSIAQKLGM

dmr5            121   VNVMIQSISDSMKRYYHSENYQAALLPP---GDAKWVNNISESSLFFSENSLVGIDAPKG
Ler eds1-2      121   VNVMIQSISDSMKRYYHSENYQAALLPP---GDAKWVNNISESSLFFSENSLVGIDAPKG
Col-0           121   VNVMIQSISDSMKRYYHSENYQAALLPPIDDGDAKWVNNISESSLFFSENSLVGIDAPKG

dmr5            178   KLIGRLLSPEPQRIVVAVVGMGGSGKTTLSANIFQSQSVQRHFESYAWVTISKSYVIEDV
Ler eds1-2      178   KLIGRLLSPEPQRIVVAVVGMGGSGKTTLSANIFQSQSVRRHFESYAWVTISKSYVIEDV
Col-0           181   KLIGRLLSPEPQRIVVAVVGMGGSGKTTLSANIFKSQSVKRHFESYAWVTISKSYVIEDV

dmr5            238   FRTMIKEFYKEADTQIPAELYSLGYRELVEKLVEYLQSKRYIVVLDDVWTTGLWREISIA
Ler eds1-2      238   FRTMIKEFYKEADTQIPAELYSLGYRELVEKLVEYLQSKRYIVVLDDVWTTGLWREISIA
Col-0           241   FRTMIKEFYKEADTQIPAELYSLGYRELVEKLVEYLQSKRYIVVLDDVWTTGLWREISIA

dmr5            298   LPDGIYGSRVMMTTRDMNVASFPYGIGSTKHEIELLKEDEAWVLFSNKAFPASLEQCRTQ
Ler eds1-2      298   LPDGIYGSRVMMTTRDMNVASFPYGIGSTKHEIELLKEDEAWVLFSNKAFPASLEQCRTQ
Col-0           301   LPDGIYGSRVMMTTRDMNVASFPYGIGSTKHEIELLKEDEAWVLFSNKAFPASLEQCRTQ

dmr5            458   NLEPIARKLVERCQGLPLAIASLGSMMSTKKFESEWKKVYSTLNWELNNNHELKIVRSIM
Ler eds1-2      458   NLEPIARKLVERCQGLPLAIASLGSMMSTKKFESEWKKVYSTLNWELNNNHELKIVRSIM
Col-0           461   NLEPIARKLVERCQGLPLAIASLGSMMSTKKFESEWKKVYSTLNWELNNNHELKIVRSIM

dmr5            418   FLSFNDLPYPLKRCFLYCSLFPVNYRMKRKRLIRMWMAQRFVEPIRGVKAEEVADSYLNE
Ler eds1-2      418   FLSFNDLPYPLKRCFLYCSLFPVNYRMKRKRLIRMWMAQRFVEPIRGVKAEEVADSYLNE
Col-0           421   FLSFNDLPYPLKRCFLYCSLFPVNYRMKRKRLIRMWMAQRFVEPIRGVKAEEVADSYLNE
```

```
dmr5          478  LVYRNMLQVILWNPFGRPKAFKMHDVIWEIALSVSKLERFCDVYNDDSDGDDAAETMENY
Ler eds1-2    478  LVYRNMLQVILWNPFGRPKAFKMHDVIWEIALSVSKLERFCDVYNDDSDGDDAAETMENY
Col-0         481  LVYRNMLQVILWNPFGRPKAFKMHDVIWEIALSVSKLERFCDVYNDDSDGDDAAETMENY

dmr5          538  GSRHLCIQKEMTPDSIRATNLHSLLVCSSAKHKMELLPSLNLLRALDLEDSSISKLPDCL
Ler eds1-2    538  GSRHLCIQKEMTPDSIRATNLHSLLVCSSAKHKMELLPSLNLLRALDLEDSSISKLPDCL
Col-0         541  GSRHLCIQKEMTPDSIRATNLHSLLVCSSAKHKMELLPSLNLLRALDLEDSSISKLPDCL

dmr5          598  VTMFNLKYLNLSKTQVKELPKNFHKLVNLETLNTKHSKIEELPLGMWKLKKLRYLITFRR
Ler eds1-2    598  VTMFNLKYLNLSKTQVKELPKNFHKLVNLETLNTKHSKIEELPLGMWKLKKLRYLITFRR
Col-0         601  VTMFNLKYLNLSKTQVKELPKNFHKLVNLETLNTKHSKIEELPLGMWKLKKLRYLITFRR

dmr5          658  NDGHDSNWNYVLGTRVVPKIWQLKDLQVMDCFNAEDELIKNLGCMTQLTRISLVMVRREH
Ler eds1-2    658  NDGHDSNWNYVLGTRVVPKIWQLKDLQVMDCFNAEDELIKNLGCMTQLTRISLVMVRREH
Col-0         661  NEGHDSNWNYVLGTRVVPKIWQLKDLQVMDCFNAEDELIKNLGCMTQLTRISLVMVRREH

dmr5          718  GRDLCDSLNKIKRIRFLSLTSIDEEEPLEIDDLIATASIEKLFLAGKLERVPSWFNTLQN
Ler eds1-2    718  GRDLCDSLNKIKRIRFLSLTSIDEEEPLEIDDLIATASIEKLFLAGKLERVPSWFNTLQN
Col-0         721  GRDLCDSLNKIKRIRFLSLTSIDEEEPLEIDDLIATASIEKLFLAGKLERVPSWFNTLQN

dmr5          778  LTYLGLRGSQLQENAILSIQTLPRLVWLSFYNAYMGPRLRFAQGFQNLKILEIVQMKHLT
Ler eds1-2    778  LTYLGLRGSQLQENAILSIQTLPRLVWLSFYNAYMGPRLRFAQGFQNLKILEIVQMKHLT
Col-0         781  LTYLGLRGSQLQENAILSIQTLPRLVWLSFYNAYMGPRLRFAQGFQNLKILEIVQMKHLT

dmr5          838  EVVIEDGAMFELQKLYVRACRGLEYVPRGIENLINLQELHLIHVSNQLVERIRGEGSVDR
Ler eds1-2    838  EVVIEDGAMFELQKLYVRACRGLEYVPRGIENLINLQELHLIHVSNQLVERIRGEGSVDR
Col-0         841  EVVIEDGAMFELQKLYVRACRGLEYVPRGIENLINLQELHLIHVSNQLVERIRGEGSVDR

dmr5          898  SRVKHIPAIKHYFRTDNGSFYVSLSS
Ler eds1-2    898  SRVKHIPAIKHYFRTDNGSFYVSLSS
Col-0         901  SRVKHIPAIKHYFRTDNGSFYVSLSS
```

## References

**Ade, J., DeYoung, B.J., Golstein, C. and Innes, R.W.** (2007). Indirect activation of a plant nucleotide binding site-leucine-rich repeat protein by a bacterial protease. Proc Natl Acad Sci U S A. 104, 2531-6.

**Allemeersch, J., Durinck, S., Vanderhaeghen, R., Alard, P., Maes, R., Seeuws, K., Bogaert, T., Coddens, K., Deschouwer, K., Van Hummelen. P., Vuylsteke, M., Moreau, Y., Kwekkeboom, J., Wijfjes, A.H., May, S., Beynon, J., Hilson, P., and Kuiper, M.T.** (2005). Benchmarking the CATMA microarray. A novel tool for Arabidopsis transcriptome analysis. Plant Physiol. 137, 588-601.

**Aravind, L., Iyer, L.M., Leipe, D.D. and Koonin, E.V.** (2004). A novel family of P-loop NTPases with an unusual phyletic distribution and transmembrane segments inserted within the NTPase domain. Genome Biol. 5:R30.

**Belkhadir, Y., Nimchuk, Z., Hubert, D.A., Mackey, D. and Dangl, J.L.** (2004). Arabidopsis RIN4 negatively regulates disease resistance mediated by RPS2 and RPM1 downstream or independent of the NDR1 signal modulator and is not required for the virulence functions of bacterial type III effectors AvrRpt2 or AvrRpm1. Plant Cell. 16, 2822-35.

**Bell, J.K., Mullen, G.E., Leifer, C.A., Mazzoni, A., Davies, D.R. and Segal, D.M.** (2003). Leucine-rich repeats and pathogen recognition in Toll-like receptors. Trends Immunol. 24, 528-33.

**Clough, S.J. and Bent, A.F.** (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735-43.

**Crowe, M.L., Serizet, C., Thareau, V., Aubourg, S., Rouzé, P., Hilson, P., Beynon, J., Weisbeek, P., van Hummelen, P., Reymond, P., Paz-Ares, J., Nietfeld, W. and Trick, M.** (2003). CATMA: a complete Arabidopsis GST database. Nucleic Acids Res. 31, 156-8.

**Dangl, J.L. and Jones, J.D.** (2001). Plant pathogens and integrated defence responses to infection. Nature. 411, 826-33.

**Dinesh-Kumar, S.P., Tham, W.H. and Baker, B.J.** (2000). Structure-function analysis of the tobacco mosaic virus resistance gene N. Proc Natl Acad Sci U S A. 97, 14789-94.

**Dong, X.** (2004). NPR1, all things considered. Curr Opin Plant Biol. 7, 547-52.

**Feys, B.J., Parker, J.E.** (2000). Interplay of signaling pathways in plant disease resistance. Trends Genet. 16, 449-55.

**Glazebrook, J., Rogers, E.E. and Ausubel, F.M.** (1996). Isolation of Arabidopsis mutants with enhanced disease susceptibility by direct screening. Genetics. 143, 973-82.

**Grant, M.R., Godiard, L., Straube, E., Ashfield, T., Lewald, J., Sattler, A., Innes, R.W. and Dangl, J.L.** (1995). Structure of the Arabidopsis RPM1 gene enabling dual specificity disease resistance. Science. 269, 843-6.

**Greenberg, J.T., Yao, N.** The role and regulation of programmed cell death in plant-pathogen interactions. Cell Microbiol. 6, 201-11.

**Hilson, P., Allemeersch, J., Altmann, T., Aubourg, S., Avon, A., Beynon, J., Bhalerao, R.P., Bitton, F., Caboche, M., Cannoot, B., Chardakov, V., Cognet-Holliger, C., Colot, V., Crowe, M., Darimont, C., Durinck, S., Eickhoff, H., de Longevialle, A.F., Farmer, E.E., Grant, M., Kuiper, M.T., Lehrach, H., Leon, C., Leyva, A., Lundeberg, J., Lurin, C., Moreau, Y., Nietfeld, W., Paz-Ares, J., Reymond, P., Rouze, P., Sandberg, G., Segura, M.D., Serizet, C., Tabrett, A., Taconnat, L., Thareau, V., Van Hummelen, P., Vercruysse, S., Vuylsteke, M., Weingartner, M., Weisbeek, P.J., Wirta, V., Wittink, F.R., Zabeau, M., and Small, I.** (2004). Versatile gene-specific sequence tags for Arabidopsis functional genomics: transcript profiling and reverse genetics applications. Genome Res. 14, 2176-89.

**Holub, E.B., Beynon, J.L. and Crute, I.R.** (1994). Phenotypic and genotypic characterization of interactions between isolates of Peronospora parasitica and accessions of Arabidopsis thaliana. Mol. Plant Microbe Interact. 7, 223-39.

**Inohara, N., Koseki, T., Lin, J., del Peso, L., Lucas, P.C., Chen, F.F., Ogura, Y. and Núñez, G.** (2000). An induced proximity model for NF-kappa B activation in the Nod1/RICK and RIP signaling pathways. J Biol Chem. 275, 27823-31.

**Jaroszewski, L., Rychlewski, L., Reed, J.C. and Godzik, A.** (2000). ATP-activated oligomerization as a mechanism for apoptosis regulation: fold and mechanism prediction for CED-4. Proteins. 39, 197-203.

**Karimi, M., Inzé, D. and Depicker, A.** (2002). GATEWAY vectors for Agrobacterium-mediated plant transformation. Trends Plant Sci. 7, 193-5.

**Kim, M.G., da Cunha, L., McFall, A.J., Belkhadir, Y., DebRoy, S., Dangl, J.L. and Mackey, D.** (2005). Two Pseudomonas syringae type III effectors inhibit RIN4-regulated basal defence in Arabidopsis. Cell. 121, 749-59.

**Kobe, B. and Kajava, A.V.** (2001). The leucine-rich repeat as a protein recognition motif. Curr Opin Struct Biol. 11, 725-32.

**Leipe, D.D., Koonin, E.V. and Aravind, L.** (2004). STAND, a class of P-loop NTPases including animal and plant regulators of programmed cell death: multiple, complex domain architectures, unusual phyletic patterns, and evolution by horizontal gene transfer. J Mol Biol. 343, 1-28.

**Lu, H., Rate, D.N., Song, J.T. and Greenberg, J.T.** (2003). ACD6, a novel ankyrin protein, is a regulator and an effector of salicylic acid signaling in the Arabidopsis defence response. Plant Cell. 15, 2408-20.

**Mestre, P. and Baulcombe, D.C.** (2006). Elicitor-mediated oligomerization of the tobacco N disease resistance protein. Plant Cell. 18, 491-501.

**Meyers, B.C., Dickerman, A.W., Michelmore, R.W., Sivaramakrishnan, S., Sobral, B.W. and Young, N.D.** (1999). Plant disease resistance genes encode members of an ancient and diverse protein family within the nucleotide-binding superfamily. Plant J. 20, 317-32.

**Pan, Q., Wendel, J. and Fluhr, R.** (2000). Divergent evolution of plant NBS-LRR resistance gene homologues in dicot and cereal genomes. J Mol Evol. 50, 203-13.

**Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D. and Daniels, M.J.** (1996). Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora parasitica specified by several different RPP genes. Plant Cell. 8, 2033-46.

**Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S. and Greenberg, J.T.** (1999). The gain-of-function Arabidopsis acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defences, and cell growth. Plant Cell. 11, 1695-708.

**Reuber, T.L. and Ausubel, F.M.** (1996). Isolation of Arabidopsis genes that differentiate between resistance responses mediated by the RPS2 and RPM1 disease resistance genes. Plant Cell. 8, 241-9.

**Saraste, M., Sibbald, P.R. and Wittinghofer, A.** (1990). The P-loop--a common motif in ATP- and GTP-binding proteins. Trends Biochem Sci. 15, 430-4.

**Sinapidou, E., Williams, K., Nott, L., Bahkt, S., Tör, M., Crute, I., Bittner-Eddy, P. and Beynon, J.** (2004). Two TIR:NB:LRR genes are required to specify resistance to Peronospora parasitica isolate Cala2 in Arabidopsis. Plant J. 38, 898-909.

**Takken, F.L., Albrecht, M., Tameling, W.I.** (2006). Resistance proteins: molecular switches of plant defence. Curr Opin Plant Biol. 9, 383-90.

**Tameling, W.I., Elzinga, S.D., Darmin, P.S., Vossen, J.H., Takken, F.L., Haring, M.A. and Cornelissen, B.J.** (2002). The tomato R gene products I-2 and MI-1 are functional ATP binding proteins with ATPase activity. Plant Cell. 14, 2929-39.

**Tameling, W.I., Vossen, J.H., Albrecht, M., Lengauer, T., Berden, J.A., Haring, M.A., Cornelissen, B.J. and Takken, F.L.** (2006). Mutations in the NB-ARC domain of I-2 that impair ATP hydrolysis cause autoactivation. Plant Physiol. 140, 1233-45.

**Tao, Y., Yuan, F., Leister, R.T., Ausubel, F.M. and Katagiri, F.** (2000). Mutational analysis of the Arabidopsis nucleotide binding site-leucine-rich repeat resistance gene RPS2. Plant Cell. 12, 2541-54.

**Tornero, P. and Dangl, J.L.** (2001). A high-throughput method for quantifying growth of phytopathogenic bacteria in Arabidopsis thaliana. Plant J. 28, 475-81.

**Tornero, P., Chao, R.A., Luthin, W.N., Goff, S.A. and Dangl, J.L.** (2002). Large-scale structure-function analysis of the Arabidopsis RPM1 disease resistance protein. Plant Cell. 14, 435-50.

**Traut, T.W.** (1994). The functions and consensus motifs of nine types of peptide segments that form different types of nucleotide-binding sites. Eur J Biochem. 222, 9-19.

**Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J.** (1992). Acquired resistance in Arabidopsis. Plant Cell. 4, 645-56.

**Van Damme, M., Andel, A., Huibers, R.P., Panstruga, R., Weisbeek, P.J. and Van den Ackerveken, G.** (2005). Identification of arabidopsis loci required for susceptibility to the downy mildew pathogen Hyaloperonospora parasitica. Mol Plant Microbe Interact. 18, 583-92.

**Van Damme, M., Huibers, R.P., Elberse, J. and Van den Ackerveken, G.** (2008). Arabidopsis DMR6 encodes a putative 2OG-Fe(II) oxygenase that is defence-associated but required for susceptibility to downy mildew. Plant J. 54, 785-93.

**Van der Biezen, E.A. and Jones, J.D.** (1998). The NB-ARC domain: a novel signalling motif shared by plant resistance gene products and regulators of cell death in animals. Curr Biol. 8, R226-7.

**Walker, J.E., Saraste, M., Runswick, M.J. and Gay, N.J.** (1982). Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J. 1: 945-51.

# Chapter 4

# Arabidopsis *DMR6* encodes a putative 2OG-Fe(II) oxygenase that is defence-associated but required for susceptibility to downy mildew

**Robin P. Huibers‡, Mireille van Damme‡, Joyce Elberse and Guido Van den Ackerveken**

‡**These authors contributed equally to the work.**

Plant-Microbe Interactions, Department of Biology, Science Faculty, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands.

Adapted from Plant Journal. 2008. 54, 785-93.

**Abstract**

**The Arabidopsis mutant *downy mildew resistant 6* (*dmr6*) carries a recessive mutation that results in loss of susceptibility to *Hyaloperonospora parasitica*. Here we describe the map-based cloning of *DMR6* (*At5g24530*) which was found to encode a 2OG-Fe(II) oxygenase of unknown function. *DMR6* transcription is locally induced during infections with both compatible and incompatible *H. parasitica* isolates. High *DMR6* transcript levels were also observed in constitutive defence mutants and after treatment with salicylic acid analog BTH, suggesting that *DMR6* has a role during plant defence. Expression analysis of *dmr6* mutants, using DNA microarrays and quantitative PCR, showed the enhanced expression of a subset of defence-associated genes including *DMR6* itself, suggesting *dmr6*-mediated resistance results from the activation of plant defence responses. Alternatively, resistance could be caused by the accumulation of a toxic DMR6 substrate, or by the absence of a DMR6 metabolic product that is required for *H. parasitica* infection.**

*Additional Keywords: Arabidopsis thaliana, Hyaloperonospora parasitica, disease susceptibility, 2OG-Fe(II) oxygenase, negative regulator of plant defence, oxidoreductase.*

**Introduction**

In their natural environment, plants are challenged with a large variety of biotic and abiotic stresses. Despite their sessile 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. Host proteins involved in establishing this basic compatibility can be considered susceptibility factors. The absence of such host susceptibility proteins, e.g. as a result of mutation, could lead to resistance or reduced susceptibility. This 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 that they infect, which are thought to be important for nutrient uptake. In the rust pathogen *Uromyces fabae*, 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 haustorial membrane of the pathogen. If and how the plant contributes to the transport of nutrients over the plant cell membrane (the extrahaustorial membrane) is still an enigma. Besides the obvious feeding relationship, 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 *Golovinomyces cichoracearum* (previously known as *Erysiphe cichoracearum*) and the downy mildew oomycete *Hyaloperonospora parasitica*. *Golovinomyces cichoracearum* grows epicuticularly, forming haustoria from the outside of the epidermal cells. To identify the compatibility genes required for powdery mildew susceptibility, Vogel and colleagues have isolated 26 recessive Arabidopsis *powdery mildew resistance* (*pmr*) mutants (Vogel and Somerville, 2000). Four of the corresponding *PMR* genes have been cloned. *PMR4* (*GLS5* = *CalS12*) encodes for a callose synthase (Nishimura et al., 2003). The *pmr4* mutant can no longer induce a callose response, and shows enhanced activation of salicylic acid-dependent defence genes. *PMR6* is 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* mutant plants is altered, in particular the levels of pectin are increased (Vogel et al., 2002, 2004). *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 the successful entry of the powdery mildew pathogen *Blumeria graminis* f. sp. *hordei* into the host cell (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 has resulted in the identification of 20 *downy mildew resistant* (*dmr*) mutants, and eight of these that corresponded to six different loci, *dmr1–dmr6*, were studied in more detail (Chapter 2; 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 to be mutants in which cellular processes that are required for downy mildew infection are disturbed. The corresponding Arabidopsis *DMR* gene products could contribute to the *H. parasitica* infection process and promote disease susceptibility. Here we describe the cloning and characterization of *DMR6* that encodes an oxidoreductase of the 2-oxoglutarate (2OG)-Fe(II) oxygenase superfamily. Although mutation of *DMR6* leads to downy mildew resistance, expression of the gene is induced during plant defence. Furthermore, *dmr6* mutants express enhanced levels of a subset of defence-associated genes, indicating that *DMR6* negatively affects plant defence.

**Results**

*DMR6 encodes a putative 2OG-Fe(II) oxygenase*

Previously *dmr6-1* was identified as a recessive trait mediating *H. parasitica* resistance, and was mapped near marker nga139 on chromosome 5 to a region predicted to encompass 74 genes (Chapter 2; Van Damme et al., 2005). Fine mapping reduced the *dmr6-1* interval to a chromosomal region between the markers IND_K16H17 and CAP_At5g24590 located in the genes *At5g24420* and *At5g24590*, respectively. Comparative analysis of the coding sequences within this chromosomal region in *dmr6-1* and the parental line, L*er eds1-2*, revealed a single point mutation in the second exon of *At5g24530*. This single base change of G to A, typical for an EMS mutation, changes TGG (trp codon) to TGA (premature stop codon) at nucleotide position 691 of the genomic sequence (Figure 1A). The *At5g24530* gene is predicted to encode a 2OG-Fe(II)-dependent oxygenase with a mass of 39.4 kDa, the biological function of which has not yet been described. The premature stop codon truncates the predicted oxidoreductase enzyme of 342 amino acids, at position 141 before the conserved catalytic domain, as defined by Pfam03171, suggesting that *dmr6-1* is a null allele.

A second allele, *dmr6-2*, was identified in a T-DNA insertion line (FLAG 445D09) from the mutant collection of INRA, Versailles (Samson et al., 2002). The presence and location of the T-DNA insert in the second intron of *At5g24530* (Figure 1A) was confirmed by PCR and sequence analysis. FLAG 445D09 lines homozygous for the T-DNA insertion showed strongly reduced susceptibility to *H. parasitica* isolate Waco9, whereas the parental line (Ws-4) was highly susceptible (Figure 1B). RT-PCR, using a *DMR6* and T-DNA primer, on cDNA synthesized from *dmr6-2* mRNA resulted in the amplification of a DNA fragment (data not shown). This

indicates that the second intron, containing the T-DNA, is not spliced out correctly in *dmr6-2*. However, the correctly spliced *DMR6* transcripts could still be detected in *dmr6-2* by quantitative PCR (Q-PCR), although at a strongly reduced level (~11-fold, data not shown). This suggests that *dmr6-2* is not a complete null allele. Nevertheless, the *dmr6-2* mutant is nearly as resistant to *H. parasitica* as *dmr6-1*.

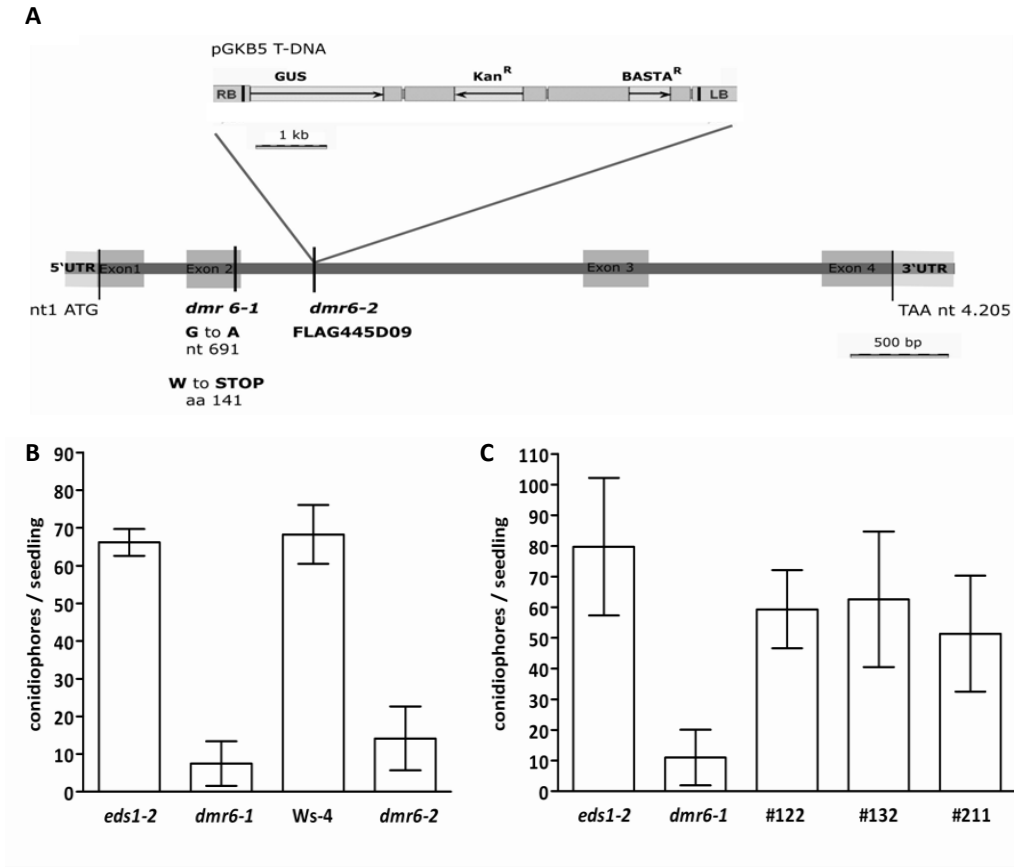


**Figure 1**. Mutations in the *DMR6* gene and their effect on susceptibility to *H. parasitica*. (**A**), the *DMR6* gene contains four exons that form a coding sequence of 1026 bases. The two alleles are indicated: *dmr6-1* with a base change in exon 2 and *dmr6-2* with a T-DNA insertion in intron 2. (**B**), quantification of conidiophores of *H. parasitica* isolate Waco9 on the *dmr6-1* mutant, compared with its parental line L*er eds1-2*, and on the *dmr6-2* mutant (FLAG 445D09 T-DNA line), compared with its parental line Ws-4. (**C**), restoration of susceptibility by complementation with the *At5g24530* cDNA under the control of the 35S promoter in the *dmr6-1* mutant. *H. parasitica* conidiophores per seedling were quantified on L*er eds1-2*, *dmr6-1* and three complementation lines, #122, #132 and #211.

To corroborate that *At5g24530* is required for *H. parasitica* growth, the *dmr6-1* mutant was transformed with the cDNA sequence of *At5g24530* under the control of the 35S promoter. In multiple independent *dmr6-1* transformants, overexpression of *At5g24530* was confirmed by Q-PCR. Three independent T3 lines, #122, #132 and #211, that overexpress *DMR6* (Figure S1) were almost fully restored for *H. parasitica* susceptibility (Figure 1C). The complementation data, together with the identification of two independent *dmr6* mutants, clearly demonstrates that a functional *At5g24530* gene is required for susceptibility of Arabidopsis to *H. parasitica*.

*DMR6 transcription is activated during H. parasitica infection*

To investigate whether *DMR6* transcription is altered during infection with *H. parasitica*, relative transcript levels were determined by Q-PCR. Arabidopsis Ler seedlings were sprayed with either a compatible (Cala2) or incompatible (Waco9) isolate, and *DMR6* transcript levels were measured at different time points after inoculation on three biological replicates. As shown in Figure 2, transcript levels increased in both the compatible and incompatible interactions at 1 day post inoculation (dpi), suggesting that *DMR6* activation is a general response to *H. parasitica* that is not compatibility specific. At the early time points (1–3 dpi) *DMR6* was more highly expressed in the incompatible interaction than in the compatible interaction, although this was only significant at 3 dpi. The early activation of *DMR6* suggests that it is activated as part of the defence response, which is in general more strongly activated in incompatible interactions. *DMR6* expression levels did not increase in the incompatible interaction after 3 dpi. This correlates well with the arrest of pathogen growth that occurs between day 1 and 3. In contrast, in the compatible interaction at 4–5 dpi, when the pathogen has colonized the leaf, the level of *DMR6* transcript was elevated by almost 40-fold, whereas in the incompatible interaction it was elevated by 20-fold.

To study the localization of *DMR6* expression during *H. parasitica* infection, transgenic lines were generated containing a construct with the *DMR6* promoter linked to the *uidA* (*GUS*) reporter gene ($Pro_{DMR6}$:*GUS*). In each of the backgrounds tested, Col-0, L*er*, L*er eds1-2* and *dmr6-1*, five independent $Pro_{DMR6}$:*GUS* lines were analysed. As the localization of *GUS* expression in Col-0 and L*er* was essentially the same as that observed in L*er eds1-2*, only

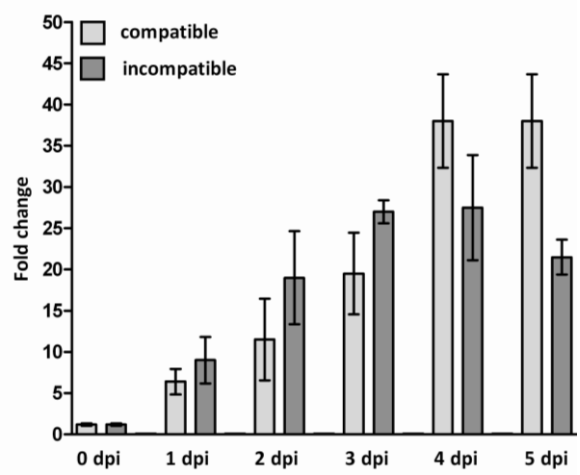


**Figure 2.** Transcript levels of *DMR6* after inoculation with a compatible (Cala2) or incompatible (Waco9) *Hyaloperonospora parasitica* isolate. Transcript levels were determined at different days post inoculation (dpi), as indicated. *DMR6* transcript levels were measured in three independent biological replicates. Bars represent the mean induction relative to mock treated plants, with error bars representing the standard deviation.

results from this latter line are shown. *H. parasitica* hyphae were stained with trypan blue, and GUS activity was visualized using magenta-Xgluc as a GUS substrate, resulting in a magenta precipitate. GUS activity was specifically detected in cells containing haustoria or directly surrounding the intercellular hyphae, in both the compatible and incompatible interaction (Figure 3A-F), indicating that *H. parasitica* induced *DMR6* expression is strictly localized to sites that are in direct contact with the pathogen. Infection sites were smaller, and the number of cells showing GUS activity was lower in the incompatible interaction compared with the compatible interaction at 2 dpi (Figure 3A,C). However, our Q-PCR data showed that the overall level of *DMR6* transcript is higher in the incompatible interaction at 2 dpi (Figure 2). As plants were inoculated with an equal dose of spores resulting in a similar number of infection sites, this suggests that the *DMR6* transcript level is relatively higher in incompatible infection sites at 2 dpi. Interestingly, GUS activity was higher in cells containing the first formed haustoria than in cells with newly or recently formed haustoria in the compatible interaction (Figure 3C,E), indicating that *DMR6* becomes activated following haustoria formation by the pathogen. This could also explain why *DMR6* transcript levels are higher in the compatible interaction at later stages of the infection (4–5 dpi), as more tissue is colonized by the pathogen. No difference was observed in the localization of *DMR6* expression in response to *H. parasitica* infection between L*er eds1-2* and *dmr6-1* plants (Figure 3A–F), although GUS activity was generally higher in the *dmr6-1* mutant background.

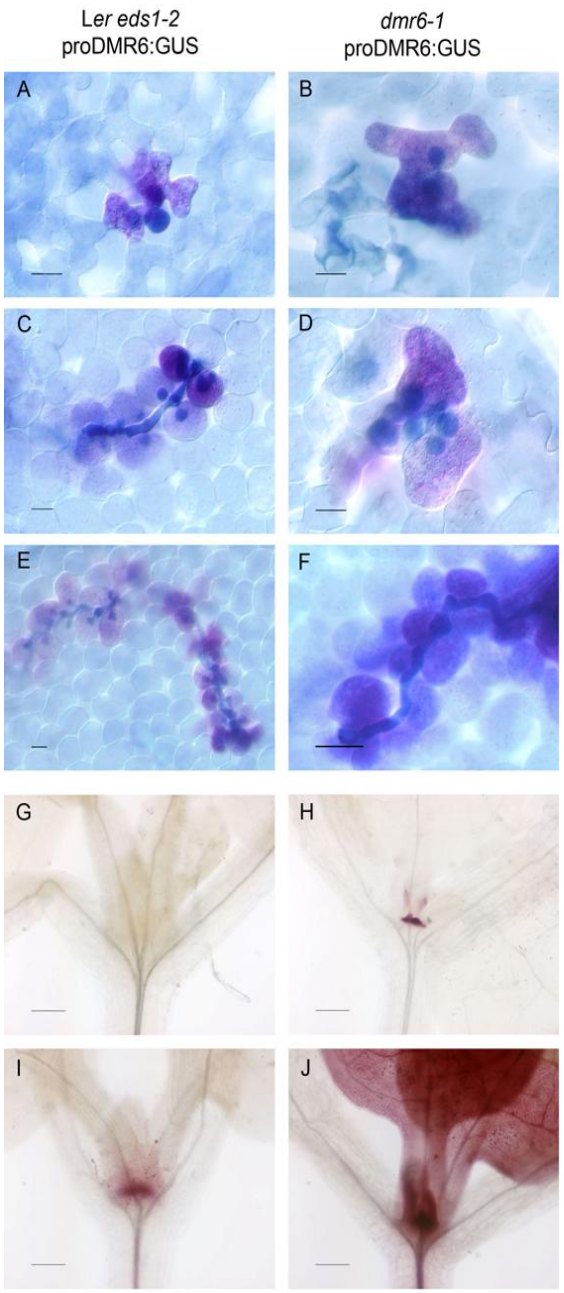


**Figure 3**. Analysis of a promoter *DMR6*-GUS fusion (ProDMR6:GUS) in transgenic L*er eds1-2* (left panels) and *dmr6-1* (right panels) seedlings. GUS activity was visualized with Magenta-X-gluc as substrate, and *Hyaloperonospora parasitica* growth was visualized with trypan blue staining. The scale bars in the upper six panels (**A-F**) correspond to 10 µm; scale bars in the lower panels correspond to 100 µm. (**A**) and (**B**), GUS activity in Arabidopsis cells that are in contact with the incompatible *H. parasitica* isolate Emoy2 in L*er eds1-2* and *dmr6-1* transgenic lines, respectively at 2 days post inoculation (2 dpi). (**C**) and (**E**), GUS activity in the infected cells of the L*er eds1-2* transgenic lines with the compatible *H. parasitica* isolate Cala2, at 2 and 3 dpi, respectively. (**D**) and (**F**), GUS activity of the *dmr6-1* transgenic lines with the compatible *H. parasitica* isolate Cala2, at 2 and 3 dpi respectively, is restricted to the infected cells. (**G**), no GUS activity is detected in L*er eds1-2* transgenic lines 3 days after mock treatment. (**I**), GUS activity is detected in the leaf primordia and shoot apical meristem in L*er eds1-2* transgenic seedlings 3 days after BTH treatment. (**H**), GUS activity in *dmr6-1*, 3 days after mock treatment, in the leaf primordial and shoot apical meristem. (**J**), GUS activity is present in the true leaves, in addition to the apical meristem and leaf primordia, in the *dmr6-1* transgenic lines 3 days after BTH treatment.

*DMR6 expression is defence-associated*

The elevated *DMR6* transcript levels during both compatible and incompatible *H. parasitica* interactions suggest that *DMR6* transcription becomes activated as part of a defence

response. As the activation of many defence genes is strongly impaired in plants unable to accumulate or respond to salicylic acid (SA), we tested *DMR6* levels in the *sid2* and *npr1* mutant. *sid2-1* (SA induction-deficient; Wildermuth et al., 2001) does not accumulate SA, whereas the *npr1-1* (non-expressor of *PR* genes) mutant (Cao et al., 1994) shows strongly impaired defence gene expression in response to SA. The induction of *DMR6* in response to a compatible (Waco9) or incompatible (Cala2) isolate was compared with wild-type Col-0 at 1 dpi in three independent biological replicates. Figure 4 shows that induction of *DMR6* is higher in the incompatible than in the compatible interaction, confirming the data from Figure 2. Interestingly, the difference in *DMR6* expression between compatible and incompatible is larger in Col-0 than in L*er*, which could be because of the difference in the *R* gene that mediates resistance: *RPP2* in Col-0 versus *RPP5* in L*er*. *DMR6* transcript levels were not significantly altered in the *sid2-1* and *npr1-1* mutants, as compared with Col-0 upon inoculation with the incompatible isolate Waco9 or compatible isolate Cala2. This indicates that SA accumulation and *NPR1* function are not important for the early transcriptional activation of *DMR6* in response to *H. parasitica* infection. The responsiveness of *DMR6* to SA or its analog BTH was tested on the L*er eds1-2* and *dmr6-1* reporter lines containing the $Pro_{DMR6}$*:GUS* construct. No GUS activity could be detected in untreated L*er eds1-2* plants (Figure 3G). Interestingly, untreated *dmr6-1* plants show GUS activity in the shoot apical meristem and leaf primordia (Figure 3H), indicating that *dmr6-1* mutants have constitutively enhanced *DMR6* transcription in these tissues. After BTH treatment, GUS activity was also detected in L*er eds1-2*, and was primarily localized in the same tissues (the leaf primordia and shoot apical meristem) as in untreated *dmr6-1* mutants (Figure 3I), indicating that *DMR6* expression in these tissues is particularly sensitive to BTH. *dmr6-1* shows an increase in GUS activity upon BTH treatment, with the GUS activity no longer localized strictly to the shoot apical meristem but also detected in true leafs (Figure 3J), indicating that in the *dmr6-1* mutant *DMR6* expression is more sensitive to BTH.

To further investigate the correlation between the transcriptional activation of *DMR6* and plant defence, *DMR6* expression levels were analysed in the *dmr3*, *dmr4* and *dmr5* mutants (Chapter 2; Van Damme et al., 2005), which have constitutively activated defence responses in the absence of pathogen infection. In all three dmr mutants high basal levels of *DMR6* expression were observed compared with the parental line L*er eds1-2* (Figure 4B). This again supports the idea that expression of *DMR6* is associated with plant defence.

*DMR6* expression was highest in *dmr3* (approximately 97-fold induction) and *dmr4* (approximately 56-fold induction), and was somewhat lower in *dmr5* (approximately 34-fold induction). The combined data show that *DMR6* expression is activated during various defence responses that are either pathogen- or chemical-induced.

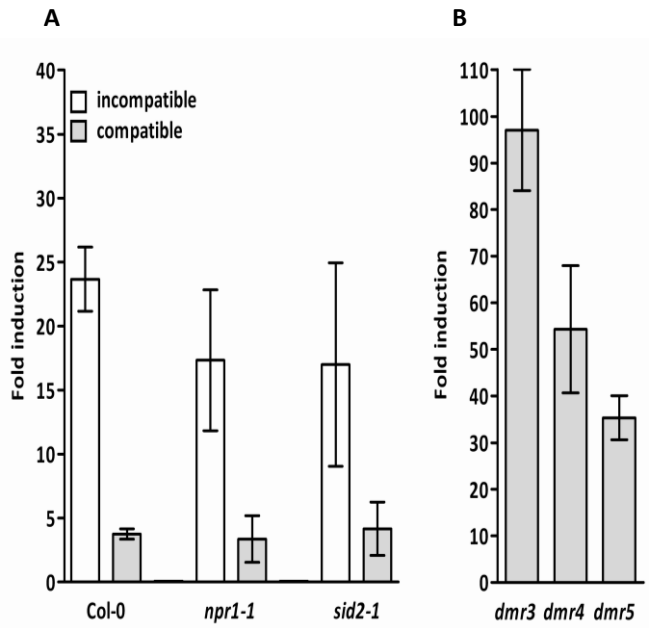


**Figure 4**. Transcript levels of *DMR6* in defence regulatory and constitutive defence mutants. (**A**), fold induction of *DMR6* transcript levels in *npr1-1* and *sid2-1*, inoculated with either a compatible or incompatible isolate, relative to Col-0. (**B**), transcript levels of *DMR6* in the constitutive defence mutants *dmr3*, *dmr4* and *dmr5*, relative to the parental line L*er* *eds1-2*. *DMR6* transcript levels were measured in three independent biological replicates. Bars represent the mean fold change compared with the parental lines, with error bars representing the standard deviation.

*DMR6 negatively affects expression of defence-associated genes*

To investigate whether *dmr6* mutants have altered expression of more genes than *DMR6* itself, changes in gene expression between *dmr6-1* and its parental line were analyzed using DNA microarrays. Probes were synthesized from RNA extracted from the above-ground parts of healthy 14-day-old seedlings and were hybridized on 25k CATMA arrays (Allemeersch et al., 2005; Hilson et al., 2004). A total of 59 different CATMA probes were identified that showed significant differences in hybridization ($P < 0.05$, fold change > 2) between *dmr6-1* and the parental line. The 59 CATMA probes corresponded to 57 unique AGI-IDs, of which 50 (including *DMR6*) showed an increased transcript level and seven showed a reduced transcript level in *dmr6-1* compared with L*er* *eds1-2* (Tables S1 and S2). Several genes that show induced expression in *dmr6-1* have previously been described to be associated with plant defence e.g. *ACD6* (Lu et al., 2005), *PR-4* (Potter et al., 1993) and *PR-5* (Uknes et al.,

1992). As the samples used for array hybridization were derived from a single biological replicate, strong conclusions cannot be drawn from this experiment. In order to obtain statistically sound data, a selection of genes, including the defence-associated genes *ACD6*, *PR-1*, *PR-2*, *PR-4* and *PR-5*, were verified by Q-PCR on three biological replicates. Expression levels were measured in the *dmr6-1* mutant, the *dmr6-2* mutant and three *dmr6-1* complementation lines (data is shown for one representative line #122). All genes tested were more highly expressed in both *dmr6-1* and *dmr6-2* compared with the parental lines (Figure 5A, B). Moreover, the complemented *dmr6-1* mutant (with the *35S::DMR6* construct) showed significantly lower expression of defence-associated genes than the mutants, demonstrating that the loss of a functional *DMR6* gene is responsible for the elevated expression levels of a number of defence-associated genes. It is unclear why the defence gene expression levels are still higher in the complemented mutant than in the parental line. The difference in defence gene expression between *dmr6-2* compared with Ws-4 is larger then the difference between *dmr6-1* compared with L*er eds1-2*, and could be the result of differences in the genetic background of the two *dmr6* mutants. The enhanced expression of *DMR6* in *dmr6-1* (Figure 3H) was also detected in the microarray experiment and confirmed by Q-PCR (Figure 5C). Interestingly, *DMR6* transcript levels in *dmr6-1* (approximately 11-fold induction) were not as high as in *dmr3*, *dmr4* and *dmr5* (97-, 56- and 34-fold induction, respectively; Figure 4B). The transcript data clearly indicate that *DMR6* has a negative effect on the expression of the subset of the defence-associated genes tested.

**Discussion**

The *DMR6* locus was identified in a genetic screen for loss of susceptibility to the downy mildew pathogen *H. parasitica* (Chapter 2; Van Damme et al., 2005). Map-based cloning identified *DMR6* as *At5g24530*, which encodes an oxidoreductase, for which no biological function has been described. Overexpression of *At5g24530* restored susceptibility to *H. parasitica* in the *dmr6-1* mutant, thereby confirming that this gene is required for susceptibility to *H. parasitica*. An additional allele, *dmr6-2*, was identified in the line FLAG 445D09 (Samson et al., 2002) that has a T-DNA insertion in the *At5g24530* gene. Both the *dmr6-1* and *dmr6-2* mutants exhibit reduced susceptibility to *H. parasitica*.

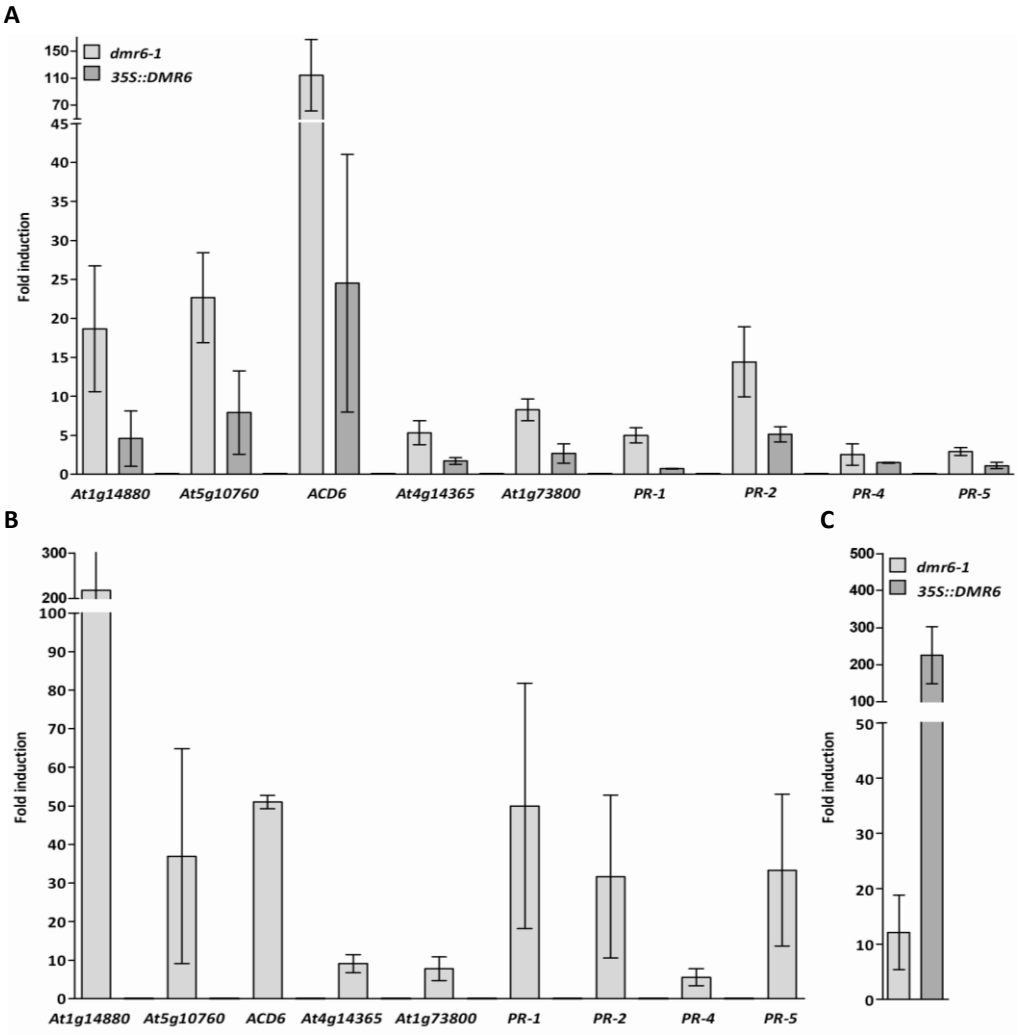


**Figure 5**. Relative transcript levels of 10 defence-associated genes in *dmr6-1* and *dmr6-2*. Gene IDs are as indicated. (**A**), transcript levels of nine defence-associated genes in *dmr6-1* and *dmr6-1* complemented with a *35S::DMR6* construct, relative to the parental line L*er eds1-2*. (**B**), transcript levels of nine defence-associated genes in *dmr6-2* relative to the parental line Ws-4. (**C**), transcript levels of *DMR6* in *dmr6-1* and *dmr6-1* complemented with a *35S::DMR6* construct relative to L*er eds1-2*. Gene transcript levels were measured in three independent biological replicates. Bars represent the mean fold change relative to the parental lines, with error bars representing the standard deviation.

The DMR6 protein is a member of the 2OG-Fe(II) oxygenase superfamily of oxidoreductases. The catalytic domain, as defined by Pfam PF03171, is located from amino acid 188 to 288 in DMR6, and contains the three Fe(II) ion-binding residues (His212, Asp214 and His269) (Roach et al., 1995). Molecular oxygen is reduced at the Fe(II) ion, where it reacts with 2- oxoglutarate and a specific substrate through the incorporation of one atom of

oxygen in each compound. The 2OG-Fe(II) oxygenases are widespread in bacteria and eukaryotes (Aravind and Koonin, 2001). In plants, these enzymes catalyze different hydroxylation and desaturation steps. Examples are the gibberellin 20-oxidases, gibberellin 3b-hydroxylases and gibberellin 2-oxidases in the biosynthesis of gibberellins (Hedden and Phillips, 2000), ACC oxidase in the final step in ethylene synthesis (Wang et al., 2002), and flavanone 3b-hydroxylase (F3H) in the biosynthesis of flavanoids, cathechins and anthocyanidins (Lukacin and Britsch, 1997).

Expression analysis by Q-PCR and promoter GUS lines demonstrated that *DMR6* is strongly induced during the interaction with both compatible and incompatible *H. parasitica* isolates. The activation of *DMR6* is locally induced within cells that are in direct contact with the pathogen. Induction of *DMR6* at early stages of infection (1 dpi), in both compatible and incompatible interactions, was independent of SA and *NPR1* function. However, under other conditions, SA and *NPR1* were found to be important for *DMR6* expression, as observed in the DNA microarray data by Mosher et al. (2006), in which *DMR6* expression was measured as being derepressed in *sni1* (suppressor of *npr1*, inducible), as well as being BTH non responsive in the *npr1* mutant. As SNI1 is thought to function as a negative regulator of systemic acquired resistance (SAR), *DMR6* expression can be considered as being SAR induced. This is supported by the fact that *DMR6* expression is induced by BTH, as shown in the $Pro_{DMR6}$:*GUS* lines. *DMR6* is induced to higher levels in incompatible than in compatible interactions with *H. parasitica* during early time points post inoculation. In general, defence responses are more strongly induced during incompatible interactions, leading to higher transcript levels of defence genes that are often dependent on the accumulation of SA (Cao et al., 1994; Lamb et al., 1992; Tao et al., 2003). High basal expression levels of *DMR6* in the constitutive defence mutants, *dmr3*, *dmr4* and *dmr5*, in the absence of pathogen infection support the link between *DMR6* transcription and plant defence. Interestingly, an ortholog of *DMR6* in wheat, which was described as an *F3H* gene, showed a similar pattern of expression during compatible and incompatible interactions with the Hessian fly (Giovanini et al., 2006), suggesting that the monocot *DMR6* ortholog has a similar function.

It is unlikely that *DMR6* has direct antimicrobial activity or has a role in the biosynthesis of antimicrobial compounds, as plants that lack a functional *DMR6* gene have reduced susceptibility. Moreover, overexpression of *DMR6* in the *dmr6-1* mutant restores susceptibility. The constitutive activation of defence-associated genes including *PR*- genes in

*dmr6-1* and *dmr6-2* strongly suggests that *DMR6* negatively affects the expression of these genes. The enhanced expression of defence-associated genes could be responsible for the observed *dmr6*-mediated resistance. The question remains why the *dmr6-1* mutant is only resistant to *H. parasitica* and *Colletotrichum higginsianum* (O'Connell and Panstruga, 2006), and not to *Pseudomonas syringae*, *Golovinomyces orontii* (Chapter 2; 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 *dmr6* mutants to be resistant to a broad range of pathogen species. However, many constitutive defence mutants that show this type of broad-range resistance show dwarfism, e.g. *dmr3* and *dmr4* (Chapter 2; Van Damme et al., 2005). *dmr6* mutants do not show dwarfism and this could indicate that constitutive defence activation is less strong. This is supported by the fact that a number of defence genes are expressed to a much higher level in *dmr3* and *dmr4* than in *dmr6*. Possibly, the subset of defence genes and their level of expression in the *dmr6* mutants only provide protection against *H. parasitica* and *C. higginsianum*. The mechanism of upregulation of this subset of genes through mutation of *DMR6* is unknown.

In the *dmr6* mutants, the substrate of the putative *DMR6* encoded 2OG-Fe(II) oxygenase is expected to accumulate, and could either be directly toxic to the pathogen or act indirectly by stimulating the expression of host defence genes. Possibly, the accumulated substrate in the *dmr6* mutant triggers and enhances the expression of *DMR6*. Also, in wild-type plants, in reponse to *H. parasitica* infection or BTH treatment, DMR6 substrate levels could be increased, resulting in the transcriptional activation of *DMR6* as part of the plant defence response. In addition, this would explain the high sensitivity of *dmr6-1* mutants to BTH-induced *DMR6* expression, as *dmr6* mutants may be unable to metabolize the BTH-induced substrate, resulting in enhanced activation of *DMR6*. In this scenario, the function of *DMR6* would be to control DMR6 substrate levels during plant defence, thereby acting as a negative regulator. Alternatively, it could be that the product of the DMR6 enzyme is either negatively regulating defence-associated gene expression or positively affecting susceptibility to *H. parasitica*. However, it is difficult to understand why plants would activate *DMR6* transcription during defence if the product of the DMR6 enzyme or DMR6 itself would be beneficial for the pathogen. In this scenario, plants would only maintain *DMR6* if it had an evolutionary advantage, e.g. an important role in plant defence. However, this is unlikely as *dmr6* mutants are not impaired in basal, *R* gene-mediated or BTH-induced resistance.

Genetic studies using defence regulatory mutants will reveal whether the enhanced defence gene expression is an essential component of *dmr6*-mediated resistance, or a secondary effect. Future studies aimed at identifying the DMR6 substrate and product, as well as genetic suppressor studies, will increase our understanding of the role of the *DMR6*-encoded 2OG-Fe(II) oxygenase in both defence and susceptibility to downy mildew.

**Experimental procedures**

*Plant growth conditions and H. parasitica inoculations*

Plants were grown on potting soil in a growth chamber (Snijders, http://www.snijders tilburg.nl) at 22 ˚C with 16 h of light (100 µE $m^{-2}$ $sec^{-1}$) and a relative humidity of 75% before inoculation. *Hyaloperonospora parasitica* isolates Waco9 (kindly provided by Dr M. Aarts, WUR, http://www.wur.nl) and Cala2 (kindly provided by Dr E. Holub, Warwick HRI, http://www2.warwick.ac.uk/fac/sci/13 whri) were maintained on Arabidopsis accessions Ws-0 and L*er*, respectively. Inocula (4 · 105 spores ml)1) were transferred weekly to 10-day-old healthy seedlings (Holub et al., 1994) by use of a spray gun. Seedlings were air-dried for approximately 45 min and incubated under a sealed lid at 100% relative humidity in a growth chamber at 16˚C with 9 h of light per day (100 µE $m^{-2}$ $sec^{-1}$). Sporulation levels were quantified 6 dpi by counting the number of conidiophores per seedling (n >40).

*dmr6 mutant backgrounds*

*dmr6-1* was identified previously and crossed to the Col-0 *rpp2* mutant *FN2* (Sinapidou et al., 2004) to create a mapping population (Chapter 2; Van Damme et al., 2005). For all other experiments, a *dmr6-1* line was used that was back-crossed twice to the parental line L*er eds1-2* (Parker et al., 1996). *dmr6-2* was identified among the segregating offspring of the T-DNA insertion line FLAG 445D09 in the Ws-4 accession (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 borders, in combination with the gene-specific primers LP or RP (5'-ATGTCCAAGTCCAATAGCCACAAG-3').

*Map-based cloning*

INDEL- and CAPs-based markers were used for fine mapping. Primers were designed around the flanking sites of known insertion and deletion polymorphisms between Col-0 and L*er*, as obtained from the Cereon database administered by Monsanto (Jander et al., 2002). The *dmr6-1* interval mapped between the INDEL-marker IND_K16H17 within gene *At5g24420* (forward primer 5'-TGGGGTTGTGGTTTATTCTGTTGAC-3' and reverse primer 5'-TGGCCAATAGTAGTTGATACGCAAGA-3') and CAPs-marker CAP_At5g24590 (forward primer 5'-GCATCATTTGTACCGTACTGAGTC-3' and reverse primer 5'-TAGTGGATACTCTGTCCCTGAGGT-3', restriction enzyme *PdmI*). Candidate genes were sequenced to identify the *dmr6-1* mutation.

Complementation lines

The *At5g24530* coding sequence of Col-0 was PCR-amplified with primers that included restriction sites that were used for directional cloning; a forward primer (5'-TTCTGGGATCCAATGGCGGCAAAGCTGATATC-3') containing a *Bam*HI restriction site near the start codon (ATG), and a reverse primer (5'-GATATATGAATTCTTAGTTGTTTAGAAAATTCTCGAGGC-3') containing an *Eco*RI site after the stop codon (TTA). The fragment was cloned directionally between the 35S promoter and the Nos terminator, and the 35S-DMR6-Tn fragment was cloned into the pGreenII0229 (Hellens et al., 2000). Complementation lines were generated by transforming *dmr6-1* plants by the floral-dip method with the *Agrobacterium tumefaciens* strain C58 (Clough and Bent, 1998). D,L-Phosphinothricin-resistant (BASTA, 300 µM) seedlings were selected and analyzed for overexpression of *DMR6* and *H. parasitica* susceptibility.

*Promoter DMR6:GUS transgenic lines*

A 2.5-kb fragment of the *DMR6* promoter was amplified using proDMR6F (5'-GACTCTGTCTGAGTCTGCAGTCCCAAACCATG-3') and proDMR6R (5'-GCCGCCATTGGATCCCAGAAAATTGAAGAAG-3'), generating *Pst*I and *Bam*HI restriction sites, respectively. The two restriction sites allowed cloning of the fragment into the pGREENII0229G plasmid (Hellens et al., 2000) in front of the *GUS* gene. $Pro_{DMR6}$*:GUS* transgenic lines were generated by transforming Ler eds1-2 and dmr6-1 plants by the floral-dip method (Clough and Bent, 1998) with the *A. tumefaciens* C58 strain. $Pro_{DMR6}$*:GUS*

transgenic (T3) seedlings were infected with *H. parasitica* and at 3 dpi the seedlings were vacuum infiltrated with Magenta-Xgluc solution [50 mM NaPO4 (pH 7.0), 0.5 mM K3FE(CN)6, 0.5 mM K4FE(CN)6, 0.1% Triton X-100, 0.5 mg ml)1 Magenta-GlcA; Duchefa, http://www.duchefa.com] and stained overnight at 37˚C. Seedlings were cleared with 70% EtOH. *H. parasitica* was stained in lactophenol (1:1:1:1 volume of lactic acid/glycerol/phenol/$H_2O$) containing 1 mg $mL^{-1}$ trypan blue, by boiling for 1–2 min and destaining overnight in chloral hydrate. Trapped air bubbles were removed by 1 min of speed vacuum infiltration. *Hyaloperonospora parasitica* growth and *GUS* expression was visualized by interference contrast microscopy.

*DNA microarray and Q-PCR analysis*

Total RNA was extracted with an RNeasy kit (Qiagen, http://www.qiagen.com) and treated with the RNase-free DNase set (Qiagen). RNA was quantified using a UVmini-1240 spectrophotometer (Shimadzu, http://www.shimadzu.com). All procedures regarding the microarray analysis are detailed in Appendix S1. For Q-PCR, cDNA was synthesized with SuperScript-III reverse transcriptase (Invitrogen, http://www.invitrogen.com) and oligo(dT)15 (Promega, http://www.promega.com) from total RNA. Cycle thresholds were determined per transcript in triplicate in multiple biological replicates using the ABI PRISM 7700 sequence detection system (Applied Biosystems, http://www.appliedbiosystems.com) using SYBR Green I (Applied Biosystems) as the reporter dye. The data are normalized using Arabidopsis *ACT2* levels (*At3g18780*). The used primer sets for the transcripts generating 99–101 base-pair fragments are listed in Table S3.

**Acknowledgements**

We thank F. Kindt and R. Leito for artwork, P.J. Weisbeek for his support and for critically reading the manuscript, and E. Holub for testing *Albugo candida* on the *dmr* mutants. This research was initiated with a fellowship of the Royal Netherlands Academy of Arts and Sciences to G. Van den Ackerveken.

**Supplementary Material**

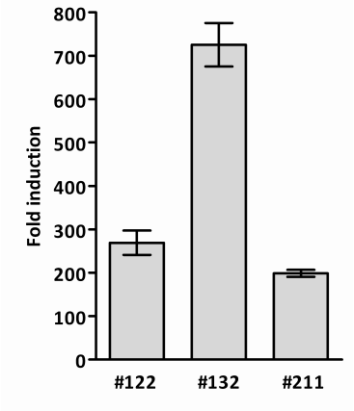


**Figure S1**. Transcript levels of *DMR6* in three independent complementation lines: #122, #132 and #211.

**Table S1**. Arabidopsis transcripts that are more than twofold induced in the *dmr6-1* mutant compared with L*er eds1-2* (P < 0.05).

| AGI code | CATMA ID | Log ratio | p-value | Annotation |
|---|---|---|---|---|
| At1g14880 | 1a13910 | 4.044 | 0.001 | expressed protein, similar to PGPS/D12 (Petunia x hybrida) GI:4105794 |
| At1g31580 | 1a29810 | 3.652 | 0.001 | expressed protein, identical to ORF1 (Arabidopsis thaliana) gi:457716:emb:CAA50905 |
| At5g10760 | 5a09500 | 3.132 | 0.001 | aspartyl protease family protein |
| At3g48640 | 3a41600 | 2.91 | 0.003 | hypothetical protein |
| At2g25510 | 2a23850 | 2.85 | 0.001 | expressed protein |
| At5g45380 | 5a41330 | 2.696 | 0.008 | sodium:solute symporter family protein |
| At4g14400 | 4a14770 | 2.686 | 0.002 | ACD6, ankyrin repeat family protein |
| At1g35710 | 1a33890 | 2.681 | 0.003 | leucine-rich repeat transmembrane protein kinase |
| | 4a06910 | 2.384 | 0.004 | |
| At5g54610 | 5a50460 | 2.123 | 0.004 | Induced in response to salicylic acid,belongs to the ankyrin repeat protein family. |
| At2g41090 | 2a39435 | 2.12 | 0.001 | calmodulin-like calcium-binding protein, 22 kDa (CaBP-22) |
| At5g24530 | 5a22130 | 1.91 | 0.003 | oxidoreductase, 2OG-Fe(II) oxygenase family protein, DMR6 |
| 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 |

| AGI code | CATMA ID | Log ratio | p-value | Annotation |
|---|---|---|---|---|
| At3g23120 | 3a23080 | 1.603 | 0.004 | leucine-rich repeat family protein, contains leucine rich-repeat (LRR) domains |
| At1g32750 | 1a31080 | 1.597 | 0.006 | HAC13 protein (HAC13), identical to HAC13 (Arabidopsis thaliana) |
| At1g75040 | 1a64376 | 1.557 | 0.006 | 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 | hevein-like protein (HEL) Encodes a protein similar to the antifungal chitin-binding protein hevein latex (PR-4) |
| At3g04210 | 3a03160 | 1.452 | 0.005 | disease resistance protein (TIR-NBS class) |
| At1g69730 | 1a59040 | 1.446 | 0.007 | WAK-like kinase (WLK) |
| At1g03850 | 1a02710 | 1.401 | 0.03 | 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 |

**Table S2**. Arabidopsis transcripts that are more than twofold repressed in the *dmr6-1* mutant compared with L*er eds1-2* ($P < 0.05$).

| AGI code | CATMA ID | Log ratio | p-value | Annotation |
|---|---|---|---|---|
| At2g35200 | 2a33340 | -1.374 | 0.02 | hypothetical protein |
| At5g15260 | 5a13540 | -1.301 | 0.007 | genomic putative protein |
| At5g10430 | 5a09183 | -1.301 | 0.015 | arabinogalactan-protein (AGP4) |
| At3g25190 | 3a25040 | -1.247 | 0.02 | 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 | -1.01 | 0.025 | putative glycine-rich protein |

**Table S3**. Primers used for Q-PCR analysis of *dmr6*-affected gene expression.

| ***Gene/Transcript*** | **Primer name** | **Primer sequence** |
|---|---|---|
| *DMR6* | QDMR6F | 5'-TGTCATCAACATAGGTGACCAG-3' |
| | QDMR6R | 5'-CGATAGTCACGGATTTTCTGTG-3' |
| *At1g14880* | QAt1g14880F | 5'-CTCAAGGAGAATGGTCCACA-3' |
| | QAt1g14880R | 5'-CGACTTGGCCAAATGTGATA-3' |
| *At5g10760* | QAt5g10760F | 5'-GGAGCAAAACCCGACTCGT-3' |
| | QAt5g10760R | 5'-TGCAGGGAATGATGACCTT-3' |
| *At1g73800* | QAt1g73800F | 5'-ACCCTTCGTTTACTATCTCCAA-3' |
| | QAt1g73800R | 5'-CGAGCCACTACCAAAACATC-3' |
| *At4g14365* | QAt4g14365F | 5'TGGTTTTCTGAGGCATGTAAA-3' |
| | QAt4g14365R | 5'-AGTGCAGGAACATTGGTTGT-3' |
| *ACD6* | QACD6F | 5'-TGGACAGTTCTGGAGCAGAT-3' |
| | QACD6R | 5'-CAACTCCTCCGCTGTGAG-3' |
| *PR-4* | QPR-4F | 5'-TTGCTGCATTGGTCCACTAT-3' |
| | QPR-4R | 5'-AGGACCTCGTGGTCAAGC-3' |
| *PR-5* | QPR-5F | 5'-GGCAAATATCTCCAGTATTCACA-3' |
| | QPR-5R | 5'-GGTAGGGCAATTGTTCCTTAGA-3' |
| *PR-2* | QPR-2F | 5'-CCCGTAGCATACTCCGATTT-3' |
| | QPR-2R | 5'-AAGGAGCTTAGCCTCACCAC-3' |
| *PR-1* | QPR-1F | 5'-GAACACGTGCAATGGAGTTT-3' |
| | QPR-1R | 5'-GGTTCCACCATTGTTACACCT-3' |
| *ACT-2* | QACT2F | 5'-AATCACAGCACTTGCACCA-3' |
| | QACT2R | 5'- GAGGGAAGCAAGAATGGAAC-3' |

**Appendix S1**. Supplemental experimental procedures regarding CATMA arrays.

*CATMA arrays*

Microarray analysis was performed with CATMA version 2 arrays (complete Arabidopsis transcriptome microarray; Allemeersch et al., 2005; Hilson et al., 2004). Arrays were spotted with 24411 gene-specific tags (GSTs). The GSTs are between 150 and 500 bp in length and show less then 70% identity with any other sequence in the genome. GSTs were spotted on GAPSII glass slides (Corning Incorporated, Acton, MA, USA) using a BioRobotics Microgrid II TAS spotter (Genomics Solutions, Ann Arbor, MI, USA) and cross-linked for 4 hours at 80˚C. Detailed information about CATMA and database access can be found at http://www.catma.org/ (Crowe et al., 2003) and http://genomics.bio.uu.nl/.

*Labelling, hybridization and scanning*

mRNA from total RNA extracts from L*er eds1-2* and *dmr6-1* were amplified with the MessageAmp aRNA kit (Ambion). Amplified RNA (5 µg) was used as template to synthesize modified cDNA, by incorporation of 5-(3-aminoallyl)-dUTP (Ambion; ratio dUTP/dTPP of 7/3) with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random nonamers (Gene Link, Westchester County, NY, USA) for 2 hours at 42˚C. RNA template was removed by hydrolysis using 3 µl 2.5 M NaOH per 30 µl reaction volume for 15 min at 37˚C. Hydrolysis was stopped by adding 15 µl 2 M MOPS buffer per 30 µl reaction volume. Modified cDNA was purified using the MineLute PCR purification kit (Qiagen) and labeled with Cy3 or Cy5 mono-reactive dye (Amersham, Buckinghamshire, UK). Modified cDNA derived from L*er eds1-2* and *dmr6-1* RNA was labeled twice with Cy3 and twice with Cy5. The reaction was quenched after 60 min using 4.5 µl 4 M hydroxylamine (Sigma-Aldrich) and incubated in the dark for 15 min. Labeled cDNA was purified using the MineLute PCR purification kit (Qiagen) and incorporation of Cy3 or Cy5 was determined using a UVmini-1240 spectrophotometer at 550 or 650 nm, respectively.

CATMA arrays were denatured in boiling demineralized water for 3 min and dipped in ethanol afterwards. Slides were spun dry for 3.5 min at 300 g in 50 ml tubes, covered with a LifterSlip (Erie Scientific Company, Portsmouth, NH, USA) and subsequently prehybridized with 100 µl filtered prehybridization solution containing 25% formamide (Merck, Whitehouse Station, NJ, USA), 5x SSC, 0.1% SDS and 1% BSA, fraction V, minimum 96% (Sigma-Aldrich) for 15 min at 55˚C. Microarrays were dipped 5 times in demineralized water and once in isopropanol and spun dry for 3.5 min at 300 g in 50 ml tubes and subsequently covered with a LifterSlip. For each hybridization, 50 µl filtered (0.2 lm) 2x hybridization mix was made, containing 50% deionized formamide (Sigma-Aldrich), 10x SSC and 0.2% SDS. Herring-sperm DNA (1 µl of 11 mg/ml stock; Sigma-Aldrich) was added, and the mix was heated to 42 ˚C to prevent precipitation of SDS. A fixed amount of 1450 ng cDNA was used for all hybridizations, corresponding to an incorporation of Cy3 or Cy5 between 259 and 590 pmol. Each pair of labeled cDNA (2 times L*er eds1-2* labelled with Cy3 and *dmr6-1* labelled with Cy5 and 2 times vica versa) was concentrated in a Speed Vac (type SC100; Savant Instruments, Hollbrook, NY, USA) to a volume of 50 µl and added to 50 µl 2x hybridization mix. The probes were denatured for 5 min at 95˚C and centrifuged for 2 min at 15000g in a standard tabletop centrifuge and immediately applied to the arrays. The arrays were put in hybridization

chambers (Corning Incorporated) containing one drop of 20 µl water on each side, covered by foil, and placed for 16–20 h at 42_C in a water bath. After the hybridization, the arrays were washed twice in a lowstringency wash solution containing 1x SSC, 0.2% SDS and 0.1 mM DTT for 4 min at 55_C. The arrays were subsequently washed in a high-stringency wash solution containing 0.1x SSC, 0.2% SDS and 0.1 mM DTT for 4 min at 55_C and in a final wash solution containing 0.1x SSC and 0.1 mM DTT each for 4 min at room temperature. The slides were dipped five times in demineralized water and immediately submerged in isopropanol. The slides were spun dry as described above and scans of the arrays were made using a ScanArray Express HT (PerkinElmer, Wellesley, MA, USA). Spot intensities of the scans were determined by ImaGene software version 6.5.1 (BioDiscovery, El Segundo, CA, USA).

*Statistics*

Spot intensities from the 4 CATMA arrays were analysed by LIMMA (Smyth, 2004) version 1.7.2 and limmaGUI version 1.2.5 (Wettenhall and Smyth, 2004) software packages from Bioconductor (Gentleman et al., 2004) running in R version 1.9.1 (CR Foundation, Vienna, Austria; Ihaka and Gentleman, 1996). The intensities were normalized by the print tip LOESS to correct for possible within array, dye and print-tip effects. Subsequently, all arrays were normalized between arrays by scaling to obtain the same median absolute deviation for each array, thereby enhancing the comparison between them (Smyth and Speed, 2003). A linear statistical model was created to estimate the log2(*dmr6-1*/L*er eds1-2*) effect and a moderated ttest (empirical Bayes) was performed to determine differential transcripts between L*er eds1-2* and *dmr6*.

## References

**Allemeersch, J., Durinck, S., Vanderhaeghen, R., Alard, P., Maes, R., Seeuws, K., Bogaert, T., Coddens, K., Deschouwer, K., Van Hummelen. P., Vuylsteke, M., Moreau, Y., Kwekkeboom, J., Wijfjes, A.H., May, S., Beynon, J., Hilson, P. and Kuiper, M.T.** (2005). Benchmarking the CATMA microarray. A novel tool for Arabidopsis transcriptome analysis. Plant Physiol. 137, 588-601.

**Aravind, L. and Koonin, E.V.** (2001). The DNA-repair protein AlkB, EGL-9, and leprecan define new families of 2-oxoglutarate- and iron-dependent dioxygenases. Genome Biol. 2, RESEARCH0007.

**Buschges, R., Hollricher, K., Panstruga, R., Simons, G., Wolter, M., Frijters, A., van Daelen, R., van der Lee, T., Diergaarde, P., Groenendijk, J., Topsch, S., Vos, P., Salamini, F. and Schulze-Lefert, P.** (1997). The barley Mlo gene: a novel control element of plant pathogen resistance. Cell. 88, 695-705.

**Cao, H., Bowling, S.A., Gordon, A.S. and Dong, X.** (1994). Characterization of an Arabidopsis Mutant That Is Nonresponsive to Inducers of Systemic Acquired Resistance. Plant Cell. 6, 1583-92.

**Clough, S.J. and Bent, A.F.** (1998). Floral dip: a simplified method for Agrobacterium-mediated transformation of Arabidopsis thaliana. Plant J. 16, 735-43.

**Consonni, C., Humphry, M.E., Hartmann, H.A., Livaja, M., Durner, J., Westphal, L., Vogel, J., Lipka, V., Kemmerling, B., Schulze-Lefert, P., Somerville, S.C. and Panstruga, R.** (2006). Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. Nat. Genet. 38, 716-20.

**Crowe, M.L., Serizet, C., Thareau, V., Aubourg, S., Rouze, P., Hilson, P., Beynon, J., Weisbeek, P., van Hummelen, P., Reymond, P., Paz-Ares, J., Nietfeld, W. and Trick, M.** (2003). CATMA: a complete Arabidopsis GST database. Nucleic Acids Res. 31, 156-8.

**de Jong, M., van Breukelen, B., Wittink, F.R., Menke, F.L., Weisbeek, P.J. and Van den Ackerveken, G.** (2006). Membrane-associated transcripts in Arabidopsis; their isolation and characterization by DNA microarray analysis and bioinformatics. Plant J. 46, 708-21.

**Giovanini, M.P., Puthoff, D.P., Nemacheck, J.A., Mittapalli, O., Saltzmann, K.D., Ohm, H.W., Shukle, R.H. and Williams, C.E.** (2006). Gene-for-gene defence of wheat against the Hessian fly lacks a classical oxidative burst. Mol. Plant-Microbe Interact. 19, 1023-33.

**Hedden, P. and Phillips, A.L.** (2000). Gibberellin metabolism: new insights revealed by the genes. Trends Plant Sci. 5, 523-30.

**Hellens, R.P., Edwards, E.A., Leyland, N.R., Bean, S. and Mullineaux, P.M.** (2000). pGreen: a versatile and flexible binary Ti vector for Agrobacterium-mediated plant transformation. Plant Mol. Biol. 42, 819-32.

**Hilson, P., Allemeersch, J., Altmann, T., Aubourg, S., Avon, A., Beynon, J., Bhalerao, R.P., Bitton, F., Caboche, M., Cannoot, B., Chardakov, V., Cognet-Holliger, C., Colot, V., Crowe, M., Darimont, C., Durinck, S., Eickhoff, H., de Longevialle, A.F., Farmer, E.E., Grant, M., Kuiper, M.T., Lehrach, H., Leon, C., Leyva, A., Lundeberg, J., Lurin, C., Moreau, Y., Nietfeld, W., Paz-Ares, J., Reymond, P., Rouze, P., Sandberg, G., Segura, M.D., Serizet, C., Tabrett, A., Taconnat, L., Thareau, V., Van Hummelen, P., Vercruysse, S., Vuylsteke, M., Weingartner, M., Weisbeek, P.J., Wirta, V., Wittink, F.R., Zabeau, M. and Small, I.** (2004). Versatile gene-specific sequence tags for Arabidopsis functional genomics: transcript profiling and reverse genetics applications. Genome Res. 14, 2176-89.

**Holub, E.B., Beynon, J.L. and Crute, I.R.** (1994). Phenotypic and genotypic characterization of interactions between isolates of Peronospora parasitica and accessions of Arabidopsis thaliana. Mol. Plant-Microbe Interact. 7, 223-39.

**Jander, G., Norris, S.R., Rounsley, S.D., Bush, .F., Levin, I.M. and Last, R.L.** (2002). Arabidopsis map-based cloning in the post-genome era. Plant Physiol. 129, 440-50.

**Koch, E. and Slusarenko, A.** (1990). Arabidopsis is susceptible to infection by a downy mildew fungus. Plant Cell. 2, 437-45.

**Lamb, C.J., Ryals, J.A., Ward, E.R. and Dixon, R.A.** (1992). Emerging strategies for enhancing crop resistance to microbial pathogens. Biotechnology (N. Y.). 10, 1436-45.

**Lu, H., Liu, Y. and Greenberg, J.T.** (2005). Structure-function analysis of the plasma membrane- localized Arabidopsis defence component ACD6. Plant J. 44, 798-809.

**Lukacin, R. and Britsch, L.** (1997). Identification of strictly conserved histidine and arginine residues as part of the active site in Petunia hybrida flavanone 3beta-hydroxylase. Eur. J. Biochem. 249, 748-57.

**Mosher, R.A., Durrant, W.E., Wang, D., Song, J. and Dong, X.** (2006). A comprehensive structure-function analysis of Arabidopsis SNI1 defines essential regions and transcriptional repressor activity. Plant Cell. 18, 1750-65.

**Nishimura, M.T., Stein, M., Hou, B.H., Vogel, J.P., Edwards, H. and Somerville, S.C.** (2003). Loss of a Callose Synthase Results in Salicylic Acid-Dependent Disease Resistance. Science. 301, 969-72.

**O'Connell, R.J. and Panstruga, R.** (2006). Tete a tete inside a plant cell: establishing compatibility between plants and biotrophic fungi and oomycetes. New Phytol. 171, 699-718.

**Panstruga, R.** (2005). Serpentine plant MLO proteins as entry portals for powdery mildew fungi. Biochem. Soc. Trans. 33, 389-92.

**Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D. and Daniels, M.J.** (1996). Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora parasitica specified by several different RPP genes. Plant Cell. 8, 2033-46.

**Potter, S., Uknes, S., Lawton, K., Winter, A.M., Chandler, D., DiMaio, J., Novitzky, R., Ward, E. and Ryals, J.** (1993). Regulation of a hevein-like gene in Arabidopsis. Mol. Plant-Microbe Interact. 6, 680-5.

**Roach, P.L., Schofield, C.J., Baldwin, J.E., Clifton, I.J. and Hajdu, J.** (1995). Crystallization and preliminary X-ray diffraction studies on recombinant isopenicillin N synthase from Aspergillus nidulans. Protein Sci. 4, 1007-9.

**Samson, F., Brunaud, V., Balzergue, S., Dubreucq, B., Lepiniec, L., Pelletier, G., Caboche, M. and Lecharny, A.** (2002). FLAGdb/FST: a database of mapped flanking insertion sites (FSTs) of Arabidopsis thaliana T-DNA transformants. Nucleic Acids Res. 30, 94-7.

**Sinapidou, E., Williams, K., Nott, L., Bahkt, S., Tor, M., Crute, I., Bittner-Eddy, P. and Beynon, J.** (2004). Two TIR:NB:LRR genes are required to specify resistance to Peronospora parasitica isolate Cala2 in Arabidopsis. Plant J. 38, 898-909.

**Struck, C., Ernst, M. and Hahn, M.** (2002). Characterization of a developmentally regulated amino acid transporter (AAT1p) of the rust fungus Uromyces fabae. Mol. Plant Pathol. 3, 23-30.

**Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G. and Katagiri, F.** (2003). Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen Pseudomonas syringae. Plant Cell. 15, 317-30.

**Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J.** (1992). Acquired resistance in Arabidopsis. Plant Cell. 4, 645-56.

**Van Damme, M., Andel, A., Huibers, R.P., Panstruga, R., Weisbeek, P.J. and Van den Ackerveken, G.** (2005). Identification of arabidopsis loci required for susceptibility to the downy mildew pathogen Hyaloperonospora parasitica. Mol. Plant-Microbe Interact. 18, 583-92.

**Voegele, R.T., Struck, C., Hahn, M. and Mendgen, K.** (2001). The role of haustoria in sugar supply during infection of broad bean by the rust fungus Uromycesfabae. Proc. Natl. Acad. Sci. USA. 98, 8133-8.

**Vogel, J. and Somerville, S.** (2000). Isolation and characterization of powdery mildew-resistant Arabidopsis mutants. Proc. Natl. Acad. Sci. USA . 97, 1897-902.

**Vogel, J.P., Raab, T.K., Schiff, C. and Somerville, S.C.** (2002). PMR6, a Pectate Lyase-Like Gene Required for Powdery Mildew Susceptibility in Arabidopsis. Plant Cell. 14, 2095-106.

**Vogel, J.P., Raab, T.K., Somerville, C.R. and Somerville, S.C.** (2004). Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. Plant J. 40, 968-78.

**Wang, K.L., Li, H. and Ecker, J.R.** (2002). Ethylene biosynthesis and signaling networks. Plant Cell. 14, Suppl, S131-S51.

**Wildermuth, M.C., Dewdney, J. Wu, G. and Ausubel, F.M.** (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature. 414, 561-5.

# Chapter 5

# Disease-specific expression of host genes during downy mildew infection of Arabidopsis

**Robin P. Huibers‡, Mark de Jong‡, Rene Dekter and Guido Van den Ackerveken**

‡**These authors contributed equally to the work.**

Plant-Microbe Interactions, Department of Biology, Science Faculty, Utrecht University, Padualaan 8, 3584 CH Utrecht, the Netherlands.

Manuscript submitted for publication

**Abstract**

**Here we report on the identification of Arabidopsis genes that are induced during compatible but not during incompatible interactions with the downy mildew pathogen *Hyaloperonospora parasitica*. This set of so-called *compatible specific* (*CS*) genes contrasts the large group of defence-associated genes that is differentially expressed during both compatible and incompatible interactions. From the 17 identified *CS*-genes, 6 belong to the ERF family of transcription factor genes, suggesting that these ERFs have a role during compatibility. The majority of *CS*-genes are differentially regulated in response to various forms of abiotic stress. *In silico* analysis of the *CS*-genes revealed an over-representation of DREB1A/CBF3 binding sites and EveningElement motifs in their promoter regions. The CS-ERFs are closely related to the CBF transcription factors and could potentially bind the DREB1A/CBF3 promoter elements in the *CS*-genes. Transcript levels of *CS*-genes peak at 2-3 days post inoculation, when pathogen growth is highest, and decline at later stages of infection. The identified *CS*-genes could be the direct or indirect targets of downy mildew effector proteins that promote disease susceptibility or could be induced as a physiological response of the plant to downy mildew infection.**

*Additional keywords: Abiotic stress, Compatibility, Defence, Disease susceptibility, Infection-related gene expression*

**Introduction**

Most plant species are non-hosts for a given pathogen due to the effective activation of a basal defence response that is triggered by the detection of microbe- or pathogen-associated molecular patterns (MAMPs or PAMPs, respectively) by predominantly cell surface localised receptors (Chisholm *et al.*, 2006). MAMPs/PAMPs, such as bacterial flagellin, trigger a signal transduction cascade that results in the transcriptional activation of defence-associated genes, production of antimicrobial compounds and cell wall reinforcements (Nurnberger *et al.*, 2004; Zipfel and Felix, 2005). Basal defence responses do not result in resistance in compatible plant-pathogen interactions. Pathogens actively suppress MAMP/PAMP-triggered responses via so-called effector proteins.

For bacterial effector proteins it has been shown that they can interfere with a large variety of cellular defence responses including cell wall-mediated defence responses, hormone signaling, programmed cell death and non-host disease resistance (Grant *et al.*, 2006). Recently, the abscisic acid (ABA) signaling pathway was discovered as a major target of effectors secreted by *Pseudomonas syringae.* Transcript profiling identified a prominent group of effector-induced genes that are associated with the biosynthesis of, and responsiveness to ABA. Levels of ABA increase during bacterial colonisation and bacterial growth is reduced in an ABA biosynthetic mutant, suggesting that ABA signaling suppresses activated defence responses (Truman *et al.*, 2006; de Torres-Zabala *et al.*, 2007). Alternatively, pathogen effectors can directly interfere with host gene transcription. The nuclear localised AvrBs3 effector protein of *Xanthomonas* induces more then 20 genes in susceptible pepper plants (Marois *et al.*, 2002; Kay *et al.*, 2007).

For fungal and oomycete pathogens hardly anything is known about host genes that are specifically activated during the infection process and not during plant defence. In flax, the *fis1* gene was shown to be induced by rust fungi (Ayliffe *et al.*, 2002). However, over-expression and silencing of *fis1*, which is involved in proline catabolism, did not have an effect on the development of rust disease (Mitchell *et al.*, 2006). Gene profiling of a compatible interaction between potato and *Phytophthora infestans* revealed the specific down-regulation of a plastidic carbonic anhydrase gene (Restrepo *et al.*, 2005). Silencing of the gene resulted in more pathogen growth, suggesting that down-regulation aids the infection process.

Several reports have shown that changes in host gene-expression induced upon inoculation with virulent or avirulent pathogen isolates or strains are remarkably similar. Differences are predominantly seen in timing and level of gene expression (Tao *et al.*, 2003; Li *et al.*, 2006). These reports support the view that *R*-gene mediated responses accelerate and amplify MAMP/PAMP-triggered responses, resulting in the transcriptional activation of predominantly similar defence-associated genes (Jones and Dangl, 2006). Little attention, however, has been paid to the specific differences between compatible and incompatible interactions.

Here we report on the changes in gene-expression in Arabidopsis in response to infection with an avirulent and virulent isolate of *H. parasitica*. The gene expression changes in both interaction types were found to be very similar. More then 97% of the genes that

become activated or repressed do so in both the compatible and incompatible interactions. However, 17 genes were found to be compatible specific (CS), including six *ERF* transcription factor genes. *In silico* promoter analyses revealed that *CS*-gene promoters are enriched for EveningElements and DREB1a/CBF3 binding sites. Several *CS*-genes were highly induced during the initial phase of infection whereas expression levels declined at later stages of infection. The transcriptional activation of *CS*-genes could be a physiological response to pathogen infection or result from the activity of effector proteins that possibly aid pathogen growth.

**Results**

*Transcriptome changes in Arabidopsis in response to infection with compatible and incompatible downy mildew isolates*

To identify compatible-specific genes expression profiles were generated from Arabidopsis seedlings (L*er*) inoculated with a compatible (Cala2) and an incompatible (Waco9, recognized by *RPP5*) *H. parasitica* isolate and compared to a common mock treatment. Plant material was harvested at 3 days post inoculation (dpi). At this time point pathogen growth was fully arrested in the incompatible interaction whereas in the compatible interaction hyphal growth was extensive and many haustoria were established. The experiment was carried out in triplicate and RNA of each biological replicate was hybridised on 4 CATMA arrays. Genes were considered differentially expressed within each biological replicate when they had a $\log_2$ ratio of ≤ -0.75 or ≥ 0.75 (~1.68-fold repression or induction respectively) and a q-value < 0.05 (p-value corrected for multiple testing errors using the false discovery rate). The number of differentially expressed genes for each interaction and the overlap between triplicates is shown in Figure 1A. For further analysis, only those genes were studied that were differentially expressed in at least two out of three biological replicates in either the compatible or incompatible interaction (Figure 1A, light grey middle segments). This resulted in a compatible gene set of 852 genes and an incompatible gene set of 1052 genes. An overlay was made between both gene sets (Figure 1B) resulting in 192 genes only found in the compatible interaction and 392 genes only in the incompatible interaction. In total, 660 genes were identified as differentially expressed in both interactions. Many of these genes are known to be related to host-defence and a selection of genes with their corresponding $\log_2$ ratios (average fold change) is listed in supplementary table S1.

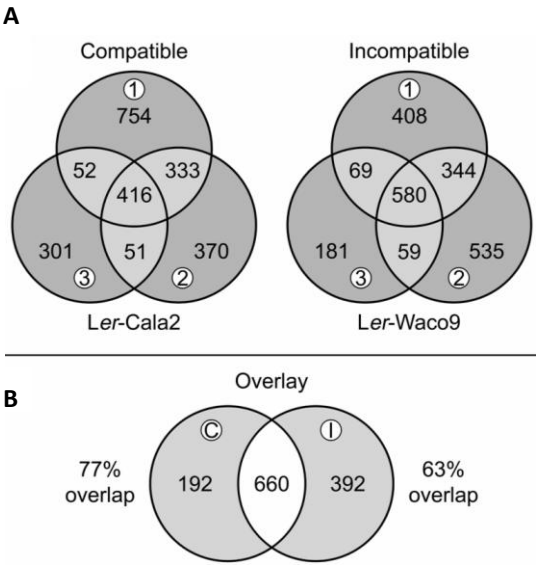


**Figure 1.** Venn diagrams and overlay of differentially expressed genes in compatible and incompatible *H. parasitica* - Arabidopsis interactions. (**A**), differentially expressed (DE) genes were selected based on a $\log_2$ ratio of ≤ -0.75 or ≥ 0.75 and q-values < 0.05. The venn diagrams show the DE genes for each biological replicate (1, 2 and 3). The middle segment of each diagram (shown in light grey) represents DE genes at least present in 2 out of 3 replicates and these were selected for further analysis for either a compatible (C, 852 genes) or incompatible (I, 1052 genes) interaction. (**B**), overlay of the 852 DE genes from a compatible interaction (C) and 1052 DE genes from an incompatible interaction (I). 660 transcripts are DE in both interactions.

Genes that were identified as differentially expressed in both interactions could be transcriptionally activated in one interaction and be transcriptionally repressed in the other. To investigate whether the genes were regulated in a similar manner in both interactions, the $\log_2$-ratios (based on the average fold change over 3 replicates) of all 660 genes from the overlay between the compatible and incompatible interaction were plotted and the Pearson correlation between the interactions was calculated. The overlapping 660 genes are similarly induced or repressed in both interactions and have an overall correlation which is greater than 0.9 (Figure 2A).

Genes that are differentially expressed in only one of the interactions could be specific for that interaction. Alternatively, they could have been selected as being differentially expressed only in one interaction because their measured expression is just below the lower limit of the selection criteria used ($\log_2$ ratio of ≤ -0.75 or ≥ 0.75 and a q-value < 0.05) in the other interaction. To verify this, we calculated the correlation between the $\log_2$-ratio (based on the average fold change over 3 replicates) of the non-overlapping genes (192 compatible and 392 incompatible) (Figure 1B). A modest correlation between them was found (r=0.67, Figure 2B). Figure 2B shows that the majority of genes have $\log_2$ ratios around the used selection criteria and that induction or repression does not differ much between the compatible and incompatible interaction, showing that these genes are also non-specifically induced or repressed in both interactions.

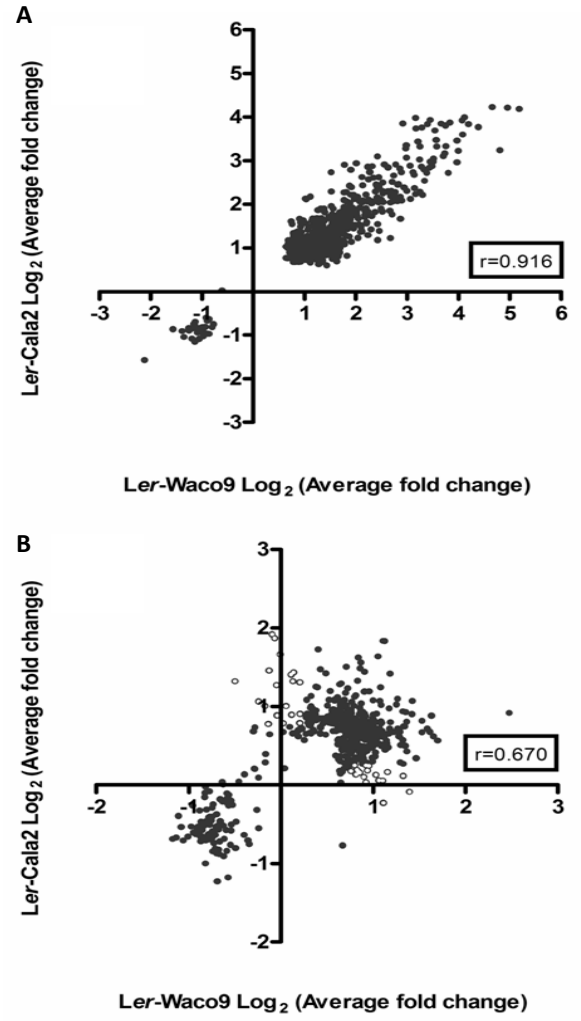


**Figure 2.** Correlations between the $\log_2$ ratios of differentially expressed genes in a compatible and incompatible interaction. The average fold-change of the overlapping and non-overlapping transcripts was calculated from 3 biological replicates. The values for compatible and incompatible interactions were log-transformed and plotted. (**A**), correlation between overlapping transcripts (660) from figure 1B. (**B**), correlation between non-overlapping transcripts (192 and 392) from figure 1B. Transcripts in (**A**) are strongly correlated and are highly differentially expressed (DE) as opposed to (**B**) where the correlation is modest and $\log_2$ ratios are around the selection criteria (≤ -0.75 or ≥ 0.75). Several transcripts in (**B**) show a specific differential expression for either a compatible or incompatible interaction (shown as open dots). These interaction specific DE genes were selected for a compatible interaction based on L*er*-Cala2 $\log_2$ ratio ≥ 0.75 and L*er*-Waco9 $\log_2$ ratio ≤ 0.25 or vice versa for an incompatible interaction.

Interestingly, a small number of genes could be identified as specifically differential expressed for either interaction. These genes were selected using a L*er*-Cala2 log2 ratio ≥ 0.75 and L*er*-Waco9 log2 ratio ≤ 0.25 for a compatible interaction or vice versa for an incompatible interaction. These selection criteria were based on the rationale that specifically differential expressed genes should be up-regulated in one interaction ($\log_2$ ratio ≥ 0.75) and remain relatively unchanged or down-regulated in the other interaction (log2 ratio ≤ 0.25) or vice versa. Based on these selection criteria 36 transcripts showed elevated levels in one interaction only (represented as open dots in Figure 2B). Of these 36 transcripts, 19 were specific for the compatible (*CS*-genes) interaction and 17 for the incompatible (IS-

genes) interaction. These 36 transcripts are of major interest as they are not generally activated in response to *H. parasitica*, and could therefore provide valuable information on the differences between both interactions.

*Incompatible- and compatible-specific gene expression*

The 36 transcripts that were identified as relatively non-changing ($Log_2$ ratio ≤ 0.25) in either the compatible or incompatible interaction had relatively high q-values (> 0.05) in the non-changing condition. This is because the q-values are based on the probability that $Log_2$ ratios deviate from 1 (not differentially expressed). These microarray measurements were therefore less reliable and needed verification by Q-PCR.

CATMA probes (Crowe *et al.*, 2003) for all tested genes were converted to AGI-identifiers (Arabidopsis Genome Initiative; TIGR 6.0 – The Institute for Genome Research; http://www.tigr.org/tdb/e2k1/ath1/) for convenience (see supplementary data). CATMA probes do not always correspond in a one-to-one relation to AGI-identifiers, which can result in a different number of genes with unique AGI codes. Conversion of the 19 *CS*- transcripts based on CATMA probes resulted in 18 AGI-codes; 16 of them matched 16 AGI-codes, 1 of them matched 2 AGI-codes and 2 had no matches at all. The 16 IS-transcripts based on CATMA probes gave 16 AGI-codes; 12 of them matched unique AGI-codes, 2 of them matched 2 AGI-codes each and 2 had no matches. Non-matching CATMA probes were discarded and for the double-matching CATMA probes unique Q-PCR primers were designed for the genes with an AGI-code.

For the Q-PCR analysis, 14 genes that were activated in both interactions were taken along as controls. *NPR1*, *PR-1* and *PDF1.2* are not present on the CATMA array, but are associated with the defence response of the host (Reuber *et al.*, 1998) and were therefore included in the analysis. Figure 3A shows that all 14 genes are induced in both interactions, thereby confirming the microarray data. In addition, *NPR1*, *PR-1* and *PDF1.2* show the expected expression pattern for a compatible and incompatible interaction, with *NPR1* showing similar induction in both interactions, and *PR-1* and *PDF1.2* being induced to a higher level in the incompatible interaction. These results show that the microarray data is reliable and confirm that defence-associated expression occurs both in the incompatible as in the compatible interaction.

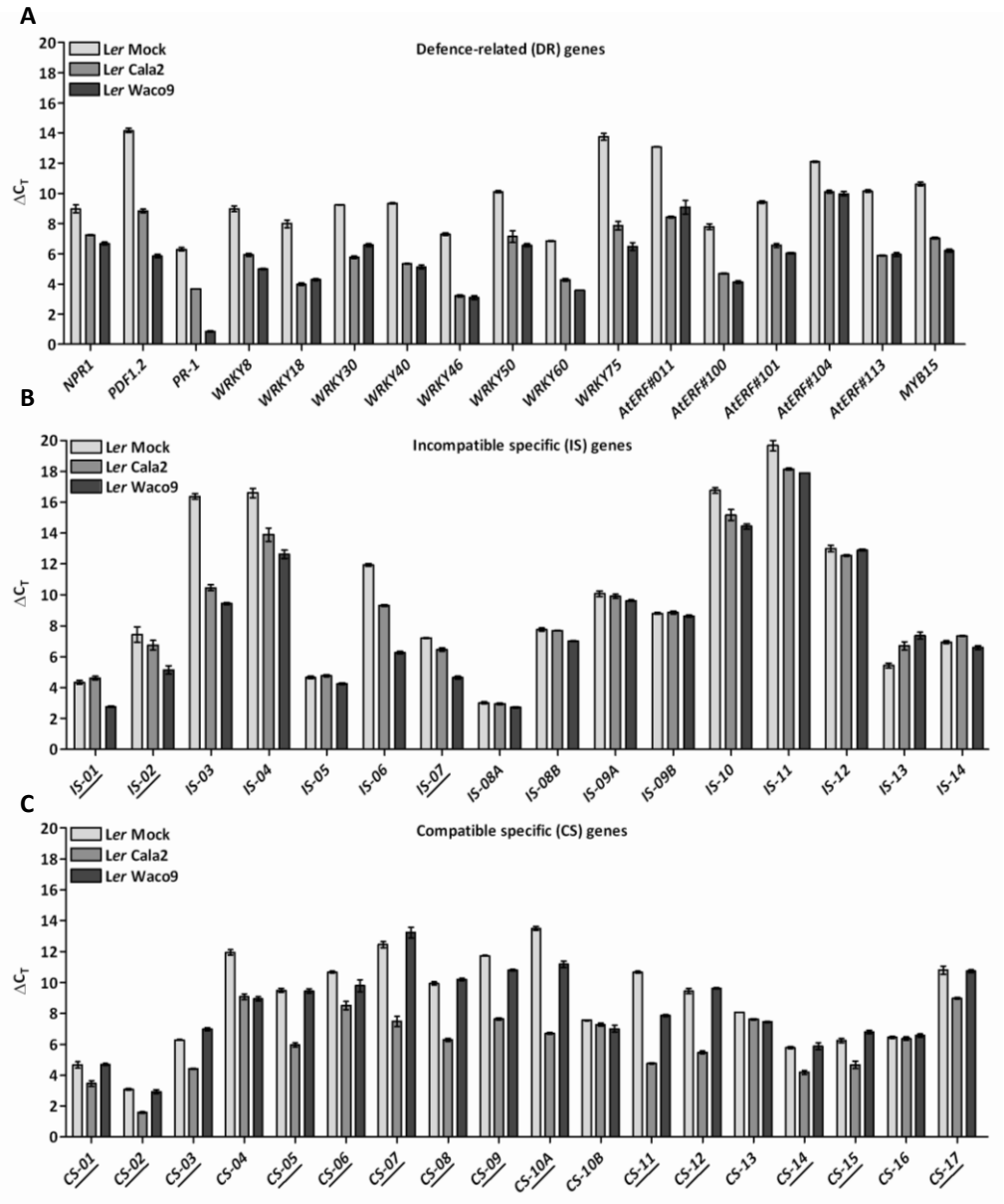


**Figure 3.** Relative transcript abundance of defence-related and potential compatible- and incompatible-specific genes. RNA extracted from each biological replicate for each interaction was pooled (see material and methods) and cDNA was synthesized. Note that a lower bar indicates higher transcript abundances in all diagrams and that all measurements were normalised for Arabidopsis *ACT2*-levels (*AT3G18780*). The error-bars are given as standard error of mean (SEM). Three treatments were investigated being Landsberg *erecta* (L*er*), (i) mock-inoculated, (ii) inoculated with the compatible isolate Cala2 and (iii) inoculated with the incompatible isolate Waco9. A and B versions of several potential IS or CS-genes are due to individual CATMA probes that detect two different transcripts simultaneously on the array and two qPCR-primer sets were made to discriminate between two mRNAs. (**A**), defence-related (DR) transcripts. Q-PCRs were

performed on several transcription factors from table S1 and on *NPR1*, *PDF1.2* and *PR-1*, which were not present on the CATMA array. All genes show similar levels of induction in both interactions, confirming the microarray data. Only *PR-1* and *PDF1.2* are higher expressed in an incompatible interaction but not exclusively induced. (**B**), incompatible specific (IS) genes. Three genes, *IS-01*, *IS-02* and *IS-07*, show a modest induction during an incompatible interaction. (**C**), compatible specific (CS) genes. The majority of genes show a specific differential expression in the L*er*-Cala2 (compatible) interaction and are highlighted by underlining, exceptions are *CS-04*, that seems to be defence-related and *CS-13* and *CS-16* showing no differential expression at all.

Incompatible-specific gene-expression was confirmed for only 3 IS-genes by Q-PCR (Figures 3B, underlined genes). The other IS-genes were also induced in the compatible interaction or no induction was observed at all. Interestingly, for the majority of *CS*-genes (14 out of 18) up-regulation in the compatible- but not in the incompatible interaction could be confirmed by Q-PCR (Figure 3C, underlined genes). This implies that interaction specific gene-expression occurs mainly in the compatible interaction as far more *CS*-genes were found as opposed to IS-genes, when similar microarray selection criteria are applied.

*Compatible specific ERF transcription factor genes*

From the 14 identified *CS*-genes, 3 genes (*CS-12*, *CS-10A* and *CS-09*) belong to the family of ethylene response factor (ERF) transcription factors. The ERF family in Arabidopsis comprises 122 genes divided in 10 clades based on domain architecture (Nakano *et al.* 2006). *AtERF#26* (*CS-12*) belongs to clade III, *AtERF#111* (*CS-10A*) and *AtERF#114* (*CS-09*) belong to clade X (Figure S1). Not all family members in clades III and X are present on the CATMA array. To investigate the gene expression response of members of clade III and clade X to *H. parasitica* infections, they were tested by Q-PCR (tested genes are shown in bold, Figure S1). In addition to *AtERF#026* (*CS-12*) in subclade III-b, two homologous *ERF* genes, *AtERF#024* and *AtERF#025* showed compatible specific expression (designated *CS-18* and *CS-19*, Figure 4A). Of the 8 ERFs in clade X, three showed compatible-specific expression; the already identified *AtERF#114* (*CS-09*) and *AtERF#111* (*CS-10A)* and one additional gene *AtERF#109* (designated *CS-20*, Figure 4B). Interestingly, *AtERF#108*, *AtERF#112* and *AtERF#113* show strong induction in both the compatible and incompatible interaction (Figure 4B), indicating that members in this clade respond in various ways to downy mildew. Members showed either no induction, activation by compatible and incompatible downy mildew or showed compatible specific expression. The 3 additional compatible-specific genes from the ERF clades III and X bring the number of downy mildew-induced *CS*-genes to 17.

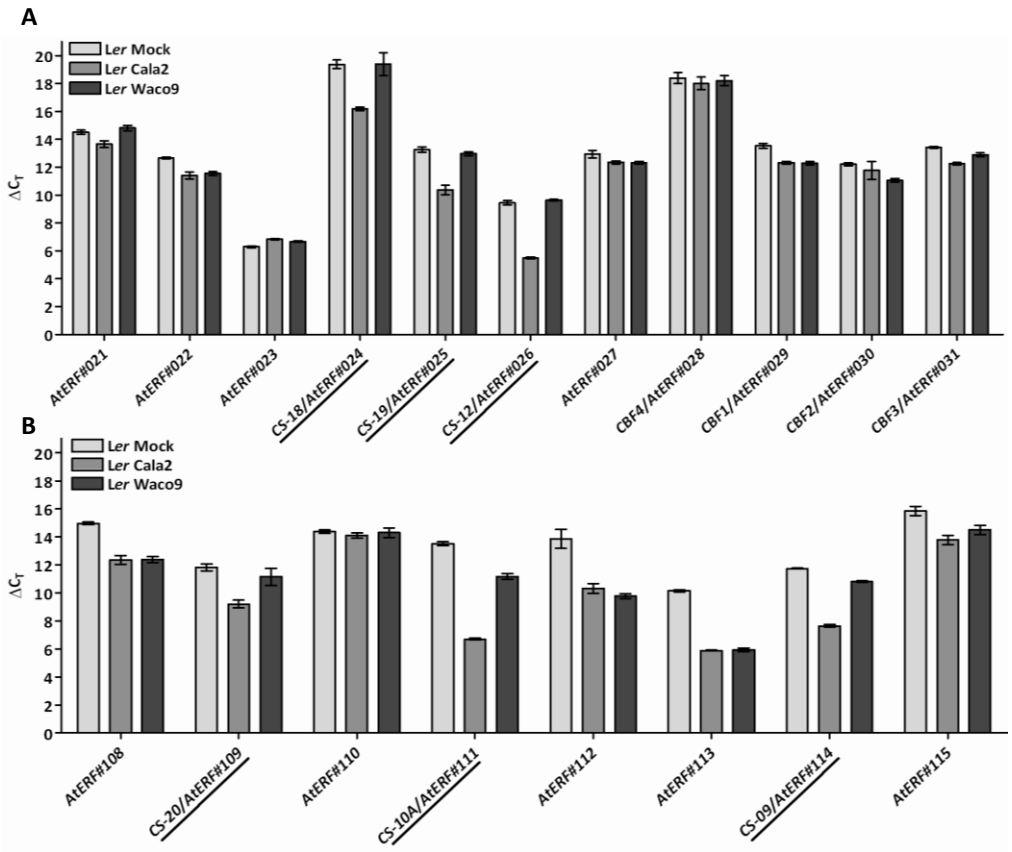


**Figure 4.** Relative transcript levels of ERF family members homologous to the identified compatible specific ERF transcription factors. Plants were either mock treated or inoculated with a compatible (Cala2) or incompatible (Waco9) *H. parasitica* isolate. The error-bars are given as standard error of mean (SEM) and ERFs that show induction specific for the compatible interaction (Cala2) are underlined. Note that lower bars represent higher transcript abundance. (**A**), clade III of the ERF gene-family (Nakano *et al.*, 2006) homologous to *CS-12/AtERF#26*. The diagram shows that *AtERF#024* and *AtERF#025* are also specifically induced during a compatible interaction, whereas the other genes are not. (**B**), differential expression of clade X members of the ERF family homologous to *CS-10A*, *CS-09* and *AtERF#113* which is also induced during incompatible interactions. *AtERF#109* is also a CS gene whereas *AtERF#108*, *AtERF#112* and *AtERF#115* are induced in both interactions and therefore defence-related (DR).

**Table 1.** Description of compatible- and incompatible-specific genes that have been confirmed by qPCR. For a selection of genes (of which the qPCR- and AGI-IDs are given) their induction is described in literature. In contrast to the incompatible-specific (IS) genes, many of the compatible-specific (CS) genes are induced by various abiotic stress responses like low temperature, dehydration, high salt and ABA. References (1) (Stotz *et al.*, 2000) (2) (Kimura *et al.*, 2003) (3) (Nylander *et al.*, 2001) (4) (Okamoto *et al.*, 2006) (5) (Kushiro *et al.*, 2004) (6) (Bailey *et al.*, 2003) (7) (Kolukisaoglu *et al.*, 2004) (8) (Nakano *et al.*, 2006) (9) (Pandey *et al.*, 2005) (10) (Capel *et al.*, 1997) (12) (Medina *et al.*, 2001) (12) (Medina *et al.*, 2005) (13) (Mitsuya *et al.*, 2005) (14) (Wilhelm and Thomashow, 1993).

| *Q-PCR-ID* | *AGI-ID* | *Name* | *Induced by* | *Reference(s)* |
|---|---|---|---|---|
| *IS-01* | *At1g52400* | *BGL1* | JA | 1 |
| *IS-02* | *At1g52410* | *Caldesmon-related* | - | - |
| *IS-07* | *At1g67865* | *Expressed protein* | - | - |
| | | | | |
| **Compatible specific (CS) genes** | | | | |
| | | | | |
| *Q-PCR-ID* | *AGI-ID* | *Name* | *Induced by* | *Reference(s)* |
| *CS-01* | *At1g01470* | *LEA14* | HL, DH, LT, Salt | 2 |
| *CS-02* | *At1g20440* | *COR47* | LT | 3 |
| *CS-03* | *At1g51090* | *Heavy-metal associated* | - | - |
| *CS-05* | *At3g50970* | *Xero2/LTI30* | LT | 3 |
| *CS-06* | *At4g19230* | *CYP707A1* | ABA | 4,5 |
| *CS-07* | *At4g20970* | *bHLH162* | - | 6 |
| *CS-08* | *At5g25110* | *CIPK25* | Calcium | 7 |
| *CS-09* | *At5g61890* | *AtERF#114* | - | 8 |
| *CS-10A* | *At5g64750* | *AtERF#111/ABR1* | ABA, LT, DH, Salt | 8,9 |
| *CS-11* | *At1g15010* | *Expressed protein* | - | - |
| *CS-12* | *At1g63040* | *AtERF#026* | - | 8 |
| *CS-14* | *At4g30650* | *LTI6A/RCI2AS* | LT, ABA, DH, Salt | 10,11,12,13 |
| *CS-15* | *At2g42530* | *COR15b* | LT, ABA | 14 |
| *CS-17* | *At2g15890* | *Expressed protein* | - | - |
| *CS-18* | *At2g36450* | *AtERF#024* | - | - |
| *CS-19* | *At5g52020* | *AtERF#025* | - | - |
| *CS-20* | *At4g34410* | *AtERF#109* | - | - |

*In silico promoter analysis of compatible specific genes*

For many *CS*-genes it has been described that they respond to abiotic stress conditions e.g. dehydration, low temperature and abscisic acid (ABA) (summarised in table 1). Accordingly, *CS*-genes show enrichment for the class “response to abiotic or biotic stimulus” in the GO category “biological process”; using the TAIR gene ontology functional categorisation tool (http://www.arabidopsis.org/tools/bulk/go/index.jsp). The publicly available dataset abiostress of AtGenExpress (visualisation tool (AVT), http://jsp.weigelworld.org/expviz/expviz.jsp) revealed that 6 *CS*-genes are induced during multiple abiotic stress conditions (listed in the upper part of table 2), e.g. cold, osmotic stress, salt, drought, wounding, and ABA. Other *CS*-genes are less generally induced by abiotic stress condition (listed in the lower part of table 2). The observations from literature,

gene ontologies and AtGenExpress datasets clearly indicate that a subset of *CS*-genes is responsive to both *H. parasitica* infection and to abiotic stress.

Genes that are co-regulated in a given biological process often share common regulatory elements in their promoters. As all 17 *CS*-genes are induced during a compatible downy mildew infection and many are responsive to abiotic stress, there could reside similar promoter elements in their respective promoters. To investigate this, the web-based tool Athena (http://www.bioinformatics2.wsu.edu/cgi-bin/Athena/cgi/home.pl) (O'Connor *et al.*, 2005) was used to identify and visualise known regulatory elements overrepresented in the promoter regions of the *CS*-genes 1000 bp upstream of the start codon. Two promoterelements were significantly enriched when all *CS*-genes were analysed together. The EveningElement (AAAATATCT, $P<10^{-5}$) was found once in 8 different promoters and is involved in evening specific transcription (Harmer *et al.*, 2000). The DREB1A/CBF3 (Dehydration Responsive Element/ C-repeat Binding Factor (CBF)) element (consensus sequence RCCGACNT, $P<10^{-3}$) was found 1-3 times in 6 different promoters. Importantly, enrichment for both promoter elements was also found when the 14 initially identified *CS*-genes were analysed separately. Moreover, analyses of all differentially expressed genes in the compatible or/and incompatible interaction showed no enrichment for these elements. Separate promoter analysis of the 7 *CS*-genes that are responsive to a broad range of abiotic stresses (table 2) showed that these genes are enriched for more elements. A more significant enrichment was found of the EveningElement ($P<10^{-6}$) and DREB1A/CBF3 ($P<10^{-4}$) and several other elements, like the DRE core motif ($P<10^{-4}$) (consensus sequence RCCGAC) (Chen *et al.*, 2002), which is a relaxed form of DREB1A/CBF3, the CBF1 binding site (BS) ($P<10^{-3}$) (consensus sequence TGGCCGAC) (Hao *et al.*, 2002) and the Z-box motif ($P<10^{-3}$) (consensus sequence ATACGTGT) (Ha and An, 1988). Although many *CS*-genes show responsiveness towards ABA, the ABA responsive element ABRE (consensus sequence YACGTGGC) (Choi *et al.*, 2000) was not overrepresented and only found once in the promoter of *CS-20*/*AtERF#109*. However, 9 *CS*-genes contain the ABRE-like binding site motif (consensus sequence BACGTGKM) (Shinozaki and Yamaguchi-Shinozaki, 2000). The occurrence of the above mentioned binding sites is summarised for all *CS*-genes in table 3. That CS-gene promoters are enriched for DRE/Evening elements suggests that expression during compatible *H. parasitica* interactions is controlled by these elements.

**Table 2.** AtGenExpress microarray data of compatible-specific genes. Differential expression of 17 CS-genes was investigated with the visualisation tool of AtGenExpress in the abiostress dataset to find inducers or repressors of them. *CS-07* (*At4g20970*) and *CS-10A* (*At5g64750*) are not present in the AtGenExpress dataset and are therefore given a not present (np) classification. All measurements were background corrected and the induction or repression was classified according to the following rules: 0-500, "0" (no difference); 500-1000, "+"; 1000-5000, "++"; 5000-10 000, "+++" and >10 000, "++++"; Identical for repression but with minus signs and minus classification. Some genes have gained double classifications indicating that they are first repressed and then induced in time (e.g. ---/++++) or vice versa (e.g. ++++/-).

| **AtGenExpress induction/repression of *CS*-genes** | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | |
| **Q-PCR-ID** | **Cold** | **Osmotic - 300 mM Mannitol** | **Salt - 150 mM NaCl** | **Drought - Dry air stream** | **Wounding - Leave punctuation** | **10 µM ABA** |
| | | | | | | |
| *CS-01* | ++++ | ++++ | +++ | ++ | +++ | ++++ |
| *CS-02* | ++++ | ++++ | ++++ | ++ | ++++ | ++++ |
| *CS-03* | ++++ | ++ | ++ | 0 | ++ | 0 |
| *CS-05* | ++++ | ++++ | +++ | + | +++ | ++++ |
| *CS-10A* | np | np | np | np | np | np |
| *CS-14* | ---/++++ | ++/-- | +++ | +/-- | ++++/- | ++ |
| *CS-15* | ++++ | +++ | +++ | ++/-- | ++/-- | +++ |
| | | | | | | |
| *CS-06* | 0 | 0 | 0 | 0 | 0 | +++ |
| *CS-07* | np | np | np | np | np | np |
| *CS-08* | ++ | + | 0 | 0 | 0 | + |
| *CS-09* | 0 | 0 | 0 | 0 | 0 | 0 |
| *CS-11* | 0 | 0 | 0 | 0 | ++ | 0 |
| *CS-12* | 0 | 0 | 0 | 0 | 0 | 0 |
| *CS-17* | --- | +/-- | -- | -/++ | +/-- | -- |
| *CS-18* | 0 | 0 | 0 | 0 | 0 | 0 |
| *CS-19* | 0 | 0 | 0 | 0 | + | 0 |
| *CS-20* | 0 | 0 | 0 | 0 | ++ | 0 |

**Table 3.** Promoter elements in the compatible specific genes. The classifications for induction were combined with the presence of the number of overrepresented promoter elements as indicated by Athena. A subset of 7 compatible specific (CS) genes (top list) responded towards all kinds of inducers like cold, osmotic stress, salt and ABA and their promoters have in general combinations of the ABRE-like and DREB1A/CBF3 motif. Other CS-genes (bottom list) show a specific induction or a modest differential expression as shown by AtGenExpress. Note that *CS-07* (*At4g20970*) and *CS-10A* (*At5g64750*) are not present in the AtGenExpress dataset, but *CS-10A* is also induced by abiotic stress according to literature. *CS-07* (*bHLH162*), *CS-12* (*AtERF#026*), *CS-18* (*AtERF#024*) and *CS-19* (*AtERF#025*) are only differentially expressed (DE) by compatible *H. parasitica* and do not hold ABRE-like and/or DRE core elements in their promoters.

| | **ABRE-like** | **DRE core** | **DREB1A/CBF3** | **CBF1** | **EveningElement** | **Z-box** |
|---|---|---|---|---|---|---|
| | | | | | | |
| *CS-01* | 1 | 3 | 2 | | 1 | 1 |
| *CS-02* | 1 | 3 | 2 | | | 1 |
| *CS-03* | 1 | 2 | 2 | | 1 | |
| *CS-05* | | 3 | 3 | 1 | 1 | 1 |
| *CS-10A* | 1 | 3 | | | | |
| *CS-14* | | 1 | 1 | | 1 | |
| *CS-15* | 2 | 2 | 2 | 1 | 1 | |
| | | | | | | |
| *CS-06* | 2 | | | | 1 | |
| *CS-07* | | | | | 1 | |
| *CS-08* | 1 | | | | | |
| *CS-09* | | 2 | | | | |
| *CS-11* | 1 | | | | | |
| *CS-12* | | | | | | |
| *CS-17* | | | | | 1 | |
| *CS-18* | | | | | | |
| *CS-19* | | | | | | |
| *CS-20* | 1 | | | | | |

The gene-products of *AtERF#029* (*CBF1*), *AtERF#030* (*CBF2*) and *AtERF#031* (*CBF3*) are known to bind the identified DREB/CBF elements (Agarwal *et al.*, 2006) and belong to subclade III-c of the ERF family (Nakano *et al.*, 2006). However, as shown in Figure 5A, none of these *CBF* genes is induced by downy mildew. In contrast, the related genes from subclade III-b, *AtERF#024 (CS-18)*, *AtERF#025 (CS-19)* and *AtERF#026* (*CS-12)* are activated upon downy mildew infection. The *CBFs*, *AtERF#024, AtERF#025* and *AtERF#026* belong to clade III,

suggesting that their encoded proteins could have similar functions based on homology of the AP2/ERF domain (Nakano *et al.*, 2006).

The promoters of *AtERF#024 (CS-18)*, *-#025 (CS-19)* and *-#026 (CS-12)* do not contain any of the discussed promoter elements (table 3), suggesting that they are regulated in a different way than other *CS*-genes. Moreover, *AtERF#024 (CS-18)*, *-#025 (CS-19)* and *-#026 (CS-12)* are not induced by a broad range of abiotic stress conditions according to AtGenExpress data (table 2), whereas *CBF1, -2* and *-3* are highly induced by cold stress. As *AtERF#024 (CS-18)*, *-#025 (CS-19)* and *-#026 (CS-12)* become induced during compatible *H. parasitica* infections it could be that they control expression of other *CS*-genes with DRE elements in their promoters.

*Temporal expression of compatible specific genes during downy mildew infection*

To study *CS*-gene expression during infection with *H. parasitica*, transcript levels of 5 *CS*-genes were measured by Q-PCR on inoculation series from 0 to 5 dpi with intervals of 1 day. Pathogen colonisation was monitored by measuring *H. parasitica Actin2* transcript levels. The highest growth-rate of the pathogen was observed between 1 and 3 dpi and the growth rate declines at 4 to 5 dpi, around the same time that sporulation starts (Figure 6A). In addition, expression of *PR-1*, *At1g14880* and *DMR6* were studied, as those genes are highly induced in both the compatible and incompatible interaction (Figure 6B, C and D). The defence-related genes *PR-1* and *At1g14880*, which show a similar expression pattern, are higher induced in the incompatible interaction than in the compatible interaction at early time points (1-3 dpi). Induction of *DMR6* (*At5g24530*) (Chapter 4; Van Damme et al., 2008) is similarly induced in both interactions (1-3 dpi), although expression is also slightly higher at 1 dpi in the incompatible interaction. Transcript levels of all 3 defence-related genes decline in the incompatible interaction after 3 dpi. This is expected as the early defence responses have arrested *H. parasitica* growth already at 1-2 dpi. In the compatible interaction, where pathogen growth is abundant at later stages of the infection (4-5 dpi) transcript levels of the defence-related genes are higher (Figure 6B, C and D).

All 5 tested *CS*-genes are induced only during the compatible interaction and not during an incompatible interaction (Figure 6E-I) although a slight induction is observed for *CS-05* (*Xero2*) at 3 and 4 dpi during an incompatible interaction (Figure 6E). The *CS*-genes

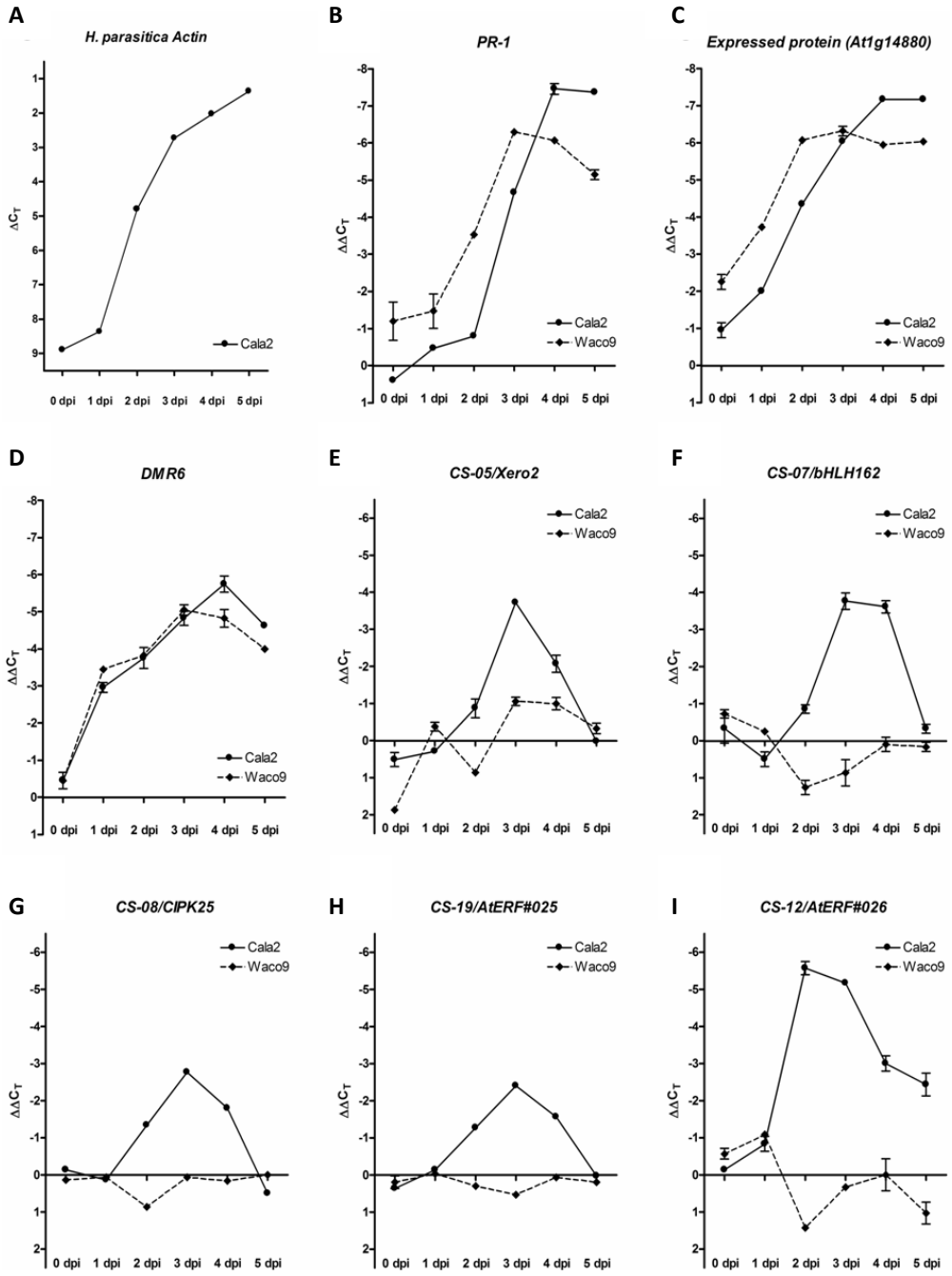


**Figure 5.** Temporal expression of three defence-related and five compatible-specific genes in a compatible (Cala2) and incompatible (Waco9) interaction. Transcripts levels for all qPCR measurements were normalised for Arabidopsis *ACT2* and the mock-treatment giving $\Delta\Delta C_T$-values except for *H. parasitica Actin2* levels. The error-bars are given as the standard error of mean (SEM) and the scale is reversed to ease interpretation. (**A**), *H. parasitica Actin* levels were used as a measure of pathogen growth from 0 to 5 dpi. The highest growth-rate was observed

between 1 and 3 dpi. (**B**, **C** and **D**), three defence-related (DR) transcripts were followed in time. *At1g14880* and *PR-1* are higher expressed in an incompatible interaction during early time points, indicating a fast induction. Relative higher transcript levels are observed at 4 and 5 dpi for a compatible interaction. *DMR6* transcript levels are very similar for both interactions with identical starting points. (**E**, **F**, **G**, **H** and **I**), temporal expression of 5 CS-genes: *Xero2*, *bHLH162*, *CIPK25*, *AtERF#025* and AtERF#26. Transcript levels are enhanced and peak at 2/3 dpi in the compatible interaction whereas this is not observed in an incompatible interaction. Interestingly, their specific induction disappears at 5 dpi. *AtERF#026* shows a very strong induction already at 2 dpi and this diminished at 5 dpi.

show a similar pattern in time that is different from that of the defence-related genes. *CS-05* (*Xero2*), *CS-07* (*bHLH162*), *CS-08* (*CIPK25*) and *CS-19* (*AtERF#025*) are most highly expressed at 3 dpi and their transcript levels decline after this time point, to reach the same level as in mock-treated plants at 5 dpi. Remarkably, both pathogen growth and *CS*-gene expression are highest at 3 dpi, but when at 4-5 dpi *H. parasitica* starts sporulating, their specific induction completely disappears. *CS-12* (*AtERF#026*) reaches its peak level already at 2 dpi with a much stronger induction than the other *CS*-genes. This induction also declines towards 5 dpi but does not completely return to expression levels of mock-treated plants. The expression patterns of the *CS*-genes show that they are truly specifically induced during a compatible interaction and that their highest transcript levels are obtained when *H. parasitica* has it largest growth rate at 2-3 dpi.

*Isolate-specific induction of CS-genes*

The induction of *CS*-genes during compatible Cala2 interactions but not during incompatible Waco9 interactions indicates that *CS*-gene expression is related to pathogen colonization and not to defence. However, it could also be due to specific differences between the isolates. Although the identified *CS*-genes are not induced during incompatible Waco9 interactions, they may become induced during incompatible Cala2 or compatible Waco9 interactions. To test this, Ws-4 was inoculated with Waco9 and Cala2 and expression of *CS*-genes analysed at 3 dpi. In these interactions Waco9 is compatible and Cala2 incompatible. Figure 6A shows that none of the *CS*-genes become induced during the incompatible Cala2 interaction. This demonstrates that *CS*-gene induction is due to colonization by Cala2. Half of the *CS*-genes showed induction in the compatible Waco9 interaction, demonstrating that both isolates induce expression of these genes during compatible and not during incompatible interactions. The other half of the *CS*-genes was not induced during the compatible Waco9

interaction, of these *CS-1* and *CS-17* showed no induction, *CS-2* and *CS-5* were slightly lower expressed in both interactions, and *CS-8*, *CS-14*, *CS-15* and *CS-18* showed lower expression levels in the compatible Waco9 interaction. There are different explanations possible for the different expression patterns in the L*er* and Ws-4 interactions. It may be that Waco9 does not induce some of the *CS*-genes or even down-regulates them. Alternatively, induction could be low, thereby being difficult to detect, e.g. *CS-1* and *CS-17* or genes may not be induced specifically in WS-4, irrespective of the isolate. For three *CS*-genes, *CS-8* (*CIPK25*), *CS-14* (*LTI6A*/*RCI2AS*) and *CS-15* (*Cor15B*), these different possibilities were sorted out. L*er eds1-2*, Col-*eds1* and Ws-*eds1* plants were inoculated with both Cala2 and Waco9. Due to mutation of *EDS1*, all interactions were compatible. *CS-8*, *CS-14* and *CS-15* were specifically induced by Cala2 and not by Waco9, irrespective of the Arabidopsis accession (Figure 6B, C, D). *CS-9* was induced by both isolates as expected and confirms the data from Figure 6A. However, no specific down-regulation of *CS-8*, *CS-14* and *CS-15* was seen in Waco9-challenged Ws-*eds1* plants (Figure 6D) as was seen in the other compatible Waco9 interactions (Figure 6A, B, C). Nevertheless, it is clear that several *CS*-genes are induced in an isolate-specific manner.

*T-DNA lines disrupted in compatible-specific genes*

To verify the requirement of the genes listed in Supplementary table S2 for susceptibility to downy mildew, corresponding sequence-tagged insertion mutants were investigated. Development of pathogen growth was monitored in time by measuring the level of sporulation as well as the number of days it takes *H. parasitica* to start sporulating. No T-DNA lines were available for *CS-01* (*LEA14*) and *CS-18* (*AtERF#024*). Unfortunately, none of the T-DNA insertion mutants showed alterations in disease susceptibility. The absence of observable phenotypes could be due to redundant roles of several *CS*-genes e.g. the three homologous ERFs from clade IIIb. Moreover, the available T-DNA lines were all in the Col-0 accession (with exception of FLAG_486C09 (Ws-4)) and could therefore not be tested with the Cala2 isolate. All T-DNA lines were therefore tested with the compatible Waco9 isolate. As only half of the *CS*-genes become induced by Waco9 in Ws-4 (Figure 6A) it is possible that these *CS*-genes are not involved in compatible Waco9 interactions (e.g. *CS-8*, *CS-14* and *CS-15* (Figure 6 B, C, D)) and hence give no phenotype when disrupted.

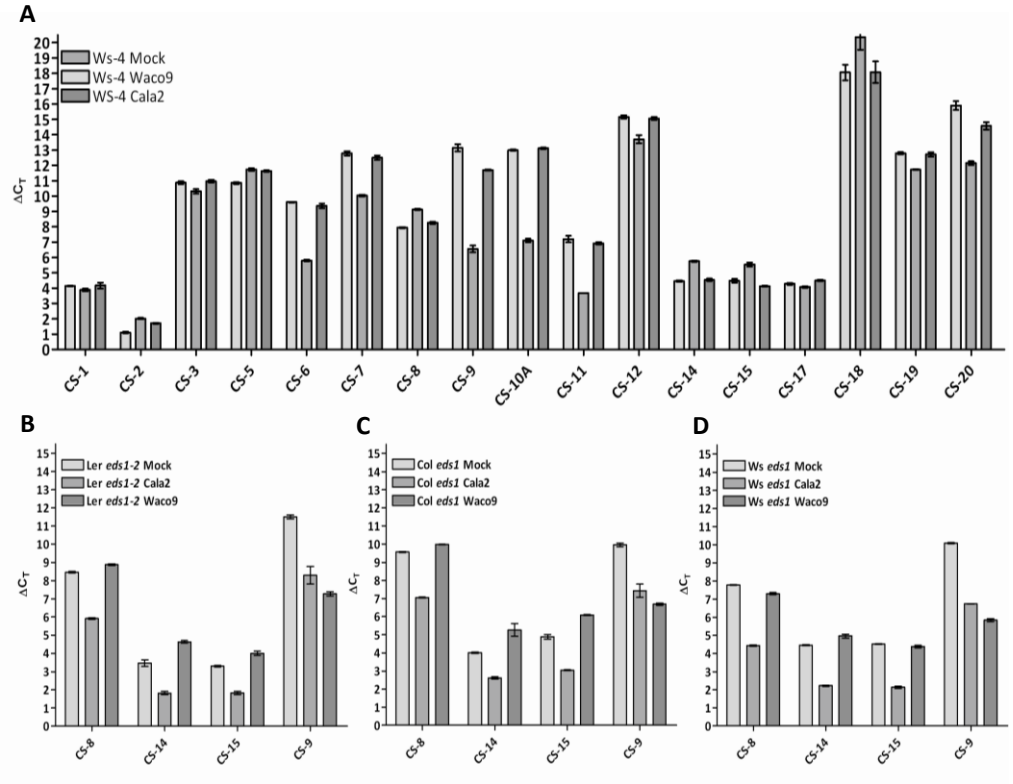


**Figure 6.** Relative transcript abundance of CS-genes 3 dpi in different Arabidopsis accessions inoculated with different isolates. Note that a lower bar indicates higher transcript abundances in all diagrams and that all measurements were normalised for Arabidopsis *ACT2*-levels (*AT3G18780*). The error-bars are given as standard error of mean (SEM). (**A**), three treatments were investigated being Ws-4, (i) mock-inoculated, (ii) inoculated with the compatible isolate Waco9 and (iii) inoculated with the incompatible isolate Cala2. Relative expression of CS-8, CS-9, CS-14 and CS-15 was analysed by Q-PCR in L*er eds1-2* (**B**), Col-*eds1* (**C**) and WS-*eds1* (**D**) plants respectively either mock treated or inoculated with Cala2 or Waco9.

**Discussion**

Arabidopsis genes were identified that are differentially expressed only during compatible but not during incompatible interactions with the downy mildew pathogen *H. parasitica*. This is in contrast to the majority of genes that show induction or repression in both interactions upon *H. parasitica* inoculation. Many genes that are differentially expressed in both interactions have been associated with plant defence, and are likely activated due to a general plant response upon pathogen recognition (Maleck *et al.*, 2000). The defence-related genes *PR-1*, *At1g14880* and *DMR6* were higher induced at early timepoints of infection during the incompatible interaction with Waco9, indicating that *R* gene-mediated responses

(triggered by *RPP5*) are faster in general. After 3 dpi, pathogen growth is fully arrested in the incompatible interaction and defence-related transcript levels decline. In the compatible interaction, on the other hand, pathogen growth is exponential, and non-specific defence responses are triggered resulting in higher transcript levels at later stages of infection. These quantitative differences have also been found for other plant pathogen interactions (Tao *et al.*, 2003; Li *et al.*, 2006) and support the view that *R* gene-mediated responses amplify MAMP/PAMP-triggered responses (Jones and Dangl, 2006) resulting in the transcriptional activation of a similar set of defence-related genes. In contrast, *CS*-genes were highest expressed around 2-3 dpi strictly in the compatible interaction and expression levels did not follow the increase in *H. parasitica* biomass as the defence-related transcripts do. This clearly indicates that *CS*- and defence-related genes are differently regulated.

Based on publicly available micro-array data it was found that most *CS*-genes are responsive to a broad range of abiotic stress conditions like cold, salinity, drought, wounding and ABA, suggesting that compatible *H. parasitica* infections provoke an abiotic stress-like response. This could be the result of absorption of water and nutrients by the pathogen. One would expect that water and nutrient uptake increases with pathogen biomass. However, *CS*-gene expression does not increase with *H. parasitica* biomass but was highest expressed at 2-3 dpi. Promoter analyses of all *CS*-genes revealed an overrepresentation of the DREB1A/CBF3 (Dehydration Responsive Element/C-repeat Binding Factor (CBF)) element and the EveningElement involved in evening specific transcription (Harmer *et al.*, 2000). The ABRE-like element (Shinozaki and Yamaguchi-Shinozaki, 2000) was slightly below the significance threshold but was present in 9 *CS*-genes, which is interesting as many *CS*-genes are ABA responsive. The overrepresentation of promoter motifs or presence in the case of the ABRE-like element is in good agreement with the expression of *CS*-genes during abiotic stress conditions.

*CS-12* (*AtERF#026*), *CS-18* (*AtERF#024*) and *CS-19* (*AtERF#025*) did not show responsiveness to different abiotic stress conditions according to AtGenExpress and none of the discussed promoter elements were present in their promoters. These 3 genes belong to subclade III-b of the ERFs and could be involved in the transcriptional regulation of other *CS*-genes containing DRE-like elements similarly as described for members of subclade III-c. The subclade III-c members, *CBF1, CBF2* and *CBF3* are induced by low temperature and activate transcription of genes with DRE elements in their promoters (Gilmour *et al.*, 1998; Gilmour *et*

*al.*, 2004). Interestingly, cold induction of these 3 *CBF* genes is controlled by the circadian clock (Fowler *et al.*, 2005) and links circadian rhythmicity with the transcriptional activation of genes containing DRE elements in their promoters. However, *CBF1*, *CBF-2* and *CBF-3* did not show induction during *H. parasitica* infections and it could be that ERFs from subclade III-b activate the induction of *CS*-genes containing DRE(-like) and EveningElements in their promoters during compatible downy mildew infections.

Several *CS*-genes are responsive to ABA and contain ABRE-like elements in their promoters, suggesting a role for ABA in compatible *H. parasitica* interactions. *CS-6* (*CYP707A1*) belongs to the ABA 8'-hydroxylases and is involved in catabolism of ABA and could dampen ABA levels (Kushiro *et al.*, 2004; Okamoto *et al.*, 2006). A similar observation was made for *CS-10A* (*AtERF#111/ABR1*) which is a transcription factor serving as repressor of ABA-regulated gene expression (Pandey *et al.*, 2005). Induction of these genes could lower endogenous ABA levels and/or ABA sensitivity. *CS-08* (*CIPK25*) is probably involved in calcium signaling which could be involved in various processes like stomatal closure (Klusener *et al.*, 2002). Recently, it was shown that ABA plays a role in Arabidopsis susceptibility to *P. syringae* (de Torres-Zabala *et al.*, 2007). *P. syringae* virulence factors specifically manipulate the ABA biosynthetic pathway and response machinery. Enhanced ABA levels were detected during bacterial growth and ABA hypersensitive mutants show enhanced susceptibility. Moreover, alterations in ABA levels are not just a physiological response as *in planta* expression of the bacterial effector AvrPtoB increased ABA levels (de Torres-Zabala *et al.*, 2007). There could be a role for ABA in susceptibility to *H. parasitica*. The ABA-deficient mutant *aba1-1* showed partial resistance to compatible *H. parasitica* isolates however, ABA-insensitivity had no effect on virulence (Mohr and Cahill, 2003; Mauch-Mani and Mauch, 2005), suggesting that ABA levels are important for *H. parasitica* virulence whereas a functional ABA signaling cascade is not. Responsiveness to ABA was found for 8 *CS*-genes according to AtGenExpress data. However, many of the defence-related genes also contain ABRE(-like) elements. Therefore, genes responsive to ABA are not specific for a compatible interaction.

Recently it was shown that there is crosstalk between cold and osmotic stress pathways. Genes transcriptionally activated during cold stress are regulated by DRE elements in their promoters and during osmotic stress by ABRE elements, which are ABA dependent (Shinozaki and Yamaguchi-Shinozaki, 2000; Yamaguchi-Shinozaki and Shinozaki, 2006). A model was proposed for *RD29A* (also known as *COR78* or *LTI78*) in which DRE and ABRE

elements in the promoter of this gene are required for the subsequent stress tolerance. Several *CS*-genes (*CS-01, -02, -03, -10A* and *-15*) hold combinations of ABRE-like and DRE elements like DRE-core, DREB1a/CBF and/or CBF1. It could be that ABA responsive *CS*-genes are compatible specific expressed because their promoters contain this combination of elements.

*CS*-genes could primarily respond to the physiological consequences of *H. parasitica* infections e.g. due water and nutrient loss. Alternatively, the pathogen could secrete effectors targeting the expression of *CS*-genes. Three *CS*-genes, *CS-8* (*CIPK25*), *CS-14* (*LTI6A*/*RCI2AS*) and *CS-15* (*Cor15B*) become specifically induced by compatible interactions with the Cala2 isolate but not in interactions with the Waco9 isolate. This suggests that their expression is induced by pathogen produced effectors or compounds. How this would aid the pathogen in growth and/or nutrient retrieval is unclear. It may be that the CS-proteins enhance nutrient production of the host or they could lower plant defences by mimicking a certain type of abiotic stress. Knock-out and overexpressing lines of *CS*-genes are being studied to elucidate their role in compatible *H. parasitica* infections and disease susceptiblity in general.

**Experimental procedures**

*Growth and inoculation of Arabidopsis*

Arabidopsis ecotype Landsberg-*erecta* (L*er*) was grown on potting soil for 11 days (Primasta, Asten, The Netherlands) in a growth chamber (Snijders Scientific, Tilburg, The Netherlands) at 22 ºC with 16 h of light (~100 µmol photons $m^{-2}$ $sec^{-1}$) and a relative humidity of 70%. Plants were subsequently mock-inoculated or treated with 50 sporangia $\mu l^{-1}$ Cala2 or Waco9 respectively using a spray gun. After inoculation plants were allowed to dry for 2 hours and subsequently incubated under a sealed lid with 100% relative humidity in a growth chamber at 16 ºC with 9 h of light (~100 µmol photons $m^{-2}$ $sec^{-1}$).

*RNA extraction*

RNA was extracted with an RNeasy kit (Qiagen, Venlo, The Netherlands) and treated with the RNase-free DNase set (Qiagen) yielding approximately 50 µg RNA per isolation. The quantity of RNA was measured using an UVmini-1240 spectrophotometer (Shimadzu, Kyoto, Japan)

and the quality with a bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA, USA) using the RNA 6000 Nano Assay kit (Agilent Technologies).

*CATMA arrays*

Microarray analysis was performed with CATMA version 2 arrays (complete Arabidopsis transcriptome microarray) Allemeersch et al., 2005; (Hilson *et al.*, 2004; Allemeersch *et al.*, 2005). CATMA version 2 contained 24 411 gene-specific tags (GSTs). The GSTs (which are between 150 and 500 bp in length and show no more than 70% identity with any other sequence in the genome) were spotted on GAPSII glass slides (Corning Incorporated, Acton, MA, USA) using a BioRobotics Microgrid II TAS spotter (Genomic Solutions, Ann Arbor, MI, USA) and cross-linked for 4 h at 80 ºC. Detailed information about CATMA and database access can be found at http://www.catma.org/ (Crowe *et al.*, 2003) and http://genomics.bio.uu.nl/.

*Labelling, hybridisation and scanning*

The complete microarray procedure used in this article is extensively described before (de Jong *et al.*, 2006). Briefly, mRNA from isolated RNA was amplified with the MessageAmp II aRNA kit (Ambion, Austin, TX, USA). Amplified mRNA was used as template to synthesize modified cDNA with SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and random nonamers (Gene Link, Westchester County, NY, USA) with incorporation of 5-(3-aminoallyl)-dUTP (Ambion). Obtained cDNA was labelled with either Cy3 or Cy5 mono-reactive dye (Amersham, Buckinghamshire, UK) and incorporation was determined using an UVmini-1240 spectrophotometer at 550 or 650 nm, respectively. The probes were hybridised overnight on CATMA arrays and scans were made using a ScanArray Express HT (PerkinElmer, Wellesley, MA, USA). Spot intensities of the scans were determined by ImaGene software version 6.5.1 (BioDiscovery, El Segundo, CA, USA).

*Statistics*

Analysis of spot intensities from the CATMA arrays and applied statistics were performed as described before (de Jong *et al.*, 2006). Biological replicates of the mock-treated and Waco9 or Cala2 inoculated materials were analysed separately to visualise the variation between them. Differentially expressed (DE) genes in the Cala2 or Waco9 interactions at 3 dpi were

selected based on -0.75≥M≥0.75 ($M=Log_2(A/B)$) and q-values<0.05. Pearson correlations were calculated from the average M-values ($log_2$-ratios) obtained from DE genes present in the overlay between the interactions.

*Quantitative PCR*

Extracted RNA from biological replicates for each interaction (mock, Cala2 or Waco9) was pooled in equal amounts and cDNA was subsequently synthesized with SuperScript III reverse transcriptase (Invitrogen) and oligo(dT)15 (Promega, Madison, WI, USA). Timeseries for each interaction from 0 dpi to 5 dpi with 1 day intervals were made from pooled RNA from three biological replicates per time point. Cycle tresholds ($C_T$) were determined in triplicate per transcript by the ABI PRISM 7700 sequence detection system (Applied Biosystems, Foster City, CA, USA) using SYBR Green I (Applied Biosystems) as reporter dye. Primer sets with amplicons between 99 and 101 bases for the $C_T$ determination of all transcripts discussed in this paper are given in the supplementary material (Table S3).

**Acknowledgements**

We would like to thank the following people from our microarray facility for their general help and providing us with the CATMA arrays: Frank L.H. Menke, Sharon C. Mithoe and Annemiek Andel. This research was funded by a VIDI innovational research grant from The Netherlands Organization for Scientific Research (NWO) to Guido Van den Ackerveken and by the Centre for BioSystems Genomics (CBSG), which is part of the Netherlands Genome Initiative.

**Supplementary Material**

**Table S1.** Selection of defence-related genes induced during compatible and incompatible *H. parasitica* interactions. The 660 genes from Figure 1b contain many known defence-related (DR) genes from Arabidopsis. The average fold-change ($log_2$-ratios) for these genes is given for the compatible interaction (C) and the incompatible interaction (I). (Note that three important DR-genes like *PR-1* (*AT2G14610*), *NPR1/NIM1* (*AT1G64280*) and *PDF1.2* (*AT5G44420*) are not present on the CATMA version 2 array). References (1) (Nakano *et al.*, 2006) (2) (Berrocal-Lobo *et al.*, 2002) (3) (McGrath *et al.*, 2005) (4) (Eulgem *et al.*, 2000) (5) (Dong *et al.*, 2003) (6) (Zhang and Wang, 2005) (7) (Chen and Chen, 2002) (8) (Xu *et al.*, 2006) (9) (Yanhui *et al.*, 2006) (10) (Bensmihen *et al.*, 2002) (11) (Jakoby *et al.*, 2002) (12) (Bensmihen *et al.*, 2005) (13) (He *et al.*, 1999) (14) (Rate *et al.*, 1999) (15) (Parker *et al.*, 1996) (16) (Bartsch *et al.*, 2006) (17) (Mishina and Zeier, 2006) (18) (Jambunathan and Mahalingam, 2006) (19) (Glazebrook *et al.*, 1997) (20) (Thomma *et al.*, 2002) (21) (Nawrath *et al.*, 2002) (22) (Dong *et al.*, 1991) (23) (Potter *et al.*, 1993) (24) (Uknes *et al.*, 1992) (25) (Wildermuth *et al.*, 2001) (26) (Weigel *et al.*, 2001) (27) (Weigel *et al.*, 2005) (28) (Reuber and Ausubel, 1996) (29) (Chapter 4; Van Damme *et al.*, 2008) (30) (Chini and Loake, 2005) (31) (Hua *et al.*, 2001) (32) (Chen *et al.*, 2006) (33) (Consonni *et al.*, 2006) (34) (Sanderfoot *et al.*, 2000) (35) (Collins *et al.*, 2003) (36) (Schaller *et al.*, 2000).

| **Defence-related (DR) genes** | | | | |
|---|---|---|---|---|
| | | | | |
| **AGI-ID** | **Name** | **C** | **I** | **Ref** |
| | | | | |
| *At3g50260* | *AtERF#011* | 1.8 | 1.41 | 1 |
| *At1g28370* | *AtERF#076/AtERF-11* | 1.02 | 1.27 | 1 |
| *At2g44840* | *AtERF#099/AtERF-13* | 0.83 | 1.35 | 1 |
| *At4g17500* | *AtERF#100/AtERF-1* | 2.23 | 2.55 | 1,2 |
| *At5g47220* | *AtERF#101/AtERF-2* | 2.13 | 2.77 | 1,3 |
| *At5g47230* | *AtERF#102/AtERF-5* | 1.63 | 1.27 | 1 |
| *At5g61600* | *AtERF#104* | 1.64 | 1.69 | 1 |
| *At5g13330* | *AtERF#113* | 1.66 | 1.5 | 1 |
| *At5g46350* | *WRKY8* | 2.03 | 2.14 | 4,5,6 |
| *At2g23320* | *WRKY15* | 1.39 | 1.29 | 4,5,6 |
| *At4g31800* | *WRKY18* | 2.26 | 1.83 | 4,7,5,6,8 |
| *At2g30250* | *WRKY25* | 0.86 | 0.75 | 4,5,6 |
| *At4g18170* | *WRKY28* | 0.84 | 1.05 | 4,5,6 |
| *At5g24110* | *WRKY30* | 2.11 | 1.04 | 4,5,6 |
| *At5g22570* | *WRKY38* | 1.22 | 1.44 | 4,5,6 |
| *At1g80840* | *WRKY40* | 1.27 | 1.57 | 4,5,6,8 |
| *At2g46400* | *WRKY46* | 2.03 | 2.46 | 4,5,6 |
| *At5g26170* | *WRKY50* | 1.81 | 2.4 | 4,5,6 |

| AGI-ID | Name | C | I | Ref |
|---|---|---|---|---|
| *At2g40750* | *WRKY54* | 1.14 | 0.99 | 4,5,6 |
| *At2g25000* | *WRKY60* | 1.92 | 1.79 | 4,5,6,8 |
| *At5g13080* | *WRKY75* | 2.16 | 2.78 | 6 |
| *At2g47190* | *MYB2* | 1.19 | 1.5 | 9 |
| *At4g09460* | *MYB6* | 0.81 | 0.94 | 9 |
| *At3g23250* | *MYB15* | 2.13 | 2.43 | 9 |
| *At1g49010* | *MYB-Like* | 0.81 | 1.02 | 9 |
| *At2g42660* | *MYB-Like* | 1.3 | 1.4 | - |
| *At5g23650* | *MYB-Like* | 1.2 | 1.01 | - |
| *At2g41070* | *bZIP12* | 0.88 | 1.01 | 10,11,12 |
| *At1g42990* | *bZIP60* | 0.74 | 0.86 | 11 |
| *At1g21270* | *WAK2* | 2.14 | 2.44 | 13 |
| *At4g14400* | *ACD6* | 3.09 | 3.73 | 14 |
| *At3g48090* | *EDS1* | 1.49 | 1.69 | 15 |
| *At1g19250* | *FMO1* | 2.12 | 2.87 | 16,17 |
| *At4g12720* | *NUDT7/GFG1* | 2.37 | 2.47 | 16,18 |
| *At3g52430* | *PAD4* | 3.31 | 3.27 | 19 |
| *At1g19610* | *PDF1.4* | 1.83 | 2.68 | 20 |
| *At4g39030* | *EDS5/SID1* | 2.05 | 2.1 | 21 |
| *At3g57260* | *PR-2/BGL2* | 1.36 | 1.58 | 22 |
| *At3g04720* | *PR-4/HEL* | 2.95 | 3.99 | 23 |
| *At1g75040* | *PR-5* | 1.75 | 2.73 | 24 |
| *At1g74710* | *ICS1* | 2.44 | 2.42 | 25 |
| *At1g02450* | *NIMIN-1* | 2.2 | 1.92 | 26,27 |
| *At3g25882* | *NIMIN-2* | 3.82 | 4.22 | 26 |
| *At1g33960* | *AIG1* | 4.21 | 4.68 | 28 |
| *At3g28930* | *AIG2* | 2.35 | 3.16 | 28 |
| *At5g24530* | *DMR6* | 3.31 | 3.6 | 29 |
| *At4g33300* | *ADR1-L1* | 1.49 | 1.63 | 30 |
| *At3g61190* | *BAP1* | 2.9 | 2.8 | 31 |
| *At5g61900* | *BON1* | 1.55 | 1.69 | 31 |
| *At1g11310* | *MLO2* | 1.09 | 1.07 | 32,33 |
| *At2g39200* | *MLO12* | 0.98 | 0.77 | 32,33 |
| *At3g11820* | *PEN1/SYP121* | 1.18 | 1.06 | 34,45 |
| *At3g52400* | *SYP122* | 1.11 | 1.15 | 34 |
| *At2g06050* | *OPR3* | 1.1 | 1.43 | 36 |

**Table S2.** Sequence-tagged insertion mutants in CS genes. T-DNA lines inserted in CS genes were investigated, when available, for requirement in compatibility. Several inserted lines were tested to minimize the chance to find false-positives. The mutated lines were obtained from (i) NASC (Scholl *et al.*, 2000) or (ii) Génétique et amélioration des plantes, respectively (Bechtold and Pelletier, 1998).

| **T-DNA lines in *CS*-genes** | | |
|---|---|---|
| | | |
| ***Q-PCR-ID*** | ***AGI-ID*** | ***T-DNA lines*** |
| *CS-01* | *At1g01470* | x |
| *CS-02* | *At1g20440* | N103649 (E), N850911 (5) |
| *CS-03* | *At1g51090* | N842310 (E), N825426 (E) |
| *CS-05* | *At3g50970* | N614915 (5), N614914 (5) |
| *CS-06* | *At4g19230* | N569127 (E), N502069 (E/3), N854291 (E) |
| *CS-07* | *At4g20970* | N806504 (E), N102664 (E), N818753 (I), N847069 (E) |
| *CS-08* | *At5g25110* | N529271 (E), N579011 (E), N559092 (5) |
| ***Q-PCR-ID*** | ***AGI-ID*** | ***T-DNA lines*** |
| *CS-09* | *At5g61890* | N500833 (P) |
| *CS-10A* | *At5g64750* | N542372 (I), N512151 (E), N618861 (I) |
| *CS-11* | *At1g15010* | N564541 (5) |
| *CS-12* | *At1g63040* | FLAG_486C09 (E), N121567 (E), N603737 (5) |
| *CS-14* | *At4g30650* | N101822 (3), N618610 (5), N608033 (5) |
| *CS-15* | *At2g42530* | N580284 (I), N585398 (3), N103788 (E), N106713 (E) |
| *CS-17* | *At2g15890* | x |
| *CS-18* | *At2g36450* | x |
| *CS-19* | *At5g52020* | N593012 (5), N510482 (5) |
| *CS-20* | *At4g34410* | N650615 (E), N650614 (E), N650622 (E) |

*Abbreviations: E: Exon; I: Intron; P: 1000-Promoter; 5: 300-UTR5; 3: 300-UTR3.

**Table S3.** Primer sets with amplicons between 99-101 bases for $C_T$ determination of all transcripts discussed in this chapter.

| ***Compatible specific (CS)*** | | | | | |
|---|---|---|---|---|---|
| **AGI-ID** | **Q-PCR-ID** | | **Sequence (5'-3')** | | **Sequence (5'-3')** |
| *AT1G01470* | *CS-01* | Fw | ATATCAAGAGCCGTCATGTCC | Rv | GATCAGTTTCACTTTCCACAGC |
| *AT1G20440* | *CS-02* | Fw | ATCCTCTGCTTTCTCGTCGT | Rv | AGAGTGTGGTGGAGCATGAC |
| *AT1G51090* | *CS-03* | Fw | GAAAGCTATCCGCAAATTCC | Rv | AGCCTCTCAGGATCGTAACAA |
| *AT3G20410* | *CS-04* | Fw | AAACACGAATGCAATGAGAAT | Rv | TTTTGATTCATCATACTTACGCAAT |
| *AT3G50970* | *CS-05* | Fw | ATGGATCACACCAAACTGGA | Rv | TTCTCCATAACTTTTTCGGTCA |
| *AT4G19230* | *CS-06* | Fw | GGGGAAAATGTTAGTACAAGTCTC | Rv | GAAGTCTCAATCCAGGAGGAA |
| *AT4G20970* | *CS-07* | Fw | ATGATGCTGTCTTCCACACC | Rv | AACACTGTTCACAATCTTCTCCA |
| *AT5G25110* | *CS-08* | Fw | CGACCACACTATCTCCTCCA | Rv | GCCGTCGTTGAGTTGTGTAA |
| *AT5G61890* | *CS-09* | Fw | AGAGCCACCAGAAGACGAAG | Rv | TAATTTGGGCGGTGGGTAT |
| *AT5G64750* | *CS-10A* | Fw | AAGCGGTTAAGGCAGCTAAT | Rv | CGGACCTGACATGTTACTCG |
| *AT5G64760* | *CS-10B* | Fw | CCACTGAATCACCTCCATTG | Rv | GAGCCTACTCAATGCAACCTT |
| *AT1G15010* | *CS-11* | Fw | AAACGTTTCTCCCATTGCAT | Rv | ACGAGAACATGGTGGTCCTT |
| *AT1G63040* | *CS-12* | Fw | ATACGGCGGAATCTTCAACT | Rv | GAGCCACCATCATCCCTTT |
| *AT2G34960* | *CS-13* | Fw | CACACTTTCGGTGTTCGTTG | Rv | AAGGGATCATGGATCGGTTA |
| *AT4G30650* | *CS-14* | Fw | TTGCTTGGTGCTGACAATCT | Rv | AGCTCCAAGTTCGGTAGAGC |
| *AT2G42530* | *CS-15* | Fw | CCTCAGTCGCAGTTTCATTG | Rv | TCGTGACGGATAAGACGAAG |
| *AT1G73830* | *CS-16* | Fw | GCTTACAGCAGCAAGTTCGT | Rv | CTTGACCCATCTCCACTGC |
| *AT2G15890* | *CS-17* | Fw | GTCAGGAGCGTGAGAGAACC | Rv | TTGGAGGAAGAAGAGAAATCG |
| *AT2G36450* | *CS-18* | Fw | GAAAACCATCAGCGTCGTAA | Rv | TTCGACATGCCTAATGTGCT |
| *AT5G52020* | *CS-19* | Fw | TTCCATCGTCGGATGTATCA | Rv | GATGAGGAAGCGATGTTGAA |
| *AT4G34410* | *CS-20* | Fw | GAGCGCCACAGATTCAGTT | Rv | CCCAAAATCCATCATCATCA |
| ***Incompatible specific (IS)*** | | | | | |
| ***AGI-ID*** | ***Q-PCR-ID*** | | **Sequence (5'-3')** | | **Sequence (5'-3')** |
| *AT1G52400* | *IS-01* | Fw | TGAGTGGCAAGATGGGTACA | Rv | GAATACCATTTGCCCGAAAC |
| *AT1G52410* | *IS-02* | Fw | AAACTGTTGGCGAAACTTG | Rv | CTCAGAAGCATCAGCCAGAA |
| *AT1G17615* | *IS-03* | Fw | CAACTTCTTGTTGCGTTTGG | Rv | TTCCCTAAAGCCGATTTGAC |
| *AT1G26200* | *IS-04* | Fw | TTTCTGGATCCTATGGAGCA | Rv | TCTAGAGATCAATTCCATAAACAACA |
| *AT1G29965* | *IS-05* | Fw | CTGTGTCTTGACGCCATCTC | Rv | CAGAGCCGTACAGGTTACCA |
| *AT1G61120* | *IS-06* | Fw | CAAAACCGTGCAAGGAGATT | Rv | CATGAAGCATCTCCGTGTTT |
| *AT1G67865* | *IS-07* | Fw | CCGGACTCGTCATATTGTTG | Rv | GATTCTTGGAACGGTCGAT |
| *AT2G07671* | *IS-08A* | Fw | AGCCTGCCTCCACTTTCTTA | Rv | TGGCCTTTTTGATCTTATTCG |
| *ATMG01080* | *IS-08B* | Fw | GACCCTAGCTACGAGTCATTCC | Rv | CCACGGAGTGGTAGACTGAA |
| *ATMG00980* | *IS-09A* | Fw | GCCAGGTGTAAAATCCCATT | Rv | TTGGGTTTTTCTGCACCATA |
| *AT2G07675* | *IS-09B* | Fw | TCGAAGAAGAGGCAGATCAA | Rv | TGATTGTTCCACCGACTGAC |
| *AT4G11230* | *IS-10* | Fw | AGGAACGCAAGAATTGGAGT | Rv | AACCTGGTAATTCCCGTCTG |
| *AT5G27905* | *IS-11* | Fw | AGTCTGGCTGAAAAGTTTGCT | Rv | ACTCGCAAAGTGAGATGGTG |
| *AT5G38750* | *IS-12* | Fw | CGCAGTGAAACTCCGTACTC | Rv | ACTGCTGCAATCATGGAGGT |
| *AT5G62670* | *IS-13* | Fw | TTCAACGAGCTTTCCCAAAT | Rv | CAACAGATTCAACATGACCCTTA |
| *AT5G67380* | *IS-14* | Fw | TGCAGGGATGATATTCCGTA | Rv | TTCAATTCGTCAGTCCCAAG |
| | | | | | |
| ***Defence-related (DR)*** | | | | | |
| | | | | | |
| **AGI-ID** | **Q-PCR-ID** | | **Sequence (5'-3')** | | **Sequence (5'-3')** |
| *AT5G46350* | *WRKY8* | Fw | GAGCTCGAACTCACTCTCCTG | Rv | TGATGATCTCTTCCGTGTGC |
| *AT4G31800* | *WRKY18* | Fw | AAGGGACGCATAACCACTTG | Rv | GACAGCCATTAACCAGATCCA |
| *AT5G24110* | *WRKY30* | Fw | AGCCAGATGCTGGAAAATTG | Rv | TGCTTCAAATGCGAGATCAA |
| *AT1G80840* | *WRKY40* | Fw | TCATGGTGGTTCAGCTTCAA | Rv | TCGTCACTTTCTTCGATTCAA |

| AGI-ID | Q-PCR-ID | | Sequence (5'-3') | | Sequence (5'-3') |
|---|---|---|---|---|---|
| *AT2G46400* | *WRKY46* | Fw | CAACCAATCCTGTCCGAAAT | Rv | GCGAGGTTTTATCTGCACCT |
| *AT5G26170* | *WRKY50* | Fw | GTGGGCTGTTCTTCACCATC | Rv | AAAGGGAGAGTTGCGTTCAA |
| *AT2G25000* | *WRKY60* | Fw | GAGGGACGATTCAAGAGGTTT | Rv | CAATCTCCCGGAAATAGCAG |
| *AT5G13080* | *WRKY75* | Fw | GTGGATTTCTCGATGGGATG | Rv | GGATGCAATGTGAAGAAGCA |
| *AT3G50260* | *AtERF#011* | Fw | GTGGTGCAGAACAACAAACG | Rv | CGGGTCAGGTAACTTGTTCA |
| *AT4G17500* | *AtERF#100* | Fw | TTGTTGAATTTTCCGTTGAGA | Rv | GGAGCTCCGTTCTCGTTAGA |
| *AT5G47220* | *AtERF#101* | Fw | ACCTCCGACGTCAGATTCTC | Rv | ATCTTCTTCGTCGTCGTCGT |
| *AT5G61600* | *AtERF#104* | Fw | CCGTCGTCTCCGACGTATTA | Rv | TTAGGGAAACGAAAGCGAGA |
| *AT5G13330* | *AtERF#113* | Fw | CTTCAGTCAACCTTTTTCAACG | Rv | TTCTTCTTCACGCTGCTGTT |
| *AT3G23250* | *MYB15* | Fw | CTTGGGCTCCGCTAATAATG | Rv | CCGGCTAAGAGATCTTGTTCC |
| | | | | | |
| **Miscellaneous** | | | | | |
| | | | | | |
| **AGI-ID** | **Q-PCR-ID** | | **Sequence (5'-3')** | | **Sequence (5'-3')** |
| *AT3G18780* | *ACT2* | Fw | AATCACAGCACTTGCACCA | Rv | GAGGGAAGCAAGAATGGAAC |
| Not available | *HpACT* | Fw | GTGTCGCACACTGTACCCATTTAT | Rv | ATCTTCATCATGTAGTCGGTCAAGT |
| *AT2G14610* | *PR-1* | Fw | GAACACGTGCAATGGAGTTT | Rv | GGTTCCACCATTGTTACACCT |
| *AT5G44420* | *PDF1.2* | Fw | TGCTTCCATCATCACCCTTA | Rv | ACTTGGCTTCTCGCACAAC |
| *AT1G64280* | *NPR1/NIM 1* | Fw | TCCTAATTCCAAATTGTCCTCA | Rv | GACGACACTGCTGAGAAACG |
| *AT5G24530* | *DMR6* | Fw | CCACAAGTATGTCAATGAGTGG | Rv | CCTCTATTTTAAATCCCACTTCTCTT |
| *AT1G14880* | *Expressed protein* | Fw | CGACTTGGCCAAATGTGATA | Rv | CTCAAGGAGAATGGTCCACA |
| *AT1G71450* | *AtERF#021* | Fw | CAAGCGATCAACGAGTCAAC | Rv | CCAAGCATTCATCTCCACAT |
| *AT1G33760* | *AtERF#022* | Fw | GGCCGGAAAGCTCTAGTTC | Rv | CAGACTCGAGAGCTGGTTCA |
| *AT1G01250* | *AtERF#023* | Fw | CGTCATCATCTCCCATTTTG | Rv | GATCTACCTCGACCGTCCAC |
| *AT1G12630* | *AtERF#027* | Fw | AAGGGGAGGAGGAGAAAAAG | Rv | ACGACGAAGATCCAACATCA |
| *AT5G51990* | *AtERF#028 /CBF4* | Fw | AACACACCACCATTCTGCTC | Rv | CGACGGAGGGATCTAAAACT |
| *AT4G25490* | *AtERF#029 /CBF1* | Fw | GCCAAATCCTGGTTTTCTTG | Rv | GTGAGACTCGTCACCCAATTT |
| *AT4G25470* | *AtERF#030 /CBF2* | Fw | TCTCCTCCATGTCAAGACCA | Rv | TGCCAAGGAAATCCAAAAG |
| *AT4G25480* | *AtERF#031 /CBF3* | Fw | ACGTCTCCTCCATGTCGAA | Rv | CGCTAAGGACATCCAAAAGG |
| *AT1G43160* | *AtERF#108* | Fw | ACTCTCGATGTATGGAGCAACA | Rv | GATCATATTCGGTCCATGGTT |
| *AT5G50080* | *AtERF#110* | Fw | GTTGCAAAGTTCAGGCCAAC | Rv | GAAGGAGGCAGAGGTTGTTC |
| *AT2G33710* | *AtERF#112* | Fw | ACAAGTTGGCACTGGAATCA | Rv | CGTGACACCACCTAGTCCAC |
| *AT5G07310* | *AtERF#115* | Fw | CCGGAGGCTCAATGTATAATC | Rv | CTGGCGTAATCTTGTTCATCA |

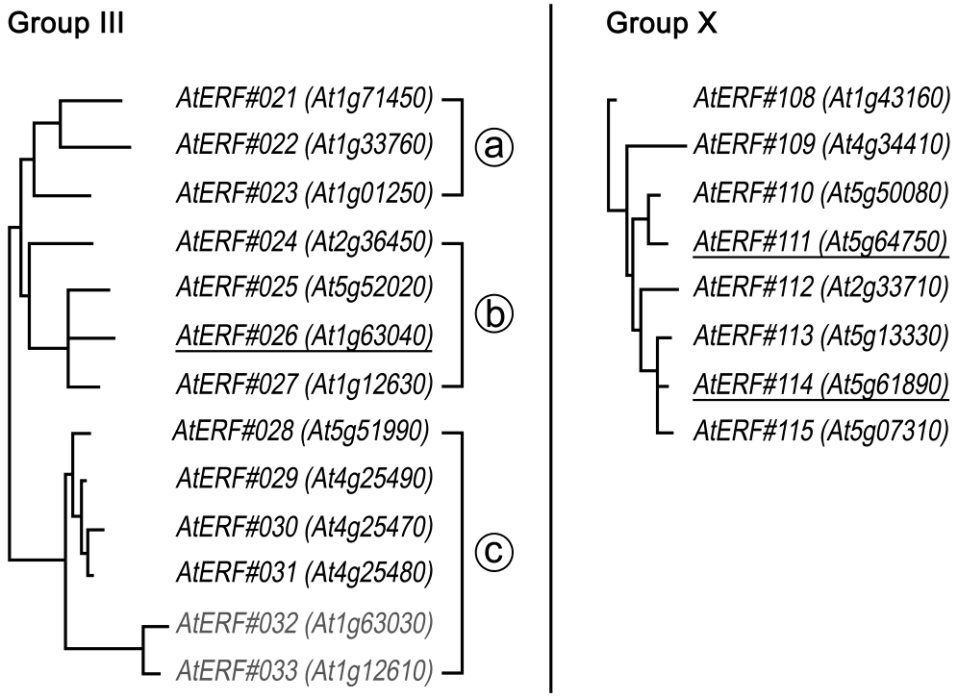


**Figure S1.** Phylogenetic relationship between ERF transcription factors in group III and X (according to Nakano *et al.,* 2006). The underlined ERFs in group III and X were identified to be specifically differentially expressed (DE) in the compatible interaction by microarray analysis. *CS-12*/*AtERF#026* belongs to subclade III-b whereas *CS-10A*/*AtERF#111* and *CS-09*/*AtERF#114* belong to clade X. ERFs marked in light gray in subclade III-c were not taken along for further analysis.

## References

**Agarwal, P.K., Agarwal, P., Reddy, M.K. and Sopory, S.K.** (2006). Role of DREB transcription factors in abiotic and biotic stress tolerance in plants. Plant Cell Rep. 25, 1263-74.

**Allemeersch, J., Durinck, S., Vanderhaeghen, R., Alard, P., Maes, R., Seeuws, K., Bogaert, T., Coddens, K., Deschouwer, K., Van Hummelen, P., Vuylsteke, M., Moreau, Y., Kwekkeboom, J., Wijfjes, A.H., May, S., Beynon, J., Hilson, P. and Kuiper, M.T.** (2005). Benchmarking the CATMA microarray. A novel tool for Arabidopsis transcriptome analysis. Plant Physiol. 137, 588-601.

**Ayliffe, M.A., Roberts, J.K., Mitchell, H.J., Zhang, R., Lawrence, G.J., Ellis, J.G. and Pryor, T.J.** (2002). A plant gene up-regulated at rust infection sites. Plant Physiol. 129, 169-80.

**Bailey, P.C., Martin, C., Toledo-Ortiz, G., Quail, P.H., Huq, E., Heim, M.A., Jakoby, M., Werber, M. and Weisshaar, B.** (2003). Update on the basic helix-loop-helix transcription factor gene family in Arabidopsis thaliana. Plant Cell. 15, 2497-502.

**Barrett, T., Suzek, T.O., Troup, D.B., Wilhite, S.E., Ngau, W.C., Ledoux, P., Rudnev, D., Lash, A.E., Fujibuchi, W. and Edgar, R.** (2005). NCBI GEO: mining millions of expression profiles--database and tools. Nucleic Acids Res. 33, D562-6.

**Bartsch, M., Gobbato, E., Bednarek, P., Debey, S., Schultze, J.L., Bautor, J. and Parker, J.E.** (2006). Salicylic acid-independent ENHANCED DISEASE SUSCEPTIBILITY1 signaling in Arabidopsis immunity and cell death is regulated by the monooxygenase FMO1 and the Nudix hydrolase NUDT7. Plant Cell. 18, 1038-51.

**Bensmihen, S., Rippa, S., Lambert, G., Jublot, D., Pautot, V., Granier, F., Giraudat, J. and Parcy, F.** (2002). The homologous ABI5 and EEL transcription factors function antagonistically to fine-tune gene expression during late embryogenesis. Plant Cell. 14, 1391-403.

**Bensmihen, S., Giraudat, J. and Parcy, F.** (2005). Characterization of three homologous basic leucine zipper transcription factors (bZIP) of the ABI5 family during Arabidopsis thaliana embryo maturation. J Exp Bot. 56, 597-603.

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

**Capel, J., Jarillo, J.A., Salinas, J. and Martinez-Zapater, J.M.** (1997). Two homologous low-temperature-inducible genes from Arabidopsis encode highly hydrophobic proteins. Plant Physiol. 115, 569-76.

**Chen, C. and Chen, Z.** (2002). Potentiation of developmentally regulated plant defence response by AtWRKY18, a pathogen-induced Arabidopsis transcription factor. Plant Physiol. 129, 706-16.

**Chen, W., Provart, N.J., Glazebrook, J., Katagiri, F., Chang, H.S., Eulgem, T., Mauch, F., Luan, S., Zou, G., Whitham, S.A., Budworth, P.R., Tao, Y., Xie, Z., Chen, X., Lam, S., Kreps, J.A., Harper, J.F., Si-Ammour, A., Mauch-Mani, B., Heinlein, M., Kobayashi, K., Hohn, T., Dangl, J.L., Wang, X. and Zhu, T.** (2002). Expression profile matrix of Arabidopsis transcription factor genes suggests their putative functions in response to environmental stresses. Plant Cell. 14, 559-74.

**Chen, Z., Hartmann, H.A., Wu, M.J., Friedman, E.J., Chen, J.G., Pulley, M., Schulze-Lefert, P., Panstruga, R. and Jones, A.M.** (2006). Expression analysis of the AtMLO gene family encoding plant-specific seven-transmembrane domain proteins. Plant Mol Biol. 60, 583-97.

**Chini, A. and Loake, G.J.** (2005). Motifs specific for the ADR1 NBS-LRR protein family in Arabidopsis are conserved among NBS-LRR sequences from both dicotyledonous and monocotyledonous plants. Planta. 221, 597-601.

**Chisholm, S.T., Coaker, G., Day, B. and Staskawicz, B.J.** (2006). Host-microbe interactions: shaping the evolution of the plant immune response. Cell. 124, 803-14.

**Choi, H., Hong, J., Ha, J., Kang, J. and Kim, S.Y.** (2000). ABFs, a family of ABA-responsive element binding factors. J Biol Chem. 275, 1723-30.

**Collins, N.C., Thordal-Christensen, H., Lipka, V., Bau, S., Kombrink, E., Qiu, J.L., Huckelhoven, R., Stein, M., Freialdenhoven, A., Somerville, S.C. and Schulze-Lefert, P.** (2003). SNARE-protein-mediated disease resistance at the plant cell wall. Nature. 425, 973-7.

**Consonni, C., Humphry, M.E., Hartmann, H.A., Livaja, M., Durner, J., Westphal, L., Vogel, J., Lipka, V., Kemmerling, B., Schulze-Lefert, P., Somerville, S.C. and Panstruga, R.** (2006). Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. Nat Genet. 38, 716-20.

**Crowe, M.L., Serizet, C., Thareau, V., Aubourg, S., Rouze, P., Hilson, P., Beynon, J., Weisbeek, P., van Hummelen, P., Reymond, P., Paz-Ares, J., Nietfeld, W. and Trick, M.** (2003). CATMA: a complete Arabidopsis GST database. Nucleic Acids Res. 31, 156-8.

**de Jong, M., van Breukelen, B., Wittink, F.R., Menke, F.L., Weisbeek, P.J. and Van den Ackerveken, G.** (2006). Membrane-associated transcripts in Arabidopsis; their isolation and characterization by DNA microarray analysis and bioinformatics. Plant J. 46, 708-21.

**de Torres-Zabala, M., Truman, W., Bennett, M.H., Lafforgue, G., Mansfield, J.W., Rodriguez Egea, P., Bogre, L. and Grant, M.** (2007). Pseudomonas syringae pv. tomato hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. Embo J. 26, 1434-43.

**Dong, J., Chen, C. and Chen, Z.** (2003). Expression profiles of the Arabidopsis WRKY gene superfamily during plant defence response. Plant Mol Biol. 51, 21-37.

**Dong, X., Mindrinos, M., Davis, K.R. and Ausubel, F.M.** (1991). Induction of Arabidopsis defence genes by virulent and avirulent Pseudomonas syringae strains and by a cloned avirulence gene. Plant Cell. 3, 61-72.

**Edgar, R., Domrachev, M. and Lash, A.E.** (2002). Gene Expression Omnibus: NCBI gene expression and hybridization array data repository. Nucleic Acids Res. 30, 207-10.

**Eulgem, T., Rushton, P.J., Robatzek, S. and Somssich, I.E.** (2000). The WRKY superfamily of plant transcription factors. Trends Plant Sci. 5, 199-206.

**Fowler, S.G., Cook, D. and Thomashow, M.F.** (2005). Low temperature induction of Arabidopsis CBF1, 2, and 3 is gated by the circadian clock. Plant Physiol. 137, 961-8.

**Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M. and Thomashow, M.F.** (1998). Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J. 16, 433-42.

**Gilmour, S.J., Fowler, S.G. and Thomashow, M.F.** (2004). Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol Biol. 54, 767-81.

**Glazebrook, J., Zook, M., Mert, F., Kagan, I., Rogers, E.E., Crute, I.R., Holub, E.B., Hammerschmidt, R. and Ausubel, F.M.** (1997). Phytoalexin-deficient mutants of Arabidopsis reveal that PAD4 encodes a regulatory factor and that four PAD genes contribute to downy mildew resistance. Genetics. 146, 381-92.

**Grant, S.R., Fisher, E.J., Chang, J.H., Mole, B.M. and Dangl, J.L.** (2006). Subterfuge and manipulation: type III effector proteins of phytopathogenic bacteria. Annu Rev Microbiol. 60, 425-49.

**Ha, S.B. and An, G.** (1988). Identification of upstream regulatory elements involved in the developmental expression of the Arabidopsis thaliana cab1 gene. Proc Natl Acad Sci U S A. 85, 8017-21.

**Hao, D., Yamasaki, K., Sarai, A. and Ohme-Takagi, M.** (2002). Determinants in the sequence specific binding of two plant transcription factors, CBF1 and NtERF2, to the DRE and GCC motifs. Biochemistry. 41, 4202-8.

**Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A. and Kay, S.A.** (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science. 290, 2110-3.

**He, Z.H., Cheeseman, I., He, D. and Kohorn, B.D.** (1999). A cluster of five cell wall-associated receptor kinase genes, Wak1-5, are expressed in specific organs of Arabidopsis. Plant Mol Biol. 39, 1189-96.

**Hilson, P., Allemeersch, J., Altmann, T., Aubourg, S., Avon, A., Beynon, J., Bhalerao, R.P., Bitton, F., Caboche, M., Cannoot, B., Chardakov, V., Cognet-Holliger, C., Colot, V., Crowe, M., Darimont, C., Durinck, S., Eickhoff, H., de Longevialle, A.F., Farmer, E.E., Grant, M., Kuiper, M.T., Lehrach, H., Leon, C., Leyva, A., Lundeberg, J., Lurin, C., Moreau, Y., Nietfeld, W., Paz-Ares, J., Reymond, P., Rouze, P., Sandberg, G., Segura, M.D., Serizet, C., Tabrett, A., Taconnat, L., Thareau, V., Van Hummelen, P., Vercruysse, S., Vuylsteke, M., Weingartner, M., Weisbeek, P.J., Wirta, V., Wittink, F.R., Zabeau, M. and Small, I.** (2004). Versatile gene-specific sequence tags for Arabidopsis functional genomics: transcript profiling and reverse genetics applications. Genome Res. 14, 2176-89.

**Hua, J., Grisafi, P., Cheng, S.H. and Fink, G.R.** (2001). Plant growth homeostasis is controlled by the Arabidopsis BON1 and BAP1 genes. Genes Dev. 15, 2263-72.

**Jakoby, M., Weisshaar, B., Droge-Laser, W., Vicente-Carbajosa, J., Tiedemann, J., Kroj, T. and Parcy, F.** (2002). bZIP transcription factors in Arabidopsis. Trends Plant Sci. 7, 106-11.

**Jambunathan, N. and Mahalingam, R.** (2006). Analysis of Arabidopsis growth factor gene 1 (GFG1) encoding a nudix hydrolase during oxidative signaling. Planta. 224, 1-11.

**Jones, J.D. and Dangl, J.L.** (2006). The plant immune system. Nature. 444, 323-9.

**Kay, S., Hahn, S., Marois, E., Hause, G. and Bonas, U.** (2007). A bacterial effector acts as a plant transcription factor and induces a cell size regulator. Science. 318, 648-51.

**Kimura, M., Yamamoto, Y.Y., Seki, M., Sakurai, T., Sato, M., Abe, T., Yoshida, S., Manabe, K., Shinozaki, K. and Matsui, M.** (2003). Identification of Arabidopsis genes regulated by high light-stress using cDNA microarray. Photochem Photobiol. 77, 226-33.

**Klusener, B., Young, J.J., Murata, Y., Allen, G.J., Mori, I.C., Hugouvieux, V. and Schroeder, J.I.** (2002). Convergence of calcium signaling pathways of pathogenic elicitors and abscisic acid in Arabidopsis guard cells. Plant Physiol. 130, 2152-63.

**Kolukisaoglu, U., Weinl, S., Blazevic, D., Batistic, O. and Kudla, J.** (2004). Calcium sensors and their interacting protein kinases: genomics of the Arabidopsis and rice CBL-CIPK signaling networks. Plant Physiol. 134, 43-58.

**Kushiro, T., Okamoto, M., Nakabayashi, K., Yamagishi, K., Kitamura, S., Asami, T., Hirai, N., Koshiba, T., Kamiya, Y. and Nambara, E.** (2004). The Arabidopsis cytochrome P450 CYP707A encodes ABA 8'-hydroxylases: key enzymes in ABA catabolism. Embo J. 23, 1647-56.

**Li, C., Bai, Y., Jacobsen, E., Visser, R., Lindhout, P. and Bonnema, G.** (2006). Tomato defence to the powdery mildew fungus: differences in expression of genes in susceptible, monogenic- and polygenic resistance responses are mainly in timing. Plant Mol Biol. 62, 127-40.

**Maleck, K., Levine, A., Eulgem, T., Morgan, A., Schmid, J., Lawton, K.A., Dangl, J.L. and Dietrich, R.A.** (2000). The transcriptome of Arabidopsis thaliana during systemic acquired resistance. Nat Genet. 26, 403-10.

**Marois, E., Van den Ackerveken, G. and Bonas, U.** (2002). The xanthomonas type III effector protein AvrBs3 modulates plant gene expression and induces cell hypertrophy in the susceptible host. Mol Plant Microbe Interact. 15, 637-46.

**Mauch-Mani, B. and Mauch, F.** (2005). The role of abscisic acid in plant-pathogen interactions. Curr Opin Plant Biol. 8, 409-14.

**McGrath, K.C., Dombrecht, B., Manners, J.M., Schenk, P.M., Edgar, C.I., Maclean, D.J., Scheible, W.R., Udvardi, M.K. and Kazan, K.** (2005). Repressor- and activator-type ethylene response factors functioning in jasmonate signaling and disease resistance identified via a genome-wide screen of Arabidopsis transcription factor gene expression. Plant Physiol. 139, 949-59.

**Medina, J., Catala, R. and Salinas, J.** (2001). Developmental and stress regulation of RCI2A and RCI2B, two cold-inducible genes of arabidopsis encoding highly conserved hydrophobic proteins. Plant Physiol. 125, 1655-66.

**Medina, J., Rodriguez-Franco, M., Penalosa, A., Carrascosa, M.J., Neuhaus, G. and Salinas, J.** (2005). Arabidopsis mutants deregulated in RCI2A expression reveal new signaling pathways in abiotic stress responses. Plant J. 42, 586-97.

**Mishina, T.E. and Zeier, J.** (2006). The Arabidopsis flavin-dependent monooxygenase FMO1 is an essential component of biologically induced systemic acquired resistance. Plant Physiol. 141, 1666-75.

**Mitchell, H.J., Ayliffe, M.A., Rashid, K.Y. and Pryor, A.J.** (2006). A rust-inducible gene from flax (fis1) is involved in proline catabolism. Planta. 223, 213-22.

**Mitsuya, S., Taniguchi, M., Miyake, H. and Takabe, T.** (2005). Disruption of RCI2A leads to over-accumulation of Na+ and increased salt sensitivity in Arabidopsis thaliana plants. Planta. 222, 1001-9.

**Mohr, P.G. and Cahill, D.M.** (2003). Abscisic acid influences the susceptibility of Arabidopsis thaliana to Pseudomonas syringae pv. tomato and Peronospora parasitica. Funct Plant Biol. 30, 461-9.

**Nakano, T., Suzuki, K., Fujimura, T. and Shinshi, H.** (2006). Genome-wide analysis of the ERF gene family in Arabidopsis and rice. Plant Physiol. 140, 411-32.

**Nawrath, C., Heck, S., Parinthawong, N. and Metraux, J.P.** (2002). EDS5, an essential component of salicylic acid-dependent signaling for disease resistance in Arabidopsis, is a member of the MATE transporter family. Plant Cell. 14, 275-86.

**Nurnberger, T., Brunner, F., Kemmerling, B. and Piater, L.** (2004). Innate immunity in plants and animals: striking similarities and obvious differences. Immunol Rev. 198, 249-66.

**Nylander, M., Svensson, J., Palva, E.T. and Welin, B.V.** (2001). Stress-induced accumulation and tissue-specific localization of dehydrins in Arabidopsis thaliana. Plant Mol Biol. 45, 263-79.

**O'Connor, T.R., Dyreson, C. and Wyrick, J.J.** (2005). Athena: a resource for rapid visualization and systematic analysis of Arabidopsis promoter sequences. Bioinformatics. 21, 4411-3.

**Okamoto, M., Kuwahara, A., Seo, M., Kushiro, T., Asami, T., Hirai, N., Kamiya, Y., Koshiba, T. and Nambara, E.** (2006). CYP707A1 and CYP707A2, which encode abscisic acid 8'-hydroxylases, are indispensable for proper control of seed dormancy and germination in Arabidopsis. Plant Physiol. 141, 97-107.

**Pandey, G.K., Grant, J.J., Cheong, Y.H., Kim, B.G., Li, L. and Luan, S.** (2005). ABR1, an APETALA2-domain transcription factor that functions as a repressor of ABA response in Arabidopsis. Plant Physiol. 139, 1185-93.

**Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D. and Daniels, M.J.** (1996). Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora parasitica specified by several different RPP genes. Plant Cell. 8, 2033-46.

**Potter, S., Uknes, S., Lawton, K., Winter, A.M., Chandler, D., DiMaio, J., Novitzky, R., Ward, E. and Ryals, J.** (1993). Regulation of a hevein-like gene in Arabidopsis. Mol Plant Microbe Interact. 6, 680-5.

**Rate, D.N., Cuenca, J.V., Bowman, G.R., Guttman, D.S. and Greenberg, J.T.** (1999). The gain-of-function Arabidopsis acd6 mutant reveals novel regulation and function of the salicylic acid signaling pathway in controlling cell death, defences, and cell growth. Plant Cell. 11, 1695-708.

**Restrepo, S., Myers, K.L., del Pozo, O., Martin, G.B., Hart, A.L., Buell, C.R., Fry, W.R. and Smart, C.D.** (2005). Gene profiling of a compatible interaction between Phytophthora infestans and Solanum tuberosum suggests a role for carbonic anhydrase. Mol Plant Microbe Interact. 18, 913-22.

**Reuber, T.L. and Ausubel, F.M.** (1996). Isolation of Arabidopsis genes that differentiate between resistance responses mediated by the RPS2 and RPM1 disease resistance genes. Plant Cell. 8, 241-9.

**Reuber, T.L., Plotnikova, J.M., Dewdney, J., Rogers, E.E., Wood, W. and Ausubel, F.M.** (1998). Correlation of defence gene induction defects with powdery mildew susceptibility in Arabidopsis enhanced disease susceptibility mutants. Plant J. 16, 473-85.

**Sanderfoot, A.A., Assaad, F.F. and Raikhel, N.V.** (2000). The Arabidopsis genome. An abundance of soluble N-ethylmaleimide-sensitive factor adaptor protein receptors. Plant Physiol. 124, 1558-69.
**Schaller, F., Biesgen, C., Mussig, C., Altmann, T. and Weiler, E.W.** (2000). 12-Oxophytodienoate reductase 3 (OPR3) is the isoenzyme involved in jasmonate biosynthesis. Planta. 210, 979-84.
**Shinozaki, K. and Yamaguchi-Shinozaki, K.** (2000). Molecular responses to dehydration and low temperature: differences and cross-talk between two stress signaling pathways. Curr Opin Plant Biol. 3, 217-23.
**Stotz, H.U., Pittendrigh, B.R., Kroymann, J., Weniger, K., Fritsche, J., Bauke, A. and Mitchell-Olds, T.** (2000). Induced plant defence responses against chewing insects. Ethylene signaling reduces resistance of Arabidopsis against Egyptian cotton worm but not diamondback moth. Plant Physiol. 124, 1007-18.
**Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G. and Katagiri, F.** (2003). Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen Pseudomonas syringae. Plant Cell. 15, 317-30.
**Thomma, B.P., Cammue, B.P. and Thevissen, K.** (2002). Plant defensins. Planta. 216, 193-202.
**Truman, W., de Zabala, M.T. and Grant, M.** (2006). Type III effectors orchestrate a complex interplay between transcriptional networks to modify basal defence responses during pathogenesis and resistance. Plant J. 46, 14-33.
**Uknes, S., Mauch-Mani, B., Moyer, M., Potter, S., Williams, S., Dincher, S., Chandler, D., Slusarenko, A., Ward, E. and Ryals, J.** (1992). Acquired resistance in Arabidopsis. Plant Cell. 4, 645-56.
**Van Damme, M., Huibers, R.P., Elberse, J. and Van den Ackerveken, G.** (2008). Arabidopsis DMR6 encodes a putative 2OG-Fe(II) oxygenase that is defence-associated but required for susceptibility to downy mildew. Plant J. 54, 785-93.
**Weigel, R.R., Bauscher, C., Pfitzner, A.J. and Pfitzner, U.M.** (2001). NIMIN-1, NIMIN-2 and NIMIN-3, members of a novel family of proteins from Arabidopsis that interact with NPR1/NIM1, a key regulator of systemic acquired resistance in plants. Plant Mol Biol. 46, 143-60.
**Weigel, R.R., Pfitzner, U.M. and Gatz, C.** (2005). Interaction of NIMIN1 with NPR1 modulates PR gene expression in Arabidopsis. Plant Cell. 17, 1279-91.
**Wildermuth, M.C., Dewdney, J., Wu, G. and Ausubel, F.M.** (2001). Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature. 414, 562-5.
**Wilhelm, K.S. and Thomashow, M.F.** (1993). Arabidopsis thaliana cor15b, an apparent homologue of cor15a, is strongly responsive to cold and ABA, but not drought. Plant Mol Biol. 23, 1073-7.
**Xu, X., Chen, C., Fan, B. and Chen, Z.** (2006). Physical and functional interactions between pathogen-induced Arabidopsis WRKY18, WRKY40, and WRKY60 transcription factors. Plant Cell. 18, 1310-26.
**Yamaguchi-Shinozaki, K. and Shinozaki, K.** (2006). Transcriptional regulatory networks in cellular responses and tolerance to dehydration and cold stresses. Annu Rev Plant Biol. 57, 781-803.
**Yanhui, C., Xiaoyuan, Y., Kun, H., Meihua, L., Jigang, L., Zhaofeng, G., Zhiqiang, L., Yunfei, Z., Xiaoxiao, W., Xiaoming, Q., Yunping, S., Li, Z., Xiaohui, D., Jingchu, L., Xing-Wang, D., Zhangliang, C., Hongya, G. and Li-Jia, Q.** (2006). The MYB transcription factor superfamily of Arabidopsis: expression analysis and phylogenetic comparison with the rice MYB family. Plant Mol Biol. 60, 107-24.
**Zhang, Y. and Wang, L.** (2005). The WRKY transcription factor superfamily: its origin in eukaryotes and expansion in plants. BMC Evol Biol. 5, 1.
**Zipfel, C. and Felix, G.** (2005). Plants and animals: a different taste for microbes? Curr Opin Plant Biol. 8, 353-60.

# Chapter 6

## General Discussion

The mechanism by which host plants contribute to their colonization by biotrophic and hemibiotrophic fungal and oomycete pathogens is one of the interesting themes in molecular plant pathology. A limited number of genes have been identified previously (see introduction) that are specifically required for compatibility to powdery mildew. Knowledge regarding genes involved in basic compatibility to other groups of biotrophic and hemibiotrophic pathogens is lacking. This research aimed to identify Arabidopsis genes involved in basic compatibility to the biotrophic downy mildew pathogen *Hyaloperonospora parasitica*.

***H. parasitica* resistance in *dmr* mutants is associated with defence responses**

To study basic compatibility a screen for loss of compatibility to *H. parasitica* was performed. The six *dmr* mutants identified (Chapter 2; Van Damme et al., 2005) can be divided in those showing broad range disease resistance (*dmr3* and *dmr4*) and those showing specific resistance to *H. parasitica* (*dmr1*, *dmr2*, *dmr5* and *dmr6*). Recently it was shown that *dmr2* is allelic with *dmr1* (Van den Ackerveken, unpublished data), showing that both mutants are affected in the same gene. Broad range disease resistance mutants have been known for years and loss of compatibility in these mutants is tightly associated with high SA and *PR-1* expression levels (Bowling et al., 1994; Rate and Greenberg, 2001; Greenberg and Ausubel, 1993; Dietrich et al., 1994; Yu et al., 1998). In addition, the majority of these mutants show stunted growth phenotypes and develop spontaneous necrotic lesions. Not surprisingly, dwarfism and constitutive high expression of *PR-1* accompanied broad range disease resistance in *dmr3* and *dmr4*. Pathogen-specific loss of compatibility mutants could potentially mediate resistance in absence of known defence responses. Consequently these mutants could identify novel defence mechanisms or host components required for pathogen growth and development. *H. parasitica* resistance mediated by *dmr1*, *dmr5* and *dmr6* is however, associated with enhanced activation of known defence responses albeit that these mutants show *H. parasitica*-specific loss of compatibility.

*dmr5* was shown to be a gain-of-function allele of *RPM1* (Chapter 3). RPM1 is normally activated upon infection with *P. syringae* strains carrying *avrB* or *avrRpm1* and confers full resistance (Bisgrove et al., 1994). However, *dmr5* does not mediate resistance to *P. syringae*. This suggests that constitutive RPM1 activity is sufficient to mediate *H. parasitica* resistance, but too low to mediate resistance to *P. syringae*. In addition, it suggests that *H.*

*parasitica* is more sensitive to *R* gene mediated defences compared to *P. syringae*. Only 64 genes were found to be differentially expressed in *dmr5*. In contrast, around 2000 genes become induced or repressed during incompatible interactions with *P. syringae*, including those mediated by *RPM1* (Tao et al., 2003), indicating that defence activation is indeed low in *dmr5*. The constitutive high expression of *PR-1*, among other defence-related genes, in *dmr5* was *NPR1* dependent. Resistance to *H. parasitica* was also largely dependent on *NPR1*, indicating that it results from the enhanced expression of defence-related genes. The constitutive high expression of *PR-1* in *dmr6* mutants was significantly lower compared to *dmr3*, *dmr4* and *dmr5* as *PR-1* transcripts in *dmr6* were not detected by Northern blot analyses (Chapter 2; Van Damme et al., 2005). Expression profiles of *dmr6* compared with those of *dmr3* and *dmr4* showed that defence-related gene expression is in general much lower in *dmr6* (Van den Ackerveken, unpublished results). Possibly, *dmr6* is not altered in its susceptibility to *P. syringae* due to lower defence-related gene expression. *DMR6* encodes a putative 2OG-Fe(II) oxygenase that is highly induced upon inoculation with virulent and avirulent *H. parasitica* isolates (Chapter 4; Van Damme et al., 2008). Moreover, high *DMR6* transcript levels were observed in *dmr3*, *dmr4* and *dmr5* and after treatment with the SA-analog BTH, sugesting that *DMR6* functions in plant defence. The enhanced expression of defence-related genes, including *PR-1*, in *dmr6* mutants, indicates that DMR6 negatively modulates plant defences.

Plant cells often react to invading hyphae of fungal and oomycete pathogens by the local formation of cell wall appositions (papillae). These cell wall appositions typically contain phytoalexins, phenolics, callose, silicon, $H_2O_2$ and proteins (Hardham, 2007; Hückelhoven, 2005). Formation of papillae is thought to block penetration of the pathogen and or slow down pathogen growth enabling the plant to activate additional defences (Hardham, 2007; Hückelhoven, 2005). Loss of compatibility in *dmr1* was accompanied with the enhanced formation of papillae during *H. parasitica* infections (Chapter 2; Van Damme et al; 2005). *DMR1* encodes a homoserine kinase (HSK) that converts homoserine to O-Phospho-homoserine (Van Damme, 2007). Homoserine accumulated in all different *dmr1* mutants. Exogenous application of L-homoserine to wild-type plants resulted in *H. parasitica* resistance and accompanied with enhanced papillae formation (Van Damme, 2007). The mechanism by which homoserine enhances *H. parasitica* induced papillae formation as well

as whether papillae formation is the cause of *H. parasitica* resistance in *dmr1* mutants is unknown.

**_dmr_ versus _pmr_ mutants**

*H. parasitica*-specific loss of compatibility in *dmr1*, *dmr5* and *dmr6* is associated with enhanced defence responses of the plant. Loss of compatibility to powdery mildew is also associated with enhanced defence activation. The *powdery mildew resistant (pmr) 4* mutant accumulates SA and shows enhanced *PR-1* expression. Moreover, blocking of the SA pathway, in *pmr4*/*sid2* double mutants, restored susceptibility to the powdery mildew *G. cichoracearum*, demonstrating that high SA levels are the cause of *pmr4*-mediated resistance (Nishimura et al., 2003). Interestingly, *pmr4* mutants show no reduction in susceptibility to *P. syringae* but slightly reduced susceptible to *H. parasitica* (Vogel and Somerville, 2000). Similarly, *dmr5* and *dmr6* show a slight reduction in their susceptibility to the powdery mildew *G. orontii* but not to *P. syringae* (Chapter 2; Van Damme et al., 2005). This suggests that downy and powdery mildews are more sensitive to SA-dependent defences compared to *P. syringae*. Similarly to *pmr4*, *pmr1-1* mutants expressed higher levels of *PR-1* compared to wild-type plants after challenge with *G. cichoracearum*. However, *pmr1-1* mutants were not altered in their susceptibility to the closely related species *G. orontii* as was observed for all other *pmr* mutants or to *H. parasitica* (Vogel and Somerville, 2000).

*mlo*-mediated powdery mildew resistance in barley results from the failure of the pathogen to penetrate the epidermal cell wall. This type of resistance is accompanied by enhanced deposition of callose-containing papillae at the site of pathogen entry (Piffanelli et al., 2002; Peterhansel et al., 1997; Wolter et al., 1993). Papillae are also formed in healthy unchallenged *mlo* plants indicating that in wild-type plants MLO negatively modulates this response. Disruption of the homologous Arabidopsis gene *AtMLO2/PMR2* gave similar results and papillae formation was increased in *Atmlo2/6*, *Atmlo2/12* and *Atmlo2/6/12* double and triple mutants (Consonni et al., 2006). In contrast, papillae formation in *dmr1* was only seen after pathogen challenge. Interestingly, *mlo* mutants showed no reduction in susceptibility to *H. parasitica* (Consonni et al., 2006). Visa versa, *dmr1* mutants were normally susceptible to powdery mildew (Chapter 2; Van Damme et al., 2005), indicating that both responses are quite different from each other.

The plant cell wall functions as a constitutive defence layer of the plant. Powdery mildew resistance was associated with alterations in cell wall composition in the Arabidopsis *pmr5* and *pmr6* mutants. Increased levels of pectin (among other alterations) were discovered in isolated cell walls of *pmr5* and *pmr6* by Fourier transform infrared spectroscopy (Vogel et al., 2002; Vogel et al., 2004). *PMR5* is a gene of unknown function belonging to a large family of plant-specific genes (Vogel et al., 2004). *PMR6* encodes a pectate lyase like protein, suggesting a pectin degrading activity for PMR6 (Vogel et al., 2002). Although *pmr5* and *pmr6* show cell wall alterations including increased levels of pectin, penetration and haustoria formation by the powdery mildew fungus is not blocked. The observed powdery mildew resistance in *pmr5* and *pmr6* is therefore likely not caused by a decrease in penetration efficiency. An alternative explanation could be that the extrahaustorial matrix has a different composition, especially of modified pectins, decreasing nutrient transport to the fungus. Whatever the precise basis for resistance, the *pmr5* and *pmr6* mutants highlight the importance of cell wall constitution in plant-powdery mildew interactions and compatibility. No alterations in cell wall constitution were detected in the identified *dmr* mutants. However, changes in cell wall composition were not closely examined and can therefore not be excluded.

The identification of *dmr* and *pmr* mutants has been the first step in studying basic compatibility to biotrophic pathogens. In the majority of these mutants, loss of compatibility is associated with alterations (enhanced activation) of constitutive and inducible defence mechanisms of the host. This suggests that the structural and regulatory organization of plant defence mechanism is the major determinant of basic compatibility to these pathogens. However, new screens for loss of compatibility are being performed and these studies will provide new insights into basic compatibility (O'Connell and Panstruga, 2006). Although forward genetic approaches are very useful in identifying host genes involved in compatibility, these approaches are limited as only genes can be identified that give a visible phenotype when disrupted. Compatibility genes that are vital for plant life, make small contributions to compatibility, or that act redundantly can not be identified using forward genetics. Therefore, a different approach to study basic compatibility was taken by identifying host genes that become induced or repressed during compatible *H. parasitica* infections. This strategy was applied in Chapter 5 and will be discussed below.

**Host genes specifically induced during compatible interactions with *H. parasitica***

Transcriptional profiling was used as a tool to identify host genes that become induced or repressed during compatible and/or incompatible *H. parasitica* interactions. Changes in host gene-expression in both interactions were remarkably similar. Such observations have also been reported for other plant-pathogen interactions (Tao et al., 2003; Li et al., 2006). Together these data indicate that *R*-gene mediated defences accelerate and amplify MAMP/PAMP-triggered defences resulting in the transcriptional activation or repression of a similar set of defence-related genes. Genes that become transcriptionally activated during these responses could make a negative or a positive contribution to the defence response and consequently on pathogen growth. For example the *EDS1* and *DMR6* genes are both transcriptionally activated during compatible (and incompatible) interactions (Chapter 5). However, disruption of *DMR6* results in reduced susceptibility whereas disruption of *EDS1* results in enhanced disease susceptibility (Parker et al., 1996). It is clear, also with respect to the previous section, that modulation of defence responses influence compatibility and that negative regulators of defence can be identified as compatibility genes. The identified *CS*-genes (Chapter 5) are therefore of interest as they are likely not induced due to an activated defence response. These genes could provide insight into non defence-related processes during compatible interactions.

The 17 identified *CS*-genes have so far not been associated with plant-pathogen interactions before. Interestingly promoter analyses of all *CS*-genes revealed an overrepresentation of the DREB1A/CBF3 (Dehydration Responsive Element/C-repeat Binding Factor (CBF)) and the EveningElement involved in evening specific expression (Harmer et al., 2000). However, both elements were not found in the promoter of *CS-12* (*AtERF#26*), *CS-18* (*AtERF#24*) and *CS-19* (*AtERF#25*). These three ERF transcription factors belong to subclade III-b of the ERF transcription factor family. The subclade IIIc genes, *CBF1*, *CBF2* and *CBF3* become induced by low temperature and activate transcription of genes with DRE elements in their promoter (Gilmour et al., 1998; Gilmour et al., 2004). Interestingly, induction of these *CBF* genes by low temperature was controlled by the circadian clock (Fowler et al., 2005) and links circadian rhythm with DRE element controlled transcription. However, the three *CBF* genes were not induced during *H. parasitica* infections and therefore it was suggested that subclade III-b members are responsible for the activation of DRE element containing *CS*-genes during *H. parasitica* infections (Chapter 5).

The link between low temperature, circadian rhythm and compatible *H. parasitica* interactions is not clear. However, expression of several *CS*-genes has been associated with multiple forms of abiotic stress and/or ABA and suggests that compatible *H. parasitica* interactions provoke an abiotic stress-like response. One could envision that the flow of water and nutrients from host to pathogen provokes a similar response as encountered during dehydration or osmotic stress. It is remarkable that several *CS*-genes were found to be expressed at their highest level when the pathogen had its highest growth rate, suggesting that *CS*-genes become induced due to the physiological consequences of disease. However, the correlation with abiotic stress is an intriguing lead towards understanding susceptibility, because ABA has recently been associated with suppression of defences by *P. syringae* effectors (de Torres-Zabala et al., 2007). Exogenous application ABA was shown to suppress SA accumulation and the expression of defence-related genes during incompatible interactions with *P. syringae* (Mohr and Cahill, 2007). In addition increased susceptibility was observed to avirulent *P. syringae* strains upon exogenous application of ABA (Mohr and Cahill, 2003). There could be a role for ABA in susceptibility to *H. parasitica* as the ABA-deficient mutant *aba1-1* showed partial resistance to compatible *H. parasitica* isolates (Mohr and Cahill, 2003). ABA-insensitivity had no effect on virulence (Mohr and Cahill, 2003; Mauch-Mani and Mauch, 2005), suggesting that ABA levels are important for *H. parasitica* virulence whereas a functional ABA signaling cascade is not.

Three *CS*-genes, *CS-8* (*CIPK25*), *CS-14* (*LTI6A*/*RCI2AS*) and *CS-15* (*Cor15B*) become specifically induced by compatible interactions with the Cala2 isolate but not in interactions with the Waco9 isolate. If *CS*-genes become induced due to the physiological consequences of disease one would not expect to find isolate-specific induction. However, isolate-specific induction could result from a difference in the type and number of effector proteins that are encoded in the genomes of different isolates. There is still very limited knowledge about *H. parasitica* effector proteins and the molecular function they play during infections. The *H. parasitica* effector proteins ATR1 and ATR13 trigger *RPP1*-Nd/WsB- and *RPP13*-Nd-dependent resistance, respectively, in Arabidopsis. The potential role these effector proteins play during compatible interactions was assessed by using a novel delivery system using *P. syringae* type III secretion, via fusions of *ATR*s to the N terminus of the *P. syringae* effector protein AvrRPS4 (Sohn et al., 2007). Multiple alleles of *ATR1* and *ATR13* conferred enhanced virulence to *P. syringae* on susceptible Arabidopsis accessions. Two *ATR13* alleles suppressed

MAMP/PAMP-triggered callose deposition and another allele of *ATR13* suppressed MAMP/PAMP-triggered ROS production in addition to callose deposition (Sohn et al., 2007).

The four oomycete effector proteins that have been characterised in more detail to date, Avr1b, Avr3a, ATR13 and ATR1A, share a common RxLR sequence motif (Armstrong *et al.*, 2005; Rehmany *et al.*, 2005). This sequence motif was shown to be equivalent to the *Plasmodium* host-targeting motif in its ability to translocate proteins from *Plasmodium* to human erythrocytes (Bhattacharjee et al., 2006). Avr3a becomes secreted by haustoria and requires its RxLR motif for translocation into the host cell (Whisson et al., 2007). Using bioinformatics, several hundred RxLR motif containing proteins have been identified to be encoded in the genomes of single oomycete species (Whisson et al., 2007; Win et al., 2007; Jiang et al., 2008). All these proteins are potential effectors as they are likely translocated to the host cell during the infection process. Possibly, several effector proteins will be involved in transcriptional reprogramming of the host to promote virulence. However, from the more then 850 Arabidopsis genes that were identified as differentially expressed during compatible interactions at 3 dpi, only 14 *CS*-genes were identified by CATMA microarrays (Chapter 5). This suggests that *H. parasitica* effectors do not activate the transcription of many Arabidopsis genes that are not part of the plants defence response and that the majority of effectors will function in suppressing plant defences to establish compatibility. Alternatively, the low number of identified *CS*-genes could result from detection limitations of the used technique. Genes that become induced or repressed specifically in host cells that contain haustoria are more difficult to detect. RNA used for transcriptional profiling was derived from total leaf extracts. Therefore, RNA derived from haustoria-containing cells will be a minor fraction of the total RNA pool. Consequently, the level of induction or repression of these genes will be underestimated or will not be detected at all. However, differential expression of *DMR6* was clearly detected in the microarray experiments, despite the fact that *DMR6* is specifically induced in haustoria-containing cells. Several techniques are available that would allow isolation of haustoria-containing cells e.g. laser dissection or marker based cell sorting after protoplasting leaf cells. Using these techniques one could study processes specificly occurring in these cells.

**Conclusion**

This study identified eight downy mildew resistant mutants that corresponded to six different loci. The identified *dmr* mutants could be divided in those showing broad range disease resistance (*dmr3* and *dmr4*) and those showing resistance specific to *H. parasitica* (*dmr1*, *dmr2*, *dmr5* and *dmr6*). Defence responses are not only associated with *dmr3* and *dmr4* but also with *dmr1*, *dmr5* and *dmr6*. *dmr5* encodes an weak autoactive form of RPM1 and defence activation in *dmr6* is due to disruption of a putative 2OG-Fe(II) oxygenase that negatively modulates defence responses. Enhanced defence activation is also seen in loss of compatibility mutants specific to powdery mildews, suggesting that the structural and regulatory organization of plant defence mechanism is the major determinant of basic compatibility to both downy and powdery mildews. Using a reverse genetics approach, 17 Arabidopsis genes were identified that become induced during compatible but not incompatible *H. parasitica* interactions. So far it is unclear which roles these so called *CS*-genes play during the infection process. They may become induced due to the physiological consequences of disease or alternatively, become specifically induced by *H. parasitica* effector proteins. The later argument is favored for three *CS*-genes, *CS-8* (*CIPK25*), *CS-14* (*LTI6A/RCI2AS*) and *CS-15* (*Cor15B*) as they showed isolate-specific induction. The identification of *CS*-genes is a first step in unraveling disease specific processes and their role in compatibility.

## References

**Armstrong, M.R., Whisson, S.C., Pritchard, L., Bos, J.I., Venter, E., Avrova, A.O., Rehmany, A.P., Böhme, U., Brooks, K., Cherevach, I., Hamlin, N., White, B., Fraser, A., Lord, A., Quail, M.A., Churcher, C., Hall, N., Berriman, M., Huang, S., Kamoun, S., Beynon, J.L. and Birch, P.R.** (2005). An ancestral oomycete locus contains late blight avirulence gene Avr3a, encoding a protein that is recognized in the host cytoplasm. Proc Natl Acad Sci USA. 102, 7766-71.

**Bhattacharjee, S., Hiller, N.L., Liolios, K., Win, J., Kanneganti, T.D., Young, C., Kamoun, S. and Haldar, K.** (2006). The malarial host-targeting signal is conserved in the Irish potato famine pathogen. PLoS Pathogens. 2, e50.

**Bisgrove, S.R., Simonich, M.T., Smith, N.M., Sattler, A. and Innes, R.W.** (1994). A disease resistance gene in Arabidopsis with specificity for two different pathogen avirulence genes. Plant Cell. 6, 927-33.

**Bowling, S.A., Guo, A., Cao, H., Gordon, A.S., Klessig, D.F. and Dong, X.** (1994). A mutation in Arabidopsis that leads to constitutive expression of systemic acquired resistance. Plant Cell. 6, 1845-57.

**Consonni, C., Humphry, M.E., Hartmann, H.A., Livaja, M., Durner, J., Westphal, L., Vogel, J., Lipka, V., Kemmerling, B., Schulze-Lefert, P., Somerville, S.C. and Panstruga, R.** (2006). Conserved requirement for a plant host cell protein in powdery mildew pathogenesis. Nat Genet. 38, 716-20.

**De Torres-Zabala, M., Truman, W., Bennett, M.H., Lafforgue, G., Mansfield, J.W., Rodriguez Egea, P., Bogre, L. and Grant, M.** (2007). Pseudomonas syringae pv. tomato hijacks the Arabidopsis abscisic acid signalling pathway to cause disease. Embo J. 26, 1434-43.

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

**Fowler, S.G., Cook, D. and Thomashow, M.F.** (2005). Plant Physiol. 137, 961-8.

**Gilmour, S.J., Zarka, D.G., Stockinger, E.J., Salazar, M.P., Houghton, J.M. and Thomashow, M.F.** (1998). Low temperature regulation of the Arabidopsis CBF family of AP2 transcriptional activators as an early step in cold-induced COR gene expression. Plant J. 16, 433-42.

**Gilmour, S.J., Fowler, S.G. and Thomashow, M.F.** (2004). Arabidopsis transcriptional activators CBF1, CBF2, and CBF3 have matching functional activities. Plant Mol Biol. 54, 767-81.

**Greenberg, J.T. and Ausubel, F.M.** (1993). Arabidopsis mutants compromised for the control of cellular damage during pathogenesis and aging. Plant J. 4, 327-41.

**Hardham, A.R.** (2007). Cell biology of plant-oomycete interactions. Cell Microbiol. 9, 31-9.

**Harmer, S.L., Hogenesch, J.B., Straume, M., Chang, H.S., Han, B., Zhu, T., Wang, X., Kreps, J.A. and Kay, S.A.** (2000). Orchestrated transcription of key pathways in Arabidopsis by the circadian clock. Science. 290, 2110-3.

**Hückelhoven, R.** (2005). Powdery mildew susceptibility and biotrophic infection strategies. FEMS Microbiol Lett. 245, 9-17.

**Jiang, R.H., Tripathy, S., Govers, F. and Tyler, B.M.** (2008). RXLR effector reservoir in two Phytophthora species is dominated by a single rapidly evolving superfamily with more than 700 members. Proc Natl Acad Sci U S A. 105, 4874-9.

**Li, C., Bai, Y., Jacobsen, E., Visser, R., Lindhout, P. and Bonnema, G.** (2006). Tomato defence to the powdery mildew fungus: differences in expression of genes in susceptible, monogenic- and polygenic resistance responses are mainly in timing. Plant Mol Biol. 62, 127-40.

**Mauch-Mani, B. and Mauch, F.** (2005). The role of abscisic acid in plant-pathogen interactions. Curr Opin Plant Biol. 8, 409-14.

**Mohr, P.G. and Cahill, D.M.** (2003). Abscicic acid influences the susceptibility of Arabidopsis thaliana to Pseudomonas syringae pv. Tomato and Peronospora parasitica. Functional Plant Biology. 30, 461-9.

**Mohr, P.G. and Cahill, D.M.** (2007). Suppression by ABA of salicylic acid and lignin accumulation and the expression of multiple genes, in Arabidopsis infected with Pseudomonas syringae pv. tomato. Funct Integr Genomics. 7, 181-91.

**Nishimura, M.T., Stein, M., Hou, B.H., Vogel, J.P., Edwards, H. and Somerville, S.C.** (2003). Loss of a callose synthase results in salicylic acid-dependent disease resistance. Science. 301, 969-72.

**O'Connell, R.J. and Panstruga, R.** (2006). Tête à tête inside a plant cell: establishing compatibility between plants and biotrophic fungi and oomycetes. New Phytol. 171, 699-718.

**Parker, J.E., Holub, E.B., Frost, L.N., Falk, A., Gunn, N.D. and Daniels, M.J.** (1996). Characterization of eds1, a mutation in Arabidopsis suppressing resistance to Peronospora parasitica specified by several different RPP genes. Plant Cell. 8, 2033-46.

**Peterhansel, C., Freialdenhoven, A., Kurth, J., Kolsch, R. and Schulze-Lefert, P.** (1997). Interaction Analyses of Genes Required for Resistance Responses to Powdery Mildew in Barley Reveal Distinct Pathways Leading to Leaf Cell Death. Plant Cell. 9, 1397-1409.

**Piffanelli, P., Zhou, F., Casais, C., Orme, J., Jarosch, B., Schaffrath, U., Collins, N.C., Panstruga, R. and Schulze-Lefert, P.** (2002). The barley MLO modulator of defence and cell death is responsive to biotic and abiotic stress stimuli. Plant Physiol. 129, 1076-85.

**Rate, D.N. and Greenberg, J.T.** (2001). The Arabidopsis aberrant growth and death2 mutant shows resistance to Pseudomonas syringae and reveals a role for NPR1 in suppressing hypersensitive cell death. Plant J. 27, 203-11.
**Rehmany, A.P., Gordon, A., Rose, L.E., Allen, R.L., Armstrong, M.R., Whisson, S.C., Kamoun, S., Tyler, B.M., Birch, P.R. and Beynon, J.L.** (2005). Differential recognition of highly divergent downy mildew avirulence gene alleles by RPP1 resistance genes from two Arabidopsis lines. Plant Cell. 17, 1839-50.
**Sohn, K.H., Lei, R., Nemri, A. and Jones, J.D.** (2007). The downy mildew effector proteins ATR1 and ATR13 promote disease susceptibility in Arabidopsis thaliana. Plant Cell. 19, 4077-90.
**Tao, Y., Xie, Z., Chen, W., Glazebrook, J., Chang, H.S., Han, B., Zhu, T., Zou, G. and Katagiri, F.** (2003). Quantitative nature of Arabidopsis responses during compatible and incompatible interactions with the bacterial pathogen Pseudomonas syringae. Plant Cell. 15, 317-30.
**Van Damme**. (2007). Genetic analyses of disease susceptibility in the Arabidopsis-Hyaloperonospora parasitica interaction (Ph. D. thesis, Utrecht University, The Netherlands (ISBN: 978-90-393-4515-3)).
**Vogel, J. and Somerville, S.** (2000). Isolation and characterization of powdery mildew-resistant Arabidopsis mutants. Proc Natl Acad Sci U S A. 97, 1897-902.
**Vogel, J.P., Raab, T.K., Schiff, C. and Somerville, S.C.** (2002). PMR6, a pectate lyase like gene required for powdery mildew susceptibility in Arabidopsis. Plant Cell. 14, 2095-106.
**Vogel, J.P., Raab, T.K., Somerville, C.R. and Somerville, S.C.** (2004). Mutations in PMR5 result in powdery mildew resistance and altered cell wall composition. Plant J. 40, 968-78.
**Whisson, S.C., Boevink, P.C., Moleleki, L., Avrova, A.O., Morales, J.G., Gilroy, E.M., Armstrong, M.R., Grouffaud, S., van West, P., Chapman, S., Hein, I., Toth, I.K., Pritchard, L. and Birch, P.R.** (2007). A translocation signal for delivery of oomycete effector proteins into host plant cells. Nature. 450, 115-8.
**Win, J., Morgan, W., Bos, J., Krasileva, K.V., Cano, L.M., Chaparro-Garcia, A., Ammar, R., Staskawicz, B.J. and Kamoun, S.** (2007). Adaptive evolution has targeted the C-terminal domain of the RXLR effectors of plant pathogenic oomycetes. Plant Cell. 19, 2349-69.
**Wolter, M., Hollricher, K., Salamini, F. and Schulze-Lefert, P.** (1993). The mlo resistance alleles to powdery mildew infection in barley trigger a developmentally controlled defence mimic phenotype. Mol Gen Genet. 239, 122-8.
**Yu, I.C., Parker, J. and Bent, A.F.** (1998). Gene-for-gene disease resistance without the hypersensitive response in Arabidopsis dnd1 mutant. Proc Natl Acad Sci U S A. 95, 7819-24.

# Summary

The mechanisms by which host plants contribute to their colonization by hemibiotrophic and biotrophic fungal and oomycete pathogens is a poorly understand aspect of molecular plant pathology. The aim of this study was to identify Arabidopsis genes controlling cellular processes that influence susceptibility to the biotrophic oomycete *Hyaloperonospora parasitica*. Chapter 2 describes a forward genetic approach to identify Arabidopsis mutants that show loss of compatibility to *H. parasitica*. Six of these so called *downy mildew resistant* (*dmr*) mutants were characterized in more detail. *dmr3* and *dmr4* show resistance to a broad range of pathogens whereas *dmr1*, *dmr2*, *dmr5* and *dmr6* show a type of resistance more specific to *H. parasitica*. Chapter 3 shows that constitutive high expression of defense related genes in the *dmr5* mutant contributes to resistance to *H. parasitica*. Surprisingly, it was found that *dmr5* mutants were mutated in *RPM1*, a resistance gene of the NBS-LRR class that confers resistance to *Pseudomonas syringae* bacteria carrying the *avrB* or *avrRPM1* genes. RPM1 function in *dmr5* mutants is largely, but not completely, lost. Genetic data indicate that *dmr5* encodes a mild autoactive form of RPM1. Further studies on the mutant RPM1 protein could provide more insight to R protein function. Chapter 4 describes the map based cloning of *DMR6* that encodes a putative 2OG-Fe(II) oxygenase. *DMR6* is strongly induced in infected host cells during both compatible and incompatible interactions with *H. parasitica. DMR6* is also highly induced after application of BTH and is constitutively high expressed in broad range disease resistance mutants. Absence of a functional *DMR6* gene results in resistance to *H. parasitica,* that is accompanied with high expression of several defense-related genes including *DMR6* itself. Together, the obtained data indicate that *DMR6* is a negative regulator of defense. Chapter 5 describes the identification of a group of Arabidopsis genes that is induced during compatible interactions but not during incompatible interactions. Most of these so called compatible-specific genes (*CS*-genes) are also induced during various abiotic stress conditions such as cold, dehydration, high salt and high light, but also in response to ABA. The activation of *CS*-genes can either reflect a physiological response on pathogen infection or be triggered by pathogen effector proteins. As the *CS*-genes are not induced as part of a defense response they provide a first glimpse into non-defense related processes during compatible interactions.

# Samenvatting

De manier waarop de plant bijdraagt aan zijn kolonisatie door hemibiotrofe en biotrofe schimmels en oomyceten is een onderbelicht aspect in de moleculaire plantenziektekunde. Het doel van het in dit proefschrift beschreven onderzoek was het identificeren van genen van Arabidopsis (Zandraket) die de vatbaarheid voor het valse meeldauw pathogeen *Hyaloperonospora parasitica* beïnvloeden. Hoofdstuk 2 beschrijft een genetische aanpak om mutanten te identificeren die hun vatbaarheid voor *H. parasitica* hebben verloren. Zes van deze zogenaamde *downy mildew resistant* (*dmr*) mutanten werden in meer detail gekarakteriseerd. *dmr3* en *dmr4* zijn resistent tegen een groot aantal verschillende pathogenen, terwijl *dmr1*, *dmr2*, *dmr5* en *dmr6* een type resistentie laten zien wat meer specifiek is voor *H. parasitica*. Hoofdstuk 3 laat zien dat de basale hoge expressie van afweer-gerelateerde genen in de *dmr5* mutant bijdragen aan de waargenomen resistentie tegen *H. parasitica*. Verrassend was de vondst dat *dmr5* gemuteerd is in *RPM1*, een resistentiegen van de NBS-LRR klasse dat resistentie geeft tegen *Pseudomonas* bacterien die het *avrB* of *avrRPM1* gen bevatten. De werking van RPM1 in de *dmr5* mutant is grotendeels verloren. De verkregen genetische data geeft aan dat *dmr5* voor een milde autoactive vorm van RPM1 codeerd. Verdere analyse van het mutante RPM1 eiwit zou meer inzicht kunnen geven in hoe resistentie-eiwitten functioneren. Hoofdstuk 4 beschrijft de klonering van het *DMR6* gen dat codeerd voor een 2OG-Fe(II)-afhankelijke oxygenase. *DMR6* is sterk geïnduceerd in geïnfecteerde planten cellen tijdens zowel compatibele als incompatibele interacties met *H. parasitica. DMR6* is ook sterk geïnduceerd na behandeling met BTH en komt basaal hoog tot expressie in mutanten die een brede resistentie tegen pathogenen hebben. Afwezigheid van een functioneel *DMR6* gen resulteert in resistentie tegen *H. parasitica* wat samengaat met een verhoogde expressie van afweer-gerelateerde genen, inclusief *DMR6*. De verkregen data geeft aan dat *DMR6* functioneert als een negatieve regulator van de afweer. Hoofdstuk 5 beschrijft de identificatie van genen die geïnduceerd worden tijdens compatibele maar niet tijdens incompatibele interacties. Deze zogenoemde compatibel-specifieke genen (*CS*-genen) worden ook geïnduceerd door verschillende abiotische stress-condities zoals koude, uitdroging, zout en intens licht, maar ook door ABA. De activatie van *CS*-genen tijdens het infectieproces kan een fysiologische reactie op infectie door het pathogeen zijn, of gestimuleerd worden door effector eiwitten van het pathogeen. Omdat de *CS*-genen niet

geactiveerd worden als onderdeel van de afweer van de plant geven ze een eerste blik op niet-afweer gerelateerde processen tijdens compatibele interacties.

# Dankwoord

Een aantal mensen wil ik persoonlijk bedanken voor hun bijdrage aan mijn proefschrift. Als eerste mijn co-promoter, Guido bedankt voor je geduld en de vrijheid die ik kreeg om mijn 'eigen' dingen te doen. Al was ik koppig en eigenwijs, ik heb zeker van je geleerd! Peter, bedankt voor je getoonde interesse en corrigerende werk aangaande mijn proefschrift. Karin, het is al weer een tijd terug dat je bij ons in het lab werkte. Je rondleiding door ENZA heb ik erg gewaardeerd. Mireille, ik vond het altijd erg gezellig om met jou samen te werken. Het resultaat daarvan is duidelijk aanwezig in dit boekje. Op mijn beurt hoop ik, dat jij als paranimf met jouw eeuwige optimisme, mij nu een beetje vrolijk door mijn promotie-dag kan loodsen. Inez, na de verhuizing heb ik je gemist, jij was zeker leuker dan die witte muur die ik voor je in de plaats kreeg. Ik hoop en denk dat je me niet te serieus hebt genomen als 'R.P. Bakellende'. Veel sucses met het afronden van je proefschrift. Adriana, het was altijd goed babbelen met jou, we zien elkaar vast nog wel een keer in Köln. Mark, ik ben best tevreden met het resultaat van "The Final Experiment". Het is een mooi hoofdstuk geworden. Gelukkig ben jij er ook bij, als paranimf tijdens mijn 'Final hour'. Hopelijk vinden we daarna nog wat tijd voor het schrijven van wat extra hoofdstukken. Tieme, samen 'brommers kieken' en een strandwandeling (van 15 km!) bij maanlicht in Italië. Wiens metabolisme zou er niet van op hol slaan, één en al romantiek (gelukkig hadden we bier en broodjes giros). Veel succes met het afronden van het DMR6 metabolieten verhaal. Rene, jij blijft een collega!, ook nu we het niet meer zijn. Zonder jou waren alle Sinterklaasfeestjes, Kerst- en andere borrels een stuk saaier geweest en je hebt nog een mooie toevoeging aan het laatste hoofdstuk gegeven; bedankt! Miek en Joyce, die lach-mascotte werkt ook in Duitsland. Nico, we hebben wat af geouwehoerd, althans jij, ik zei meestal een stuk minder terug. Soms serieus, vaak ook niet, vaak met bier, soms ook niet. Hoe dan ook, het was altijd goed. Zet hem op in Leiden en maak dat proefschrift af! Joost, ik hoop dat Bremia zijn effectoren aan je zal prijsgeven. Uiteraard 'mijn' studenten Hélène, Juan en Tina, de fyto's, wortels, suikers, microbiologen, het keukenpersoneel, beeldverwerking en de bollenboer. Verder natuurlijk mijn familie en vrienden. En tot slot: alle groene wezens die het waard zijn te bestuderen!

Robin

# Curriculum vitae

Robin Paul Huibers was born on Februari 27th, 1980 at Alphen aan den Rijn, the Netherlands. He followed his secondary education at the Farel College in Amersfoort. After graduation in 1998 he started with the study biology at Utrecht University, the Netherlands. As part of his education he participated in research projects in the Molecular Genetics group of Prof. Dr. P.J. Weisbeek at Utrecht University, supervised by Dr. G. van den Ackerveken and in the Bacterial Genetics group of Prof. J. van der Oost, supervised by Dr. ir. J. Ettema at Wageningen University, the Netherlands. He continued working for 3 months as a junior researcher in the Bacterial Genetics group at Wageningen University before obtaining his MSc diploma in November 2003. From Januari 2004 until April 2008 he worked as a PhD-student in the sections Plant-Microbe Interactions and Molecular Genetics at Utrecht University. During this period, the research as described in this thesis was carried out under supervision of Prof. Dr. J. Weisbeek and Dr. G. van den Ackerveken. As of June 2008, Robin holds a position as a post-doc in the group of Dr. J. Parker at the Max-Planck-Institut für Züchtungsforschung in Cologne, Germany.