Functional and applied aspects of the *DOWNY MILDEW RESISTANT 1* and *6* genes in Arabidopsis

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Functionele en toegepaste aspecten van de *DOWNY MILDEW RESISTANT 1* en *6* genen van Arabidopsis

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General introduction and thesis outline

Plants in their natural environment face constant exposure to micro-organisms. The majority of micro-organisms are harmless to the plant and do not cause disease. Some micro-organisms are even beneficial to the plant, e.g. symbiotic mycorrhiza fungi that supply plants with phosphates. On the other hand, pathogenic micro-organisms interact with plants antagonistically and are able to cause disease. It is quite remarkable that even though plants are continuously exposed to many potential harmful micro-organisms, very few are able to cause disease. A first barrier of protection is the presence of physical barriers like the cuticle and cell wall. Plants also possess several inducible layers of defence that are activated when required. To be successful, pathogens have to overcome constitutive barriers, suppress the plant's immune response and manipulate host metabolism. Pathogens causing disease have major impact on crop yield and quality in agriculture, demonstrating the importance of the research on this topic. In this general introduction I will focus on how plants are able to defend themselves from potential harmful pathogens and how successful pathogens achieve to overcome host immunity and establish plant disease. Furthermore, I will introduce forms of recessive resistance that could result from loss of host genes required for disease (so called susceptibility genes), with special emphasis on resistance to fungal and oomycete (hemi-)biotrophs.

Plant immunity

Plants are sessile organisms and are unable to escape biotic and abiotic stresses. In order to be able to detect and respond to potential pathogenic micro-organisms, plants rely on an ancient innate immune response which has features in common with the innate immune system of animals e.g. that of *Drosophila* (Ausubel 2005). Vertebrates have, in addition to their innate immune response, an adaptive immune system for the synthesis of antibodies and activation of antigen-responsive circulating cells (Nürnberger et al. 2004). Plants do not have the ability to adapt to pathogens via antigen-specific antibodies. Instead, plant immunity involves cell-autonomous responses and systemic signaling from the infection site onward. When a potential pathogen is capable of accessing the plant's interior, which is achieved through stomatal openings or by direct penetration of the epidermis, it is faced by the plant cell. On the surface of the host cell, plants carry receptor proteins that recognize conserved components of the microbial surface called pathogen-associated molecular patterns (PAMPs). This PAMP perception initiates PAMP-triggered immunity (PTI, Figure 1A). The PTI response is the plant's first active response to microbial attack. Only very few, often highly specialized, microbes are able to suppress PTI via the secretion of effector molecules into the host cell and interfere with immune signaling leading to effector-triggered susceptibility (ETS, Figure 1B). Plants, in turn, have evolved sophisticated mechanisms to recognize, often indirectly, effector molecules and activate resistance. This is the second active pathogen-sensing response of the plant and is termed effector-triggered immunity (ETI, Figure 1C).

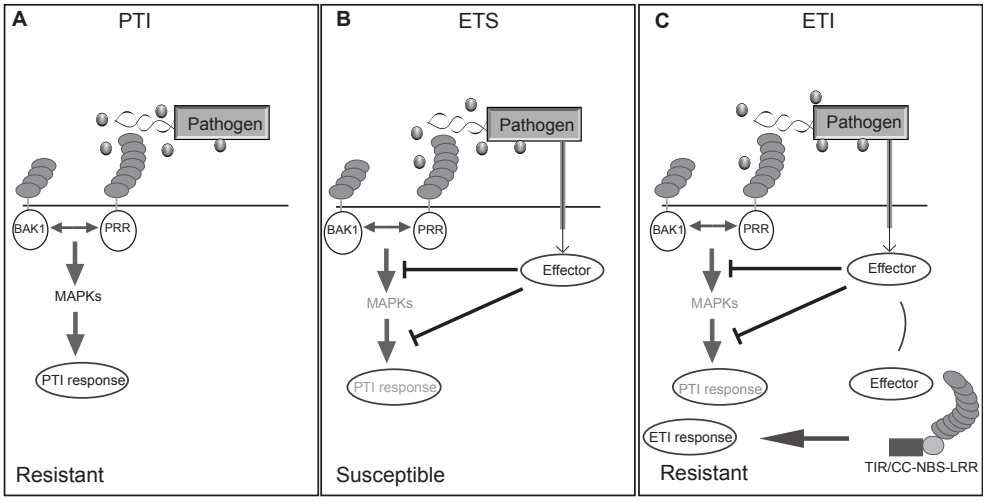

Figure 1. Model for plant immunity. (**A**) Pathogens release pathogen-associated molecular patterns (PAMPs, indicated by the balls) that are perceived by host pattern recognition receptors (PRRs). Upon recognition, BAK1 associates with the PRR, e.g. FLS2, and initiates signaling via mitogen-activated protein kinases (MAPKs) thereby inducing PTI. (**B**) Host targeted effectors are able to suppress PTI leading to ETS. (**C**) Some effectors are recognized by ETI-inducing plant resistance proteins of the TIR/CC-NBS-LRR class. Adapted from Dodds and Rathjen (2010), and Chisholm et al. (2006).

Effector recognition is mediated by resistance (R) proteins and the activation of defence differs from PTI in that it acts faster and stronger e.g. in triggering a hypersensitive response (HR) (excellent reviews from Dodds and Rathjen 2010; Chisholm et al. 2006; Jones and Dangl 2006; Zipfel 2008; Gohre and Robatzek 2008).

PAMP perception and PTI response

PTI generally triggers changes in the host cell at the molecular and physiological level already within minutes after PAMP recognition. Changes in Ca^{2+} levels, reactive oxygen species (ROS) production, and activation of MAP kinase cascades have been reported to constitute the early PAMP response (Asai et al. 2002). Within the hour after activation, major transcriptional changes have been reported which comprise around 3% of the transcriptome (Zipfel et al. 2004). Well known bacterial PAMPs that trigger innate immune responses include lipopolysaccharides (LPS), peptidoglycans (PGN) and flagellin. Recognition of these PAMPs occurs through pattern recognition receptors (PRR) of the host. These receptors contain an extracellular leucine rich repeat (LRR) and an intracellular kinase domain. A well studied Arabidopsis PRR is Flagellin Sensitive 2 (FLS2) (Gómez-Gómez and Boller 2000). FLS2 physically interacts with the N-terminal domain of flagellin, more specifically with the 22 amino acid epitope flg22. Within minutes after the FLS2/flg22 interaction, another receptor kinase, BAK1, binds to FLS2 and forms a heteromeric complex (Chinchilla et al. 2007). BAK1 functions as central regulator of plant innate immunity as it links PRR perception with the

activation of defence responses (Chinchilla et al. 2009). Although BAK1 forms a complex with FLS2 and other PRRs, the response of the *bak1* mutant to flg22 is lower but not completely absent suggesting additional proteins are involved in signaling. Overall, PAMP perception contributes to plant disease resistance at very early stages of infection preventing build up of high pathogen populations.

Another well studied PAMP is the bacterial protein elongation factor Tu (EF-Tu) that is recognized by the EF-Tu receptor (EFR) in Arabidopsis and other members of the family *Brassicaceae,* but not in members of the *Solanaceae* (Kunze et al. 2004). EF-Tu is thought to facilitate bacteria adherence to host cells. PTI triggered by EF-Tu activates a response that highly overlaps with that triggered by flg22, indicating a common downstream signaling pathway (Zipfel et al. 2006). Recently, *EFR* from Arabidopsis was stably expressed in transgenic tomato and *Nicotiana benthamiana* plants that showed increased resistance to several pathogenic bacteria that are recognized through EF-Tu (Lacombe et al. 2010). This demonstrates that tomato and *N. benthamiana* are equipped with all the EFR-signaling components required for PTI. This knowledge can enable the establishment of durable broad-spectrum resistance in the field against important bacterial pathogens.

ETI: The second line of active defence

Highly specialized pathogens are able to suppress PTI by deploying microbe-derived effector molecules that are active in the host cytoplasm. A well studied case is suppression of PTI by the type III secreted AvrPto and AvrPtoB proteins from *Pseudomonas syringae* pv. *tomato* DC3000. When brought into the host, these effector molecules interact with FLS2 and prevent its association with BAK1 (Göhre et al. 2008). By this mechanism pathogens are able to suppress the plant's PTI response and cause ETS. Plants have evolved an elaborate mechanism to recognize, often indirectly, effector molecules and initiate an effective immune response, ETI (Jones and Dangl 2006). Effector molecules were first isolated based on their ability to trigger immune responses in resistant plants (ETI), but it was generally assumed that these effectors also positively contribute to pathogen fitness on susceptible hosts (ETS). It was not until the discovery of the *Pseudomonas hrp* (hypersensitive response and pathogenicity) mutants, which lost HR induction on resistant plants and pathogenesis on susceptible ones, that both avirulence and virulence functions were mediated by the same effector molecules (reviewed by Alfano and Collmer 2004).

Not only bacteria, also fungi and oomycetes contain effector molecules that target plant processes, e.g. *Phytophthora infestans* Avr3a is recognized by the potato R-protein R3a leading to host cell death (Bos et al. 2006). Recently, Avr3a was found to target and stabilize a host CMPG U-box E3 ligase. To induce INF1-induced cell death, CMPG is degraded by the 26S proteasome. However, by Avr3a-induced stabilization, cell death is prevented in the biotrophic phase. Also, when *Avr3a* was silenced it compromised pathogenicity demonstrating its role

in virulence (Bos et al. 2010). Fungi and oomycetes do not contain a type III secretion system like bacteria, but transport proteins through the secretory pathway that is mediated by their N-terminal signal peptide sequences. Apoplastic effector proteins remain, after being secreted from the pathogen, in the extracellular space between host and pathogen cells. There they can contribute to pathogenesis, e.g. by inhibiting host enzymes (Tian et al. 2004). On the other hand, host translocated effectors are, after their secretion from the pathogen, taken up into the host cell. Many host-translocated effectors of oomycetes have a conserved and characteristic RXLR motif after the N-terminal signal sequence (Morgan and Kamoun 2007). This motif is required for the entry of effector molecules into the host cells (Whisson et al. 2007), even in the absence of the oomycete indicating no other pathogen-encoded machinery is necessary for effector delivery (Dou et al. 2008). Two host-targeted oomycete RXLR effectors are ATR1 and ATR13 of *Hyaloperonospora arabidopsidis*, the downy mildew pathogen of *Arabidopsis thaliana*, which are recognized by the resistance proteins RPP1 and RPP13, respectively (recently reviewed by Coates and Beynon 2010). In absence of recognition by the R-protein, these host-targeted effectors increase virulence and decrease callose formation. The translocation of RXLR effector molecules is proposed to occur by binding to cell surface phosphatidylinositol-3-phosphate (PI3P), followed by endocytosis. Surprisingly, also fungal effector molecules are suggested to be translocated into the host plant cell via RXLR-mediated PI3P binding as shown for the basidiomycete *Melampsora lini* AvrL567 (Kale et al. 2010).

The largest class of the host R-proteins contains a nucleotide binding site (NBS) and leucine rich repeat (LRR) domain. Depending on the class of R-protein the N-terminal part consists of a coiled coil (CC) or a TIR domain. The LRR domain is believed to mediate protein-protein interactions, while the N-terminal domain is important for activation of downstream responses (DeYoung and Innes 2006). One would expect that recognition of the cognate effector molecule would be direct through binding to the LRR domain of the R-protein. However, there are only few examples where a direct interaction has been reported, e.g. in rice where the LRR domain of the R-protein Pi-ta binds the AvrPi-ta protein from the fungus *Magnaporthe grisea* (Jia et al. 2000). In most cases there is no direct interaction observed between the R-protein and its cognate effector counterpart. Rather, recognition seems to be based on indirect interactions that can be subdivided in two models. In the guard model, the effector protein binds to a host target that, in turn, undergoes a conformational change. R-proteins that “guard” the target recognize the change and become activated leading to the triggering of resistance. Implicit in this guard model is that the host target is required for virulence function in the absence of the cognate R-protein (Dangl and Jones 2000). For example, the Arabidopsis RIN4 protein (target) forms a complex with the R-proteins RPM1 and RPS2 (guards). Degradation and phosphorylation of RIN4 by the *P. syringae* effectors AvrRpt2 and AvrRPM1, respectively, activates RPM1 and RPS2 (Kim et al. 2005). Both effector

proteins, although sequence unrelated, affect RIN4 which is guarded by RPM1 and RPS2. A second model, the decoy model, deals with the proposed evolutionary unstableness of the host target in plant populations polymorphic for *R*-genes. In other words, depending on the absence or presence of an *R*-gene, the host target is subject to opposing selection. Creation of a duplicated target mimic that solely acts as effector recognizer would be more evolutionary stable (van der Hoorn and Kamoun 2008).

Following the activation of ETI, a signaling cascade is activated that deploys essentially the same signaling components as used during the PTI response. Using a transcriptomics approach, Navarro et al. (2004) compared genes induced in nonhost, compatible and incompatible *P. syringae* pv. *tomato* interaction to flg22-induced genes. They found that 45% of the genes induced by flg22 were also induced by *Pst*-AvrB or AvrRpt2, suggesting a common set of responses during PTI and ETI. The plant hormones salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) are important for defence signaling and immunity against different types of pathogens, but their precise roles in PTI and ETI are not clear. During PTI, the production of SA is triggered locally and systemically (Mishina and Zeier 2007) and mutants impaired in SA signaling are compromised in resistance induced by flg22 treatment against *P. syringae* pv *tomato* DC3000 *hrcC* (hrcC, deficient in type III secretion system) (Tsuda et al. 2008).

The SA pathway is especially important for immunity against biotrophic pathogens like *H. arabidopsidis* and *P. syringae* (reviewed by Glazebrook 2005). Induction of SA synthesis after PAMP or effector perception requires isochorismate synthase 1 / SA induction deficient 2 (ICS1/SID2) function. *ICS1* is highly induced after pathogen attack and does not require SA (Wildermuth et al. 2001). Increase of SA levels e.g. as a result of pathogen infection, but also through exogenous application, results in activation of SA-dependent pathogenesis-related (PR) gene expression and enhances resistance to (hemi-)biotrophic pathogens. Blocking SA accumulation by degrading SA to catechol in transgenic plants expressing the bacterial salicylate hydroxylase gene (*NahG*) or by preventing its synthesis, e.g. in the *sid2* mutant, effectively eliminates the activation of SA-dependent defences and causes hypersusceptibility to biotrophic pathogens (Durrant and Dong 2004; Nawrath and Métraux 1999). SA-induced redox changes cause reduction of cytosolic inactive oligomeric NPR1 (non-expressor of pathogenesis-related genes 1), to active monomers that translocate to the nucleus (Mou et al. 2003). NPR1 has a central role in the activation of pathogenesis-related gene expression. In the nucleus, NPR1 interacts with TGA transcription factors (TFs) that in turn activate a plethora of SA-dependent genes. Also genes involved in the protein secretory pathway have been indicated as direct targets of NPR1 (Wang et al. 2005). From the 72 WRKY genes in the Arabidopsis genome, 49 are responsive to SA or avirulent *P. syringae* pv *tomato* indicating extensive transcriptional activation and repression during defence (Dong et al. 2003). These WRKY TFs are known to bind the W-box motif which is over-represented in promoters of

defence associated genes, e.g. *PR-1* and *NPR1* (Maleck et al. 2000; Yu et al. 2001). Certain WRKY TFs act as positive regulator of defence, e.g. WRKY70 (Li et al. 2006), while other WRKYs act as negative regulator of defence like the structurally related WRKY18, 40, and 60 (Xu et al. 2006). The group of *PR*-genes includes SA-dependent defence-related genes that are induced during infection (van Loon and van Strien 1999). The corresponding PR-proteins not only accumulate locally at the infection site, but are also induced in systemic tissue where they are associated with systemic acquired resistance (SAR) development. SAR and the accumulation of PR-proteins in distal parts of the plant is characterized by perception of a systemic signal after an invading pathogen is recognized (Durrant and Dong 2004).

Compatibility and the role of the haustorium

When a pathogen is able to penetrate and proliferate on a specific host, thereby suppressing defence via effector molecules and containing the necessary tools to obtain the nutrients from the host, it can be considered as an evolved state of symbiosis resulting in basic compatibility. Especially in the case of obligate biotrophs like the powdery and downy mildews, the pathogen has to make sure to redirect the host's metabolism without disturbing host cell homeostasis since these pathogens need living plant tissue for completion of their lifecycle and can not be cultured *in vitro* (O'Connell and Panstruga 2006). Obligate biotrophic pathogens have evolved specialized infection structures called haustoria that develop as branches from hyphae. Haustoria are found in obligate biotrophic oomycetes (e.g. *H. arabidopsidis*), ascomycetes (e.g. *Erysiphe cichoracearum*) and in basidiomycetes (e.g. *Uromyces fabae*) indicating that this structure is remarkable in terms of convergent evolution in these different groups. The haustorium penetrates the plant cell wall and invaginates the plasma membrane where it serves as major interface for the uptake of nutrients (O'Connell and Panstruga 2006). Haustoria are not intracellular, a derivative of the host plasma membrane, the extrahaustorial membrane, separates the haustorium from the host cytoplasm. In between the extrahaustorial membrane and pathogen plasma membrane is a layer called the extrahaustorial matrix. A hexose transporter (HXT1) of the rust pathogen *Uromyces fabae* is expressed specifically in the haustorial complex indicating the haustorium mediates the uptake of glucose and fructose (Voegele et al. 2001). Expression analysis of a haustorium-specific *U. fabae* cDNA library of *in planta* induced genes resulted in the identification of several genes involved in metabolism and nutrient transport (Jakupovic et al. 2006). In oomycetes, little information is available on the metabolic and transport events in haustoria. However, data regarding *U. fabae* suggest that haustoria function in active nutrient uptake. Besides acquisition of nutrients, haustoria are also the place where secretion of effector molecules occurs in order to suppress host defences and manipulate host metabolism (Mendgen and Deising 1993).

There is limited information on host genes that are required for basic compatibility and successful infection by biotrophic pathogens. Host genes could become activated and function as negative regulator of defence. Many Arabidopsis mutants have been identified that display activated defence without the actual presence of a pathogen. These mutants generally display resistance to a broad spectrum of pathogens, e.g. the *cpr* (Bowling et al. 1994), *lsd* (Dietrich et al. 1994), *dnd* (Yu et al. 1998) and *acd* (Greenberg and Ausubel 1993) mutants. Also, these mutant plants display various altered phenotypes like dwarfism and spontaneous HR. Although the mutants are providing insight into plant defence signaling, they do not contribute to a better understanding of disease susceptibility. On the other hand, host genes could function as susceptibility factors and lack of these factors could lead to a form of resistance that is not based on the activation of known defence responses. This type of resistance is recessively inherited since it is based on loss of function alleles. Identifying these susceptibility genes can provide insight in how the pathogen is able to manipulate the host cell for its own needs thereby establishing basic compatibility (Pavan et al. 2010).

Loss of MLO function mediate powdery mildew resistance in monocots and dicots

A candidate susceptibility gene is Barley *MLO*, which is required for successful penetration of the compatible powdery mildew fungus *Blumeria graminis* f. sp. *hordei* (*Bgh*). Absence of a functional MLO protein, due to mutation in the gene, leads to broad spectrum resistance against all known *Bgh* isolates. For the last 30 years, *mlo* has been successfully implemented in agriculture where it was introduced in elite barley cultivars. Resistance results from the failure of the fungal spores to enter the epidermal host cell. However, there are also several pleiotropic effects that reduce grain yield of *mlo* mutants compared to wildtype. *mlo* mutants exhibit spontaneous deposition of callose in leaf mesophyll cells and early leaf senescence, which indicates that MLO negatively regulates these processes (Piffanelli et al. 2002). In a genetic suppressor screen in the *mlo* mutant background, *ROR1* and *ROR2* were identified that compromise *mlo*-based resistance. The identity of ROR1 is so far not known but ROR2 is an orthologue of the Arabidopsis PEN1 plasma membrane-resident syntaxin (Collins et al. 2003). Apparently, for *mlo*-based resistance syntaxin activity is required. For a long time, barley was considered the only plant species where powdery mildew was effectively controlled by *mlo*. Only recently, *mlo*-based powdery mildew resistance has been discovered in other plant species. Arabidopsis contains 15 *MLO*-like genes and in a thorough study Consonni et al. (2006) identified the *mlo2* mutant as being less susceptible to *Golovinomyces orontii*. Using phylogenetic analysis, they identified *MLO6* and *MLO12* as being in the same clade as *MLO2*. The triple *mlo2/6/12* mutant showed full resistance indicating there is functional redundancy. Furthermore, Arabidopsis *mlo2* resistance to powdery mildew is dependent on indolic metabolites, that are antimicrobial, as shown by

Consonni et al. (2010) indicating MLO acts as negative regulator of defence. Also in tomato, a functional *MLO* orthologue has been identified. Mutation in *SlMlo1* shows full resistance against the powdery mildew *Oidium neolycopersici* (Bai et al. 2008). In pea, the *er1* gene, originating from wild accessions, has been introgressed in elite cultivars to provide durable resistance against powdery mildew. Recently, Humphry et al. (2011) showed that wild-type *Er1* is in fact *PsMLO1* and that loss of *PsMLO1* is responsible for powdery mildew resistance. Powdery mildew resistance by MLO disruption in Arabidopsis, barley, tomato and pea indicates that MLO use by the pathogen for pathogenesis is ancient as the divergence of monocots and dicots occurred approximately 200 million years ago (Wolfe et al. 1989).

PMR functionality is required for susceptibility to powdery mildew

Besides MLO, more host proteins contribute to susceptibility to powdery mildew. In a forward genetic screen, six recessively inherited *powdery mildew resistant (pmr)* mutations were identified that lead to resistance to *Erysiphe cichoracearum* (Vogel and Sommerville 2000). *PMR2* turned out to encode for *MLO2*, which was discussed in the previous section. PMR6 is considered a host susceptibility factor since resistance is independent of HR and the activation of known defence pathways. Instead, the *pmr6* mutant has an altered cell wall composition by increased pectin content. This altered cell wall composition and increased pectin content could well affect the nutrient availability for the powdery mildew (Vogel et al. 2002). Another *pmr* mutant that highlights the important role of cell wall composition in basic compatibility is *pmr5*. *pmr5*-based resistance, like *pmr6*, does not depend on activation of defence responses. PMR5 encodes a predicted ER-targeted protein of unknown function. The *pmr5* mutant exhibits pectin enrichment and contains smaller cells (Vogel et al. 2004). In the same screen, *pmr4* was identified as a mutant lacking pathogen-inducible callose. *PMR4* encodes for a glucan synthase that is responsible for callose depositions at infection sites (Nishimura et al. 2003). Normally, callose depositions provide a physical barrier that stops pathogen penetration. This mutant was lacking an important defence mechanism, but turned out to become resistant to powdery mildew attack. Nishimura et al. (2003) discovered that this mutant developed lesions of HR cell death at infection sites regulated by the SA pathway since *pmr4-1_NahG* plants were restored for susceptibility to powdery mildew. This regained susceptibility was not accompanied by callose formation indicating that callose itself is not a compatibility factor. Apparently, lack of callose in *pmr4* resulted in derepression of SA-dependent defences. Clearly, forward genetics has revealed several host genes required for powdery mildew infection but our current knowledge of genes required for disease susceptibility is still very limited.

Arabidopsis *dmr* mutants are resistant to *H. arabidopsidis*

Arabidopsis is an excellent model organism for genetic studies on basic compatibility to pathogens. A natural occurring downy mildew disease of Arabidopsis is caused by the oomycete *H. arabidopsidis* (Coates and Beynon 2010). Infection starts with the germination of a spore on the leaf where it forms an appresorium. The appresorium penetrates between the anticlinal walls of two epidermal cells allowing the pathogen to grow further intercellularly, forming haustoria in adjacent mesophyll cells. After approximately one week conidiophores grow out of the stomata that bear new conidia which are dispersed and can infect neighbouring tissues or plants (Koch and Slusarenko 1990). In an analogous approach that identified the *pmr* mutants, several downy mildew resistant (*dmr*) mutants were identified in a genetic screen. Using the highly susceptible L*er eds1-2* parental line, EMS mutants were generated and screened for loss of susceptibility. Six different downy mildew resistance loci *dmr1-dmr6* were identified (van Damme et al. 2005). Further analysis revealed *dmr3*, *dmr4*, and *dmr5* as enhanced defence response mutants and are likely mutated in a negative regulator of defence. Further research identified *dmr5* as an auto-activation mutant of *RPM1*, encoding an R-protein required for resistance to *Pseudomonas* expressing avrRPM1 (Huibers 2008). In contrast, the *dmr1*, *dmr2*, and *dmr6* mutants do not display enhanced defence responses (van Damme et al. 2005). Using map based cloning, DMR6 was identified and found to encode for a 2-oxoglutarate Fe(II)-dependent oxygenase of unknown biological function. *DMR6* expression is induced by compatible as well as incompatible *H. arabidopsidis* isolates and also when treated with SA. The *dmr6* mutant shows enhanced expression of a subset of defence-associated genes, suggesting DMR6 negatively regulates plant immunity in Arabidopsis (van Damme et al. 2008). The research described in this thesis provides deeper insights into the forward genetic screen set up to identify the *dmr* mutants and the role the *DMR* genes play in the infection process. Functional data on the *DMR* genes could provide more insight into plant disease susceptibility.

Thesis outline

The aim of this study was to identify and functionally analyze the *DMR1* and *DMR6* genes required for disease susceptibility in the Arabidopsis-*Hyaloperonospora arabidopsidis* interaction.

Chapter 2 describes the cloning and functional analysis of the *DMR1* gene. *DMR1* encodes for homoserine kinase, a key enzyme in the production of the amino acids methionine, threonine and isoleucine. The six identified *dmr1* mutants contain high levels of homoserine which are absent in the parental line while the levels of Met, Thr, and Ile are not reduced. Also, exogenous application of homoserine is sufficient to induce resistance. Our results indicate that homoserine accumulation in the chloroplast induces resistance independent of known defence pathways. In **chapter 3** the HSK gene is analyzed in more detail. Using reverse genetics through TILLING, 37 additional missense mutations are identified in *HSK* that lead to amino acid substitutions in the HSK protein. One of the TILLING mutants is resistant to downy mildew coinciding with high levels of homoserine. For another TILLING mutant, no viable homozygous mutant lines were obtained suggesting *HSK* is an essential plant gene. We demonstrate that TILLING is a valuable approach to obtain novel alleles providing recessive resistance to downy mildew. Furthermore, *HSK* orthologs of different crop species were identified and the enzyme activity of the corresponding HSK proteins of tomato and cucumber could be confirmed. **Chapter 4** describes the functional analysis of *DMR6* that encodes an 2-oxoglutarate Fe(II)-oxygenase for which the substrate is not known yet. *dmr6*-mediated resistance requires functional NPR1 and ICS1 proteins indicating that the salicylic acid pathway is required. Also, *DMR6*-overexpression plants become more susceptible to *H. arabidopsidis* suggesting DMR6 behaves as negative regulator of defence. Metabolite analysis reveals several candidate substrates of the DMR6 oxygenase. A model of the DMR6 protein allowed the identification of functionally important residues in the substrate pocket. **Chapter 5** provides more insight into the family of DMR6-LIKE oxygenases (DLOs) and their involvement in plant immunity. Using phylogenetic analysis of 19 flowering plant species we identified 68 DLOs, out of which two (DLO1 and DLO2) from *A. thaliana* were studied in more detail. Both *DLO1* and *DLO2* are able to complement *dmr6*-mediated resistance indicating they have a molecular function similar to that of *DMR6* and can act as negative regulators of defence. We propose a specific motif that can be used for the identification of DMR6/DLO protein family members. **Chapter 6** provides a general discussion of the obtained results.

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2

Downy mildew resistance in Arabidopsis by mutation of *HOMOSERINE KINASE*

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ABSTRACT

Plant disease resistance is commonly triggered by early pathogen recognition and activation of immunity. An alternative form of resistance is mediated by recessive *downy mildew resistant 1* alleles in Arabidopsis. Map-based cloning revealed that *DMR1* encodes for homoserine kinase (HSK). Six independent *dmr1* mutants each carry a different amino acid substitution in the HSK protein. Amino acid analysis revealed that *dmr1* mutants contain high levels of homoserine that is undetectable in wild-type plants. Surprisingly, the level of the amino acids, downstream in the aspartate pathway, were not reduced in *dmr1* mutants. Exogenous homoserine does not directly affect pathogen growth, but induces resistance when infiltrated in Arabidopsis. We provide evidence that homoserine accumulation in the chloroplast triggers a novel form of downy mildew resistance that is independent of known immune responses.

INTRODUCTION

Major plant diseases, such as powdery mildews, rusts, and downy mildews, are caused by obligate biotrophic fungi and oomycetes (fungal-like members of the kingdom *Stramenopila*). The obligate biotrophs are often highly specialized, and for their growth and reproduction they fully depend on particular host plants. Obligate biotrophs do not cause disease on nonhost plant species, as infection is obstructed by the plant-controlled process of nonhost resistance that involves both pre- and postinvasion defences (Lipka et al. 2005). In host plants, pathogens can circumvent or actively suppress nonhost resistance (Chisholm et al. 2006). However, a second layer of defence has evolved that is mediated by single dominant resistance (*R*) genes. The encoded R proteins mediate direct or indirect detection of pathogen determinants followed by the activation of plant immunity (Jones and Dangl 2006). Despite these effective intrinsic defence mechanisms, plants are still vulnerable to pathogen attack. The molecular mechanisms underlying disease susceptibility, and more specifically the role of host-specific processes that sustain pathogen development and growth, are still largely unknown. In recent years, genetic studies on the model plant *Arabidopsis thaliana* have resulted in the identification of a number of genes that are involved in susceptibility to biotrophic pathogens (Vogel and Somerville 2000; O'Connell and Panstruga 2006). Mutations in the *POWDERY MILDEW RESISTANT* genes *PMR6* and *PMR5*, encoding a pectate-lyase and a protein of unknown function, respectively, result in plants with an altered cell wall that is thought to underlie their phenotype (Vogel et al. 2002, 2004). The *pmr4* mutant has a lesion in a callose synthase gene that leads to enhanced defence responses upon pathogen infection (Nishimura et al. 2003). A fourth example of powdery mildew resistance is the loss-of-function of the *Arabidopsis PMR2/MLO2* gene (Consonni et al. 2006), a co-ortholog of the barley (*Hordeum vulgare*) *Mildew resistance locus O* (*Mlo*) gene (Panstruga 2005). Most of the *pmr* mutants are pathogen specific, reducing susceptibility to powdery mildew, but not to the bacterial pathogen *Pseudomonas syringae* and the downy mildew pathogen *Hyaloperonospora arabidopsidis* (Vogel and Somerville 2000). Pathogen-specific resistance was also found for the Arabidopsis *downy mildew resistant 1* (*dmr1*) mutant, which is resistant to *H. arabidopsidis* but susceptible to infection by other pathogens, for example, the powdery mildew fungus *Golovinomyces orontii* and *P. syringae* bacteria (van Damme et al. 2005). The *dmr1*-mediated resistance to *H. arabidopsidis* could be due to the absence of a specific host protein required for infection or to a hitherto unknown defence mechanism. Here, we demonstrate that accumulation of the amino acid homoserine in *dmr1* mutant plants is sufficient to provide resistance to *H. arabidopsidis*. The basis of this discovery was the map-based cloning of the *DMR1* gene, which was found to encode homoserine kinase (HSK; Arabidopsis gene At2g17265), a key enzyme in primary amino acid metabolism. Surprisingly, biosynthesis of the amino acids Thr, Met, and Ile is not reduced in the *dmr1*

mutants, whereas homoserine accumulates to high levels. Homoserine-induced resistance is independent of known defence signaling pathways and could provide a novel method for protecting crops against downy mildew disease.

RESULTS

dmr1-mediated resistance to *H. arabidopsidis*

Multiple independent alleles of *dmr1*, *dmr1-1*, *dmr1-2*, *dmr1-3*, and *dmr1-4*, were initially identified from a genetic screen for loss of susceptibility to *H. arabidopsidis* (van Damme et al. 2005). These mutants were each backcrossed twice (BC_2) to the parental line Landsberg *erecta* (L*er*) *eds1-2* to reduce the number of unlinked ethyl methanesulfonate (EMS)-induced mutations. The *dmr1* BC_2 mutants retained strong resistance to *H. arabidopsidis* compared with the parental line L*er eds1-2* (Figures 1A and 1B). Of the mutants, *dmr1-3* still supports a low level of sporulation, suggesting that it carries a weak *dmr1* allele. Microscopy analysis of the infection process in the different *dmr1* BC_2 mutants showed that most *H. arabidopsidis* hyphae were arrested after formation of the first haustoria. The inhibition of growth was associated with the encasement of haustoria by papillae, translucent structures surrounding the feeding structures that are absent in the susceptible parental line (Figure 1C, papillae are indicated by the arrows). Aniline blue staining confirmed that the papillae contain callose, a polysaccharide that is commonly present in these pathogen-induced physical barriers (Aist 1976), which are thought to prevent further penetration and nutrient uptake by the pathogen. Besides papillae formation, no other detectable defence responses, such as production of reactive oxygen species or activation of defence gene expression (Van Damme et al. 2005), were observed in the *dmr1* mutants.

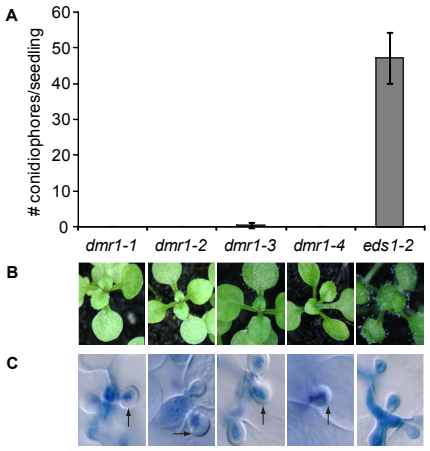

Figure 1. Quantification and visualization of *H. arabidopsidis* growth on the *dmr1* mutants (BC_2). The *dmr1* mutants confer nearly complete resistance to the downy mildew pathogen *H. arabidopsidis*.
(**A**) Conidiophore formation 5 d after inoculation on 10-d-old Arabidopsis seedlings. The average number (with standard deviation, n = 40) of conidiophores/seedling is displayed.
(**B**) Macroscopy images of conidiophores on Arabidopsis seedlings 8 d after inoculation with *H. arabidopsidis*.
(**C**) Microscopy examination of trypan blue–stained hyphae and haustoria in leaves of Arabidopsis seedlings 5 d after inoculation with *H. arabidopsidis*. The arrest of *H. arabidopsidis* growth and appearance of papillae (indicated by the arrows) in the *dmr1* mutants was observed in multiple independent experiments.

DMR1 encodes HSK

The recessive *dmr1* locus was previously mapped on the long arm of chromosome 2 (van Damme et al. 2005). More precisely, *dmr1* could be assigned to a chromosomal region of 130 kb that is covered by the BACs F6P23, T23A1, and F5J6. Five recombinants (from 650 F2 plants) between markers designed on BACs F6P23 to F5J6 were instrumental in the fine mapping of *dmr1* to a region encompassing eight genes: At2g17230 to At2g17290. Single point mutations in the At2g17265 gene were identified by nucleotide sequencing of the *dmr1-1*, *dmr1-2*, *dmr1-3*, and *dmr1-4* mutants. In addition, two resistant mutants, *dmr1-5* and *dmr1-6*, that were isolated later, also contained single point mutations in the At2g17265 coding sequence (Figure 2).

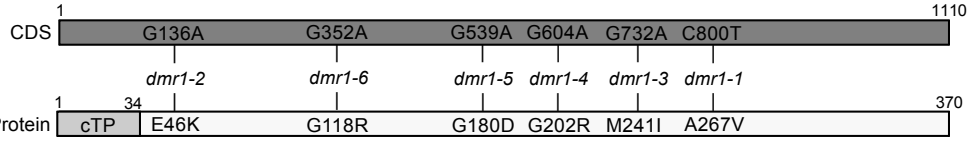

Figure 2. *dmr1* mutations affect the HSK protein. The position of the nucleotide changes (GC-to-AT transitions, typical of EMS-induced mutations) in the *HSK* coding sequence (CDS) and corresponding amino acid substitutions in the HSK protein are indicated for the *dmr1-1*, *dmr1-2*, *dmr1-3*, *dmr1-4*, *dmr1-5*, and *dmr1-6* mutants. The amino acid substitutions are in the mature protein, which has recently been confirmed to be localized in the chloroplast (Zybailov et al. 2008). Based on prediction and proteomics data, the chloroplast transit peptide (cTP) is cleaved between positions 34 and 35.

At2g17265 encodes HSK, a key enzyme in the Asp pathway for the biosynthesis of the essential amino acids Met, Thr, and Ile. All six mutations result in amino acid substitutions in the HSK protein that could reduce or abolish the activity of the enzyme. Confirmation that mutations in the *HSK* gene are responsible for the *dmr1* resistance phenotype was obtained by *Agrobacterium tumefaciens*–mediated transformation of the *dmr1-1* and *dmr1-3* mutants with the wild-type Arabidopsis *HSK* coding sequence, which reestablished susceptibility to *H. arabidopsidis* (Supplemental Figure 1).

The nuclear-encoded Arabidopsis HSK protein carries a predicted N-terminal transit sequence for chloroplast targeting. Using the ChloroP algorithm (Emanuelsson et al. 1999), the HSK protein of Arabidopsis is predicted to be cleaved after amino acid 62 (cleavage site [CS] score 3.97). As this position falls within motif 1, involved in substrate and cofactor binding (Supplemental Figure 2), this predicted site is highly unlikely to be the cleavage site. Motif 1 is highly conserved and is also present in HSK proteins from bacteria (e.g., *Escherichia coli* THRB) and fungi (e.g., *Saccharomyces cerevisiae* THR1) that are cytoplasmic and lack a chloroplast transit peptide. The transit peptide of the homologous HSK protein of *Brassica oleracea* (of which the first 171 amino acids that were predicted from EST sequences are 92% identical to HSK of Arabidopsis) is predicted to be cleaved after amino acid 34 with a CS score of 5.324. For the Arabidopsis HSK protein, the second best transit peptide cleavage site

score is also after amino acid 34 (CS score 2.787). Experimental support for this cleavage site is provided by a high-throughput proteomic study using mass spectrometry (Baerenfaller et al. 2008). The HSK-derived tryptic peptide ASVQTLVAVEPEPVFVSVK (position 37 to 55) was identified multiple independent times from different Arabidopsis tissues, indicating that the mature protein contains this peptide and supporting a cleavage site before position 37.

Amino acid substitutions in DMR1 are at conserved positions in plant HSKs

HSK is a single-copy gene in Arabidopsis, rice (*Oryza sativa*), grapevine (*Vitis vinifera*), and the moss *Physcomitrella patens*, based on their annotated genome sequences. In addition, for many other plant species, ESTs were identified that correspond to single orthologous *HSK* genes. However, in poplar (*Populus trichocarpa*) and potato (*Solanum tuberosum*), two putatively orthologous genes appear to be present that give a reciprocal best hit to Arabidopsis *HSK*. Twelve plant HSK protein sequences, including the best orthologs of potato and poplar, are depicted in a multiple alignment generated by ClustalW (Supplemental Figure 2). Except for the N-terminal part, which is the chloroplast transit peptide sequence, the plant HSKs show high amino acid similarity. The first conserved position in these 12 plant HSK proteins is a negatively charged residue (E or D) at position 46 in the Arabidopsis HSK protein. In the *dmr1-2* mutant, this acidic residue is substituted with Lys and is predicted to be located in the mature chloroplast-localized enzyme. Similarly, the other five *dmr1* amino acid substitutions are all at positions that are identical or similar in all 12 plant HSK proteins, suggesting that they are functionally important residues. However, the substitutions do not overlap with the three motifs (indicated by the dashed lines in Supplemental Figure 2) that are highly conserved in all members of the GHMP kinase superfamily, which includes galacto kinase, HSK, mevalonate kinase, and phosphomevalonate kinase (Zhou et al. 2000). In addition, the *dmr1* mutations do not overlap with the conserved amino acids that bind ATP or are in the active site (indicated by the diamonds in Supplemental Figure 2), based on alignment of the Arabidopsis HSK amino acid sequence with that of the HSK protein of *Methanococcus jannashii*, of which the crystal structure has been described (Zhou et al. 2000). Therefore, we investigated if the *dmr1* mutations cause a reduction or loss of HSK enzyme activity.

***dmr1* mutations strongly affect HSK activity**

The *dmr1* mutations all lead to amino acid substitutions in residues that are conserved in plant HSK proteins, suggesting that they correspond to important positions in the enzyme and possibly affect its activity. We tested this *in vitro* by measuring the activity of recombinant HSK enzyme from the wild type and four *dmr1* mutants, produced and purified from *E. coli*. In this assay, the consumption of ATP, which is required for the phosphorylation of homoserine, is an indirect measure of HSK activity. As shown in Figure 3, the relative activity of the

DMR1-2, 1-3, and 1-4 mutant enzymes was strongly reduced (5 to 10% of wild-type activity), whereas DMR1-1 enzyme activity was undetectable. This strong reduction or complete loss of *in vitro* activity is expected to have a profound effect on the level of soluble amino acids in the *dmr1* mutants *in vivo*, as HSK is a key enzyme in the Asp pathway (Figure 4A).

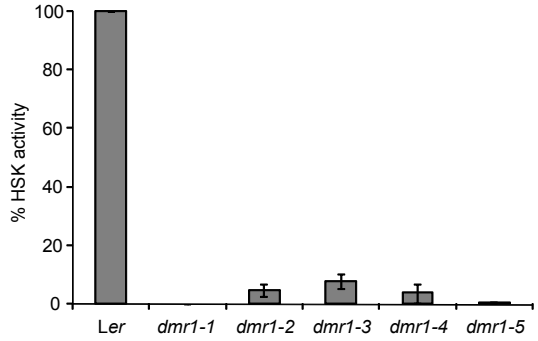

Figure 3. HSK activity of recombinant enzyme from wildtype Arabidopsis (accession L*er*) and five *dmr1* mutants produced and purified from *E. coli*. The *dmr1* mutations strongly reduce HSK activity relative to that of the wild type (set at 100% and corrected for recombinant protein input). The average activity (with SE) from three independent recombinant enzyme isolations and assays is displayed.

This could directly affect *H. arabidopsidis* as it is an obligate biotroph that could rely on host-derived amino acids for its growth and development. To test if the *dmr1* mutations in *HSK* affect the levels of amino acids in the Asp pathway, soluble amino acids were quantified in the aboveground parts of mutant and wild-type seedlings. Surprisingly, the levels of the amino acids Thr, Met, and Ile (downstream of HSK in the Asp pathway; Figure 4A) were not reduced in the *dmr1* mutants (Figures 4C to 4E), indicating that *dmr1*-resistance is not caused by a depletion of these amino acids within the host. By contrast, the levels in the *dmr1* mutants were higher than in the parental line L*er eds1-2*, with Met (Figure 4D) levels being highest in *dmr1-1* and Thr (Figure 4C) and Ile (Figure 4E) levels being highest in *dmr1-2* and *dmr1-4*, respectively. The *dmr1* mutants were found to affect free homoserine levels profoundly (Figure 4B). This amino acid is not detectable in seedlings of the parental line L*er eds1-2*. High levels of homoserine were detected in the *dmr1-2* and *dmr1-4* mutants, and lower, but still significant, levels were found in the *dmr1-1* and *dmr1-3* mutants. The high homoserine levels in the *dmr1* mutants confirm that the substitutions in HSK lead to reduced enzyme activity.

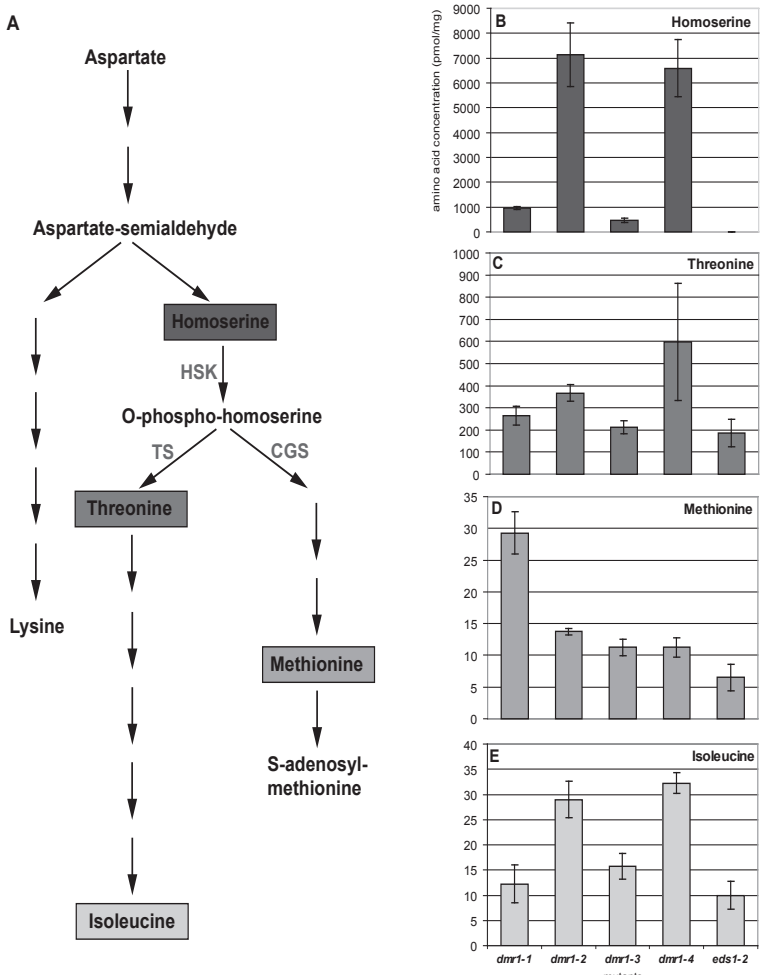

Figure 4. Mutations in the *HSK* gene in the *dmr1* mutants result in homoserine accumulation but not in depletion of the downstream amino acids Thr, Met, and Ile. (**A**) Position of HSK in the aspartate metabolic pathway in Arabidopsis (adapted from www.biocyc.org). Homoserine is the common precursor for the essential amino acids Thr, Ile, and Met. In the chloroplast stroma, HSK produces O-phospho-homoserine, which is the direct substrate for Thr synthase (TS) and cystathionine gamma-synthase (CGS). (**B**) to (**E**) The homoserine (**B**), Thr (**C**), Met (**D**), and Ile (**E**) content (in pmol/mg fresh weight) of four 14-d-old *dmr1* mutants was determined and compared with that of the parental line L*er eds1-2*. It is striking to see that Thr and Ile levels are high in the *dmr1-2* and *dmr1-4* mutants, whereas Met is particularly high in *dmr1-1*, suggesting that the different *dmr1* mutations affect interactions between HSK and enzymes further downstream in the pathway. Amino acid analysis was performed on three biological replicates per mutant. Average amino acid concentrations (with SD) are displayed. Similar relative amino acid levels were confirmed in two independent experiments.

Homoserine induces resistance to downy mildew

High homoserine levels in the *dmr1* mutants could provide direct resistance to downy mildew by inhibiting pathogen growth. However, no inhibitory effect of homoserine was found in vitro. As the obligate biotroph *H. arabidopsidis* cannot be cultured *in vitro*, we tested the effect of homoserine on radial growth of the related oomycete pathogen *Phytophthora capsici*. L-homoserine did not inhibit radial growth when compared with D-homoserine at concentrations of up to 10 mM (Supplemental Figure 3A). When tested on *H. arabidopsidis* conidiospores, L-homoserine concentrations of up to 50 mM did not negatively affect spore germination (Supplemental Figure 3B). It is likely that oomycetes can metabolize homoserine because putative HSK ortholog sequences are present in the genome sequence of *H. arabidopsidis* and other oomycete pathogens (Supplemental Figure 4). Our data indicate that homoserine, at the concentrations used, is not toxic to the pathogen, suggesting that processes in the plant mediate homoserine-induced resistance. Indeed, when L-homoserine (5 mM) was applied exogenously to wild-type Arabidopsis seedlings, by infiltration into the leaf intercellular space, resistance to *H. arabidopsidis* was observed (Figure 5A). Resistance was not induced upon infiltration with water, Met (5 mM), Thr (5 mM), Ile (5 mM), or the stereo-isomer D-homoserine (5 mM), indicating that the effect is L-homoserine specific. Microscopy analysis of the homoserine-treated leaves showed arrested *H. arabidopsidis* growth and absence of sporulation. Homoserine-induced resistance was also associated with the occurrence of encased haustoria (Figure 5B). Very similar host responses were observed in the *dmr1* mutants (Figure 1), suggesting that high homoserine levels *in planta* induce the responses.

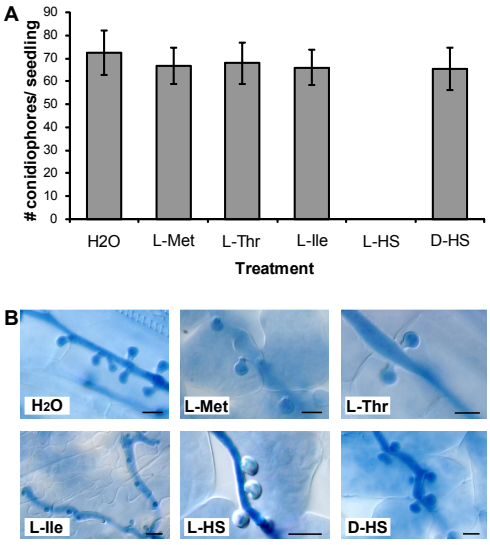

Figure 5. Exogenous application of L-homoserine leads to *H. arabidopsidis* resistance in Arabidopsis.
(**A**) Infiltration of *H. arabidopsidis*–infected seedlings with L-homoserine (5 mM) leads to resistance to *H. arabidopsidis*, whereas treatment with 5 mM of L-Met, L-Thr, L-Ile, or D-homoserine does not reduce the level of *H. arabidopsidis* sporulation. The average number (with SD, n = 30) of conidiophores/seedling is displayed. The results were confirmed in two additional independent experiments. (**B**) A high frequency of papillae formation, visible as translucent encasements surrounding the trypan blue–stained haustoria, is observed in L-homoserine (L-HS)–treated seedlings and not after treatment with the other amino acids or the D-stereo-isomer of homoserine. Bars = 10 µm.

Homoserine-induced resistance is independent of known defence pathways

The high prevalence of callose-containing papillae in the *dmr1* mutants and homoserine-treated seedlings in response to infection raised the question whether papillae formation is the primary cause of *H. arabidopsidis* resistance. To test this, we exogenously applied homoserine to seedlings of the Arabidopsis *pmr4-1* mutant, which is strongly impaired in the production of pathogen-induced callose because of a lesion in a callose synthase gene (Nishimura et al. 2003). As *pmr4* plants show enhanced activation of plant defence responses upon pathogen infection, double mutants impaired in defence signaling, *pmr4-1 npr1-1* (for *nonexpressor of PR genes*), *pmr4-1 sid2-1* (for *salicylic acid induction deficient*), and *pmr4-1 pad4-1* (for *phytoalexin deficient*), were analyzed. In all *pmr4-1* single and double mutants tested, resistance to *H. arabidopsidis* could still be induced by exogenous application of L-homoserine and not by D-homoserine (Figure 6).

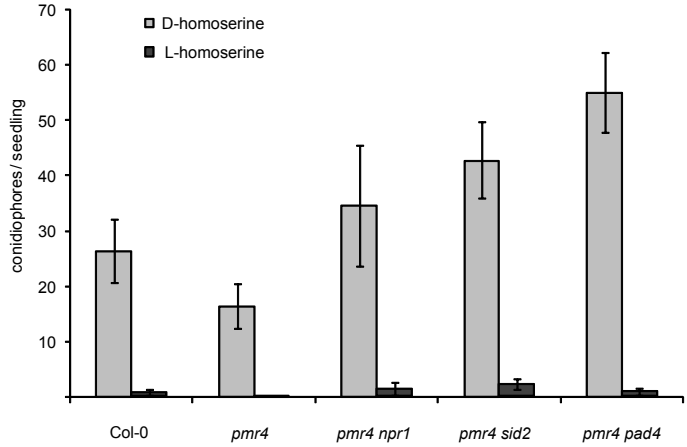

Figure 6. Sporulation of *H. arabidopsidis* on Arabidopsis *pmr4-1* single and double mutants after D- and L-homoserine (10 mM) application. L-homoserine-induced resistance is unaffected in the *pmr4* mutants (compared with wild-type Col-0), indicating that the pathogen-induced callose synthase PMR4 is not required for homoserine-induced resistance. The average number (with SE, n = 20) of conidiophores/seedling is displayed. Similar results were obtained in a separate independent experiment.

This indicates that homoserine-induced resistance does not require the pathogen-induced callose synthase PMR4 nor the defence signaling genes *NPR1*, *PAD4*, *SID2*, and *EDS1* (the *dmr1* mutants were generated in the *eds1-2* background).

A larger collection of Arabidopsis mutants impaired in immune responses (*ethylene insensitive2* [*ein2*], *non-race specific disease resistance1* [*ndr1*], *jasmonate resistant* [*jar1-1*], *mlo2*, *mlo2 mlo6 mlo12*, *penetration* [*pen1-1*], *pen2-1*, *pen3-1*, *senescence-associated gene* [*sag101-2*], *pad3-1*, *flavin dependent monooxygenase* [*fmo1-1*], *suppressor of G2 allele of skp1* [*sgt1b-3*], and *required for ML-a12 conditioned resistance* [*rar1-13*]) was tested to analyze if homoserine-induced resistance required any of the corresponding genes. In all mutants tested L-homoserine was able to induce resistance to *H. arabidopsidis* (Figure 7),

indicating that it does not rely on known immune responses. This was further supported by the fact that we did not observe activation of expression of the defence-associated genes *PR-1*, *PR-2*, and *DMR6* (van Damme et al. 2008) in uninoculated *dmr1* mutants (Supplemental Figure 5A).

As resistance in the *dmr1* mutants is already visible microscopically at 1 and 2 d postinoculation, we analyzed the expression of *PR-1* and *HSK* after inoculation with *H. arabidopsidis* Cala2. As shown in Supplemental Figure 5B, there is no strong activation of *DMR1*/*HSK* and *PR-1* in the *dmr1* mutants in response to infection with the compatible isolate Cala2. *dmr1*-mediated resistance is clearly not based on well-known defence responses.

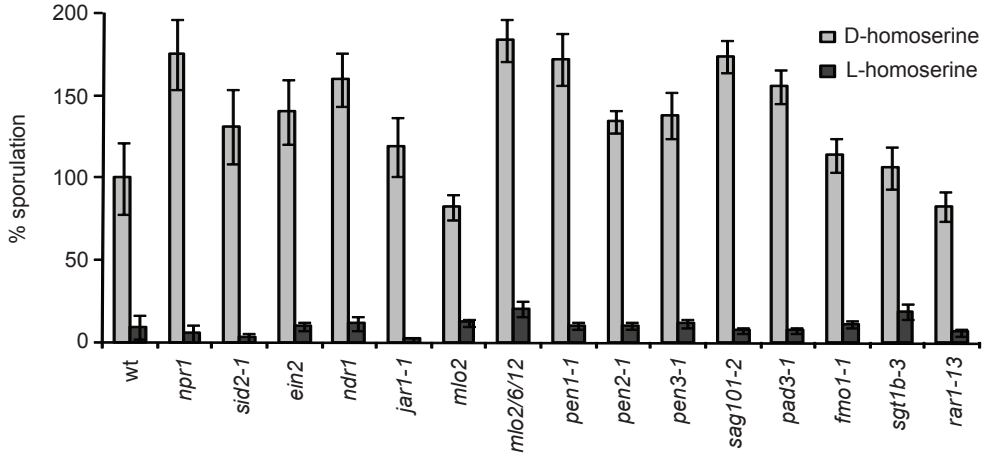

Figure 7. L-Homoserine-induced resistance is not affected in a large set of Arabidopsis immune response mutants. The level of sporulation of D-homoserine- and L-homoserine-treated (10 mM) seedlings is displayed compared with the wild-type control plant Col-0 (set to 100% sporulation). Several mutants demonstrated enhanced susceptibility to *H. arabidopsidis*, but in all cases, infiltration with L-homoserine, but not D-homoserine, strongly reduced the *H. arabidopsidis* sporulation level. The relative level (with SE, n = 20) of conidiophores/seedling is displayed. Similar results were obtained in a separate independent experiment.

Homoserine acts in the chloroplast to induce resistance

In plants, most enzymatic reactions in the Asp pathway, including the phosphorylation of homoserine, are known to take place in the chloroplast (Azevedo 2002). The nuclear-encoded HSK protein indeed carries a predicted N-terminal transit sequence for chloroplast targeting (Lee and Leustek 1999) and has been confirmed by proteomic analysis of purified chloroplasts of wild-type Arabidopsis to be present in this organelle (Zybailov et al. 2008).

To test whether *dmr1*-mediated resistance is induced by homoserine accumulation in the chloroplast or cytosol, we expressed the cytosolic HSK protein of the yeast *S. cerevisiae* (HSK or THR1 [for threonine requiring]), which lacks a chloroplast transit peptide (Mannhaupt et al. 1990), in *dmr1* plants. Transgenic plants expressing *S. cerevisiae* HSK did not gain resistance

to *H. arabidopsidis* after exogenous homoserine application (Figure 8). This suggests that exogenously applied homoserine is metabolized in the cytoplasm by *S. cerevisiae* HSK before it can induce resistance, demonstrating that the cytosolic enzyme is biologically active in the transgenic Arabidopsis lines. However, transgenic *dmr1* mutants expressing *S. cerevisiae* HSK remained as resistant as the *dmr1* mutants, whereas transgenic *dmr1* plants expressing the chloroplastic Arabidopsis HSK regained full susceptibility to *H. arabidopsidis* (Figure 9A). In addition, amino acid analysis showed that *S. cerevisiae* HSK-expressing *dmr1* plants retained a high level of homoserine, whereas in Arabidopsis HSK-expressing lines, homoserine was completely absent (Figure 9B). To rule out the possibility that the *S. cerevisiae HSK* gene was corrupted, we PCR amplified the gene from genomic DNA of the *S. cerevisiae HSK* transgenic plants. Sequence analysis confirmed that the coding sequence was unaltered. HSK enzyme assays on recombinant *S. cerevisiae* HSK protein, produced in *E. coli* from the cloned PCR product, showed that the recombinant yeast protein had an activity that was comparable to that of recombinant Arabidopsis HSK protein (which is shown in Figure 4). We conclude that *dmr1*-mediated resistance is a consequence of homoserine accumulation in the chloroplast.

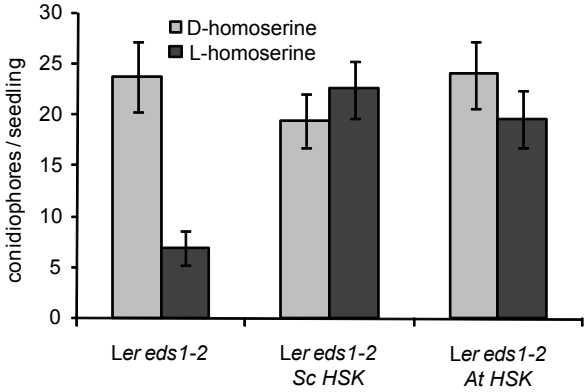

Figure 8. Exogenous application of homoserine (5 mM) does not induce resistance in transgenic lines overexpressing a cytoplasmic or chloroplastic HSK enzyme.

H. arabidopsidis sporulation (average number [with SE, n = 35] of conidiophores/seedling at 6 d after Cala2 inoculation) is strongly reduced as a result of L-homoserine in the Arabidopsis control line L*er eds1-2*. By contrast, transgenic lines overexpressing the cytoplasmic *S. cerevisiae* HSK (Sc HSK) or the chloroplastic Arabidopsis HSK (At HSK) no longer show L-homoserine-induced resistance. This shows that the transformed plants have an enhanced HSK activity, indicating that *S. cerevisiae* HSK is functional in planta. Similar results were obtained in a separate independent experiment

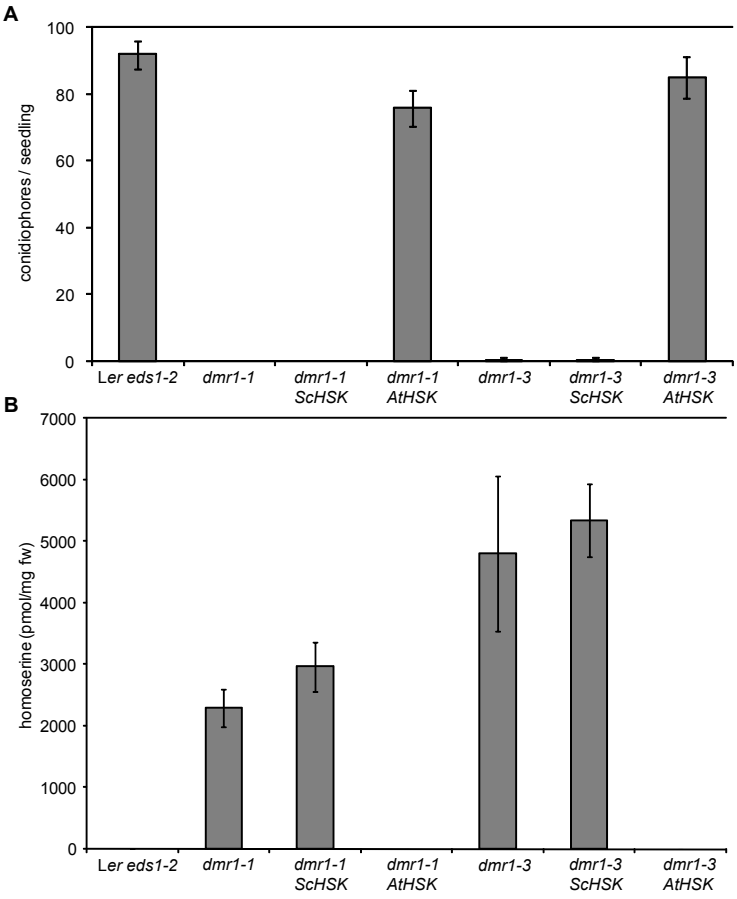

Figure 9. Downy mildew resistance and homoserine levels of *dmr1* mutants are not reduced by transgenic expression of *S. cerevisiae HSK* encoding a cytoplasmic HSK.

Transgenic *dmr1-1* and *dmr1-3* Arabidopsis lines were transformed with the wild-type Arabidopsis Col-0 *HSK* or the *S. cerevisiae HSK* coding sequence under control of a P35S promoter, and homozygous T3 plants were selected for the analyses. (**A**) Susceptibility of *dmr1* is only restored to parental levels (L*er eds1-2*) by expression of the Arabidopsis *HSK* gene encoding a chloroplastic HSK and not by *S. cerevisiae* HSK that is active in the cytoplasm. Conidiophores were counted at 6 d after *H. arabidopsidis* Cala2 inoculation. The average number (with SD, n = 20) of conidiophores/seedling is displayed. This result was confirmed in four independent T3 lines per construct. Similar results were obtained in a separate independent experiment.

(**B**) In an independent experiment, homoserine levels were measured, showing that homoserine is completely metabolized in the At *HSK* complementation lines, which express a chloroplast-targeted HSK, but not in lines expressing the cytoplasmic Sc HSK. The average concentration (with SD, n = 3) of homoserine is displayed. These data suggest that, in the *dmr1* mutants, homoserine accumulates predominantly in the chloroplast

DISCUSSION

The *DMR1* gene was map base cloned and identified as At2g17265 encoding HSK. *dmr1* alleles from six independent mutants all carried GC-to-AT transitions in the *HSK* coding sequence, typical of EMS-induced mutations (Figure 2). All six mutations resulted in amino acid substitutions in the HSK protein. The fact that no null mutations were identified suggests that HSK is an essential protein in Arabidopsis. Indeed, in other organisms, for example, *E. coli* (Theze and Saint-Girons 1974) and *S. cerevisae* (Mannhaupt et al. 1990), mutation or deletion of the HSK gene leads to Thr auxotrophy. For an autotrophic organism, such as Arabidopsis, we expect Thr auxotrophy to be lethal. How can it be that mutation of a gene involved in primary metabolism does not lead to a strong macroscopic phenotype? Amino acid analysis of the *dmr1* mutants showed that the levels of Ile, Met, and Thr, downstream of homoserine in the Asp pathway, are not reduced (Figure 4). This suggests there is sufficient HSK activity remaining in the mutants or there is an alternative, so far unknown, biosynthetic route bypassing HSK, allowing the *dmr1* mutant to grow and develop normally. However, functional redundancy in Arabidopsis is unlikely as (1) the accumulation of homoserine in the *dmr1* mutants indicates that there is a blockage that is not bypassed, and (2) only a single *HSK* gene is predicted from the Arabidopsis genome sequence. A second gene (At4g35295) is annotated as "putative *HSK*," but this gene has many stop codons and the predicted protein is only 111 amino acids, while HSK is 370 amino acids long. At4g35295 is probably a pseudogene that has degenerated from a duplicated *HSK* gene. The HSK region on chromosome 2 and the At4g35295 region on chromosome 4 are syntenic and are proposed to originate from an ancient segmental duplication (Terryn et al. 1999; Blanc et al. 2000). A single *HSK* gene was also identified in the sequenced genomes of rice, grape, and *Physcomitrella*. In poplar, two predicted *HSK* genes have been identified that could have resulted from a more recent genome duplication (Tuskan et al. 2006).

The observation that *dmr1* mutants accumulate homoserine (Figure 4B) confirms that the Asp pathway is blocked at HSK. In addition, our results confirm that homoserine is indeed the substrate of the At2g17265-encoded enzyme, as was previously shown by enzymatic analysis of recombinant HSK (Lee and Leustek 1999). Homoserine is virtually undetectable in wild-type Arabidopsis as HSK is not rate limiting. Because of this, *HSK*-overexpressing plants do not produce more Met or Thr, except when additional homoserine is applied exogenously (Lee et al. 2005). The high homoserine content of the *dmr1* mutants indicates that HSK has become rate limiting. It was expected that the reduced HSK activity would lead to lower levels of downstream amino acids. However, the levels of Met, Thr, and Ile were increased rather than decreased in the *dmr1* mutants (Figure 4). We postulate that a feedback mechanism shuttles more Asp into the pathway so that homoserine accumulates to high levels. In the presence of high substrate concentrations, the residual HSK activity

may be sufficient to produce equal amounts or even more of Met, Thr, and Ile. When tested in vitro, recombinant HSK from most *dmr1* mutants indeed retained some residual enzyme activity (Figure 3). However, mutant HSK from *dmr1-1* appeared to be completely inactive when produced in *E. coli* and tested at 37°C. The mutant enzyme could be inactive when produced at 37°C in *E. coli*, while *in vivo* (in Arabidopsis), it could retain some residual activity. Since no T-DNA insertion lines are available for *HSK*, we are currently investigating a large collection of TILLING mutants to determine if *HSK* is an essential gene.

Exogenous application of amino acids to Arabidopsis showed that only homoserine accumulation causes resistance to downy mildew (Figure 5). Resistance was not induced by exogenous application of any of the other amino acids that were increased in levels in the *dmr1* mutants. The fact that *H. arabidopsidis* spore germination or *P. capsici* mycelium growth was not inhibited by direct treatment with L-homoserine implies that the plant plays an active role in mediating homoserine-induced resistance. Homoserine synthesis and the phosphorylation of homoserine by HSK, in the Asp pathway, has been shown to take place in the chloroplast (Azevedo 2002). However, *H. arabidopsidis* is not in direct contact with chloroplasts as it is physically separated from them by the plant cell membrane and cytoplasm. It is currently unknown if homoserine is transported out of the chloroplast in Arabidopsis.

Expression of the *HSK* gene from the yeast *S. cerevisiae* (Sc *HSK*) in the cytoplasm of *dmr1* mutants did not lead to a reduction in homoserine level (Figure 9). This indicates that the amino acid is not transported from the chloroplast to the cytoplasm and that it thereby remains inaccessible to the cytoplasmic *S. cerevisiae* HSK. This observation is supported by the fact that exogenous application of homoserine to *S. cerevisiae HSK*-expressing plants does not effectively induce resistance. We postulate that in wild-type plants, which lack cytoplasmic HSK activity, exogenous homoserine is taken up by the cell and transported to the chloroplast where it induces resistance. In plants expressing a cytoplasmic yeast HSK, exogenously applied homoserine is metabolized in the cytoplasm and is thus unable to reach the chloroplast and therefore does not induce resistance.

Resistance of the *dmr1* mutants is specific to *H. arabidopsidis*, as these plants are still susceptible to other pathogens. Previously, we reported that the *dmr1* mutants are susceptible to *P. syringae* pv *tomato* and *Golovinomyces orontii* (Van Damme et al. 2005). Preliminary data indicate that *dmr1* plants are also susceptible to the anthracnose fungus *Colletotrichum higginsianum* (R. O'Connell, personal communication) and the white rust pathogen *Albugo candida* (E. Holub, personal communication). The specific resistance is directly linked to high homoserine levels and could be caused by (1) the activation of a highly specific and so far unknown defence response or (2) the sensitivity of *H. arabidopsidis* to high homoserine levels in planta. This latter explanation is unlikely because homoserine must accumulate in the chloroplast to induce resistance. As tested by homoserine infiltration

in a large collection of Arabidopsis mutants, resistance was found to be independent of known defence signaling genes (Figures 6 and 7). To identify Arabidopsis genes required for homoserine-induced resistance, we have identified suppressors of *dmr1* (so-called loss of downy mildew resistant one resistance or *ldo* mutants).

Homoserine was not detectable in wild-type Arabidopsis seedlings. However, in other plant species, such as pea (*Pisum sativum*) and other members of the legume subfamily *Vicieae*, high levels of homoserine are present. As homoserine is abundant in the phloem sap of these plants, it is thought to be a transport molecule for nitrogen and carbon allocation. Interestingly, in pea, an alternative biosynthetic route exists, by transamination of a keto acid precursor, to produce high amounts of homoserine (Joy and Prabha 1986). Aminooxyacetate, a transamination inhibitor, can inhibit this reaction, whereas homoserine synthesis through the Asp pathway remains unaffected by this drug. This alternative pathway could explain why relatively more homoserine was found in the cytoplasm than in the chloroplasts of pea plants (Mills 1980). The high endogenous homoserine level in pea was found to act as an inducer of the fungal pathogen *Nectria hematococca*. It was hypothesized that *N. hematococca* has evolved the ability to sense homoserine, but also Asn, as a signal to induce expression of virulence genes in planta (Yang et al. 2005).

We conclude that, with the isolation of the *DMR1* gene encoding HSK, we have identified an alternative form of plant disease resistance caused by the accumulation of homoserine. The molecular mechanism by which homoserine triggers resistance in the chloroplast is still an enigma. The fact that putative *DMR1*/*HSK* orthologs are present in all higher plants allows us to address the agricultural application of *dmr1* technology in breeding downy mildew resistant crops that is based on a novel mechanism of resistance caused by modulation of host amino acid metabolism.

METHODS

Plant and pathogen growth conditions

All *Arabidopsis thaliana* accessions used in this study were grown on potting soil in a growth chamber (Snijders) at 22°C with 16 h of light (100 µE/m^2/s) and a relative humidity of 75%. *Hyaloperonospora arabidopsidis* isolate Cala2 was maintained on Arabidopsis L*er* by weekly transfer to healthy 10- to 14-d-old seedlings (Holub et al. 1994). To obtain large amounts of conidiospores for bioassays, inoculum was collected from L*er eds1-2* seedlings that support abundant Cala2 growth and sporulation (Parker et al. 1996). Inoculum (4 x 10^4 spores·mL^{-1}) was applied on 14-d-old seedlings using a spray gun. After inoculation, plants were allowed to dry for ~30 min and were subsequently incubated under a sealed lid (100% relative humidity) in a growth chamber at 16°C with 9 h light/day (100 µE/m^2/s). The amount of sporulation was quantified at 5 to 6 d after inoculation by counting the number of conidiophores on the cotyledons and leaves.

Microscopy

Infections of *H. arabidopsidis* in Arabidopsis leaves were visualized by trypan blue staining. Infected seedlings or leaves were stained in lactophenol (1:1:1:1 volume of lactic acid/glycerol/phenol/water) containing 1 mg/mL trypan blue, by boiling for 1 to 2 min and destaining overnight in choral hydrate. Trapped air bubbles were removed by 1 min speed vacuum infiltration. *H. arabidopsidis* growth was visualized by differential interference contrast microscopy.

Cloning of *DMR1*

The isolation of the *dmr1* mutants, which have been generated by EMS mutagenesis, has been described previously (Van Damme et al. 2005). The *dmr1* mutants were backcrossed twice (BC_2), to reduce the number of unlinked EMS-induced mutations, to the parental line L*er eds1-2* and to L*er* containing the wild-type *EDS1* gene. A mapping population was generated by crossing the mutant to Columbia-0 (Col-0) FN2 (Sinapidou et al. 2004), and the resistant F2 plants were selected, genotyped, and rescreened for resistance in the F3. The *dmr1* phenotype was found to be linked to the ciw3 and nga1126 markers (www.arabidopsis.org) on chromosome 2. The *dmr1* mutation was fine-mapped to an ~130-kb region covered by three BACs between two IND-based markers (marker designed by use of http://www.arabidopsis.org/browse/Cereon/index.jsp site for polymorphisms), located on BAC F6P23, at 7.43 Mb, and F5J6, at 7.56 Mb, resulting in an area of 30 candidate *DMR1* genes. Additional cleaved amplified polymorphic markers in six genes, At2g17190, At2g17200, At2g17270, At2g17300, At2g17310, and At2g17360, allowed the further reduction of the *dmr1* region to

eight candidate genes At2g17230 to At2g17290. DNA sequencing of the genes in the *dmr1* mutants resulted in the finding of mutations in At2g17265, which was thereby identified as *DMR1*.

Identification of putative HSK orthologs

Putative HSK orthologs were identified from the annotated genome sequences of grape (*Vitis vinifera*), poplar (*Populus trichocarpa*), rice (*Oryza sativa*), and the moss *Physcomitrella patens* by BLASTP searches on RefSeq proteins using the *Arabidopsis* HSK protein as a query. The identified putative HSK orthologs were used as query in reciprocal BLASTP searches on all Arabidopsis proteins. Identification of Arabidopsis HSK as the highest scoring protein confirmed the finding of a reciprocal best hit; therefore, the identified protein was considered a putative ortholog. For the identification of putative orthologs from other plant species, EST databases were employed. The Arabidopsis HSK protein sequence was used as query in TBLASTN searches against the EST database. For each plant species analyzed, the high-scoring ESTs were collected and assembled into contigs using the CAP3 program (http://pbil.univ-lyon1.fr/cap3.php). Reciprocal BLASTX analysis using the contig nucleotide sequences against all Arabidopsis proteins was used to select those contigs that had the best hit with Arabidopsis HSK. All collected HSK putative orthologs formed a single clade in a phylogenetic tree generated using ClustalW (standard settings).

Amino acid analysis

Amino acids were extracted from 100 mg of aboveground parts of 14-d-old seedlings that were ground in liquid nitrogen. Amino acids were extracted with 80% methanol (twice), and the supernatant was collected in a fresh tube. The remaining pellet was extracted with 20% methanol (twice), and the extract was added to the previously collected 80% methanol extracted supernatant. Samples were lyophilized and dissolved in 150 µL of deionized water. An equal volume (150 µL) of internal standard, 750 µmol/L, *S*-amino-ethyl-cysteine was added. Prior to amino acid detection, the remaining proteins were removed by treatment with 10% sulfosalicylic acid. Plant amino acids were detected and quantified by automated ion-exchange chromatography with post column ninhydrin derivatization on a JEOL AminoTac JLC-500/V.

Homoserine treatment of Arabidopsis seedlings

The homoserine treatment was combined with an *H. arabidopsidis* pathogenicity assay. Ten-day-old Arabidopsis seedlings were spray inoculated with *H. arabidopsidis* Cala2 (4 x 10^4 spores·mL^{-1}) and 3 d later the infected seedlings were removed from the soil and submerged in L- or D-homoserine solution (or another amino acid) at the indicated concentration in an

Eppendorf tube and vacuum infiltrated. After infiltration, the seedlings were transplanted back into the soil. Pathogen growth and sporulation were monitored 5 to 6 d after infection and 2 to 3 d after infiltration.

Construction of *dmr1* transgenic lines overexpressing *HSK*

Complementation lines were generated by transforming *dmr1* plants by the floral dip method (Clough and Bent 1998) with *Agrobacterium tumefaciens* containing the *HSK* gene from Col-0 (At *HSK*) or the *HSK* gene *THR1* from *Saccharomyces cerevisiae* (Sc *HSK*) under control of the 35S promoter. The Arabidopsis *HSK* construct was generated by PCR amplification of the full-length coding sequence from Col-0 cDNA with primers For.HSKclonAT._BamHI, 5'-ctcattactggatcctcaatggcaag tct-3', containing a *Bam*HI restriction site near the start codon (ATG), and Rev.HSKclonAT._EcoRI, 5'-gttccaatcttaacgaattcaaacagcacac-3', containing an *Eco*RI site after the stop codon. The fragment was cloned directionally between the P_{35S} promoter and the Nos terminator and inserted into pGreenII0229 (http://www.pgreen.ac.uk; Hellens et al. 2000). The *S. cerevisiae HSK* gene was PCR-amplified using primers For.HSKclonYeast._BamHI, 5'-tagtgggatccgcagatggttcgtgccttc-3', and Rev.HSKclonYeast._EcoRI, 5'-ctgcagaattcctattcattgctgttcgacgc-3', and cloned as described for Arabidopsis *HSK*. Transformed seedlings containing the P_{35S}-*HSK* constructs were selected for BASTA resistance. DL-Phosphinothricin (300 µM; BASTA) was sprayed on 10-d-old seedlings, and resistant seedlings (T1) were transplanted for seed set. The T2 and T3 generations were analyzed for *H. arabidopsidis* susceptibility.

HSK recombinant protein production and enzyme assay

The coding sequences of the wild-type HSK gene (Arabidopsis accession L*er*) and that of the *dmr1* mutants were PCR amplified from genomic DNA using primers HSK_pET_F, 5'-cccccatggcaagtctttgtttccaa-3', and HSK_pET_R, 5'-cccctcgagtcatctggagacgctgttga-3'. PCR fragments were digested with *Nco*I and *Xho*I and ligated in pET-30(a)+ (Novagen). The coding sequence of the yeast HSK was PCR amplified using primers FwScHSK, 5'-ccggatccatggttcgtgccttcaa-3', and RvScHSK, 5'-cccctcgagctattcattgctgttcgacgc-3'. PCR fragments were digested with *Bam*HI and *Xho*I and ligated in pET-30(a)+. Production of recombinant enzyme and the HSK enzyme assay was performed as previously described (Lee and Leustek 1999), with several modifications, as described below. Bacterial pellets were resuspended in lysis buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 10 mM imidazole) and sonicated six times for 10 s. Cleared lysates were filtered (0.2 µm) and incubated with Ni-NTA beads (Qiagen) for 1 h at 4°C. The beads were washed three times with washing buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 20 mM imidazole) and finally eluted in elution buffer (50 mM NaH_2PO_4, pH 8.0, 300 mM NaCl, and 250 mM imidazole). HSK activity was assayed indirectly by measuring ADP levels spectrophotometrically in 96-well plates at

340 nm.

Input protein levels were quantified by protein gel blot analysis using anti-His antibodies (Amersham). Specific activities were calculated using the activity assay data and protein input levels that were plotted relative to the activity of wild-type HSK protein.

Q-PCR analysis

Total RNA was extracted using an RNeasy kit and treated with the RNase-free DNase set (Qiagen). Total RNA was quantified using a Biowave II UV/visible spectrophotometer (WPA). cDNA was synthesized with SuperScript-III reverse transcriptase (Invitrogen) and oligo(dT)15 (Promega). Cycle tresholds were determined in triplicate per transcript in three biological replicas using the ABI PRISM 7700 sequence detection system (Applied Biosystems) using SYBR Green I as the reporter dye. The data were normalized using Arabidopsis *Actin2* levels. Primers used for detecting transcripts analyzed in this study are *Actin2* (QACT2F, 5'-aatcacagcacttgcacca-3', and QACT2R, 5'-gagggaagcaagaatggaac-3'), *PR-1* (QPR-1F, 5'-gaacacgtgcaatggagttt-3', and QPR-1R, 5'-ggttccaccattgttacacct-3'), *PR-2* (QPR-2F, 5'-cccgtagcatactccgattt-3', and QPR-2R, 5'-aaggagcttagcctcaccac-3'), *DMR6* (QDMR6F, 5'-tgtcatcaacataggtgacca-3', and QDMR6R, 5'-cgatagtcacggattttctgtg-3'), and *HSK* (QHSKF, 5'-ctgctttagtcgctgctgtg-3', and QHSKR, 5'-gaatcaacggcgctctagtc-3'). The size of amplicons was between 99 and 101 bp.

Accession numbers

Sequence data from this article can be found in the Arabidopsis Genome Initiative or GenBank/EMBL databases under the following accession numbers: DMR1/HSK (At2g17265), PMR4 (At4g03550), NPR1 (At1g64280), Sid2-1 (At1g74710), PAD4 (At3g52430), EIN2 (At5g03280), NDR1 (At3g20600), JAR1 (At2g46370), PMR2/MLO2 (At1g11310), MLO6 (At1g61560), MLO12 (At2g39200), PEN1 (At3g11820), PEN2 (At2g44490), PEN3 (At1g59870), SAG101 (At5g14930), PAD3 (At3g26830), FMO1 (At1g19250), SGT1b (At4g11260), RAR1 (At5g51700), DMR6 (At5g24530), *S. cerevisiae* THR1 (NP_011890), *P. trichocarpa* HSK (XP_002328135), *V. vinífera* HSK (XP_002277887), and *O. sativa* HSK (NP_001048623).

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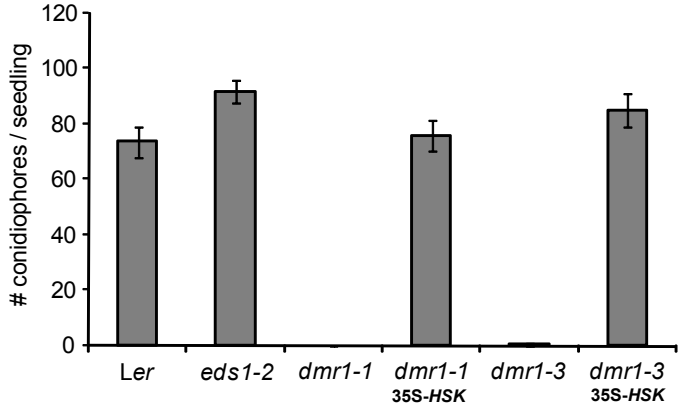
SUPPLEMENTAL DATA



Supplemental Figure 1. Complementation of *dmr1* mutants with the HSK gene.

The *dmr1-1* and *dmr1-3* mutants were transformed with the wild-type Arabidopsis Col-0 *HSK* coding sequence under control of the 35S promoter. Sporulation was quantified at six days post Cala2 inoculation of 10-day old seedlings. The average number of conidiophores/seedling (with standard deviation, n=20) is displayed. Transgenic lines have a regained susceptibility to *H. arabidopsidis*. Similar data were obtained in a separate independent experiment.


```
Arabidopsis_thaliana      1 --MASLCFQSPSKPISYFQPKSN-------PSPPLFAKVSVFRCRAS---
Citrus_sinensis           1 ---MAICFSSAVKPANHFTVFFN-------PAP----KKPIFKCSCS---
Populus_trichocarpa       1 ---MAICFLSPLKPITPTTSLTT--------NLNPKKPN-ILRCNFS---
Vitis_vinifera            1 ---MAICFHSPSKPTCISPSSNH-----YRPNLHAR----SFRCNFS---
Glycine_max               1 -MATSTCFLCPSTASLKGRARFR----------------IRIRCSSS---
Phaseolus_vulgaris        1 -MATAMSFLCPSPATFKGTEMPI----------------ARFRCCSSNTN
Nicotiana_benthamiana     1 --MAAICYQSPVKLNFTTSNAFSNPIPNNPPPLYPIKTRFSSGFNLSA--
Solanum_lycopersicum      1 ---MAITFQSPMKLSFITSNGFS-----NPPSLYPINTHFSFGFNLSS--
Solanum_tuberosum         1 ---MAITYQSPMKLNFITSNGFS-----NPPSLYPINTHFSFGFNLSS--
Physcomitrella_patens     1 ---MQVTCEAVKKAAVLTPPLKQ-----------------------------
Oryza_sativa              1 MAAAAAAAAAPSPAPCFPSTRH--------TLPGLVSVRVSRRVKVA---
Zea_mays                  1 -MAPAATSTASAPSSFHSTGRHR-------ARVGARPSLVSLRVRAAN--

                                        K(dmr1-2)          Motif1
                                           ▼               ------
Arabidopsis_thaliana     39 -VQTLVAV------EPEEVFVSVKTFAPATVANLGPGFDFLGCAVDG
Citrus_sinensis          34 -LPTVTTT------EPEEVFTSVKTFAPATVANLGPGFDFLGCAVDG
Populus_trichocarpa      36 -LPIITTT------EPEEVFTSVRSFAPATVANLGPGFDFLGCAVDG
Vitis_vinifera           36 ----KTLT-----ADPQEVFTSVKSFAPATVANLGPGFDFLGAAVDG
Glycine_max              31 -VSVNIRR------EPEEVTTLVKAFAPATVANLGPGFDFLGCAVDG
Phaseolus_vulgaris       34 SVSLNTRT------EPQEVTTFVKAFAPATVANLGPGFDFLGCAVDG
Nicotiana_benthamiana    47 -VPSKTQTTHITIPEPEEVFASVKSFAPATVANLGPGFDFLGCAVDG
Solanum_lycopersicum     41 -VSSKTQT-HITIPEPEEVFTSVKSFAPATVANLGPGFDFLGCAVDG
Solanum_tuberosum        41 -VSSKTQT-HITIPEPEEVFTSVKSFAPATVANLGPGFDFLGCAVDG
Physcomitrella_patens    21 ----LTLS------EPKLAHQFVKAFAPATVANLGPGFDFLGCAVDG
Oryza_sativa             40 ---VAIA-------DPAEAFNSVTAFAPATVANLGPGFDFLGCAVAD
Zea_mays                 41 --PNVTA-------DPAEAFQSVTTFAPATVANLGPGFDFLGCAVAD
                                          .*  .    *. ***********.*****.**..

Arabidopsis_thaliana     79 ----LGDHVTLRVDPSVRAGEVSISEITGTTT-----KLSTNPLRNC
Citrus_sinensis          74 ----LGDYVSLKVDPSVHPGEVSISEVIGPS------KLSKNPLWNC
Populus_trichocarpa      76 ----LGDFVSLRVDPSVHPGEISISDISGTKN------LSKNPLNNC
Vitis_vinifera           74 ----LGDFVSLRVDPDVRPGEISIVDIDGVGNS--AKKLSKNPLWNC
Glycine_max              71 ----LGDIVSVKVDPQVHPGEICISDISGHAPN----KLSKNPLWNC
Phaseolus_vulgaris       75 ----LGDIVSVRVDPEVRPGEIRISDITGHAPN----KLSTNPLWNC
Nicotiana_benthamiana    93 ----LGDFTTLRVDSKVKPGEVSISDISGAGG-----KLSKDPLSNC
Solanum_lycopersicum     86 ----VGDFVTLRVDPNVKAGEVSISDISGAGN-----RLSKDPLSNC
Solanum_tuberosum        86 ----LGDFVTLRVDPNVKAGEVSISDISGAGN-----RLSKDPLSNC
Physcomitrella_patens    58 ----LGDYVTAEVSNAVPAGQVCISTTTGDNG-----RLSLVAGNNC
Oryza_sativa             77 ASLSLGDTVTATLDPSLPPGTVAIASVTSPSRPTLADRLSRDPLRNC
Zea_mays                 79 ASLSLGDTVTATLDPSLPPATVSIASVTSPSRPNLAERLSRDPLRNC
                               .** ..  ... .  . . *  ...        .**  ..  **
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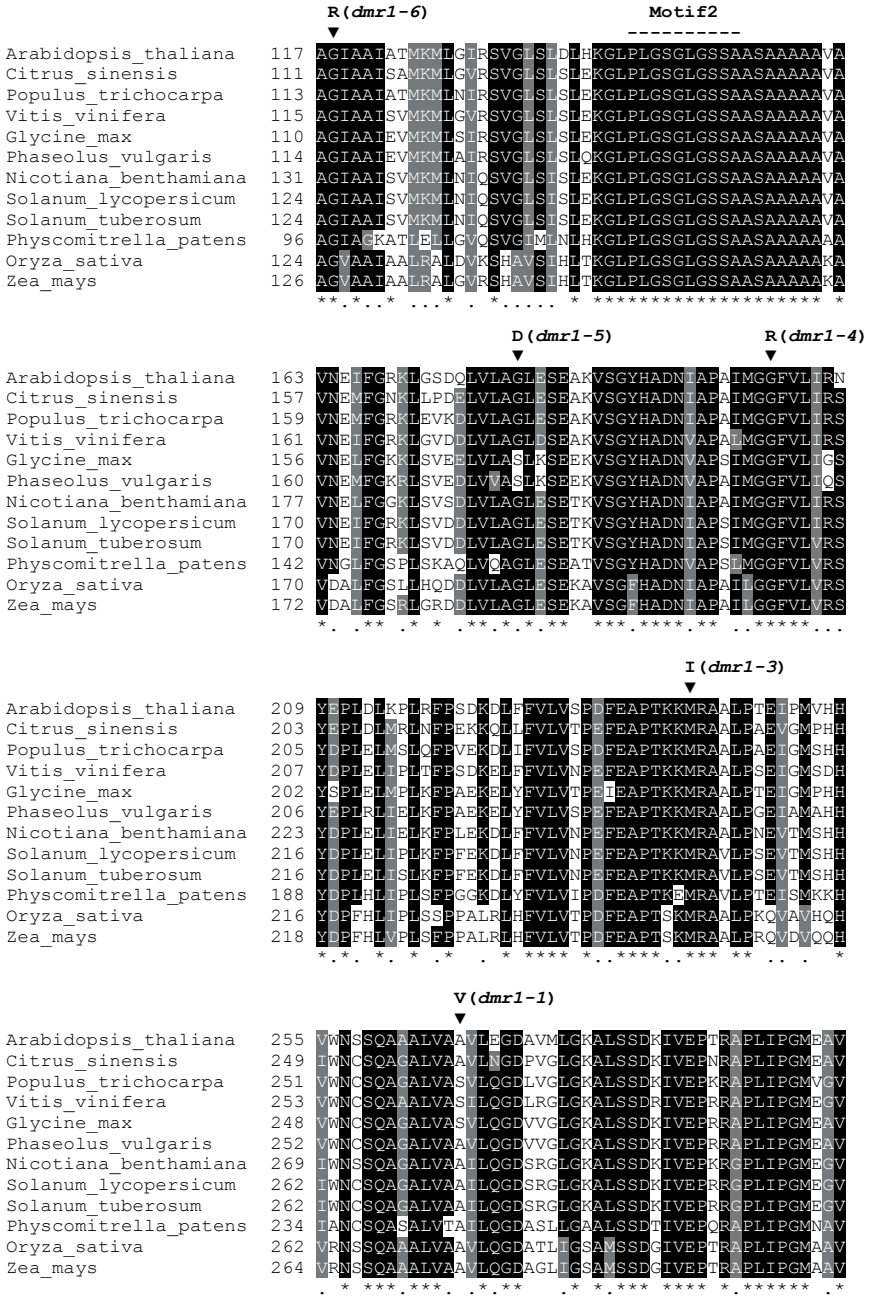
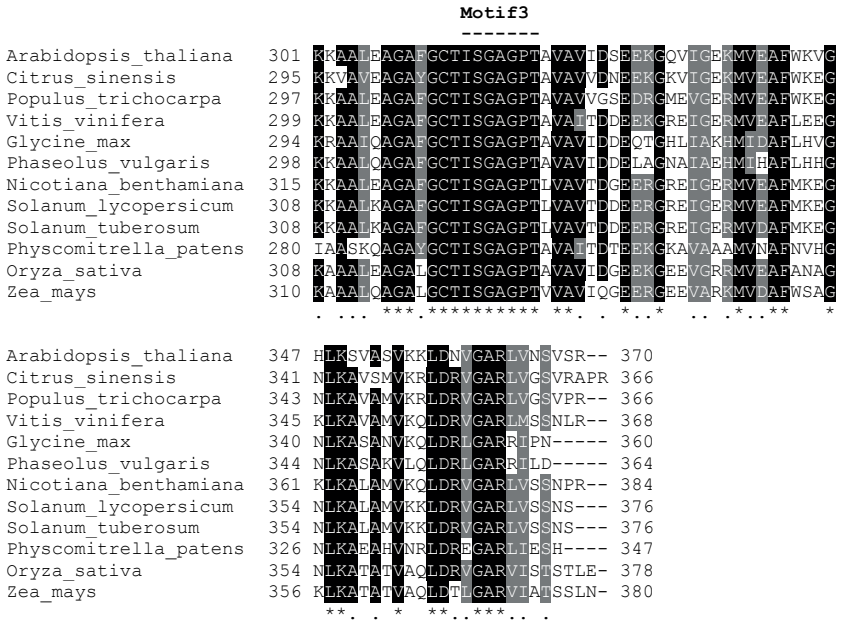
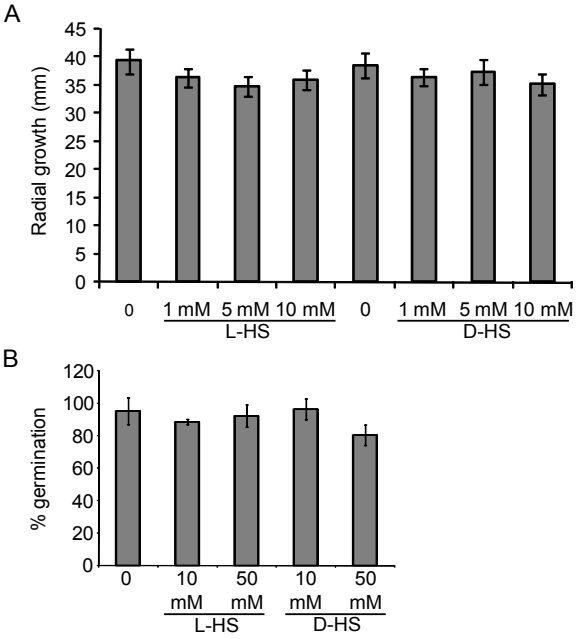

R(dmr1-6)
Motif2
D(dmr1-5)
R(dmr1-4)
I(dmr1-3)
V(dmr1-1)
Arabidopsis_thaliana
Citrus_sinensis
Populus_trichocarpa
Vitis_vinifera
Glycine_max
Phaseolus_vulgaris
Nicotiana_benthamiana
Solanum_lycopersicum
Solanum_tuberosum
Physcomitrella_patens
Oryza_sativa
Zea_mays



50 **Supplemental Figure 2.** Multiple Sequence Alignment of plant homoserine kinase amino acid sequences.
The sequence alignment was generated using the CLUSTALW program from the European Bioinformatics Institute (EBI) and conserved residues were highlighted using the BOXSHADE tool. HSK sequences are derived from mRNA, genomic or assembled EST sequences which are available from GenBank on the National Center for Biotechnology Information (NCBI) website. Below the sequence the conserved amino acids are indicated by dots, and identical amino acids by asterisks. The black triangles indicate the amino acids that are substituted in the six Arabidopsis *dmr1* mutants. The three motifs that are conserved in the superfamily of GHMP kinases are indicated by lines. The diamonds above the alignment indicate the amino acids that bind ATP or are in the active site.



Supplemental Figure 3. Oomycete hyphal growth and spore germination are not affected by L-homoserine compared to D-homoserine treatment.

A. Radial growth (average with standard deviation, n=5) of *P. capsici* was measured from day 1 to day 3 after inoculation of an agar plug of mycelium on V8 medium containing different concentrations of L- or D-homoserine. Although there is a slight reduction in radial growth on medium containing the amino acids, it is not different between L-homoserine and D-homoserine treatments. Similar results were obtained for *P. brassicae*. **B.** Spore germination of *H. arabidopsidis* Waco9 was monitored in water, 10 mM and 50 mM L- and D-homoserine and spore germination was counted after 24 h of incubation. The average percentage (with standard deviation, n=30) of germination is displayed.

```
Hyaloperonospora_arabidopsidis   MT--TTDATTPRVATQVAVAAAGAALAYYVGARTKSAKRASAQHRAKAVA 48
Phytophthora_ramorum             MAGGNSDASTQRVATQVAVAAAGAALAYYVGSRSKSARRSTAERRIQAVA 50
Phytophthora_sojae               MAGSNSDASTQRVATQVAVAAAGAALAYYVGSRSKSARRAAAKRRAQDVA 50
Arabidopsis_thaliana             ------------MASLCFQSPSKPISYFQ-PKSNPSPPLFAKVSVFRCR 36
Oryza_sativa                     ------------MAAAAAAAAAPSPAPCF-PSTRHTLPGLVSVRVSR-R 35
                                             :  .  :.. . :    . :. :    ..

Hyaloperonospora_arabidopsidis   EAMATVLPTDLAAHKLTSVVVRVPATTANMGPGFDTIGMALDIWT----- 93
Phytophthora_ramorum             EAKAAALPTDLERRKLTSVVVRVPATTANMGPGFDTIGMALDIWT----- 95
Phytophthora_sojae               QAKAAALPTDLERRKLTSVVVRVPATTANMGPGFDTIGMALDIWT----- 95
Arabidopsis_thaliana             ASVQTLVAVEPEP-VFVSVKTFAPATVANLGPGFDFLGCAVDG----LGD 81
Oryza_sativa                     VKVAVAIA-DPAP-AFNSVTAFAPATVANLGPGFDFLGCAVADASLSLGD 83
                                     . :. :     : ** . .***.**:***** :* *:

Hyaloperonospora_arabidopsidis   EIAVEIVAPLQGDALNVLRVTLTNEGEGATELPKDETNLVIVGLKAAFKA 143
Phytophthora_ramorum             EISAEVAAPLEGDAPNVRRVTLTNEGEGAKELPTDESNLVIVGIKAAFKA 145
Phytophthora_sojae               EISAEVVE----DASVAPGITLTNEGEGAKELPTDASNLVIVGIKAAFKA 141
Arabidopsis_thaliana             HVTLRVDPSVRAGEVSISEITGTT-----TKLSTNPLRNCAGIAAIATMK 126
Oryza_sativa                     TVTATLDPSLPPGTVAIASVTSPSRPTLADRLSRDPLRNCAGVAAIAALR 133
                                  ::  :      .      :* ..      .*. :  .        *

Hyaloperonospora_arabidopsidis   AGEEMPRHVKVHCKNRIPFARGLGSSSAGIVGGIIAGLALSGMRLPVQGK 193
Phytophthora_ramorum             AGEELPRHIKVHCKNRIPFARGLGSSSAGIVGGIIAGLALAGMRLPVHGR 195
Phytophthora_sojae               AGEPLPRHLKVHCKNRIPFARGLGSSSAGIVGGIIAGLALAGMRLPVRGR 191
Arabidopsis_thaliana             MLGIRSVGLSLDLHKGLPLGSGLGSSAASAAAAAVAVNEIFGRKLGSD-Q 175
Oryza_sativa                     ALDVKSHAVSIHLTKGLPLGSGLGSSAASAAAAAKAVDALFGSLLHQD-D 182
                                      .  :.:.  : :*:. *****:*. ...  *   : *  *

Hyaloperonospora_arabidopsidis   EELLQLASEIEG---HPDNVAPAIYGGLQLG-IFADNRWYSSRVQIPDGL 239
Phytophthora_ramorum             EELLQLSSEIEG---HPDNVAPAIYGGLQLG-IFADDRWYSSRVQIPDGL 241
Phytophthora_sojae               EELLQLASEIEG---HPDNVAPAIYGGLQLG-IFADDRWYSSRVQIPDGL 237
Arabidopsis_thaliana             LVLAGLESEAKVSGYHADNIAPAIMGGFVLIRNYEPLDLKPLRFPSDKDL 225
Oryza_sativa                     LVLAGLESEKAVSGFHADNIAPAILGGFVLVRSYDPFHLIPLSSPPALRL 232
                                   *  * **      *.**:**** **: *   :      .       *

Hyaloperonospora_arabidopsidis   QCVVFIPDSTGPTSVARAILPPNVSRQDAVFNIGRTAIFVNAFRSGNLDE 289
Phytophthora_ramorum             QCVVFIPDSTGPTSVARAILPPNVPRKDAVFNIGRAAIFVNAFRSGNLDE 291
Phytophthora_sojae               QCVVFIPDSTGPTSVARAILPPDVPRKDAVFNIGRAAIFVNAFRSGNLDE 287
Arabidopsis_thaliana             FFVLVSPDFEAPTKKMRAALPTEIPMVHHVWNSSQAAALVAAVLEGDAVM 275
Oryza_sativa                     HFVLVTPDFEAPTSKMRAALPKQVAVHQHVRNSSQAAALVAAVLQGDATL 282
                                   *:. **  .**.  ** ** ::.  . * * .::* :* *. .*:

Hyaloperonospora_arabidopsidis   LRFATQDMLHQPQRGAAQYPHLEPLINAALGAGAHGCFLSGAGPTVLAIT 339
Phytophthora_ramorum             LRYATQDMLHQPQRGAAQYPHLEPLMKAALGAGAHGCFLSGAGPTVLAIT 341
Phytophthora_sojae               LRYATQDMLHQPQRGAAQYPHLEPLIKAALGAGAHGCFLSGAGPTVLAIT 337
Arabidopsis_thaliana             LGKALSSDKIVEPTRAPLIPGMEAVKKAALEAGAFGCTISGAGPTAVAVI 325
Oryza_sativa                     IGSAMSSDGIVEPTRAPLIPGMAAVKAAALEAGALGCTISGAGPTAVAVI 332
                                 :  * ..        *.  * : .:  *** *** ** :******.:*:

Hyaloperonospora_arabidopsidis   SGRAGDIFTQQLAERQENKVANAMRETAAAMG-VPGCVFITNPDHRGAFI 388
Phytophthora_ramorum             SGRAGDIFTQQLAERQENKVANAMREAAAAIG-VSGCVFITNPDHRGAFI 390
Phytophthora_sojae               SGRAGDIFTQQLAERQENKVANAMREAAAALG-VSGCVFITNPDHRGAFI 386
Arabidopsis_thaliana             DS-----------EEKGQVIGEKMVEAFWKVGHLKSVASVKKLDNVGARL 364
Oryza_sativa                     DG-----------EEKGEEVGRRMVEAFANAGNLKATATVAQLDRVGARV 371
                                 ..           *.: : :.. * *:    * : . . : : *. ** :

Hyaloperonospora_arabidopsidis   VRAEPRYSDRSVARYEGDIADL 410
Phytophthora_ramorum             VRAEPRFSDRAVARYEGDVADL 412
Phytophthora_sojae               VRAEPQFSDRSVARYEGDVADL 408
Arabidopsis_thaliana             VNSVSR---------------- 370
Oryza_sativa                     ISTSTLE--------------- 378
                                 : : .
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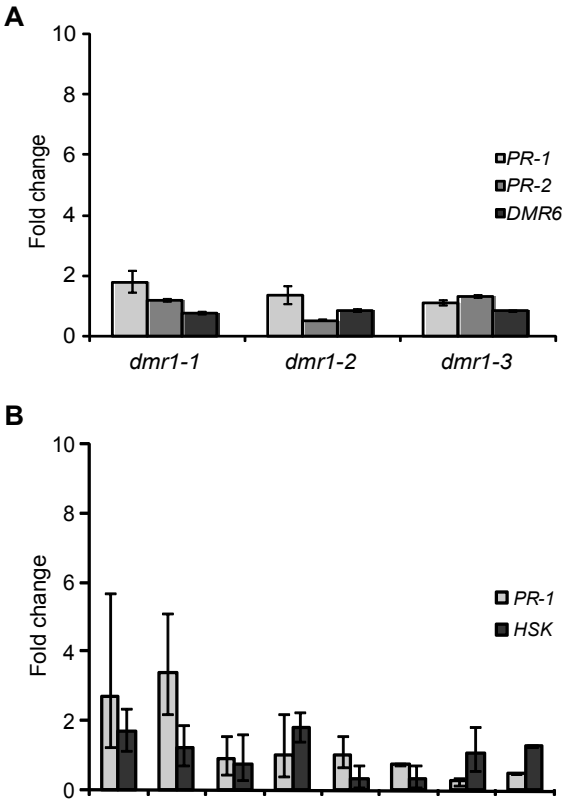
Supplemental Figure 4. Multiple alignment of three oomycete and two plant HSK protein sequences.
The putatively orthologous HSK protein sequences of the oomycete pathogens *H. arabidopsidis*, *P. sojae*, and *P. ramorum* have been identified by homology to the Arabidopsis HSK protein sequence. A reciprocal Blast search using the oomycete HSKs on all Arabidopsis proteins indicated the Arabidopsis HSK protein as best hit, suggesting they are orthologous.



Supplemental Figure 5. Defence-associated genes and HSK are not strongly activated in the mutants *dmr1-1*, *dmr1-2*, and *dmr1-3*.

A. Transcript levels of the defence-associated genes *PR-1*, *PR-2*, and *DMR6* were determined in the *dmr1-1*, *dmr1-2*, and *dmr1-3* mutants by Q-PCR and plotted relative to the parental line L*er eds1-2*. **B**. Transcript levels of *PR-1* and *HSK*, at 1 and 2 days post inoculation (dpi) with *H. arabidopsidis* Cala2, in the *dmr1-1*, *dmr1-2*, *dmr1-3* mutants and the parental line L*er eds1-2* compared to the transcript levels of material taken directly after inoculation (0 dpi). Bars represent the mean fold change in transcript level, with error bars representing the standard deviation (n=3).

3

Arabidopsis *HOMOSERINE KINASE* TILLING mutants; engineering resistance to downy mildew by mutation

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ABSTRACT

Mutations in the Arabidopsis *HOMOSERINE KINASE* (*HSK*) gene were previously shown to be responsible for resistance to the oomycete pathogen *Hyaloperonospora arabidopsidis* in six independent *downy mildew resistant 1* (*dmr1*) mutants. To explore the use of reverse genetics to obtain disease resistant plants we used TILLING to identify novel mutations in *HSK*. A total of 55 mutant alleles were obtained of which 37 were missense mutations leading to amino acid substitutions in the HSK protein. Arabidopsis mutants homozygous for these mutant alleles were analysed for downy mildew resistance and homoserine levels. One mutant (#44) proved to be resistant to *H. arabidopsidis* and accumulated high levels of homoserine, showing that resistance can be engineered by mutation. Homoserine was undetectable in wild type plants and all other TILLING mutants that also did not display resistance to downy mildew. For TILLING mutant #34 no viable homozygous mutant lines could be obtained suggesting that *HSK* is an essential plant gene. *HSK* genes were identified in genome and transcript sequences of crop species. Recombinant proteins, produced from *HSK* of tomato and cucumber, were shown to exhibit HSK enzyme activity. We have demonstrated that reverse genetics using TILLING is a valuable approach to obtain novel alleles providing of recessive resistance to downy mildew.

INTRODUCTION

Recessive forms of plant disease resistance can be found in natural populations or can be artificially obtained by chemical mutagenesis. In both cases, the homozygous presence in the host plant of alleles that encode inactivated proteins (null alleles) or proteins with reduced activity (hypomorphic alleles) can lead to reduced susceptibility to pathogen infection. We can distinguish three mechanisms by which these recessive genes mediate resistance. The first one is by removal of a negative regulator of defence like in the *cpr30* (*constitutive expressor of PR genes*), *lsd1* (*lesion stimulating disease resistance response*), or *dmr6* (*downy mildew resistant 6*) mutants that have constitutive activation of plant defence in the absence of pathogen invasion (Bowling et al. 1994; Dietrich et al. 1994; van Damme et al. 2008). A second mechanism is the absence of important factors in the host that are required for the infection process e.g. *eIF4E* in virus infection (reviewed by Robaglia and Caranta 2006) and the rice genes *Xa5* and *Xa13* to the bacterium *Xanthomonas oryzae* (Iyer-Pascuzzi and McCough 2007). The third mechanism that we distinguish is due to reduced activity of a metabolic enzyme resulting in accumulation of a substrate that then induces resistance, e.g. the accumulation of homoserine in the Arabidopsis *dmr1* mutant (van Damme et al. 2009).

In a genetic screen aimed at identifying host genes that are involved in susceptibility of Arabidopsis to the obligate biotrophic oomycete *Hyaloperonospora arabidopsidis*, several independent *downy mildew resistant 1 (dmr1)* mutants were isolated (van Damme et al. 2005). The *DMR1* gene was found to encode for homoserine kinase and the six available mutant alleles all carried missense substitutions. Recombinant enzyme produced from the *dmr1* alleles displayed reduced or almost complete lack of HSK activity *in vitro*. This reduced activity was evident in the *dmr1* mutants as they accumulated the amino acid homoserine to high levels, whereas in wild type plants this compound was not detected. However, there was no reduction in the levels of the downstream amino acids threonine, methionine, and isoleucine in the *dmr1* mutants. The high level of homoserine was shown to be responsible for disease resistance as exogenous application of the amino acid in wild type plants reduced infection by *H. arabidopsidis*. Homoserine-induced resistance was shown to be independent of known defence pathways, thereby constituting a novel form of disease resistance.

Homoserine kinase (HSK) is a key enzyme in primary metabolism. HSK catalyzes the ATP-dependent phosphorylation of Lhomoserine to *O*-phospho-homoserine. This conversion is part of the Aspartate pathway and ultimately leads to the formation of the amino acids Methionine, Threonine, and Isoleucine (Azevedo et al. 2006). HSK belongs to the GHMP kinase superfamily (**G**alactokinase, **H**omoserine kinase, **M**evalonate kinase, and **P**hospomevalonate kinase) and members of this superfamily are present in bacteria, archaea, and eukaryotes where they participate in various pivotal metabolic pathways (Bork

et al. 1993). The first HSK crystal structure published is from the archaeon *Methanococcus jannaschii* (Zhou et al. 2000). Conserved within members of the GHMP family is a unique kinase fold and a novel nucleotide binding site (Zhou et al. 2000). Apparently, HSK acts as homodimer in which it forms two active sites (Burr et al. 1976; Krishna et al. 2001). In *Escherichia coli* and *Saccharomyces cerevisae,* lack of a functional HSK protein due to mutation or deletion of the homoserine kinase gene leads to threonine auxotrophy (Kingsbury and McCusker 2008; Theze and Saint-Girons 1974). In plants, lack of threonine, methionine or isoleucine is expected to be lethal since they are autotrophic organisms. This idea is strengthened by the fact that no HSK null-mutants have been identified in Arabidopsis to date. To identify new *HSK* alleles we employed TILLING (Targeting Induced Local Lesions in Genomes) (Till et al. 2003). TILLING is a powerful reverse-genetic method involving chemical mutagenesis that results in randomly distributed induced point mutations in any gene of interest. It has been estimated that 5% of the EMS-induced mutations of a targeted coding region result in a premature stopcodon (Greene et al. 2003). Also, around 50% of all mutations is predicted to be missense and alter the protein sequence (Greene et al. 2003).

Previously, TILLING has successfully been used to identify resistance alleles of *eIF4E* in tomato. eIF4E is a translation initiation factor and loss of function of the corresponding gene leads to recessive resistance towards potyviruses. Piron et al. (2010) identified a splicing mutant in their TILLING analysis that confers resistance towards Potato virus X and Pepper mottle virus. Also in melon a similar approach resulted in the identification of mutants with improved fruit shelf life by mutation in *ACC OXIDASE 1* (Dahmani-Mardas et al. 2010). In our analysis, we assess the idea if random mutation can lead to hypomorphic *HSK* alleles that in homozygous mutant lines result in accumulation of the HSK substrate homoserine and consequential resistance to downy mildew. From the 37 missense alleles that we obtained by TILLING, no premature stop codons were identified and only 1 mutation could be linked to disease resistance showing that the frequency of hypomorphic *HSK* alleles is low. For one particular mutant, no viable homozygous mutant lines could be obtained suggesting that *HSK* is an essential plant gene. We demonstrate that the untargeted reverse-genetic approach enables the identification of a mutant which is resistant to the oomycete pathogen *H. arabidopsidis*. Furthermore, this work could serve as framework for engineering downy mildew resistance into economically important crop species.

RESULTS

HSK TILLING alleles

In order to identify hypomorphic *HSK* alleles, a collection of mutants containing nucleotide changes in the *HSK* gene were obtained by TILLING. A total of 52 mutants with nucleotide changes in the *HSK* coding sequence (CDS) (Supplemental Table 1) and 3 with nucleotide

changes in the untranslated regions (UTRs) (#1, 54, 55) were identified. The mutations are evenly distributed in the *HSK* gene and are all GC to AT substitutions that are expected for EMS-mutagenized DNA. Of these mutations, 37 were missense (non-synonymous) leading to amino acid substitutions in the encoded protein (Figure 1), while the remaining 15 were silent (synonymous substitutions). We observe slightly more missense mutations than expected according to the CODDLE prediction tool (71,2% versus 62,9%, Table 1).
Unfortunately, no nonsense mutations were identified that result in pre-mature stop codons, despite the fact that the HSK coding sequence has 12 codons that have the potential to change to stop codons by a single GC to AT transition (Table 1).

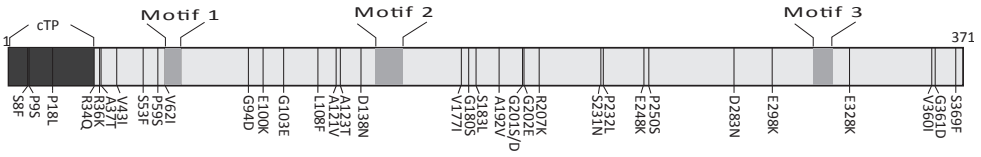


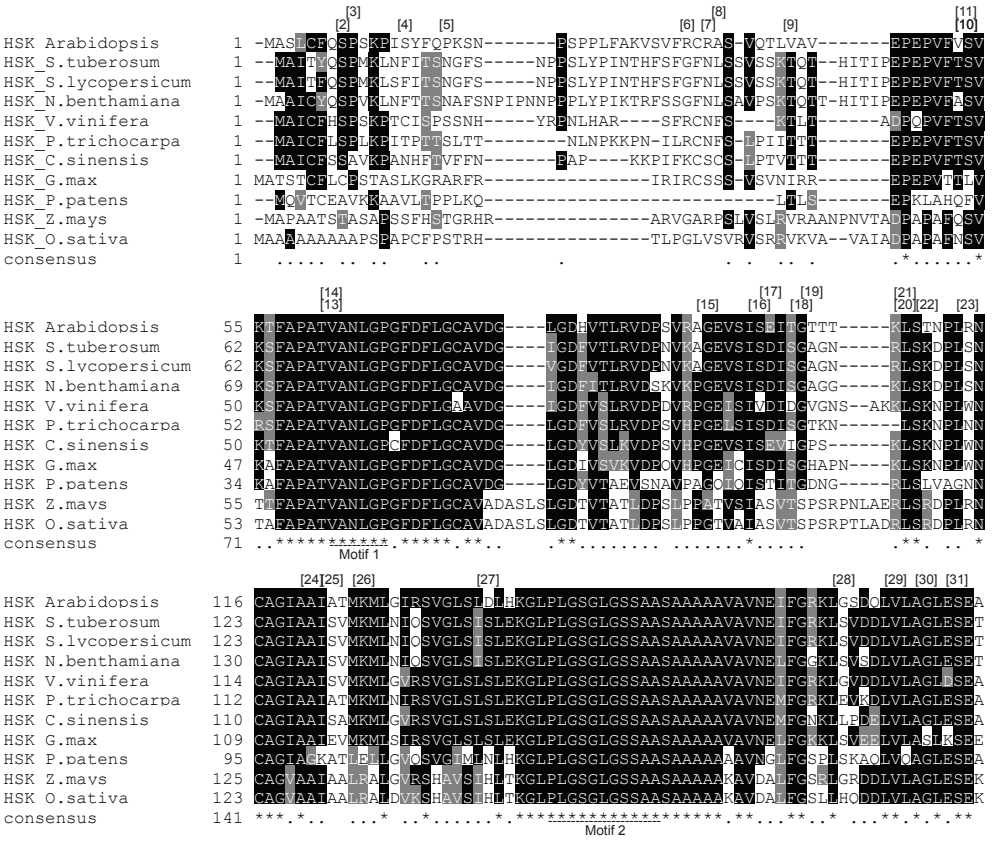
Figure 1. Non-synonymous TILLING mutations in the HSK protein. The position of the amino acid substitutions in the HSK protein. The regions for substrate and co-factor binding is indicated by the three motifs. In black the chloroplast transit peptide is shown (cTP).

Table1. Frequency of mutation types of HSK TILLING alleles (observed) compared to the expected frequency as predicted by CODDLE.

	Missense	Nonsense	Silent
Expected	62,9%	2,0%	35,1%
Observed	71,2%	0%	28,8%

HSK is a member of the GHMP kinase superfamily that is characterized by the presence of three motifs required for substrate and co-factor binding (indicated by the dark grey boxes in Figure 1). Interestingly, one amino acid substitution was identified in motif 1 where valine at position 62 is substituted for isoleucine (#13, Supplemental Table 1). No mutations have been identified in the second and third motif. The four most N-terminal acid substitutions are localized in the predicted chloroplast transit peptide (cTP) of HSK (indicated by the black box in Figure 1). The ChloroP algorithm was used to analyse if the amino acid substitutions affect the transit peptide prediction. Altered scores could indicate a failure of the protein to enter the chloroplast. The ChloroP scores of the mutant HSK proteins were similar to that of the wildtype HSK protein suggesting there is no effect of the cTP amino acid substitutions on chloroplast targeting (Supplemental Table 1). The HSK protein is highly conserved in plants as shown in a multiple alignment of HSK proteins of several dicots, monocots, and the moss *Physcomitrella patents* (Figure 2). It is striking to see that many TILLING protein alleles (numbers between square brackets in Figure 2) have an amino acid substitution at positions

that are highly conserved in plant HSK proteins, making these mutant proteins candidates with potential reduced enzyme function. To further analyze the evolutionary conservation of the HSK protein, we expanded the multiple alignment by including HSK of the bacteria *Methanococcus jannaschii* (of which the crystal structure has been elucidated), *Escherichia coli*, and the yeast *Saccharomyces cerevisiae* (Supplemental Figure 1). Compared to the plant HSK proteins, there is little conservation between the HSK proteins of the 4 species analysed besides the 3 motifs that are largely conserved. In total, 41 amino acids are conserved in all 4 species and for 7 of these, amino acids substitutions were identified in the TILLING alleles. To predict which mutations are likely to be damaging to the protein we used the SIFT prediction tool. The algorithm underlying SIFT makes use of the conservation of the amino acids within the HSK protein family and the type of amino acid substitution. Substitutions of highly conserved amino acids are more likely to affect function than non-conserved amino acids that are more tolerant to most substitutions. SIFT predicts 7 amino acid substitutions to affect protein function (Supplemental Table 1). Interestingly, from the 7 widely conserved HSK mutations only 4 are predicted to be damaging to the protein (mutation #35, #36, #44, and #52).



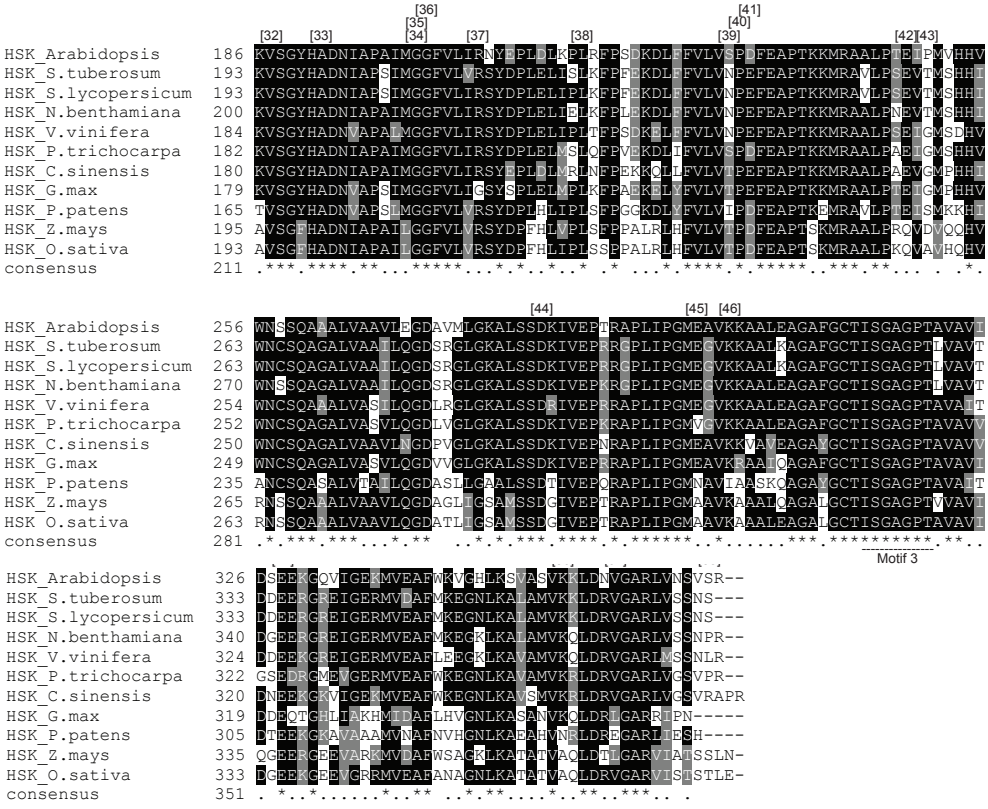


Figure 2. Multiple sequence alignment of plant homoserine kinase amino acid sequences with localization of TILLING mutations. The sequence alignment was generated using the CLUSTALW2 program from the European Bioinformatics Institute (EBI) and conserved residues were highlighted using the BOXSHADE tool. Numbers above the alignment indicate the numbers of the TILLING mutations. HSK sequences are derived from mRNA, genomic or assembled EST sequences which are available from GenBank through the National Center for Biotechnology Information (NCBI). Below the sequence similar amino acids are indicated by dots, and identical amino acids by asterisks. The three motifs that are conserved in the superfamily of GHMP kinases are indicated by lines.

Arabidopsis lines containing the TILLING alleles were obtained from the Arabidopsis stock centre (NASC) and the *HSK* CDS was fully sequenced in all lines. The presence of the expected mutations was confirmed for all *HSK* coding sequences and no other intragenic mutations were identified. Also, we were able to determine if the lines were homozygous or heterozygous for the mutations.

For one particular allele (#34) we were unable to obtain homozygous mutants, which coincided with approximately one quarter of the progeny of a heterozygous parental line not developing beyond the seedling stage. This suggests that the arrested seedlings are homozygous for a lethal mutation as these seedlings never developed true leaves and eventually died. Sequencing of the *HSK* coding sequence in these arrested seedlings revealed that they were homozygous mutant for the mutation G601A corresponding to a

glycine to serine substitution (aa 201). To further investigate this, we analysed the progeny of a selfed plant heterozygous for the #34 allele. Of 57 seedlings that were sequenced for the *HSK* mutation, only 1 mutant homozygous for the #34 mutation was identified. Of the other seedlings, 39 were heterozygous and 17 were wildtype for the #34 mutation. Instead of finding a 1:2:1 ratio, we identified a ratio of 1:39:17 indicating a deleterious effect of the homozygous mutants. The only homozygous mutant that developed true leaves showed a severe growth phenotype and was unable to produce any offspring. This suggests that mutant #34 is a null mutant in which HSK activity is completely abolished leading to lethality. We are currently investigating this phenomenon by transformation of the wildtype *HSK* in a heterozygous mutant background to test if progeny homozygous for the #34 mutation can be complemented.

Resistance of TILLING mutants

Previously, we identified six independent amino acid substitutions in HSK that were found in the *dmr1* mutants and which cause resistance to *H. arabidopsidis* (van Damme et al. 2009). Using TILLING, we have obtained 37 more missense mutations in the *HSK* gene. Likely, mutations at important positions could lead to loss or reduced function of HSK thereby resulting in homoserine accumulation and possibly resistance to *H. arabidopsidis* similar to that in the described *dmr1* mutants. Therefore, all mutants were screened for resistance towards *H. arabidopsidis*. Except for one mutant (#44) allele, all mutant lines were susceptible comparable to the parental Col-*er* line. Mutant #44 (D283N) gained resistance as plants homozygous for this mutation did not show any *H. arabidopsidis* sporulation (Figure 4A). The mutant protein D283N, does not contain an aspartic acid, but now an asparagine at position 283. According to SIFT, this mutation is predicted to affect protein function. Also, mutant #44 seedlings are slightly chlorotic (Figure 3A), similar to that of the *dmr1-1* mutant (Figure 3C), although it is unclear if this is linked to or caused by the *HSK* mutation.

Figure 3. Phenotype of TILLING mutant #44 (**A**), its parental line Col-er (**B**) and *dmr1-1* (**C**). Seedlings are 10-days old. Notice the chlorotic phenotype of #44 compared to Col-er.

To investigate if the observed resistance is accompanied by high homoserine levels, we analysed homoserine content of the TILLING mutants (Figure 4B). Interestingly, only mutant #44 contained high levels of homoserine while in all the other lines homoserine levels were too low to be detected. As control we included the *dmr1-1* mutant, which was previously shown to accumulate homoserine.

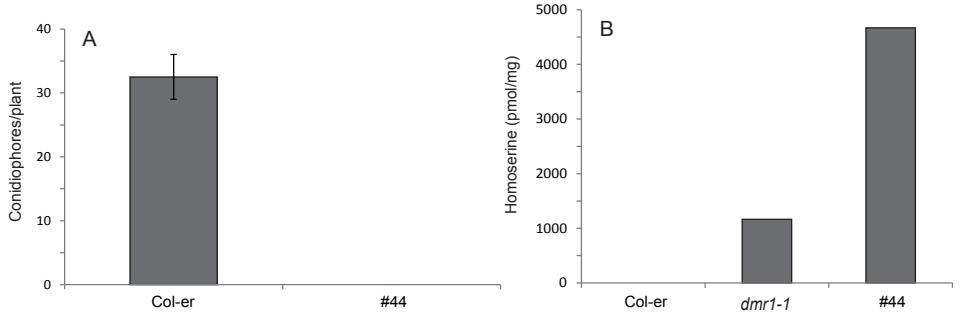


Figure 4. TILLING mutant #44 accumulates homoserine and is resistant to *H. arabidopsidis*. (**A**) Amount of conidiophores per plant at 5 dpi of 14-day old seedlings of mutant #44 and its parental line Col-*er* with standard deviation (n=20). (**B**) Homoserine content of line #44, the parental line Col-*er* and *dmr1-1* (in pmol/mg fresh weight) of 14-day old uninfected seedlings.

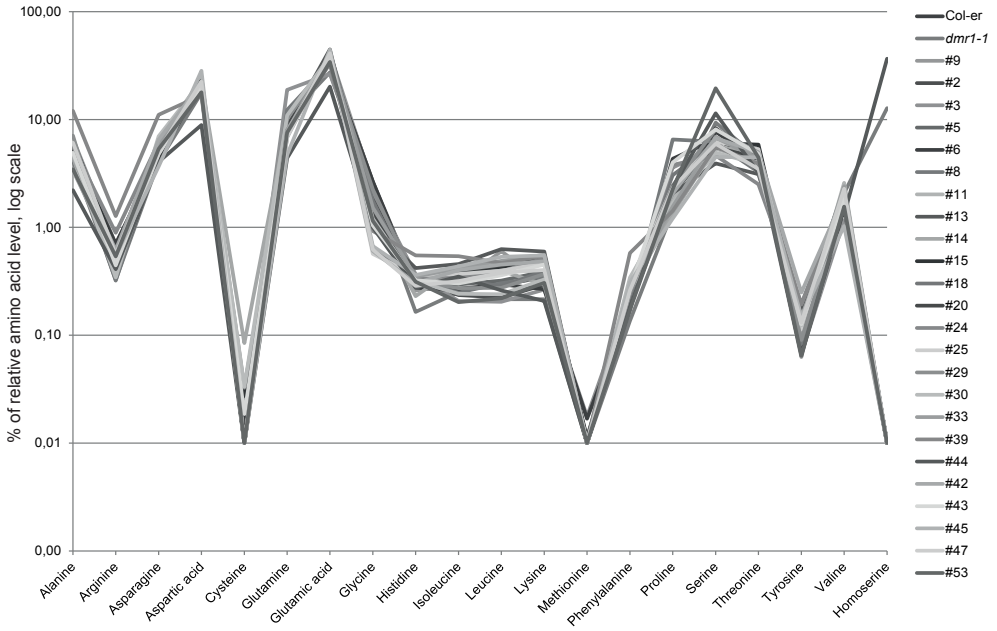


Figure 5. Relative amino acid levels (in % of total measured amino acid content) in different TILLING mutants, *dmr1-1*, and Col-er. All TILLING mutants show the same pattern across the tested amino acids. Only in *dmr1-1* and mutant #44 homoserine is detected.

This particular TILLING mutant, #44, accumulates up to 4 times more homoserine than the *dmr1-1* mutant. Figure 5 shows the relative amino acid levels of the different TILLING mutants, the parental line Col-*er* and the *dmr1-1* control. Besides homoserine, there is no other obvious difference observed in relative amino acid levels between the mutants and the control.

Application of *dmr1*-mediated resistance in crops

TILLING has successfully been applied to obtain downy mildew resistant mutants in Arabidopsis. A first step to explore if HSK mutation in crop species could result in resistance we aimed at identifying several HSK orthologues. Like Arabidopsis where *HSK* is a single copy gene, in most crop species *HSK* is encoded by a single gene based on the available genome and/or EST data. We obtained the reciprocal best hits of HSK orthologues in tomato and cucumber. To confirm functional HSK proteins were identified, recombinant HSK proteins were tested in an enzyme assay as previously described (van Damme et al. 2009). We tested the activity *in vitro* of tomato, cucumber and Arabidopsis HSK which were purified and produced from *E. coli*. As shown in figure 6, the HSK activity from tomato and cucumber exceeds the Arabidopsis HSK activity at the two tested homoserine concentrations. The enzyme assay clearly indicates that the cloned orthologues from tomato and cucumber indeed have HSK activity.

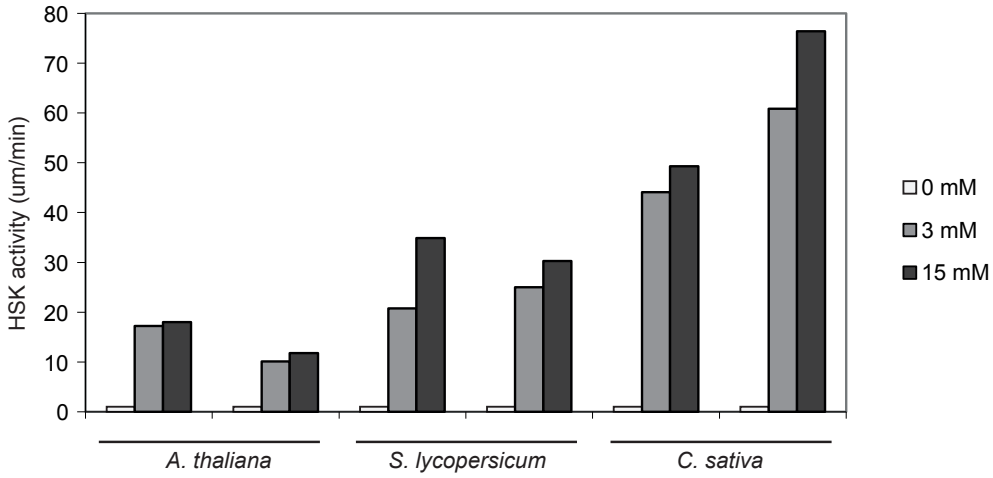


Figure 6. HSK activity of recombinant enzyme from tomato (*S. lycopersicum*), cucumber (*C. sativa*) ,and Arabidopsis (*A. thaliana*). Recombinant HSK activity was determined using equal amount of recombinant protein with 0, 3, and 15 mM of homoserine as substrate. HSK activity of two independent assays is shown.

DISCUSSION

In this study we have taken advantage of TILLING and identified an additional *hsk* mutant that gained resistance towards *H. arabidopsidis*. This mutant, #44, contains an asparagine instead of an aspartic acid at position 283. This position is conserved throughout all HSK proteins tested including *E. coli* (THRB), *S. cerevisiae* (THR1), and *M. jannashii* (HSK) suggesting that the presence of aspartic acid at this position is important for function. Also, this conservation is specific for HSK proteins since other members of the GHMP family do not contain this amino acid at that position (Krishna et al. 2001). Of a total of 37 missense mutants that we identified, only 1 proves to be resistant to *H. arabidopsidis*. This suggests that HSK is very tolerant towards mutation despite the high conservation of several amino acid residues that are substituted in the TILLING mutants. The D283N mutant is, as expected, also the only mutant that accumulates high levels of homoserine confirming that high homoserine levels are strictly linked to the observed resistance.

Previously, with the identification of the *dmr1* alleles, we have not found any mutations leading to a premature stopcodon or frameshift. This suggested that *HSK* is an essential gene and that complete loss of function would be lethal to the plant. In this study we have performed a reverse genetics approach by searching for EMS-induced mutations in the *HSK* gene. In theory you would expect to obtain null mutants since the percentage of null mutations in the total collection of possible EMS mutations in *HSK* CDS is 2% (CODDLE), but we have not identified any in our collection of 52 mutants. The seeds of the TILLING mutants that were obtained from NASC were the progeny of selfed M2 plants and are mostly heterozygous for the desired mutation. Although we did not identify mutant lines containing a premature stopcodon, one particular mutant (#34) was identified where approximately one quarter of the progeny of a heterozygous parent died in the seedling stage. Further investigation will reveal if the *hsk* mutation is the cause of the lethality and answer the question if HSK is an essential gene.

Putative HSK orthologues are identified in many other plant species. Using recombinant HSK protein production and enzyme assays, we could confirm that we obtained the functional HSK orthologues from tomato, cucumber, and other economically important crop species. By TILLING we have identified an additional allele of *HSK* in Arabidopsis providing resistance to downy mildew. Previously, TILLING has already been successfully applied to several model organisms, e.g. Arabidopsis, Zebrafish, and Drosophila (Greene et al. 2003; Wienholds et al 2003; Winkler et al. 2005). The use of TILLING in crop species has increased over the last years since it has several advantages over other reverse genetic approaches. TILLING does not require the need for transgenic modification, it can be performed high throughput, and can even be used for polyploid organisms. One example is wheat, an allohexaploid organism, in which TILLING has been successfully employed to

identify mutations in the waxy locus (Slade et al. 2005). TILLING was performed on two different wheat varieties and resulted in the identification of 246 alleles. The mutation frequency in the Arabidopsis TILLING project was approximately 1 mutation per 170 kb. In wheat 1/40 and 1/24 kb was reported, which is higher than in Arabidopsis due to higher tolerance towards mutation because of its polyploid nature. Also in melon, which is a diploid species, TILLING has proved a valuable technique for improved fruit shelf life (Dahmani-Mardas et al. 2010). They estimate to have 1 mutation per 573 kb, and identified in total 134 alleles out of which 65% were missense mutations. In tomato, the translation initiation factors *eIF4E/G* have been screened for mutations in a TILLING population. In total 5 *eIF4E/G* genes were analysed resulting in 83 different mutations (mutation frequency 1/574 kb), of which 28 appeared missense and 3 null mutations. Infection assays with three potyviruses resulted in the identification of 1 mutant that conferred immunity to virus infection. Further investigation revealed that this mutant had a mutation in an exon/intron splice site (Piron et al. 2010).

In plant breeding application, TILLING has proved to be a valuable technique for the engineering of new alleles. Since these mutants are not transgenic, all subsequent generations are, without restrictions, allowed to grow under field condition. Any interesting induced mutation can be incorporated as marker for selection by breeders. We demonstrate that via TILLING it is possible to create *HSK* mutant alleles in Arabidopsis that gained resistance towards the oomycete *H. arabidopsidis.*

METHODS

Selection of HSK TILLING mutants

A *HSK* TILLING screen was performed by the Arabidopsis TILLING project (Till et al. 2003). *HSK* was amplified using primers HSK_Fw 5'-gacaagcttaccggttcggttcagttc-3' and HSK_Rv 5'-tcccagaagccaaaaagagctaaacaa-3'. Candidate mutations were verified by sequencing. Seeds from the missense mutant lines were ordered from NASC. The *HSK* gene was sequenced using primers: HSK_F1 5'-ccaccatttacaatcatctaagc-3', HSK_F2 5'-gggaccagggtttgatttct-3', HSK_F3: 5'-caaccaaatccaatccatc-3', HSK_R1 5'-cgttgtcctttattggtgtagaaa-3', HSK_R2 5'-agctgcttggctactgttcc-3' and HSK_R3 5'-tctcctccgaatcaatcacc-3'.

Plant growth and *H. arabidopsidis* infection

Plants were grown under long light (16h) conditions at 22°C with a relative humidity of 75% (100 µE/m^2/s). *H. arabidopsidis* was maintained as described previously (van Damme et al. 2009). Plants were inoculated with 50 spores/ul of isolate Waco9 using a spray gun. After inoculation, plants left to dry for 1 h and subsequently incubated at 100% relative humidity at 16°C with 9 h of light per day (100 µE/m^2/s). The amount of sporulation was quantified at 5 days post inoculation by counting the conidiophores on cotyledons and true leaves.

Amino Acid Analysis

Amino acids were extracted as described previously (van Damme et al. 2009). Briefly, 100 mg of aboveground parts of 14-day old Arabidopsis seedlings were ground in liquid nitrogen. Amino acids were extracted twice with 80% methanol followed by two extractions with 20% methanol. Samples were lyophilized and dissolved in 150 µL of deionized water. An equal volume (150 µL) of internal standard, 750 µmol/L, *S*-amino-ethyl-cysteine was added. Prior to amino acid detection, the remaining proteins were removed by treatment with 10% sulfosalicylic acid. Plant amino acids were seperated by automated ion-exchange chromatography and detected and quantified with post column ninhydrin derivatization on a JEOL AminoTac JLC-500/V.

HSK Recombinant Protein Production and Enzyme Assay

The coding sequence of the Arabidopsis HSK gene was PCR amplified from genomic DNA using primers HSK_pET_F 5'-cccccatggcaagtctttgtttccaa-3', and HSK_pET_R 5'-cccctcgagtcatctggagacgctgttga-3', tomato (*Solanum lycopersicum*) HSK (Solyc04g008760) was PCR amplified using primers: SlHSK_pET_F 5'-cccccatggctataacctttcaatctcc-3' and SlHSK_pET_R 5'-cccctcgagcatcgatcatcatgaattgc-3', cucumber HSK was PCR amplified using primers: ScHSK_pET_F 5'-cccccatggctatgctctcctatc-3' and ScHSK_pET_R

5'-cccctcgagtgcttctcataaaactctatctaaagg-3'. Arabidopsis PCR fragments were digested with *Nco*I and *Xho*I and ligated in pET-30(a)+ (Novagen), tomato and cucumber PCR fragments were digested with *Bam*HI and *Xho*I and ligated in pET-30(a)+. Production of recombinant enzyme and the HSK enzyme assay was performed as previously described (Lee and Leustek 1999; with modifications as described by van Damme et al. 2009). HSK activity was assayed indirectly by measuring ADP levels spectrophotometrically in 96-well plates at 340 nm.

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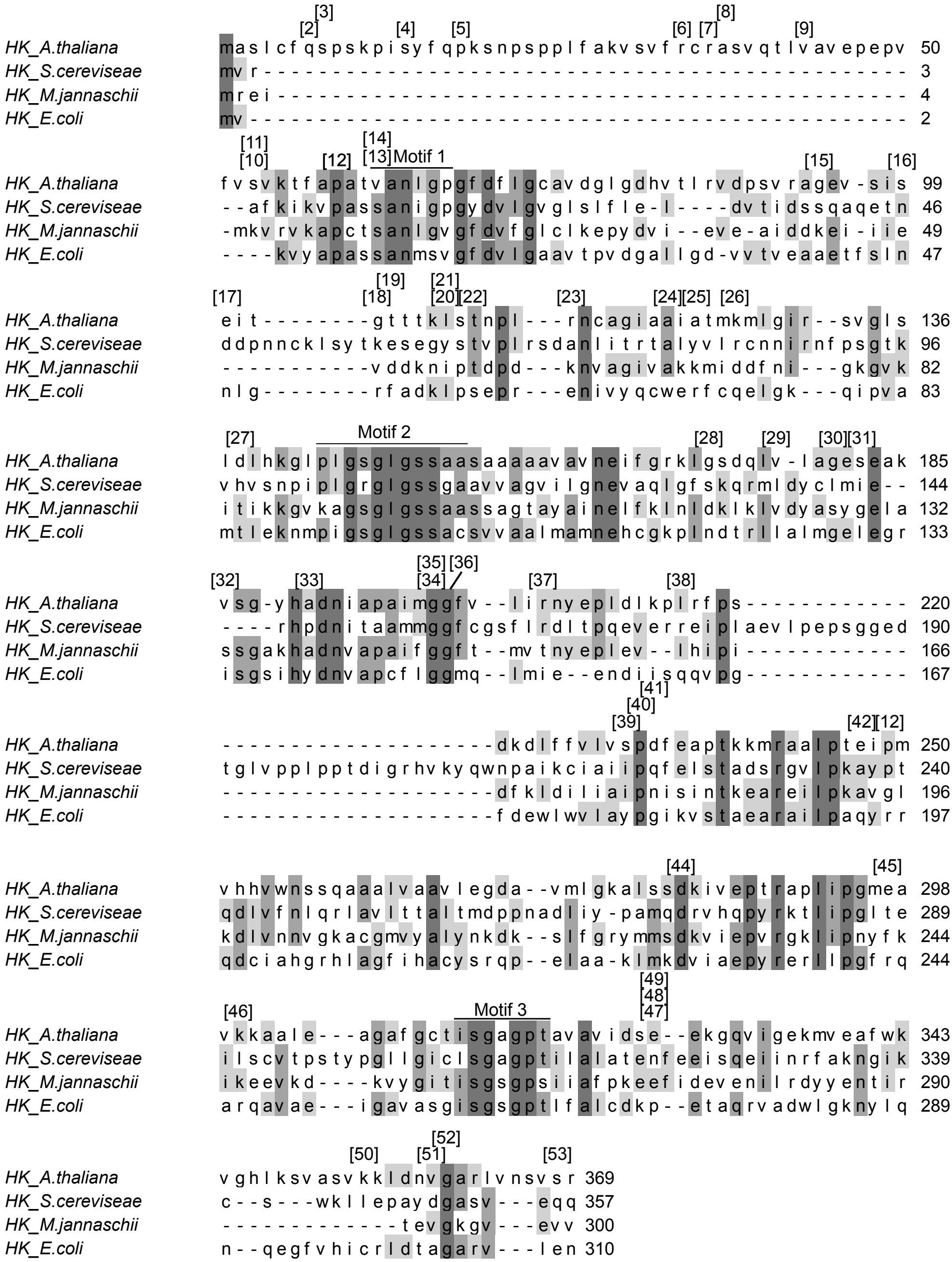
SUPPLEMENTAL DATA



Supplemental Figure 1. Multiple sequence alignment of HSK proteins of Arabidopsis (*A. thaliana*), yeast (*S. cereviseae*), *M. jannaschii* and *E. coli*. The position of the motifs 1-3 is marked. The position of the TILLING mutations is indicated by the number between square brackets. Conserved amino acids are indicated in grey.

Supplemental Table 1. Overview of HSK TILLING mutations. The position of the nucleotide changes in the HSK CDS and the corresponding amino acid substitutions in the HSK protein, including SIFT prediction is indicated. Variants 2 to 6 are predicted to be located in the chloroplast transit peptide for these the ChloroP score is shown.

Variant No.	Nucleotide Change	Change	SIFT prediction	ChloroP
2	C23T	S8F		0,576
3	C25T	P9S		0,578
4	C42T	S14=		0,578
5	C51T	P18L		0,575
6	G101A	R34Q		0,576
7	G107A	R36K	Tolerant	
8	G109A	A37T	Tolerant	
9	G127A	V43I	Tolerant	
10	C158T	S53F	Tolerant	
11	C158T	S53F	Tolerant	
12	C175T	P59S	Tolerant	
13	G184A	V62I	Tolerant	
14	C186T	V62=		
15	G281A	G94D	Tolerant	
16	G297A	S99=		
17	G298A	E100K	Tolerant	
18	G308A	G103E	Damaging	
19	C312T	T104=		
20	C322T	L108F	Damaging	
21	C324T	L108=		
22	G330A	T110=		
23	G342A	R114=		
24	C362T	A121V	Tolerant	
25	G367A	A123T	Tolerant	
26	G378A	K126=		
27	G412A	D138N	Tolerant	
28	G516A	G172=		
29	G529A	V177I	Tolerant	
30	G538A	G180S	Tolerant	
31	C548T	S183L	Tolerant	
32	C561T	V187=		
33	C575T	A192V	Damaging	
34	G601A	G201S	Tolerant	
35	G602A	G201D	Damaging	
36	G605A	G202E	Damaging	
37	G620A	R207K	Tolerant	
38	G651A	L217=		
39	G692A	S231N	Tolerant	
40	C695T	P232L	Tolerant	
41	C699T	D233=		
42	G742A	E248K	Tolerant	
43	C749T	P250S	Tolerant	
44	G847A	D283N	Damaging	
45	G892A	E298K	Tolerant	
46	G903A	K301=		
47	G982A	E328K	Tolerant	
48	G982A	E328K	Tolerant	
49	G984A	E328=		
50	G1065A	K355=		
51	G1078A	V360I	Tolerant	
52	G1082A	G361D	Damaging	
53	C1106T	S369F	Tolerant	

4

Negative regulation of plant defence by the oxygenase DMR6 acts upstream of salicylic acid accumulation

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ABSTRACT

Arabidopsis mutants lacking a functional *DMR6* gene are resistant to infection by the downy mildew *Hyaloperonospora arabidopsidis*. Resistance is associated with enhanced defence gene expression and both resistance and defence were found to require salicylic acid and signaling through the key regulator NPR1, which signals downstream of salicylic acid. The hypothesis that DMR6 is a negative regulator of defence was further supported by the finding that overexpression of *DMR6* leads to enhanced susceptibility to *H. arabidopsidis* and the bacterium *Pseudomonas syringae* pv. *tomato*. DMR6 is a 2-oxoglutarate Fe(II)-dependent oxygenase for which no substrate is known yet. Site-directed mutagenesis confirmed the requirement of conserved catalytic residues for its function as a negative regulator of defence. Metabolomic analysis of *dmr6* mutants revealed hydroxy-benzoic acids and glucosinolates as candidate substrates. However, genetic and functional studies rejected these metabolites as true DMR6 substrates. Structural modeling allowed the identification of residues important in the predicted substrate binding pocket, the mutation of which strongly reduced the biological activity of the protein.

INTRODUCTION

Plants are under constant threat of a plethora of potential microbial pathogens. To combat these harmful microbes, plants have preformed barriers like the cuticle, cell wall, and protective antimicrobial compounds. Only when the pathogen is able to overcome these barriers, it faces the activation of inducible defences. Typically, these plant responses involve major reprogramming of the host transcriptome and metabolome (Truman et al. 2006; Ward et al. 2010). The degree of defence activation should be under tight regulation as uncontrolled defence responses, e.g. spontaneous programmed cell death, could be detrimental to the plant. A good example of uncontrolled defence occurs in the Arabidopsis *lsd1* mutant, which is not able to keep in check reactive oxygen species that are produced in response to pathogen infection (Richberg et al. 1998). As a result a runaway cell death phenotype will eventually destroy the complete plant. Also, when pathogen attack is overcome, defence responses should return to basal levels. An important part of pathogen-induced defence requires the accumulation of the phytohormone salicylic acid (SA), which regulates the expression of a subset of defence-associated genes (Glazebrook 2005). Arabidopsis mutants containing elevated levels of SA display a broad range resistance to biotrophic pathogens, which is associated with constitutive defence gene expression and reduced growth. An important cause for the enhanced defence response in several of these mutants is the loss of function of a negative regulator of defence.

We can distinguish two groups of enhanced defence mutants in Arabidopsis. The first group forms spontaneous cell death-like lesions and often shows severe growth defects, e.g. in the *acd* (accelerated cell death), and *cpr* (constitutive expressor of PR genes) mutants (Greenberg et al. 1994; Bowling et al. 1994). The second group consists of mutants that show enhanced defence in the absence of spontaneous cell death or hypersensitive response, e.g. the *dnd1* (defence, no death) and *dmr6* (downy mildew resistant 6) mutants (Yu et al. 1998; van Damme et al. 2008). *dmr6* is particularly interesting since it does not exhibit dwarfism that is often associated with constitutive defence. The *dmr6-1* mutant was identified in an EMS screen for loss of susceptibility to the downy mildew pathogen *Hyaloperonospora arabidopsidis* (van Damme et al. 2005). *dmr6* mutants show enhanced expression of defence-associated genes in the absence of pathogen infection. Also, *DMR6* transcript levels are induced after SA treatment and locally after *H. arabidopsidis* infection. *DMR6* was found to encode for an oxidoreductase belonging to the superfamily of 2-oxoglutarate Fe(II)-dependent oxygenases (2OG oxygenase) (van Damme et al. 2008). The majority of enzymes belonging to this superfamily depend on iron (Fe^{2+}) and 2-oxoglutarate as cofactors and catalyze the incorporation of one oxygen atom from molecular oxygen into the substrate (Costas et al. 2004; Hewitson et al. 2005). The other oxygen atom converts 2-oxoglutarate into succinate and CO_2. Plants contain many 2OG oxygenase genes that are

involved in secondary metabolism (Prescott and Lloyd 2000). In the Arabidopsis genome 151 proteins are predicted to contain an 2-oxoglutarate/Fe(II) oxygenase domain (PF03171). 2OG oxygenases are typically involved in hydroxylation, desaturation, and epoxidation reactions, that occur in many biosynthetic pathways, e.g. as those in the biosynthesis of ethylene (Wang et al. 2002), gibberellin (Xu et al. 1995), and flavonoids (Reviewed by Prescott and John 1996). For the majority of the 2OG oxygenases, including for DMR6, the biochemical function is still unknown.

In this study, we functionally analyse the Arabidopsis DMR6 oxidoreductase. Overexpression of *DMR6* is shown to increase susceptibility to pathogen infection confirming that it acts as negative regulator of defence. We provide evidence that *dmr6*-mediated resistance requires SA and signal transduction via NPR1. Furthermore, we have measured DMR6-dependent metabolite changes and identified several candidate substrates. We constructed a structural model of DMR6 and identified key residues in the oxygenase that, when mutated, result in loss of biological activity.

RESULTS

dmr6-based resistance requires a functional salicylic acid signaling pathway

Arabidopsis mutants that do no longer produce the DMR6 protein are resistant to *H. arabidopsidis* infection. Expression analysis of the *dmr6-1 and dmr6-2* mutants showed that a subset of defence-associated genes is upregulated in the absence of pathogen infection, suggesting that DMR6 is a negative regulator of defence (van Damme et al. 2008). The defence-associated genes that are activated in the *dmr6-1* mutant largely overlap with SA-responsive genes and genes that are activated during infection with compatible and incompatible isolates of *H. arabidopsidis* (Supplemental Figure 1). To determine if *dmr6*-mediated resistance requires SA accumulation and NPR1 (Non-expressor of PR genes) function, we crossed *dmr6-2* to the *npr1-1* mutant that is blocked in signaling through the SA pathway, and to the *sid2-1* (SA-induction deficient 2) mutant that is impaired in the synthesis of pathogen-induced SA. NPR1 is a key regulator of SA-mediated systemic acquired resistance (SAR) and in the *npr1* mutant many defence-related genes including *PR-1* are no longer activated after SA treatment or pathogen infection (Cao et al. 1994 and 1997; Dong 2004). *SID2* codes for isochorismate synthase 1 (ICS1), which catalyzes the reaction from chorismate to isochorismate in the shikimate pathway, and is important for the production of defence associated SA (Wildermuth et al. 2001). Loss of function of the *NPR1* or *SID2* genes by mutation results in plants that do not activate a subset of defence genes, and consequently become more susceptible to biotrophic pathogens, e.g. to *H. arabidopsidis*. Compared to the *dmr6-2* mutant that does not support sporulation of *H. arabidopsidis*, the double mutants *npr1_dmr6-2* and *sid2_dmr6-2* are as susceptible to downy mildew infection

as the *npr1* and *sid2* single mutants (Figure 1). This indicates that SA accumulation and NPR1 function are both required for *dmr6*-based resistance. Furthermore, we can conclude that resistance of the *dmr6-2* mutant is caused by the upregulation of plant defence.

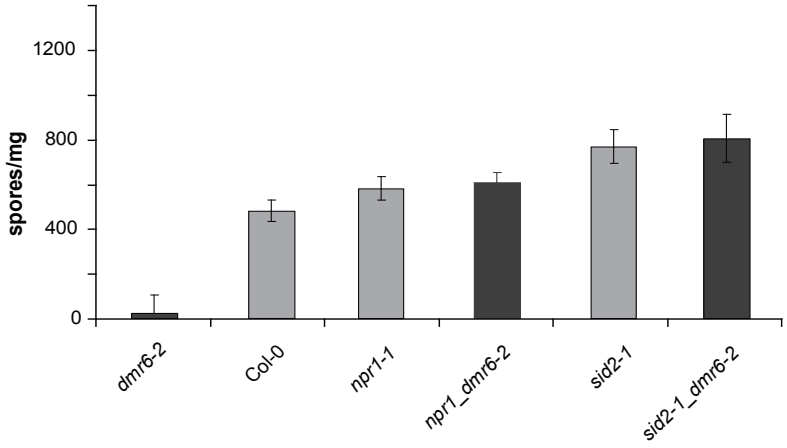


Figure 1. *dmr6*-mediated resistance requires *SID2* and *NPR1*. The amount of *H. arabidopsidis* spores per mg of leaf tissue at 5 dpi with *H. arabidopsidis* Waco9 on *dmr6-2*, Col-0, *npr1-1*, *sid2-1* as well as on the double mutants *npr1-1_dmr6* and *sid2-1_dmr6*.

The expression of *DMR6* in wild-type plants is activated during plant defence and in response to SA or the SA mimic BTH (van Damme et al. 2008). To assess if SA and NPR1 are required for the activation of *DMR6,* its transcript levels were determined in the *npr1-1* and *sid2-1* mutants. Arabidopsis Col-0 seedlings treated with BTH showed a clear induction of *DMR6* (Figure 2A). However, in the *npr1* and *sid2* mutants the induction of *DMR6* is reduced compared to that of the wild-type although the induction is not completely abolished. This indicates that NPR1 and SID2 function is important for full BTH-induced *DMR6* expression.

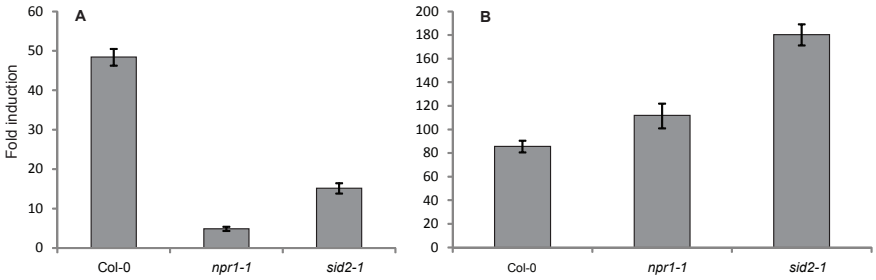


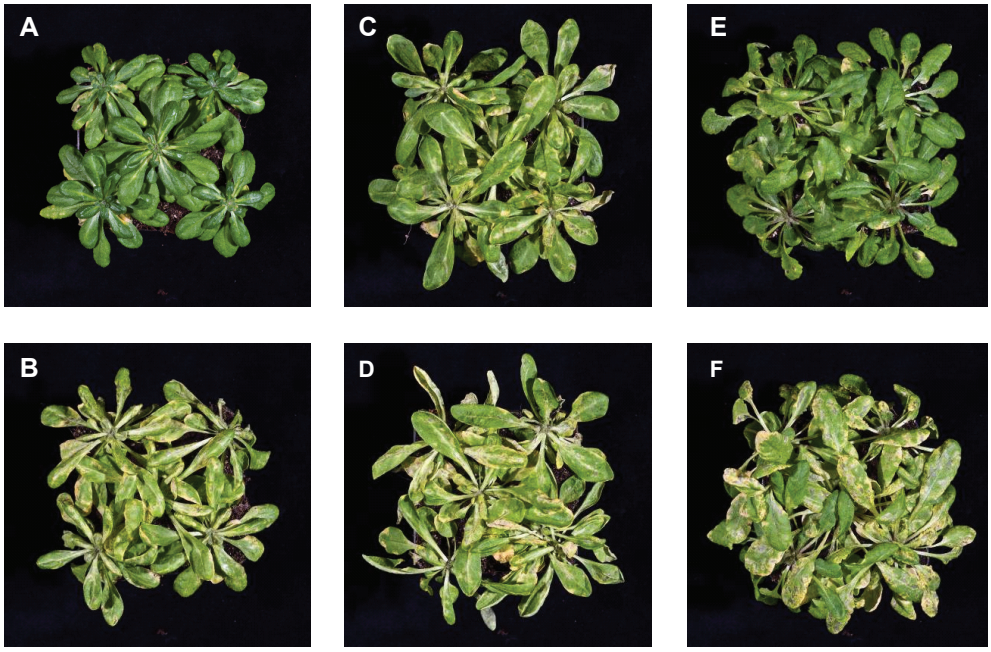
Figure 2. *DMR6* expression is reduced in the *npr1-1* and *sid2-1* mutant backgrounds after BTH treatment (**A**) but not after *H. arabidopsidis* infection (**B**). (**A**) BTH was sprayed onto 14-day old seedlings and leaf material was collected 24 hours later. (**B**) 11-day old seedlings were infected with 50 spores/ul *H. arabidopsidis* isolate Waco9 and leaf material was harvested 3 dpi. Expression was measured by Q-PCR. The vertical axis represents the fold change level relative to that of the mock treated control. Bars represent means and error bars represent standard deviation. This experiment is repeated twice with similar result.

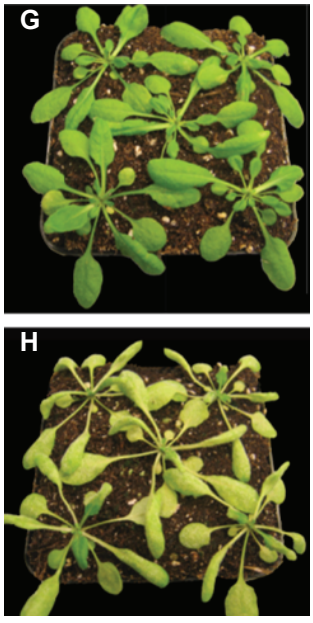
Also, we analysed *DMR6* expression in plants that were infected with the compatible *H. arabidopsidis* isolate Waco9 (Figure 2B). Surprisingly, downy mildew-induced *DMR6* activation did not depend on NPR1 or SID2 since *DMR6* expression is even higher in both mutant backgrounds compared to Col-0 wild type plants. The higher expression level could result from the enhanced susceptibility of the *npr1* and *sid2* mutants. Apparently, downy mildew-induced *DMR6* induction is independent of NPR1 and SID2 function.

Overexpression of DMR6 results in enhanced susceptibility to biotrophic pathogens

The elevated expression levels of a subset of defence-associated genes in the *dmr6* mutants suggest that in wild-type plants DMR6 negatively regulates the expression of these genes. To investigate if DMR6 indeed is a negative regulator, *DMR6* was expressed from the constitutive 35S promoter in transgenic Arabidopsis lines. *DMR6*-overexpression lines showed a clear increase in disease susceptibility to the biotrophic pathogens *H. arabidopsidis* and *Pseudomonas syringae* pv *tomato*. As shown in figure 3H, *H. arabidopsidis* infection-related chlorosis was more pronounced in *DMR6*-overexpression lines than in non-transgenic Col-0 plants (Figure 3G) that coincided with more sporulation (Supplemental Figure 2A). Even L*er eds1-2* plants, which are already enhanced disease susceptible, that overexpress *DMR6* support more sporulation of *H. arabidopsidis* (Supplemental Figure 2B). The increased susceptibility of six-week old plants to *P. syringae* pv *tomato* DC3000 bacteria was equally striking. While the control line (Col-0) showed a relatively low level of chlorosis and lesions at 3 days post inoculation (Figure 3E), the *DMR6*-overexpression lines showed more severe disease symptoms, e.g. more chlorosis and more and larger lesions (Figure 3F). L*er eds1-2* plants overexpressing *DMR6* did not show increased bacterial growth, which can be explained by the already high susceptibility of L*er eds1-2* to *P. syringae* pv *tomato* infection (Figure 3C and 3D). The increased susceptibility of *DMR6*-overexpressors to *P. syringae* pv *tomato* infection was confirmed by bacterial growth assays that showed increased bacterial titers at 3 days post inoculation (Supplemental Figure 3). A role of *DMR6* in defence to *P. syringae* pv *tomato* was confirmed in 6-week old *dmr6-1* mutant plants that showed elevated levels of resistance to bacterial infection (Figure 3A).

To investigate if basal defence is altered in the *DMR6*-overexpression lines, we analysed the expression of several defence genes and compared the expression level to Col-0. As shown in figure 3I, the expression of *PR-1*, *PR-2*, and *PR-5* is lower in the *DMR6*-overexpression line compared to Col-0. This means that under pathogen free conditions, the basal level of these *PR* genes is lower when *DMR6* is overexpressed, further explaining that these lines are more susceptible to *H. arabidopsidis* and *P. syringae* pv *tomato*. We propose that enhanced *DMR6* level results in stronger negative regulation of defence leading to enhanced susceptibility towards biotrophic pathogens.





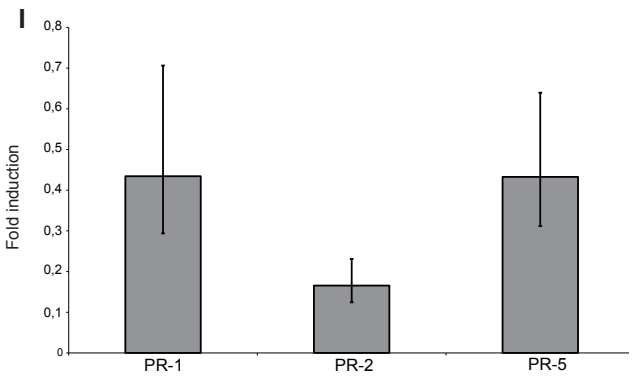


Figure 3. Overexpression of *DMR6* results in enhanced susceptibility towards *P. syringae* pv *tomato* and *H. arabidopsidis*. Disease symptoms of *dmr6-1* (**A**) and *dmr6-1* complemented with the P35S promoter *DMR6* (**B**), L*er eds1-2* (**C**) and L*er eds1-2* P35S promoter *DMR6* (**D**), Col-0 (**E**) and Col-0 P35S promoter *DMR6* (**F**) 3 days after *Pst* infection. Disease symptoms of Col-0 (**G**) and Col-0 P35S promoter *DMR6* (**H**) 7 days after *H. arabidopsidis* infection. Overexpression of *DMR6* results in more disease symptoms compared to the parental line. (**I**) Fold induction of *PR-1*, *PR-2*, and *PR-5* in Col-0 P35S promoter *DMR6*. Bars represent mean fold change compared with Col-0, with the error bars representing standard deviation.

DMR6 enzyme activity is required for plant disease susceptibility

The mechanism by which DMR6 negatively regulates defence or stimulates disease susceptibility is unknown. As *DMR6* encodes a putative oxidoreductase it is expected that its enzymatic activity is required for its function, although the protein could also have a structural role that is independent of its enzymatic function. Therefore, we mutated the three amino acids that compromise the catalytic triad that is essential for iron (FeII) binding and enzymatic activity. The two histidines (H) and one aspartic acid (D) residues were previously shown to be essential for the activity of the 2-oxoglutarate Fe(II)-dependent oxygenases flavonone 3-hydroxylase (F3H) and anthocyanidin synthase (ANS) (Lukačin and Britsch, 1997; Wilmouth et al. 2002). The corresponding amino acid residues in DMR6, H212, D214, and H269, were identified through multiple alignment with known oxidoreductases (Supplemental Figure 4). Amino acid substitutions were generated in the DMR6 protein by site-directed mutagenesis of the *DMR6* coding sequence (CDS).

The function of the mutant proteins was tested by transformation of the mutated *DMR6* CDS under control of the 35S promoter into the *dmr6-1* mutant line. As the constructs were generated to also include a C-terminal HA-tag we first tested the wild-type *DMR6* CDS. The tagged CDS could fully complement the mutant, restoring susceptibility to *H. arabidopsidis*, indicating that the C-terminal HA tag does not interfere with DMR6 function. In contrast, the mutant versions corresponding to the substitutions H212Q, H269Q and H212Q_D214A, did not complement the resistance phenotype as transgenic plants remained resistant to *H. arabidopsidis*. The amino acid substitutions did not negatively affect protein stability as the HA-tagged proteins could be detected in all transgenic lines (data not shown). This indicates that the enzyme activity of the DMR6 oxidoreductase is required for disease susceptibility and therefore also for its function as a negative regulator.

Untargeted metabolite profiling

To identify the substrate and/or product of the DMR6 enzyme, an untargeted metabolic profiling approach was taken. Arabidopsis 2OG oxygenases with known activities and substrates that are most homologous to DMR6 have a function in flavonoid or gibberellin biosynthesis. As these compounds and their precursors can be effectively separated and detected following LC of methanol/water extracts of 5-week old rosette leaves we chose LC-QTOF-MS for untargeted metabolic profiling of the *dmr6-1* and *dmr6-2* mutants and controls. For each line 3 biological replicates were analysed. The resulting data from the analysis contained 1139 intensity values (mass signals) across all samples. To analyse which compounds are differential between the mutants and their parental lines, we calculated the ratio of the mass signals of *dmr6-1* versus its parental line L*er eds1-2*, as well as the *DMR6* complementation line versus L*er eds1-2* from the three replicates (Table 1). Also, we

Table 1. Metabolites detected by LC-QTOF-MS in the untargeted metabolic screen that were significantly different (p <0.05, n=3) between the *dmr6-1* or *dmr6-2* mutants and their parental lines, as well ratios of relevant compounds. Values between 0 and 1 indicate a compound accumulating in the mutant, values above 1 means these compounds are lowered in the mutant compared to wild type. The ratio of the complementation line and wild type was taken as control and should be close to 1.

Retention time	Measured mass (m/z)	Calculated mass (m/z)	Difference measured vs. calculated (ppm) [a]	Elemental composition	Compound identity	Ratio Ler eds/dmr6-1 [b]	Ratio compl/Ler eds [d]	Ratio Ws-4/dmr6-2 [b]
11.71	285.0622			$C_{12}H_{14}O_8$	Dihydroxybenzoyl-xyloside	5.79 (0.009) [c]	1.40 (0.6)	0.98 (0.3)
13.55	285.0619	285.0616	1.09	$C_{12}H_{14}O_8$	3,4-Dihydroxybenzoic acid-xyloside	0.36 (0.024)	0.98 (0.3)	1.04 (0.6)
16.64	420.0469	420.0462	1.62	$C_{12}H_{23}NO_9S_3$	4-Methylthiobutyl glucosinolate	0.34 (0.086)	0.95 (0.8)	0.97 (0.7)
19.05		unknown		unknown	too low signal for correct accurate mass	0.29 (0.003)	1.02 (0.5)	0.94 (0.5)
24.81	477.0646	477.0643	0.60	$C_{17}H_{22}N_2O_{10}S_2$	4-Methoxyindo-3-ylmethylglucosinolate	0.30 (0.019)	0.84 (0.5)	0.71 (0.3)
24.81				$C_{17}H_{22}N_2O_{10}S_2$	isotope 477	0.39 (0.014)	0.86 (0.5)	0.78 (0.3)
24.81				$C_{17}H_{22}N_2O_{10}S_2$	isotope 477	0.40 (0.011)	0.85 (0.3)	0.79 (0.3)
24.81				$C_{17}H_{22}N_2O_{10}S_2$	fragment adduct 477	0.27 (0.018)	1.06 (0.4)	0.63 (0.2)
24.81				$C_{17}H_{22}N_2O_{10}S_2$	adduct 477 (2xM+Na)	0.24 (0.006)	0.75 (0.4)	0.67 (0.3)
24.81				$C_{17}H_{22}N_2O_{10}S_2$	isotope adduct 977	0.23 (0.006)	0.81 (0.6)	0.67 (0.3)
24.81				$C_{17}H_{22}N_2O_{10}S_2$	isotope adduct 977	0.29 (0.010)	0.93 (0.6)	0.64 (0.2)
24.82				$C_{17}H_{22}N_2O_{10}S_2$	fragment adduct 977	0.27 (0.016)	0.93 (0.2)	0.59 (0.2)
40.83	462.0930	462.0932	-0.37	$C_{15}H_{29}NO_9S_3$	7-Methylthioheptyl glucosinolate (isotope of 462)	0.21 (0.142)	1.48 (0.2)	0.96 (0.4)
9.04	315.0715	315.0722	-2.08	$C_{13}H_{16}O_9$	3,4-Dihydroxybenzoic acid-glucoside	1.04 (0.02)	0.84 (0.16)	0.07 (0.0003)
9.11				$C_{13}H_{16}O_9$	isotope 315	0.98 (0.17)	1.00 (0.47)	0.39 (0.0018)
18.57		unknown		unknown	too low signal for correct accurate mass	0.99 (0.47)	0.20(0.48)	4.13 (0.0010)
26.53	160.0407	160.0404	1.86	$C_9H_7NO_2$	fragment N-(1H-Indol-3-ylacetyl)aspartic acid	1.05 (0.03)	1.07 (0.03)	0.25 (0.0029)
12.24	137.0245			$C_7H_6O_3$	Hydroxybenzoic acid	0.70 (0.03)	1.05 (0.2)	0.39 (0.0023)

[a] Accuracy of the mass measurement,as difference between calculated and measured mass, in ppm per calculated mass
[b] Ratio of the mean mass signal intensities of each metabolite from wildtype and mutant
[c] Value of significance using student t-test.
[d] Ratio of the mean mass signal intensities of wildtype and the DMR6 complementation line

included the T-DNA insertion mutant *dmr6-2* and its parental line Ws-4 to investigate if there are differences between the different accessions used in this analysis.

We selected mass signals that were significantly different (p<0.05) and that exceeded the threshold in all three biological replicates. In total, 18 mass signals representing 10 different compounds were identified to be differentially present in *dmr6-1* (upper part) or *dmr6-2* (lower part) (Table 1). Interestingly, compounds differentially identified in the L*er eds1-2* background remained unaltered in the Ws-4 background and vice versa. Ten mass signals corresponded to glucosinolates, of which the compound 4-Methoxyindo-3-ylmethylglucosinolate (4MI3G) is represented by eight isotopes reducing the number of unique glucosinolate compounds to 3. These glucosinolates can be classified in the indol and aliphatic class. Indol glucosinolates are derived from tryptophan while aliphatic glucosinolates are mostly derived from methionine (Sønderby et al. 2010). To investigate if the accumulation of 4MI3G is caused by the inability of the *dmr6* mutant to convert this compound we crossed the *dmr6* mutant to the *cyp79b2/b3* double mutant. It is known that the *cyp79b2/b3* mutant does not accumulate any indol glucosinolates as these cytochrome enzymes convert tryptophan into indole-3-acetaldoxime which is the precursor of 4MI3G (Zhao et al. 2002) . A triple *cyp79b2/b3_dmr6-2* mutant was generated and infected with *H. arabidopsidis*. The obtained triple mutant was as resistant as the *dmr6-2* single mutant (data not shown). This indicates that, although the defence compound 4MI3G accumulates in the *dmr6* mutant, it is not the cause of resistance to downy mildew.

A second highly interesting and defence-associated class of compounds is formed by the hydroxybenzoic acids. Interestingly, SA accumulates in the mutants together with dihydroxybenzoic acid glucoside, which is a glucosylated form of SA. It is known that SA

is rapidly deactivated by coupling to glucose or xylose groups (Dean and Delaney 2008; Wildermuth 2006). On the other hand, a dihydroxybenzoic acid coupled to glucose or xylose is lower in the mutants than in wildtype. We therefore speculated that DMR6 could be involved in converting SA to an inactive compound by hydroxylation to dihydroxybenzoic acid followed by glucosylation and thereby removing a positively acting factor of defence. In *dmr6* mutants this would then lead to accumulation of SA due to reduced inactivation and subsequent activation of defence genes in the absence of pathogen infection. On the other hand, in *DMR6* overexpressing plants, the amount of active SA would be lower leading to enhanced susceptibility towards biotrophic pathogens.

To test the hypothesis that SA is a substrate of DMR6, we treated *DMR6*-overexpression lines with SA. As Col-0 35S:*DMR6* is highly susceptible to *H. arabidopsidis*, we included two other highly susceptible lines; the Col-*eds1* mutant and a Col-0 *NahG* transgenic line that expresses a bacterial SA-hydroxylase. As shown in figure 4, wild type Col-0 plants as well as the *eds1* mutant become resistant to *H. arabidopsidis* after treatment with SA. As expected, resistance could not be induced by SA in the *NahG* transgenics that convert the compound. Interestingly, the *DMR6*-overexpression lines behaved similar to the *eds1* mutant as they did respond to SA, indicating that SA is not inactivated by the DMR6 enzyme. As expected, all plants respond to the SA mimic BTH that is not inactivated by the *NahG*-encoded SA-hydroxylase.

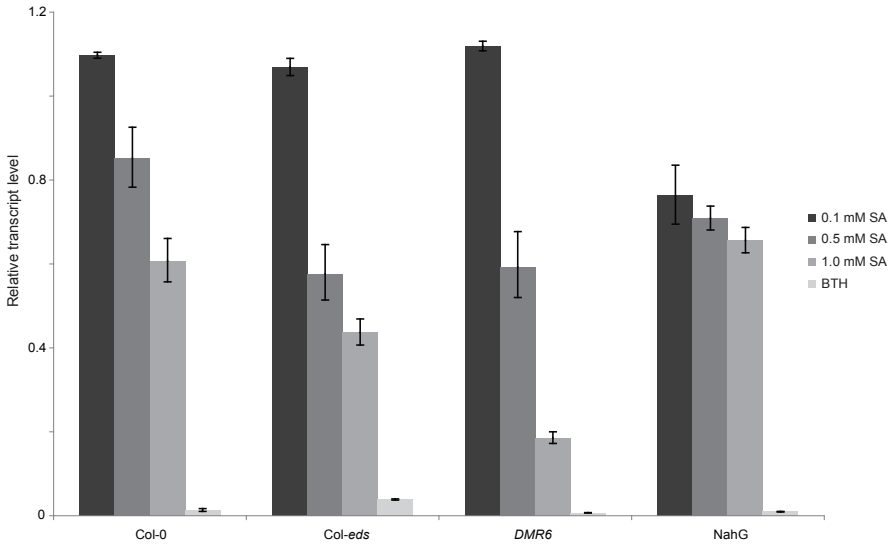


Figure 4. *DMR6* overexpression lines still respond to SA as measured by their resistance to *H. arabidopsidis*. Relative transcript level that is a level of pathogen biomass of *H. arabidopsidis Actin* in Col-0, Col-*eds1-2*, Col-0 P35S promoter *DMR6* and NahG after SA or BTH application and subsequent *H. arabidopsidis* infection. SA or BTH was sprayed on 14-day old seedlings. After 24 hours, *H. arabidopsidis* was inoculated and 3dpi seedlings were collected for Q-PCR analysis. Bars represent relative trancript level that is a measure of pathogen biomass of *H. arabidopsidis Actin* relative to mock treatment. Error bars depict standard deviation.

We conclude that SA is not the substrate of DMR6, and that differential abundance of benzoic acids is a response downstream of DMR6 that might be related to SA levels.

DMR6 structure analysis

Analyzing the structure of DMR6 could be valuable for the identification of key residues in substrate binding as well as for providing a framework to characterize the DMR6 substrate(s). Unfortunately, no experimental structure is yet available. However, the 3-dimensional structure of a related 2OG Fe(II) oxygenase of Arabidopsis anthocyanidin synthase (ANS), has been solved by X-ray diffraction (Welford et al. 2005). ANS catalyzes the reaction of leucocyadinin to anthocyanidin, which is the precursor of anthocyanin. ANS shares 51% sequence similarity and 32% sequence identity with DMR6, which allowed us to build a homology model of DMR6 using the structure of ANS (PDBid: 2brt) as a template. The essential iron binding residues in ANS (H232, H288, and D234) are strictly conserved in DMR6 (respectively: H212, H269, and D214). The DMR6 model presented in figure 5 shows how these key amino acids coordinate the iron, together with oxoglutarate. The jelly-roll motif, typical of the 2OG oxygenases and present in ANS, is indicated in the DMR6 model as yellow arrows (Figure 5 and 6).

Detailed analysis of the substrate pocket in the DMR6 model revealed the presence of six positively charged amino acids that are potentially involved in substrate binding. Among these, three (K105, R108 and R124) were subjected to site-directed mutagenesis (Figure 6) in order to investigate their role in DMR6 function.

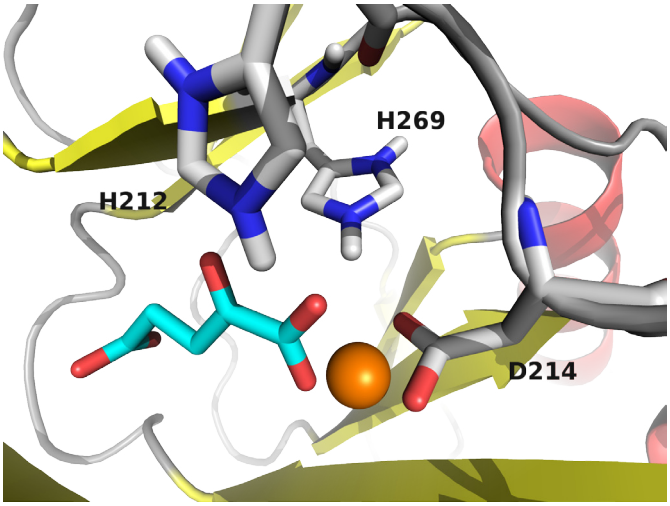


Figure 5. Close up view of the active site of DMR6. The iron is indicated by an orange sphere and the oxoglutarate as cyansticks. The iron-coordinating residues (D214, H212, and H269) are indicated in white sticks.

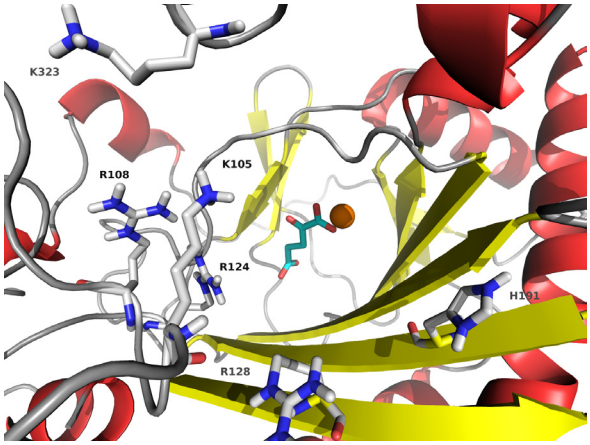


Figure 6. View of the DMR6 substrate binding pocket. The amino acids K105, R108, and R124 are predicted to be located at the entrance of the pocket, facing inward. The iron and oxoglutarate are shown deep inside of the pocket.

We replaced K105, R108, and R124 independently by alanine and transformed these constructs in the *dmr6-1* mutant background. Plants expressing DMR6 (K105A), (R108A), and (R124A) were challenged with *H. arabidopsidis* and analyzed for complementation. DMR6 (K105A) is able to complement the *dmr6-1* phenotype as plants became susceptible, indicating that the lysine at this position is not essential for DMR6 activity (Figure 7). On the other hand, plants expressing the DMR6 (R108A) and (R124A) substitutions are not able to complement the resistant phenotype when challenged with *H. arabidopsidis* (Figure 7). This indicates that these specific positions are crucial for DMR6 enzymatic function .

We investigated the potential binding of SA as a substrate for DMR6 using the homology model in combination with a flexible refinement approach (for details see the method section). The goal was to evaluate how well a given compound would fit into the binding pocket of DMR6. As shown in figure 8, the amino acids R108, R124, and K105 are in close contact with SA when modelled into the pocket. Arginine and lysine are both basic amino acids that carry positively charged groups. These are prone to interact with negatively charged groups from a substrate like SA. Indeed, the model predicts with high confidence that SA would interact with the basic residues (Figure 8). The active site (the place where the hydroxylation occurs) is, however, too far away indicating that no hydroxylation reaction could occur. The model suggests that it is unlikely that SA could be a DMR6 substrate, which is in line with the observation that *DMR6* overexpressing plants still respond to SA. Based on our modelling, the DMR6 protein is expected to accommodate a larger substrate with a negatively charged side group that is located opposite of the site where molecular oxygen is incorporated (e.g. by hydroxylation).

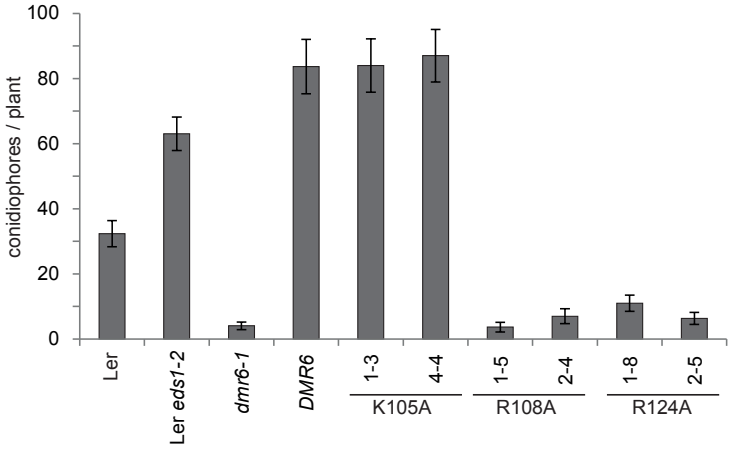


Figure 7. The amino acid positions R108 and R124, but not K105A, are essential for the function of DMR6. Amount of conidiophores per plant at 5 dpi of *H. arabidopsidis* grown on L*er*, L*er eds1-2*, *dmr6-1*, *dmr6-1* P35S promoter *DMR6*, and *dmr6-1* P35S promoter *DMR6* with amino acid substitutions K105A, R108A, and R124A is indicated.

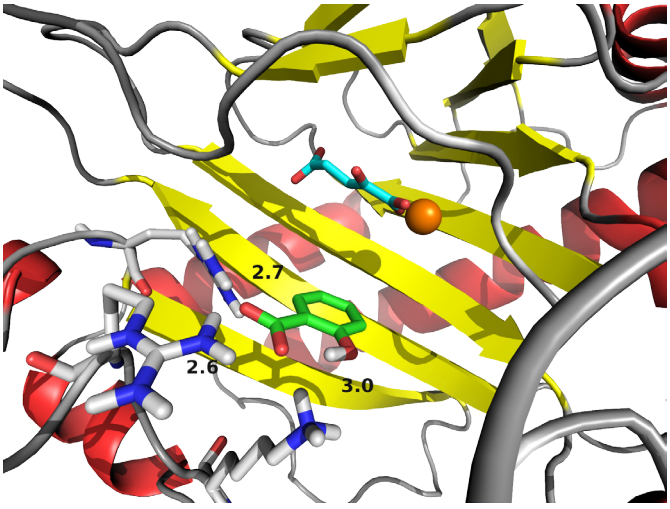


Figure 8. Salicylic acid modelled into the DMR6 pocket. K105 (3.0Å), R108 (2.6Å), and R124 (2.7Å) are in close proximity of SA (represented in green). Note the gap between SA and the active site of DMR6.

DISCUSSION

Plants carrying a null mutation in the *DMR6*-encoded 2OG oxygenase are resistant to the oomycete *H. arabidopsidis* and show a moderate constitutive expression of defence related genes. Activation of defence appeared to be responsible for the observed resistance as it requires NPR1 and SID2 function. The double mutants *npr1_dmr6* and *sid2_dmr6* have lost resistance to downy mildew that is observed in the *dmr6* single mutant. When encountered by pathogens with a biotrophic lifestyle Arabidopsis and many other plant species synthesizes SA which is an inducer of a large number of pathogenesis-related genes (*PR*-genes). These corresponding PR-proteins establish resistance through their antimicrobial activity (van Loon 1997). Lack of pathogen-induced SA accumulation, in the case of the *dmr6_sid2-1* double mutant, or SA unresponsiveness, in the case of the *dmr6_npr1-1* mutant, leads to increased susceptibility towards biotrophic pathogens demonstrating that *dmr6*-mediated resistance requires an intact SA pathway. These results suggest that DMR6 acts upstream of SID2 and NPR1.

The activated defence in the *dmr6* mutant is sufficient to provide resistance against the bacterial pathogen *P. syringae* pv *tomato*. Previously, van Damme (et al. 2005) found the *dmr6* mutant just as susceptible as L*er eds1-2* when challenged with *P. syringae* pv *tomato*. These contrasting results may be due to the age of plants used. 10-day old seedlings were screened previously, while now 5-week old plants were inoculated with this bacterial pathogen. It could be that in the *dmr6* mutant a substrate accumulates that increases with the development of the plant. That would mean that in older plants the level can be higher and therefore plants are more resistant.

DMR6 expression is induced after pathogen attack and SA or BTH treatment. Notably, *DMR6* transcript is less induced in the *npr1-1* and *sid2-1* background after BTH treatment indicating NPR1 and SID2 are required for full DMR6 induction. This was already seen in the microarray data from Mosher et al.(2006) where *DMR6* expression was not induced in the *npr1* mutant background when Arabidopsis plants were treated with BTH. Surprisingly, *DMR6* expression in the *npr1* and *sid2* background after compatible *H. arabidopsidis* infection is not altered indicating that *DMR6* expression can also be induced independent of SA accumulation and NPR1 function. Alternatively, the downy mildew pathogen could also directly induce expression of *DMR6.* It was previously shown by van Damme et al. (2008) that *DMR6* expression after *H. arabidopsidis* infection is confined to the cells harboring haustoria. One can envision that activation of *DMR6* could directly be triggered by the pathogen without the need of SA accumulation. There are examples known where host genes are activated directly by pathogen proteins, e.g. by bacterial transcriptional activator like effectors (TALEs). *Xanthomonas* bacteria produce TALEs and inject them into the plant cell where they enter the nucleus and bind specific promoter elements in order to activate

host genes to promote virulence (Boch et al. 2009).

Overexpression of *DMR6* causes enhanced susceptibility to *H. arabidopsidis.* Also, *DMR6* overexpressing plants are more susceptible to the bacterial pathogen *P. syringae* pv *tomato* in the Col-0 background but not in the L*er eds1-2* background, probably due to the very susceptible nature of the *eds1-2* mutant due to lower basal defences. Overexpression of *DMR6* enhances susceptibility towards *H. arabidopsidis* and *P. syringae* pv *tomato* while the *dmr6* mutant is resistant to both pathogens. This combined with the fact that *DMR6* expression is induced after pathogen attack, leads to the conclusion that DMR6 plays a role as negative regulator in defence. It is tempting to speculate why plants would activate *DMR6* following pathogen attack thereby increasing susceptibility to that pathogen. It could well be that by activating *DMR6*, plants are able to control the defence response and prevent uncontrolled over-activation that could lead to uncontrolled SA accumulation and defence activation with detrimental effects on to plant growth and development. Also, when pathogen attack is overcome the defence response should return to basal level. In this way, *DMR6* could balance the defence response thereby preventing unnecessary activation of defence responses. If the pathogen directly activates *DMR6*, this could well be a strategy to suppress the SA-dependent defence response thereby facilitating colonization more effectively. In this case, the pathogen hijacks the hosts defence response for its own benefit.

DMR6 enzyme activity is required for its function as negative regulator. Using multiple alignment analysis we identified H212, D214, and H269 as core amino acids required for enzyme activity. These catalytic residues are required for enzyme function as described earlier (Wilmouth et al. 2002). To analyse if mutant versions of these 3 amino acids would result in loss of DMR6 activity we replaced these amino acids independently with alanine using site directed mutagenesis and transformed these constructs in the *dmr6* mutant background. *dmr6* mutant plants expressing these mutated forms of DMR6 remain resistant to *H. arabidopsidis* confirming that enzyme activity is lost in these mutants and with this, activity is required for susceptibility.

Understanding the biochemical role, i.e. the DMR6 encoded substrate and product, would increase our understanding of *dmr6*-mediated resistance. Using untargeted metabolomic profiling of wild type and *dmr6* mutant plants, we have identified several compounds that accumulate in the *dmr6* mutant that are restored to wild type in the complementation line. The majority could be classified as glucosinolates and phenolic acids. Glucosinolates have been implicated in resistance against herbivores (reviewed by Hopkins et al. 2009) requiring glycoside activation by myrosinases in disrupted tissue cells. Recently, glucosinolates have been implicated in antifungal defence which differs from the pathway activated by herbivores (Bednarek et al. 2009). 4MI3G is required for defence against fungi, and in our analysis this compound accumulates in the *dmr6-1* mutant. This finding proposed us to further analyse glucosinolate biosynthesis. These tryptophan-derived indol

glucosinolates are synthesized through two cytochrome P450 enzymes, CYP29B2 and CYP79B3 (Zhao et al. 2002). The *cyp79b2/b3* double mutant is unable to produce 4MI3G and other tryptophan-derived metabolites like camalexin. Resistance is not altered in the *cyp79b2/b3_dmr6-2* triple mutant indicating that the accumulation of 4MI3G is not causing the observed phenotype. The fact that there is an accumulation of several glucosinolates in the *dmr6-1* mutant is likely to be part of a general defence response. Indeed, Mikkelsen et al. (2003) show that Arabidopsis plants treated with 2,6-Dichloro-isonicotinic acid (INA), which is a functional analog of SA, show a moderate increase of 4MI3G after 24 hours. Also, glucosinolates are specific for the order of the Brassicales while homologues of DMR6 are found in other plant species (Chapter 5, this thesis) as well, making it very unlikely that DMR6 is involved in glucosinolate biosynthesis. Other compounds differential in the *dmr6* mutants compared to its parental line are several benzoic acid derivatives including salicylic acid (review regarding benzoic acids from Wildermuth 2006). The observation that dihydroxybenzoic acid (DHBA), in the glucose and xylose-conjugated forms, are differential could well indicate that DMR6 is directly influencing the levels of hydroxybenzoic acids. We find a DHBA-xyloside that accumulates in the *dmr6-1* mutant but not in *dmr6-2*. Also, we identified a DHBA-xyloside, with differs in retention time, that is lower in the *dmr6-1* mutant compared to its parental line L*er eds1-2*. In the *dmr6-2* mutant, we identified a similar DHBA but in glucose-conjugated form. This indicated that DMR6 could well play a role in the regulation of these phenolic compounds. The reason we do not identify free benzoic acids other than SA could be because they conjugate immediately as a plant mechanism to control the amount of other benzoic acids. Bartsch et al. (2010) only identified free 2,3-DHBA after pathogen attack and gentisic acid was identified in concentrations around their detection limit. These results led to the idea that DMR6 might hydroxylate SA directly thereby regulating the amount of bioactive SA levels. This could explain the *dmr6*-mediated resistance (SA accumulation) and the highly susceptible *DMR6* overexpression lines (decreased active SA levels).

To test if overexpression lines of *DMR6* still respond to SA, we measured the biomass of *H. arabidopsidis* using different SA concentrations. As control, we included transgenic Arabidopsis plants expressing the salicylate hydroxylase *NahG*, isolated from *Pseudomonas putida*, because of their inability to accumulate SA. The *DMR6* overexpression lines still responded to SA in a concentration dependent manner as measured by *H. arabidopsidis* biomass increase. *NahG* transgenic plants do not respond to SA as it is converted to catechol (Delaney et al. 1994; Karegoudar and Kim 2000). Still, a minor decrease in oomycete biomass was detected after SA application indicating catechol has a negative effect on *H. arabidopsidis* growth which has been described earlier (van Wees and Glazebrook 2003). Further support that SA is not the substrate of DMR6 came from *in silico* modelling of SA into the DMR6 structure. The DMR6 model as presented in figure 5 was used to fit SA into

the pocket. Although the polar amino acids R108 and R124 were able to bind SA, the pocket was too large for SA and therefore it did not come in contact with the active site. Although several phenolic compounds accumulate in the *dmr6* mutant, DMR6 appears not be the enzyme that hydroxylates SA. Future metabolic studies aimed at identifying the DMR6 substrate will make use of a double *dmr6_sid2* mutant to reduce abundantly present SA-related compounds and other defence-related metabolites that may hinder the detection of the candidate DMR6 substrate.

METHODS

Plant growth conditions and pathogen infections

Unless noted otherwise, plants were grown on potting soil at 22°C with 16 h of light with 75% relative humidity. *H. arabidopsidis* inoculation was performed on 14-day old Arabidopsis seedlings or 5-week old plants. Inocula were transferred to plants using a spray gun and plants were air-dried for approximately 1 h before transferred into a growth chamber at 16°C with 9 h light per day at 100% relative humidity. Sporulation was quantified at 5, 6 or 7 dpi by counting the number of spores or conidiophores. The growth of *P. syringae* pv *tomato* DC3000 was performed as described by Tornero and Dangl (2001) with minor modifications. Six-week old plant leaves were sprayed with bacterial suspension (OD 0.05) and whole leaves (4 plants per line; 3 leaves per plant) were taken for colony counting at 0 and 3 days post infiltration. All experiments were repeated twice with similar results.

Chemical inductions

The induction treatment was performed by spraying 100 µM BTH onto 14-day old seedlings. After 24 hours, the seedlings were snap-frozen in liquid nitrogen. The responsiveness of Arabidopsis to SA treatment was analysed using 0.1, 0.5, and 1 mM of SA. SA was applied by using a spray gun in an aqueous solution containing 0.02 (v/v) Silwet L-77.

Generation of double mutants, triple mutant, and overexpression lines

The mutant alleles used in this analysis were *dmr6-2* (van Damme et al. 2008), *npr1-1* (Cao et al. 1994), *sid2-1* (Wildermuth et al. 2001), and *cyp79b2/b3* (Zhao et al. 2002). The *dmr6-2* mutation was confirmed using primers: LP 5'-caggtttatggcatatctcacgtc-3'; RP 5'-atgtccaagtccaatagccacaag-3'; and RB4 5'-tcacgggttggggtttctacaggac-3'. Mutants carrying the *npr1-1* mutation were identified using primers: npr1-1_F 5'-gatctccattgcagcttg-3' and npr1-1_R 5'-ggatatacggtgcttc-3'. Mutants carrying the *sid2-1* mutation were identified using sid2-1_MunI_F 5'-aagcttgcaagagtgcaaca-3' and sid2-1_MunI_R 5'-aaacagctggagttggatgc-3' followed by restriction analysis using MunI. Mutants carrying the *cyp79b2/b3* double mutation were identified as described previously (Zhao et al. 2002). *DMR6* was amplified from Col-0 using primers DMR6_Fw 5'-caccatggcggcaaagctgata-3', DMR6_Rv 5'-gacaaacacaaaggccaaaga-3' and cloned into the pENTRY vector via Gateway ® cloning (Invitrogen). Constructs were cloned into the pB7WG2 Gateway® compatible binary vector. These were transformed via *A. tumefaciens* strain C58 in Col-0, L*er eds* and *dmr6-1* plants using floral dipping. Transformants were selected using BASTA.

Q-PCR analysis

RNA isolation was conducted using RNeasy kits (Qiagen) including treatment with DNase (Qiagen). cDNA was synthesized with Superscript-III reverse transcriptase (Invitrogen) from total RNA. Cycle tresholds using the ABI PRISM 7700 system (Applied Biosystems) were determined using SYBR Green as reporter dye. The resulting Ct values were normalized using *ACTIN2* levels (At3g18780) with primers QACT2F, 5'-aatcacagcacttgcacca-3', and QACT2R, 5'-gagggaagcaagaatggaac-3'. *DMR6* expression was analysed using primers: QDMR6F 5'-tgtcatcaacataggtgaccag-3'and QDMR6R 5'-cgatagtcacggattttctgtg-3'. *PR-1, PR-2,* and *PR-5* expression were analysed using primers: QPR-1F 5'-gaacacgtgcaatggagttt-3', QPR-1R 5'-ggttccaccattgttacacct-3', QPR-2F 5'-cccgtagcatactccgattt-3', QPR2-R 5'-aaggagcttagcctcaccac-3', QPR-5F 5'-ggcaaatatctccagtattcaca-3', and QPR-5R 5'-ggtagggcaattgttccttaga-3'.

Site-directed mutagenesis

The mutations of the codons in *DMR6* were generated using the Phusion® site-directed mutagenesis protocol (Finnzymes). First the *DMR6* CDS was cloned into the pENTRY vector using primers: DMR6_CDS_pENTRY_F 5'-caccatggcggcaaagctgata-3' and DMR6_CDS_pENTRY_R 5'-gttgtttagaaaattctcgaggca-3' with Gateway® cloning (Invitrogen). Second, mutations were created using back-to-back PCR on the pENTRY *DMR6* vector with specific primers on the mutation site. The primers used for mutations in the active site were: DMR6_H212Q_F 5'-ttacctgctcaaaccgacccaaac-3', DMR6_H212Q_R 5'- accgtaagtgagctcaggttcagg-3', DMR6_H269Q_F 5'- aagtgtttggcaacgcgctgtaac-3', DMR6_H269Q_R 5'- ttgtatactccattacttaatgcc-3', DMR6_H212Q_D214A_F 5'- tacctgctcaaaccgccccaaacg-3' and DMR6_H212Q_D214A_R 5'- aaccgtaagtgagctcaggttcagg-3'. The primers used for mutation of potential substrate binding were: DMR6_K105A_F 5'- acgatccaacggcgacaacaaga-3', DMR6_K105A_R 5'-ctgaatatagcttcatttttttcttcc, DMR6_R108A_F 5'- acgaagacaacagcattatcgacg-3', DMR6_R108_R 5'- tggatcgtctgaatatagcttca-3', DMR6_R124A_F 5'- gtcaacaattgggcagactatcta-3', and DMR6_R124A_R 5'-ttcttctttcttcacattgaagctcg-3'. Mutated constructs were cloned into the pXCSG 3xHA-StrepII Gateway® compatible binary vector. *dmr6-1* mutant plants were transformed via *Agrobacterium tumefaciens* (GV3101) containing the pMP90RK helper plasmid using floral dipping (Zhang et al. 2006). Transformants were selected by spraying 0.1% (v/v) BASTA® on seedlings.

To check protein stability, proteins were extracted from 100 mg above ground plant material using SDS sample buffer. Total protein was separated on a 10% SDS-PAGE gel and transferred onto a nitrocellulose membrane. Ponceau ((0.1%(w/v) Ponceau S in 5%(v/v) acetic acid) staining was performed to visualize protein bands. After washing, the membrane was incubated with anti-HA (1:1000) antibody for 1 h and subsequently with HRP conjugated monoclonal goat anti mouse followed by visualization with the ECL detection kit (Amersham).

Untargeted metabolomic analysis

All leaves from 5 6-week old plants per line were harvested and ground to fine powder in liquid nitrogen. 100-mg ground material was used for the LC-QTOF MS analysis as described previously (de Vos et al. 2007).

Structural modelling and analysis

A structural model of DMR6 was built by homology using as a template the 3D structure of the 2OG Fe(II) oxygenase anthocyanidin synthase (ANS) (PDBid: 2brt). Thousand models were generated using the MODELLER package version 9.7 (Sali et al. 1995) and the best ranking model according to the DOPE score was selected for further analysis.

Different chemical compounds were tested against the DMR6 model for binding. We used for this the default refinement protocol of HADDOCK (Dominguez et al. 2003; de Vries et al. 2010). Each tested chemical was superimposed onto the native substrate bound in the holoprotein DMR6 and further refined in water using the HADDOCK webserver. The best model was chosen based on the HADDOCK score. Topologies and coordinates were obtained using the PRODRG server (Schüttelkopf et al. 2004).

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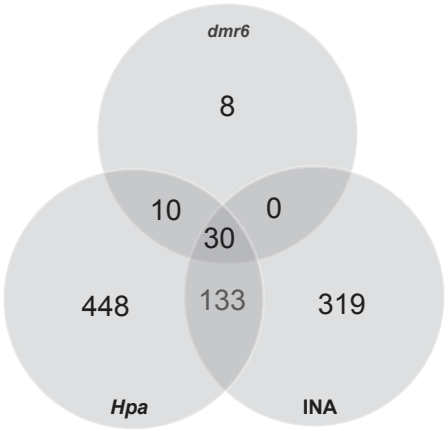
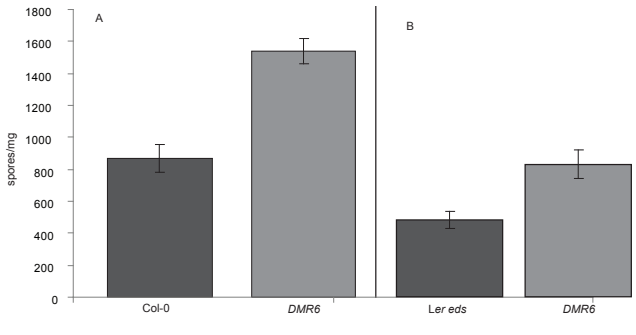
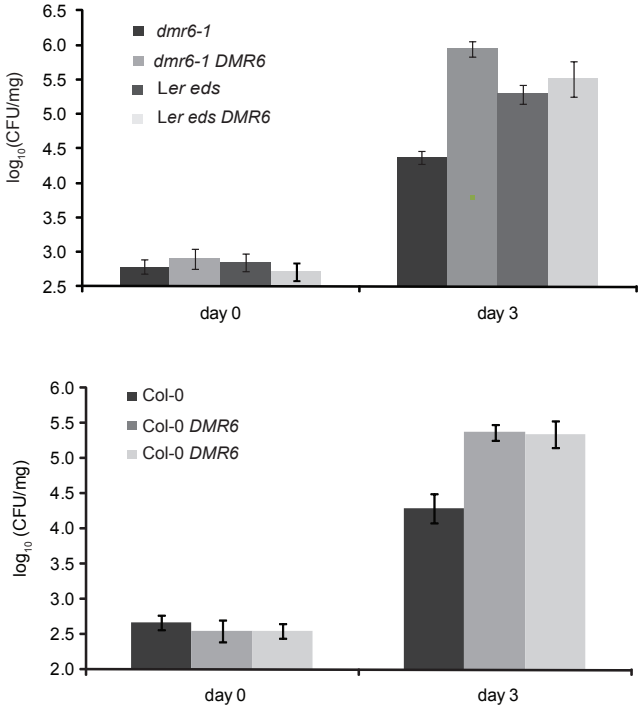
SUPPLEMENTAL DATA



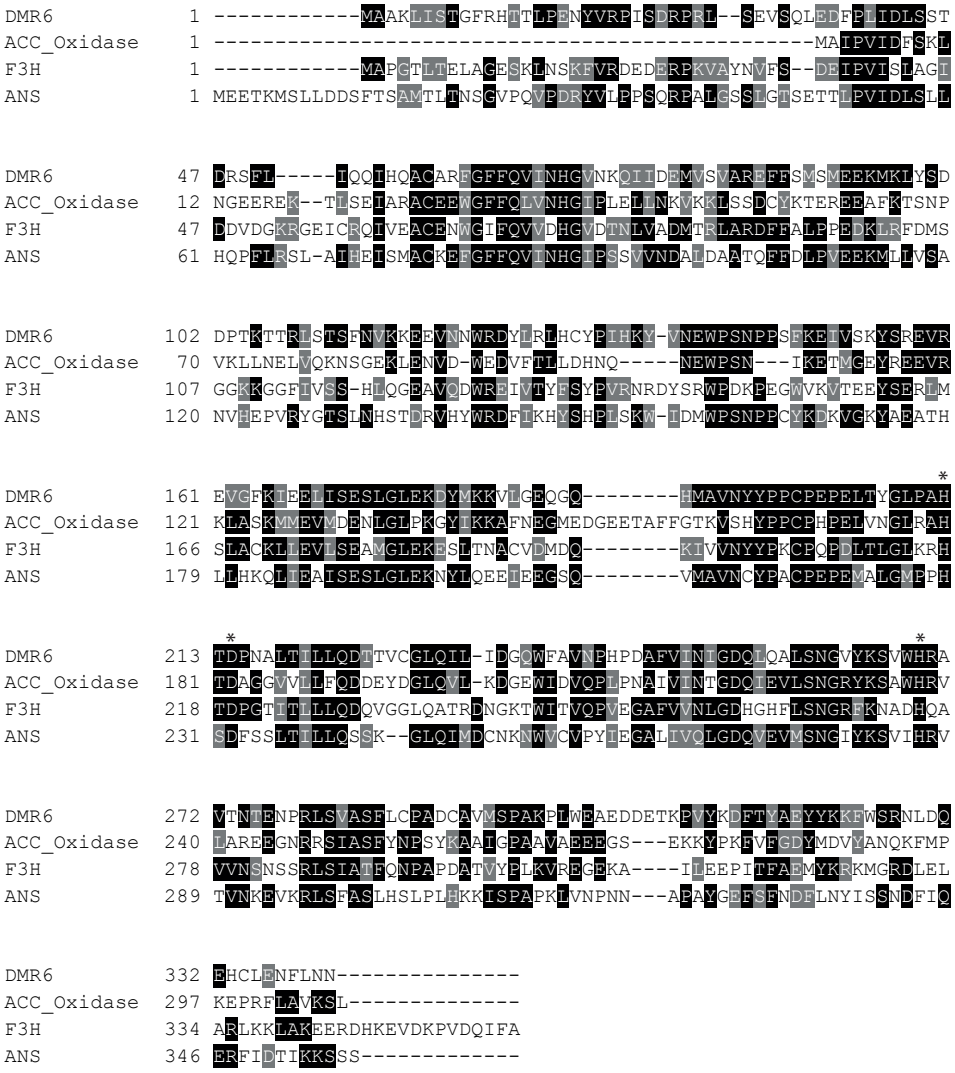
Supplemental Figure 1. Venn diagram showing the majority of *dmr6*-induced genes (48) are also induced by the SA-homologue INA (30) (P<e-41; determined with hypergeometric distribution) and during infection with *H. arabidopsidis* (*Hpa*) (40) (P<e-59). *dmr6* microarray data (van Damme et al. 2008) was compared to published expression data from Huibers et al. (2009) (Hpa) and Knoth et al. (2009) (INA).



Supplemental Figure 2. Overexpression of *DMR6* leads to enhanced susceptibility towards *H. arabidopsidis*. Quantification of *H. arabidopsidis* spores per mg fresh weight of 2-week old seedlings of (**A**) Col-0 and Col-0 P35S promoter *DMR6*, and (**B**) L*er eds1-2* and L*er eds1-2* P35S promoter *DMR6*. Note that in (**A**) spores were counted 5 dpi while in (**B**) spores were quantified at 4 dpi. Bars represents means with error bars represent standard error.



Supplemental Figure 3. Overexpression of *DMR6* leads to higher bacterial numbers compared to the parental line. Growth of *Pseudomonas syringae* pv. *tomato* DC3000 was analysed in 6-week old L*er eds1-2* and L*er eds1-2* P35S promoter *DMR6*, *dmr6-1* and *dmr6-1* P35S promoter *DMR6* (upper panel), Col-0 and Col-0 P35S promoter *DMR6* (lower panel) at 0 and 3 dpi. A bacterial suspension with OD 0.05 was sprayed on the plants. Bars represent standard error of 4 replicates.



Supplemental Figure 4. Multiple alignment of DMR6, ACC Oxidase, F3H, and ANS. Asterisks indicate iron binding residues which are conserved in all 2OG oxygenases. The sequence alignment was generated using the CLUSTALW2 program and conserved residues were highlighted using the BOXSHADE tool.

5

DMR6 and DMR6-LIKE OXYGENASE 1 have overlapping but distinct activities as negative regulators of immunity in Arabidopsis

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ABSTRACT

Plant immune responses typically involve the activation of inducible defences. These defence responses are under tight control of negative regulators. DOWNY MILDEW RESISTANT 6 is a 2OG Fe(II)-dependent oxygenase that acts as negative regulator of defence. In this study, we investigated the family of plant DMR6-like oxygenases for their involvement in immunity. Phylogenetic analysis of the large superfamily of 2OG oxygenases of 19 monocot and dicot species revealed a subgroup of 68 DMR6-related oxygenases. Within Arabidopsis, *DMR6* is most closely related to the *DMR6-like oxygenase 1* and *2* (*DLO1* and *DLO2*), which are recently duplicated and neighbouring genes. *DLO1* and *DLO2* are both capable of complementing the *dmr6* mutant indicating they can act as negative regulators of defence and have overlapping activities. *DLO1*, but not *DLO2*, is highly co-regulated with *DMR6*, showing upregulation after pathogen attack or SA treatment. Furthermore, the *dlo1_dmr6-2* mutant showed reduced *H. arabidopsidis* sporulation compared to the *dmr6-2* and *dlo1* single mutants indicating DLO1 and DMR6 have distinct activities. Finally, we identified an amino acid motif specific for DMR6 and DMR6-like oxygenases, that could be used as a tool for the identification of DMR6/DLOs in crop species so that they can be targets for gene inactivation aimed at generating disease resistant plants.

INTRODUCTION

Plants employ multiple defence responses to protect themselves from potential harmful micro-organisms. To prevent auto-immunity, these responses are kept under tight regulation by the action of negative regulators. DOWNY MILDEW RESISTANT 6 (DMR6) acts as negative regulator as its activity is required for susceptibility towards the biotrophic pathogens *Hyaloperonospora arabidopsidis* and *Pseudomonas syringae* (Chapter 4, this thesis). Inactivition of *DMR6* by mutation leads to constitutive activation of defence-related genes that causes of the resistant phenotype. Expression analysis demonstrated that *DMR6* is induced in compatible and incompatible *H. arabidopsidis* interactions, in particular in the cells harboring haustoria (van Damme et al. 2008). DMR6 belongs to the superfamily of 2-oxoglutarate Fe(II)-dependent oxygenases (2OG oxygenase) (Pfam domain PF03171). This superfamily comprises 151 members in Arabidopsis based on the Pfam domain, however, for most of the genes, including *DMR6*, the metabolic activity is unknown. 2OG oxygenases are known to catalyze a plethora of reactions that involve the oxidation of a substrate using molecular O_2 (Hewitson et al. 2005). They commonly use iron as co-factor and require 2-oxoglutarate as co-substrate for supplying two electrons (Prescott and John 1996). A general hallmark of these enzymes is the presence of the conserved HxD/Ex_nH motif located on a double-stranded beta sheet, although exceptions do exist (Clifton et al. 2006). Together with two four-stranded beta sheets (jelly roll fold) it capsulates the active center (Roach et al. 1995). 2OG oxygenases are implicated in secondary metabolism and biosynthesis of signaling molecules e.g. the biosynthesis of flavonoids, gibberellins, and alkaloids. Flavonoid biosynthesis involves 2OG oxygenase dependent hydroxylation (flavanone 3-hydroxylase; Pelletier and Shirley 1996) and desaturation (flavone synthase and flavonol synthase; Chua et al. 2008) reactions. Multiple steps in the gibberellin biosynthesis are catalyzed by 2OG oxygenases. For example, Arabidopsis contains 5 GA 2-oxidase genes all capable of inactivating active C_{19}-GAs. By the coordinated action of those 5 genes, Arabidopsis is able to control the level of active GA thereby limiting GA-responsive growth and development (Rieu et al. 2008a). Overexpression of these GA-oxidases leads to dwarfism and lower bioactive GA levels. During abiotic stress such as cold, these genes are transcriptionally activated and inactivate GA that in turn promotes the accumulation of DELLA proteins and increase freezing tolerance (reviewed by Gao et al. 2011). The regulation of GA biosynthesis is very complex and involves the action of multiple 2OG oxygenases that show various regulation patterns and overlap in substrate binding (Pimenta Lange and Lange 2006).

To identify additional 2OG oxygenases involved in resistance towards *H. arabidopsidis* we performed phylogenetic analysis to determine orthologues and close paralogues of DMR6. The cluster of *DMR6* was analyzed in more detail and identified 2 DMR6-like oxygenases (DLOs), DLO1 and DLO2. Interestingly, the *dlo1* knock-out mutant

shows reduced susceptibility towards *H. arabidopsidis* while the *dmr6-2_dlo1* double mutant is even more resistant compared to the *dmr6-2* single mutant suggesting partial redundancy. Overexpression of *DLO1* leads to more susceptibility suggesting DLO1 has a role similar to DMR6 as negative regulator of defence. In conclusion, DMR6 and DLO1 have overlapping but distinct activities as negative regulators of defence.

RESULTS

Phylogeny and classification of the 2OG oxygenase family in plants

The family of 2OG oxygenases was analyzed in more detail by phylogenetic analysis of plant proteins containing the 2OG-Fe(II) oxygenase superfamily Pfam domain PF03171. For this we used proteins of *Arabidopsis thaliana* and eighteen flowering plants of which genome sequences and protein models are available in the Phytozome v7.0 database (www.Phytozome.net). A total of 2951 proteins containing the PF03171 domain were selected using the HMMER3 algorithm. To filter small protein fragments and remove very large proteins, we only included proteins that do not exceed a 20% length difference to DMR6. Furthermore, only proteins were maintained that have a length difference of the oxidoreductase domain of less than 20% compared to DMR6. This resulted in a selection of 2038 proteins that fulfill all criteria, including 110 of 151 predicted Arabidopsis 2OG oxygenases. Phylogenetic clustering resulted in a tree (Figure 1A) in which many distinct clades representing different enzyme activities are shown. Well characterized oxygenases include flavonone-3-hydroxylase (F3H) (Pelletier and Shirley 1996), 1-aminocyclopropane-1-carboxylic acid (ACC) oxidase (Prescott and John 1996), and anthocyanidin synthase (ANS) (Wilmouth et al. 2002) which are present in distinct clades different from the DMR6 clade (indicated in red in Figure 1A). Two separate branches are distinguished in the DMR6 clade that each contain 2OG oxygenases from dicots and monocots indicating that these subclades have arisen in the ancestor of all flowering plants (82% bootstrap confidence). Figure 1B zooms in on the DMR6 clade of the 2OG oxygenase tree. For clarity we only included the well annotated genomes of 2 monocots species, rice (*Oryza sativa*) and mays (*Zea mays*), as well as 7 dicots, *A. thaliana*, papaya (*Carica papaya*), *A. lyrata*, grape vine (*Vitis vinifera*), castor bean (*Ricinus communis*), soybean (*Glycine max*), and cucumber (*Cucumis sativus*). In the upper subclade, DMR6 closely groups with orthologues from dicots (yellow vertical bar; Figure 1B) and more distantly with those from monocots (green vertical bar). The closest homologue of *A. thaliana* DMR6 is from *A. lyrata* (the divergence between *A. thaliana* and *A. lyrata* occurred approximately 10 Mya ago (Hu et al. 2011)). Gene duplications in the DMR6 clade are frequent in monocots in the upper part of the tree and in soybean and grape vine in both branches of the DMR6 clade. In the lower subclade, two *A. thaliana* DMR6 homologues cluster together (Figure 1B; indicated by the arrows) together with two

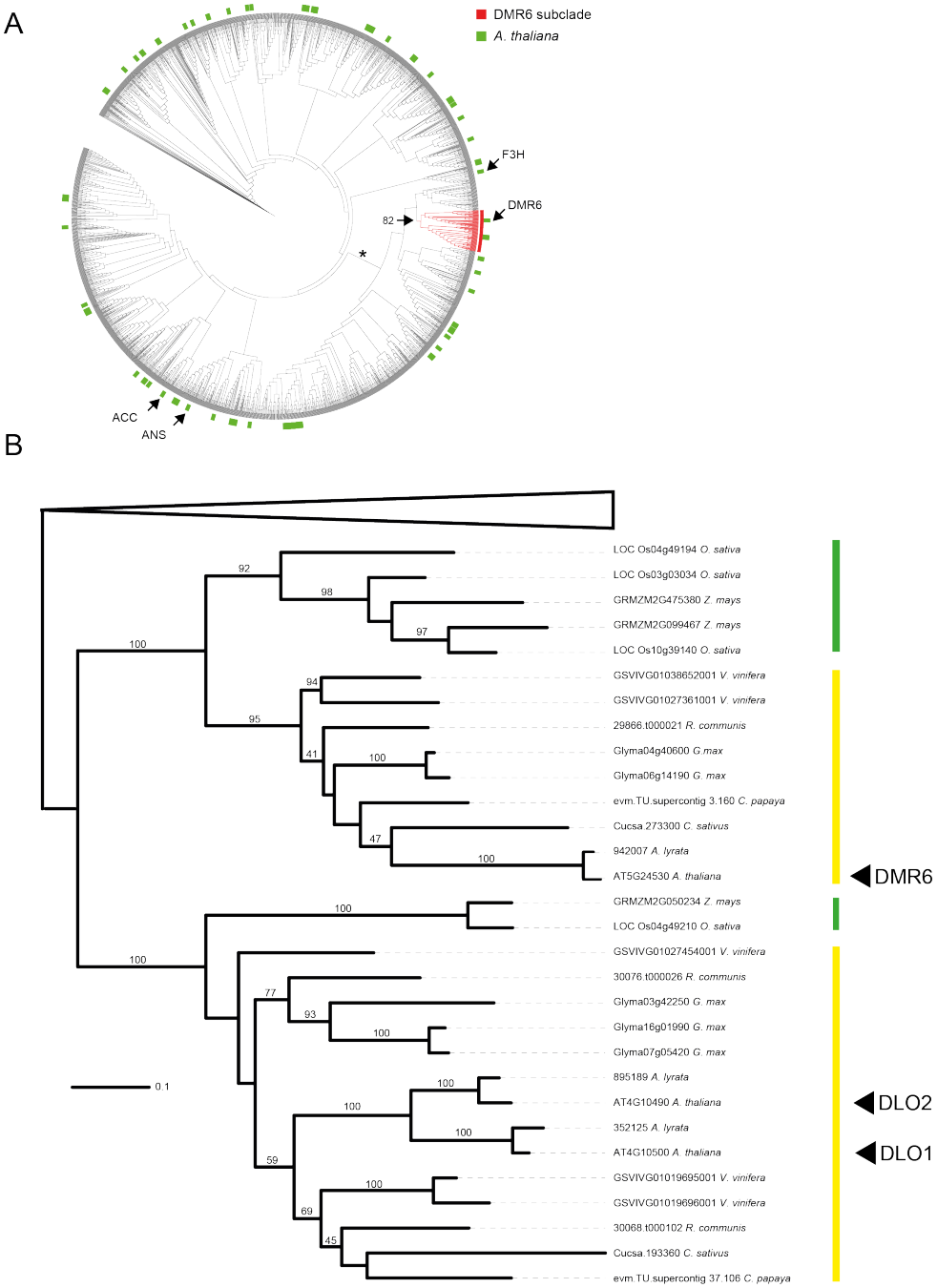

Figure 1. Phylogenetic analysis of plant 2OG oxygenases. (**A**) Phylogeny of 2OG oxygenase proteins from *A. thaliana* and 19 flowering plants from the Phytozome database. *A. thaliana* proteins are indicated with green dots. In red the DMR6 clade is indicated. (**B**) Close up of the phylogenetic tree showing the DMR6-clade of 2OG oxygenases that includes DLO1 and DLO2. Official gene identifiers and species names are indicated. Bootstrap values are shown in the tree. The scale represent branch length expressed as the relative number of amino acid substitutions.

proteins from *A. lyrata* suggesting they are the result of a relatively recent gene duplication in the common ancestor of these two species. These *A. thaliana* proteins are now designated DMR6-LIKE OXYGENASE or DLO (with gene At4g10500 encoding DLO1 and At4g10490 encoding DLO2). Also the DLO subclade shows a clear separation of the monocot (indicated by the green vertical bar) and dicot (indicated by the yellow vertical bar) proteins suggesting that the ancestor of all flowering plants already possessed a DLO besides DMR6. Grouping closely to DMR6, the DLOs form an interesting group that was subsequently analyzed in more detail, focusing on the *A. thaliana DLO1* and *DLO2* genes.

DLO1 and *DLO2* complement the *dmr6* mutant

The DLOs could have the same biochemical activity as DMR6, however, the substrate of the DMR6 enzyme is still unknown making it impossible to determine activities *in vitro*. To test if the DLOs have a function similar to that of DMR6 we tested if the *DLO1* and *DLO2* genes could complement the *dmr6* mutant. To this end, *DLO1* and *DLO2* were expressed under the constitutive 35S promoter and transformed into the *dmr6-1* mutant background. Four independent T3 transformants were analyzed for their expression level and 3 transformants showing clear transgene expression were selected. To check complementation, 2-week old plants were infected and at 5 days post inoculation (dpi) the number of spores per mg seedlings was scored as measure of susceptibility (Figure 2).

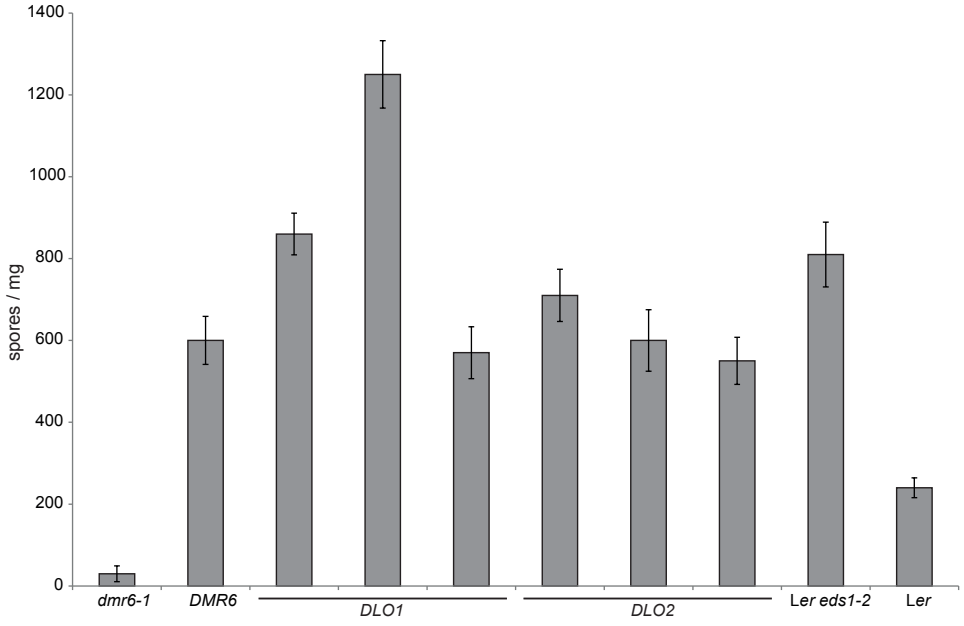

Figure 2. *DLO1* and *DLO2* complement *dmr6*-mediated resistance. Bars represent average number of *H. arabidopsidis* (Cala2) spores per mg seedling of *dmr6-1*, L*er eds1-2*, L*er* and *dmr6-1* complemented with P35S promoter *DMR6* (1 line), *DLO1* and *DLO2* (both three independent lines). Error bars represent standard error. This experiment has been repeated twice with similar results.

Intriguingly, while *dmr6-1* showed clear resistance, the 35S::*DLO1* and 35S::*DLO2* plants were highly susceptibility similar or higher than L*er eds1-2* that is the parental line of the *dmr6-1* mutant. As both *DLO1* and *DLO2* can complement the *dmr6-1* mutant phenotype we conclude that the DLOs have a biochemical function similar to that of DMR6.

Overexpression of *DLO1* and *DLO2* increases susceptibility to downy mildew

As overexpression of *DMR6* in the Col-0 background results in enhanced susceptibility to downy mildew and other pathogens, we next investigated if overexpressing *DLO1* and *DLO2* would also make Col-0 more susceptible. *DLO1* and *DLO2* coding sequences were transformed independently under the control of the CaMV 35S constitutive promoter in the Col-0 background. As control we included Col-0 overexpressing *DMR6* (Chapter 4, this thesis) and the highly susceptible Col-*eds1* mutant (Bartsch et al. 2006). Disease assays with *H. arabidopsidis* showed that overexpression of *DLO1* and *DLO2* enhanced susceptibility compared to the Col-0 parental line as shown by the higher level of sporulation (Figure 3). The observed enhanced susceptibility was comparable to the *DMR6* overexpression plants and the Col-*eds1* mutant. This confirms that the DLO1 and DLO2 protein have an activity similar or identical to DMR6 resulting in the same phenotypic effects. The question remains why the *dmr6* mutants have such a clear resistance phenotype when *A. thaliana* has two *DLO* genes that can take over *DMR6* function?

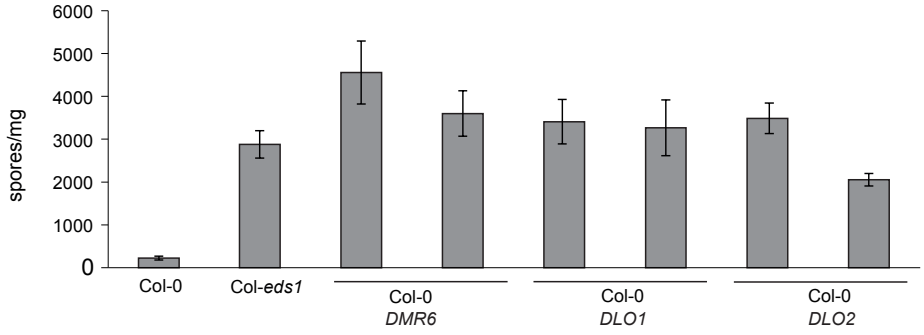

Figure 3. Overexpression of *DLO1* and *DLO2* increases susceptibility towards *H. arabidopsidis*. Amount of *H. arabidopsidis* (Waco9) spores per mg leaves of 5-week old plants at 7 dpi of Col-0, Col-*eds1*, and 2 independent T3 lines of P35S promoter *DMR6*, *DLO1*, and *DLO2* plants. Bars represent mean with error bars standard deviation. This experiment has been repeated twice with similar results.

Expression of *DLO1* but not *DLO2* is immunity-related

The DLO1 and DLO2 complementation and overexpression lines were all generated using the 35S promoter. It is, however, likely that the expression of the wild-type DLOs is highly regulated similar to that of DMR6, which is strongly activated during plant defence. Therefore, we analysed publicly-available gene expression data to determine if *DLO1* and *DLO2*

show immunity-related expression similar to *DMR6*. For this analysis, data of 22 different Affymetrix microarray experiments all dealing with transcriptional profiling after pathogen attack, defence related hormone application, elicitor/effector treatment, abiotic stress or mutants involved in defence response, were used (Supplemental Table 1). The expression analysis was focused on 30 2OG oxygenases that belong to the large clade containing the *DLO*s and *DMR6* (indicated with an asterisk in the tree; Figure 1A). Hierarchical clustering of the experiments and genes allowed to group the 2OG oxygenase genes according to their expression patterns, providing information about which genes are co-regulated during plant immune responses (Figure 4). Strikingly, *DLO1* clusters with *DMR6*, whereas *DLO2* does not show any co-regulation with *DMR6* or *DLO1*. *DMR6* and *DLO1* are downregulated for example after chitin perception and chitooctaose (purified chitin fragments) treatment while the majority of DLOs is upregulated.

Also, *DMR6* and *DLO1* are both upregulated after Type III effector and SA treatment. *DLO2* clusters well away from *DMR6* and *DLO1.* Further analysis of available microarray data using Genevestigator revealed that *DLO2* is not or hardly expressed in any tissue or in response

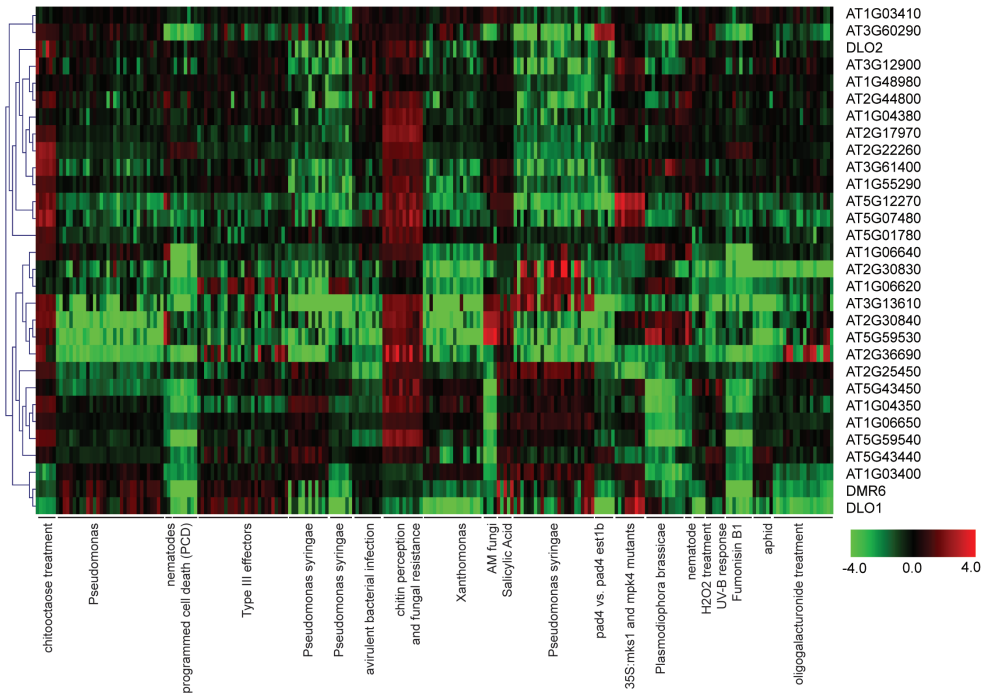

Figure 4. Hierachical clustering of the *DLO*s in response to various biotic and abiotic stresses. Hierarchical clustering of 30 *DLO*s from figure 1A (branch indicated with asterisk) on publicly available microarray data, treatments are indicated below the clusters (more detail in Supplemental Table 1). Red indicates a higher level of gene expression relative to control treatment, while green indicates downregulation.

to any treatment, except for siliques, suggesting that *DLO2* does not have a role in the plant immunity. Genevestigator analysis further confirmed that *DMR6* and *DLO1* are both upregulated after pathogen attack and after BTH or SA treatment.

The responsiveness of the *DLOs* to *H. arabidopsidis* infection was determined using quantitative PCR (qPCR). As shown in figure 5, *DMR6 and DLO1* are highly activated in plants infected with a compatible or incompatible isolate of *H. arabidopsidis*, whereas *DLO2* is completely unresponsive. Also following treatment with the SA mimic BTH, both *DMR6* and *DLO1* are strongly activated. In contrast, the *DMR6* and *DLOs* are unresponsive to methyl jasmonate (MeJA) which is known to activate jasmonic acid-induced genes. *DLO2* expression is virtually undetectable confirming it is not immunity-related and suggesting the gene does not have a role during pathogen infection. The fact that both *DMR6* and *DLO1* are activated during the plant's immune response suggests that DLO1 also acts as a negative regulator of defence. The question remains, however, why the *dmr6* mutants show enhanced resistance in the presence of an intact *DLO1* gene.

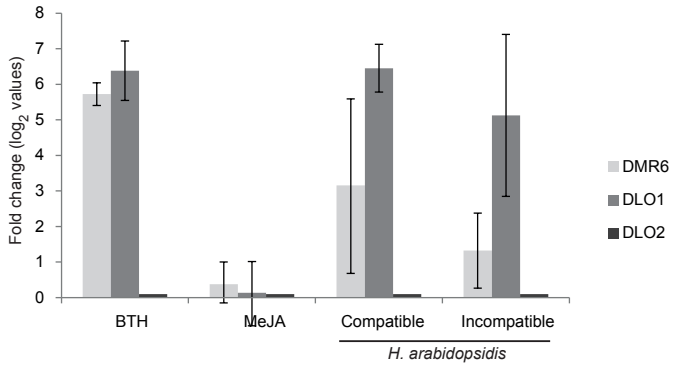

Figure 5. *DMR6* and *DLO1* but not *DLO2* are activated by BTH and *H. arabidopsidis*. Average fold change (log2-values) of *DMR6*, *DLO1* and *DLO2* after BTH and MeJA treatment (1 dpi), as well as infection with a compatible (Waco9) and incompatible (Cala2) *H. arabidopsidis* isolate (3 dpi) compared to mock-treated plants. Expression levels are the mean from 3 independent biological replicates. Error bars indicate standard deviation.

DLO1 and *DMR6* show different spatial expression in infected leaves

To analyze the tissue-specific expression of *DLO1* during downy mildew-infection, we generated transgenic lines containing a construct with the *DLO1* promoter fused to the *GUS* reporter gene (pro$_{DLO1}$:*GUS*). Since we have not observed any expression of *DLO2*, no GUS fusion with the promoter of *DLO2* was constructed. Following *H .arabidopsidis* infection, *DLO1* spatial expression was specifically detected in and around the vascular tissue of the leaves (Figure 6A). *DLO1* expression was not induced in cells that are in close contact with the pathogen. This differs from *DMR6* that is expressed in cells that are in direct contact with the pathogen and that harbor haustoria (Figure 6B).

These expression studies show that *DLO1* and *DMR6* have distinct expression patterns. The absence of *DLO1* expression in haustoria-containing cells could explain why in *dmr6* mutants *DLO1* does not complement the loss of *DMR6* function.

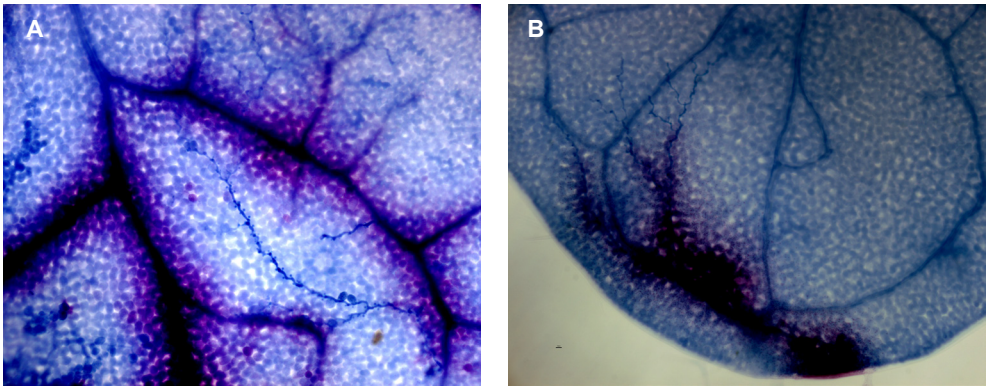

Figure 6. Expression of *DLO1* is localized in the cells near the vascular tissue after *H. arabidopsidis* infection. GUS activity in the pro$_{DLO1}$:GUS (**A**) and pro$_{DMR6}$:GUS (**B**) plant lines was visualized with Magenta-X-gluc as substrate. *H. arabidopsidis* growth was visualized with trypan blue staining. GUS activity in the pro$_{DMR6}$:GUS line is specifically located in cells containing haustoria, while that of pro$_{DLO1}$:GUS is located in cells near the vascular tissue.

DLO1 and *DMR6* function partially overlaps

The role of DLO1 as a putative negative regulator of defence was tested in a *dlo1* T-DNA insertion line. Mutants homozygous for the insertion did not show any growth-related phenotype as they developed identical to the parental line, Col-0. When infected with *H. arabidopsidis* the *dlo1* mutant showed reduced sporulation at 5 dpi (Figure 7) compared to Col-0. However, the level of resistance to *H. arabidopsidis* is lower than that of the *dmr6-2* mutant. To analyze if there is an additive effect of *dlo1* when combined with *dmr6-2*, we crossed the two mutants.

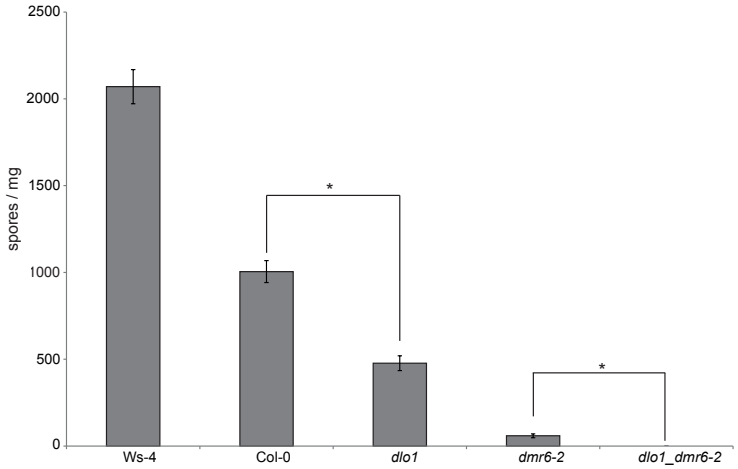

Figure 7. The double *dlo1_dmr6* mutant supports less *H. arabidopsidis* growth compared to *dmr6-2*.
Bars represent average number of spores per mg seedlings of *dlo1*, Col-0, *dmr6-2*, Ws-4 and the double mutant *dlo1_dmr6-2*. Error bars represent the standard deviation. The asterisks indicate significant differences between *dmr6* and *dlo1_dmr6* and between Col-0 and *dlo1* ($P<0.01$; two-tailed T-test).

Analysis of the homozygous *dmr6-2_dlo1* double mutant revealed that it supported significantly less sporulation compared to the *dmr6-2* or *dlo1* single mutants. This suggests that *DLO1* and *DMR6* have a partially redundant function and that both genes are required for full disease susceptibility to downy mildew.

Tomato and cucumber DMR6 complement the Arabidopsis *dmr6* mutant

Because DLOs and DMR6 are found in all flowering plants, we analyzed if DMR6 from crop species would be able to complement the *dmr6* mutant phenotype. For this, we obtained *DMR6* orthologous coding sequences as identified in the phylogenetic tree. The *dmr6-1* mutant was transformed with the tomato and cucumber *DMR6* orthologues under control of the 35S promoter. For each construct, two independent lines were tested with *H. arabidopsidis*. As shown in figure 8, both *DMR6* orthologues are able to complement the *dmr6-1* resistant phenotype. The susceptibility to *H. arabidopsidis* of Col-0 transformants with the tomato and cucumber *DMR6* gene was similar to that of the Arabidopsis 35S::*DMR6* and L*er eds1-2* plants, while the *dmr6-1* mutant hardly showed any disease symptoms. This demonstrates that the *DMR6* orthologues from crops have the same intrinsic function as *DMR6* suggesting that DMR6 function is ancestral being conserved in flowering plants.

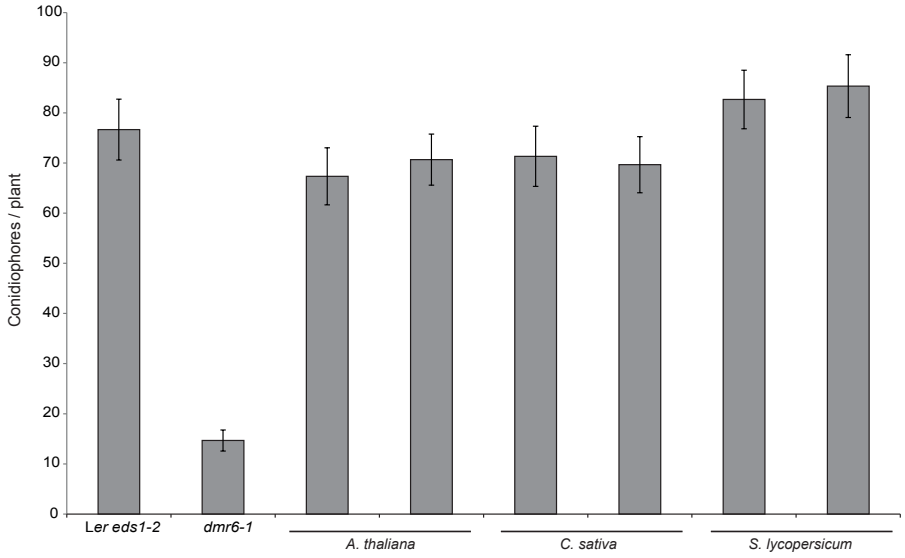

Figure 8. Cucumber and tomato *DMR6* complement the *dmr6* resistance phenotype. Quantification of conidiophores of *H. arabidopsidis* on two-week old seedlings 5 days post inoculation. Two independent lines of P35S promoter *DMR6* of Arabidopsis (*A. thaliana*), cucumber (*C. sativa*) and tomato (*S. lycopersicum*) were tested for susceptibility together with *dmr6-1* and its parental line L*er eds1-2*. This experiment has been repeated twice with similar results.

DMR6 and DLO1 share a common specific amino acid motif

Previously, we have identified three amino acids that are located inside the substrate binding pocket of DMR6 (Chapter 4, this thesis). Two of them, R108 and R124, are critical for enzyme function as substitution of these amino acids into alanine completely abolishes DMR6 activity. Further investigation shows that in Arabidopsis R124 is located on a beta sheet that starts at position 123. Figure 9 shows a multiple sequence alignment of the DMR6 and DLO homologues from figure 1B. Highlighted is the beta sheet that is conserved in all proteins of the analyzed plant species. In total, 6 amino acid residues are present in this small beta sheet (aa 123-128; indicated in grey; Figure 9). Three amino acids, R124, F/Y126 and R128 face inwards in the pocket where they could be involved in substrate binding. The other three amino acids, W123, D125, and L127 are pointing in the opposite direction making it unlikely that they are involved in substrate binding. Intriguingly, all analyzed DLO and DMR6 proteins from flowering plant species (Figure 1B) contain this WRD(F/Y)LR motif (Figure 9). Furthermore, the subclade containing DLO1 and DLO2 have a phenylalanine at position 126, while all plant species in the DMR6 subclade contain a tyrosine at this location. Since we have shown that DLO1 has a similar molecular activity as DMR6, F126 and Y126 are apparently both tolerated for enzymatic activity and substrate specificity.


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DMR6
04g49194_O.sativa                 102 ---EPSKKIRLSTSFNVRKETVHNWRDYLRLHCHPLEEFVPEWPS- 144
03g03034_O.sativa                 100 ---DPAKKIRLSTSFNVRKETVHNWRDYLRLHCYPLHRYLPDWPSN 143
GRMZM2G475380_Z.mays              100 ---DPAKKMRLSTSFNVRKETVHNWRDYLRLHCHPLEQYVP----- 138
GRMZM2G099467_Z.mays              100 ---DPARKIRLSTSFNVRKETVHNWRDYLRLHCHPLDEFLP----- 138
10g39140_O.sativa                 106 ---DPAKKIRLSTSFNVRKETVHNWRDYLRLHCYPLHQFVPDWPS- 148
GSVIVG01038652001_V.vinifera      102 ---DPTKTMRLSTSFNVNKEKVHNWRDYLRLHCYPLDQYT------ 139
01027361001_V.vinifera            101 ---DPSKTMRLSTSFNVKKEKVHNWRDYLRLHCHPLEQYM------ 138
t000021_R.communis                101 ---DPTKTMRLSTSFNMKKEKVHNWRDYLRLHCYPLDKYISE---- 140
04g40600_G.max                    101 ---DPSKTMRLSTSFNVKKETVHNWRDYLRLHCYPLDK-------- 136
106g14190_G.max                   101 ---DTSKTMRLSTSFNVKKETVRNWRDYLRLHCYPLEKY------- 137
3.160_C.papaya                    101 ---DPAKTTRLSTSFNVKKEKVHNWRDYLRLHCHPLHKYMP----- 139
273300_C.sativus                  105 ---DPSKTVRLSTSFNVRKEQFRNWRDYLRLHCYPLSNYTP----- 143
942007_A.lyrata                   101 ---DPTKTTRLSTSFNVKKEEVNNWRDYLRLHCYPIHK-------- 136
DMR6_A.thaliana                   101 ---DPTKTTRLSTSFNVKKEEVNNWRDYLRLHCYPIHK-------- 136
DLO
GRMZM2G050234_Z.mays              111 ---DPNKAIRLSTSFNVRTEKVSNWRDFLRLHCYPLQSF------- 147
04g49210_O.sativa                 111 ---DPKKAIRLSTSFNVRTEKVSNWRDFLRLHCYPLESFI------ 148
01027454001_V.vinifera            109 ---DPLKTMRLSTSFNVKTEQVSNWRDFLRLYCYPLEDYIQE---- 148
t000026_R.communis                109 ---DPMMRTRLSTSFNVRTEKTSNWRDFLRLHCYPLDDYMQE---- 148
03g42250_G.max                    109 ---DPFKASRLSTSFNVNSEKVSSWRDFLRLHCHPIEDY------- 145
16g01990_G.max                    108 ---DPTKTTRLSTSFNVKTEKVSNWRDFLRLHCHPLEDYIQE---- 147
07g05420_G.max                    108 ---DPSKTTRLSTSFNVKTEKVSNWRDFLRLHCHPLEDY------- 144
895189_A.lyrata                   111 ---DTKKTTRLSTSFNVSKEKVSNWRDFLRLHCYPIE--------- 145
DLO2_A.thaliana                   108 ---DTKKTTRLSTSFNVSKEKVSNWRDFLRLHCYPIEDFI------ 145
352125_A.lyrata                   111 ---DPTKTTRVSTSFNIGADKILNWRDFLRLHCFPIED-------- 146
DLO1_A.thaliana                   110 ---DPTKTTRLSTSFNVGADKVLNWRDFLRLHCFPIEDF------- 146
01019695001_V.vinifera            110 ---NPSKTTRLSTSFNVKTEKVANWRDFLRLHCYPLEDYV------ 147
01019696001_V.vinifera            110 ---NPSNPVRLSTSFNVKTEKVANWRDFLRLHCYPLEDYV------ 147
t000102_R.communis                108 ---DPTKTTRLSTSFNVKTEKVSNWRDFLRLHCYPLADYIQE---- 147
193360_C.sativus                  150 ---DPTKKTRLSTSFNVKTEKVANWRDFLRLHCY------------ 181
37.106_C.papaya                   109 ---DPAKTTRLSTSFNVKTEKFSNWRDFLRLHCYPVQDYIHE---- 148
                                          :.    *:*****:  :   .***:***:*.
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Figure 9. Multiple sequence alignment of a DMR6 segment (in bold) indicating the conserved motif signature in grey. DLOs from the same plant species as in Figure 1B were used in this multiple alignment. This alignment was generated using the CLUSTALW2 program and conserved residues were highlighted under the sequence. Asterisk indicate identical amino acids while dots indicate conserved amino acids. Indicated in grey is the specific DLO motif.

The presence of this conserved motif could determine the specificity for a particular substrate that is shared between different species. Oxygenases from other plant species that are obtained by similarity searches with DMR6 or DLOs can be checked for the conservation of WRD(F/Y)LR that is only conserved in the DMR6-like 2OG oxygenases. Presence of this motif means that the protein likely has the same function as DMR6 or the DLOs.

DISCUSSION

The 2-oxoglutarate Fe(II)-dependent oxygenases are widely present in all flowering plant species. In this study we focused on identifying oxygenases that share high sequence conservation with Arabidopsis DMR6. We considered an oxygenase as DMR6-like based on its presence in the same subclade as DMR6 in our phylogenetic analysis (Figure 1). The selection was based on sequence homology and additionally (1) the presence of the oxygenase domain and (2) size similarity, being within 20% length difference of DMR6. A total of 68 2OG oxygenase proteins from monocot and dicot species are present in the same subclade as DMR6 and therefore considered as DMR6 and DMR6-like (Figure 1A). These DMR6-like oxygenases were analyzed in more detail since they could play a similar role in defence as DMR6. From the phylogenetic analysis it is evident that DMR6 comprises a clade containing family members of dicots as well as monocots (Figure 1B). The closest orthologue (homolog separated by speciation) is in *A. lyrata*. The monocot species are clearly represented by a separate branch in this clade, indicating that the *DMR6* gene in Arabidopsis already existed before the monocot-dicot divergence. In the same clade, 2 *A. thaliana* paralogues (homologs generated by gene duplication) are present but located on another branch, designated *DMR6*-like oxygenase 1 and 2 (DLO1 and DLO2). These 2 neigbouring DLOs are the result of a recent duplication event that occurred before the divergence of *A. thaliana* and *A. lyrata* since both genes have orthologues in *A. lyrata*. The fact that DMR6 and DLO1 are on different branches both containing monocot and dicot members indicate that the ancestor of flowering plants already contained both a *DMR6* and *DLO* gene. We investigated the *A. thaliana DLO1* and *DLO2* genes further since they may possess similar molecular activities. Indeed, *DLO1* and *DLO2* are capable of complementing *dmr6*-mediated resistance when transgenically expressed from the constitutive 35S promoter. This suggests that DLO1 and DLO2 have a molecular activity similar to DMR6 thereby able to negatively regulate defence. The fact that the wild-type *DLO1* and *DLO2* under their native promoters are not able to suppress *dmr6*-mediated resistance indicates that the *DLOs* have a role different from *DMR6* in the plant. Analysis of genome-wide expression data related to pathogen, elicitor and hormone treatment, showed that *DLO1* is regulated similar to *DMR6* (Figure 4). A clear induction after pathogen attack and salicylic acid treatment is observed for these genes. As *DMR6*, *DLO1* is transcriptionally activated in compatible and incompatible interactions with *H. arabidopsidis*. *DLO2* on the other hand is not expressed during *H. arabidopsidis* infection nor after BTH treatment, making it very unlikely this gene is involved in immunity.

To further elucidate the role of *DLO1*, we analyzed its spatial expression. We show that the localization of expression of *DLO1* is not associated with the growth of the pathogen. The spatial expression of *DMR6* is mostly in cells harboring the haustoria, while *DLO1* spatial expression is more pronounced around the main veins. This could explain why *DLO1* is

unable to suppress the *dmr6* mutant phenotype since activation of *DMR6* and *DLO1* occurs in different cells of the infected leaf tissue. This would suggest that, although they share the same molecular activity, each gene has a specific function. This phenomenon is not uncommon as exemplified by regulation of the hormone gibberellin through the concerted action of several GA-oxidase families belonging to the 2OG oxygenase superfamily (Pimenta Lange and Lange 2006). These family members exhibit different expression patterns depending on developmental processes and environmental cues (e.g. mediating the effect of low temperature (Yamauchi et al. 2004)) thereby controlling the GA level during the development of the plant (Pimenta Lange and Lange 2006). For example, it has been shown that three of the five GA 20-oxidases, GA20ox1-3, catalyse the oxidation reaction from C_{20}-GA to C_{19}-GA, a precursor of bioactive GA (Rieu et al. 2008b). These three genes show different tissue-specific expression; *GA20ox1* is mainly expressed in stems, *GA20ox2* in flowers and siliques, *GA20ox3* only in siliques. Although these three genes have the same molecular function, they show a different expression pattern indicating that the contribution of each of these genes to GA biosynthesis is organ-specific.

Previously, we showed that DMR6 acts as a negative regulator of defence (chapter 4, this thesis). To study if DLO1 is involved in defence we analyzed the *dlo1* mutant for resistance to *H. arabidopsidis*. We observed that the *dlo1* mutant is more resistant compared to wild-type plants, although to a lesser extent as the *dmr6* mutant. Furthermore, when overexpressed, plants become more susceptible to *H. arabidopsidis* infection, confirming that also DLO1 acts as a negative regulator of defence. This was further supported by the fact that it is only activated after pathogen attack or defence-related hormone treatment. Many negative regulators are repressed during normal plant growth and activated only when encountered by pathogens. One example is *CPR30* which is repressed under pathogen free conditions. The *cpr30* mutant is resistant to virulent and avirulent bacterial pathogens which coincides with activated defence responses (Gou et al. 2009). The negative regulation of *CPR30,* but is only partially SA-dependent and NPR1-independent (Gou et al. 2009).

The *dlo1_dmr6* double mutant supports even less *H. arabidopsidis* growth compared to the single *dmr6* and *dlo1* mutants indicating both *DMR6* and *DLO1* are required for full susceptibility. The fact that the double mutant provides a higher level of resistance compared to the single mutants indicate DMR6 and DLO1 have distinct activities. This is not a dosage effect since heterozygous mutants in either gene behave like wild type. It is not uncommon that genes of the same family are involved in similar processes with distinct but overlapping molecular functions. So are several members of the WRKY transcription factor (TF) gene family involved in defence-associated transcriptional reprogramming. WRKY11 and WRKY17 are in the same subclade and share 72% amino acid identity. Mutation of *wrky11* enhanced resistance to *P. syringae* pv *tomato*, while the *wrky17* mutant has no phenotypic effects. The double *wrky11_wrky17* mutant showed enhanced resistance towards *P. syringae* pv *tomato*,

even more than the *wrky11* single mutant showing WRKY 11 and WRKY17 act redundantly as negative regulator of defence (Journot-Catalino et al. 2006). Another WRKY family, comprising WRKY18, WRKY40, and WRKY60, is involved in resistance towards *P. syringae* (Xu et al. 2006). Single mutants do not or hardly show an effect on disease resistance, but the triple *wrky18_wrky40_wrky60* mutant shows reduced growth of the bacterial pathogen *P. syringae*. Interestingly, overexpression of *WRKY18* enhanced *PR-1* expression and increased resistance to *P. syringae*. But when *WRKY18* combined with *WRKY40* or *WRKY18* combined with *WRKY60* are coexpressed, plants are enhanced susceptible to *P. syringae* indicating these genes function in a complex manner as negative regulator of defence (Xu et al. 2006).

DMR6 function is not specific for Arabidopsis since we have identified functional DMR6 orthologues in crop species like tomato and cucumber by their ability to complement the *dmr6* mutant phenotype. Also, we identified a amino acid sequence motif specific for DMR6 and DMR6-like oxygenases. This WRD(F/Y)LR motif is predicted to be located on a small beta sheet of 6 amino acids. Three of the amino acid residues in this beta sheet are in close proximity of the substrate and could be involved in substrate binding. Indeed, previously we showed that R124 (the first R in the motif) is indispensible for DMR6 function (chapter 4, this thesis). The presence of this particular motif in DMR6 and DMR6-like oxygenases suggest that a similar substrate is shared between different plant species. The identification of the WRD(F/Y)LR motif which is specific for DMR6 and DMR6-like oxygenases, can be valuable in the identification of DMR6 orthologues in economically important crop species. This tool can be used for the identification of DMR6/DLOs in crop species so that they can be targets for gene inactivation for the generation of resistant plants.

METHODS

Plant and Pathogen Growth Conditions

Arabidopsis plants were grown on potting soil at 22°C with 16 hours of light and a relative humidity of 75%. Infection assays with *H. arabidopsidis* was performed as described previously (van Damme et al. 2009). *H. arabidopsidis* sporulation was quantified by counting the spores per 100 mg fresh weight 5 days post infection or by counting the conidiophores per plant. *dmr6-2* was genotyped as described previously (van Damme et al. 2008). *dlo1* was obtained through NASC (N559907) and genotyped using primers: dlo1_LB 5'-attccatccctctgatcgatc-3'; dlo1_RB 5'-tcaacaaacgggtaggttctg-3' and LBb1.3 5'-attttgccgatttcggaac-3'.

Phylogenetic analysis and data mining

The described phylogenetic analysis was performed using the monocot species *Brachypodium distachyon*, *Oryza sativa*, *Setaria italica*, *Zea mays*, and *Sorghum bicolor*. Dicot species used were *Manihot esculenta*, *Ricinus communis*, *Medicago truncatula*, *Glycine max*, *Cucumis sativus*, *Prunus persica*, *Arabidopsis thaliana*, *Arabidopsis lyrata*, *Carica papaya*, *Citris clementina*, *Eucalyptus grandis*, *Vitis vinifera*, *Mimulus guttatus*, and *Aquilegia coerulea* from the Phytozome v7.0 database (www.Phytozome.net). Proteins containing the PF03171 domain were selected using the HMMER3 algorithm. Proteins exceeding 20% length difference compared to *A. thaliana* DMR6 as well as domain predictions that differ more than 20% in length to the DMR6 prediction were removed from the selection. If proteins were 100% identical, only one of the proteins was included. The alignment was performed using MAFFT applying the LINSi algorithm option (Katoh et al. 2002). The tree was calculated using RAxML (v.7.0.4), with the WAG substitution matrix and GAMMA model of rate heterogeneity (Stamatakis 2006).

Publicly available pathogen-related microarray experiments showing differential expression for the 30 genes were obtained as processed data from Array Express (http://www.ebi.ac.uk/arrayexpress/). A list of experiments used is provided (Supplemental Table 1). Fold change was calculated per gene, and $\log_2$ values were then plotted using MeV 4.5 (Saeed et al. 2006). Data was clustered using pearson correlation with average linkage.

Generation of overexpression lines

DLO1 (At4g10500) and *DLO2* (At4g10490) were amplified from Col-0 using primers: DLO1_Fw 5'-caccatggcaacttctgcaatatctaag-3', DLO1_Rv 5'- gagccaattcaagattgattacaa-3', DLO2_Fw 5'- caccatggcagcatcaaaactcctc-3' and DLO2_Rv 5'- ttaggcggtggatgctttgaa-3' and cloned into the pENTRY vector using Gateway ® cloning (Invitrogen). Constructs were cloned into the pB7WG2 Gateway ® compatible binary vector (Karimi et al. 2002). These were transformed

using *A. tumefaciens* strain C58C1 containing pGV2260 in Col-0 and *dmr6-1* plants using floral dipping (Zhang et al. 2006). Transformants were selected for resistance to BASTA. Cucumber (Cucsa.273300) and tomato (NP_001233840.1) *DMR6* were cloned using primers: Cuc_Fw 5'- caccatgagcagtgtgatggagat-3', Cuc_Rv 5'- tgggccaaaaagtttatcca-3', Slyc_Fw 5'-caccatggaaaccaaagttatttctagc-3' and Slyc_Rv 5'- gggacatccctatgaaccaa-3' and transformed into *dmr6-1* plants as described above.

Plant treatments and quantitative PCR

The induction treatments were performed by spraying 100 µM BTH or 100 µM MeJA onto 14-day old seedlings. After 24 hours, the seedlings were harvested for RNA isolation. *H. arabidopsidis* isolates Waco9 and Cala2 were sprayed (50 spores/µl) onto 14-day old seedlings that were harvested 3 days post inoculation. RNA was extracted following the instructions of the RNeasy kit (Qiagen). From total RNA, a DNase treatment was performed (Qiagen) and subsequent cDNA was synthesized using Superscript-III reverse trascriptase (Invitrogen). QPCR was performed on a ABI PRISM 7700 sequence detection system using SYBR green I as reporter (Applied biosystems). Arabidopsis *ACTIN2* levels were used for normalization. *DMR6*, *DLO1* and *DLO2* expression was analysed using primers QDMR6F 5'-tgtcatcaacataggtgaccag-3' QDMR6R 5'-cgatagtcacggattttctgtg-3', QDLO1_Fw 5'-aatatcggcgaccaaatgc-3', QDLO1_Rv 5'-cgctcgttctcggtgtttac-3', QDLO2_Fw 5'-tcctgttcccaaccactttca-3', and QDLO2_Rv 5'-gctgttcacaaccgctctg-3'.

Promoter GUS transgenic lines

Pro_{DMR6}:GUS transgenic lines were generated as described previously (van Damme et al. 2008). The Pro_{DLO1}:GUS transgenic line was generated using primers: pDLO1_Fw 5'-cacctgtaaagatccaaataacatggt-3' and pDLO1_Rv 5'-ttaatgtgtttggtaatgtaat-3' and Gateway® cloned into the pENTRY vector. The promoter was cloned into the pBGWFS7 binary vector in front of the GUS gene (Karimi et al. 2002) that was transformed using *A. tumefaciens* strain C58C1 in Col-0. Transformants were selected for resistance to BASTA. Pro_{DMR6}:GUS and Pro_{DLO1}:GUS transgenic seedlings (T3) were first infected with *H. arabidopsidis* and at 3 days post inoculation vacuum infiltrated with Magenta-Xgluc solution to visualize GUS activity as described previously (van Damme et al. 2008).

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SUPPLEMENTAL DATA

Supplemental Table 1. List of microarray experiments related to biotic and abiotic stress and used for expression analysis. Experiment ID is shown together with title of the experiments.

ID	Text for figure	Title
E-GEOD-4746	chitooctaose treatment	Transcription profiling of response of Arabidopsis seedlings to chitooctaose treatment
E-GEOD-5685	Pseudomonas	Transcription profiling of Arabidopsis treated with Pseudomonas - AtGenExpress: Pathogen Series
E-GEOD-5724	nematodes	Transcription profiling of Arabidopsis exposed to H. schachtii nematodes
E-GEOD-5735	programmed cell death (PCD)	Transcription profiling of Arabidopsis to identification of core genes regulating plant programmed cell death (PCD)
E-GEOD-6176	Type III effectors	Transcription profiling of Arabidopsis to assess the impact of Type III effectors on plant defense responses
E-GEOD-6556	Pseudomonas syringae	Transcription profiling of Arabidopsis wild type Columbia-0 and mutant gh3.5-1D in response to pathogen Pst DC3000(avrRpt2)
E-GEOD-6829	Pseudomonas syringae	Transcription profiling of Arabidopsis wild type and WRKY T-DNA knockout mutants challenged with Pseudomonas syringae
E-GEOD-6831	avirulent bacterial infection	Transcription profiling of Arabidopsis systemic response to avirulent bacterial infection
E-GEOD-8319	chitin perception and fungal resistance	Transcription profiling of Arabidopsis ALys RLK1 mutant reveals LysM receptor-like kinase mediates chitin perception and fungal resistance
E-GEOD-9674	Xanthomonas	Transcription profiling of Arabidopsis plants mis-expressing AtMYB30 AFTER Xanthomonas inoculation AT early timepoints
E-GEOD-10323	AM fungi	Transcription profiling of Arabidopsis Col-0 inoculated with AM fungi to test for the presence of arbuscular mycorrhizal signalling pathways
E-GEOD-14961	Salicylic Acid	Transcription profiling of Arabidopsis Seedlings treated with Salicylic Acid
E-GEOD-18978	Pseudomonas syringae	Arabidopsis thaliana mutant leaves treated with Pseudomonas syringae ES4326
E-GEOD-18979	pad4 vs. pad4 est1b	pad4 vs. pad4 est1b
E-MEXP-173	35S:mks1 and mpk4 mutant plants	Transcription profiling of Arabidopsis wild type, 35S:mks1 and mpk4 plants
E-MEXP-254	Plasmodiophora brassicae infection of Arabidopsis thaliana	Transcription profiling of Plasmodiophora brassicae infection of Arabidopsis thaliana
E-NASC-3	nematodes	Transcription profiling of Arabidopsis infected with the nematode of HHeterodera schachtii
E-NASC-5	H2O2 treatment	Transcription profiling of Arabidopsis OX1 protein kinase knock out vs wild type (ecotype WS) following H2O2 treatment
E-NASC-16	UV-B response	Transcription profiling of Arabidopsis UV-B responses in light grown plants
E-NASC-22	Fumonisin B1	Transcription profiling of Arabidopsis protoplasts are treated with 20mM Fumonisin B1 (FB1)
E-NASC-46	aphid	Transcription profiling of Arabidopsis aphid infested vs control plants
E-NASC-76	oligogalacturonide treatment	Transcription profiling of Arabidopsis seedlings treated with oligogalacturonides to assess how recognition of these compounds may contribute to defense against fungal pathogens

6

General discussion

The research described in this thesis was aimed at identifying, characterizing and functionally study Arabidopsis genes involved in disease susceptibility to the oomycete pathogen *Hyaloperonospora arabidopsidis*. The focus was on two *DOWNY MILDEW RESISTANT (DMR)* genes; *DMR1* encoding for homoserine kinase (HSK) and *DMR6* encoding for a 2-oxoglutarate Fe(II)-dependent oxygenase. The cloning of *DMR1* was facilitated by the availability of six *dmr1* alleles that all encoded mutant HSK proteins with different single amino acid substitutions. HSK catalyses the phosphorylation of homoserine to O-phospho-1-homoserine, an intermediate in the aspartate pathway, leading to the biosynthesis of methionine, threonine, and isoleucine. Unexpectedly, resistance was not caused by a depletion of these amino acids but by the accumulation of homoserine. Known immune responses were not activated in the *dmr1* mutants, and homoserine-induced resistance was shown to be independent of known defence signalling pathways, indicating that homoserine triggers a novel form of resistance to downy mildew.

The second gene that we identified, *DMR6*, encodes for an 2-oxoglutarate Fe(II)-dependent oxygenase of unknown biochemical function. Resistance of the *dmr6* mutants was correlated with enhanced expression of defence-associated genes. Genetic analysis revealed that *dmr6*-mediated resistance requires the activation of SA-dependent defence responses. Furthermore, Arabidopsis plants overexpressing *DMR6* are more susceptible to *H. arabidopsidis*. Taken together, this strongly suggests that DMR6 acts as a negative regulator of plant immunity (Figure 1). In wild-type plants (Figure 1A), the intrinsic role of *DMR6* is to prevent overactivation of defence e.g. by reducing the level of *DMR6* substrate. In *dmr6* mutant plants (Figure 1B) this would result in the accumulation of the substrate thereby positively affecting plant defence. Alternatively, the product of the DMR6 enzyme could negatively affect defence.

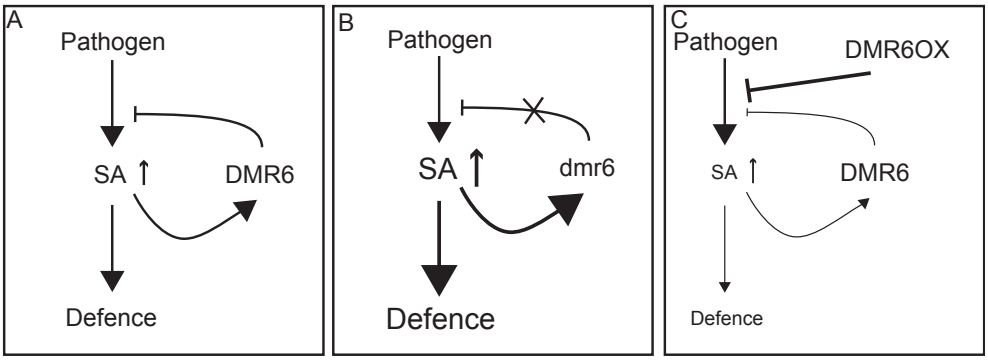

Figure 1. Model for negative regulation of defence by DMR6 in Arabidopsis wildtype (**A**), *dmr6* mutant (**B**) and *DMR6* overexpression (**C**) plants. In the wildtype, DMR6 is activated upon pathogen infection and then negatively regulates SA-dependent defence. In *dmr6* mutant plants this negative regulation is blocked leading to enhanced SA accumulation and defence activation. In *DMR6* overexpression plants (DMR6OX), the SA pathway is constantly negatively regulated leading to reduced defence and associated enhanced disease susceptibility.

In plants overexpressing *DMR6* (Figure 1C) there is a constant negative regulation of defence associated with low SA levels and reduced immune responses. Since SA accumulation is required for *dmr6*-mediated resistance we propose that the negative regulation by DMR6 occurs upstream of SA accumulation, but exactly at what level and what stage remains unclear.

The mechanism by which SA biosynthesis is induced upon pathogen infection is still largely unknown. I will discuss the current knowledge on SA biosynthesis and regulation, and focus on the molecular players that function between pathogen recognition and SA accumulation. In plants, SA is produced in the chloroplast from isochorismate that is produced by isochorismate synthase (ICS) or from benzoic acid that is produced through the phenylalanine ammonia lyase (PAL) pathway (Chen et al. 2009a). The major route of pathogen-induced SA production in Arabidopsis is via ICS1 (Wildermuth et al. 2001). It is well described that SA accumulation, endogenously produced or externally applied, enhances resistance towards several biotrophic pathogens (reviewed by Glazebrook 2005), while SA depletion, e.g. by expression of a bacterial SA hydroxylase (NahG) increases susceptibility (Gaffney et al. 1993). SA accumulation induced by pathogen attack leads to a cascade of reactions many of which require the NPR1 protein (reviewed by Vlot et al. 2009). Upon SA stimulation, NPR1 is translocated to the nucleus where it is required for activating multiple transcription factors (TFs), including TGA TFs (reviewed by Pieterse and van Loon 2004) and WRKY TFs (Wang et al. 2006), ultimately leading to the production of pathogenesis-related proteins (PR-proteins) and other antimicrobial compounds (van Loon and van Strien 1999). SA is also required for systemic acquired resistance (SAR). Another small molecule, glycerol-3-phosphate (G3P), was recently found to be required for SAR as well. The metabolite G3P is synthesised by the *GLY1*-encoded glycerol-3-phosphate dehydrogenase through reduction of dihydroxyacetone phosphate and by the *GLI1*-encoded glycerokinase from glycerol. Both the *gly1* and *gli1* mutant have reduced G3P levels and are compromised in SAR. Exogenous application of G3P to the *gly1 and gli1* mutants fully restored the SAR phenotype (Chanda et al. 2011).

Various mechanisms regulate the amount of active SA in the plant, as to prevent uncontrolled accumulation of SA that is detrimental for growth and development. An important inactivation method is transfer of a glucose moiety to SA forming SA-glucoside, which is sequestered in the vacuole (Dean et al. 2005). These coupling reactions are reversible so that SA can be released again from the vacuolar pool when required. Another mechanism to mobilize SA is methylation by SA methyl transferase to form methyl-SA (Park et al. 2007). The majority of the produced methyl-SA is emitted into the atmosphere where it can function in plant-to-plant communication (Attaran et al. 2009). Other SA breakdown mechanisms likely exist but are, so far, unknown. However, it is obvious that the level and

activity of the ICS1 enzyme, that produces the precursor of SA, is a major step determining SA levels in Arabidopsis. *ICS1* transcript levels correlate with SA production suggesting that transcriptional activation of the *ICS1* gene is a major regulatory step (Wildermuth et al. 2001).

Processes upstream of SA signalling

Plants can recognize potential pathogens through pathogen-associated molecular patterns (PAMPs) resulting in PAMP-triggered immunity (PTI). PTI is the first active plant response to invaders and should be under tight control to avoid fitness costs. Early PTI responses include the accumulation of ethylene, ROS production, deposition of callose, and downstream activation of defence genes. These downstream defences are amplified through a MAPK cascade, of which the last step is the activation of MPKs (reviewed by Dodds and Rathjen 2010). WRKY transcription factors (TFs), involved in defence gene activation, are then phosphorylated and activated by MPK3 and MPK6 (Asai et al. 2002). In a similar way, the phosphorylation of 1-aminocyclopropane-1-carboxylic acid synthases (ACS2/6) by MPK6 activates ethylene biosynthesis (Liu and Zhang 2004). MPK4 is activated after treatment with the flagellin-derived PAMP flg22, and negatively regulates SA accumulation (Brodersen et al. 2006). Recently, it was shown that the TFs Ethylene insensitive3 (EIN3) and Ethylene insensitive3-like1 (EIL1) negatively regulate PTI. By mutating *EIN3* and *EIL1*, plants become more susceptible to *P. syringae* while plants overaccumulating EIN3 show enhanced susceptibility to the same pathogen. Specifically, EIN3 and EIL1 bind to the *ICS1* promoter, thereby negatively regulating the biosynthesis of pathogen-induced SA (Chen et al. 2009b). Another recent study identified the WKRY28 TF as being rapidly induced after pathogen infection, directly followed by the binding of the protein to the *ICS1* promoter leading to *ICS1* activation and SA production (van Verk et al. 2011). Despite these examples, there are still major gaps in our knowledge on the activation and regulation of SA biosynthesis.

The question remains where DMR6 acts as a negative regulator upstream of SA. One approach, to further pinpoint where in the signal transduction pathway DMR6 plays a role, is by using mutants of genes that are negatively regulating early defence signalling (e.g. the *mpk4* mutant). These gain of resistance mutants can be transformed with the *DMR6* overexpression construct and analysed for disease susceptibility. Another approach is to make use of mutants of genes that positively regulate SA accumulation and cross these with the resistant *dmr6* mutant. The obtained double mutants can then be compared to the parental lines in disease tests to study epistatic effects and position the genes in the signalling network.

Resistance through accumulation of metabolites

A striking feature of the two *DOWNY MILDEW RESISTANT* genes studied in this thesis is that they both encode metabolic enzymes. In addition, the accumulation of the enzyme substrate is likely responsible for the induction of disease resistance. In the case of the *dmr1* mutant homoserine accumulates that can also induce resistance when applied exogenously. The substrate that accumulates in the *dmr6* mutant is still unknown. Nevertheless, it is clear that the accumulation of metabolites can lead to disease resistance. Below, I will discuss several examples of metabolic genes that when mutated activate resistance to pathogens (as a result of accumulation of a metabolite). These metabolic genes are interesting targets for disease resistance breeding. Classic and novel methods for disrupting such genes in plants are discussed.

The accumulation of homoserine in *dmr1* mutant plants was shown, in chapter 2, to induce a novel form of resistance. Besides HSK, mutation of three other genes encoding enzymes of the Aspartate pathway, affect defence. In an EMS screen, in the *rar1* mutant background, for Arabidopsis mutants conferring resistance to *H. arabidopsidis*, 2 non-allelic *rar1 suppressors* (*rsp1* and *rsp2*) were identified (Stuttmann et al. 2011). *rsp1* is mutated in *ASPARTATE KINASE2* and is impaired in lysine dependent inhibition while *RSP2* encodes for *DIHYDRODIPICOLINATE SYNTHASE2.* The RSP2 enzyme catalyses the first committed step for the production of lysine. Mutation of these genes results in constitutive high levels of methionine, threonine, and isoleucine. Spray application of these amino acids on Arabidopsis seedlings revealed that threonine accumulation is likely responsible for the observed resistance of the *rsp* mutants to *H. arabidopsidis*. In contrast, we did not find any effect of threonine application on *H. arabidopsidis* infection (chapter 2). This could be explained by the fact that Stuttmann et al. (2011) first applied threonine, by spraying, and subsequently *H. arabidopsidis* while we first inoculated *H. arabidopsidis* and then infiltrated threonine. It is quite possible that threonine, as used by Stuttmann et al. (2011), has a direct negative effect on *H. arabidopsidis* spore germination or penetration of the host disabling successful infection of Arabidopsis. We have shown that homoserine does not inhibit *H. arabidopsidis* spore germination nor *in vitro* mycelium growth of the related oomycete *Phytophthora capsici*. The third aspartate pathway-related gene is *ABERRANT GROWTH AND DEATH2* (*AGD2*), which is involved in lysine biosynthesis (Hudson et al. 2006). The *agd2* mutant displays resistance to *H. arabidopsidis* together with dwarfism and altered leaf morphology (Song et al. 2004). However, the *agd2* mutant has high SA levels and therefore differs from the *dmr1*, *rsp1*, and *rsp2* mutants that do not have constitutive activation of SA-dependent defence.

Also the amino acid glutamine (Gln) has been shown to affect disease resistance. Gln is the main substrate of the amino acid transporter LYSINE HISTIDINE TRANSPORTER1 (LHT1) (Liu et al. 2010). The *lht1* mutant displayed broad-spectrum resistance, amongst others to *Pseudomonas syringae* and *Colletotrichum higginsianum,* which coincided with accelerated callose depositions and programmed cell death (PCD). In *lht1* mutant plants Gln was no longer translocated into the chloroplast. Lack of chloroplastic Gln is thought to imbalance the cellular redox state leading to uncontrolled ROS (reactive oxygen species) and SA production, suggesting Gln homeostasis is vital for survival of plant cells (Liu et al. 2010). Besides glutamine, also asparagine (Asn), another nitrogen rich amino acid, was shown to affect disease resistance. When the pepper asparagine synthase gene (*CaAS1*) was silenced, plants became more susceptible to *Xanthomonas campestris* infection that was correlated with the absence of nitric oxide (NO) accumulation and strong reduction of ROS production (Hwang et al. 2011). Overexpression of *CaAS1* in transgenic Arabidopsis plants increased resistance to *P. syringae* and *H. arabidopsidis*. While amino acid levels in the *CaAS1* silenced plants remained unaltered in unchallenged plants, in infected plants, the level of Asn did not increase whereas it accumulated in wildtype plants. This suggests that, during infection, *CaAS1* silenced plants are impaired in the conversion of Asp to Asn. Apparently, CaAS1 is specifically required for Asn biosynthesis during infection and defence (Hwang et al 2011).

A link to the amino acid proline was described in a study on Arabidopsis, in which the expression of *Proline Dehydrogenase* (*ProDH*) was found to be upregulated specifically in the cells undergoing the hypersensitive response after avirulent pathogen attack. This response was shown to depend on SID2 and NPR1 during early time points (6 hours), but independent at later time points (24 hours). Plants silenced for *ProDH* showed reduced HR and enhanced the susceptibility to avirulent pathogens. This suggests that the conversion of proline to pyrroline-5-carboxylate by ProDH contributes to HR and disease resistance (Cecchini et al. 2011).

Exogenous application of certain amino acids can induce disease resistance, as described above and in chapter 2. Also the non-protein amino acid, ß-aminobutyric acid (BABA), is known to activate resistance towards a range of different biotic and abiotic stresses when applied to the roots of Arabidopsis plants. These stresses include *P. syringae* pv. *tomato* DC3000 infection (Zimmerli et al. 2000), salt stress, drought stress (Jakab et al. 2005) and even heat shock stress (Zimmerli et al. 2008). BABA is not toxic to plants and it is not metabolized *in planta*, ruling out that derivatives of BABA induce resistance. Low BABA doses do not directly activate defence gene expression but condition the plant for augmented activation of defence responses, a phenomenon called priming (Zimmerli et al. 2000). Primed plants are able to respond faster and stronger upon pathogen attack, without wasting valuable energy under pathogen-free conditions (van Hulten et al. 2006). Multiple mechanisms

are primed in plants treated with BABA, e.g. SA-dependent defences are augmented after challenge with *P. syringae* or *B. cinerea* (Zimmerli et al. 2000, 2001). Also, BABA-treated plants are primed for faster and stronger callose depositions at infection sites, a response that is particularly effective towards *P. cucumerina* and *A. brassicicola* (Ton and Mauch-Mani 2004). A mutagenesis screen was performed to identify mutants impaired in BABA responses. Three T-DNA insertion mutants were identified for impaired BABA-induced sterility (*ibs1*, *ibs2*, and *ibs3*). The *ibs1* mutant is impaired in BABA-induced resistance to *H. arabidopsidis* and *P. syringae* that coincided with reduced priming of SA-dependent responses, whereas the *ibs2* and *ibs3* mutants display reduced priming for callose depositions. *IBS1* encodes a cyclin-dependent kinase-like protein, *IBS2* a polyphosphoinositide phosphatase, and IBS3 a zeaxanthin epoxidase, suggesting priming requires specific but different cellular signalling components (Ton et al. 2005).

Application of *DMR* technology in crop species

The knowledge obtained on the *DMR* genes in Arabidopsis offers possibilities for application in crop plants. Downy mildews and other oomycetes are important pathogens on many crop species leading to major yield losses. Current methods to control infection often include the use of fungicides with detrimental effects on the environment. Especially since we are facing a growing human population and rapid global environmental changes, the challenge will be to sustain and even increase crop yields. To achieve this there is an important role for effective and durable forms of disease resistance in crops (Tester and Langridge 2010). Classical methods for improving disease resistance make use of resistance genes identified in the species' gene pool or from related wild species. By traditional breeding methods the identified resistance genes are introgressed into elite cultivars, a process that can take up to 10-15 years. A major drawback of this method is the limited durability of most resistance gene (Rommens and Kishore 2000). In many cases, pathogens rapidly evolve to overcome new resistance genes, thereby making them ineffective. It often only takes mutation or deletion of one gene in the pathogen, encoding an effector protein, to be no longer recognized by the corresponding resistance protein. On the other hand, recessively inherited resistance could well prove to be more durable in the field against pathogens, as this type of resistance is not based on effector recognition in most cases. Resistance of the Arabidopsis mutants described in this thesis, *dmr1* and *dmr6*, also inherits in a recessive way. To generate and identify loss-of-function alleles in plant genomes there are several methods possible.

I will discuss methods based on random mutagenesis followed by selection, but also more recent technologies for targeted modification of genomes. Random mutagenesis of plants is applied in breeding since many decades. Mutations can be induced by chemical or physical damage of the plants' DNA, as well as through insertional methods and RNA silencing (reviewed by Gilchrist and Haughn (2010). Physical mutagens include ionizing

radiation which makes DNA breaks that often result in a high frequency of deletions. The most common method of chemical mutagenesis of plants is done by treating seeds with EMS, which leads to GC-AT basepair substitutions randomly in the genome (reviewed by Henikoff and Comai 2003). Insertional mutagenesis has been exploited to disrupt target genes either by transformation or transposon activity. For several plant species, populations have been created containing insertional knockouts which are easily identified using PCR in any gene of interest (e.g in rice; Jeon et al. 2000). A drawback of insertional mutagenesis is the unavailability of insertions in all genes. Indeed, even in the model organism Arabidopsis, in which insertional mutagenesis has been performed on large scale, not all genes contain inserts, e.g. in *HSK* (chapter 3). Also, insertional mutagenesis requires plant transformation that, for many plant species, includes the need for tissue culture, which is a source of newly introduced mutations. RNA-based silencing (also known as RNA-mediated interference; RNAi) makes use of the plants' RNA-silencing pathway that is triggered by dsRNA corresponding to the gene of interest. Several methods exist to activate RNAi, but the most popular currently is microRNA-based. Gene-specific constructs are generated to produce precursor miRNAs that are then processed to give miRNAs that target the complementary mRNAs that in turn are degraded through the RNA-silencing pathway (Ossowski et al. 2008). It is possible to silence multiple genes from the same family with a single microRNA construct (Alvarez et al. 2006). Disadvantages include that silencing efficiency is variable making it difficult to obtain genes that are completely silenced, and that for every gene of interest a new construct needs to be generated and transformed into the plant (Small 2007). Major drawbacks of insertional and silencing mutagenesis are that, 1) it is not developed for all plant species, 2) it requires plant transformation which is not developed for several economically important plant species, and 3) it involves the creation of genetically modified organisms through transgenic technologies which is highly restricted due to regulations, in particular in the EU.

On the other hand, chemical mutagenesis is valuable as reverse genetics technique when coupled to screening methods for the discovery of mutations that allow screens for point mutations in any gene of interest. These methods are of great interest to industry, as it does not involve any transgenic technology. There are several strengths regarding EMS mutagenesis, 1) it does not require gene transformation techniques, 2) EMS provides allelic series of mutations, even for small genes depending on the density of the mutations, 3) only target gene sequences have to be known, which is particularly convenient if genomic sequence data is limited, 4) it is broadly applicable to all crop species and 5) it is possible to obtain different sorts of alleles (loss-of-function, gain-of–function, loss-of-inhibition etc). In the last decade, Targeting Induced Local Lesions In Genomes (TILLING) has been introduced combining the advantage of EMS-mutagenesis with the availability of enzyme-mediated detection based on the CEL I nuclease (Till et al. 2003). Chapter 3 describes TILLING of Arabidopsis HSK that resulted in the identification of 37 missense mutations. One HSK

mutation resulted in *H. arabidopsidis* resistance that was accompanied with high homoserine levels. For several crop species, TILLING populations have been generated including rice (Till et al. 2007), maize (Till et al. 2004), soybean (Cooper et al. 2008), tomato (Minoia et al. 2010), and melon (Dahmani-Mardas et al. 2010). Although many advantages, the drawback of using TILLING is often the poor efficiency of the endonuclease. A new development is the use of next-generation sequencing for the identification of EMS-induced mutants or polymorphisms. Using the Keypoint™ technology, high throughput parallel sequencing of mutated and non-mutated populations easily identifies mutations in a potential important gene, e.g. as has been shown for tomato *eIF4E* (Rigola et al. 2009).

Recently, new technologies have been developed that no longer require random genome mutagenesis. I will focus on two developed methods for genome engineering that both make use of the FokI nuclease. The first method is Zinc-finger nucleases (ZFN) that contain an endonuclease, FokI, bound to a zinc-finger protein engineered to bind a specific target of interest in any genome of interest. The ZFNs are able to bind the target DNA where it induces a double-strand break, via the FokI nuclease. The break is in turn is repaired by the plants intrinsic repair mechanism leading to specific sequence modifications at any desired target (reviewed by Carroll 2011). These modifications could be mutations but also gene replacements have been reported. This method has been developed almost a decade ago in the model organism Drosophila (Bibikova et al. 2002), but has more recently been applied to many other model species like Arabidopsis (Lloyd et al. 2005), *C. elegans* (Morton et al. 2006), and human cell cultures (reviewed by Urnov et al. 2010). This opened up the possibility for the use of ZFN in crop species to modify any gene of interest. Indeed, this has received much attention lately with ZFN-induced genome modifications in maize (Shukla et al. 2009) and tobacco (Townsend et al. 2009). In maize, the *IPK1* gene was targeted that encodes an enzyme involved in phytate biosynthesis (Shukla et al. 2009), a process that is of agricultural importance for its polluting effects to the environment. The authors show that *IPK1* was effectively targeted and reduced the phytate content of maize both by mutagenesis (nonhomologous end joining) and by gene replacement (homologous recombination) methods. Furthermore, these genetic alterations were successfully transmitted to next generations.

The second genome editing method is based on the Transcription activator-like (TAL) effectors that were first identified in *Xanthomonas* that injects these effectors into the plant cell after which they are imported in the nucleus. There these TAL effectors target specific gene promoters to regulate the expression of downstream genes (reviewed by Boch and Bonas 2010). They achieve this through their characteristic tandem repeats that determine specificity by the number of repeats and the amino acid sequence in those repeats (Boch et al. 2009). Especially two adjacent amino acids at position 12 and 13 are very variable (these are called the repeat-variable diresidues; RVDs) and are responsible for DNA recognition

where 1 specific RVD corresponds to 1 specific basepair (Moscou and Bogdanove 2009). Repeats with different RVDs are used for creating the specificity required for targeting a given DNA sequence. When the TAL effectors were fused to the FokI nuclease to generate TALENs, it became possible to use them in a similar way as the ZFNs and target any sequence of interest (Christian et al. 2010). Cermak et al. (2011) used the *ADH1* gene from Arabidopsis for the assembly of specific TALENs. They identified 6 independent mutations containing small (4-15 bp long) deletions. The available tools to find new mutations by high-throughput sequencing methods or to create them by genome engineering, is opening up a whole new ballgame in the plant breeding field. Time will tell if *DMR*-technology will work in crop species and if it will deliver a more durable form of disease resistance in the field.

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Summary

Plants are constantly exposed to micro-organisms including pathogens. They are resistant to the vast majority of potential pathogens due to a multilayered defence system. Susceptibility to plant diseases, on the other hand, is determined by virulence factors from the pathogen, their targets in the host and the successful suppression of plant defences. To gain insight into the molecular mechanisms underlying disease susceptibility, the interaction between the oomycete pathogen *Hyaloperonospora arabidopsidis*, causing downy mildew, and its host plant Arabidopsis was studied. A forward genetic screen resulted in the identification of several *downy mildew resistant* (*dmr*) Arabidopsis mutants of which *dmr1* and *dmr6* were further characterized. Chapter 2 describes the cloning of *DMR1* that encodes for homoserine kinase (HSK), a key enzyme in the Aspartate pathway. Mutation of *HSK* in the *dmr1* mutants resulted in accumulation of the amino acid homoserine. Exogenous application of homoserine proved to be sufficient to induce resistance against *H. arabidopsidis*. No known defence-related pathways were associated with the observed resistance, indicating homoserine accumulation triggers a novel form of plant resistance. Chapter 3 describes a reverse genetics approach (TILLING) that resulted in additional mutant *hsk* alleles. One mutant was found to accumulate homoserine and to be resistant to downy mildew infection. Another mutant did not develop beyond the seedling stage suggesting *HSK* is an essential plant gene. We also identified *HSK* orthologs in a number of crop species making them candidates for mutagenesis to further explore *dmr1*-mediated resistance. Chapter 4 describes the functional characterization of the *DMR6* gene, coding for a 2-oxoglutarate Fe(II)-dependent oxygenase. Absence of a functional *DMR6* gene resulted in resistance that requires a functional salicylic acid pathway. On the other hand, overexpression of *DMR6* resulted in enhanced susceptibility towards biotrophic pathogens indicating *DMR6* acts as negative regulator of defence. A 3-dimensional model of the DMR6 protein was constructed that facilitated the identification of important residues in the substrate pocket of the enzyme. Chapter 5 describes the family of DMR6-like oxygenases (DLOs) and their role in plant defence. Two Arabidopsis *DLO*s, identified through phylogenetic analysis, have a similar molecular function as *DMR6*. Also, *DMR6* orthologs were identified in several crop species that were able to complement *dmr6*-mediated resistance. A DMR6/DLO-specific motif was identified that can be used for the identification of crop DMR6 and DLO proteins. The presence of this conserved motif could determine the specificity for a particular substrate that is shared between different species.

Samenvatting

Planten staan constant bloot aan micro-organismen waaronder ziekteverwekkers. Het merendeel van deze potentiële ziekteverwekkende micro-organismen is niet in staat planten te koloniseren en ziekte te veroorzaken. Vatbaarheid van planten, daarentegen, wordt bepaald door factoren van het micro-organisme die succesvol de afweerreactie van de plant kunnen onderdrukken. Inzicht krijgen in de manier waarop planten bijdragen aan kolonisatie door ziekteverwekkers blijft een onderbelicht aspect van de plantenziektekunde. Het doel van dit onderzoek was om inzicht te krijgen welke genen van de zandraket (*Arabidopsis thaliana*) invloed hebben op de infectie door valse meeldauw (*Hyaloperonospora arabidopsidis*). Oftewel, welke genen van de zandraket zijn noodzakelijk voor vatbaarheid voor *H. arabidopsidis*. Genetische analyse resulteerde in de identificatie van een aantal *downy mildew resistant* (*dmr*) mutanten waarvan *dmr1* en *dmr6* verder werden onderzocht. Hoofdstuk 2 beschrijft de identificatie van het *DOWNY MILDEW RESISTANT 1* (*DMR1*) gen dat codeert voor homoserine kinase (HSK). Homoserine kinase is een enzym dat de reactie van homoserine naar fosfo-homoserine katalyseert in de aspartaat route. Dit enzym is in de *dmr1* mutant verminderd actief, wat leidt tot accumulatie van het aminozuur homoserine, dat afwezig is in wildtype planten. Homoserine infiltratie in een vatbare Arabidopsis lijn resulteerde in resistentie. Dit betekent dat de accumulatie van homoserine verantwoordelijk is voor de resistentie tegen *H. arabidopsidis*. Ook opereert *dmr1* resistentie onafhankelijk van de huidige, bekende afweermechanismen. Dit bewijst dat door modulatie van de aminozuursynthese van de plant, valse meeldauw resistentie geïnduceerd kan worden. Om meer inzicht te krijgen in het *DMR1/HSK* gen, is een mutagenese uitgevoerd om zoveel mogelijk verschillende mutaties in het *HSK* gen te bemachtigen. Dit wordt beschreven in hoofdstuk 3. Deze verschillende mutaties kunnen meer inzicht geven over het feit of *HSK* een essentieel gen is. Van de 37 additionele mutaties vertoont 1 mutant homoserine accumulatie alsmede resistentie tegen valse meeldauw. Een andere mutant ontwikkelt zich niet verder na het zaailing stadium wat zou kunnen betekenen dat *HSK* een essentieel gen is. *HSK* genen van enkele belangrijke gewassen zijn geïdentificeerd en zijn belangrijke kandidaten voor mutagenese om te onderzoeken of homoserine-geïnduceerde resistentie ook in gewassen toepasbaar is. Hoofdstuk 4 beschrijft de karakterisatie van het *DMR6* gen dat codeert voor een oxidoreductase enzym. In de afwezigheid van een functioneel *DMR6* gen, in de *dmr6* mutant, worden met name afweer-gerelateerde genen geactiveerd. Resistentie is afhankelijk van het plantenhormoon salicylzuur dat de activatie van bepaalde afweer-gerelateerde genen regelt. Aan de andere kant, wanneer *DMR6* tot overexpressie wordt gebracht, worden planten juist vatbaarder voor *H. arabidopsidis* infectie. Dit impliceert dat DMR6 als negatieve regulator betrokken is bij de afweer. Een geconstrueerd 3-dimensionale model van DMR6 heeft het mogelijk gemaakt de positie van belangrijke

aminozuren te vinden die betrokken zijn bij de substraatbinding. Hoofdstuk 5 beschrijft de familie van DMR6-achtige oxidoreductases en de rol die deze familieleden mogelijk spelen in de afweer. Via een uitgebreide fylogenetische analyse is het DMR6 eiwit gegroepeerd met DMR6-achtigen van verschillende plantensoorten. Twee Arabidopsis familieleden zijn nader onderzocht en_ dezelfde moleculaire functie te bezitten. Sequentie analyse naar aanleiding van een 3-D model van DMR6 in de groep van DMR6-achtigen resulteert in de identificatie van een specifiek aminozuurmotief dat ook gebruikt kan worden om DMR6 of DMR6-achtigen te identificeren in gewassen.

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Op naar nieuwe uitdagingen!

Curriculum vitae

Tieme Zeilmaker werd geboren op 7 augustus 1980 te Rheden. Na het behalen van zijn diploma op de middelbare school is hij begonnen met de HBO studie Plantenbiotechnologie aan Hogeschool Larenstein te Velp. Tijdens deze studie werd een onderzoeksstage vervuld bij Plant Research International, waar onder begeleiding van Ing. Ronny Joosen en Dr. Kim Boutilier werd gewerkt aan "Optimization of Real Time PCR as verification for *Brassica napus* microarray expression profiles". Na afronding van deze studie in 2003 werd aangevangen met de Master Plantbiotechnology aan de Universiteit van Wageningen. Tijdens deze studie werden twee onderzoeksstages verricht: allereerst bij de leerstoelgroep Fytopathologie in Wageningen waar onder begeleiding van Dr. Renier van der Hoorn werd gewerkt aan "Activity Profiling of Serine Proteases in Plants". De volgende stage vond plaats in het buitenland aan de University of British Colombia te Vancouver in Canada waar onder begeleiding van Prof. Jim Kronstad gewerkt werd aan "Identifying genes involved in virulence in the corn smut pathogen *Ustilago maydis*". Tevens is daar gewerkt aan de "Genomic characterization of *Cryptococcus gatti* strain WM276". In augustus 2006 studeerde hij af, om vervolgens in september van datzelfde jaar te beginnen als promovendus bij de leerstoelgroep Moleculaire Genetica dat per 1 januari 2007 gefuseerd verder ging onder de naam Plant-Microbe Interactions aan de Universiteit Utrecht. Daar werd onder begeleiding van Dr. Guido van den Ackerveken en Prof. Corné Pieterse het onderzoek uitgevoerd dat in dit proefschrift is beschreven.