the plant journal

SEB Society for Experimental Biology

The Plant Journal (2015) **81,** 210–222 doi: 10.1111/tpj.12719

DOWNY MILDEW RESISTANT 6 and DMR6-LIKE OXYGENASE 1 are partially redundant but distinct suppressors of immunity in Arabidopsis

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Received 25 July 2014; revised 4 October 2014; accepted 27 October 2014; published online 7 November 2014.
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SUMMARY

Arabidopsis *downy mildew resistant 6 (dmr6)* mutants have lost their susceptibility to the downy mildew *Hyaloperonospora arabidopsidis*. Here we show that *dmr6* is also resistant to the bacterium *Pseudomonas syringae* and the oomycete *Phytophthora capsici*. Resistance is accompanied by enhanced defense gene expression and elevated salicylic acid levels. The suppressive effect of the DMR6 oxygenase was confirmed in transgenic Arabidopsis lines overexpressing *DMR6* that show enhanced susceptibility to *H. arabidopsidis*, *P. capsici*, and *P. syringae*. Phylogenetic analysis of the superfamily of 2-oxoglutarate Fe(II)-dependent oxygenases revealed a subgroup of DMR6-LIKE OXYGENASEs (DLOs). Within Arabidopsis, DMR6 is most closely related to DLO1 and DLO2. Overexpression of *DLO1* and *DLO2* in the *dmr6* mutant restored the susceptibility to downy mildew indicating that DLOs negatively affect defense, similar to DMR6. *DLO1*, but not *DLO2*, is co-expressed with *DMR6*, showing strong activation during pathogen attack and following salicylic acid treatment. *DMR6* and *DLO1* differ in their spatial expression pattern in downy mildew-infected Arabidopsis leaves; *DMR6* is mostly expressed in cells that are in contact with hyphae and haustoria of *H. arabidopsidis*, while *DLO1* is expressed mainly in the vascular tissues near infection sites. Strikingly, the *dmr6-3_dlo1* double mutant, that is completely resistant to *H. arabidopsidis*, showed a strong growth reduction that was associated with high levels of salicylic acid. We conclude that DMR6 and DLO1 redundantly suppress plant immunity, but also have distinct activities based on their differential localization of expression.

Keywords: downy mildew resistant 6, *Arabidopsis thaliana*, negative regulator, immunity, DMR6-like oxygenase, suppressor.

INTRODUCTION

Plant immunity is activated upon detection of invading microbes by the neighbouring host cells. A first line of defense is triggered by membrane-bound pattern recognition receptors that sense microbe-associated molecular patterns (MAMPs) in the extracellular environment (He *et al.*, 2007). During attempted penetration of plants by microbes, MAMPs become exposed and recognized by the plant leading to the activation of MAMP-triggered immunity (MTI). Adapted pathogenic microbes are able to suppress MTI by deploying effector molecules that interfere with plant immunity at the level of detection, signaling or production of defensive compounds (Dou and Zhou, 2012). Plants have evolved a second class of immune receptors, the nucleotide-binding and leucine-rich repeat (NLR) proteins, that detect pathogen effectors directly, or through their modifying effect on host targets. The resulting effector-triggered immunity (ETI) involves the activation of plant defense responses, involving major reprogramming of the host transcriptome and metabolome (Truman *et al.*, 2006; Ward *et al.*, 2010), and is often associated with programmed cell death (PCD). It is evident that PCD is a strictly controlled process, as deregulated cell death

responses are detrimental to the host. Also other plant immune responses need to be under strict control as they are energy-demanding and negatively affect plant growth and development (Bolton, 2009). In addition, strong immune responses pose a fitness cost for the plant (reviewed by Heil and Baldwin, 2002). This is obvious in many Arabidopsis mutants with constitutively activated immunity, e.g. the *defense, no death 1* (*dnd1*) mutant (Clough *et al.*, 2000) and several *constitutive expressor of PR-genes* (*cpr*) mutants (Clarke *et al.*, 2000), that show strongly impaired growth and dwarfed phenotypes.

Negative regulators of immunity in Arabidopsis belong to very diverse functional classes, e.g. ubiquitin ligases and MAP kinase cascades. Several plant U-box proteins (PUBs) with E3 ubiquitin ligase activity are known to regulate the levels of immune receptors and signaling proteins, thereby acting as negative regulators of plant defense. PUB12 and PUB13 attenuate the activated flagellin receptor FLS2, so that immune signaling is controlled (Lu *et al.*, 2011). The *pub13* mutant also shows increased cell death, resistance to biotrophic pathogens, and enhanced levels of the defense hormone salicylic acid (SA) (Li *et al.*, 2012). The PUB22/23/24 proteins negatively affect MTI (Trujillo *et al.*, 2008); it was recently shown that PUB22 interferes with exocytosis by mediating the degradation of the Exo70B2 protein (Stegmann *et al.*, 2012). The MAP kinase MPK4 is known to suppress immunity as the *mpk4* mutant shows constitutive SA-dependent defense (Petersen *et al.*, 2000). Expression of a constitutively active form of MPK4 in Arabidopsis resulted in reduced defense levels and enhanced susceptibility to pathogen infection, confirming a role as negative regulator of immunity (Berriri *et al.*, 2012). Recently it was shown that the MAP kinase kinase kinase MEKK2 is negatively regulated by the MEKK1–MKK1/MKK2–MPK4 kinase cascade, reducing activated levels of MEKK2 that would activate immunity (Kong *et al.*, 2012).

Several genes encoding negative regulators of immunity are activated during pathogen infection so that the inducible defense response is controlled and down-regulated to prevent over-activation. Examples are the Nudix hydrolase-encoding *NUDT7* (Ge *et al.*, 2007), and transcription factor-encoding *WRKY48* (Xing *et al.*, 2008) that are induced upon infection or MAMP treatment. Similarly, the *DOWNY MILDEW RESISTANT 6* gene (*DMR6*, Van Damme *et al.*, 2008) is activated during infection with compatible and incompatible isolates of the downy mildew *Hyaloperonospora arabidopsidis.* Inactivation of *DMR6* by mutation leads to a low constitutive activation of defense-related genes and resistance to the downy mildew *H. arabidopsidis* (Van Damme *et al.*, 2008).

DMR6 belongs to the superfamily of 2-oxoglutarate Fe(II) dependent oxygenases (2OG oxygenases, Pfam domain PF03171). This superfamily comprises 151 members in Arabidopsis (The Arabidopsis Information Resource, www.arabidopsis.org, March 2014). However, for most of these proteins, including DMR6, their 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$_n$H motif located on a double-stranded beta sheet (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.

In this study, we functionally analyze the Arabidopsis DMR6 oxygenase and related DMR6-LIKE OXYGENASE (DLO) 1 and 2. Overexpression of *DMR6*, *DLO1*, and *DLO2* increased disease susceptibility indicating the three proteins can act as suppressors of immunity. *DLO1* is highly co-regulated with *DMR6*, whereas *DLO2* is not expressed in leaves. During downy mildew infection, *DMR6* and *DLO1* are highly activated, but in different parts of the leaf. The *dmr6-3_dlo1* double mutant was found to be completely resistant to *H. arabidopsidis* and showed a strongly reduced growth associated with high levels of SA. Our data indicate that DMR6 and DLO1 have redundant, but also distinct, roles as suppressors of plant immunity.

RESULTS

Overexpression of *DMR6* results in enhanced susceptibility to (hemi-)biotrophic pathogens

Seedlings of the *dmr6-1* mutant were previously described to be more resistant to *H. arabidopsidis*, but not to *P. syringae* (Van Damme *et al.*, 2008). When tested on adult *dmr6-1* plants, however, strong resistance to *P. syringae* DC3000 was observed (Figure S1). Compared to seedlings, adult *dmr6-1* plants were also more resistant to the obligate biotroph *H. arabidopsidis.* In addition, strong resistance to the hemi-biotrophic oomycete *Phytophthora capsici* was evident in *dmr6-1* plants when compared to its highly susceptible parental line L*er eds1-2;* whereas all *dmr6-1* mutant plants survived *P. capsici* infection, the vast majority of plants of the parental line and of the *DMR6*-complemented *dmr6-1* mutant was destroyed by this pathogen (Figure S2). The resistance of the *dmr6-1* mutant to different (hemi-)biotrophs suggests that in wild-type plants DMR6 suppresses immunity to these pathogens.

To investigate this idea, the *DMR6* coding sequence was expressed from the constitutive 35S promoter in transgenic Col-0 lines. The *DMR6* overexpression lines showed a clear increase in disease susceptibility to *H. arabidopsidis* and

P. syringae (Figure 1). The level of *H. arabidopsidis* sporulation, which is a measure of downy mildew infection, was doubled in *DMR6*-overexpression lines compared with the control (Figure 1c). Also the development of disease-associated chlorosis was more pronounced in *DMR6*-overexpression lines (Figure 1b) than in non-transgenic Col-0 plants (Figure 1a). The increased susceptibility of 6-week-old plants to *P. syringae* bacteria was also clearly visible. While the control line (Col-0) showed a relatively low level of chlorosis and lesions at 3 days post inoculation (Figure 1d), the *DMR6*-overexpression line showed more severe disease symptoms, i.e. more chlorosis and more and larger lesions (Figure 1e). The increased susceptibility of *DMR6*-overexpressors to *P. syringae* infection was confirmed by bacterial growth assays that showed increased bacterial titers at 1 and 3 days post inoculation compared to the Col-0 control (Figure 1f). Furthermore, expression of the defense marker genes *PR-1*, *PR-2*, and *PR-5* in uninfected leaf tissue was reduced by 50–80% in the *DMR6*-overexpression line compared with wild-type Col-0 plants that already have a very low level of expression (Figure S3). The reduced immunity of the *DMR6*-overexpression line, together with the enhanced resistance of the *dmr6-1* mutant, strongly supports the role of DMR6 as a suppressor of immunity.

As DMR6 is a putative oxygenase it is expected that its catalytic activity is required to suppress immunity. To test this, we made substitutions for two of the amino acids compromising the catalytic triad that binds the iron (FeII) atom in DMR6, consisting of two histidines (H212 and

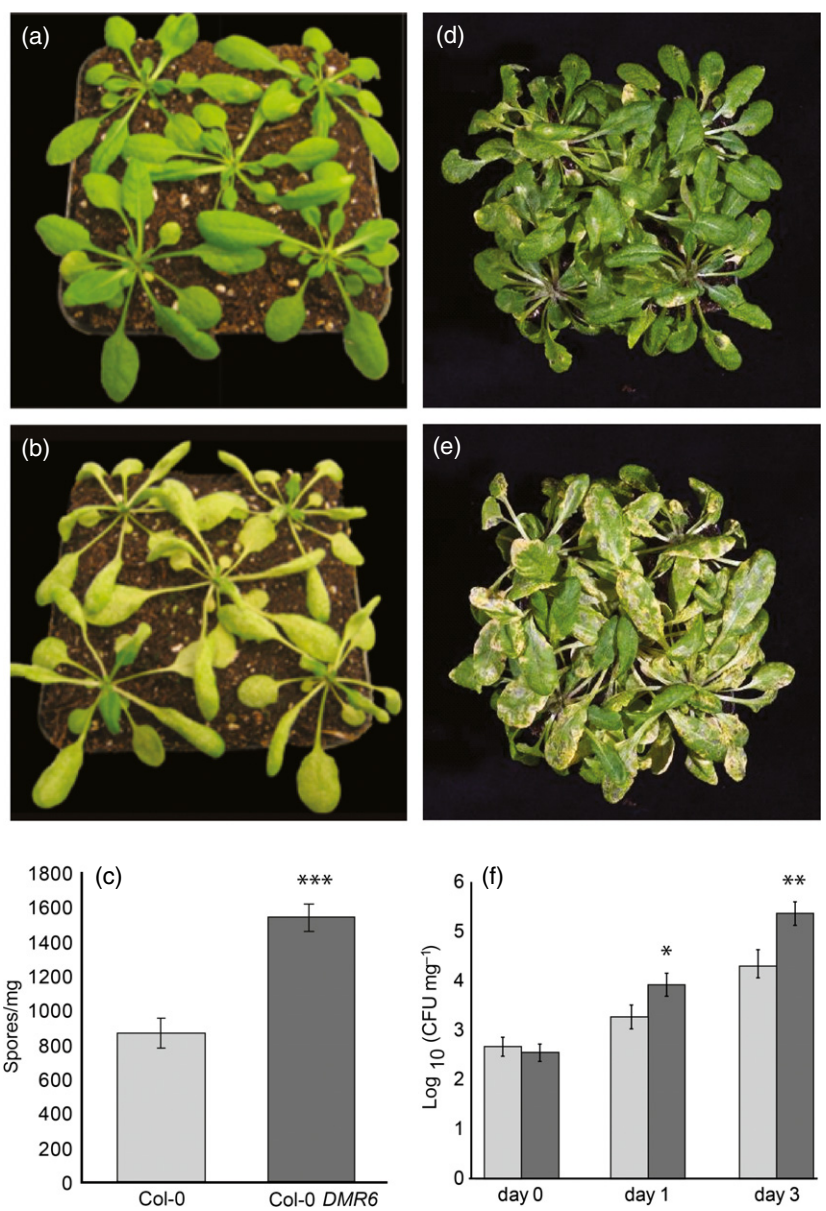


Figure 1. Overexpression of *DMR6* results in enhanced susceptibility towards *P. syringae* pv. *tomato* DC3000 *(Pst)* and *H. arabidopsidis.*
Disease symptoms of Col-0 (a, d) and Col-0 *35S:DMR6* (b, e) 7 days after *H. arabidopsidis* infection (a, b), and 3 days after *Pst* infection (d, e). Overexpression of *DMR6* results in more disease symptoms compared with the parental line for both *H. arabidopsidis* and *P. syringae* infection.
(c) Amount of *H. arabidopsidis* spores per mg fresh weight in ▫ Col-0 and ■ Col-0 *35S:DMR6.* Bars represent standard error of three replicates. Enhanced susceptibility was confirmed in multiple independent overexpression lines.
(f) *Pst* bacterial count in 6-week-old Col-0 and Col-0 *35S:DMR6* at 0, 1 and 3 days post infection (dpi). Overexpression of *DMR6* results in higher bacterial numbers. A bacterial suspension with OD 0.05 was sprayed on the plants. Bars represent standard error of four replicates. A *T*-test was used to determine the significance of difference. *, **, and *** indicate significant differences at $P < 0.05$, 0.01, and 0.001 respectively.

H269) and one aspartic acid (D214) residue. This triad was shown to be essential for the activity of the oxygenases flavonone 3-hydroxylase and anthocyanidin synthase (Lukačin and Britsch, 1997; Wilmouth *et al.*, 2002). The substituted proteins, DMR6(H212Q) and DMR(H269D), were not able to restore susceptibility to *H. arabidopsidis* (Figure S4). This suggests that the enzyme activity of the DMR6 oxygenase is required for restoring susceptibility of the *dmr6-1* mutant and therefore also for its function as a suppressor of immunity.

DMR6 and DMR6-LIKE OXYGENASEs represent separate branches of a distinct clade in flowering plants

The Arabidopsis genome contains more than 150 2OG oxygenase genes some of which are similar to *DMR6.* To analyze the evolutionary conservation of DMR6 and related oxygenases in flowering plants we phylogenetically analyzed the family of 2OG oxygenases that contain the 2OG-Fe(II) oxygenase superfamily Pfam domain PF03171. From *Arabidopsis thaliana* and eighteen other flowering plants, of which genome sequences and protein models were available in the Phytozome v7.0 database, 2912 proteins containing the PF03171 domain were selected using the HMMER3 algorithm and subsequently filtered. To remove redundancy, small protein fragments and very large proteins were excluded (for details see Experimental Procedures). This resulted in a selection of 2038 proteins that fulfil all criteria, including 106 of 151 predicted *A. thaliana* 2OG oxygenases. Phylogenetic clustering resulted in a tree (Figure 2a) 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 2a). Two separate branches can be distinguished in the DMR6 clade that each contain 2OG oxygenases from dicots and monocots indicating that these subclades were already present in the ancestor of all flowering plants or earlier (82% bootstrap confidence). Figure 2b zooms in on the DMR6 clade of the 2OG oxygenase tree. For clarity we only included the well annotated genomes of two monocots species, rice (*O. sativa*) and maize (*Z. mays*), as well as seven dicots, *A. thaliana*, papaya (*C. papaya*), *A. lyrata*, grapevine (*V. vinifera*), castor bean (*R. communis*), soybean (*G. max*), and cucumber (*C. sativus*). In the upper subclade, DMR6 closely groups with orthologues from dicots (yellow vertical bar; Figure 2b) 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 grapevine in both branches of the DMR6 clade. In the lower subclade, two *A. thaliana* DMR6 homologues cluster together (Figure 2b; indicated by the arrows) with two proteins from *Arabidopsis lyrata* suggesting they are the result of a, relatively recent, gene duplication in the common ancestor of these two species. These *A. thaliana* proteins, designated DMR6-LIKE OXYGENASE 1 (DLO1, encoded by At4g10500) and DMR6-LIKE OXYGENASE 2 (DLO2, encoded by At4g10490), show 52 and 54% identity and 70 and 72% similarity to DMR6, respectively (Figure S5). 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.

Overexpression of *DLO1* and *DLO2* restores susceptibility of the *dmr6* mutant

The DLOs could have the same biological activity as DMR6 and were tested if they could, similar to DMR6, restore susceptibility of the otherwise resistant *dmr6-1* 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 lines, transformed with *35S:DLO1* or *35S:DLO2*, were analyzed for their expression level and two lines per construct were selected that showed clear transgene expression. To check for susceptibility, 2-week-old plants were infected with *H. arabidopsidis* isolate Cala2 and at 5 days post inoculation (dpi) the number of spores per mg seedlings was scored as measure of susceptibility (Figure 3). Intriguingly, while *dmr6-1* showed clear resistance, the *35S:DLO1* and *35S:DLO2* plants were highly susceptible, similar to or higher than L*er eds1-2*, which is the parental line of the *dmr6-1* mutant. As negative controls, two oxygenase genes outside of the *DMR6/DLO* clade (At3g60290 and At1g06620) were tested and found not to restore susceptibility in the *dmr6-1* mutant background (Figure S6). The fact that DLO1 and DLO2, but not oxygenases outside of the DMR6/DLO clade, can complement the otherwise resistant *dmr6-1* mutant, suggests they have a function similar to that of DMR6.

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. Transformants expressing the *35S:DLO1* and *35S:DLO2* transgenes were selected and as controls Col-0 overexpressing *DMR6* and the highly susceptible Col *eds1-2* mutant (Bartsch *et al.*, 2006) were included. Disease assays with *H. arabidopsidis* showed that overexpression of *DLO1* and *DLO2* lead to enhanced susceptibility compared to the

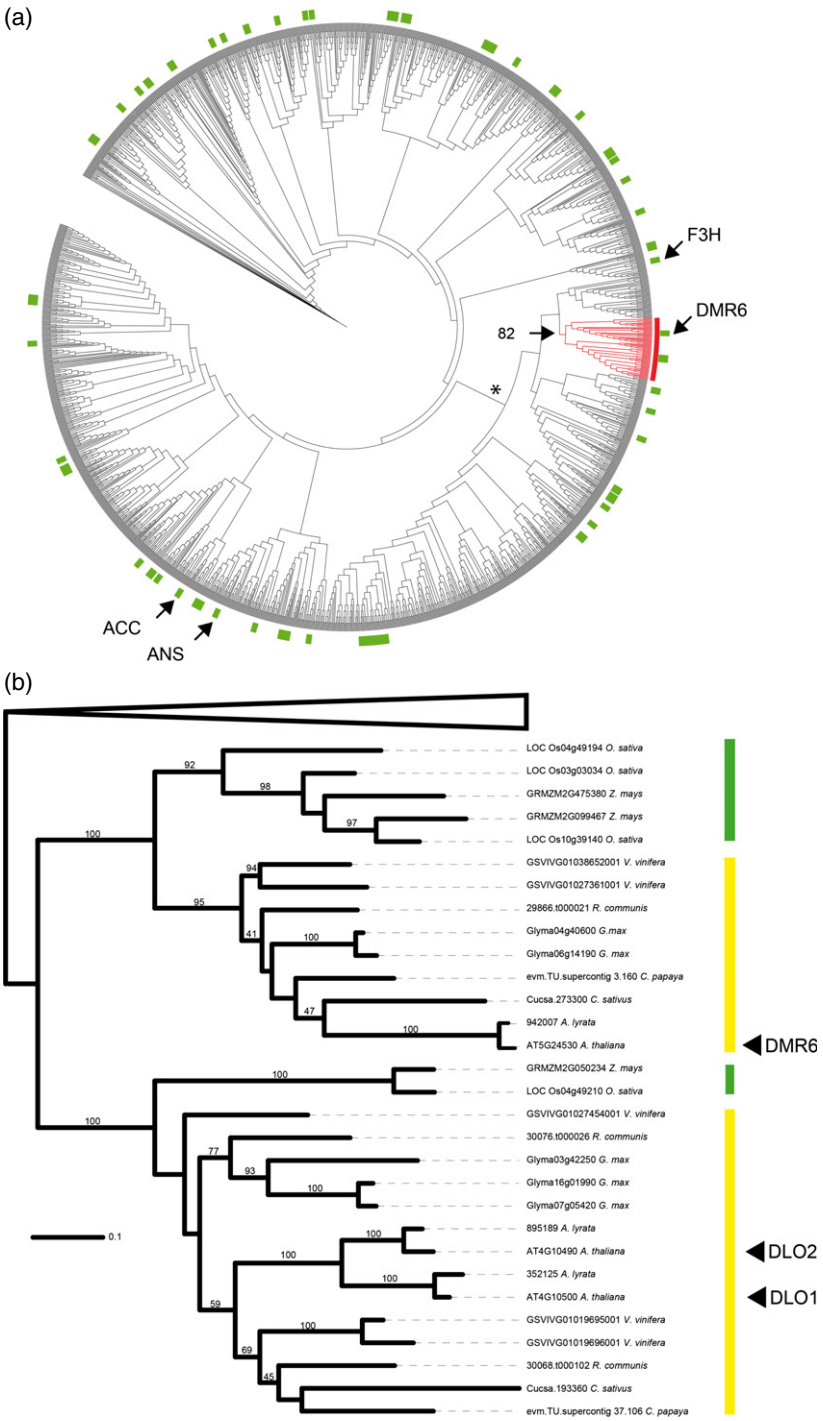


Figure 2. Phylogenetic analysis of 2OG oxygenases.
(a) Phylogeny of 2OG oxygenase proteins from *A. thaliana* and 19 flowering plants from the Phytozome database. *A. thaliana* proteins are indicated with green dots.
(b) Close up of the phylogenetic tree showing the DMR6-clade of 2OG oxygenases that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

Col-0 parental line as shown by the higher level of sporulation (Figure S7). The observed enhanced susceptibility was comparable with the *DMR6* overexpression plants and the Col *eds1-2* mutant. This confirms that the DLO1 and DLO2 proteins also act as negative regulators of defense and this suggests that they have an activity similar or identical to DMR6 resulting in the same phenotypic effects.

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 defense. Therefore, we analyzed publicly available gene expression data to determine if

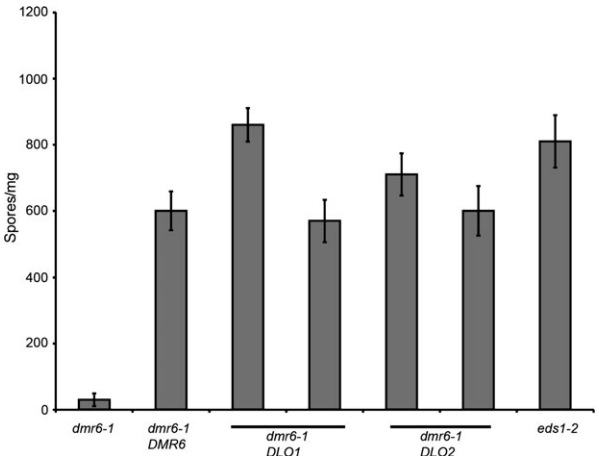
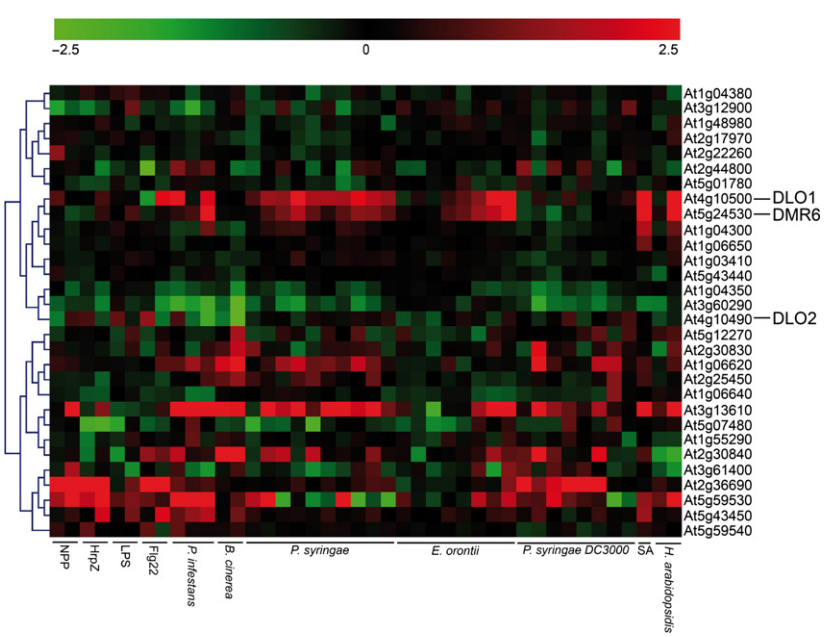


Figure 3. Overexpression of *DLO1* and *DLO2* restores susceptibility in the otherwise resistant *dmr6* mutant.
Bars represent average number of *H. arabidopsidis* spores per mg of *dmr6-1*, L*er eds1-2*, and *dmr6-1* complemented with *35S:DMR6* (1 line), *DLO1* and *DLO2* (both two independent lines). Error bars represent standard error. This experiment has been repeated twice with similar results. The expression of the transgene was confirmed by qPCR analysis.

DLO1 and *DLO2* show immunity-related expression similar to *DMR6.* For this analysis, data of nine different Affymetrix microarray experiments all dealing with transcriptional profiling after pathogen attack, defense-related hormone application and elicitor/effector treatment, were used (Table S1). 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; Figure 2a). Hierarchical clustering of these expression patterns allowed grouping of the 2OG oxygenase genes 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 both activated after infection with the downy mildew *H. arabidopsidis*, the powdery mildew *Erysiphe orontii*, and the bacterium *P. syringae* as well as SA treatment. *DLO2* clusters well away from *DMR6* and *DLO1* and appears to be unresponsive in the different experiments. Further analysis of available microarray data using Genevestigator revealed that *DLO2* is not expressed in response to any treatment or in any tissue, except for siliques, suggesting that *DLO2* does not have a role in immunity of the vegetative plant tissues.

The responsiveness of the *DLOs* to *H. arabidopsidis* infection was experimentally verified by 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.* Also following treatment with the SA mimic BTH, both *DMR6* and *DLO1* are strongly activated. In contrast, *DMR6* and *DLO1* are unresponsive to methyl jasmonate (MeJA), which is known to activate jasmonic acid-induced genes (Sasaki *et al.*, 2001). *DLO2* expression is undetectable (Ct values higher than 35) in the different experimental conditions and therefore not included in the graph, confirming the Genevestigator data. The fact that both *DMR6* and *DLO1* are activated during the plant's immune response suggests that in leaves of wild-type plants DLO1 also acts as a negative regulator of defense. However, the question remains why the *dmr6* mutants have such a clear resistance phenotype in the presence of an intact *DLO1* gene that could take over *DMR6* function?

Figure 4. Hierarchical clustering of expression profiles of a selection of 30 *A. thaliana* 2OG oxygenase genes related to *DMR6* (from Figure 2(a) – branch indicated with an asterisk) in response to biotic (*Pseudomonas syringae*, *Phytophthora infestans*, *Botrytis cinerea*, *Hyaloperonospora arabidopsidis*, *Erysiphe orontii* and elicitors) or hormone (SA) treatment.
Treatments are indicated below the clusters (more detail in Table S1). In this TREEVIEW, red indicates a higher level of gene expression relative to control or mock treatment, while green indicates downregulation.



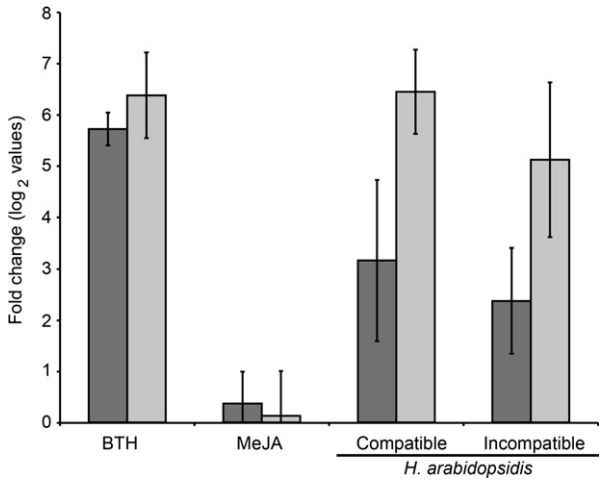


Figure 5. *DMR6* and *DLO1* are activated by BTH and *H. arabidopsidis*.
Average fold change (log_2 values) of *DMR6* and *DLO1* after BTH and MeJA treatment (1 dpi), as well as infection with a compatible (Waco9) and incompatible (Cala2) *H. arabidopsidis* isolate (3 dpi) compared with mock-treated plants. Data obtained from three independent biological replicas were combined. Error bars indicate standard error.

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 (*proDLO1:GUS*). As we did not observe any expression of *DLO2*, no GUS fusion with the promoter of *DLO2* was constructed. Following *H. arabidopsidis* infection, *DMR6* spatial expression was specifically detected to the sites that are in direct contact with the pathogen (Figure 6a,b) as has been described previously (Van Damme *et al.*, 2008). In contrast, *DLO1* expression was not induced in cells that are in close contact with the pathogen (Figure 6c,d) but only in or around the main veins of infected cotyledons and leaves. Interestingly, *DLO1* expression was observed only in areas of the leaf that are close to *H. arabidopsidis* infection sites, indicating that the activation of *DLO1* depends on the presence of the pathogen. The absence of *DLO1* activity in haustoria-containing cells could explain why the DLO1 protein, expressed from its own promoter, cannot fully complement for loss of DMR6 activity in the *dmr6* mutants. Whereas these data show distinct activities of the *DMR6* and *DLO1* genes, the extent of redundancy of these genes is unclear and was therefore further studied genetically.

DLO1 function is partially redundant with *DMR6*

Redundancy analysis in mutant lines is best performed in the same genetic background. We, therefore, obtained mutants in the Col-0 background for *DMR6* (GABI-KAT line GK-249H03.01, designated mutant *dmr6-3*) and *DLO1* (SALK line 059907, named *dlo1*). *dmr6-3_dlo1* double mutants were generated and phenotypically analyzed together with the *dmr6-3* and *dlo1* single mutants, as well as with the parental Col-0 line. The level of susceptibility to *H. arabidopsidis* Waco9 was strongly reduced in the *dmr6-3* mutant, and reduced to a lesser extent in the *dlo1* mutant (Figure 7a). Combining the two mutations in the *dmr6-3_dlo1* double mutant resulted in plants that showed complete resistance to *H. arabidopsidis*.

We next tested the level of defense gene expression in the mutants, as our previous research on the *dmr6-1* and

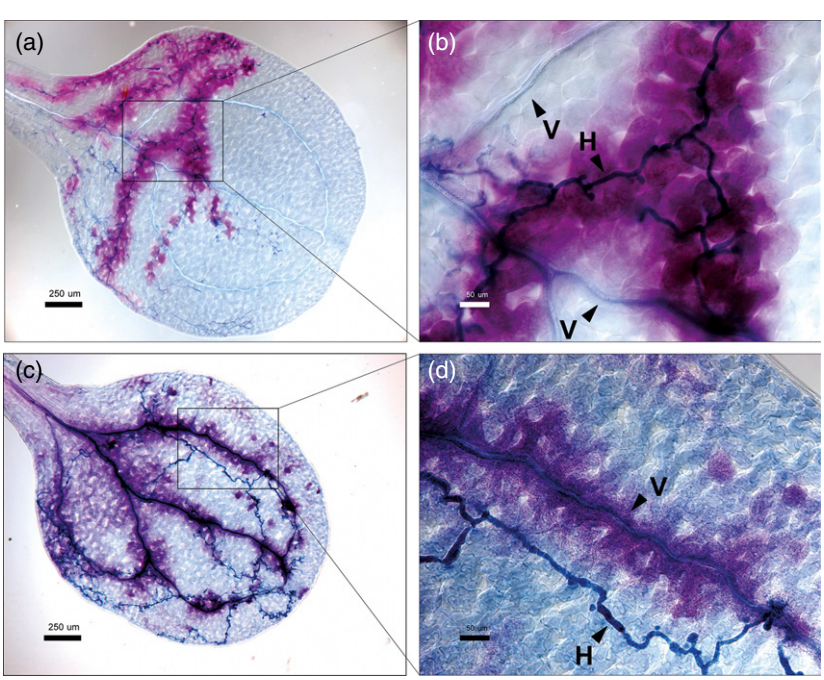


Figure 6. Expression of *DLO1* is localized in the cells near the vascular tissue 4 days after *H. arabidopsidis* infection.
GUS activity in the *proDMR6:GUS* (a, b) and *proDLO1:GUS* (c, d) plant lines was visualized with Magenta-X-gluc as substrate. *H. arabidopsidis* growth was visualized with trypan blue staining. GUS activity in the *proDMR6:GUS* line is specifically located in cells neighbouring the hyphal growth (H), while that of *proDLO1:GUS* is located in cells near the vascular tissue (V). Scale marker is 250 μm in (a) and (c), 50 μm in (b) and (d).

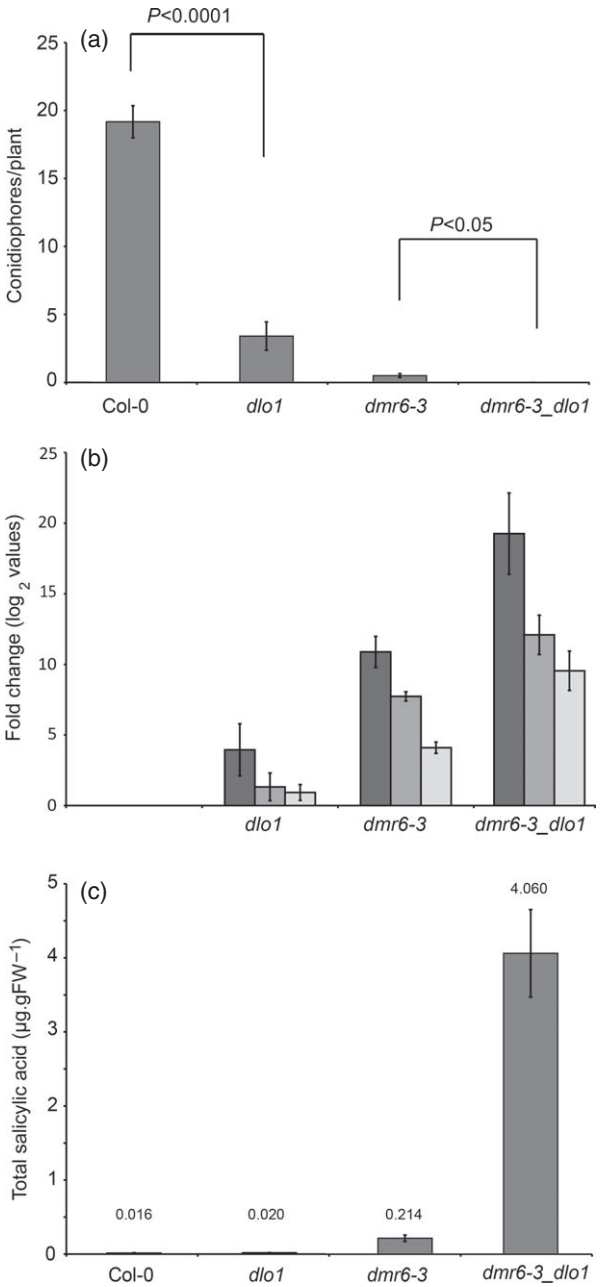


Figure 7. Redundancy of *DMR6* and *DLO1* revealed in the *dmr6-3_dlo1* double mutant that is highly resistant to *H. arabidopsidis* infection and shows high *PR*-gene expression and elevated SA levels.

(a) Amount of *H. arabidopsidis* conidiophores at 4 dpi for Col-0, *dlo1*, *dmr6-3* and *dmr6-3_dlo1* is shown. Error bars depict standard error. This experiment has been repeated multiple times with similar results. Level of significance is given following *T*-test analysis.

(b) Average fold change (log_2-values) of *PR-1*, *PR-2*, and *PR-5* expression in *dlo1*, *dmr6-3*, and *dmr6-3_dlo1* compared with the Col-0 parental line. Expression levels are the mean of two biological replicas, error bars indicating standard deviation. This experiment has been repeated twice with similar results.

(c) Total salicylic acid content (in µg g FW^{-1}) is shown for Col-0, *dlo1*, *dmr6-3*, and *dmr6-3_dlo1*. Means of three biological replicates are presented, error bars represent standard deviation. All plants were 14 days old at the moment of treatment.

dmr6-2 mutants showed increased levels of expression of *PR-1* and other defense genes (Van Damme *et al.*, 2008). Also the *dmr6-3* mutant showed elevated expression of *PR-1*, *PR-2* and *PR-5*, confirming our previous results (Figure 7b). The *dlo1* single mutant showed slight increased *PR-1* levels but no significant induction of expression of *PR-2 and PR-5.* In contrast, the *dmr6-3_dlo1* double mutant showed extremely high levels of defense gene expression. *PR-1* transcripts were more than 30 000-fold higher in the *dmr6-3_dlo1* mutant than in Col-0, and more than 100-fold higher than in the *dmr6-3* single mutant. In the tested mutants there was a clear correlation between the level of resistance to downy mildew and increase in defense gene expression, suggesting that resistance is caused by activation of plant immune responses. Our data shows that the *dlo1* mutation enhances the immunity of the *dmr6-3* single mutant, indicating DLO1 and DMR6 act partially redundant.

This was further corroborated by the growth phenotype of the mutants. Plants grown for 5 weeks under short day conditions showed striking differences between the genotypes (Figure 8). Whereas the *dlo1* mutant (Figure 8b) grows in a similar way to Col-0 (Figure 8a), and the *dmr6-3* mutant (Figure 8c) only shows a slight growth reduction, the *dmr6-3_dlo1* double mutant (Figure 8d) displayed strong growth reduction resulting in dwarfed plants. The growth reduction and level of resistance to downy mildew are correlated in the tested mutants, suggesting these two phenotypes are functionally linked. It is well known that strong activation of plant immunity can be accompanied by severe growth reduction, which in many cases can be linked to high SA levels (Scott, 2004). Indeed, levels of SA were more than 200 times higher in the *dmr6-3_dlo1* double mutant than in the Col-0 control, and approximately 20 times higher than in the *dmr6-3* mutant (Figure 7c). The single mutant *dmr6-3* showed a modest approximately 10 fold increase in SA compared with the Col-0 control, whereas the *dlo1* mutant did not accumulate more SA than Col-0.

To test if the high SA levels in *dmr6-3_dlo1* are the cause of the dwarf phenotype and high level of resistance to downy mildew, the double mutant was crossed to the *sid2-1* mutant, which is strongly compromised in SA biosynthesis as a result of loss of isochorismate synthase 1 (Wildermuth *et al.*, 2001). The triple mutant *dmr6-3_dlo1_sid2-1* (Figure 8e) showed almost complete recovery of the growth phenotype of the *dmr6-3_dlo1* double mutant (Figure 8d), although it remained slightly smaller than the *sid2-1* mutant (Figure 8f). Disease assays showed that also the high level of resistance of the *dmr6-3_dlo1* double mutant and *dmr6-3* single mutant was strongly reduced in the absence of SID2 (Figure 9a). Because of the low SA levels the *sid2-1* mutant is more susceptible to *H. arabidopsidis* than the wild-type Col-0. The level of susceptibility to *H. arabidopsidis* correlates well to the level of total SA in the mutants (Figure 9b). Both *dmr6-3* as well as the

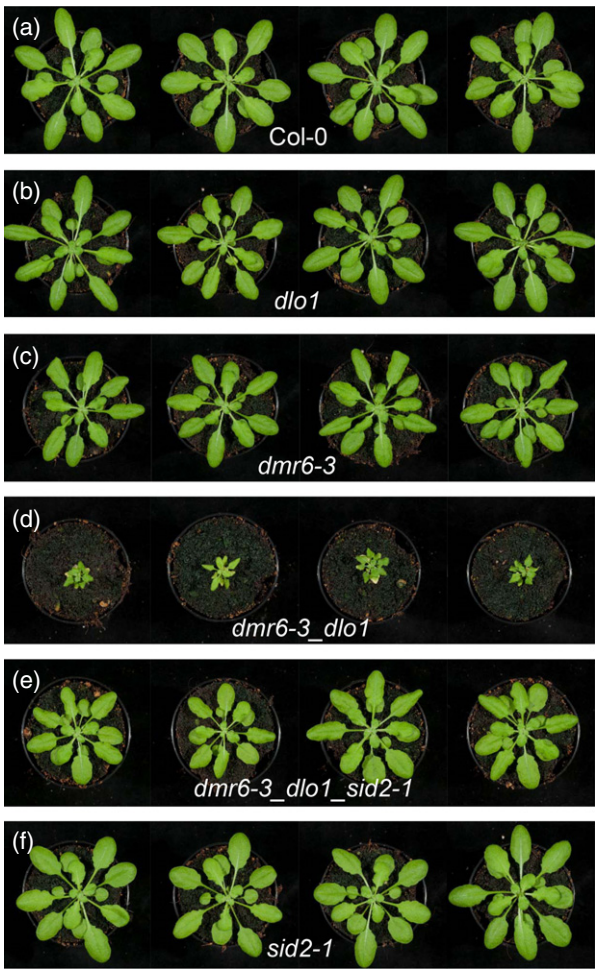


Figure 8. The *dmr6-3_dlo1* double mutant shows a severe growth phenotype that is dependent on SA and strongly reduced in the *sid2-1* background.
Representative pictures of 5-week-old plants of Col-0 (a), *dlo1* (b), *dmr6-3* (c), *dmr6-3_dlo1* (d), *dmr6-3_dlo1_sid2-1* (e), and *sid2-1* (f), grown under short day conditions. Note that *dmr6-3*, but not *dlo1*, shows a slight growth reduction compared to the Col-0 parental line. Also note the slight growth reduction of the *dmr6-3_dlo1_sid2-1* triple mutant compared with the *sid2-1* single mutant.

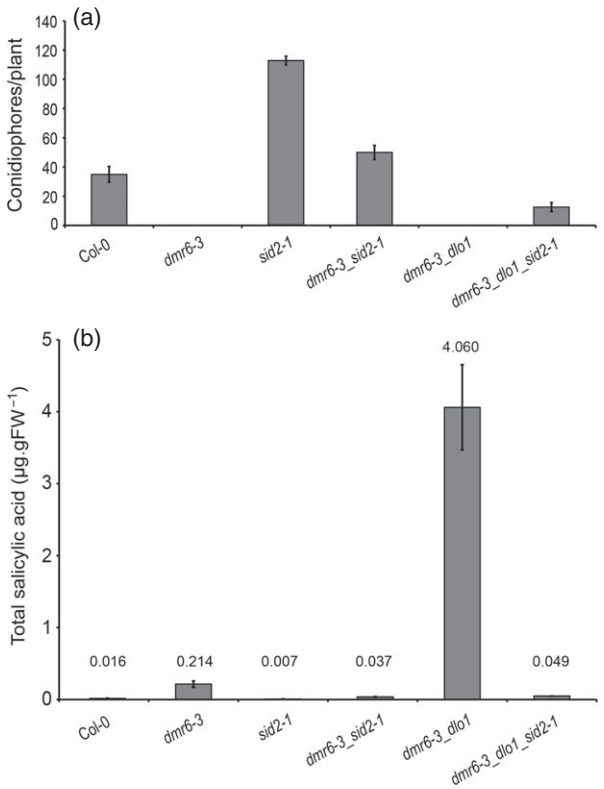


Figure 9. Resistance of *dmr6-3_dlo1* is only partially compromised in the *dmr6-3_dlo1_sid2-1* mutant, whereas SA levels are strongly reduced in the *sid2-1* background.
(a) Amount of *H. arabidopsidis* conidiophores per plant of 14-day-old seedlings 5 dpi and (b) corresponding SA levels (in µg gFW^{-1}) in Col-0, *dmr6-3*, *sid2-1*, *dmr6-3_sid2-1*, *dmr6-3_dlo1*, and *dmr6-3_dlo1_sid2-1* plants. Note that (b) is identical to Figure 7(c) with the exception of the omission of the *dlo1* mutant and the addition of the *sid2-1* single, *dmr6-3_sid2-1* double and *dmr6-3_dlo1_sid2-1* triple mutants and is added for comparison.

dmr6-3_dlo1 double mutant show no sporulation at 5 dpi and have the highest SA levels. The triple mutant *dmr6-3_dlo1_sid2-1* still contains more SA than Col-0, which might explain its lower susceptibility to downy mildew. We conclude that both the resistance to *H. arabidopsidis*, as well as the growth reduction of the *dmr6-3_dlo1* mutant is the result of increased SA levels. The extreme phenotypes of the double mutant demonstrate that the *DLO1* and *DMR6* genes act redundantly. However, the *dmr6-3* single mutant is more resistant to downy mildew than the *dlo1* mutant. Together with the observed different localization of expression of the *DMR6* and *DLO1* genes, our data indicate that the *DMR6* and *DLO1* genes have distinct but partially redundant functions as negative regulators of plant immunity.

DISCUSSION

The 2-oxoglutarate Fe(II) dependent oxygenases are widely present in all flowering plant species (Kawai *et al.*, 2014). In this study we focused on identifying oxygenases that share high sequence conservation with Arabidopsis DMR6. 2OG oxygenase proteins belonging to the DMR6 clade were found in all monocot and dicot species analyzed and grouped into two distinct subclades, one containing *A. thaliana* DMR6, and the other two closely related *A. thaliana* paralogs, designated DLO1 and DLO2. Both subclades contain dicot and monocot orthologues indicating that the ancestor of flowering plants already contained both a *DMR6* and *DLO* gene.

In this study the *A. thaliana DLO1* and *DLO2* genes were analyzed in more detail since they could play a role in plant immunity. Indeed, overexpression of *DLO1* and *DLO2* could restore susceptibility of the resistant *dmr6* mutant,

and enhance susceptibility of wild-type Col-0 plants, similar to overexpression of *DMR6.* This indicates that DLO1 and DLO2 have a molecular activity comparable with DMR6 and can act as suppressors of plant immunity. Analysis of genome-wide expression data related to pathogen, elicitor and hormone treatment, showed that *DLO1* is co-regulated with *DMR6* (Figure 4). A clear induction after pathogen attack and SA 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 or after SA or BTH treatment, making it very unlikely that this gene has a role in *H. arabidopsidis*-infected leaves. Although *DMR6* and *DLO1* are co-expressed they show distinct differences in the localization of expression. Infection of promoter GUS fusion lines showed that whereas *DMR6* is mainly expressed in cells that are in close contact to *H. arabidopsidis*, *DLO1* expression is high in the vascular tissue in the vicinity of the infection sites, but not in the interacting mesophyll cells. We speculate that, in the ancestor of all flowering plants, the *DMR6* and *DLO* genes have originated by gene duplication, followed by subfunctionalization to result in genes with a similar function but different localization of expression.

A similar phenomenon was described for several GA-oxidases belonging to the 2OG oxygenase superfamily that regulate levels of the hormone gibberellin (Pimenta Lange and Lange, 2006). The GA-oxidase genes exhibit different expression patterns depending on developmental status and environmental cues (e.g. in response to low temperature (Yamauchi *et al.*, 2004)) thereby controlling the GA level during the different growth stages of the plant (Pimenta Lange and Lange, 2006). It has been shown, e.g., that three of the five GA 20-oxidases, GA20ox1 to 3, that catalyze the oxidation reaction from C20-GA to C19-GA, a precursor of bioactive GA, show different tissue-specific expression (Rieu *et al.*, 2008a). *GA20ox1* is mainly expressed in stems, *GA20ox2* in flowers and siliques, and *GA20ox3* only in siliques (Rieu *et al.*, 2008b). Although these three genes have the same molecular function, they show a different expression pattern that contributes to organ-specific GA biosynthesis.

Our research revealed that DMR6 and DLO1 act redundantly as negative regulators of plant immunity. Whereas the *dmr6-3* mutant already showed strong resistance to *H. arabidopsidis*, and the *dlo1* mutant a lower level of resistance, the *dmr6-3_dlo1* double mutant showed complete resistance. Increased SA levels are the cause of resistance, as reduction of SA levels by mutation of the *ISOCHORISMATE SYNTHASE 1* gene (in the *sid2-1* mutant) resulted in a strong reduction of resistance. Loss of resistance was not complete, as there was still SA produced in the *dmr6-3_sid2-1* double mutant and *dmr6-3_dlo1_sid2-1* triple mutant, to a level that was slightly higher than in the Col-0 control. It is known that the *sid2-1* mutant is not completely devoid of SA. In Arabidopsis, SA is mainly derived from isochorismate produced from chorismate by isochorismate synthase that is encoded by two genes *ICS1* (*SID2*) and *ICS2* (Garcion *et al.*, 2008). SA levels in the *ics1* (*sid2-1*) mutant are roughly 10% of that in wild-type Arabidopsis Col-0 (Wildermuth *et al.*, 2001), whereas the *ics1_ics2* double mutant shows a further reduction by approx. 30% (Garcion *et al.*, 2008). An isochorismate-independent route is thought to be responsible for the minor residual level of SA present in the *ics* double mutant. Nevertheless, our data clearly demonstrate that *dmr6-3-* and *dmr6-3_dlo1*-based resistance requires SA accumulation for the activation of plant immunity. When attacked by biotrophic pathogens Arabidopsis and many other plant species synthesize SA that is an inducer of a large number of pathogenesis-related genes (*PR*-genes). Several SA-induced PR-proteins are known to have antimicrobial activity that contributes to plant disease resistance (Van Loon, 1997). We have shown that *dmr6*-based resistance is not only effective against downy mildew, but also against the bacterial pathogen *P. syringae* and the oomycete *P. capsici*, that are both known to be sensitive to SA-induced defenses.

Recently, it was described that the *DLO1* gene, which is also activated during senescence, encodes a 2OG oxygenase that can hydroxylate SA to form 2,3-dihydroxybenzoic acid (2,3-DHBA), and was therefore named SALICYLIC ACID 3-HYDROXYLASE (S3H) (Zhang *et al.*, 2013). The S3H (DLO1) protein was shown to reduce SA levels to control the onset and rate of leaf senescence by acting in a negative feedback regulation system. The *s3h* (*dlo1*) mutant accumulates SA that is responsible for its enhanced senescence (Zhang *et al.*, 2013). Since we showed that the *dmr6-3* mutant accumulates even more SA than the *s3h* (*dlo1*) mutant, it is likely that also DMR6 catabolizes SA, possibly by a similar hydroxylation reaction. We are currently investigating whether DMR6 has SA-hydroxylase activity. In the *dmr6-3_dlo1* double mutant SA accumulates to even higher levels, suggesting that DMR6 and DLO1 (S3H) together, are key players in the conversion of SA, thereby negatively regulating plant immunity. The fact that *DMR6* and *DLO1* (*S3H*) are both activated during pathogen infection indicates that they are part of a feedback regulation system to tightly control the level of SA. We envision that by activating *DMR6* and *DLO1*, the plant is able to fine tune its immune response and prevent uncontrolled SA accumulation and over-activation of defense with detrimental effects on plant growth and development.

EXPERIMENTAL PROCEDURES

Plant growth conditions and pathogen infections

Unless noted otherwise, plants were grown on potting soil at 21°C with 16 h of light with 75% relative humidity. *H. arabidopsidis*

inoculation was performed on 14-day-old Arabidopsis seedlings or 6-week-old plants as described before (Van Damme *et al.*, 2005). To measure the growth of *P. syringae* pv *tomato* DC3000 6-week-old plants were sprayed with a bacterial suspension (OD 0.05) with 0.02% silwet. Leaves (four plants per line; three leaves per plant) were taken for colony counting 0, 1 and 3 days post inoculation. For *P. capsici* inoculation plants were grown on potting soil at 21°C with 10 h of light at 70% relative humidity. Forty eight seedlings per line were transplanted at 12 days past germination (dpg) and *P. capsici* inoculation (50 zoospores μl^{-1}) was performed using a spray gun at 18 dpg. Inoculated plants were kept in the dark for 24 h and at 100% relative humidity for 7 days. Scoring was performed at 7 dpi by counting the number of dead plants. *P. capsici* LT3112 was grown on V8 agar (V8A) plates (20% v/v V8 juice, 1.5% agar, 35 mM $CaCO_3$) for 1 week at 10 h of light at 21°C. Zoospore production was performed by cutting the mycelium-containing V8A into squares and dividing it over two Petri dishes. 10 ml ddH_2O was added and refreshed after 1 h. Plates were kept at RT for 3 days and were given a cold shock for 1 h at 4°C to release zoospores. All experiments were repeated twice with similar results.

Plant treatments and QPCR analysis

RNA isolation was conducted using RNeasy kits (Qiagen, www.qiagen.com) including treatment with DNase (Qiagen). cDNA was synthesized with Superscript-III reverse transcriptase (Invitrogen, www.lifetechnologies.com) from total RNA. Cycle thresholds using the ABI PRISM 7700 system (Applied Biosystems www.appliedbiosystems.com) were determined using SYBR Green as reporter dye. The resulting Ct values were normalized using *ACTIN2* levels (At3g18780) with primers QACT2F, 5′-aatcaca gcacttgcacca-3′ and QACT2R, 5′-gagggaagcaagaatggaac-3′. *DMR6* expression was analyzed using primers: QDMR6F 5′-tgtcatcaa cataggtgaccag-3′and QDMR6R 5′-cgatagtcacggattttctgtg-3′. *PR-1*, *PR-2*, and *PR-5* expression were analyzed using primers: QPR-1F 5′-gaacacgtgcaatggagttt-3′, QPR-1R 5′-ggttccaccattgttacacct-3′, QPR-2F 5′-cccgtagc atactccgattt-3′, QPR-2-R 5′-aaggagcttagcctcacc ac-3′, QPR-5F 5′-ggcaaatatctccagtattcaca-3′, and QPR-5R 5′-gg tagggcaattgttccttaga-3′.

The induction treatments were performed by spraying 100 μM BTH or 100 μM MeJA onto 14-day-old seedlings. After 24 h, the seedlings were harvested for RNA isolation. *H. arabidopsidis* isolates Waco9 and Cala2 were sprayed (50 spores μl^{-1}) onto 14-day-old seedlings and harvested 3 days post inoculation. *DLO1* expression was analyzed using primers: QDLO1F 5′-aatatcggcgac-caaatgc-3′ and QDLO1R 5′-cgctcgttctcggtgtttac-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 (Goodstein *et al.*, 2012). Proteins that solely contain the PF03171 domain were selected using the HMMER3 algorithm. Proteins exceeding 20% length difference compared to *A. thaliana* DMR6 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). The robustness of the phylogenetic tree was assessed by bootstrapping with 100 replicated for the initial phylogeny (Figure 2a) and 1000 for the subset (Figure 2b).

Selected publicly available immunity-related microarray experiments were obtained as processed data from Array Express (http://www.ebi.ac.uk/arrayexpress/). A list of experiments used is provided (Table S1). For each gene, expression values in replicate slides were averaged. Subsequently, average expression values for treatment slides were divided by average value of the control replicates, to obtain fold change (using R 2.10.1, http://www.R-project.org). Fold change was calculated per gene, and log2 values were then plotted using MeV 4.5.1 (Saeed *et al.*, 2006). Data was clustered using Pearson correlation with average linkage.

Generation of mutants and overexpression lines

DLO1 (At4g10500), *DLO2* (At4g10490), At3g60290 and At1g06620 were amplified from Col-0 and cloned into the pENTR vector using Gateway ® cloning (Invitrogen). Constructs were cloned into the pB7WG2 Gateway® compatible binary vector. These were transformed via *A. tumefaciens* strain C58C1 containing pGV2260 in Col-0 and *dmr6-1* plants using floral dipping (Zhang *et al.*, 2006). Transformants were selected by BASTA. Site-directed mutagenesis constructs were constructed using the Phusion Site-Directed Mutagenesis Kit (Thermoscientific, www.thermoscientific.com) and cloned into the pFAST R05 vector (Shimada *et al.*, 2010) and transformed via *A. tumefaciens* strain GV3101 (pMP90) in *dmr6-1* plants using floral dipping. Western blot analysis using anti-GFP antibody (Miltenyi Biotec, www.miltenybiotec.com) was performed to verify if the protein was expressed *in planta*. *dmr6-3* was obtained through NASC (N329085) and genotyped using primers: no097 LP N329085 5′-gactggtttcgtttcatcgtc-3′; no098 RP N329085 5′-gaaagtgcttggtgaacaagg-3′ and no089 LB GK o8409 5′-atattgaccatcatactcattgc-3′. *dlo1* was obtained through NASC (N559907) and genotyped using primers: dlo1_LB 5′-attccatccctct-gatcgatc-3′; dlo1_RB 5′-tcaacaaacgggtaggttctg-3′ and LBb1.3 5′-att-tgccgatttcggaac-3′.

Promoter GUS transgenic lines

proDMR6:GUS transgenic lines were generated as described previously (Van Damme *et al.*, 2008). The *proDLO1:GUS* transgenic line was generated using primers: pDLO1_Fw 5′- cac-ctgtaaagatccaaataacatggt-3′ and pDLO1_Rv 5′-ttaatgtgtttggtaatg-taat-3′ and Gateway® cloned into the pENTR vector. This construct was cloned into the pBGWFS7 binary vector in front of the GUS gene (Karimi *et al.*, 2002). Col-0 plants were transformed via the *Agrobacterium tumefaciens* C58C1 (pGV2260) strain. Transformants were selected by BASTA. *proDMR6:GUS* and *proDLO1:GUS* transgenic seedlings (T4) were first infected with *H. arabidopsidis* and 4 days post inoculation vacuum infiltrated with Magenta-X-gluc solution following trypan blue staining as described previously (Van Damme *et al.*, 2008).

Phytohormone extraction

Phytohormone extraction was performed according to Van den Burg *et al.* (2010), with small modifications: 100 mg plant material was ground in liquid nitrogen, extracted with 500 μl 90% MeOH containing 100 ng ml^{-1} 4D-labeled SA, followed by a re-extraction with 500 μl MeOH containing 0.2 mM NaOH. Both extracts were combined, and evaporated *in vacuo* until approximately 30–50 μl was left. Next, 1 ml warm (60°C) ddH_2O was added and samples were vortexed. The samples were split; 500 μl was added to 500 μl 0.2 M sodium acetate (pH 4.5) containing 0.1 mg β-glucosidase

(Sigma-Aldrich, www.sigmaaldrich.com), and 500 μl to 0.2 M sodium acetate. Samples were incubated for 2.5 h at 37°C, acidified with 20 μl 37% HCl to pH 1, and extracted twice with 800 μl pentane/ethyl acetate/2-propanol (50/50/1). The combined extracts were evaporated *in vacuo*, taken-up in 150 μl MeOH, vortexed for 2 min, and 50 μl ddH_2O was added. The samples were centrifuged for 15 min to remove particles; 100 μl was transferred to an LC-MS vial containing an insert.

SA levels were analyzed on a Varian 320 LC-MS/MS, using instrument parameters and ions as described in Van Doorn *et al.* (2011). Samples were separated on a Kinetix C18 column (50 × 2.1 mm, 5 μM particle diameter, Phenomenex, www.phenomenex.com), using solvents A (ddH_2O/0.05% formic acid) and B (MeOH/0.05% formic acid) as mobile phase. The gradient was first isocratic at 5% (v/v) solvent B for 1.5 min, followed by a linear gradient to 95% (v/v) solvent B at 7.5 min, 95% (v/v) solvent B at 13.5 min and 5% (v/v) solvent B at 18 min, at a flow rate of 0.2 ml min^{-1}.

SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article.

Figure S1. The *dmr6-1* mutant is resistant to *P. syringae* infection.

Figure S2. Disease phenotype of L*er eds1-2*, *dmr6-1* and *dmr6-1 35S:DMR6* 7 days after *Phytophthora capsici* inoculation.

Figure S3. Overexpression of *DMR6* results in reduced levels of *PR*-gene transcript.

Figure S4. Catalytic inactive DMR6 remains resistant to *H. arabidopsidis* infection.

Figure S5. Multiple alignment of Arabidopsis DMR6, DLO1 and DLO2.

Figure S6. Overexpression of two distantly related oxygenases fails to complement *dmr6*-mediated resistance.

Figure S7. Overexpression of *DLO1* and *DLO2* increases susceptibility towards *H. arabidopsidis.*

Table S1. List of selected microarray experiments dealing with biotic and abiotic stress.

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