Transcriptome analysis highlights preformed defences and signalling pathways controlled by the *prAe1* quantitative trait locus (QTL), conferring partial resistance to *Aphanomyces euteiches* in *Medicago truncatula*

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SUMMARY

To gain an insight into the molecular mechanisms of quantitative disease resistance in *Medicago truncatula* to the root-infecting oomycete *Aphanomyces euteiches*, we selected two near-isogenic lines (NILs), NR and NS, partially resistant and susceptible, respectively, differing in the allelic state of the quantitative resistance locus (QRL) *prAe1* (partially resistant to *A. euteiches* 1). Complementary molecular and cytological phenotyping methods showed that *prAe1* alone confers quantitative resistance to *A. euteiches*. Root and stem tissues were colonized in NS plants and 80% of NS plants died by 21 days post-inoculation (dpi). In contrast, *A. euteiches* mycelium was restricted to the root cortex and the spread of symptoms was arrested in aerial parts of NR plants. A transcriptome analysis performed at 0, 1 and 6 dpi identified 1198 differentially expressed genes (DEGs) between NR and NS lines. More than 87% of the DEGs were significantly more expressed in NR. The highest number of DEGs was found in control conditions, with 723 genes over-expressed in NR versus 85 in NS. Genes belonging to secondary metabolism, pathogenesis-related (PR) proteins and kinases were significantly enriched. The significant role of the flavonoid pathway in resistance was corroborated by the detection of larger amounts of flavonoids in NR roots and the inhibition of *A. euteiches* zoospore germination by 2'-*O*-methylisoliquiritin, a compound synthesized by enzymes specifically induced in NR. Our study revealed that *prAe1*-dependent resistance relies mainly on the constitutive expression of defence-related pathways and signalling elements, which can be re-amplified in later time points of the infection.

Keywords: *Aphanomyces*, flavonoid, legume, oomycete, partial resistance, secondary metabolism.

INTRODUCTION

To detect and counter pathogen attack, plants have evolved two interconnected tiers of innate immunity (Jones and Dangl, 2006). The detection of highly conserved molecular signatures (e.g. pathogen-associated molecular patterns, PAMPs) by membrane-associated receptors allows plants to mount a defence response leading to PAMP-triggered immunity (PTI) (Monaghan and Zipfel, 2012). Early signalling events, including the production of reactive oxygen species (ROS) (Torres *et al.*, 2002), Ca²⁺ signalling (Cheval *et al.*, 2013) and mitogen-activated protein (MAP) kinase activation (Rasmussen *et al.*, 2012), trigger profound transcriptomic reprogramming. The synthesis of diverse antimicrobial metabolites, callose deposition, cell wall strengthening and the production of pathogenesis-related (PR) proteins are components of the defence response (Eulgem, 2005) underlying PTI, and are sufficient to protect plants against a broad range of potentially harmful microbes. However, successful pathogens can escape this first layer of surveillance by producing effectors that disrupt the signalling cascade leading to PTI, thus promoting virulence (effector-triggered susceptibility, ETS) (Fabro *et al.*, 2011). Co-evolution between a plant and a pathogen is often illustrated as an arms race, as plants have evolved a second immune system that relies on effector recognition by specific proteins, called resistance (R) genes, which leads to effector-triggered immunity (ETI) (Collier and Moffett, 2009). This second immune response shares common components of PTI, but is of a higher magnitude and often induces programmed cell death at the infection site, thus limiting pathogen spread and leading to full resistance. Although the molecular bases of PTI, ETS and R-gene-mediated resistance (also called qualitative resistance) are now well defined, less is known about the quantitative variation in resistance found in natural populations. Partial or quantitative disease resistance (QDR) is characterized by a reduction in disease severity rather than a total absence of symptoms. Contrary to qualitative resistance conferred by single specific R-gene products, QDR is believed to be driven by several loci (quantitative resistance loci, QRL), each contributing to the observed partial resistance pheno-

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type. The polygenic basis of QDR makes it more durable for use in agricultural systems (Palloix *et al.*, 2009; Parlevliet, 2002). Several hypotheses have been postulated to explain QDR (Poland *et al.*, 2009), including sequence polymorphism in classical defence genes, residual activity of *R* pseudogenes and resistance mechanisms related to morphological traits. Only a few QRLs have been cloned (Roux *et al.*, 2014), and detailed characterization of the genes and molecular mechanisms underlying QRLs is needed for a better understanding of QDR (St.Clair, 2010).

Aphanomyces root rot, caused by the soil-borne oomycete pathogen *Aphanomyces euteiches* Drechs., is one of the most destructive diseases of pea (*Pisum sativum*) (Gaulin *et al.*, 2007; Moussart *et al.*, 2008). Long-term avoidance of infested fields is currently the only solution to manage the disease, and highlights the need for new strategies to efficiently control *A. euteiches*. Efforts to mine the variability of resistance to *Aphanomyces* root rot in pea germplasm have been hampered by the scarcity of resistant sources (Malvick and Percich, 1999; Pilet-Nayel *et al.*, 2002) and by weak levels of polygenic partial resistance (Hamon *et al.*, 2011, 2013). A pathosystem between the model legume *Medicago truncatula* and *A. euteiches* has been developed to accelerate genetic studies and to develop a better understanding of QDR. The accessions F83005.5 (hereafter named F83) and Jemalong A17 reference line, susceptible and partially resistant, respectively, were chosen to initiate the characterization and genetic dissection of partial resistance to *A. euteiches* (Djébali *et al.*, 2009). Specific features of A17 resistance include protection of the central cylinder against pathogen invasion, associated with frequent pericycle cell divisions, lignin deposition around the pericycle and the accumulation of soluble phenolic compounds following infection. Using a recombinant inbred line population derived from a cross between the two accessions, a major QRL was identified on the top of chromosome 3. This recessive QRL, named *prAe1* (partially resistant to *A. euteiches* 1), explained 34% of the resistance phenotype. The key role of *prAe1* was recently confirmed by a genome-wide association study (GWAS), which identified two major independent loci, precisely co-localizing with *prAe1* (Bonhomme *et al.*, 2014). The most significant single nucleotide polymorphisms (SNPs) occur in a gene encoding an F-box protein, whereas the second locus contains two closely located genes, encoding an adenylate isopentenyltransferase gene and a MYB transcription factor (TF).

Here, we took advantage of two near-isogenic lines (NILs) differing in the allelic state of *prAe1* to explore the molecular mechanisms and gene network underlying QDR. By compiling cytological and molecular analyses, together with computer-assisted phenotyping, we found that *prAe1* alone is sufficient to confer partial resistance to *A. euteiches*. Results obtained using Affymetrix *Medicago* Genome arrays to monitor transcript changes in the resistant (NR) and susceptible (NS) genotypes at 0, 1 and 6 days post-inoculation (dpi) showed that resistance conferred by *prAe1* relies mainly on the constitutive expression of defence-related

pathways. Together with supplementary biochemical analyses, our results highlight the key role of secondary metabolites in the partial resistance to *A. euteiches*, and identify a chalcone-derived Nod-inducing compound as a potent inhibitor of *A. euteiches*.

RESULTS

The *prAe1* locus alone is sufficient to confer partial resistance to *A. euteiches*

Two F₁₁ NILs, NR and NS, differing only in the A17 and F83 alleles of *prAe1*, respectively, were obtained following two rounds of self-fertilization of 5A and 5F NILs described in Djébali *et al.* (2009). The seedling roots of both NILs were inoculated with *A. euteiches* zoospores and pathogen development was monitored for 21 dpi. The relative quantification of *A. euteiches* α -tubulin transcript expression showed that mean *A. euteiches* transcript abundance was two-fold higher in NS than in NR at 6 dpi (Fig. 1A). This result indicates rapid colonization and multiplication of *A. euteiches* in NS, which was confirmed by cytological analyses (Fig. 1B); cortical cells were fully invaded by *A. euteiches* mycelium in NS, whereas only a small amount of intercellular hyphae was observed in the NR cortex. In one-quarter (five of 20) of the observed root sections, mycelium began to enter the stele in NS at this time point. Another striking difference was the observation of a significantly greater number of oospores in NS than in NR (Fig. 1A, B). At 15 dpi, a dark-brown discoloration, similar to that observed in the inoculated resistant A17 parental line (Djébali *et al.*, 2009) and clearly distinct from the honey-light-brown tissues of infected susceptible plants, was displayed by NR infected roots. In addition, symptoms were arrested in NR near the hypocotyl, leaving most of the stem symptom and pathogen free (Fig. 1C). At this time point, 96% of tissues from NS were symptomatic, whereas symptoms were limited to 64% of NR plant tissues (Fig. 1D). About 80% of NS plants had died by 21 dpi, whereas all NR (like A17) plants were still alive (Fig. 1D). Taken together, these results indicate that *prAe1* alone can confer a partial level of resistance to *A. euteiches*, similar to that observed in A17 (Djébali *et al.*, 2009), relying on a slower and reduced rate of colonization associated with protection of the root stele and of the aerial parts of the plant.

Infection with *A. euteiches* induces wide transcriptional reprogramming in NR and NS

To study the molecular mechanisms involved in partial resistance to *A. euteiches*, total RNA from roots of NR and NS was collected at 0, 1 and 6 dpi and used for microarray analyses. The selected time points were chosen to collect information at an early stage of infection (1 dpi, penetration of root) and at a later stage (6 dpi) corresponding to the initiation of penetration of the root stele of susceptible plants (Djébali *et al.*, 2011). A total of 5526 genes

Fig. 1 Phenotype analyses of resistant (NR) and susceptible (NS) near-isogenic lines (NILs) validate the *prAe1* quantitative resistance locus (QRL). (A) Left: quantification of *Aphanomyces euteiches* α -tubulin transcripts *in planta* [6 days post-inoculation (dpi)]. To compare the amount of parasite in each line, a relative quantification was calculated by normalizing pathogen transcript with *Medicago truncatula* transcripts of the constitutively expressed H3L (MtG19-TC118424) gene. Right: oospore counts in transverse root sections of independent plantlets (NR, 20 sections; NS, 15 sections). (B) Assessment of root colonization by *A. euteiches* in NR and NS at 6 dpi. *Aphanomyces euteiches* mycelium and oospores are labelled in red/orange with Alexa Fluor® 633 conjugate of wheat germ agglutinin (WGA) in 100- μ m root sections. White arrowheads highlight some pathogen oospores and illustrate the greater number of these structures that can be observed in NS versus NR root sections. Scale bar, 100 μ m. (C) *In vitro* phenotypes at 21 dpi. NR plants display dark root browning and a clear arrest (white arrows) of symptom progression near the hypocotyl. (D) Left: percentage of symptomatic tissues (15 and 21 dpi) estimated by computer-assisted measurement. Following scans of Petri dishes at 15 and 21 dpi, means of ratios between the length (in pixels) of brown tissues versus whole-plant (root + stem) length were calculated. Right: percentage of dead plants (21 dpi) of NR and NS following infection with *A. euteiches*. Error bars represent standard error of the mean (SEM). Means of each line were compared by *t*-test for symptom notation, by the χ^2 test for the proportion of dead plants, and by the Mann–Whitney *U*-test for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) results. Asterisks indicate significant differences: **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

(Table S1, see Supporting Information) was detected with differential expression in at least one time point in one line. Transcriptional reprogramming was substantially greater in the susceptible than resistant NIL (Fig. 2A); 1923 and 1115 genes (1 dpi) and 3182 and 2550 genes (6 dpi) were found to show a significantly altered expression in NS and NR, respectively, compared with non-inoculated roots. Although differentially expressed genes (DEGs) of NS were mainly up-regulated at 1 and 6 dpi (86% and 59%, respectively), in NR they were mostly induced at 1 dpi (71%), but repressed (64%) at 6 dpi. Comparisons of the expression levels of these 5526 genes revealed that 1404 (25%) displayed similar transcript accumulation in both lines at both time points (Fig. 2B). This set of genes is hereafter referred to as the common response of the two lines, whereas the remaining 4122 genes (75%) belong to the NIL-specific response after pathogen inoculation.

NR and NS share a common core defence response following *A. euteiches* infection

The list of the 1404 regulated genes in the common response to *A. euteiches* is presented in Table S2 (see Supporting Information). A functional analysis of these genes is presented in Fig. 3 and Table S3 (see Supporting Information). Although the global percentage of up-regulated genes was slightly higher (51%) than that of down-regulated genes, the distribution of these two gene categories varied greatly at each time point. A similar number of up-regulated genes was detected at 1 and 6 dpi (458 and 478,

Fig. 2 Global transcriptome responses of resistant (NR) and susceptible (NS) near-isogenic lines (NILs) after *Aphanomyces euteiches* inoculation. The selected genes have $P < 9.823 \times 10^{-7}$ following normalization (see Experimental procedures). (A) Number of induced or repressed genes, compared with non-inoculated lines, in NR and NS at 1 and 6 days after inoculation with *A. euteiches*. The number of Affymetrix probes showing differential hybridizations with a significant P value is taken as a good indicator of the number of regulated genes (despite a slight overestimation as the same gene is sometimes represented by more than one probe on the chip). (B) Distribution of the 5526 regulated genes after *A. euteiches* inoculation. Genes similarly regulated at both time points in NR and NS are included in the common response to *Aphanomyces euteiches* (i.e. induced at 1 and 6 dpi, repressed at 1 and 6 dpi, induced/repressed at 1 dpi and unregulated at 6 dpi, and unregulated at 1 dpi and induced/repressed at 6 dpi).

respectively). In contrast, more than 95% of the down-regulated genes were detected at 6 dpi. To gain an insight into gene functions that were significantly altered in this set of genes shared by both NILs after *A. euteiches* infection, we used Mapman Bin and sub-Bin classification and performed a hypergeometric test to assess the distribution of regulated genes inside the functional classes determined by Mapman software. This allowed us to iden-

tify significant functions that were up- or down-regulated in response to *A. euteiches* inoculation, independent of *prAe1* alleles (Table S3).

Globally, 10 of 35 functional categories (Bins) were significantly detected with a Bonferroni-corrected P value. Following this first step, the same test was applied within each sub-Bin belonging to the 10 identified Bins in order to detect over-represented gene families or pathways (Table S3 and Fig. 3B). The induction of the flavonoid pathway, at both 1 and 6 dpi, was the most significantly detected. Following the same kinetics, the phenylpropanoid pathway, together with the biotic stress sub-Bin, which contains many genes encoding PR proteins or resistance gene analogues (RGA), was detected. Consistent with the early detection of the pathogen in both lines, genes classified in the signalling Bin, encoding for receptor-like kinases, kinases or calcium signalling-related proteins, were found to be significantly induced at either 1 dpi only or constantly during the whole time course. Early signalling also involved the induction of 36 TFs at 1 dpi. This class remained significant at 6 dpi with a large number of TF genes detected, but with a well-balanced distribution among induced and repressed genes (46 versus 50, respectively). Ethylene was the only significantly detected hormonal pathway, with several ethylene-responsive factors (ERFs) induced at both time points and ethylene synthesis-related oxido-reductases regulated at 6 dpi. Generally, genes encoding enzyme families involved in redox mechanisms were also significant. Genes coding for glutathione-S-transferases (GSTs) (1 dpi) and for lactoylglutathione lyases (6 dpi) were up-regulated, whereas genes coding for peroxidases or cytochrome P450 were significantly repressed at 6 dpi. Other genes known to be involved in activation/deactivation and storage or transport of defence-related molecules, such as ABC transporters (induced at 1 dpi); glucosyl/glucuronyl transferases and β -glucosidases (repressed at 6 dpi), were also significantly detected. Finally, it is worth noting that genes associated with primary metabolism involved in sugar or amino acid synthesis and their transport were particularly affected and down-regulated at 6 dpi.

Defence-related genes are more highly expressed in NR

The next step of our analysis aimed to identify which genes were significantly differentially expressed between NR and NS, among both those that were found to be regulated after inoculation and those that were expressed in non-inoculated NR and NS lines. The set of 4122 NIL-specific response genes identified was used to analyse the expression ratio between NR and NS at 1 and 6 dpi. This led to the identification of only 356 genes (Table S4, see Supporting Information) which were at least two-fold more strongly expressed in either NR or NS. Among these 356 DEGs after *A. euteiches* inoculation, about 70% were more highly expressed in NR than in NS. A complementary analysis was also

Fig. 3 Transcriptome analysis of the common response after *Aphanomyces euteiches* inoculation. (A) Venn diagram obtained following comparisons of genes commonly up- or down-regulated in both lines at 1 and 6 days after *A. euteiches* inoculation. (B) Significantly enriched functional subcategories as defined by Mapman software. Categories represented here were detected as significantly enriched ($P < 4.9 \times 10^{-4}$; Bonferroni correction) following hypergeometric distribution-based enrichment analysis in the common response genes set in at least one condition. Aa, amino acid; Gluc. transferase : glucosyl transferase; Beta-Gluc. : beta-glucosidase; Glut., glutathione; PR, pathogenesis-related; RGA, resistance gene analogue; RLK, receptor-like kinase.

performed to assess the differential gene expression between non-inoculated NILs (NR₀/NS₀). A total of 873 genes was detected, including 31 genes that also belong to the DEGs after *A. euteiches* inoculation. Among them, 783 genes (almost 90%) were at least two-fold more strongly expressed in NR, thus highlighting a major quantitative difference between the two NILs. Taken together, the pathogen-induced and constitutively expressed genes represent a final set of 1198 DEGs (Table S5, see Supporting Information) between the NILs, which can be associated with the allele differences at the *prAe1* locus. Functional analyses of these DEGs are presented in Fig. 4. Their distribution at the different time points is shown in Fig. 4A, which highlights the greater number of genes more strongly expressed in NR. Hierarchical clustering was then used to compare the regulation of the 1198 DEGs in NR and NS

(Fig. 4B). This revealed a close similarity between the constitutive expression of DEGs in non-inoculated plants (NR₀/NS₀) and the expression of these genes in NS at 1 dpi (NS₁) and 6 dpi (NS₆). For example, 89% of DEGs under-expressed in NR₀ were repressed in NS₁ and unchanged in NR₁. Similarly, 55% of DEGs over-expressed in NR₀ were induced in NS₁, whereas 83% were unchanged in NR₁, thus explaining the striking reduction in the number of DEGs following infection with *A. euteiches* (872 DEGs in non-inoculated lines versus 90 DEGs at 1 dpi; Fig. 4A). These results suggest that a significant proportion of the genes whose expression is modified in NS after *A. euteiches* inoculation are constitutively similarly regulated in NR.

We performed a hypergeometric test to identify the functional categories that were significantly enriched among the 1198 DEGs

Fig. 4 Transcriptome analysis of differentially expressed genes (DEGs) between resistant (NR) and susceptible (NS) lines. (A) Number of DEGs at 0 (non-inoculated), 1 and 6 days post-inoculation (dpi). The non-inoculated category corresponds to genes that were differentially expressed between NR and NS in control conditions. DEGs at 1 and 6 dpi were identified among the 4122 genes of the specific response. A gene was differentially expressed when the $\log_2$ ratio of NR_x/NS_x was greater than 1 or smaller than -1 . (B) Hierarchical clustering performed on constitutive DEGs using the Ward agglomeration method and Pearson's correlation coefficient. (C) Significance of the most enriched functional categories in the 783 and 89 genes significantly over-expressed in NR and NS, respectively, among the genes identified in non-inoculated plants. The broken line represents the Bonferroni threshold ($P < 4.9 \times 10^{-4}$). ni, non-inoculated. (D) Significance of the most enriched functional categories in genes detected as significantly over-expressed in NR or NS at 1 or 6 dpi. The broken line represents the Bonferroni threshold ($P < 4.9 \times 10^{-4}$). AGP, arabinogalactan protein; PR, pathogenesis-related; RGA, resistance gene analogue.

(Table S6, see Supporting Information). Figure 4C presents the five sub-Bins that were significantly detected under the Bonferroni threshold ($P < 4.9 \times 10^{-4}$) among the set of DEGs in non-inoculated NILs. The five detected gene categories were all more highly expressed in NR. There were no significant classes more highly expressed in NS. According to the Mapman classification, NR displayed the constitutive expression of genes directly related to plant defences: PR proteins, resistance gene analogues, flavonoid biosynthesis and a significant number of kinases. The significant detection of genes involved in transcription, together with an over-representation of genes encoding enzymes belonging to energetic processes, probably indicates that NR has constitutively higher catabolic activity than NS.

Figure 4D shows the categories of genes (sub-Bins) that are over-represented in the 356 genes that are specifically expressed in NR or NS on *A. euteiches* infection at 1 and 6 dpi. Table 1 complements Fig. 4C, D by showing the most highly DEGs with a known function between NR and NS lines at 0, 1 and 6 dpi. Many of these genes belong to the most significantly detected sub-Bins. At the early time point of infection, genes encoding enzymes involved in the modification of secondary metabolites (β -glucosidases), PR proteins and nucleotide binding site-leucine-rich repeat (NBS-LRR) genes were only detected in NR plants, together with genes encoding cell wall proteins. Interestingly, this latter category is exclusively represented by five arabinogalactan proteins (AGPs), proteins already known to be involved in the control of the early infection of pea roots by *A. euteiches* (Cannesan *et al.*, 2012). These genes are more highly expressed in NR. Genes encoding peroxidases or enzymes associated with sulfur redox reactions were the only categories detected in NS.

Six days after inoculation, over-represented genes in NR were involved in defence reactions, whereas over-represented genes in NS were related to redox reactions. The analysis highlights the importance of genes involved in secondary metabolism in NR, whether they are encoding enzymes leading to medicarpin synthesis (flavonoids) or to lignin synthesis (phenylpropanoids). More than 60% of the secondary metabolism-related genes (26 of 42) were directly involved in flavonoid biosynthesis or conjugation. These included genes involved in dihydroflavonol and flavone biosynthesis, as well as chalcone and isoflavonoid pathways, including an isoflavonol reductase, three chalcone synthases and three chalcone *O*-methyltransferases (ChOMTs), which were among the most highly DEGs at 6 dpi in NR plants. Genes encoding glucosidases and glucosyl-transferase, known to preferentially modify flavonoids (Modolo *et al.*, 2007), were also overexpressed. Cytochrome P450 genes were among the most significantly detected sub-Bins at 6 dpi in NR plants. Significant signalling Bins included kinases and genes involved in calcium-related signalling, such as calmodulins. The jasmonic acid (JA) signalling sub-Bin was significant for NR plants at 6 dpi and included genes for three lipoxigenases and a COI1 F-box protein (Mtr.41830.1.S1_at). A

hydroxyjasmonate sulfotransferase gene (Mtr.12550.1.S1_at) was among the three most DEGs in NR plants at the three analysed time points. Among the 95 genes detected as more highly expressed in NS plants at 6 dpi, three sub-Bins corresponding to ferredoxins, thioredoxin and peroxiredoxin were significantly detected, together with the sub-Bin of lipid transfer proteins. The abscisic acid sub-Bin was also identified as enriched in the susceptible NIL.

To validate microarray data, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was performed to analyse the relative expression of 15 genes belonging to major functional classes detected in the common response (Table S2) and 10 DEGs (Table S5). There was a high correlation between qRT-PCR and microarray gene expression results, as gene expression levels and variations were similar between the two methods for 93% of the analysed time points and genes (Table 2).

Flavonoids are major components in NR plant defences

The induction of genes involved in secondary metabolism, especially in the flavonoid pathway, was highlighted by microarray and qRT-PCR experiments. These genes were strongly induced after *A. euteiches* inoculation at 1 and 6 dpi in both NILs (Fig. 3B), and their differential expression between NR and NS was a hallmark in the transcriptome of the resistant NIL, which showed a significantly greater number of DEGs in this class of genes in non-inoculated plants and at the two time points after inoculation. To further investigate the role of flavonoids in defence, we determined the total flavonoid content in NR and NS roots at 0, 1 and 6 dpi using the aluminium chloride titration method on the same material as used for Affymetrix RNA analysis. Total flavonoid levels were significantly higher in resistant roots for every time point tested (Fig. 5A). Basal flavonoid content ranged from 2.3 $\mu\text{g}/\text{mg}$ dry weight in NS to 3.85 $\mu\text{g}/\text{mg}$ dry weight in NR. After infection, a slight decrease in flavonoid content was seen for both lines, but NR still displayed a two-fold greater amount of flavonoid than NS. The higher constitutive flavonoid level was confirmed in younger roots by diphenylboric acid (DPBA) staining of root endogenous flavonoids (Fig. 5B). DPBA signal intensity was quantified using image analysis software, and significantly higher levels of fluorescence were observed in NR relative to NS roots. DPBA conjugated compounds were homogeneously distributed in NR root tips and the elongation zone, whereas, in NS, DPBA fluorescence was restricted to the root meristem. Taken together, transcriptomic and biochemical analyses stress the significant differences in flavonoid content between the resistant and susceptible NILs, which are directly associated with the resistance alleles at the *prAe1* locus.

To assess the putative role of these compounds in resistance to *A. euteiches*, we measured the antimicrobial activity of one flavonoid compound which is likely to be differentially synthesized by NR given the transcriptome results. Three genes encoding

Table 1 Most highly differentially expressed annotated genes between resistant (NR) and susceptible (NS) lines in the non-inoculated condition (0) and/or at 1 and 6 days post-inoculation.

Gene ID*	NR vs. NS†			Sub-Bin name‡	Description (homology)
	0 dpi	1 dpi	6 dpi		
Mtr.16873.1.S1_s_at	-0.04	0	2.39	Secondary metabolism.phenylpropanoids	O-Methyltransferase family 2 protein
Mtr.18067.1.S1_at*	1.24	0.63	2.6	Secondary metabolism.phenylpropanoids	Caffeate O-methyltransferase (ATOM1)
Mtr.9254.1.S1_at	0	0	1.67	Secondary metabolism.phenylpropanoids	Phenyl ammonia lyase (PAL)
Mtr.24231.1.S1_at	0	0.31	2.18	Secondary metabolism.isoflavonoids	Isoflavone reductase
Mtr.27396.1.S1_at*	1.91	-0.58	0	Secondary metabolism.flavonoids.chalcones	Isoliquiritigenin 2'-O-methyltransferase
Mtr.10220.1.S1_s_at*	3.4	0.57	2.6	Secondary metabolism.flavonoids	Isoliquiritigenin 2'-O-methyltransferase
Mtr.9392.1.S1_at	0	0.44	2.96	Secondary metabolism.flavonoids	Flavonoid 3'-hydroxylase
Mtr.43075.1.S1_at	4.1	3.92	0	Secondary metabolism.Beta glucosidases	β-Glucosidase
Mtr.27713.1.S1_at	0	0	1.99	Misc.cytochrome P450	Cytochrome P450 (CYP71A23 family)
Mtr.28255.1.S1_at	0	0	1.91	Misc.cytochrome P450	Cytochrome P450 (CYP71A26 family)
Mtr.1553.1.S1_at	2.19	2.63	0.55	Misc.cytochrome P450	Cytochrome P450 (CYP93D1 family)
Mtr.51253.1.S1_at*	2.53	2.94	1.01	Stress.biotic.PR-proteins	Disease resistance protein (CC-NB ARC-LRR)
Mtr.10871.1.S1_at	2.26	-0.04	0.3	Stress.biotic.PR-proteins	Disease resistance protein (TIR class)
Mtr.37670.1.S1_at	2.21	1.6	1.36	Stress.biotic.PR-proteins	Disease resistance protein (NBS-LRR class)
Mtr.21036.1.S1_at	1.96	1.83	1.9	Stress.biotic.PR-proteins	Disease resistance protein (RPS5)
Mtr.37670.1.S1_x_at	1.89	1.71	1.12	Stress.biotic.PR-proteins	Disease resistance protein (NBS-LRR class)
Mtr.10285.1.S1_at	1.64	2.21	0.88	Stress.biotic.PR-proteins	Disease resistance protein
Mtr.50996.1.S1_at*	1.37	1.09	0.35	Stress.biotic.PR-proteins	Disease resistance protein (CC-NB ARC-LRR)
Mtr.23865.1.S1_at	0	-1.35	-0.78	Stress.biotic.PR-proteins	Disease resistance protein (NBS-LRR class)
Mtr.20427.1.S1_at	0.15	0	3.75	Stress.biotic.PR-proteins	Disease resistance protein (CC-NB ARC-LRR)
mtr.19911.1.s1_at	0	0.03	3.7	Stress.biotic.PR-proteins	β-1,3-Glucanase 2 (BGL2)
Mtr.10750.1.S1_at	6.66	5.9	4.77	Kinase	Serine/threonine protein kinase
Mtr.11970.1.S1_at	0.03	0.64	1.88	Signalling.receptor kinases.DUF 26	Lectin protein kinase family protein
Mtr.26331.1.S1_at	2.31	1.98	2.25	Signalling.receptor kinases.DUF 26	Protein kinase
Mtr.21535.1.S1_at	0.26	0	-1.47	Protein.posttranslational modification	Protein kinase (STN8)
Mtr.27929.1.S1_at	0.06	0	1.63	Signalling.calcium	Calmodulin-dependent protein kinase (CPK2)
Mtr.45488.1.S1_at	0	0	2.08	Signalling.calcium	Calmodulin binding
Mtr.14057.1.S1_s_at	3.46	0.1	0	RNA.transcription	RNA polymerase β' subunit-1
Mtr.20282.1.S1_s_at	2.77	1.45	0	RNA.transcription	RNA polymerase β' subunit-2
Mtr.18232.1.S1_s_at	4.85	1.67	0	PS.lightreaction.ATP synthase	β Subunit of ATP synthase
Mtr.45597.1.S1_s_at	2.56	0.86	0	PS.cyclic electron flow-chlororespiration	NADH dehydrogenase ND1
Mtr.45606.1.S1_s_at	2.7	1.2	0	Protein.synthesis.ribosomal protein	50S Ribosomal protein
Mtr.12550.1.S1_at*	6.05	4.87	4.41	Lipid metabolism	Hydroxyjasmonate sulfotransferase 2A (ST2A)
Mtr.41830.1.S1_at	0.85	0.55	1.57	Protein.degradation.ubiquitin.FBOX	Fbox-Coronatine Insensitive 1 (COI1)
Mtr.8439.1.S1_s_at	0	0.37	1.82	Hormone metabolism.jasmonate.synthesis	Lipoxygenase
Mtr.44955.1.S1_s_at	0.98	-0.33	-1.64	Hormone metabolism.abscisic acid.regulated	ABA-responsive protein-related
Mtr.51607.1.S1_at	0	1.27	0	Cell wall.cell wall proteins.AGPs	Fasciclin-like arabinogalactan protein (FLA12)
Mtr.13136.1.S1_s_at	-0.68	1.31	0	Cell wall proteins.AGPs	Fasciclin-like arabinogalactan protein (FLA12)
Mtr.17361.1.S1_at	0	1.26	0	Cell wall proteins.AGPs	Fasciclin-like arabinogalactan protein (FLA11)
Mtr.45615.1.S1_s_at	2.77	1.49	0	Protein.degradation	Caseinolytic protease
Mtr.40121.1.S1_at	0	-1.06	0	Misc.peroxidases	Peroxidase 22 (PER22)-class III
Mtr.8179.1.S1_at	0.09	-1.15	0	Misc.peroxidases	Haem peroxidase
Mtr.45535.1.S1_at	-0.01	-1.16	-0.76	S-assimilation	Adenylylsulfate kinase
Mtr.50907.1.S1_at	0	-1.46	-0.61	Misc.gluco-. galacto- and mannosidases	Glycoside hydrolase
Mtr.27921.1.S1_at	0.03	-0.25	-1.05	Seed storage/lipid transfer protein	Lipid transfer protein-related
Mtr.40926.1.S1_at	0.32	0.06	-1.07	Redox.thioredoxin	Thioredoxin family protein
Mtr.42854.1.S1_at	0.38	-0.11	-1.14	Redox.thioredoxin	Thioredoxin (TRX-M4)
Mtr.22013.1.S1_s_at	0.32	0	-1.04	Redox.peroxiredoxin	ATPRX Q antioxidant/peroxiredoxin
Mtr.12512.1.S1_at	0.24	-0.86	-1.1	Misc.glutathione S transferases	Glutathione-S-transferase (ATGSTF9)
Mtr.12060.1.S1_at	1.84	1.39	0.66	Cell.organization	Annexin (ANNAT7) calcium ion binding
Mtr.5869.1.S1_at	-0.06	-1.08	-0.84	Transport.sugars	Carbohydrate transmembrane transporter
Mtr.10081.1.S1_s_at	0.69	-1.38	-0.41	Transport.metal'	Metal transporter family protein

ABA, abscisic acid; AGP, arabinogalactan protein; ARC, CC, coiled coil; LRR, leucine-rich repeat; NB, nucleotide binding; NBS, nucleotide binding site; PR, pathogenesis-related; PS, photosystem; TIR, Toll Interleukin-1 receptor-like domain.

*Gene identities are the Affymetrix ID for each selected probe. Selected genes are genes belonging to the top 20 of the most highly differentially expressed (and annotated) genes between resistant (NR) and susceptible (NS) near-isogenic lines (NILs) in at least one of the three analysed conditions [non-inoculated plants, 1 or 6 days post-inoculation (dpi)].

†Indicated values are log₂ expression ratios. A value of >1 indicates that the gene is at least two-fold more strongly expressed in NR than in NS, whereas a value of <-1 shows a gene two-fold more strongly expressed in the susceptible line. Bold highlight genes with ratios (NR/NS) that are among the first twenty highest positive or negative values at each analysed time point.)

‡Sub-Bin classification was provided by Mapman software.

Table 2 Validation of transcriptome results by quantitative reverse transcription-polymerase chain reaction (qRT-PCR).

Genes commonly regulated (selected from Table S3)										
Affymetrix ID	Functional class	Gene function	NR1/NR0		NR6/NR0		NS1/NS0		NS6/NS0	
			RT-PCR*	Array†	RT-PCR*	Array†	RT-PCR*	Array†	RT-PCR*	Array†
Mtr.14428.1.S1_at	Flavonoid	Chalcone synthase	1.83	2.47	1.41	2.01	2.09	3.42	1.98	3.37
Mtr.17610.1.S1_at	Flavonoid	Chalcone synthase	2.73	2.29	2.03	2.11	2.80	2.82	1.75	2.33
Mtr.19752.1.S1_at	Flavonoid	Anthocyanin acyltransferase	2.57	3.00	2.00	2.24	3.96	4.06	2.76	2.80
Mtr.10968.1.s1_at	PR proteins	Osmotin/thaumatin	2.56	2.40	3.78	4.54	2.83	3.24	5.46	6.37
Mtr.40555.1.s1_at	PR proteins	Osmotin/thaumatin	2.20	2.23	3.16	3.38	2.19	2.65	2.25	3.05
Mtr.30325.1.s1_at	Resistance gene	Resistance protein	0.81	0.05	3.80	2.97	1.40	0.68	3.67	3.42
Mtr.34600.1.s1_at	PR proteins	Peroxidase	-0.18	-0.27	-2.51	-3.03	-0.60	0.44	-2.78	-2.72
Mtr.37598.1.s1_at	PR proteins	Peroxidase	-1.10	-0.90	-4.49	-4.67	-0.51	-0.22	-3.73	-3.94
Mtr.17914.1.s1_at	PR proteins	Thaumatin	-0.78	-1.19	-1.94	-2.54	-1.33	-1.43	-1.88	-2.41
Mtr.23284.1.s1_at	Ca ²⁺ signalling	Calcium-binding EF hand	2.00	2.10	2.89	2.90	1.86	2.40	1.15	1.25
Mtr.19570.1.s1_at	Ethylene signalling	ERF1	3.88	3.39	3.99	3.16	3.74	4.13	3.07	3.38
Mtr.27549.1.s1_at	Regulation of transcription	WRKY72	0.84	0.18	-3.58	-2.73	-0.67	0.05	-4.78	-2.71
Mtr.20368.1.S1_at	Cell organization	ANNAT4 annexin	0.32	-0.11	-2.79	-4.01	-0.26	-0.27	-4.11	-4.40
Mtr.13100.1.s1_at	Transporter	Amino acid transporter	0.24	0.21	-2.36	-2.95	0.38	-0.08	-2.96	-4.21
Mtr.7226.1.s1_at	Transporter	Amino acid transporter	0.49	0.33	-2.53	-2.72	0.55	0.25	-3.25	-3.68
Genes differentially expressed in NR or NS (selected from Table S6)										
Affymetrix ID	Functional class	Gene function	NR0/NS0		NR1/NS1		NR6/NS6			
			RT-PCR*	Array†	RT-PCR*	Array†	RT-PCR*	Array†		
Mtr.10220.1.S1_s_at	Flavonoid	Chalcone- <i>O</i> -methyltransferase	3.80	3.40	0.23	0.57	2.17	2.60		
Mtr.18067.1.S1_at	Flavonoid	Chalcone- <i>O</i> -methyltransferase	2.82	1.24	1.94	0.63	0.78	2.60		
Mtr.27396.1.S1_at	Flavonoid	Isoliquiritigenin 2'- <i>O</i> -methyltransferase	1.96	1.91	1.61	-0.58	-0.57	0.00		
Mtr.28714.1.S1_at	Flavonoid	Chalcone synthase	1.09	1.08	0.62	0.12	-0.80	-0.58		
Mtr.19752.1.S1_at	Flavonoid	Anthocyanin acyltransferase	1.07	1.18	-0.32	0.12	0.31	0.62		
Mtr.13960.1.S1_at	Flavonoid	Flavonoid 3' hydroxylase	0.53	0.00	-0.72	0.40	9.23	2.44		
Mtr.51253.1.s1_at	Resistance gene	Resistance protein	4.47	2.53	4.01	2.94	3.06	1.01		
Mtr.19911.1.s1_at	PR protein	β-1,3-Glucanase 2 (BGL2)	1.07	0.00	0.63	0.03	4.60	3.70		
Mtr.12550.1.s1_at	Lipid metabolism	Hydroxyjasmonate sulfotransferase 2A (ST2A)	4.86	6.05	4.55	4.87	5.43	4.41		
Mtr.44234.1.s1_at	Not assigned	Unknown	5.39	4.43	4.71	4.33	4.69	4.09		

ERF1, Ethylene Response Factor 1; PR, pathogenesis-related.

*Indicated numbers are log₂ ratios calculated for qRT-PCR with the 2^{-ΔΔCt} method of Livak and Schmittgen (2001).†Numbers were reported from Tables S3 and S6 following normalization of the raw microarray data and can therefore be compared with log₂ ratios.Induced genes (log₂ > 1) are in dark green boxes, whereas repressed genes (log₂ < -1) are in bold. Ratios inside light green boxes show selected genes that are at least two-fold more strongly expressed in resistant (NR) than in susceptible (NS) lines.

ChOMT were indeed more highly expressed in NR roots, one being the most differentially expressed flavonoid-related gene. This enzyme catalyses the methylation of isoliquiritigenin to produce 2'-O-methylisoliquiritigenin (Maxwell *et al.*, 1993). The effects of these two compounds were therefore tested on the germination of *A. euteiches* zoospores in two replicated experiments. The low concentration of 18.5 μM isoliquiritigenin had no inhibitory effect on the germination of *A. euteiches* zoospores, whereas 2'-O-methylisoliquiritigenin caused a 72%–86% decrease in the rate of germination (Fig. 4C), illustrating the antimicrobial activity of this flavonoid on *A. euteiches*.

DISCUSSION

Mycelium spread and *A. euteiches* life cycle are hampered in NR

The main objective of this work was to better understand the molecular basis underlying the partial resistance to *A. euteiches*

linked to the major QTL *prAe1* (Djébali *et al.*, 2009). To achieve this goal, we compared two F₁₂ NILs, NR and NS, whose genomes differ only by the presence of the parental A17 (partially resistant) or F83005.5 (susceptible) allele in the first 5 Mb of the distal part of chromosome 3 (about 2% of the genome) spanning *prAe1*. The analysis of symptom development, molecular detection of *A. euteiches* and cytological observations discriminated between the responses of NR and NS after *A. euteiches* inoculation. These results confirmed that the A17 allele at the *prAe1* locus was sufficient to confer a partial resistance phenotype, associated with a delay in mycelium spread and reproduction of *A. euteiches*, as no or only a very few oospores were observed in NR at 6 dpi. Protection of the stele against *A. euteiches* invasion, observed in the A17 parental line (Djébali *et al.*, 2009), was conserved in NR, together with a marked restriction of symptomatic brown root tissues. These features were never observed in NS, in which roots and stems were fully colonized by mycelium, indicating that hyphae need to first invade the stele to spread into aerial tissues. The massive invasion of mycelium led to a high mortality rate (80%) in NS plants.

The *prAe1* QRL is associated with constitutive expression of defence and signalling genes

To unravel the molecular mechanisms involved in *prAe1*-mediated resistance, transcriptomes of both NILs were compared between

Fig. 5 Differential accumulation of flavonoids in roots of resistant (NR) and susceptible (NS) lines. (A) AlCl_3 quantification of total flavonoid levels in roots of NR and NS following methanol extraction. Root samples used for flavonoid quantification and Affymetrix RNA extraction were collected in the same triplicate experiments. Each bar represents the mean of three independent experiments with at least 15 plants per sample. (B) Diphenylboric acid (DPBA) staining and quantification of flavonoids in non-inoculated young seedlings of NR and NS. Left: bright field and short-pass green fluorescent protein (GFP) filter images of seedling roots (bar, 1 mm). Right: DPBA signal intensity quantified using Image J image analysis software. (C) Effect of isoliquiritigenin and 2'-O-methylisoliquiritigenin treatment ($18.5 \mu\text{M}$) on germination of *A. euteiches* zoospores. Results of two separate experiments in which 20 and 15 drops of zoospores mixed with the different compounds were observed to calculate a germination rate. Means of each condition were compared by a χ^2 test. (** $P < 0.01$).

non-inoculated plants and plants at 1 and 6 dpi, two key time points of infection in which the mycelium penetrates the rhizodermis and then the central cylinder in susceptible plants. Both NILs were able to mount a common defence response which included genes encoding receptor-like kinases, calcium-associated proteins and calmodulin-related proteins, all well known to be involved in early biotic stress signalling (Ma and Berkowitz, 2011), together with a large number of TF gene families. Genes for 15 WRKY TFs were identified, including homologues shown to be positive regulators of plant immunity, such as WRKY75 (Encinas-Villarejo *et al.*, 2009) and WRKY33 (Birkenbihl *et al.*, 2012). The activation of this latter gene might be linked to ethylene signalling, as observed in *Arabidopsis* (Li *et al.*, 2012). This would be consistent with the regulation of this hormonal pathway 1 and 6 days after *A. euteiches* infection, with the activation of PR proteins (chitinases, glucanases or osmotins) and genes involved in secondary metabolite pathways. Genes in the flavonoid and phenylpropanoid pathways were the most significantly induced and enriched at both time points, and constitute a well-studied defensive arsenal in *M. truncatula* (Naoumkina *et al.*, 2008; Uppalapati *et al.*, 2009). The strong induction of secondary metabolism in common responses between both NILs was accompanied by a significant down-regulation of primary nitrogen and carbon metabolism, probably illustrating a trade-off between defence and growth (Neilson *et al.*, 2013).

Although this common defence-like response indicates that both lines are able to perceive *A. euteiches*, the susceptibility of NS clearly shows that it is not sufficient to control this oomycete. Searching among the genes that were differentially expressed (DEGs) between NR and NS gave clues to the mechanisms that could explain the partial resistance of NR. The analysis of non-inoculated NILs identified a large group of DEGs. The majority of genes constitutively expressed at a high level in NR were induced at 1 and/or 6 dpi in NS and, conversely, genes found to be constitutively under-expressed in NR were repressed in NS following infection. Taken together, these results suggest that a significant part of

the defence responses induced in NS on *A. euteiches* inoculation are already active in NR. The identification of a large set of genes more highly expressed in one NIL versus another is not common. A comparison of resistant and susceptible NILs of tomato indicated that their transcriptomes were indistinguishable in non-inoculated lines, whereas several hundreds of DEGs were revealed after inoculation with *Phytophthora infestans* (Cai *et al.*, 2013). Similarly, DEGs between NILs were identified only after inoculation of *Fusarium graminearum* in wheat (Schweiger *et al.*, 2013) or in maize (Ye *et al.*, 2013). However, in an RNA-seq analysis of soybean NILs partially resistant or susceptible to *Xanthomonas axonopodis* pv. *glycines*, a set of 1654 DEGs was significantly detected in non-inoculated plants, among which 86% were more expressed in the resistant NIL (Kim *et al.*, 2011). Among the genes that were constitutively more highly expressed in NR, we detected significant enrichment in functional classes directly related to biotic stress resistance. This included genes associated with pathogen perception and signalling [receptor-like kinases (RLKs), NBS-LRR genes, NADPH oxidases, TFs, etc.] or genes that led to the synthesis of antimicrobial compounds (PR proteins, secondary metabolites, etc.). A strong correlation was therefore observed between NR partial resistance and a preformed expression of defence-related genes. A similar feature was also observed for partial resistance to rice blast by analysing the expression of a small set of 21 defence genes in 23 natural accessions displaying various levels of resistance to *Magnaporthe oryzae* (Vergne *et al.*, 2010). Natural variations in the level of defence induction have been reported among plants and could be at the origin of several defence strategies proposed by Ahmad *et al.* (2010), which allow plants to cope with the biotic pressure of their environment. In our case, the defence strategy conferred by *prAe1* leads to a 'constitutively primed immune system', which can allow plants to quickly respond to an aggressive and fast-spreading pathogen.

prAe1* regulates secondary metabolism pathways, a key weapon to control *A. euteiches

Constitutively expressed defences were not the only strategy used against *A. euteiches*, as partial resistance was also linked to a higher level of defence gene expression at 6 dpi. This elicitation might be specifically triggered by JA, as genes associated with this hormonal pathway were significantly enriched in NR, but not in NS, after *A. euteiches* infection. Jasmonate is, for example, a well-known elicitor of the phenylpropanoid/flavonoid pathway, as reported previously in a cell suspension of *M. truncatula* (Naoumkina *et al.*, 2008). In agreement with this, among the NR-specific functional categories detected after *A. euteiches* inoculation, this secondary metabolism pathway was the most significantly enriched (Fig. 4D). Twenty-five genes of this class, mainly involved in the first steps of the flavonoid pathway (chalcone synthase, ChOMT, isoflavone reductase, flavonoid synthase, flavonoid hydroxylase, etc.), were more expressed in NR

compared with only two in NS. The key role of this pathway in partial resistance was also strengthened by the significant detection of 19 genes coding for cytochrome P450 overexpressed in NR at 6 dpi (versus zero in NS), as members of this class of protein are known to convert flavones into isoflavones, or to participate in the synthesis and modification of flavonoids/isoflavonoids (Modolo *et al.*, 2007). Several compounds produced in this well-known pathway have been found to show antimicrobial properties (Naoumkina *et al.*, 2010), including against *A. euteiches*, as demonstrated with the phytoalexin pisatin in the border cells of pea (Cannesan *et al.*, 2011). We have shown previously that the phenylpropanoid pathway leading to the synthesis of lignin plays a major role in *A. euteiches* resistance in A17, scavenging the ROS produced during the interaction, strengthening the endodermis and preventing pathogen penetration into the stele (Djébali *et al.*, 2009, 2011). In this work, we validated the key role of the genes involved in the flavonoid pathway that were overexpressed in NR by confirming their higher level of expression through qRT-PCR and showing the higher synthesis/accumulation of these compounds in NR at each time point. To go further and illustrate the potential antimicrobial action of this category of compounds on *A. euteiches*, we focused notably on ChOMTs, because three genes encoding these enzymes were found to be more strongly expressed in NR. Moreover, one of the corresponding proteins has been specifically detected in A17 roots infected by *A. euteiches* through a proteomic analysis (Colditz *et al.*, 2004). In this work, we showed that 2'-*O*-methylisoliquiritigenin, the metabolite produced by this enzyme, also known to be a powerful inducer of *Rhizobium meliloti* nodulation genes (Maxwell *et al.*, 1993), can significantly delay *A. euteiches* development at 18.5 μM . The antimicrobial property of this compound therefore seems to be more efficient than pisatin, as this latter compound had no effect on oospore germination or mycelium development of *A. euteiches* at about 80 μM (25 $\mu\text{g/mL}$) (Cannesan *et al.*, 2011).

CONCLUSIONS

Successful infection by *A. euteiches* in NS probably relies on rapid penetration into root cortical cells, enabling further access to the root vascular system before a threshold of antimicrobial compounds has accumulated. The pre-activation of pathogen monitoring systems and defence signalling elements, together with preformed defence metabolites in NR, enable these plants to rapidly deploy an efficient defensive state that hampers pathogen development. Defences can be re-amplified following pathogen penetration of the cortex to protect the central cylinder, ultimately leading to partial resistance.

Given the close genetic similarity between NR and NS, we therefore infer that differential basal and induced defences are conferred by *prAe1*, which spans two close, but independent, loci. The best candidate genes for these loci are genes encoding an F-box protein and a MYB TF (Bonhomme *et al.*, 2014). Either

protein could be responsible for the large number of DEGs: the F-box gene by controlling the specific degradation of TFs or resistance genes that activate the defence responses, such as *CPR1/CPR30* in *Arabidopsis* (Cheng *et al.*, 2011); or the MYB gene by direct transcriptional regulation, especially of genes involved in secondary metabolism pathways, as already shown following an elicitation by flg22 (Schenke *et al.*, 2011). Although GWAS showed that both loci were involved in resistance, a future challenge will be to identify whether resistance is explained by one or both genes. Functional analyses of candidate genes are currently underway to address this issue.

EXPERIMENTAL PROCEDURES

Plant material and growth conditions

NR and NS are *M. truncatula* (Gaertn.) F₁₂ NILs that were obtained following three rounds of self-fertilization of the F₉ heterogeneous inbred family (HIF) #LR5-5A and #LR5-5F lines, previously described by Djébali *et al.* (2009). Genotyping results of NR and NS plants with some molecular markers of the (A17 × F83005.5) genetic map (Ben *et al.*, 2013) are presented in Table S8 (see Supporting Information), and indicate that the two NILs differ only in the very distal part of chromosome 3 (approximately 5 Mb) which spans the *prAe1* region. NR has alleles from the A17 partially resistant parental line at the *prAe1* locus, whereas NS has alleles of the F83005.5 susceptible parental line in the same region. The rest of the genome (approximately 98%) is identical and homozygous (at the tested loci) between the two NILs and contains a combination of A17 and F83005.5 loci. Plants were grown *in vitro* on M medium under conditions of 16 h light at 22 °C and 8 h darkness at 20 °C, as described previously (Djébali *et al.*, 2009).

Inoculation procedure

Aphanomyces euteiches Drechs. strain ATCC 201684, a pea isolate, was used for the infection of *M. truncatula* seedlings. Zoospores were produced according to Badreddine *et al.* (2008) and adjusted to 10⁵ spores/mL. For *in vitro* assays, seedlings were inoculated 1 day after transfer onto M medium with a 5-μL droplet of zoospore suspension deposited on the middle of the root. To compare NR and NS resistance, three spatially and temporally independent repeats were performed with 15 inoculated plants per genotype in each repeat. For microarray experiments, plants were grown for 15 days before inoculation to allow sufficient root mass for RNA extraction. The whole root system was inoculated with 1 mL of zoospore suspension per plant for homogeneous infection of the root.

Phenotyping

Plates with infected plants were scanned at 6, 15 and 21 dpi at 24 bits and 300 dots per inch resolution with a Perfection V700 photo scanner (Seiko EPSON, Nagano, Japan). The percentage of symptomatic tissues from the bottom of the primary root to the top of the shoot was calculated by image analysis software (Image Pro-Plus, Media Cybernetics, Silver Spring, MD, USA) using the trace feature function for every single plant. The number of dead plants was obtained by counting plants without any remaining green tissues.

Sample preparation for microscopy

Confocal images were acquired with a spectral confocal laser scanning system (SP2 AOBS, Leica, Solms, Germany) equipped with an upright microscope (DM 6000, Leica). Observations were made using 20× (HCX APO, N.A. 0.5) water objectives. A laser diode (emission, 405 nm) and an argon laser (emission, 488 nm) were used to collect autofluorescence of the cell wall in the range 420–480 nm, blue look-up table (LUT), and 500–550 nm, green LUT, respectively. Cell walls of *A. euteiches* were labelled using wheat germ agglutinin (WGA) coupled with Alexa Fluor® 633 [or fluorescein isothiocyanate (FITC), as described previously (Djébali *et al.*, 2009)]. Samples were immersed in 50 μg/mL WGA–Alexa (or WGA–FITC) for 60 min and then washed three times in phosphate-buffered saline for 10 min. Fluorescence was collected in the range 635–720 nm after excitation by an He–Ne laser at 633 nm. Autofluorescence and fluorescence images were acquired simultaneously in dual mode; the overlay images were computed by projection of 30–40 plan-confocal images acquired in the Z dimension with an increment of 1 μm.

Total flavonoid quantification by the AlCl₃ method

Root tissues were ground in liquid nitrogen and resuspended in 80% methanol (10 mL/g fresh weight). After 30 min of incubation at room temperature, samples were sonicated for 2 min (Bioruptor Scientific 88169 Illkirch, France) and centrifuged at 2000 *g* for 20 min. The pellet was used for the estimation of dry weight and the supernatant was used for the quantification of flavonoids. The total flavonoid content was estimated using the AlCl₃ method (according to Ayoola *et al.*, 2008). Briefly, 1 mL of the supernatant was added to 1 mL of 2% AlCl₃ and incubated for 30 min at room temperature. The absorbance at 430 nm was recorded and total flavonoid contents were obtained from the regression equation of the calibration curve of rutin, and expressed as rutin equivalent.

DPBA staining and quantification in root tips

Roots of germinated *M. truncatula* seeds (1-day-old) were immersed in 0.25% diphenylboric acid 2-amino ethyl ester with 2% dimethyl sulfoxide, 1% sucrose and 10 mM sodium phosphate, pH 6, for 3 min and immediately visualized with a fluorescent macroscope (AxioZoom V16, Zeiss, Oberkochen, Germany) equipped with a Zeiss AxioCam Hrc camera. The DPBA signal was acquired with a Zeiss Filter 38 (excitation BP, 440–470 nm; emission, 525–550 nm) and extracted by RGB split using Image J software. The mean pixel intensity of the green signal corresponding to DPBA was then quantified on a 1000-pixel root length on every image from two independent replicates of 20 and 15 plants, respectively.

RNA extraction and qRT-PCR

Total RNA was isolated from roots with the RNeasy Mini kit (Qiagen, Venlo, The Netherlands) followed by DNase treatment. RNA integrity was checked using a bioanalyser (Agilent Technologies, Santa Clara, CA, USA) and reverse transcription was performed with 3 μg of total RNA for each sample and SuperScript reverse transcriptase III (Invitrogen-Life Technologies, Carlsbad, CA, USA). qRT-PCR was performed in an ABI Prism SDS 7900HT system (Applied Biosystems, Foster City, CA, USA) as described previously (Djébali *et al.*, 2009). Each qRT-PCR was conducted in triplicate.

qRT-PCR primer sequences are given in Table S7 (see Supporting Information). An *M. truncatula* gene encoding histone-3 like (Medtr4g097170) was used to normalize plant gene expression or relative pathogen transcript abundances in each analysed sample, as described previously (Rey *et al.*, 2013). The gene encoding an *A. euteiches* α -tubulin was used to detect the amount of pathogen at 6 dpi. The $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001) was used to measure the relative amount of pathogen transcript accumulation.

Affymetrix array hybridization and data analyses

Total RNA was extracted using the RNeasy Kit (Qiagen). One microgram of total RNA was transcribed (as described in Experimental procedures S1) to produce labelled cRNA, which was used to hybridize to Affymetrix GeneChip® Medicago genome arrays at INRA URGV (Evry, France). The raw CEL files were imported in R software for data analysis (<http://www.r-project.org/>). Following normalization (as described in Experimental procedures S1), a gene was declared to be differentially expressed in a given condition if its *P* value was lower than the Bonferroni-corrected *P* value threshold of $0.05/N$, where *N* represents the number of *M. truncatula* genes (50 900) present on the chip. Genes with a fold change of more than unity were considered to be differentially regulated. Affymetrix datasets were deposited on Gene Expression Omnibus (GEO) under the accession number GSE31198. The Mapman BIN structure (Goffard and Weiller, 2006; Rey *et al.*, 2013) was used to classify and count statistically identified genes into each functional class. On the basis of these results, we performed a hypergeometric test to assess the significant enrichment of genes in different Bins or sub-Bins.

Test of zoospore germination

Commercial stock solutions (Biobiopha, Kunming, China) of isoliquiritigenin and methyl-isoliquiritigenin (12 mg/mL in 75% methanol) were diluted to 10 µg/mL with 2% bacto-peptone and 0.5% glucose liquid medium. The same dilution was applied to a 75% methanol solution (mock treatment). Each solution was mixed with an equivalent volume of zoospore suspension (85 000 zoospores/mL) in 1.5-mL sterile microcentrifuge tubes for a final concentration of 5 µg/mL for each compound. Each tube was vigorously vortexed for 30 s to induce zoospore encystment. For each test, 15–20 10-µL drops (one replicate) were spotted onto separate microscope glass slides, and incubated for 4 h at 21 °C. Glass slides were then stored at 4 °C to prevent further germination during counting. The zoospore germination rate was calculated on a 10× microscopic field located at the centre of each drop.

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SUPPORTING INFORMATION

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Experimental procedures S1 Microarray hybridization and analysis.

Table S1 Genes significantly induced or repressed in at least one time point in one line.

Table S2 List of 1404 genes belonging to the common response after *Aphanomyces euteiches* inoculation.

Table S3 Identification by a hypergeometric test of Bins and sub-Bins significantly enriched in genes belonging to the common response.

Table S4 List of 4122 genes belonging to the specific response after *Aphanomyces euteiches* inoculation at 1 and 6 days post-inoculation (dpi), and identification of the 356 differentially expressed genes (DEGs) among them.

Table S5 List of the 1198 genes differentially expressed between resistant (NR) and susceptible (NS) lines in non-inoculated conditions and/or in the specific response after inoculation.

Table S6 Identification of significantly enriched sub-Bins in resistant (NR) and susceptible (NS) lines with the 1198 differentially expressed genes (DEGs).

Table S7 Sequence of primers used for quantitative reverse transcription-polymerase chain reaction (qRT-PCR) experiments.

Table S8 Genotyping results of resistant (NR) and susceptible (NS) lines.