Partial Resistance of *Medicago truncatula* to *Aphanomyces euteiches* Is Associated with Protection of the Root Stele and Is Controlled by a Major QTL Rich in Proteasome-Related Genes

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Submitted 17 December 2008. Accepted 4 May 2009.

A pathosystem between *Aphanomyces euteiches*, the causal agent of pea root rot disease, and the model legume *Medicago truncatula* was developed to gain insights into mechanisms involved in resistance to this oomycete. The F83005.5 French accession and the A17-Jemalong reference line, susceptible and partially resistant, respectively, to *A. euteiches*, were selected for further cytological and genetic analyses. Microscopy analyses of thin root sections revealed that a major difference between the two inoculated lines occurred in the root stele, which remained pathogen free in A17. Striking features were observed in A17 roots only, including i) frequent pericycle cell divisions, ii) lignin deposition around the pericycle, and iii) accumulation of soluble phenolic compounds. Genetic analysis of resistance was performed on an F7 population of 139 recombinant inbred lines and identified a major quantitative trait locus (QTL) near the top of chromosome 3. A second study, with near-isogenic line responses to *A. euteiches* confirmed the role of this QTL in expression of resistance. Fine-mapping allowed the identification of a 135-kb sequenced genomic DNA region rich in proteasome-related genes. Most of these genes were shown to be induced only in inoculated A17. Novel mechanisms possibly involved in the observed partial resistance are proposed.

To counteract pathogen attack, plants have evolved a two-branched innate immune system (Jones and Dangl 2006). In the first branch, plant responses are linked to the detection of pathogen-associated molecular patterns (PAMPs) by trans-membrane pattern recognition receptors (Zipfel and Felix 2005), leading to PAMP-triggered immunity (PTI). Pathogenic microbes secrete effectors, molecules that can interfere with PTI and promote virulence. Coevolution of pathogens with their hosts resulted in the establishment of the second system that involves resistance (*R*) gene products that are able to detect pathogen effectors, leading to effector triggered immunity (ETI). ETI is very effective, usually pathogen-specific and generally associated with the onset of a hypersensitive cell death response (HR) at the infection site that rapidly halts the invading pathogen and leads to complete resistance.

While tremendous progress occurred during the past ten years towards deciphering the molecular bases of *R* gene resistance (Glazebrook 2005; Jones and Dangl 2006), the mechanisms underlying quantitative or partial resistance are poorly understood. In such a resistance, pathogen growth is significantly reduced, when compared with a fully susceptible line. Quantitative trait loci (QTL) associated with partial resistance to pathogens have been detected in numerous cultivated species (Ballini et al. 2008; Young 1996), but there is still a lack of studies aimed at identifying the gene or genes underlying the resistance mechanisms associated with these QTL. Since insufficient genomic data in the analyzed crops are probably the main limitations to such studies, establishing pathosystems between model plants and pathogens is a valuable strategy i) to decipher the mechanisms underlying resistance QTL and ii) to provide new molecular targets that may be used in crop breeding programs.

To gain insights into legume responses to pathogens, *Medicago truncatula* has emerged as a suitable model plant (Cook 1999). This diploid and autogamous alfalfa relative is a host for major grain and forage legume pathogens. Contrasted responses were detected among *M. truncatula* accessions follow-

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*The e-Xtra logo stands for “electronic extra” and indicates that two supplementary tables and two supplementary figures are published online.

ing inoculation with fungi and oomycetes (Colditz et al. 2005; Ellwood et al. 2006; Moussart et al. 2007; Tivoli et al. 2006; Torregrosa et al. 2004) or bacteria (Vailleau et al. 2007). These examples illustrate the high degree of biological variability of *M. truncatula* and justify the development of genetic maps and recombinant inbred lines (RIL) to identify genetic components of resistance to each biotic stress, as recently illustrated for major fungal legume pathogens (Ameline-Torregrosa et al. 2008b). The combination of genetic analyses with abundant genomic data (VandenBosch and Stacey 2003; Young et al. 2005) allows identification of the molecular components underlying quantitative resistance to legume pathogens. Among them, *Aphanomyces euteiches*, a soilborne oomycete (*Saprolegniaceae*), has a broad host range on crop and forage legumes (Gaulin et al. 2007; Moussart et al. 2008) and is the most limiting factor for pea production in Europe and, notably, in France (Pilet-Nayel et al. 2005). Genetic improvement of legume resistance remains the most promising approach to control *A. euteiches*. Several QTL for resistance to this pathogen were identified in pea (Pilet-Nayel et al. 2002, 2005). However, polygenic inheritance, a low level of resistance, a high level of pathogenic variability, and interactions with other soilborne pathogens hindered pea breeding programs (Wicker et al. 2003).

In this study, the *M. truncatula*/*A. euteiches* pathosystem was used to enhance understanding of legume defense mechanisms and make links between genetic and molecular components of quantitative resistance. Cytological analyses of two *M. truncatula* lines expressing partial resistance and a susceptible response following inoculation with *A. euteiches* revealed several mechanisms allowing the protection of the root central cylinder against pathogen invasion in the resistant line. Genetic analyses highlighted a major recessive QTL, the location of which was narrowed to a 135-kb genomic region that is rich in proteasome-associated genes. Based on the cytological, genetic, and molecular data, novel mechanisms that may account for the observed resistance are proposed.

RESULTS

Differential responses of *M. truncatula* lines to *A. euteiches*.

To follow the onset and development of symptoms on several lines, an in vitro bioassay was developed. To this end, seedlings were grown on a sugar-free solid-culture medium and were inoculated with a drop of *A. euteiches* zoospores and subsequently phenotyped. Four lines (A17, F83005.5, DZA315.16, and DZA45.5), parents in different RIL populations (Ameline-Torregrosa et al. 2008b), Pilet-Nayel et al. 2009) were analyzed. A kinetic study of six parameters was undertaken on inoculated plants in three independent experiments. Results obtained for each recorded parameter at the most discriminating time after inoculation are shown in Figure 1A. A susceptible line (F83005.5) turned a yellow-gray color at the site of inoculation 3 days postinoculation (dpi), which then encompassed the entire root by 7 dpi. Thereafter, plant growth progressively stopped and the hypocotyls became macerated and soft brown. Between 15 and 21 dpi, cotyledons

turned yellow before wilting. Green tissues finally disappeared on the aerial parts, leading to plant death. The extent of root browning (3 dpi), which assessed the speed of pathogen colonization in the early stages of infection, was significantly lower in DZA45.5 than in F83005.5, indicating that symptoms and pathogen development were delayed in DZA45.5. Intermediate values obtained for the two other lines did not allow discrimination of responses in the early stages of infection. However, an obvious difference observed for root symptoms between F83005.5 and the other lines was the intensity of the root coloration (Fig. 1B, lines F83005.5 and A17). While infected F83005.5 root tissues showed a faint honey-gray color, dark brown tissues were observed in response to *A. euteiches* in DZA45.5, A17, and DZA315.16. Following entire root colonization, the symptoms that developed on the aerial parts also separated F83005.5 from the other lines, as pathogen spread was faster, and consequently, there was a higher proportion of yellow cotyledons (more than 80%) and of dead plants (about 60% of inoculated plants) in F83005.5. By comparison, A17, DZA315.16, and particularly DZA45.5 were not severely affected by *A. euteiches* (with 30, 29, and 16% of dead plants, respectively). Finally, the initiation and development of secondary roots as well as plant fresh weight showed that the pathogen spread observed in F83005.5 severely impaired plant growth. In contrast, the development of the three other lines was only slightly delayed upon infection (Fig. 1B, controls and inoculated plants for A17). In inoculated A17, the number of secondary roots was higher than that observed in the other inoculated lines, and this root number was even significantly higher in inoculated than in A17 control plants (Fig. 1A), suggesting that this phenomenon might be one of the mechanisms helping infected plants limit pathogen colonization. Thus, the F83005.5 line was found to be the most susceptible line, pathogen colonization was rapid and usually led to death (Fig. 1B). Of the three other lines, DZA 45.5 showed the highest level of resistance. A17 and DZA315.16 exhibited an intermediate level of resistance, which was, nevertheless, different from F83005.5. When young seedlings grown in vermiculite were inoculated with *A. euteiches*, the four lines displayed phenotypes in agreement with the in vitro assay results (Fig. 1B, A17 and F83005.5) and with the disease score index obtained for the same lines tested by a conventional inoculation protocol (Moussart et al. 2007). Therefore, these observations validated our in vitro assay for assessing *M. truncatula* resistance to *A. euteiches*. Based on these studies, two lines, F83005.5 (susceptible) and A17 (partially resistant), were selected for further analyses of the components underlying partial resistance to *A. euteiches*. The A17 line was retained because of i) the wealth of genomic data available for this line; ii) the availability of a RIL population generated from an initial cross between F83005.5 and A17, along with an associated genetic map; and iii) the possibility to compare responses to *A. euteiches* with responses of these same RIL to other pathogens, including *Ralstonia solanacearum* (Vailleau et al. 2007) and *Colletotrichum trifolii* (Ameline-Torregrosa et al. 2008b).

Fig. 1. Comparisons of disease symptoms on four *Medicago truncatula* lines with different levels of resistance to *Aphanomyces euteiches* following inoculation. **A**, Measured parameters (extent of root browning, length of symptoms on stem, yellowing on cotyledons, percentages of dead plants, secondary root number, and plant fresh weight) after inoculation on A17, F83005.5, DZA315.16, and DZA45.5. Means of each scored parameter, corresponding to three independent experiments, are presented in the different graphs. Error bars represent standard errors. Different letters above each bar indicate significant differences obtained after mean comparisons by a Duncan multiple range test (MRT). The asterisk (*) indicates that Ni = the mean number of secondary roots for noninoculated A17 and F83005.5 controls, recorded 23 days following their transfer on M medium, from two independent experiments (10 plants per experiment). Mean comparisons by MRT indicated significant differences with root numbers of the same lines inoculated with *A. euteiches*. **B**, Phenotypes of the two selected lines (A17 and F83005.5) (bar = 1 cm) from in vitro experiments or from growth chamber assays (15 dpi) following inoculation with *A. euteiches*.

A **B**

Cytological analysis of partial resistance.

Major differences between A17 and F83005.5 responses following *A. euteiches* inoculation were observed using cytological analyses. We took advantage of the recent identification of *N*-acetyl glucosamine polymers in *A. euteiches* cell walls (Badreddine et al. 2008) to stain pathogen mycelium and oospores with a wheat germ agglutinin (WGA)-fluorescein isothiocyanate (FITC) conjugate. By using epifluorescence microscopy and plant tissue autofluorescence in the blue range, pathogen development and plant defense responses were simultaneously observed in a real-time course of infection (Fig. 2A). In F83005.5, the susceptible line, all root cortical cells were invaded 6 dpi and the vascular tissues were totally colonized 15 dpi. A faint blue autofluorescence was dispersed within cortical cells 6 dpi and progressively decreased in intensity following colonization. In contrast, the A17 roots exhibited a strong autofluorescence, mainly located within cortical cells. The higher level of fluorescence in A17 was confirmed by image analyses (Supplementary Fig. S2). A very good correlation was found between intensity of autofluorescence and accumulation of soluble phenolic compounds. The mycelia were restricted to cortical cells, which did not autofluoresce and were never detected in the A17 stele. Finally, taking advantage of the spectral confocal microscopy, we observed plant and pathogen development in our bioassay at 21 dpi. All F83005.5 root cells were shown to be colonized and most were dead. Cell walls were generally degraded, leading to tissue disorganization. Conversely, plant cell walls were finely depicted within A17 roots, some of them notably reinforced in cell layers surrounding the stele. Mycelium detection was still restricted to cortical cells only, while blue autofluorescence was observed inside the A17 central cylinder (Fig. 2A).

Further analyses were conducted to search for specific features that might account for the limited infection of A17. Clear differences between inoculated A17 and F83005.5 roots were visible 6 dpi (Fig. 2B). Bright-field microscopy analyses of transverse root sections revealed that, in most cases, the pericycle of A17 roots was subjected to additional cell divisions, leading to supplementary cell layers surrounding the A17 stele. This phenomenon was not observed in inoculated F83005.5 roots or in control noninoculated A17 roots (Supplementary Fig. S1). In epifluorescence microscopy, a strong fluorescence occurred in the outer walls of the A17 pericycle cells but was not detected in F83005.5 roots. The purple color obtained with phloroglucinol staining indicated that the observed wall fluorescence was associated with the deposition of lignin-like compounds. These compounds were detected from 3 dpi in A17

(not shown) and formed a ring (6 dpi) around the stele (Fig. 2B). In F83005.5 inoculated roots, or in A17 noninoculated control roots, the purple color was only restricted to the cell walls of xylem vessels.

These observations showed that partial resistance observed in A17 was associated with a release of soluble phenolic compounds, as confirmed by biochemical analyses, inside the cortex and reinforcement of physical barriers surrounding the stele. These mechanisms are likely to play a key role in the protection of the central cylinder against *A. euteiches* invasion.

Genetic analysis of resistance to *A. euteiches* in the LR5 RIL population.

A total of 139 F₇ *M. truncatula* lines in the LR5 (A17 × F83005.5) RIL population were phenotyped after inoculation with *A. euteiches*, using the in vitro bioassay. Symptoms on stems (15 dpi), cotyledon yellowing, and plant death, along with fresh weight (21 dpi) were recorded for each plant. Analysis of variance (ANOVA) showed significant genetic variation ($P < 0.0001$) for each measured parameter. Heritability (h^2) was found to be between 0.41, for plant weight, and 0.58, for symptoms on stems (Table 1), indicating moderate levels of heritability. Segregation data for each of the scored measurements and molecular markers used to establish the LR5 genetic map were calculated by composite interval mapping (CIM) with PLABQTL software. The QTL-mapping results obtained for each symptom are shown in Table 1. For each measured parameter analyzed at 15 or 21 dpi, only a single QTL, located at the top of chromosome 3, was detected. An 8-cM maximal confidence interval (log of the likelihood ratio [LOD] = 3.72) was obtained for fresh weight. This interval was reduced to 4 cM when calculated with symptoms on stem, percentages of dead plants, as well as with cotyledon yellowing. QTL for each of these measurements had respective LOD scores of 10.87, 8.81, and 6.71 (Table 1). For these three latter parameters, the same major QTL of resistance, named *prAe1* (for partial resistance to *Aphanomyces euteiches* 1), was identified. It was characterized by a peak of maximum likelihood centered on 6 cM, a confidence interval of 4 cM, and *mtic846* as the left marker. The highest R^2 value was observed for symptoms on stem. In this case, *prAe1* explained more than one third ($R^2 = 34.1\%$) of the observed resistance.

Validation of *prAe1* in near-isogenic lines (NIL).

NIL were selected from two F₇ inbred lines (numbers 5 and 184) still retaining heterozygosity in the *prAe1* region. Progenies of these lines segregated for the *prAe1* locus and repre-

Fig. 2. Cytological analyses to compare responses of A17 and F83005.5 after infection by *Aphanomyces euteiches*. **A**, Observation of *A. euteiches* development (green) and plant phenolic compound accumulation (blue) in the two selected lines. Cross-sections of inoculated root stained with wheat germ agglutinin-fluorescein isothiocyanate were observed by epifluorescence microscopy (at 6 and 15 dpi) or by spectral confocal microscopy (at 21 dpi). The concentration of mycelium in root tissue was inversely proportional to the plant level of fluorescence, and the mycelium was not observed inside the A17 central cylinder. At 21 dpi, plant cell walls (red) were finely depicted within A17 roots and were reinforced in the cell layers surrounding the root stele. Autofluorescence in the blue range was mainly restricted inside the stele, in which no mycelia were present. In contrast, all F83005.5 root tissues were entirely colonized by *A. euteiches* mycelium, and many cell walls were disrupted, including some around the stele, leading to the pathogen invasion. Bar = 200 μ m. **B**, Histological changes observed at 6 dpi in inoculated A17 roots vs. A17 control or F83 inoculated roots. Additional cell layers were observed all around the A17 stele due to additional pericycle cell divisions (open arrowheads). The pericycle outer cell walls were reinforced with fluorescent compounds under UV light in A17 that were identified as lignin-like compounds through phloroglucinol staining. None of these features were ever observed in F83005.5 infected roots Bar = 200 μ m.

Table 1. Characteristics for quantitative trait loci (QTL) of resistance to *A. euteiches* detected with data recorded on the LR5 RIL population.

Scored measurements	Heritability (h^2)	Chromosome	Left marker	Position (cM)	Confidence interval (cM)	LOD score	R^2
Stem browning (15 dpi)	0.58	3	mtic 826	6	4 to 8	10.87	34.1
Cotyledon yellowing (21 dpi)	0.44	3	mtic 826	6	4 to 8	6.71	19.9
Plant death (21 dpi)	0.51	3	mtic 826	6	4 to 8	8.81	28.8
Fresh weight (21 dpi)	0.40	3	mtic 136	6	0 to 8	3.72	11.7

sented heterogeneous inbred families (HIF) of NIL (Tuinstra et al. 1997). Four NIL were selected (Fig. 3A) and their F₉ progenies were inoculated to validate the *prAe1* effect on resistance. Means of symptoms on stems, cotyledons, and dead plants

were compared between each line (Fig. 3B). Statistical analyses showed that the NIL with A17 alleles in the *prAe1* region were more resistant than A17 (05A) or as resistant as A17 (184A). Conversely, NIL with fixed F83005.5 alleles in *prAe1*

Fig. 3. Use of near-isogenic lines (NIL) to confirm the involvement of the *prAe1* quantitative trait loci (QTL) in resistance to *Aphanomyces euteiches*. **A**, Distribution of parental alleles on chromosome 3 for the selected NIL. The curves of the log of the likelihood ratio scores calculated for three parameters, i.e., yellow areas on cotyledons (speckled columns); relative length of stem tissues with symptoms (solid light gray), and percentages of dead plants (solid dark gray) identify the same QTL on the top of chromosome 3, named *prAe1*. Beneath is a schematic representation of the two F₇ heterogeneous inbred family genotypes (05 and 184) on chromosome 3, still heterozygous in the *prAe1* region, that were selected to generate F₈ NIL (05A and 05F and 184A and 184F, each of them differing only in the *prAe1* genomic region). A is for A17 alleles, F is for F83005.5 alleles, and H is for heterozygous. **B**, Means obtained for three independent repeats of the four tested NIL and the control parental lines. Means of stem tissues with symptoms (light gray column), cotyledon yellowing (speckled), and dead plants (dark gray) were compared by a Duncan multiple range test. Different letters above each bar indicate significant differences obtained after mean comparisons. Error bars represent standard error. Results indicated that NIL fixed with A17 alleles in the *prAe1* region were at least as resistant as A17, while F83005.5 alleles in the *prAe1* region conferred susceptibility. Plants retaining heterozygosity inside *prAe1* displayed a susceptible phenotype, indicating that resistance was recessive.

were as susceptible (184F) or even more susceptible (05F) than the F83005.5 parental line (Fig. 3B). Heterozygous F₉ lines (05-H and 184-H) displayed a susceptible phenotype with intermediate values between those of A17 and F83005.5 but closer to the ones observed for F83005.5 (Fig. 3B).

Taken together, these results confirmed that the *prAe1* locus played a major part in the partial resistance displayed by A17 to *A. euteiches* and indicated that this resistance was recessive, as already suggested by results obtained on a few F₁ plants inoculated with *A. euteiches* that died at the end of the test (not shown). The finding of transgressive phenotypes observed among the NIL also suggests epistatic interactions between A17 *prAe1* alleles and F83005.5 genes located in other parts of the NIL genome, which may modulate plant resistance. Such an explanation of transgressivity has already been observed in cotton inbred lines that were more resistant to a nematode than was the resistant parental line (Wang et al. 2008).

Cytological analyses of NIL.

To know whether the previously observed A17 resistance characteristics were linked to *prAe1*, two NIL, 5A and 5F, respectively resistant and susceptible, were microscopically analyzed. Thin root sections from eight plants per NIL were observed at each of the three selected timepoints after inoculation. The most frequently observed features for each NIL are shown in Figure 4. Mycelium colonization was markedly more reduced in 5A than in 5F root cortex (eight out of eight plants) at 7 dpi (Fig. 4B and F). At this stage, a faint phloroglucinol staining was observed around both 5A and 5F steles (Fig. 4A and E). Intensity of phloroglucinol staining was generally stronger (five of eight plants) in 5A plants, 15 days following inoculation (Fig. 4C and G). Supplementary pericycle cell divisions were observed all around the 5A central cylinder (six of eight plants), leading to a larger stele size in 5A than in 5F roots. Most 5F roots were invaded by *A. euteiches* 21 dpi (seven

of eight plants), while the 5A stele remained pathogen free (eight of eight plants) at the end of the test (Fig. 4D and H).

Fine-mapping and physical localization of the *prAe1* locus.

Fine-mapping of the *prAe1* locus was based on phenotyping data obtained from the RIL that were recombinant within the QTL confidence interval and on an enrichment of the locus with new molecular markers. Genotyping the LR5 RIL population identified six lines (numbers 53, 59, 60, 82, 183, and 222) in which crossovers occurred between 4 and 8 cM on chromosome 3. To avoid scoring errors in resistance assessments and to have phenotyping results for all these lines (including A17 and F83005.5) together in the same inoculation experiments, two supplementary repeats were performed with them. Means of symptoms on stems and cotyledons and of dead plants were recorded and compared with the ones simultaneously obtained for parental lines and an F₇ heterozygous line (number 199) (Fig. 5A and B).

As the genome of the A17 parental line is being sequenced, we took advantage of the available A17 bacterial artificial chromosomes (BAC) that were located within the *prAe1* genomic region to design new simple sequence repeats (SSR) markers. Of the 12 SSR tested, only five (mtic1151, 1158, 1149, 742 and 811) were polymorphic. The LR5 population was then genotyped with these new SSR and a new genetic map was calculated to integrate these markers. Combining phenotyping and genotyping results on the six selected RIL (Fig. 5C) narrowed the QTL to a maximal interval size of 1.2 cM between SSR mtic1149 and mtic742.

Gene content of the *prAe1* locus and gene expression data.

Markers mtic1149 and mtic742 defined a 135-kb interval located over a two-BAC contig formed by AC135103 and CR940308. A total of 26 putative genes were found in this region according to International Medicago Genome Annotation

Fig. 4. Cytological analyses to compare responses of near-isogenic lines (NIL) following *Aphanomyces euteiches* inoculation. Two NIL that differ only for the *prAe1* locus, 5A and 5F, are resistant and susceptible, respectively, to *A. euteiches*. They were analyzed at **A, B, E,** and **F**, 7 days; **C** and **G**, 15 days, and **D** and **H**, 21 days following *A. euteiches* inoculation. Root sections were observed in Bright field, following phloroglucinol staining (**A, C, E,** and **G**) or by epifluorescence microscopy following wheat germ agglutinin-fluorescein isothiocyanate labeling (**B, D, F,** and **H**). Eight plants per NIL were observed at each timepoint. Selected photos were chosen to illustrate the most frequently observed features at each analyzed timepoint for each NIL. Phloroglucinol staining was observed on endoderm or pericycle cell walls in both inoculated susceptible and resistant lines at 7 dpi (**A** and **E**). This staining was reinforced 15 dpi, mainly in the 5A line, all around the stele (**C** and **G**). A higher density of brown cells in the cortex, a stronger accumulation of fluorescent soluble compounds, and supplementary pericycle cell divisions all around the stele (**C** and **D**) were only observed in the 5A line. At 21 dpi, all the root tissues were invaded in 5F, while the stele was protected against *A. euteiches* invasion in 5A.

Group (IMGAG) annotation; nine of them encode hypothetical proteins with unknown function, while the remaining 17 have homologies with known proteins (Table 2). A striking feature was the presence of a cluster of nine genes encoding putative cyclin-like F-box proteins together with a gene encoding an ubiquitin-associated enzyme. Of the remaining genes, three of them (CR940308_46, CR940308_47, and CR940308_49) encode putative transporters; two were related to the cell wall, namely AC135103_35, which showed strong similarities with a cinnamyl alcohol dehydrogenase gene (CAD), and CR940308_39, which shared homologies with a vegetative cell wall protein gp1 precursor. Finally, one gene had homologies with ethylene insensitive 3 (*EIN3*), a gene encoding a transcription factor in-

involved in ethylene regulation, and the last gene contained polynucleotidyl transferase and RNaseH domains.

To assess gene expression, quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) experiments were conducted for some of the genes; those encoding four F-box proteins, the ubiquitin-associated enzyme, CAD, and EIN3 were evaluated at 1, 3, and 6 dpi. Six genes showed differential gene expression between A17 and F83005.5 (Fig. 6). The gene encoding an ubiquitin-associated enzyme was strongly induced (about seven times higher than in control plants) in early infection stages (1 dpi) in the A17 line and showed a similar pattern as AC135103_24, which encodes one of the cyclin-like F-box proteins. Among this latter protein family, AC135103_31 was

Fig. 5. Fine-mapping of the *prAe1* locus. **A**, Phenotype comparisons of the recombinant inbred lines (RIL) that showed a recombination event inside the *prAe1* locus. Means of percentages of stem tissues with symptoms; cotyledon yellowing, and dead plants obtained for the selected RIL were calculated from two independent experiments. A total of 25 to 45 plants for each tested line were inoculated in these experiments. Bars represent standard errors. **B**, Mean comparisons obtained for parental and selected recombinant lines for each recorded symptom following a Duncan multiple range test. Each selected RIL could be compared with A17 (resistant) and F83005.5 (susceptible). Different letters in each line indicate significant differences for one kind of scored symptom. **C**, Combining genotyping and phenotyping results of the selected RIL to narrow the *prAe1* locus. A indicates the A17 alleles and F indicates F83005.5 alleles for each marker inside the *prAe1* locus. Rectangle indicates the maximal genome interval containing gene(s) responsible for *Aphanomyces euteiches* resistance. This interval is flanked by the mtic1149 and mtic742 simple sequence repeat markers.

induced (ratio >2) at all analyzed times in A17, CR940308_45 was induced 3 dpi in A17 and strongly repressed (ratio = 0.13) in F83005.5 at 6 dpi, and no expression was detected in either of the two lines for CR940308_38 (data not shown). Kinetics of the *CAD* gene indicated an induction of this gene at 3 and 6 dpi in A17 but not in F83005.5. *EIN3* was also strongly induced in A17, about eight and four times higher than in control plants at 3 and 6 dpi, respectively.

DISCUSSION

To gain insights in basic knowledge dealing with partial resistance, we have characterized a pathosystem that involves the oomycete *Aphanomyces euteiches* and the model legume *Medicago truncatula*. Although the pathogen infected and colonized both selected lines, symptoms and pathogen development were markedly reduced in A17 as compared with F83005.5. Complementary cytological, genetic, and molecular analyses revealed different features associated with the partial resistance of A17.

Partial resistance is associated to changes in root architecture and to tissue modifications around the stele.

Changes in root architecture were the first visible differences between A17 and susceptible F83005.5 phenotypes after inoculation. While a limited number of secondary roots developed in inoculated F83005.5 plants, this number was almost four times greater in A17. That the production of supplementary roots in A17 accounted for their ability to survive pathogen infection was illustrated by a comparison with noninoculated plants. Indeed, A17 roots were significantly more numerous 21 dpi than in control plants (Fig. 1A), indicating that the plants responded to pathogen attack by expanding their root system.

There was also a major contrast in colonization and oospore production within root tissues of the two lines following inoculation. For F83005.5, all root tissues were entirely colonized by

A. euteiches, while the stele of A17 remained pathogen free. This was associated with potential defense responses that might prevent pathogen ingress, as illustrated by a strong accumulation of soluble fluorescent compounds as soon as 24 h postinoculation in A17 cortical cells. This fluorescence, which was found to be associated with the presence of phenolic compounds, was detected throughout the test and spread to the stele 21 dpi. Legumes and, notably, *M. truncatula* are known to accumulate such defense-related compounds, mostly belonging to the isoflavonoid and phenylpropanoid families (Dixon et al. 2002) in response to pathogen attack (Foster-Hartnett et al. 2007; Kamphuis et al. 2008; Torregrosa et al. 2004) or to elicitor treatments (Farag et al. 2008; Naoumkina et al. 2007). In comparison, the accumulation of these compounds was low in F83005.5 inoculated roots, most likely insufficient to hinder *A. euteiches* colonization and to mount efficient structural barriers to protect the stele. Mechanisms allowing reinforcement of the cells surrounding the central cylinder were indeed the most striking differences between the two inoculated lines. A novel feature that had not yet been described, to our knowledge, during a plant-pathogen interaction was the induction of cell divisions in the pericycle, leading to additional cell layers around the stele. Among the root cells that have left the meristematic zone, the pericycle cells are well known to have the unique ability to reenter the cell cycle and become founder cells (Dubrovsky et al. 2000) of lateral root meristems or be involved in legume nodule development (Timmers et al. 1999). Depending on the species, the founder cells are located at the xylem or phloem poles. In inoculated A17 roots, divisions were observed all around the stele, without any preferential location. Up to three supplementary pericycle cell layers were found around the stele of A17 but not in F83005.5. This suggests that pericycle cell divisions might provide additional structural barriers to either prevent stele colonization, allow an increased secondary root production, or both. This would explain why secondary roots are more numerous in inoculated A17 than in noninoculated control plants. Besides this effect, additional cytological features contribute to protec-

Table 2. Genes with a putative known function in the *prAe1* region, between mtic742 and mtic1149

Gene/call and markers	BAC start position	Predicted size (nt)	Putative function (IMGAG annotation)
mtic742	66,607		
AC135103_21	66,801	5,965	Ubiquitin-associated enzyme
AC135103_35 ^a	78,774	2,188	CAD
AC135103_31 ^b	82,963	1,112	F-box protein
AC135103_24	88,238	2,200	Cyclin-like F-box
CR940308_37	13,867	1,775	F-box protein
CR940308_38	23,246	3,86	Cyclin-like F-box
CR940308_39	36,425	423	Vegetative cell wall protein gp1 precursor
CR940308_41	41,644	818	Cyclin-like F-box
CR940308_42	50,423	4,444	Cyclin-like F-box
CR940308_43	55,539	1,754	Cyclin-like F-box
CR940308_15	66,960	2,387	Cyclin-like F-box
CR940308_45	70,326	949	Cyclin-like F-box
CR940308_46	78,722	1,970	Transporter-like protein
CR940308_47	81,936	3,210	ZIFL1
CR940308_7	86,847	225	Polynucleotidyl transferase, ribonuclease H fold
CR940308_49	92,117	4,537	Major facilitator superfamily MFS_1
CR940308_10	11,1909	3,194	Ethylene insensitive 3
mtic1149	11,5102		

^a This gene is presently annotated by International Medicago Genome Annotation Group (IMGAG) (v2.0) as a dihydroflavonol-4-reductase (DFR). However BLAST searches performed with AC135103_35 do not confirm this functional annotation. BLASTP against the National Center for Biotechnology Information nonredundant database mainly returned matches to putative cinnamyl alcohol dehydrogenase (CAD) in several organisms, the best one being AAC06319.1 in *Malus domestica* (identity = 75%, E-value = 4e-41), while the first hit with a DFR appeared far down in the list (BAC78578.1 in *Oryza sativa*, identity = 47%, E-value = 5e-19). The same figure was obtained with TBLASTN against the The Institute for Genomic Research Gene Index (all plants selected) in which the best match is TC100392 with an E-value = 1e-41 (annotated as a CAD in *M. truncatula*), while matches with DFR had much lower E-values (1.2e-19 with TC236822 in wheat and 3e-13 with TC103465 in *Medicago*).

^b This gene was first detected by IMGAG (v1.0) annotation as a gene encoding an F-box protein. It was probably considered as a pseudogene by IMGAG (v2.0) and was removed in the present annotation. However, given our quantitative reverse transcriptase-polymerase chain reaction results, this gene was found to be expressed and was, therefore, maintained in this table.

tion of the A17 stele, including endoderm as well as pericycle cell-wall thickenings and lignin deposition on the outer pericycle cell wall, a phenomenon already observed in the nonhost interaction between broomrape (*Orobanche crenata*) and *M. truncatula* (Lozano-Baena et al. 2007).

Identification of *prAe1*, a major recessive QTL of resistance to *A. euteiches*.

The genetic dissection of resistance to *A. euteiches* was undertaken with a population of 139 F₇ RIL. The disease index scale used in greenhouse (or in field) assays to assess the resistance of pea or other legumes is mainly based on the proportion of root-rot symptoms observed on the root of each inoculated plant at the end of the test (Moussart et al. 2007, 2008; Pilet-Nayel et al. 2002, 2005; Wicker et al. 2003). The in vitro bioassay allowed precise measurement of several symptoms on various parts of the plant, including roots, stems, and cotyledons, at different timepoints after pathogen inoculation. Given the early stage of inoculation that we used (i.e., 2 days after germination), we could not discriminate responses of the two selected lines using development of root symptoms. However, we found that the extent of symptoms on stems were the most significant parameter to identify a QTL of resistance. With this parameter, one major recessive QTL, named *prAe1*, that explained approximately one third of the total calculated variance for resistance was found near the top of chromosome 3. No other significant QTL was found elsewhere on the genome. Nevertheless, such an incomplete explanation of the observed resistance is not rare when the CIM method is used (Young 1996). Very similar results were recently reported for another recessive resistance QTL of *M. truncatula* to *Phoma medicaginis* (Kamphuis et al. 2008). The major role played by the *prAe1* QTL in resistance to *A. euteiches* was confirmed by inoculating NIL differing only in the *prAe1* locus. Homozygous NIL with A17 alleles in the *prAe1* locus were at least as resistant as the A17 parental line, while NIL with F83005.5 alleles in the *prAe1* region were fully susceptible. The distal part of *M. truncatula* chromosome 3, in which *prAe1* was detected, was previously associated with other QTL or genes for resistance to biotic stresses. A recent study with the LR3 (DZA45.5 × F83005.5) *M. truncatula* RIL population showed that *AER1*, a major dominant resistance gene to another *A. euteiches* pea isolate, was also located on chromosome 3 (Pilet-Nayel et al. 2009). Genetic analyses are in progress to precisely compare *AER1* and *prAe1* loci. If the same one or more genes are involved in resistance to *A. euteiches* in the two analyzed RIL populations, a major issue would be to explain why resistance is dominant in one case and recessive in the other. Among the other interesting loci on chromosome 3 that were found to be associated with resistance to other pathogens or pests, a 7-cM minor QTL, also covering the *prAe1* region, contributed to the expression of partial resistance of *M. truncatula* to the root bacterium *Ralstonia solanacearum* (Vailleau et al. 2007). Two resistance genes against aphids were also detected in close vicinity to the *prAe1* locus, using different F₂ *M. truncatula* populations (Klingler et al. 2005, 2007). Furthermore, a recent report identified several clusters of resistance gene analogs (RGA) in this

Fig. 6. Expression levels of selected genes in the two inoculated parental lines. Three independent quantitative reverse transcriptase-polymerase chain reaction experiments were performed to measure expression of selected genes inside the *prAe1* genomic region. The bars indicate fold change values between inoculated vs. noninoculated plants at each timepoint, calculated with the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001), using an EF1 α gene as endogenous control. A gene was considered induced or repressed when the ratio between inoculated and control plants was >2 or <0.5, respectively. Error bars represent standard errors.

region that belong to the coiled-coil nucleotide binding site-leucine-rich repeat family (Ameline-Torregrosa et al. 2008a). To investigate whether such genes might be associated to resistance against *A. euteiches*, fine-mapping of *prAe1* was initiated to narrow the QTL to a 135-kb sequenced genome region.

Proteasome-associated genes are overrepresented in the *prAe1* region.

Within the *prAe1* genomic DNA fragment, there were no RGA identified. Among the 17 annotated genes with identified homologies in the *prAe1* region, the most striking feature was the presence of a cluster of nine F-box protein-encoding genes and of one gene encoding a ubiquitin-associated enzyme. The qRT-PCR results that showed that this latter gene and at least three of the F-box-encoding genes were induced in A17 following inoculation but not in the susceptible line also suggested a putative role of proteasome-related genes in the resistance to *A. euteiches*. This hypothesis is in agreement with previously reported proteomic data indicating that two proteasome alpha subunits were differentially induced in another *M. truncatula* line partially resistant to *A. euteiches* (Colditz et al. 2005). F-box proteins are also known to be involved in hormone regulation and in plant immunity (Lechner et al. 2006). Homologies with cyclin-like F-box protein-encoding genes in the *prAe1* locus would be consistent with a role in the regulation of pericycle cell divisions, as already observed for another F-box protein in *Arabidopsis* (del Pozo et al. 2006). Alternatively, as lateral root production is known to be regulated by auxin and ethylene (Ivanchenko et al. 2008), one of the F-box candidates might play a role in one of these hormone pathways, as demonstrated for *EBF1* and *EBF2* for ethylene. These two F-box proteins were reported to negatively regulate the ethylene-response pathway by addressing the transcription factor EIN3 to the proteasome (Guo and Ecker 2003). Interestingly, a putative homolog of the *Arabidopsis thaliana* EIN3 gene was also present in *prAe1* and is highly induced 3 dpi, which correlates with the onset of first pericycle cell divisions.

Among the other genes of *prAe1*, two could also be involved in A17 resistance, given one or both cytological and the qRT-PCR results. *CR940308_37* showed homologies with a hydroxyproline-rich glycoprotein family, known to be responsible for cell-wall strengthening in response to pathogen attacks (Esquerré-Tugayé et al. 1999), and *AC135103_35* showed very strong homologies with an atypical cinnamyl alcohol dehydrogenase (Goffner et al. 1998), an enzyme directly involved in lignification, which might therefore be responsible for lignin deposition in the outer pericycle cell walls.

In conclusion, our study presents novel data aimed at identifying mechanisms involved in partial resistance to an important root pathogen. For the first time, we provide genomic sequences of putative candidate genes that are associated with limiting the extent of *A. euteiches* colonization of legume roots. This work opens new applied and basic approaches to control a very damaging pathogen that has a broad host range among the legume family (Gaulin et al. 2007) as well as to gain insights into novel mechanism expression of partial resistance. Several strategies based on the use of reverse and forward genetic tools along with a transcript-based cloning approach are presently under way to formally identify one or more *prAe1* genes that are responsible for partial resistance to *A. euteiches*.

MATERIALS AND METHODS

Plant material and growth conditions.

A17, F83005.5, DZA45.5, and DZA 315.16 seeds were provided by J. M. Prosperi (Institut National de la Recherche Agronomique SGAP Laboratory, Mauguio, France). Seeds of

the A17 × F83005.5) F₇ RIL population, named LR5, were produced by T. Huguet as previously reported (Ameline-Torregrosa et al. 2008b). Heterogeneous inbred families (Tuinstra et al. 1997) of NIL were selected from progenies of two F₇ RIL (5 and 184) still segregating for the *prAe1* region. Ten plants from each of the two RIL were grown separately and were genotyped with markers covering the entire region of the QTL. From each HIF, two F₈ NIL containing either A17 or F83005.5 alleles in the *prAe1* region were selected and their progenies were used for resistance analyses.

After a sulphuric acid scarification treatment, seeds were washed with sterile water, sterilized in bleach (2.5%) for 4 min., and were washed again in sterile water. Following germination, seeds were either transferred into petri dishes on M medium without any carbon source (Bécard and Fortin 1988) for in vitro bioassays or into pots filled with vermiculite for growth-chamber infection assays. Plantlets were grown with a 16-h light at 22°C and 8-h dark at 20°C.

Inoculation procedure.

The strain *Aphanomyces euteiches* Drechs. ATCC 201684, a pea isolate, was provided by F. Krajinski (University Hanover, Germany). Zoospores were produced using the protocol described by Badreddine and associates (2008), and their concentration was adjusted to 10⁵ spores per milliliter to inoculate plantlets 1 day after germination. A 4-μl droplet of spore suspension was deposited in the middle of the root for the in vitro bioassay, while 5 ml of spore suspension per plant were used in the growth-chamber infection assay, as described by Moussart and associates (2007).

Recorded symptoms and analysis of resistance.

To assess resistance in the four parental lines, three independent in vitro infection assays were performed with 15 inoculated plants per line in each repeat. Size (in mm) of brown tissues on roots (3 dpi) and on hypocotyls (15 dpi), visual estimation (in %) of yellow or dry area on cotyledons (21 dpi), along with plant death (21 dpi) were recorded for each inoculated plant. Fresh plant weights and the number of secondary roots (more than 1 mm long) were recorded 21 dpi. Similar parameters were recorded to assess resistance of the 139 RIL in two independent experiments with 5 to 15 plants per RIL in each repeat. These data were completed by measuring size of the whole stem (15 dpi) in order to calculate relative length (in percentage) of stem tissues displaying symptoms.

The scored measurements for each symptom recorded on parental lines, NIL, and RIL with crossovers inside the *prAe1* locus were compared through statistical ANOVA analyses using the package stats of R software (version 2.7.2). For significant factor, means were compared using a Duncan multiple range test (Duncan 1955) available in Laercio package of R.

Sample preparations for microscopy.

For optical microscopy, 150-μm sections of fresh samples were made from roots embedded in 5% low-melting point agarose, using a vibratome (VT1000S; Leica, Rueil-Malmaison, France) mounted on a glass slide in a drop of distilled water and observed in Bright-field microscopy using an inverted microscope (DMIRBE, Leica). Some sections were stained using phloroglucinol (Wiesner reagent) to visualize lignin content. Other sections were labeled for 30 min with WGA-FITC (50 μg/ml diluted in PBS), to localize *A. euteiches* hyphae and oospores using epifluorescence illumination (excitation filter, BP 450 to 490 nm). Plant fluorescent metabolites were also observed with epifluorescence (excitation filter BP 340 to 380 nm, suppression filter LP 430 nm). All the images were acquired using a CCD camera (color Coolview, Photonic

Science, Robertsbridge, U.K.). To compare levels of autofluorescence in both inoculated (and control) lines at 6 dpi, all the acquisition settings (the gain of the CCD camera and the number of integration) were adjusted on the higher signal intensity to avoid overexposure of the detector. All these parameters were then unchanged to acquire images of the different root samples. Fluorescence measurements were then performed using image analysis software (Image Pro-Plus, Media Cybernetics, Silver Spring MD, U.S.A.). For each condition, measurements were done on root sections from 12 plants.

Confocal images were acquired with a spectral confocal laser scanning system (SP2 SE, Leica, Wetzlar, Germany) equipped with an upright microscope (DM 6000, Leica, Germany). Observations were made using 10× (HC PL Fluotar, N.A. 0.3) dry objectives. A 405- and a 561-nm diode laser were used to collect the emitted autofluorescence, collected in the range of 410 to 470 nm. The 488-nm ray line of an argon laser was used to detect WGA-FITC fluorescence in a range between 510 and 540 nm.

Extraction and dosage of soluble phenolic compounds.

Control and inoculated (6 dpi) root samples (300 mg) were ground in liquid nitrogen and were homogenized in 1 ml of 80% aqueous methanol. Following centrifugation (10,000 × g, 5 min.), each supernatant was transferred in a fresh tube. An additional extraction from each remaining pellet was performed in the same conditions, and the resulting supernatant was added to the first one. Each 2-ml sample was then dried in a SpeedVac, and the resulting residue was resuspended in 1 ml of 50% aqueous methanol. Total soluble phenolic compounds were determined using the Folin–Ciocalteu reagent, and the absorbance was measured at 760 nm, according to the method of Singleton and Ross (1965). The amount of soluble phenolic compounds was calculated from a standard curve obtained with different amounts of ferulic acid. Two independent experiments with 10 plants per analyzed root sample were performed for these measurements.

QTL analysis and fine mapping.

The genetic map used, based on 105 microsatellite markers, along with the QTL analysis procedure have already been described in other works (Ameline-Torregrosa et al. 2008b; Vailleau et al. 2007). Replicate and genotype effects were examined by ANOVA with the PROC GLM procedure of SAS (SAS Institute Inc., Cary, NC, U.S.A.) software. Genetic variance was estimated using the PROC VARCOMP procedure of the SAS software, in which the genetic effect was assumed to be random. Heritability was estimated as $h^2 = \sigma^2_g / [\sigma^2_g + (\sigma^2_e/r)]$, with σ^2_g being the genetic variance, σ^2_e the residual variance, and r the number of replicates. QTL were detected by CIM (Jansen 1993), using the software PLABQTL V1.1 (Utz and Melchinger 1996) to combine the different sets of experiments. A purely additive model was employed. Individual cofactor sets were selected via stepwise regression for each trait. Final selection was for the model that minimized Akaike's information, a measure of the goodness-of-fit of the regression model (Jansen 1993). Empirical threshold values for the LOD scores were determined by computing 10,000 permutations (Churchill and Doerge 1994) using the "permute" command of the PLABQTL software. The critical LOD score to indicate QTL significance was therefore 2.3. QTL positions were determined at the local maxima of the LOD-curve plot in the region under consideration. Confidence intervals were set as the map interval corresponding to a 1-LOD decline on either side of the LOD peak. The proportion of phenotypic variance explained by a single QTL was obtained by the square of the partial correlation coefficient (R^2). Estimates of

the additive effects of the QTL were computed by fitting a model including all putative QTL for a given trait.

For QTL fine-mapping, new SSR markers (Fig 4B; Supplementary Table S1) covering the *prAel* region were designed from the *M. truncatula* sequence, using SSRIT software (Temnykh et al. 2001).

RNA extraction and quantitative RT-PCR.

Roots from inoculated and noninoculated (control) plants were ground in liquid nitrogen, and total RNA was isolated with the "SV total RNA isolation system" kit (Promega Corporation, Madison, WI, U.S.A.), according to the manufacturer's recommendations. Reverse transcription was performed with 500 ng of total RNA using SuperScript reverse transcriptase III (Invitrogen, Carlsbad, CA, U.S.A.). RNA was then eliminated from cDNA samples by an RNaseH treatment. qRT-PCR was carried out in an ABI Prism SDS 7900HT (Applied Biosystems, Foster City, CA, U.S.A.) system with the following conditions: 5 min. at 95°C, followed by 40 cycles (15 s at 95°C, 1 min at 56°C). For each sample, a mix containing primers (0.15 μM each), SYBR Green I (Eurogentec, Liège, Belgium), and an appropriate dilution of cDNA was prepared. Specific primers (Supplementary Table S2) were designed for different genes encoding F-box proteins, ubiquitin-associated enzyme, EIN3, and CAD that are located on the genomic sequence between the mtic742 and mtic 1149 SSR markers. To normalize qPCR data, an elongation factor-1α (TC111470), whose expression was found to be more stable than actin or tubulin genes during *A. euteiches* infection in *M. truncatula*, was used as endogenous control. To determine the effect of infection on the analyzed genes, the $2^{-\Delta\Delta C_T}$ method of Livak and Schmittgen (2001) was applied. Here, $\Delta\Delta C_T = (C_T \text{ of the gene of interest} - C_T \text{ of EF1}\alpha) \text{ at time } x \text{ after inoculation} - (C_T \text{ of the gene of interest} - C_T \text{ of EF1}\alpha) \text{ at the same time in a noninoculated plant}$. Each qRT-PCR reaction was conducted in triplicate for each cDNA and was performed for three biological replications. Specificity of the amplifications was verified by melting curve analysis. Efficiency of the amplification was verified by the analysis of standard curves.

ACKNOWLEDGMENTS

The authors thank D. A. Samac (University of Minnesota, U.S.A.) for critically reviewing the manuscript and F. Krajinski (Max-Planck-Institute of Molecular Plant Physiology, Potsdam, Germany) for providing the *A. euteiches* strain. Financial support for this work was provided by the FP6 European Grain Legume Integrated Project and by a Tunisian-French collaborating program (CMCU 07G 0907). N. Djebali was supported by grants from the Tunisian research ministry and from the University of Toulouse.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The R Project for Statistical Computing website: www.r-project.org
Medicago truncatula Sequencing Resources website:
medicago.org/genome