The *RAP1* Gene Confers Effective, Race-Specific Resistance to the Pea Aphid in *Medicago truncatula* Independent of the Hypersensitive Reaction

Sophie Alice Stewart,¹ Simon Hodge,¹ Nurul Ismail,¹ John W. Mansfield,¹ Bart J. Feys,¹ Jean-Marie Prospéri,² Thierry Huguet,³ Cécile Ben,³ Laurent Gentzbittel,³ and Glen Powell¹

¹Division of Biology, Imperial College London, South Kensington Campus, London, SW7 2AZ, U.K.; ²INRA, UMR DIAPC 1097, Montpellier, F34130 Mauguio, France; ³INP-ENSAT, Symbiose et Pathologie des Plantes–SP2, Pôle de Biotechnologie Végétale, Chemin Borde Rouge, 31326, Castanet Tolosan Cedex, France

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Plant resistance to pathogens is commonly associated with a hypersensitive response (HR), but the degree to which the HR is responsible for incompatibility is subject to debate. Resistance to aphids is likely to share features with resistance to pathogens but is less well understood. Here, we report effective resistance to the pea aphid *Acyrtosiphon pisum* in *Medicago truncatula*. Aphids lost weight and died rapidly (within two days) on the resistant genotype Jemalong, which developed necrotic lesions following infestation. Lesions were induced by nonvascular intracellular stylet punctures by aphids, remained localized to the site of stylet entry, stained for the presence of reactive oxygen species, and were similar to the HR induced by the bacterial pathogen *Pseudomonas syringae* pv. *phaseolicola*. The implication that aphid-induced lesions confer resistance was tested by quantitative trait loci analysis using recombinant inbred lines derived from a cross between Jemalong and the susceptible genotype DZA315.16. One major locus, *RAP1*, was identified that was sufficient to confer race-specific resistance against the pea aphid and was mapped to the middle of chromosome 3. Surprisingly, a separate locus, mapping to the top of chromosome 3, governed aphid-induced HR, indicating that the HR-like lesions are not required for *RAP1*-mediated aphid resistance.

Aphids are widespread crop pests; their numbers build up rapidly on susceptible plants through parthenogenetic reproduction, they tap into the sieve elements, draining plant resources, and can cause significant crop losses due to the transmission of phytopathogenic viruses. The majority of plants are capable of defending themselves from aphids and other phloem-feeding insects through a variety of means (Goggin 2007; Kaloshian and Walling 2005; Smith and Boyko 2007; Walling 2000), ranging from glandular trichomes at the plant surface to P-protein dispersal and occlusion of phloem sieve elements (Kehr 2006; Neal et al. 1990; Will and van Bel 2006). Conversely, aphids have evolved counter-mechanisms to avoid or suppress such defenses.

For example, fast wound responses involving the mobilization and clogging of phloem sieve pores by P proteins (Knoblauch and Van Bel 1998) are thought to be suppressed by aphids in compatible interactions (Will et al. 2007). In order to minimize crop damage by aphids and develop resistant plant cultivars, it is important to improve our understanding of the interactions between plants and aphids and elucidate the processes and mechanisms that define incompatibility.

In 1955, Flor proposed a genetic model for the basis of plant resistance to pathogens, which led to the theory of gene-for-gene resistance (Flor 1955, 1971; Keen 1990). This model describes how actively induced plant responses and resistance are conferred by resistance (*R*) genes on recognition of a pathogen-derived molecule (avirulence [*Avr*] gene product). The *R*-*Avr* recognition system that underlies incompatibility between plants and pathogens has also been proposed as a mechanism behind avirulence in plant-aphid interactions (Kaloshian 2004; Kessler and Baldwin 2002; Smith and Boyko 2007). Research in this area has uncovered a number of plant *R* genes that provide resistance against aphid pests (Klingler et al. 1998, 2005, 2007; Monet and Massoné 1994; Reinink and Dieleman 1989; Smith et al. 1999; Teetes et al. 1999). Significantly, the *Mi-1.2* gene in tomato and the *Vat* gene in melon, conferring protection against the potato aphid (*Macrosiphum euphorbiae*) and the melon aphid (*Aphis gossypii*), respectively, have been cloned and found to belong to the predominant class of plant *R* proteins with nucleotide binding site and leucine-rich-repeat (NBS-LRR) motifs (Dogimont et al. 2007; Kaloshian et al. 1998; Milligan et al. 1998). Aphid resistance genes in other plant species also show tight linkage with NBS-LRR gene clusters (Klingler et al. 2005).

A common feature of *R* gene-mediated defense against pathogens is the hypersensitive response (HR). The HR has been described as a form of programmed cell death, accompanied by the early presence of reactive oxygen species (ROS) and biosynthesis of secondary metabolites within surrounding living tissues (Lamb and Dixon 1997; Mansfield 2000). The requirement for cell death and lesion formation for the restriction of microbial pathogens during gene-for-gene mediated resistance has long been the subject of debate (Dangl et al. 1996; Ingram 1978; Stakman 1915). Signals leading to resistance have been demonstrated to be effective in the absence of the HR (Grün et al. 2006; Mittler 2002; Ryals et al. 1996). Microscopy and physiological experiments, however, have revealed

Corresponding author: Glen Powell; E-mail: g.powell@imperial.ac.uk

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that hypersensitive cell death is an essential component of resistance to obligately biotrophic rust fungi and the oomycete downy mildews (Ellis et al. 2007; Heath 1974; Maclean et al. 1974; Mansfield et al. 1997). In addition, when bacterial avirulence genes have been cloned using in planta bioassays, it has proved impossible to separate restriction of bacterial multiplication from the induction of the HR (Tsiamis et al. 2000). By contrast, inhibition of *Potato virus X* determined by the *Rx* resistance gene does not appear to require the HR (Bendahmane et al. 1999), but the *Rx* protein does regulate plant cell death (Moffet et al. 2002).

The role of the HR is less obvious during incompatible interactions with insects. Mobile leaf-chewing herbivores such as caterpillars of the order Lepidoptera may be unlikely to be deterred by such an induced, local response. Conversely, for aphids and galling insects whose feeding habits are largely sedentary, lesion formation may be a successful deterrent (Cornelissen et al. 2002; Fernandes and Negreiros 2001; Larsson and Wingsle 2005). Some aphids, for example, can remain feeding on a single sieve element for several hours or even days (Tjallingii 1995) and could, therefore, be affected by locally induced plant defense. Hypersensitive-like responses have been proposed as a mechanism of plant defense against aphids (Boyko et al. 2006; Chen 2008); hypersensitive-like phenotypes have been associated with aphid resistance in apple (Lyth 1985), barley (Belefant-Miller et al. 1994), and melon (Sarria et al. 2009). Interestingly, *Mi*-mediated resistance in tomato does not exhibit HR in response to avirulent potato aphids, but the HR is associated with *Mi*-mediated resistance to the root-knot nematode (Martinez de Ilarduya et al. 2003). When the generalist aphid *Myzus persicae* feeds on *Arabidopsis* plants, symptoms of premature leaf senescence (including localized cell death) are induced and contribute to basal resistance (Pegadaraju et al. 2005, 2007). The hyper-senescent mutant plants *constitutive expresser of PR genes5* (*cpr5*) and *suppressor of SA insensitivity2* (*ssi2*) exhibit sponta-

neous cell death and enhanced resistance to aphids, whereas the delayed senescence in *phytoalexin deficient4* (*PAD4*) mutants involves enhanced susceptibility to aphids in the absence of detectable aphid-induced cell death (Pegadaraju et al. 2005).

In the past, the lack of an appropriate model in which to study plant resistance to aphids has been a limiting factor in the identification and characterization of aphid resistance. Two independent aphid resistance genes have been identified in the model legume *Medicago truncatula*, namely, *AKR* and *TTR* (*Acyrtosiphon kondoi* resistance and *Therioaphis trifolii* f. *maculatus* resistance genes) confer resistance to the blue-green aphid and the spotted alfalfa aphid, respectively (Klingler et al. 2005, 2007). In addition, a degree of resistance against the pea aphid has also been reported in *M. truncatula* and may be governed by the *AKR* locus (Gao et al. 2008) or, possibly, a separate but tightly-linked locus (Guo et al. 2009). In this study, we describe highly effective, race-specific resistance to the pea aphid in *M. truncatula*. Both these organisms have been developed as model species (Cook 1999; May and Dixon 2004; Tagu et al. 2008) and, therefore, offer huge potential for dissecting the mechanisms behind specificity in plant resistance to aphids.

We found that the *M. truncatula* accession Jemalong A17 (the reference genotype in the *M. truncatula* genome-sequencing project) developed HR-like lesions when infested with an avirulent pea aphid clone, PS01. Lesions were absent from compatible interactions between Jemalong A17 and a virulent aphid genotype LL01. Although a clear HR-like phenotype was associated with the incompatible interaction in Jemalong A17, the use of quantitative trait loci (QTL) analysis with recombinant inbred lines (RIL) revealed that two separate *Medicago* loci independently control the HR and aphid resistance phenotypes, thereby excluding the HR as the causal agent for aphid resistance in this interaction.

RESULTS

M. truncatula cv. Jemalong shows resistance against pea aphid clone PS01.

A comparison of growth and survival of two aphid clones on selected *M. truncatula* lines revealed that a clone of the pea aphid (PS01) lost weight and died (within 3 days) on the cultivar Jemalong A17 (Figs. 1 and 2). Jemalong 6 (isogenic sister line

Fig. 1. Survival of pea aphid clones PS01 and LL01 on *Medicago truncatula* lines. **A**, Jemalong A17 ($P = <0.001$) and **B**, DZA315.16 ($P = 0.037$); $n = 20$. Aphids were 2 days old when plants were infested.

Fig. 2. Mean relative growth rate (MRGR) of pea aphid clones PS01 and LL01 on *Medicago truncatula* lines Jemalong 6, Jemalong A17, and DZA315.16. Bars show one standard error. Significantly different means are labeled with different letters ($P < 0.0001$). Negative growth-rate values (PS01 on Jemalong 6 and Jemalong A17) reflect aphid weight loss.

of Jemalong A17 and parent in the RIL mapping population) showed an equivalent resistant phenotype as indicated by a similar failure of aphids to grow. By contrast, a second aphid clone (LL01) performed well on both Jemalong A17 and 6. Both aphid clones gained weight and survived well on the Algerian accession DZA315.16, although there was a slight reduction in survival of clone PS01 on DZA315.16 compared with clone LL01.

Aphid-induced lesions (AIL) in the resistant line are HR-like responses.

Jemalong A17 plants infested with PS01 aphids developed localized necrotic lesions on leaves and petioles that were visible from 24 h postinfestation (hpi). No lesions were observed in virulent aphid-plant interactions (data not shown). Diamino-benzidine reaction with endogenous H_2O_2 in Jemalong A17

leaves exposed to 2 h of aphid activity displayed indicative localized staining at 24 and 48 h (Fig. 3A and B, respectively) in response to PS01 aphid stylet probes. No staining was observed in response to interaction with the LL01 aphid nor in interactions with the susceptible plant line. The stylet track where PS01 entered the leaf and triggered localized ROS production inside Jemalong A17 epidermal and mesophyll cells and the cell wall is illustrated in Figure 3B.

AIL that developed in leaves of Jemalong A17 were also characterized by the accumulation of autofluorescent compounds in mesophyll cells. Autofluorescence was retained after clearing tissue in methanol as shown in Supplementary Figure S1. The timing of AIL formation after aphid exposure was comparable to the appearance of the HR after inoculation with the bean pathogen *Pseudomonas syringae* pv. *phaseolicola*, which is avirulent on *M. truncatula*. The lesions around aphid

Fig. 3. Characterization of hypersensitive-like responses. Leaves (Jemalong A17) stained with 3,3-diaminobenzidine tetrahydrochloride show localized reactive oxygen species (ROS) (dark staining) surrounding the stylet penetration point. **A**, At 24 h postinfestation (hpi), ROS staining localized to the epidermal cell next to the stylet penetration point and, to a lesser degree, in the adjacent cells. **B**, At 48 hpi, ROS staining is visible in the mesophyll cells and cell walls of the epidermal cells surrounding the stylet penetration site. The arrowheads in each case indicate the site of stylet penetration. Scale bars = 200 μ m; SP = stomatal pore. **C** through **F**, Comparison of lesion appearance in *Medicago truncatula* Jemalong A17 challenged with aphid clone PS01 (**C** and **D**) or the avirulent bacterium *Pseudomonas syringae* pv. *phaseolicola* (**E** and **F**). **C** and **E**, Appearance under tungsten, visible light. **D** and **F**, Autofluorescence of the same lesions under UV excitation with green fluorescent protein filter set. Scale bars = 2 mm.

feeding sites expanded to produce clear macroscopically visible flecks. The similar appearance of bacterially and aphid induced lesions under visible light and their epifluorescence under UV is shown in Figure 3C through F. As an indicator of biochemical changes occurring within defined lesions, we recorded the emission spectra of fluorescence from responding cells (Fig. 3C through F). Very similar emission spectra were obtained, peaking in intensity around 540 nm, from lesions caused by challenge with incompatible aphids or bacteria (Supplementary Fig. S2). The distinctive autofluorescence observed indicated that the plant response to aphid stylet penetration is comparable to a bacterially induced HR and suggests that similar autofluorescent secondary metabolites accumulate in the lesions (Hutzler et al. 1998).

Superficial cell punctures from aphid stylets lead to HR.

To characterize aphid stylet activities that lead to AIL, individual aphid ‘probes’ of leaf tissue were monitored using the electrical penetration graph (EPG) technique (Tjallingii 1988). A total of 137 Jemalong A17 leaves were exposed to a single, recorded PS01 aphid probe. Each leaf was examined under UV for autofluorescent lesions 24 h after exposure to the aphid. No phloem-specific waveforms were recorded at any time during the experiment, showing that phloem penetration was not required for lesion induction. Figure 4A and B summarizes the stylet penetration behavior of PS01 aphids on *M. truncatula*

Fig. 4. The mean activity recorded for probes on resistant *Medicago truncatula* Jemalong A17 by avirulent PS01 pea aphids. **A**, Total duration of probe and **B**, total time with stylet tips inside cells were analyzed according to probes that led to necrotic lesions and probes that had no effect. Bars show means $\pm$ standard error.

Jemalong A17 for probes that either led to an AIL or did not. On average, longer probes with greater time spent puncturing cells were more likely to result in lesion development. Quantitative analysis of cell punctures during individual probes (Fig. 5) demonstrated that the development of AIL was linked to multiple cell punctures; stylet penetrations with >1 cell puncture were very likely to induce a lesion. Conversely, the majority of single cell punctures failed to trigger a reaction.

QTL analysis: HR unnecessary for aphid resistance.

The genetic control of pea aphid resistance was investigated by QTL analysis using RIL from the LR4 reference mapping population derived from Jemalong 6 (a sister line of Jemalong A17, with $>4,000$ amplification fragment length polymorphism markers showing no differences between these lines; Thoquet et al. 2002) and DZA315.16. Mapping data for 86 RIL ($F_{7:8}$) were integrated with phenotypic data to identify molecular markers linked to aphid resistance. A quantitative phenotypic screen was developed in which individual RIL plants were scored according to an aphid survival index (ASI) and the number of AIL. The ASI incorporated assessments of aphid survival and reproduction to give each plant a score from 0 (highly resistant) to 5 (highly susceptible). The ASI of all parental control plants was 0 and 5 for Jemalong and DZA315.16, corresponding to 0 and 100% survival, respectively. The mean ASI ($\pm$ standard error) for all RIL plants was 2.46 ± 0.19 . Lesions were present on all Jemalong and absent from all DZA315.16 parental plants. Approximately half of the RIL developed AIL and half did not (45 and 41, respectively; $\chi^2 = 0.09$, $P = 0.9$).

Unexpectedly, the phenotypic screening of RIL showed there was no relationship ($r = -0.25$) between aphid performance (ASI) and the number of AIL (Fig. 6). Some RIL were resistant to PS01 aphids in the absence of lesions and many RIL showed susceptibility with lesions present. This was confirmed in QTL analysis, which revealed separate loci controlling AIL and aphid resistance. A single QTL for AIL mapped to linkage group (LG) 3, positioned at 15 cM with a logarithm of odds (LOD) score of 14. The differences between the genotypes of RIL at this locus are clearly correlated with the phenotypes as shown in Table 1, which shows the mean number of AIL for RIL with the Jemalong genotype is greater than that of RIL with the DZA315.16 genotype. Aphid resistance, on the other hand, was linked to two QTL. A major resistance QTL mapped to 47 cM on LG 3 with a LOD score of 14 and R^2 (explained variance) of 53%. We propose the name *RAP1* (resis-

Fig. 5. The distribution of individual stylet probes by avirulent pea aphids (clone PS01) on *Medicago truncatula* Jemalong A17 according to the number of cell punctures recorded by electrical penetration graph (total = 137) that either induced lesions or did not. $\chi^2 = 31.7$, $P < 0.001$. The label “75” is the frequency of cases in which probes with two cell punctures did not induce a lesion.

tance to *Acyrtosiphon pisum* 1) for this major resistance locus. A second QTL (*RAP2*) on LG 6 mapped to 63 cM with a LOD score of 4 and R^2 of 20%. The allelic means in Table 1 clearly show that the ASI of RIL with the Jemalong genotype at resistance loci is reduced compared with RIL with the DZA315.16 genotype at the same position.

Aphid survival on selected RIL.

An aphid survival assay was carried out subsequently to confirm the major findings from the QTL analysis: i) *RAP1* confers resistance to pea aphid clone PS01 in Jemalong, and ii) AIL are not directly associated with aphid resistance. Individual RIL were selected that, according to marker data, fell into one of four categories, each with the DZA315.16 genotype at *RAP2* but differing in genotype at *RAP1* and the *AIL* locus on chromosome 3: i) resistant (Jemalong genotype at *RAP1*) with lesions (Jemalong genotype at *AIL*), ii) resistant (Jemalong genotype at *RAP1*) without lesions (DZA315.16 genotype at *AIL*), iii) susceptible (DZA315.16 genotype at *RAP1*) with lesions (Jemalong genotype at *AIL*), and iv) susceptible (DZA315.16 genotype at *RAP1*) without lesions (DZA315.16 genotype at *AIL*). Poor aphid survival was recorded on all plants with the Jemalong genotype at *RAP1*, and good survival was recorded in all cases with the DZA315.16 genotype at *RAP1* (Fig. 7). Aphid survival was again unaffected by the presence or absence of AIL.

Mapping of *RAP1* using residual heterozygous lines (RHL).

QTL analysis mapped the *RAP1* locus to 47 cM on chromosome 3. To further map *RAP1* relative to genetic markers, we exploited the availability of two RIL with a defined residual heterozygous region containing the *RAP1* locus. Both lines (LR4-70 and LR4-174) were confirmed to segregate for resistance to PS01 in a 3:1 ratio (totals = 221 resistant, 84 susceptible; $\chi^2 = 1.12$, $P = 0.25$). Markers flanking the *RAP2* locus

Fig. 6. Relationship between the number of aphid-induced lesions and the average aphid survival index (ASI) for each recombinant inbred line phenotyped for quantitative trait loci analysis ($r = -0.25$).

Table 1. The linkage map location, coefficient of determination (R^2), and phenotypic means $\pm$ standard error (SE) (according to genotype) of loci significantly associated with aphid-induced lesions (AIL) and pea aphid resistance^a

QTL position	LOD	R^2 (%)	LG	Allelic means					
				Average number of AIL ($\pm$ SE)			Average survival index ($\pm$ SE)		
				Jemalong	Het	DZA315	Jemalong	Het	DZA315
15	14	35	3	6.41 $\pm$ 0.64	1.00 ^b	0.32 $\pm$ 0.15			
47	14	53	3				0.21 $\pm$ 0.08	^c	3.11 $\pm$ 0.197
63	4	20	6				1.92 $\pm$ 0.22	2.88 $\pm$ 0.82	2.97 $\pm$ 0.309

^a Quantitative trait loci (QTL) position shown in cM. LOD = logarithm of odds. LG = linkage group.

^b Single data value.

^c *Data missing for heterozygotes where no data available.

were used to confirm that both lines are fixed for the DZA315.16 genotype at the *RAP2* locus. Individual progeny from lines LR4-70 and LR4-174 were scored for aphid resistance and were genotyped using simple sequence repeat (SSR) markers across the heterozygous region, corresponding to the predicted QTL map position. The genotype at the *RAP1* locus was confirmed by progeny testing. *RAP1* was mapped 3.8 cM north of the SSR marker mtic505 (identical to marker h2_180m21a) and 5.7 cM south of marker mtic51 (Fig. 8). The h2_180m21a marker maps to 48.4 cM on chromosome 3, according to the integrated genetic map of *Medicago truncatula* (available online from the United States and European Union *M. truncatula* genome sequencing project), which gives a map position for *RAP1* in close agreement with the QTL data. The genetic distance between the *RAP1* flanking markers mtic51 and h2_180m21a calculated from our mapping data is 10.4 cM, which is again in close agreement with the 10.7 cM distance previously assigned on the integrated *Medicago* map (Fig. 8). This places *RAP1* approximately 9 cM south of marker BE187590, which has previously been mapped 0.8 cM south of the locus controlling resistance to *T. trifolii* f. *maculata* (Fig. 8) (Klinger et al. 2007).

DISCUSSION

Here, we report extremely effective race-specific resistance to the pea aphid in *M. truncatula*, leading to rapid weight loss and death of avirulent aphids. Surprisingly, AIL with hallmarks of a classical HR that develop during the incompatible interaction were shown to be ineffective in generating aphid resistance. Despite evidence that HR-like necroses may provide protection against aphids (Belefant-Miller et al. 1994; Moloi and van der Westhuizen 2006), phenotypic scoring of

Fig. 7. Survival of pea aphids (clone PS01) on progeny plants of selected recombinant inbred lines with Jemalong (P39, P59, P62, P83) or DZA315.16 (P4, P6, P32, P143) genotype at *RAP1*. P4, P6, P59, and P62 have the Jemalong genotype at the locus for aphid-induced lesions, whereas P32, P39, P83, and P143 have the DZA315.16 genotype at this locus.

aphid resistance and lesion formation in this study revealed that aphid performance varied on plants irrespective of lesion induction. Genetic analysis confirmed that a single locus (*AIL*) maps to chromosome 3 and controls the HR phenotype in the F_(7:8) RIL population. QTL analysis also revealed the presence of two independent loci (*RAP1* and *RAP2*) controlling resistance to PS01 in Jemalong. *RAP1* alone is sufficient for full resistance to the PS01 clone (Fig. 7). The independence of the aphid resistance and HR lesion phenotypes raises questions regarding the role and trigger of the HR. Indeed, lesions were shown to be directly associated with aphid activity, thereby rejecting the possibility that aphid probing initiates systemic processes that induce nonlocalized lesions (Miles 1999). *AIL* had characteristics reminiscent of the HR, with rapid, localized necrosis of cells at the infection site (Kombrink and Schmelzer 2001). Lesions also shared features with the HR in terms of ROS production as well as timing of cell death and autofluorescence (Foster-Hartnett et al. 2007; Torregrosa et al. 2004) and were indistinguishable from the *M. truncatula* response to an incompatible *P. syringae* pv. *phaseolicola* strain. Using the confocal microscope to record emission spectra revealed nearly identical fluorescence profiles from cells within the lesions induced by aphids and by bacteria. Characterization of the fluorescent emission spectra in this way indicates that similar biochemical changes occur after aphid or bacterial challenge leading to the HR. The cause of fluorescence is likely to be the accumulation of phenylpropanoid metabolites within dead cells (Dixon et al. 2002).

In our experiments, HR-like lesions tended to be induced only after extended probes and multiple brief punctures of

nonvascular cells by avirulent aphids (Figs. 4 and 5). Lesions were initiated by brief stylet puncture of mesophyll cells adjacent to the stylet pathway. Although such a cellular response may have been elicited by mechanical stylet damage, different pea aphid clones show very similar stylet pathway activities (Wilkinson and Douglas 1998). The specific induction of the HR and associated ROS by only one of the two clones tested here implicates clone-specific salivary elicitors in triggering the cellular response. During plant penetration, aphids secrete two types of saliva. The saliva secreted as the aphid stylets move between cells in the apoplast gels to form a sheath around the stylets. Gelling saliva therefore forms a barrier between the aphid and the host as well as a channel through which the stylets can move (Miles 1999). During the intracellular punctures that punctuate the stylet pathway, aphids briefly switch to secretion of 'watery' saliva, delivering nongelling salivary material directly into each punctured cell (Powell 2005). Within 24 h, the HR initiated in individual cells adjacent to the stylet pathway spreads rapidly, forming a macroscopic lesion around the stylet pathway. This rapid cascade reaction might be expected to disadvantage sedentary phloem feeders such as aphids, but QTL and correlation analyses show that the HR phenotype has no relationship with resistance to aphids in this model system. On the aphid-susceptible RIL that show *AIL*, it is possible that the insects are able to avoid potentially detrimental effects of the HR spatially or temporally. Significantly, because the response appears to be initiated in peripheral tissues relative to the sieve element feeding site, phloem feeding may continue unaffected during lesion development. It is also possible that the plant cellular response occurs over a time-scale too great to affect aphids, which unlike microbial pathogens, are able to move from an unsuccessful inoculation site and establish a new site of challenge on the same or an adjacent leaf.

Alternatively, necrotic lesions may be a by-product of aphid activity rather than direct plant defense (Kombrink and Schmelzer 2001). For example, the development of plant necroses might be an adaptation brought about by aphids to work to their advantage under some conditions. Many aphid species are capable of inducing physiological changes in their host plant that enhance insect performance. For example, feeding by the pea aphid on alfalfa results in the redirection of nitrogen to the aphid feeding sites (Girousse et al. 2005). Kerns and associates (1989) however, compared the performance of various aphid clones on plants with variable reactions to feeding and were unable to show any consistent advantage of lesion-formation to the insects. The redox hypothesis (Miles and Oertli 1993) suggests that an imbalance between antioxidants and plant- and aphid-derived oxidases can lead to overproduction of ROS, which can result in tissue necrosis (Miles 1999). Resistance in wheat to the Russian wheat aphid is thought to be conferred by the HR and involves delicate control of ROS and possesses mechanisms to control their levels to minimize plant-toxic effects (Moloi and van der Westhuizen 2006). It has also been suggested that aphid gelling saliva may be capable of minimizing potentially harmful plant ROS by neutralizing them with salivary oxidases (Miles 1999).

The HR does not provide Jemalong with resistance against PS01 aphids. Instead, resistance is mediated in this interaction by two resistance loci, which were identified in the *M. truncatula* RIL population derived from Jemalong and DZA315.16. Resistance in both cases was a Jemalong-inherited trait. The major resistance-conferring locus (*RAP1*) mapped to chromosome 3 and progeny from heterozygous lines segregated in accordance with resistance conferred by a single dominant gene. A survival assay using selected RIL that are homozygous Jemalong or DZA315.16 at *RAP1* (all selected lines had the

Fig. 8. Genetic map position of *RAP1* relative to simple sequence repeat markers in the chromosomal region defined by the quantitative trait loci analysis. Results were aligned with data from the integrated genetic map of *Medicago truncatula* to allow relative positioning of the *Therioaphis trifolii* f. *maculatus* resistance gene (*TTR*) locus (Klinger et al. 2007). Marker mtic51 lies on bacterial artificial chromosome (BAC) mth2-34f1 (GenBank accession AC147712), which has an approximately 48-kb overlap with BAC mth2-39a22 (GenBank accession CT009615) containing the marker h2_39a22a. The physical distance between markers mtic51 and h2_39a22a is approximately 109 kb, and their positions on the genetic map are shown as approximately equivalent (dotted line).

DZA315.16 genotype at *RAP2*, the minor aphid resistance locus on chromosome 6) indicates that *RAP1* alone provides the full Jemalong resistance phenotype against the PS01 aphid clone. RIL with the DZA315.16 genotype at *RAP1* were indistinguishable from the susceptible parental line DZA315.16.

Two other *R* loci have been identified in *M. truncatula*, conferring resistance to *A. kondoi* (*AKR*) and *T. trifolii* f. *maculata* (*TTR*) (Klinger et al. 2005, 2007). Both loci also map to chromosome 3 but act separately to reduce survival and growth of their target species (Klinger et al. 2007). *AKR* maps to the top of chromosome 3 and is clearly separate from *RAP1*. *TTR* maps to a region 0.8 cM north of marker BE187590 (Klinger et al. 2007), whereas our genetic mapping positions *RAP1* nearly 9 cM south of BE187590. Genetic distances obtained from our mapping population indicate that *RAP1* can be confidently positioned relative to *TTR* as shown in Figure 8. Therefore, we conclude that *RAP1* and *TTR* constitute independent resistance loci on *Medicago* chromosome 3.

Resistance conferred by *RAP1* in *M. truncatula* seems more effective than in other recently identified *R* genes, causing 100% mortality of pea aphid clone PS01 after just 3 days. By comparison, survival of aphids over 5 days on resistant vs. susceptible *M. truncatula* lines was 50% vs. 91% for *T. trifolii* f. *maculata* and 54% vs. 93% for *A. kondoi*, respectively (Klinger et al. 2007). *Medicago truncatula* plants with *A. kondoi* resistance also reduced the population score of pea aphids between 6 and 15 days after infestation compared with the susceptible plant line (Gao et al. 2008).

Using the EPG technique, resistance to *A. kondoi* was identified as phloem-specific, with similar prephloem activities in the Jester (resistant) and Jemalong (susceptible) lines but dramatically reduced phloem ingestion on resistant plants (Klinger et al. 2005). Other examples of aphid resistance also appear to be mediated by a phloem-specific resistance mechanism, as the insects are able to reach sieve elements on resistant plants but are unable to sustain prolonged phloem feeding (Kaloshian et al. 2000; Klinger et al. 1998, 2005; Pegadaraju et al. 2007; Sauge et al. 1998; Tjallingii 2006). Our preliminary data using the EPG technique to monitor aphid stylet activities over 7 h suggest that the PS01-Jemalong incompatible interaction also fits this pattern, with regular salivation into sieve elements but only limited sap ingestion (Supplementary Table S1). These observations are consistent with the speed and high level of aphid mortality, probably reflecting death due to starvation, as seen with resistant *Mi-1* plants and the potato aphid (Kaloshian et al. 2000). The resistance in the *Mi* gene case, however, is developmentally regulated, and plants remain susceptible until after they reach 5 weeks of age (Kaloshian et al. 1995). In contrast, comparison of PS01 performance on 2-, 3-, and 4-week-old Jemalong A17 plants indicated that plant age is not a factor in *RAP1* resistance (data not shown).

The specificity of *RAP1* resistance for aphid clone PS01 as opposed to LL01 is interesting. Within-species or race-specific differences in host plant range for aphids are well known (Kerns et al. 1989; Loxdale 2008), and variation in specificity of resistance genes between different aphid clones has been found in some cases (Burd et al. 2006; Goggin et al. 2001; Kim et al. 2008; Rossi et al. 1998). However, the molecular processes that govern specificity are not yet understood. *R* gene-mediated plant defense is thought to involve recognition of an insect-derived molecule or elicitor, but to date, no elicitors have been identified for phloem-feeding insects. Nevertheless, several advances have been made in recent years, employing a range of approaches to identify components in insects that affect their interaction with plants. For example, extracts from the Russian wheat aphid (*Diuraphis noxia*) that were in-

jected into two wheat genotypes induced defensive enzyme activity in resistant plants and infestation symptoms, such as leaf rolling, in a susceptible line (Lapitan et al. 2007). Aphid saliva contains elements that are vital for feeding. The knockdown of a pea aphid salivary transcript, *C002*, by RNAi caused previously virulent aphids to become unable to feed on host plants (Mutti et al. 2006, 2008). Proteomics approaches have identified enzymes in saliva of the polyphagous aphid *Myzus persicae* with demonstrated roles in manipulating plant defense in other insects (Harmel et al. 2008). Significantly, glucose oxidase (GOX) activity in the aphid saliva was found to be in the same range as in several caterpillar species, in which, for example, GOX from *Helicoverpa zea* inhibits defensive nicotine production in tobacco (Harmel et al. 2008; Musser et al. 2005). Experiments with another aphid species, *Megoura viciae*, revealed that secreted saliva includes proteins with Ca^{2+} -binding domains, allowing aphids to remove free Ca^{2+} from sieve tubes and therefore prevent plugging by P proteins (Will et al. 2007). A recently-identified putative calcium-binding protein (regucalcin) in the saliva of the pea aphid (Carolan et al. 2009) probably plays a key role in the suppression of such defensive processes within sieve tubes. While these recent studies have provided fascinating insights into the potential subversion of plant defenses by aphid saliva, the role of salivary factors in determining race specificity in *R* gene-mediated interactions remains unexplored.

We have demonstrated extremely effective resistance in *Medicago* spp. to an important crop pest, the pea aphid, and shown that the HR is not required for resistance. The *M. truncatula*–pea aphid interaction provides a powerful model with which to explore plant defense mechanisms to an obligate biotroph. Although plant incompatibility with pathogens and aphids likely share multiple features of the *R-Avr* recognition system, the mechanisms by which avirulence is achieved may well be very different. Simply inherited resistance to *A. pisum* provides opportunities for studying the aphid side of such race-specific interactions, particularly using the availability of genetically diverse aphid clones within the species (Ferrari et al. 2008) as well as the recently completed pea aphid genome sequence to explore the nature of aphid salivary effectors.

MATERIALS AND METHODS

Plants.

M. truncatula seeds were scarified (with sandpaper), soaked, and refrigerated at 4°C for 24 h prior to germination in the dark at room temperature. Germinated seeds were transferred to compost (mixture of 4:1 organic compost (Levington's F2 with sand and Perlite) and were grown in the glasshouse (temperature 20 ± 5°C, with supplementary lighting to provide a minimum day length of 16 h). Seedlings at the cotyledon stage were transplanted into 6-cm diameter pots and were used in experiments at 2 to 3 weeks old with at least two fully expanded trifoliolate leaves. During experiments, plants were held under constant conditions (19 ± 1°C, 16 h light and 8 h dark) in a growth chamber. Prior to use in experiments, plants used for microscopy were scanned by eye to ensure there were no visible signs of stress or necroses.

For QTL analysis, the LR4 RIL population (T. Huguet, *unpublished*) was provided by the Institut National de la Recherche Agronomique (INRA) Centres de Ressources Biologique in Montpellier, France. Seven seeds were sown per RIL, but due to variability in germination, the number of plants tested per line varied from one to seven. In addition, 33 plants of Jemalong A17 and 53 plants of DZA315.16 were used as controls. Jemalong 6 plants were also phenotyped to demonstrate equivalence with its sister line, Jemalong A17.

Aphids.

Two green clones of the pea aphid, *Acyrtosiphon pisum*, were used in this study. Clone LL01 was originally collected from *M. sativa* and clone PS01 was collected from *Vicia faba*. Both clones were maintained on tic bean (*V. faba* var. *minor*) as described by Hodge and associates (2005). To obtain aphids of a particular age for use in experiments, adults were allowed to reproduce on tic beans for no longer than 24 h, at which point, they were removed and the remaining nymphs left to grow until they reached the required age. Infestation of *M. truncatula* was carried out using a fine paintbrush to transfer aphids from the tic bean subculture to the youngest, fully expanded trifoliate leaf of the plant and was restricted to the plant using a perforated plastic bag secured to the plant pot using elastic bands. For EPG experiments, fourth-instar nymphs (approximately 7 days old) of pea aphid clone PS01 were used.

Aphid performance.

Aphid survival was measured by counting live aphids on plants over 4 days subsequent to infestation with a single 2-day-old nymph per plant (20 plants per treatment).

The mean relative growth rate (MRGR) of aphids was calculated using the formula $MRGR = (\log_e W_{\text{final}} - \log_e W_{\text{orig}}) / t$, where t = time in days between original and final measurements of aphid fresh weight (W) (Leather and Dixon 1984). Due to the rapid mortality of the 2-day-old aphids used in the survival assessments, 4-day-old aphids were individually weighed on a microbalance (Mettler/Toledo MX5) before being placed onto the plant. The final weight of each aphid was taken 2 days after infestation. At least 12 replicates per treatment were used.

For the purpose of quantifying aphid resistance in RIL, the ASI was developed. Three-day-old nymphs ($n = 2$) were placed onto the upper surface of the youngest trifoliate leaf of each plant. Aphid survival and reproduction was assessed 4, 7, and 11 days after infestation. Using these data, each plant from all lines was assigned a survival score based on the following criteria: 0 = all aphids dead four days postinfestation (dpi), 1 = all aphids dead 7 dpi, 2 = all aphids dead 11 dpi or one alive but no offspring, 3 = at least one aphid alive at 11 dpi with offspring or no adults alive with >15 offspring, 4 = at least two aphids alive at 11 dpi with some offspring (<15) or one adult alive with >15 offspring, 5 = at least two adult aphids alive at 11 dpi with >15 offspring.

To measure the number of AIL in the RIL screen, a single, 7-day-old nymph was placed onto the upper surface of the elder (of two) fully-expanded trifoliate leaves and contained using a tube cage (1.5-cm-diameter, 5.5-cm-long clear plastic tube with a single open end, secured with cotton wool and supported by a metal wire, which was inserted into the compost) to prevent the aphid escaping. To standardize aphid exposure and lesion development times, the aphid was removed after 24 h but the tube cage was left in place to prevent any subsequent feeding or probing from the aphids tested for performance. At 4 dpi, AIL on the contained leaf were counted.

Microscopy.

Localization of the stylet entry point was performed using detached trifoliate leaves of *M. truncatula* Jemalong A17. Individual leaves were placed, adaxial surface up, onto damp filter paper in petri dishes before each leaf was infested with six 4-day-old PS01 aphids for 24 h. At 48 hpi, leaves were fixed in methanol and were cleared in saturated chloral hydrate solution before they were examined using a Zeiss Axioplan microscope for stylet entry and autofluorescence, with filter sets 01 and 09 (for UV and blue excitation). Intact Jemalong A17 and DZA315.16 leaves used for staining with 3,3-diaminobenzidine tetrahydrochloride (DAB) for ROS were exposed to

aphid probing for 2 h only. After 24 and 48 h, leaves were detached from the plant and vacuum infiltrated with a 0.2% DAB (Sigma-Aldrich, Ltd., Poole, U.K.) solution and were left in the dark at room temperature (approximately 22°C) for 8 h. Leaves were then fixed and cleared as above before they were examined using a Zeiss Axioplan microscope for stylet entry and localized brown staining.

Jemalong A17 plants were used for the comparison of aphid- and bacterial-induced lesions. For the aphid induction treatment, individual leaves were infested with three aphids of clone PS01 for 2 h. At the same time, plants used for bacterial induction were infiltrated with *Pseudomonas syringae* pv. *phaseolicola* (5×10^7 bacteria per milliliter in 10 mM $MgCl_2$) into young leaves using a 1-ml syringe and 25G needle. Two and three days later, the treated leaves were examined using a Leica TCS4D dissecting microscope with transmitted light or UV excitation with a green fluorescent protein (GFP) filter. The emission spectra of autofluorescent materials accumulating within dead cells in the lesions were recorded using a Leica TCS-SP2-DM IRE2 confocal laser scanning microscope (Leica Microsystems GmbH, Wetzlar, Germany). Single optical sections were collected using a 20-nm window and scans in xyλ mode for emission between 505 and 670 nm from excitation at 488 nm. Analysis of images was performed using Leica software TCS SP2.

Leaves examined following EPG recording were viewed at 24 hpi, using a Leica MZ16F microscope with a GFP filter.

EPG technique.

The DC EPG method (Tjallingii 1978) was used to monitor activity of PS01 aphid stylets during individual probes of Jemalong A17 leaves to analyze activities that led to lesion induction. Individual apterous adult aphids (nine in total) were used consecutively in recordings, tethered to an amplifier input using gold wire. The aphid was presented with an intact Jemalong A17 leaf until a single probe was monitored using the EPG equipment. At this point, a fresh leaf was presented to the aphid for the monitoring of the next probe, and so on. Leaves were then examined under the microscope 24 hpi for the presence or absence of lesions. As a control measure, all aphids used in experiments were placed onto individual plants for 24 h (after the EPG recordings), under perforated bags to confirm each was capable of inducing lesions. All aphids used in EPG experiments induced lesions on control plants subjected to a 24-h exposure period. Control leaves that had not been exposed to aphid probes showed no autofluorescence; therefore, all lesions were considered directly associated with the probes made by the aphids during EPG monitoring.

The information received from the EPG monitor was displayed and analyzed using PROBE 3.0 software (W. F. Tjallingii, Wageningen University, The Netherlands). Waveforms were scored according to Tjallingii and Hogen Esch (1993).

Genetic map, QTL analysis, and marker-based selection of RIL.

The F6 LR4 genetic map was constructed using a set of 316 SSR markers (T. Huguet, *unpublished*), using Carthagene software (de Givry et al. 2004). The map spans 910 Kosambi cM, with an average of 3 cM between markers. The eight linkage groups have different genetic sizes ranging from 90 to 131 cM.

The phenotypes of individual RIL were scored using five quantitative traits characterizing aphid resistance. These included plant height and number of nonsenescent leaves (reduced by heavy infestation), number of offspring, ASI, and number of AIL. RIL were also categorized as those with AIL and those without. For simplicity, we present data using ASI and number of AIL, although all data showed good heritability

(Supplementary Table S2). QTL were detected using PLABQTL software (available online from the Plant Breeding Institute, University of Hohenheim) with the composite interval mapping procedure with cofactors. Association was considered significant if minimum LOD values were greater than 2.19. A chi-square test was used to test markers for segregation distortion to determine if the population's genotype followed the expected 1:1 (A:B) segregation ratio. Genotypic means were calculated from the means of the marker genotypes in combination with the adjusted RIL means from the aphid phenotyping screen.

RIL with the DZA315.16 genotype at *RAP2* and contrasting genotypes at *RAP1* and *AIL* were selected, based on having a Jemalong or DZA315.16 homozygous genotype at the nearest SSR marker: mtic93 for *RAP2*, mtic673 for *RAP1*, and mtic47 for the *AIL* locus.

Genetic mapping using RHL.

Progeny from lines LR4-70 (F₈) and LR4-174 (F₆) were scored for aphid resistance as described above and were genotyped using SSR markers mtic51 and h2_180m21a/mtic505 (latter markers detect the same simple sequence length polymorphism between nucleotide positions 119,310 and 119,624 bp on BAC mth2-16m9, GenBank CU468244). Primer details are available online (United States and European Union *M. truncatula* genome sequencing project). Recombinants in the mtic51-mtic505 interval were progeny-tested for aphid resistance to confirm *RAP1* genotype.

Other statistical analyses.

Survival data were analyzed using log-rank tests. The mean relative growth rates were compared using one-way analysis of variance, followed by post-hoc Tukey tests, which separated treatments into two groups at *P* < 0.001. The chi-square test was used as indicated.

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AUTHOR RECOMMENDED INTERNET RESOURCES

Medicago truncatula Sequencing Resources, an organizational center for the United States and European Union *Medicago truncatula* genome sequencing project: www.medicago.org/genome

AphidBase, the aphid genome database: www.aphidbase.com

Plant Breeding Institute, University of Hohenheim software packages: www.uni-hohenheim.de/plantbreeding/software

The INRA *Medicago truncatula* Stock Center: www.montpellier.inra.fr/BRC-MTR