Characterization of pea aphid resistance in *Medicago truncatula*

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ABSTRACT

To achieve a thorough understanding of plant-aphid interactions, it is necessary to investigate in detail both the plant and insect side of the interaction. The pea aphid (PA), Acyrthosiphon pisum has been selected by an international consortium as the model species for genetics and genomics studies, and the model legume Medicago truncatula is a host of this aphid. In this study we identified resistance to pea aphid in a M. truncatula line, Jester, with well-characterized resistance to a closely-related aphid, the bluegreen aphid (A. kondoi). The biology of resistance to the two aphid species shared similarity, with resistance in both cases occurring at the level of the phloem, requiring an intact plant and involving a combination of antixenosis, antibiosis and plant tolerance. In addition, pea aphid resistance co-segregated in Jester with a single dominant gene for bluegreen aphid resistance. These results raised the possibility that both resistances may be mediated by the same mechanism. This was not supported by the results of gene induction studies and resistance induced by bluegreen aphid had no effect on pea aphid feeding. Moreover, different genetic backgrounds containing a bluegreen aphid resistance gene from the same resistance donor, differ in resistance to PA. These results suggest that distinct mechanisms are involved in resistance to these two aphid species. Resistance to pea aphid and bluegreen aphid in the same genetic background in M. truncatula makes this plant an attractive model for the study of both plant and aphid components of resistant and susceptible plant-aphid interactions.
INTRODUCTION

Phloem-sucking insects, such as aphids, whiteflies, scales and psyllids of the Hemiptera suborder Sternorrhyncha, represent an important area in studies of plant interaction with biotic stress. Aphids are ubiquitous and serious pests that cause substantial losses to agriculture worldwide by draining plant nutrients, injecting plant elicitors, and transmitting pathogenic viruses (Ng and Perry, 2004). In many cases, the highly specialized mode of aphid feeding causes little apparent damage to the plant. With their stylets, aphids penetrate plant tissues by probing intercellularly through epidermal and mesophyll cell layers and ultimately feed specifically from the phloem sieve element where aphids have a long-lasting association with their host. In many instances aphids are able to evade plant defenses while moving their stylets intercellularly; recent evidence suggests they are able to manipulate the host through secretion of saliva into the phloem sieve elements (Will et al., 2007). A thorough understanding of plant-aphid interactions, including the molecular mechanisms underlying plant resistance and those employed by aphids to evade these defenses, is of importance to agriculture and to our understanding of plant defense against biotic stresses.

Until recently, very little was known about the molecular mechanisms underlying aphid resistance. An important advance was the cloning of the Mi gene in tomato, which confers resistance to potato aphid (Rossi et al., 1998). This gene belongs to the NBS-LRR class of plant R (resistance) genes but differs from the normal high specificity associated with other family members in that it also confers resistance to nematodes (Milligan et al., 1998), whiteflies (Nombela et al., 2003) and psyllids (Casteel et al., 2006). Products of such genes have been shown to generally act near the top of a signal transduction cascade.
within the host cell, leading to an appropriate defense response. Aphid resistance encoded by such genes may be common in plants; genetic analysis of other plant-aphid interactions has shown tight linkage between resistance loci and NBS-LRR gene sequences (Seah et al., 1998; Brotman et al., 2002; Cevik and King, 2002; Klingler et al., 2005). Gene expression profiling associated with R-gene mediated defense has shown that aphids can cause up-regulation of both salicylic acid (SA)- and jasmonic acid (JA)-responsive genes as well as other aphid-responsive pathways (Thompson and Goggin, 2006). Recently, the SA signaling pathway was found to be important for Mi-1-mediated resistance to potato aphid (Li et al., 2006) while for bluegreen aphid (BGA) interacting with Medicago truncatula, induction of the JA pathway was exclusively associated with resistance (Gao et al., 2007a).

Although progress is being made in our understanding of plant resistance against aphids, very little is known about the aphid side of the interaction. Relatively few molecular tools have been developed to study the aphids for which plant resistance has been studied. This has made obtaining a balanced picture of a plant-aphid interaction difficult. The adoption of model species has been successful in focusing the development of molecular tools for many systems. Acyrthosiphon pisum (Harris) (pea aphid, PA) possesses a number of characteristics that are driving its development as a model aphid species. PA is a major agricultural pest, a virus vector, and is a relatively large, easily cultured aphid that is well-suited to laboratory based studies (http://www.hgsc.bcm.tmc.edu/projects/aphid/PeaAphidGenomeWhitePaper.pdf).

Furthermore, PA has proved to be a good model for studying aphid-bacterial symbiosis and aphid mediated virus transfer (van der Wilk et al., 1997; Nakabachi et al., 2005;
Scarborough et al., 2005). A number of molecular tools such as the genome sequence, an EST database, microarrays, BAC libraries and genetic maps have recently been developed for PA (http://www.hgsc.bcm.tmc.edu/projects/aphid). In addition, studies involving PA transformation and the use of RNA interference show promise (Jaubert-Possamai et al., 2007). However, to fully capitalize on these developments, clear resistance to this aphid must also be identified in a tractable model plant species for which complementary genomic tools have been developed. Arabidopsis is not a host of PA and resistance to other aphid species has not yet been identified in this model plant species, although quantitative variation in aphid performance exists among ecotypes (Cabrera y Poch et al., 1998).

PA is one of a number of aphid species that are serious pests for many legume crops (Edwards and Singh, 2006). It is an important pest of *Pisum sativum* (field pea), *Medicago sativa* (alfalfa) and *Lens culinaris* (lentils) (Maiteki et al., 1986; Mackay et al., 1993; Blackman and Eastop, 2000). Resistance to PA has been identified in some crop and pasture legumes (Andarge and Westhuizen, 2004; Julier et al., 2004; Ali et al., 2005), but to date no resistance has been identified in a tractable model plant species. Bournoville et al., (2004), found that *Medicago truncatula*, a pasture crop and model species was a host to PA. The *M. truncatula* scientific community has developed many molecular tools that are useful for the study of plant-aphid interactions such as a genome sequence, EST libraries, transformation systems, and various mutagenized populations for the identification of gene function through forward and reverse genetic approaches (http://www.noble.org/MedicagoHandbook/). We have previously used *M. truncatula* to
study resistance to an aphid species closely related to PA, *Acrythosiphon kondi* (bluegreen aphid, BGA), and have shown that BGA resistance is mediated by a single dominant gene called *AKR* (Klingler et al., 2005; Klingler et al., 2007).

In this paper we report on the identification of resistance to PA in a well characterized BGA resistant and susceptible pair of near isogenic *M. truncatula* lines: Jester (resistant to PA and BGA) and A17 (susceptible to PA and BGA and a reference genotype). Mapping data revealed co-segregation of BGA and PA resistance, raising the possibility that one gene may confer resistance to both aphids. However, a different genetic background containing a BGA resistance gene from the same resistance donor differs in resistance to PA. In addition, in Jester the downstream defense responses to PA and BGA differ. We have characterized this powerful model system further to investigate the mechanisms underlying resistance to the model aphid.

**RESULTS**

**Jester has some resistance to pea aphid compared to a near isogenic line.**

South Australian plant breeders created a pair of near-isogenic lines of *M. truncatula* with either susceptibility or resistance to *A. kondoi* (bluegreen aphid, BGA) (Hill, 2000). Specifically, A17 (the reference genotype for *M. truncatula*) is susceptible and its near-isogenic line, Jester, is resistant to BGA. We have previously characterized the interactions of these lines with BGA (Klingler et al., 2005; Gao et al., 2007a; Gao et al., 2007b; Klingler et al., 2007). In an attempt to identify sources of resistance to PA, a close relative of BGA, plant damage and aphid performance on A17 and Jester was assessed in a time course experiment where the aphids were confined to a single host.
The aphids quickly spread across the entire aerial regions of plants of each line. In the case of A17, plants rapidly developed damage symptoms, such as yellowing and distortion of developing leaflets followed by severe stunting and eventually the plants died (Fig. S1). In contrast, Jester showed significantly less (P<0.05) damage and a much lower aphid population as measured by aphid population score from day 6 to day 15 after aphid infestation (Fig. 1). The significantly lower aphid population (P<0.05) was also confirmed by the measurement of aphid weight per plant at day 15, which was 178.0 (±15.3) mg on the susceptible A17 versus 53.5 (±11.6) mg on the resistant Jester. At day 15, the mean plant dry weight of Jester (0.50 ±0.02 g) was significantly (P<0.05) higher than that of A17 (0.34 ±0.04 g), reflecting the lower plant damage due to the resistance of Jester against PA. Similar results were obtained from follow-up experiments where the aphids were free to move among plants (Fig. S1 or data not shown).

**Resistance to pea aphid in Jester co-segregates with resistance to BGA**

We were interested in understanding the genetic basis for PA resistance in *Medicago truncatula*. It was noted that Jester, which is resistant to BGA, was also resistant to the closely related PA, while the near isogenic line, A17 (roughly 90% identical to Jester), was susceptible to both aphids. Given this and that the major region introgressed into A17 to form Jester contains the BGA resistance gene, *AKR*, we first tested the hypothesis that PA resistance would co-segregate with the dominant BGA resistance trait. To test this hypothesis we used F₃ families from 10 F₂ plants, descended from the cross A17 x Jester, with recombination events tightly linked to the *AKR* locus controlling BGA resistance. The infestation of individual plants with PA in the greenhouse showed a clear difference
in plant reaction between the parental controls, A17 and Jester while no differences were observed between non-infested A17 and Jester (Klingler et al., 2005). The tolerance index (TI), recorded for each plant, showed non-overlapping ranges for the 12 plants of each parental genotype: for Jester plants, the TI ranged from 21 to 53; for A17 plants, the TI ranged from -9 to 10. The F3 progeny had TI values that tended, almost exclusively, to fall within one range or the other. Family D449 had one plant with a TI of 18, well above the range of the susceptible controls; this plant was considered qualitatively similar to Jester. The clear separation of TI scores into discrete ranges led us to assign qualitative characters (resistant, R; susceptible, S) to each F3 plant. In some cases, an F3 family fell entirely within one of the categories; in other cases, plants in a family segregated among the two categories.

Table 1 summarizes the correlation between F2 genotypes for molecular markers and the segregation ratios for PA resistance in F3 families. The molecular markers in the table are those that appear in the map reported by Klingler et al. (2005), with the addition of a new marker, 176P5, which maps between 004H01 and R1109. Marker 004H01 has been estimated to be located only 0.2 cM from AKR (Klingler et al., 2005), suggesting that a plant’s genotype for this marker is very likely to be its genotype for AKR. One rare exception was found in F2 plant F092, which we assume was a consequence of a recombination event between this marker and the AKR locus. In this case, the F3 progeny showed a reaction to PA that paralleled the F2 progenitor’s BGA phenotype (susceptible) rather than its genotype for 004H01 (heterozygous).

Our mapping data from two different F2 populations indicate that AKR lies at a genetic distance of no more than 2-3 cM from the distal edge of the major introgressed
segment of Chromosome 3 in Jester (Gao et al., 2007a). This is based on the position of CAPS marker DK258L, which is known to lie outside this introgression (Klingler et al. unpublished data) so the actual distance of \textit{AKR} from the introgression border could be significantly less than 2-3 cM. Studies comparing cytogenetic and genetic data have produced multiple estimates of the ratio between physical and genetic distance for \textit{M. truncatula}. These estimates range from 200 kb/cM in a region of chromosome (LG) 5 (Ane et al., 2002) to 1,000 kb/cM in a region of chromosome (LG) 4 (Schnabel et al., 2003), with an assumption of 300 kb/µm of euchromatin (Kulikova et al., 2001). Based on this range of figures, we estimate that the maximum distance of 2-3 cM separating \textit{AKR} and the introgression border represents approximately 400 to 3,000 kb of physical distance if the introgression border is tightly linked to DK258L. If not \textit{AKR}, the gene mediating resistance to pea aphid is located in this same region bounded by marker 004H01 and the introgression border. In summary, each of the 10 F\textsubscript{3} families segregated for PA tolerance as predicted under a model in which either the dominant gene \textit{AKR}, or a closely linked gene, controls resistance to PA. In the following sections we further characterize the resistance to PA in Jester and compare these results to BGA resistance mediated by \textit{AKR}.

**PA shows a preference for A17 over Jester**

Observation of host choice by alatae (the winged, migratory morph) can reveal clues to mechanisms of aphid resistance, such as whether antixenotic (deterrent) factors are present and the speed with which they influence behavior of a foraging aphid. In the host-choice test, alatae quickly dispersed from the point of release and most flew to the
tops of cages before settling on a plant. As shown in Fig. 2 the average number of settled alatae increased in both A17 and Jester plants up to 24 h after PA release suggesting there was no immediate effect of an antixenotic factor. After 24 h the number of aphids on Jester remained stable while the number of alatae settled on A17 throughout the 72h time course increased significantly ($P < 0.001$ at 48 and 72 h) suggesting a host preference by PA. This is in contrast to the response of BGA in similar experiments where a clear preference for the susceptible A17 was visible within 6 hours of release (Klingler et al., 2005).

**Resistance to PA in Jester is exerted through the phloem**

The electrical penetration graph (EPG) method is a powerful means of discerning, in real time, the locations and activities of aphid stylets during probing including their salivation into sieve elements and passive uptake of phloem sap (Walker, 2000). Representative EPG traces produced by PA probing A17 and Jester are shown in Fig. 3A & B. The proportions of time that tethered apterae spent outside the cuticle (non-probing), penetrating between cells *en route* to the vascular tissue (pathway phase), contacting xylem, salivating into sieve elements, or briefly puncturing cells (of unknown cell types) did not differ significantly between A17 and Jester (Fig. 3C). The similarities between the behavior of the aphids for these activities suggest that neither surface features (e.g. epicuticular waxes or trichomes) nor cell wall properties play a role in Jester’s resistance mechanism.

In contrast to these pre-ingestion activities, the proportion of time aphids spent ingesting phloem sap (E2 phase) was dramatically reduced on Jester plants (Fig. 3C).
While the sap ingestion occupied an average of 15% of total recorded activity on A17 plants, it occupied only 0.5% on Jester plants. This reduction in phloem ingestion phase, in contrast to pre-feeding activities, indicates the resistance mechanism to PA in Jester is exerted through the phloem.

In addition, the repeated potential drop (R-pd) waveform was observed during PA penetration of both A17 and Jester (Fig. 3A & B). This waveform was first described by (Tjallingii and Gabrys, 1999) for PA and some other aphids, and has also been termed “X-waves” using an AC EPG system (Reese et al., 2000). It appeared that the R-pd was a prerequisite for phloem feeding but did not necessarily always lead to phloem feeding. The number of R-pds, and therefore the total duration of R-pds, differed significantly (P<0.001) between Jester and A17 (Fig. 3C). This result again indicates that phloem feeding of PA is impaired in Jester compared to A17.

Similar experiments conducted with BGA on Jester and A17 revealed a significant reduction in phloem feeding on the resistant Jester (Klingler et al., 2005) indicating that resistance to both PA and BGA is phloem-based, although no R-pds were observed during probing by BGA.

**Resistance in Jester requires an intact plant**

We tested aphid performance on shoots excised from the host plant in comparison with an intact plant. Excision and maintenance of shoots on nutrient-supplemented agar did not cause any visible wilting or other signs of damage during the 3 day assay. Aphids settled on excised shoots, deposited honeydew and produced nymphs as they would on an intact plant. There was no significant difference (P>0.05) in aphid survival in any of the
treatments (data not shown). However, the aphid population growth rate (PGR) on intact plants was significantly lower on the resistant Jester than on the susceptible A17 (P<0.001) (Fig. 4). This resistance in Jester was lost on excised shoots, with aphids growing as well as they did on A17. Excision did not significantly affect the PA PGR on A17. These results are similar to those obtained from BGA infestation of A17 and Jester (Klingler et al. 2005). Possible explanations for the loss of resistance in excised shoots include a role of mobile resistance factors or changes in metabolism due to the leaf excision.

**Defense-related responses to pea aphid in Jester differ to the responses to BGA**

The similarity of resistance to PA and BGA in Jester suggested that similar defense mechanisms may be at play in the response to both aphids. We previously investigated transcriptional changes occurring in A17 and Jester in response to BGA feeding (Gao et al., 2007a). Genes associated with the SA pathway were induced in both resistant and susceptible lines, although in some cases with differing induction kinetics. Genes associated with the jasmonate pathway were exclusively or predominantly induced in the resistant line, Jester. To test the activation of these pathways in A17 and Jester in response to PA, the expression of selected genes from these pathways were analyzed using quantitative real time PCR.

**Salicylic acid and ethylene pathway genes**

The expression of SA-responsive genes (*BGL* and *PR5*; Gao et al., 2007a) was studied at three time points at 24, 36 and 72 h after PA infestation. As shown in Fig. 5A, the transcript levels of BGL increased in both the susceptible and resistant plants...
following PA infestation with expression induced at 24 h in Jester while in A17 an increase was not observed until 36 h. The transcript levels were not significantly different between the two genotypes at 36 h and 72 h following aphid infestation. In the case of \textit{PR5}, expression patterns were similar in both interactions, with the higher transcript levels remaining constant in both infested genotypes at all three time points (Fig. 5B).

Similar to the SA-responsive genes, the transcript levels of genes involved in the ethylene signaling pathway increased in both resistant and susceptible plants following PA infestation, with some differences in the kinetics of induction (Fig. 5C & D). For the gene encoding a hevein-like protein (\textit{HEL}), higher transcript levels were observed in infested relative to uninfested controls at all three time points for both resistant and susceptible lines (Fig. 5C). Similarly, ACC oxidase gene (\textit{ACC}), expression increased in both infested genotypes at all three time points (Fig. 5D). However, there were significant differences in transcript levels between infested Jester and A17 at 24h and 72 h. The basal expression of \textit{BGL}, \textit{PR5}, \textit{HEL} and \textit{ACC} prior to infestation were shown not to differ significantly between A17 and Jester (Gao et al 2007a).

\textit{Jasmonate pathway genes}

PA infestation did not induce large changes in the expression of genes of the octadecanoid pathway, which leads to the production of jasmonic acid (among other compounds). This was in contrast to the response to BGA infestation where genes involved in the jasmonate pathway are exclusively or predominantly induced in the resistant Jester (Gao et al. 2007a). For example, \textit{LOX2} was induced in both resistant and susceptible plants following PA infestation (Fig. 6A). A moderate increase of approximately 2-3 fold was seen at 24 h in both genotypes and in the susceptible plants at
36 h. The transcript level of \textit{LOX3} was not significantly changed by aphid infestation (Fig. 6B). The expression of other members of the LOX family (\textit{LOX1}, \textit{LOX5} and \textit{LOX6}) also did not show significant differences between A17 and Jester (data not shown).

To investigate whether JA-regulated genes were induced following PA infestation, we studied the expression of vegetative storage protein (VSP) and proteinase inhibitor (\textit{PI}) genes. Both genes previously showed large inductions following BGA infestation in the resistant line Jester while the susceptible A17 showed no response (Gao et al 2007a). In contrast to BGA infestation, PA infestation did not induce either VSP or \textit{PI} genes in either line (Fig. 6C & D).

\textbf{Resistance mechanisms in Jester are aphid specific.}

Despite similarities in the resistance phenotype against PA and BGA in Jester, the difference in the expression patterns of defense genes, particularly the jasmonate pathway, suggests different mechanisms may be responsible for resistance to each aphid. To compare the cross-effectiveness of the resistance responses we compared the ability of systemic defenses induced by one aphid species to inhibit the population growth rate of the other aphid in a second infestation. Prior infestation of Jester with BGA was shown to reduce the performance of BGA on uninfested systemic leaves (Klingler et al., 2005). To determine whether PA feeding causes systemic effects on BGA performance and vice-versa, we compared the aphid population growth rates (PGR) of BGA and PA either with or without prior infestation of A17 and Jester by these two types of aphids. Consistent with previous results in Klingler et al. (2005), prior infestation by BGA had no significant systemic effect on the PGR of BGA on susceptible A17 but caused a significant reduction.
in the PGR of BGA on the resistant Jester (Fig. 7A). In contrast, prior infestation by PA showed no effect on the PGR of BGA on either susceptible A17 or resistant Jester (Fig. 7A). Similarly, prior infestation by BGA showed no significant effect on the PGR of PA on either A17 or Jester (Fig. 7B). Interestingly, prior infestation with PA also had no effect on the PGR of a second PA infestation, suggesting that in contrast to the effect of BGA, PA does not induce a systemic resistance response effective against subsequent infestations of the same aphid species.

**Resistance to bluegreen aphid can occur in the absence of resistance to pea aphid in**

*M. truncatula*

To further investigate the similarities and differences between resistance to BGA and PA, other lines of *M. truncatula* possessing resistance to BGA and their susceptible near isogenic partners were tested for resistance to PA. Plants were grown in a growth chamber and plants individually caged. BGA resistant line Caliph and its susceptible recurrent-backcross parent Cyprus both showed equally (P>0.05) high aphid population scores and mean aphid weight per plant (Figs. 8A & E) indicating the aphids are able to grow and reproduce effectively on these plants. However, as shown in Figs. 8B & F, Caliph showed significantly lower (P<0.05) plant damage scores and significantly higher (P<0.05) mean plant dry weight than Cyprus, suggesting that although Caliph supports large numbers of aphids, it possesses tolerance to PA. This differs from the results observed in Jester where a reduced aphid population score and plant damage score indicated that in addition to tolerance, Jester is able to restrict aphid population growth. Caliph has gained resistance to BGA from the same resistance donor as Jester so the
different responses to PA suggest there may be an effect of genetic background on the PA resistance gene, or that Caliph contains a PA tolerance gene distinct from the PA resistance gene in Jester.

BGA resistant line Mogul and its susceptible near isogenic partner Borung both showed high aphid population and plant damage scores following PA infestation, which were not significantly different between the two lines (P<0.05) (Fig. 8C &D). Further measurements of aphid weight (Fig. 8E) and plant dry weight (Fig. 8F) again indicated both Borung and Mogul are susceptible to PA. While the BGA resistance donors for Mogul and Jester were different, mapping studies and allelism experiments strongly suggest that Mogul contains the same BGA resistance gene (AKR) as Jester (Klingler et al, 2007). The absence of resistance in Mogul suggests either different resistance genes function in Jester to provide resistance to PA and BGA or the overall genetic background in Mogul, which is quite different from Jester, is unable to mediate resistance to PA in spite of the presence of AKR.

A follow-up experiment conducted in a glasshouse, where the aphids were free to move among plants of all six lines (A17, Jester, Cyrpus, Caliph, Borung and Mogul), yielded similar results (Fig. S1 or data not shown).

DISCUSSION

The value of *M. truncatula* as a model for studying aphid defense has been demonstrated in studies with BGA and spotted alfalfa aphid (Klingler et al., 2005; Gao et al., 2007a; Gao et al., 2007b; Klingler et al., 2007). Single gene resistance against each aphid species has been characterized and mapped (Klingler et al., 2005; 2007). The utility
of *M. truncatula* as a model for studying aphid-plant interactions would be substantially improved if genomic resources were also available to study the aphid side of the interaction. The international aphid research community has selected PA, another legume-feeding aphid as its model species for genomics studies including a genome sequencing project that will be completed by 2008. PA can feed successfully on *M. truncatula* (Bournoville et al. 2004) but resistance to PA in *M. truncatula* had not yet been identified.

In this study, we have identified resistance to PA in the *M. truncatula* cultivar Jester as compared to its near-isogenic recurrent parent line A17. Resistance to BGA, a close relative of PA, has been well-characterized in this same line (Klingler et al. 2005), including a survey of defense gene induction (Gao et al. 2007a). In this study we have shown that the biology of resistance to PA in Jester has a number of similarities with resistance to BGA. For example, PA population growth is suppressed (Fig. 1A), and exposed plants show less damage (Fig. 1B). In choice tests, winged adult PAs prefer A17 to Jester (Fig. 2). However, it should be noted that resistance in Jester to PA appears to be more moderate than resistance to BGA. When the effects of Jester resistance on the two aphids are measured in the same experiment, PA population growth and plant damage are less affected than those of BGA (Gao et al. unpublished data).

While both PA and BGA show a preference for A17 over Jester, BGA exhibited this preference as early as 6h after exposure (Klingler et al. 2005), while PA showed no preference until after 24 h. This suggests that the mechanisms of non-preference are different, or that the effect of antixenotic factors varies between the two species. It is possible that BGA feeding induces an antixenotic factor within 6h, while PA non-
preference results from an inability to establish an effective feeding connection to the phloem over 24-48h. Delayed effects of resistance on aphid preference have been documented previously. For example, alate *Aphis gossypii* (cotton-melon aphid) showed no significant preference between unrelated resistant and susceptible lines of *Cucumis melo* (melon) until 24 and 48 h after release (Kennedy and Kishaba 1977). Like PA resistance in Jester, the resistance mechanism in melon was later shown to be phloem localized (Kennedy et al., 1978; Chen et al., 1997; Klingler et al., 1998), suggesting that time was spent probing phloem tissue before alatae showed a preference between resistant and susceptible hosts.

This study has demonstrated that resistance to PA in Jester is phloem-based (Fig. 3) and is not present in excised leaf tissue (Fig. 4), both of which are also characteristics of BGA resistance in this line (Klingler et al. 2005). Unlike BGA, PA exhibits an unusual EPG waveform called “repetitive potential drops” (R-pds), which is thought to represent a series of sieve element punctures (Tjallingii and Gabrys 1999). In A17, these R-pds were always followed by phloem salivation (E1) and feeding (E2) (Fig. 3A). In contrast, when aphids fed on Jester, phloem contact was often terminated before any feeding had occurred (Fig. 3B) and the duration of these R-pds was significantly reduced compared to A17 (Fig. 3C). This is consistent with the hypothesis that R-pds are important in conditioning a plant for PA feeding, proposed by Tjallingii and Gabrys (1999); and R-pds may be a prerequisite in *M. truncatula* for the achievement of phloem sap ingestion. It is interesting to note that the effect of resistance on aphid ingestion (E2) seems disproportional to the strength of the resistance as measured in the longer feeding and preference bioassays (Figs 1, 2). This difference could be due to the assay conditions.
during EPG, as the tether may restrict the aphids from locating the most suitable feeding sites on Jester – which can differ between resistant and susceptible plants (Klinger et al. 2007). However, it may also indicate that PA is eventually able to condition Jester plants and ingest more successfully after the 9 hour duration of the EPG experiments.

The biology of resistance to PA and BGA shared similarity, with resistance in both cases occurring at the level of the phloem, requiring an intact plant and involving a combination of antixenosis, antibiosis and plant tolerance. This suggested PA resistance might be mediated by the same gene as BGA resistance, AKR, which is thought to be a member of the NBS-LRR class of resistance genes (Klingler et al. 2005). NBS-LRR genes are usually species-specific (Ellis et al., 2000), but this is not always the case. For example, the Mi gene in tomato confers resistance to an aphid, a nematode, a whitefly species and a psyllid (Rossi et al. 1998, Milligan et al. 1998, Nombela et al. 2003; Casteel et al., 2006). Therefore it is reasonable to hypothesize that the same NBS-LRR protein could be active against these two closely-related aphid species. This hypothesis was further supported by the fact that BGA resistance is inherited as a single, dominant gene that maps to a cluster of R gene analogs (Klingler et al., 2005), and that PA resistance co-segregated with this trait (Table 1). However, because NBS-LRR genes typically reside in clusters in plant genomes the resolution of our current map of the AKR locus does not allow us to distinguish whether the same gene mediates resistance to BGA and PA in Jester, or whether closely linked genes mediate resistance to specific aphid species.

The biological evidence for a single resistance gene acting against both PA and BGA was not supported by the results on defense-related gene expression. The resistant interaction of Jester with BGA is characterized by the specific up-regulation of genes
involved in the jasmonate pathway (Gao et al., 2007a), which was not observed in the interaction with PA (Fig. 6). The jasmonate pathway is normally associated with defense against chewing insects and mechanical wounding which, unlike aphid feeding, involve maceration of plant tissue (Howe et al., 1996; McConn et al., 1997). In response to PA, JA-responsive genes were not induced or showed only moderate induction in both resistant and susceptible interactions (Fig. 6). Thus, different signal transduction pathways appear to be involved in resistance against BGA and PA in Jester, which suggests resistance is not regulated by the same resistance gene or the response is modified by other factors.

Despite the differences in jasmonate-related gene expression, other defense responses were consistent with responses to other aphids. Consistent with previous results for BGA on Jester (Gao et al. 2007a), genes involved in the SA and ethylene pathways were induced in both the susceptible and resistant interactions with PA, with some interesting quantitative difference in some cases (e.g. $BGL$, Fig. 5A). In tomato, earlier and stronger induction of SA-responsive genes has been associated with the presence of the $Mi1-1$ resistance gene (de Ilarduya et al., 2003). Further evidence for the involvement of SA in resistance to some aphids was provided by the use of the SA-metabolizing $NahG$ as a transgene in the $Mi1$ background to demonstrate that SA is essential for potato aphid resistance (Li et al., 2006). In Jester, early and strong induction of specific SA-responsive genes may potentiate resistance against infestation by both BGA and PA.

Despite the association of SA and ET in resistance to both PA and BGA, the involvement of different resistance mechanisms in Jester was indicated by reciprocal preinfestation studies (Fig. 7). As has been observed previously (Klingler et al. 2005),
preinfestation with BGA on Jester causes increased systemic resistance against BGA feeding (Fig. 7A). In this study, preinfestation with PA did not affect subsequent PA feeding (Fig. 7B). Also, preinfestation with BGA did not affect subsequent PA feeding nor vice versa (Figs. 7A & B), suggesting that different downstream resistance mechanisms may be involved in resistance against these two closely-related aphid species.

Further support for the presence of two independent resistance genes was obtained from experiments comparing PA performance and plant damage on two additional resistant lines (Fig. 8). There is strong evidence from mapping studies and allelism experiments that one of these lines, Mogul, contains the same BGA resistance gene (AKR), or a different allele at the same locus, as Jester (Klingler et al., 2006), yet Mogul shows no resistance against PA (Fig. 8C-F). Resistance to BGA in the second line, Caliph, is believed to be derived from the same source as that in Jester (Lake, 1993; Hill, 2000). Caliph also shows no resistance to PA (Fig. 8A & E), although it shows damage tolerance to PA feeding (Fig. 8B & F). It is possible that a single resistance gene controls resistance to both BGA and PA, but that other modifying genes are regulating the resistance response differently against the two aphid species. These modifying genes could be absent or different in the Mogul and Caliph backgrounds, leading to different responses to PA. If AKR encodes a NBS-LRR protein that mediates resistance to both aphid species in Jester, this would require that the resistance protein elicit different responses to different effector molecules. Under the “guard hypothesis” of NBS-LRR function (Dangl and Jones, 2001), this might occur if, for example, the AKR protein simultaneously guarded distinct targets of effectors from each aphid species.
With the development of PA genomics resources, in particular EST libraries from salivary gland tissue, the *M. truncatula* system becomes an even more attractive model for the study of aphid-plant interactions. A major focus of future work in this system will be on the identification and characterization of the aphid salivary secretome, and in particular the effector proteins or their targets recognized by *M. truncatula* aphid resistance proteins. PA is a highly attractive model for these studies, as this species is known to form races with restricted host ranges (Via, 1999) and effectors in salivary secretions can be collected *in vitro* using artificial diets (Madhusudhan and Miles, 1998). Only some European clones of PA collected off alfalfa/lucerne (*M. sativa*) can feed successfully on *M. truncatula* (Bournoville et al., 2004) and based on a pathogen-plant model, one would expect differences in secreted effector proteins to contribute to this difference in feeding ability. Salivary comparisons of PA and BGA should help to clarify their interactions with resistance gene(s) in Jester. As a whole, these studies should contribute significantly to a fuller understanding of plant resistance against aphids and other phloem-feeding insects.

**MATERIALS AND METHODS**

**Plants**

Two genotypes of *M. truncatula* were the primary focus of this study: bluegreen aphid (BGA)-susceptible genotype A17 and BGA-resistant cv. Jester. In addition two other pairs of closely-related lines of *M. truncatula* (cvs. Cyprus-Caliph, Borung-Mogul) were used. The genetic background and the origins for all these lines have been described in detail in (Klingler et al., 2005; Gao et al. 2007a; 2007b).
Prior to planting, seeds were scarified and germinated in the dark on moist filter paper for two days at room temperature. For most experiments, plants were grown in a growth chamber with 16 h light (22°C)/8 h dark (20°C) under metal halide and incandescent lamps producing 300 µE m⁻² s⁻¹. Plants were watered with a half-strength Hoagland’s solution every second day.

**Aphids**

The aphid species used were the pea aphid (*Acyrthosiphon pisum* (Harris)) - PA and the bluegreen aphid (*Acyrthosiphon kondoi*) – BGA. Aphids of each species were obtained from colonies initiated from single aphid clones collected in Western Australia and were reared on field pea (*Pisum sativum*) for PA and alfalfa-lucerne (*M. sativa*) for BGA with 14 h light (23°C)/10 h dark (20°C) under high pressure sodium lamps and fluorescent light at 280 µE m⁻² s⁻¹. Aphids were transferred to experimental plants with a fine paintbrush. Single clones were used for each aphid species to minimize within-treatment variability, but additional clones have been tested and produced similar results in separate experiments with the same plant genotypes (data not shown).

**Plant damage experiments**

To assess the susceptibility or resistance of *M. truncatula* lines against pea aphids, three experiments were conducted, two in glasshouses and one in growth chamber at CSIRO (Floreat, Western Australia). In both glasshouse and growth chamber experiments, plants were grown in individual 0.9 L pots and six replicate plants of each *M. truncatula* line were randomly arranged. For the glasshouse experiments, plants were
grown in an aphid screening chamber. For the growth chamber experiment, individual plants and aphids were caged separately. For all three experiments, two weeks after planting, each plant was infested with three apterous adults of PA. For the initial glasshouse experiment (A17 and Jester), the damage of each plant was visually assessed at three weeks after aphid infestation. Following this experiment, two experiments (A17, Jester, Cyprus, Caliph, Borung, Mogul) were conducted in the growth chamber or glasshouse where the damage of plants were assessed at a 3-day interval up to 15 days or 21 days for growth chamber experiment or the glasshouse experiment, respectively. The damage of each plant was scored on a 1 to 5 scale (1: no visual damage; 2: plants slightly stunted, no leaf discoloration; 3: leaf yellowing; 4: heavily stunted; 5: a dead plant) as described by Nair et al. (2003). For the growth chamber experiment, the aphid population on each individual plant was also scored on a 1 to 5 scale (1: <20 aphids; 2: 21-50 aphids; 3: 51 to 100 aphids; 4: 101-250 aphids; 5: >250 aphids).

**Genetic analysis of PA resistance**

The relation between resistance to BGA and PA was tested using an experimental plant population developed specifically for mapping BGA resistance in Jester. Previously, F2 plants from the cross A17 x Jester were phenotyped for BGA resistance by assessing the degree of feeding damage and aphid numbers on individual plants, followed by aphid removal and rescue of plants for the purpose of DNA analysis and for harvesting at least a few pods containing F3 seed (Klingler et al., 2005). In some cases, for the purpose of high-resolution mapping, seed from F3 families were grown and resulting plants infested with BGA to determine the aphid resistance genotype at the AKR locus. In
the present study, ten of these F3 families were selected for testing their response to PA infestation. These families were chosen from rare F2 progenitors that had recombination events tightly linked to the AKR locus, based on molecular marker genotypes (Klingler et al., 2005).

Pilot experiments had shown that Jester suffered more damage from PA if infested at 14 days after planting (DAP), compared with damage from BGA. Specifically, PA caused necrotic lesions on both A17 and Jester (but to a lower degree on Jester), whereas BGA had been shown to cause lesions only on the susceptible line A17 (Klingler et al., 2005). In order to maximize the difference in reaction to PA between A17 and Jester, plants were infested at 19 DAP instead of 14 DAP. Seedlings were grown in individual 1.2 L pots in a controlled environment chamber as described by Klingler et al. (2005), and then moved to a greenhouse before infestation. From six to 16 individual F3 plants were included from each of the 10 families, depending on the BGA resistance phenotype of the F2 progenitor and the amount of seed available. Twelve plants each of A17 and Jester were included as controls. Pots were arranged in a completely randomized design. The experiment took place under natural light during March and April in Perth, Western Australia, with temperature ranging from 17°C at night to 24°C during the day. Two third or fourth-instar PA nymphs were placed on each plant with a fine brush, and the entire plant was then covered with a clear plastic, ventilated cage as described by Klingler et al. (2007). Three days after infestation, the cages were removed and aphids were free to move among the plants. Twenty-six days after infestation, plants were phenotyped for PA resistance.
Unlike plant reaction to BGA in this population, which showed a clear contrast between resistant and susceptible plants, based on a single damage score (Klingler et al., 2005), the response to PA was more quantitative and required the development of a tolerance index using several plant characteristics recorded on the day of phenotyping. An index was developed that showed a pronounced, non-overlapping difference between the range of reactions for the parental controls, A17 and Jester. This tolerance index (TI) was calculated for each plant as follows:

$$TI = [(\text{longest stem length in cm}) + (\text{number of pods}) + (\text{number of stems > 20 cm})] - [(\text{number of leaves with necrotic lesions}) + \text{(damage score from 1-10)}],$$

where the damage score was a subjective rating based on plant damage (Klingler et al., 2005), in which 1 = little or no visible necrosis or stunting and 10 = a dead plant. For counts of leaves with necrotic flecks, up to 10 leaves were counted, and then simply noted as “> 10” leaves if more. For data analysis, these "> 10" were assigned a value of 11, which greatly under-represented the true number of necrotic leaves on many of the plants. Thus, TI was overestimated for many of the susceptible plants. After phenotyping, F3 plants were chemically treated to remove aphids, and then were cultured for DNA analysis and production of F4 seed.

**Host Selection Behavior**

The experiment to examine host choice by PA alatae was set up as described for BGA in Klingler et al., (2005) with modifications. Settling of aphids on each plant was observed at 3, 6, 12, 24, 48 and 72 h after release. The numbers of alatae that settled on the two plants of A17 or Jester in each cage were pooled. The significance of the difference in the settling of aphids (pooled data) between A17 and Jester and between
time points was analyzed with two-way ANOVA (genotype × time points), using the Genstat 6.2 software (Lawes Agricultural Trust, Rothamsted Experimental Station).

**Aphid development on intact plants and excised leaves**

The survival and growth rate of pea aphids were measured after 3 d on individual intact plants or excised shoots of each *M. truncatula* line, A17 or Jester with six replicates for each treatment. The plant growth, the leaf excision and culture and the measurement of the survival and the growth rate have been previously described in Klingler et al., (2005) and Gao et al., (2007b), with some modifications. For the excised leaves, three weeks after planting, a stem tip with one node was excised from each plant of A17 or Jester and inserted into agar supplemented with soluble fertilizer in an inverted 90-mm diameter petri dish. A cohort of eight first or second instar nymphs were placed on each of the intact plants or excised leaves of A17 or Jester plants. The proportion of aphids that survived and aphid population growth rate were analyzed by two-way ANOVA (genotype, A17 and Jester; treatment, leaf excision and intact plant) using Genstat 6.2.

**Aphid Feeding Behavior**

The feeding behavior of PA on the plants of A17 and Jester was studied using the DC EPG technique (Tjallingii, 1987) as described in Klingler et al., (2005) with modifications. Plants were grown with 16 h light (22°C)/8 h dark (20°C) under metal halide and incandescent lamps producing 300 µE m⁻² s⁻¹. When plants were 3 to 5 weeks old, a single aperous adult pea aphid was placed on a single trifoliate leaf and the feeding behavior was monitored. Ten biological replicates were included for each A17 and Jester
genotypes. A four-channel amplifier simultaneously recorded four individual aphids on separate plants, two A17 and two Jester. Waveform patterns in this study were scored according to categories described by Tjallingii and Esch (1993): nonpenetration; pooled pathway phase activities; salivary secretion into sieve elements; phloem sap ingestion; xylem ingestion; cell puncture events of several seconds duration (referred as potential drop, pd). The number and the duration of the repetitive pd (R-pd), similar to the type of potential drops described by Tjallingii and Gabrys (1999), were also recorded. The mean proportional time spent in each behavior on each plant of the two cultivars were analyzed by one-way ANOVA using GenStat 6.2.

**Transcriptional analysis of defense-related genes**

For the transcriptional analysis of defense-related genes in *M. truncatula* genotypes of A17 and Jester following PA infestation, the plant growth, aphid infestation and sampling, RNA isolation and cDNA synthesis, PCR primer design, real-time quantitative PCR conditions and analysis were similar to those used in the study of BGA with modifications (Gao et al., 2007a). Relative gene expression was derived from using $2^{\Delta CT}$, where $\Delta C_T$ represents $C_T$ of the gene of interest minus $C_T$ of tubulin. Where required, the significance of differences between relative gene expressions was analyzed by two-way ANOVA (genotype: A17 and Jester; treatment: infested and non-infested control) and compared by the LSD test at a 5% significance level using GenStat 6.2.

**Aphid performance on preinfested plants**
To assess the effect of the preinfestation of BGA or PA on the performance of these two aphid species on A17 and Jester, aphid survival and growth were measured after 4 days on preinfested and control plants of each cultivar using cohorts of 10 preweighed, early-instar nymphs as described in Klingler et al. (2005). Plants were grown in individual 0.9 L pots in a growth chamber. Four weeks after sowing, a linen mesh cage (35 x 200 mm) was placed on a single trifoliate leaf of each plant. A wooden stake supported the stem and cage. The cage was placed on either the fourth or fifth trifoliate leaf to emerge on the primary stem of each plant. Plants were randomly placed into one of three treatments: control (with cage but without aphids), preinfested with 15 adults of bluegreen aphids or pea aphids inside the cage. Aphids had access to the stem, a single trifoliate leaf, and its petiole.

At the end of the 2 d preinfestation treatment, a mesh cage was placed on the next trifoliate leaf distal to (younger than) the original caged leaf on the same stem. A cohort of 10 preweighed, early-instar nymphs of BGA or PA was placed inside this second cage, while the original aphids remained in their cage on the other leaf. Four days after the second infestation, the number and weight of surviving aphids in the second cage were recorded. The PGR of surviving nymphs was calculated as described above. The proportion of aphids that survived and PGR were compared by two-way ANOVA (genotype, A17 and Jester; treatment, preinfestation and no preinfestation) and compared by the LSD test at a 5% significance level using GenStat.

**SUPPLEMENTAL MATERIAL**
Figure S1. Photograph of near isogenic *M. truncatula* lines three weeks after three apterous adult PAs were placed on each plant and allowed to move among plants.

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Figure legends

**Figure 1.** Mean aphid population score (1: <20 aphids to 5: >250 aphids per plant) (A) and mean plant damage score (1: no visual damage to 5: dead plant) (B) for *M. truncatula* lines A17 and Jester over a time course observation following pea aphid infestation. Values are mean and standard error of six biological replicates.

**Figure 2.** Settling of pea aphid alatae in a choice test. Values are mean and standard error of six biological replicates. Means labeled with (*) for *M. truncatula* line Jester are significantly different from those for line A17 (P < 0.05).

**Figure 3.** EPG showing representative waveform patterns produced when pea aphid (*A. pisum*) apterae feed on susceptible line A17 (A) or on resistant line Jester (B). The horizontal axis represents a 1-h time period; the vertical axes represent voltage. Histological studies of plant-aphid interactions have correlated stylet positions in plant tissues with specific EPG waveforms (Walker, 2000). ‘‘Nonpenetration’’ indicates stylets are outside the plant. ‘‘Pathway’’ indicates mostly intramural probing activities between mesophyll or parenchyma cells. ‘‘Sieve element contact,’’ consisting primarily of sap ingestion with short periods of salivation into sieve elements, was frequently seen with plants of A17 and only rarely seen with plants of Jester. Sharp, downward spikes, named potential drop, indicate cell puncture events by stylets, each lasting approximately 5 s. ‘‘Repetitive potential drop’’, as also described in Tjallingii and Gabryś (1999), indicate repeated punctures of phloem sieve elements. The percentage of time aphids spent in various activities on A17 or Jester during 9 h exposure to the host plants is shown in
Figure C. Each value represents the mean and standard error of 12 biological replicates. Means for the cultivars labeled with * are significantly different (P < 0.001).

**Figure 4.** Effects of plant genotype and shoot excision on the population growth rate of pea aphid (*A. pisum*). Each value represents the mean and standard error of six biological replicates. Means labeled with the same letter are not significantly different (P < 0.05).

**Figure 5.** Differential expression of genes representing the salicylic acid- (A and B) or ethylene- (C and D) signaling pathways in petiole tissues of non-infested control or pea aphid-infested A17 and Jester plants, at 24, 36 or 72 h after aphid infestation. A. β-1,3-glucanase (*BGL*); B. PR5; C. hevein-like protein (*HEL*); D. ACC oxidase (*ACC*). The relative transcript abundance of each gene was normalized to a tubulin gene, and calculated using the formula $2^{-\Delta CT}$, where $\Delta CT$ represents $CT$ of the gene of interest minus $CT$ of tubulin. Values are the mean and standard error of three biological replicates.

**Figure 6.** Expression of genes representing the jasmonic acid signaling pathway in petiole tissues of non-infested control or pea aphid-infested A17 and Jester plants, at 24, 36 or 72 h after aphid infestation. A. lipoxygenase (*LOX2*); B. lipoxygenase (*LOX3*); C. vegetative storage protein (*VSP*); D. proteinase inhibitor (*PI*). The relative transcript abundance of each gene was normalized to a tubulin gene, and calculated using the formula $2^{-\Delta CT}$, where $\Delta CT$ represents $CT$ of the gene of interest minus $CT$ of tubulin. Values are the mean and standard error of three biological replicates.
**Figure 7.** Effects of plant genotype and prior infestation with bluegreen aphid (*A. kondoi*) or pea aphid (*A. pisum*) on population growth rate of bluegreen (A) and pea aphid (B). Population growth rate was measured as described in Materials and Methods. Values represent the mean and standard error of six biological replicates. Means labeled with the same letter are not significantly different (P < 0.05).

**Figure 8.** Mean aphid population score (1: <20 aphids to 5: >250 aphids per plant) and mean plant damage score (1: no visual damage to 5: dead plant) for *Medicago truncatula* lines Cyprus and Caliph (A and B); Borung and Mogul (C and D) over a time course of observations following pea aphid (*A. pisum*) infestation. Mean aphid weight (mg) per plant (E) and mean plant dry weight (g) (F) for all four *M. truncatula* lines at day15. Values are mean and standard error of six biological replicates.
Figure 1
Figure 2

Settled alatae per cage

Hours following pea aphid alatae release

A17
Jester
A: A17 (Susceptible)

B: Jester (Resistant)

C

Figure 3
Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

Gao et al., Resistance to pea aphid in Medicago truncatula
Table 1. Molecular marker genotypes and aphid resistance phenotypes of A17 x Jester F2 plants and their F3 progeny. Markers are listed in their order on a genetic map (Klingler et al., 2005), with 004H01 tightly linked to AKR. F2 plants were phenotyped for BGA resistance while F3 progeny were tested for BGA and/or PA resistance. Expected ratios for PA resistance phenotype are based on the model of a single dominant gene, or closely linked genes, conferring resistance to both aphid species.

<table>
<thead>
<tr>
<th>F2 progenitor plant</th>
<th>Marker genotype* near AKR locus</th>
<th>BGA resistance (AKR) genotype of F2 generation#</th>
<th>Segregation of PA phenotype in F3 generation&amp;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>R6M23L</td>
<td>R1109L</td>
<td>176P5</td>
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<td>C133</td>
<td>H</td>
<td>H</td>
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</tr>
<tr>
<td>C094</td>
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<td>B</td>
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</tbody>
</table>

*A = homozygous for A17 allele; B = homozygous for Jester allele; H = heterozygous; C = resistant, with either B or H genotype (F3 progeny not tested).
#Results listed in bold are based on phenotyping F3 progeny for BGA resistance.
&R = resistant; S = susceptible.