Characterization of Pea Aphid Resistance in *Medicago truncatula*[^W][^OA]


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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 (*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 PA in a *M. truncatula* line, 'Jester', with well-characterized resistance to a closely related aphid, the bluegreen aphid (BGA; *Acyrthosiphon 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, PA resistance cosegregated in 'Jester' with a single dominant gene for BGA 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 BGA had no effect on PA feeding. Moreover, different genetic backgrounds containing a BGA 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 PA and BGA 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.

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 styles, 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 styles 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 (*Solanum lycopersicum*), which confers resistance to potato aphid (*Macrosiphum euphorbiae*; Rossi et al., 1998). This gene belongs to the nucleotide-binding site-Leu-rich repeat (NBS-LRR) class of plant resistance (*R*) 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; Čevik 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), whereas for bluegreen aphid (BGA; *Acyrthosiphon kondoi*) 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. Pea aphid (PA; *Acyrthosiphon pisum*) 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, bacterial artificial chromosome 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 (*Arabidopsis thaliana*) 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 field pea (*Pisum sativum*), alfalfa (*Medicago sativa*), and lentils (*Lens culinaris*; 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; Julié et al., 2004; Ali et al., 2005), but to date no resistance has been identified in a tractable model plant species. Bournowille et al. (2004) found that *M. 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, BGA, and have shown that BGA resistance is mediated by a single dominant gene, called AKR (Klingler et al., 2005, 2007).

In this article, 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 cosegregation 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 R 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 PA 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 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, 2007; Gao et al., 2007a, 2007b). 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 (Fig. 1). 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 (Supplemental 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 (Supplemental Fig. S1; data not shown).

Resistance to PA in ‘Jester’ Cosegregates with Resistance to BGA

We were interested in understanding the genetic basis for PA resistance in *M. truncatula*. It was noted that ‘Jester’, which is resistant to BGA, was also resistant to the closely related PA, whereas 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 R gene AKR, we first tested the hypothesis that PA resistance would cosegregate with the dominant BGA resistance trait. To test this hypothesis, we used F3 families from 10 F2 plants, descended from the cross A17 × ‘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’, whereas no differences were observed between non-infested A17 and ‘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/susceptible) 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 I summarizes the correlation between F3 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 to 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 cleaved amplified polymorphic sequence marker DK258L, which is known to lie outside this introgression (J. Klingler, O. Edwards, and K. Singh, unpublished data), so the actual distance of AKR from the introgression border could be significantly less than 2 to 3 cM. Studies comparing cytogenetic and genetic data have produced multiple estimates of the ratio between physical and genetic distance for 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 maximal distance of 2 to 3 cM separating 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, AKR, the gene mediating resistance to PA, is located in this same region bounded by marker 004H01 and the introgression border. In summary, each of the 10 F3 families segregated for PA tolerance as predicted under a model in which either the dominant gene, 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 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 Figure 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, whereas the number of alatae settled on A17 throughout the 72-h 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 h 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 Figure 3, A and B. The proportions of time that tethered apterae spent outside the cuticle (nonprobing), 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 the resistance mechanism of ‘Jester’. In contrast to these preingestion activities, the proportion of time aphids spent ingesting phloem sap (E2 phase) was dramatically reduced in ‘Jester’ plants (Fig. 3C). Whereas 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 prefeeding activities, indicates the resistance mechanism to 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-d assay. Aphids

#### Table 1. Molecular marker genotypes and aphid resistance phenotypes of A17 × ‘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, whereas 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†</th>
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<tr>
<td></td>
<td>R6M23L</td>
<td>R1109L</td>
<td>176P5</td>
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<tr>
<td>C133</td>
<td>H</td>
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<td>D592</td>
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<td>D606</td>
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<td>F051</td>
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<tr>
<td>F013</td>
<td>A</td>
<td>A</td>
<td>A</td>
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<tr>
<td>C094</td>
<td>B</td>
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<td>B</td>
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*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.

Figure 2. Settling of pea aphid alatae in a choice test. Values are mean ± se of six biological replicates. Means labeled with * for *M. truncatula* line ‘Jester’ are significantly different from those for line A17 (P < 0.05).
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 PA in ‘Jester’ Differ from 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 JA pathway were exclusively or predominately 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.

SA 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 Figure 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’,...
of other members of the LOX family (LOX1, LOX5, and 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 (PI) genes. Both genes previously showed large inductions following BGA infestation in the resistant line ‘Jester’, whereas the susceptible A17 showed no response (Gao et al., 2007a). In contrast to BGA infestation, PA infestation did not induce VSP or PI genes in either line (Fig. 6, C and D).

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 JA 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 PGRs 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 BGA Can Occur in the Absence of Resistance to PA 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 (Fig. 8, A and E), indicating that aphids are able to grow and reproduce effectively on these plants. However, as shown in Figure 8, B and F, ‘Caliph’ showed signifi-
cantly 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 $R$ gene, or that ‘Caliph’ contains a PA tolerance gene distinct from the PA $R$ 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 that were not significantly different between the two lines ($P < 0.05$; Fig. 8, C and D). Further measurements of aphid weight (Fig. 8E) and plant dry weight (Fig. 8F) again indicated both ‘Borung’ and ‘Mogul’ are susceptible to PA. Whereas the BGA resistance donors for ‘Mogul’ and ‘Jester’ were different, mapping studies and allelism experiments strongly suggest that ‘Mogul’ contains the same BGA $R$ gene ($AKR$) as ‘Jester’ (Klingler et al., 2007). The absence of resistance in ‘Mogul’ suggests either different $R$ 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’, ‘Cyprus’, ‘Caliph’, ‘Borung’, and ‘Mogul’), yielded similar results (Supplemental Fig. S1; 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 (*Theroaphis trifolii f. maculate*; Klingler et al., 2005, 2007; Gao et al., 2007a, 2007b). 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 end of 2008. PA can feed successfully on *M. truncatula* (Bournoville et al., 2004), but resistance to PA in *M. truncatula* has not yet been identified.

In this study, we have identified resistance to PA in *M. truncatula* ‘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 (L. Gao, O. Edwards, and K. Singh, unpublished data).

Whereas both PA and BGA show a preference for A17 over ‘Jester’, BGA exhibited this preference as early as 6 h after exposure (Klingler et al., 2005), whereas PA showed no preference until after 24 h. This suggests that the mechanisms of nonpreference 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 6 h, whereas PA nonpreference results from an inability to establish an effective feeding connection to the phloem over 24 to 48 h. Delayed effects of resistance on aphid preference have been documented previously. For example, alate cotton-melon aphids (*Aphis gossypii*) showed no significant preference between unrelated resistant and susceptible lines of melon (*Cucumis melo*) until 24 and 48 h after

![Figure 6. Expression of genes representing the JA-signaling pathway in petiole tissues of noninfested control or PA-infested A17 and ‘Jester’ plants, at 24, 36, or 72 h after aphid infestation. A, Lipoxygenase (LOX2). B, Lipoxygenase (LOX3). C, VSP. D, PI. The relative transcript abundance of each gene was normalized to a tubulin gene and calculated using the formula 2^(-ΔΔCT), where ΔΔCT represents CT of the gene of interest minus CT of tubulin. Values are the mean and se of three biological replicates.](image-url)
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 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 that 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 and 2). This difference could be due to the assay conditions during EPG because the tether may restrict the aphids from locating the most suitable feeding sites on ‘Jester’, which can differ between resistant and susceptible plants (Klingler et al., 2007). However, it may also indicate that PA is eventually able to condition ‘Jester’ plants and ingest more successfully after the 9-h 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 R 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 (Milligan et al., 1998; Rossi 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 cosegregated with this trait (Table I). 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 R 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 JA pathway (Gao et al., 2007a), which was not observed in the interaction with PA (Fig. 6). The JA 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 R gene or the response is modified by other factors.
Despite the differences in JA-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 ET 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 Mi-1 R 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 Mi-1 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 resis-

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 *M. truncatula* lines 'Cyprus' and 'Caliph' (A and B) and 'Borung' and 'Mogul' (C and D) over a time course of observations following PA (*A. pisum*) infestation. E and F, Mean aphid weight (mg) per plant (E) and mean plant dry weight (g; F) for all four *M. truncatula* lines at day 15. Values are mean and se of six biological replicates.
tance 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 (Fig. 7, A and 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 R 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 R gene (AKR) or a different allele at the same locus as ‘Jester’ (Klingler et al., 2007), yet ‘Mogul’ shows no resistance against PA (Fig. 8, C–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. 8, A and E), although it shows damage tolerance to PA feeding (Fig. 8, B and F). It is possible that a single R 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 R 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 R proteins. PA is a highly attractive model for these studies because 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 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 R genes 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 *Medicago truncatula* were the primary focus of this study: BGA-susceptible genotype A17 and BGA-resistant ‘Jester’. In addition, two other pairs of closely related lines of *M. truncatula* (‘Cyprus’-‘Caliph’, ‘Borung’-‘Mogul’) were used. The genetic background and the origins for all these lines have been described in detail by Klingler et al. (2005) and Gao et al. (2007a, 2007b).

Prior to planting, seeds were scarified and germinated in the dark on moist filter paper for 2 d 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 one-half-strength Hoagland solution every second day.

**Aphids**

The aphid species used were PA (*Acyrthosiphon pisum*) and BGA (*Acrithosiphon kondoi*). Aphids of each species were obtained from colonies initiated from single aphid clones collected in Western Australia and were reared on *Pisum sativum* for PA and alfalfa-lucerne (*Medicago 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 paint brush. 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 PAs, three experiments were conducted, two in glasshouses and one in a growth chamber at CSIRO. 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 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, 2 weeks after planting, each plant was infested with three aperiodic adults of PA. For the initial glasshouse experiment (A17 and ‘Jester’), the damaging plants were visually assessed at 3 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 was assessed at a 3-d interval up to 15 d or 21 d for the 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, 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, 51–100 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 × ‘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 *F₂* seed (Klingler et al., 2005). In some cases, for the purpose of high-resolution mapping, seeds from *F₂* families were grown and resulting plants infested with BGA to determine the aphid resistance genotype at the AKR locus. In this study, 10 of these *F₂* 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 d after planting (DAP), compared with damage from BGA. Specifically, PA caused necrotic lesions on both A17 and 'Jester' (but to a lesser degree on 'Jester'), whereas BGA had been shown to cause lesions only on the susceptible line A17 (Klingler et al., 2005). 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 F2 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 TI using several plant characteristics recorded on the day of phenotyping. An index was developed that showed a pronounced, nonoverlapping difference between the range of reactions for the parental controls, A17 and 'Jester'. This TI was calculated for each plant as TI = [(longest stem length in cm) + (no. of pods) + (no. of stems >20 cm)] - [(no. of leaves with necrotic lesions) + (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 underrepresented the true number of necrotic leaves on many of the plants. Thus, TI was overestimated for many of the susceptible plants. After phenotyping, F2 plants were chemically treated to remove aphids and then were cultured for DNA analysis and production of F3 seed.

Host Selection Behavior

The experiment to examine host choice by PA alatae was set up as described for BGA by 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 (poled data) between A17 and 'Jester' and between time points was analyzed with two-way ANOVA (genotype × time points), using GenStat 6.2 software (Lawes Agricultural Trust, Rothamsted Experimental Station).

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 d on preinfested and control plants of each cultivar using cohorts of 10 preweighed, early-instar nymphs as described by 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 × 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), or preinfested with 15 adults of BGA or PA 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, whereas 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 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^(-ΔΔCT), where ΔΔCT represents ΔCT of the gene of interest minus ΔCT of tubulin. Where required, the significance of differences between two relative gene expressions was analyzed by two-way ANOVA (genotype: A17 and 'Jester'; treatment: infested and noninfested control) and compared by the LSD test at a 5% significance level using GenStat 6.2.

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