A single gene, AIN, in *Medicago truncatula* mediates a hypersensitive response to both bluegreen aphid and pea aphid, but confers resistance only to bluegreen aphid

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Abstract

Biotic stress in plants frequently induces a hypersensitive response (HR). This distinctive reaction has been studied intensively in several pathosystems and has shed light on the biology of defence signalling. Compared with microbial pathogens, relatively little is known about the role of the HR in defence against insects. Reference genotype A17 of *Medicago truncatula* Gaertn., a model legume, responds to aphids of the genus *Acyrthosiphon* with necrotic lesions resembling a HR. In this study, the biochemical nature of this response, its mode of inheritance, and its relationship with defence against aphids were investigated. The necrotic lesion phenotype and resistance to the bluegreen aphid (BGA, *Acyrthosiphon kondoi* Shinji) and the pea aphid (PA, *Acyrthosiphon pisum* (Harris)) were analysed using reference genotypes A17 and A20, their F2 progeny and recombinant inbred lines. BGA-induced necrotic lesions co-localized with the production of H2O2, consistent with an oxidative burst widely associated with hypersensitivity. This HR correlated with stronger resistance to BGA in A17 than in A20; these phenotypes cosegregated as a semi-dominant gene, AIN (*Acyrthosiphon*-induced necrosis). In contrast to BGA, stronger resistance to PA in A17, compared with A20, did not cosegregate with a PA-induced HR. The AIN locus resides in a cluster of sequences predicted to encode the CC-NBS-LRR subfamily of resistance proteins. AIN-mediated resistance presents a novel opportunity to use a model plant and model aphid to study the role of the HR in defence responses to phloem-feeding insects.

Key words: *Acyrthosiphon*, bluegreen aphid, H2O2, hypersensitive response, *Medicago truncatula*, pea aphid, phloem, plant–insect interactions, resistance, R gene.

Introduction

Biotic stressors such as pathogens and herbivores induce a broad spectrum of reactions in host plants, ranging from transcriptional changes to macroscopic symptoms including alterations in growth, chlorosis, and tissue death. One of the best studied of these reactions is the hypersensitive response (HR), a phenomenon observed in many types of plant–pest interactions. Hypersensitivity is generally defined as the programmed death of plant cells at the point of pathogen infection, correlated with host resistance (Mur et al., 2008). Depending on the nature of the interaction, a HR can encompass a microscopic area of just a few cells, or can spread over a much broader, macroscopic area of necrosis such as an entire leaf.

The frequent association of hypersensitivity with monogenic resistance to microbial pathogens has facilitated the study of this plant response at the genetic, molecular, and physiological levels. The highly specific interaction between HR-associated resistance genes in flax against strains of rust fungi led to Flor’s gene-for-gene concept of plant resistance (Flor, 1955, 1971). In this model, resistance acts through the
combined presence of two gene products, one encoded by a resistance (R) gene in the host and the other by an avirulence (Avr) gene in the pathogen. A vast literature has supported and elaborated the model, showing that recognition of an avirulence factor(s) by the R protein leads to a suite of host cell responses, including ion fluxes and an oxidative burst, often resulting in a HR (Thatcher et al., 2005). This form of programmed cell death may serve to prevent the pathogen’s spread to other host cells, although in many cases a direct causal link between cell death and the prevention of pathogen spread is not entirely clear (Jones and Dangl, 2006).

A wide range of pests can cause a HR in plants, including viruses, bacteria, fungi, nematodes, and herbivorous insects. Some interactions involve known plant R genes that condition incompatibility between host and pest, while other interactions show quantitative variation in resistance among host genotypes, wherein a HR does not completely prevent the pest from establishing on the host. Of all these interactions, the least understood involve those insects that induce a HR (Fernandes, 1990; Fernandes and Negreiros, 2001). These insect species generally require intimate contact with the host during most or critical portions of their life cycle. With respect to a HR involving a gene-for-gene relationship, the best studied interactions involve gall midges that attack cereals such as rice (Oryza sativa L.) and wheat (Triticum aestivum L.) (Grover, 1995; Sardesai et al., 2001; Harris et al., 2003). The HR has also been associated with resistance to oviposition by insects in black mustard (Brassica nigra L.) (Shapiro and Devay, 1987; Little et al., 2007), potato (Solanum tuberosum L.) (Balbyshev and Lorenzen, 1997), and common bean (Garza et al., 2001), although genetic variation for insect virulence has not been reported in these interactions.

Hypersensitivity can occur in response to piercing–sucking insects of the order Hemiptera (Fernandes, 1990). Members of the suborder Sternorrhyncha, which includes adelgids, aphids, psyllids, and whiteflies, have particularly close and long-lasting contact with their host; some species use their styllets to feed on cell sap from macerated parenchyma cells while others tap directly into the sap of the translocation stream within phloem sieve tubes. Adelgids feed on the sap from cortical parenchyma cells of conifers, and can induce a more rapid HR on resistant tree genotypes than on susceptible genotypes (Hain and Cook, 1988; Rohfritsch, 1988). In contrast to feeding by parenchyma maceration, phloem sap feeding involves adaptations that minimize or actively suppress plant defences (Will et al., 2007; Walling, 2008), although many distinctive damage symptoms can occur depending on the specific plant–insect combination. Aphids are the most economically important phloem feeders, attacking a wide range of crops and spreading pathogenic viruses. Monogenic resistance to aphids is common and in some cases can involve a HR. The best examples are resistance against the Russian wheat aphid (RWA, Diuraphis noxia Mordvilko) in barley (Hordeum vulgare L.) and in wheat (Belefant-Miller et al., 1994; Botha et al., 2006; Moloi and van der Westhuizen, 2006). In the case of the potato aphid (Macrosiphum euphorbiae Thomas) and the Mi-1 resistance gene of tomato (Solanum lycopersicum L.), a gene-for-gene interaction may exist despite the absence of hypersensitivity against this aphid (Goggin et al., 2001; de Ilarduya et al., 2003; Hebert et al., 2007).

Interactions between the model legume M. truncatula and aphids of the genus Acyrthosiphon have been studied and developed as a model system for mechanisms of plant defence against insect herbivory (Klingler et al., 2005, 2007; Gao et al., 2007a, b, 2008). These studies, particularly that of Gao et al. (2007b), revealed a broad range of phenotypic variation in levels of plant resistance to different legume-feeding aphid species. M. truncatula–aphid interactions present models for complete resistance (or incompatibility) as well as models for quantitative resistance.

One example of quantitative resistance is observed in the interactions of M. truncatula genotypes with two Acyrthosiphon species. These species also cause distinctive damage symptoms on one particular genetic background of M. truncatula. Both the bluegreen aphid (BGA, Acyrthosiphon kondoi Shinji) and the pea aphid (PA, Acyrthosiphon pisum Harris) induce necrotic lesions and severe stunting in the reference genotype Jemalong-A17 of M. truncatula, hereafter referred to as A17 (Klingler et al., 2005; Gao et al., 2008). These necrotic lesions are reminiscent of a HR, although A17 is relatively susceptible to both aphids compared to cv. Jester, a line that is near-isogenic with A17. Jester contains a dominant resistance gene, AKR, which protects against BGA without conferring complete resistance (Klingler et al., 2005; Gao et al., 2008; Guo et al., 2009). A17 and another plant genotype, A20, were both used by Klingler et al. (2005) as relatively-susceptible parental lines in a genetic analysis of BGA resistance in Jester. In the course of that study it was noticed that A17 and A20 differ in their reactions to BGA; whereas discrete necrotic lesions, stunting, and deformation were observed in A17, no obvious damage symptoms occurred in A20 aside from mild, general chlorosis of shoot tips at high BGA population levels. Moreover, it was clear that A20 exhibited higher BGA population levels than A17. Indeed, under high aphid pressure it was observed that many A20 plants were completely killed by BGA while adjacent A17 plants remained alive, albeit stunted in growth.

In the present study, a genetic analysis of Acyrthosiphon spp. colony development and plant reaction to infestation was undertaken using reference genotypes A17 and A20. The results indicate that A17 exhibits hypersensitivity in response to both BGA and PA, and that the trait is conditioned by a single genetic locus that also confers a significant level of resistance (relative to A20) to BGA but not PA. The similar HR produced by these two aphid species in the presence of this gene, combined with the gene’s specificity in defending against only one of these aphids, presents a novel system for the molecular dissection of the role of the plant HR in defence against insects. Since PA, like M. truncatula, is a model species, these findings create a significant opportunity to elucidate mechanisms underlying plant–aphid interactions.
Materials and methods

Plants and aphids

Plants used in this study were *M. truncatula* genotypes A17 and A20, both described by Pennetsa and Cook (2000), or progeny derived from crosses between these inbred lines. Prior to laboratory or greenhouse experiments, seeds were scarified and germinated in the dark on moist filter paper, and then kept at 4 °C for 10–14 d to synchronize radicle growth before transfer to soil. For all experiments, plants were grown in 1.2 l pots in either a growth chamber under high pressure sodium and incandescent light at 225–250 μmol m⁻² s⁻¹ or in natural light in a greenhouse with temperatures ranging from 15–30 °C. The aphids used in this study were asexual, parthenogenetic strains of BGA and PA collected in Western Australia, derived from single-aphid isolates, and cultured in the laboratory as described by Gao et al. (2007a). Aphids were transferred to experimental plants with a fine paintbrush.

Performance of BGA confined to individual plants

In a test of BGA colony growth on genotypes A17, A20, and their F₁, eight individual 2-week-old seedlings were each infested with two adult apterae in a growth chamber. Each plant was then covered with a whole-plant cage made from a clear plastic bottle modified with a cut-off base and large, mesh-covered ventilation holes. Nineteen days after infestation the bottles were removed and damage symptoms were recorded. Aphids on each plant were gently brushed off and immediately weighed. The aphid-free plant was then cut at soil level, dried in an oven, and weighed. Means of aphid fresh weight per plant fresh weight were subjected to one-way ANOVA and compared using the Tukey–Kramer Honestly Significant Difference test with JMP 7.0 software (SAS Institute Inc.).

Analysis of necrosis induced by BGA

Test for H₂O₂ production: Three plants each of genotypes A17 and A20 were infested on the second fully expanded trifoliate leaf with 16 adult apterae, confined to leaf cages. An equal number of plants received aphid-free cages to serve as negative controls. Three days after infestation, when all infested leaves of A17 were beginning to show macroscopic lesions induced by BGA, the caged leaves were excised, aphids were removed, and leaves were placed individually in vials containing a solution of 3,3′-diaminobenzidine (DAB; Sigma-Aldrich, Inc.) at 1 mg ml⁻¹, pH 3.8, based on the methods of Thordal-Christensen et al. (1997) and Orozco-Cardenas and Ryan (1999). The DAB solution was vacuum-infiltrated into leaf tissue for 90 min at room temperature; leaves were then left in the solution at room temperature overnight, under constant light, with no vacuum applied. The next day, the leaves were boiled in 95% ethanol for 20–30 min until they were cleared of pigment, and then stored in 70% ethanol and photographed.

Test for local versus systemic production of necrotic lesions: Plants of genotype A17 were grown in a growth chamber to analyse damage from BGA infestation. Three weeks after sowing, the fifth trifoliate leaf to develop on each plant, which was still expanding on all plants, was covered with a transparent leaf cage to protect it from contact with aphids, or to serve as a negative control on non-infested plants. Plants were then randomly assigned to one of two large cages within the growth chamber, with eight replicate plants per cage. The design of these leaf cages and that of large, multi-plant cages were described by Klingler et al. (2005). Plants in the large cage receiving the infestation treatment were immediately infested with 16 apterae by placing the aphids at the lowest part of the stem, from which they climbed upward to settle and feed from various parts of the plant within approximately 30 min. Plants inside the other large cage, adjacent to the first cage, were kept completely free of aphids. Damage symptoms on all plants were scored 8 d after infestation.

Genetic analysis of aphid–plant interactions

Flowers of genotype A17 were emasculated and fertilized with pollen from A20 to produce F₁ plants, based on the method of Pathipanawat et al. (1994). F₂ seed were produced from these self-fertilized F₁ plants.

Inheritance of BGA-induced necrotic lesions: F₂ seedlings and their parental genotypes were phenotyped for BGA-induced damage symptoms during growth in individual pots in a greenhouse, with two separate rounds of phenotyping performed for a total of 192 F₂ plants analysed. Two apterous adult aphids were placed on each seedling 22 d after sowing. Aphids were allowed to develop, reproduce, and move freely among plants for 22 d before feeding damage was assessed. Each round of phenotyping included 12 plants of each parental genotype, A17 and A20, placed randomly among the F₂ plants to serve as controls. Plants were scored as either having or not having aphid-induced necrotic lesions on any of their leaves. Approximate aphid density on each plant was also noted, using the clearly visible, white exuviae (exoskeletons) that are shed after each aphid moult, and that typically adhere to the plant at the spot where they were shed. This was a much easier visual indicator of aphid density than the aphids themselves, which closely match the colour of the host plant and can be difficult to observe. Aphid density was rated on a subjective scale, using a score of 1 (lowest density) to 10 (highest density). After plants were scored for damage symptoms and aphid density, they were treated with insecticide to remove aphids and grown to maturity to produce healthy leaf tissue (for genomic DNA analysis) and self-fertilized F₃ seed. In cases where the presence versus absence of the necrotic lesion phenotype of the F₂ progenitor was ambiguous, selected families of F₃ progeny (8–18 plants per
family) were tested for BGA damage phenotype in the same manner as for the F2 generation. DNA from each phenotyped A17×A20 F2 plant was tested for molecular polymorphisms (Klingler et al., 2005) using PCR-based markers developed and mapped in a population of F2 plants derived from crossing these same genotypes, A17 and A20 (Pennmetsa and Cook, 2000), by the Medicago truncatula genome sequencing project (Cannon et al., 2005; http://medicago.org/genome/). Genetic distances between molecular markers and aphid resistance phenotypes were determined with Mapmaker software (Lander et al., 1987), using the Kosambi function, with a maximum recombination fraction, θ, of 0.40 and a minimum LOD score of 3.

Identification of open reading frames near the AIN locus (BAC contig 1065) was performed using Medicago Genome Sequence Consortium release version 2.0, finalized on 10 August 2007 (Young et al., 2005; http://www.medicago.org/genome/) and the Legume Information System (Gonzales et al., 2005; http://www.comparative-legumes.org/lis/).

Quantitative analysis of plant damage and BGA performance in F2 and recombinant inbred populations: Eighty randomly chosen F2 seedlings and eight seedlings each of parental genotypes A17 and A20 were infested as in the experiment described above. Eighteen days after infestation, aphids were washed from each plant, stored in 95% ethanol, and later dried in an oven and weighed. Plants that were washed free of aphids were lyophilized (for genomic DNA analysis) and weighed. Due to inequality of variances, counts of damaged leaves were transformed as log(X+1) and ratios of colony dry weight per plant dry weight were transformed as arcsine(\[X^{0.5}\]) prior to one-way ANOVA. Multiple comparisons were performed using Tukey–Kramer Honestly Significant Difference tests. In addition, a recombinant inbred population was developed for better control of environmental variation in the aphid-plant interaction phenotypes. This population was derived from A17×A20 F2 plants that were advanced to the F6 generation by random selection and growth of a single seed at each generation to create 93 recombinant inbred lines (RILs). Tissue samples from F2,6 individuals were collected and lyophilized for genomic DNA analysis. Seed were also collected from each of these plants (representing individual RILs) for performing experiments with aphids. Twelve replicate plants from each RIL, along with 12 replicate plants of each parent and their F1, were grown in separate 5 cm pots in a greenhouse and divided into two groups of six replicates, with each group of replicates positioned on benches on opposite sides of the room. In each group, the six replicates of each RIL were positioned with six replicate plants of A17, A20, and their F1 in a completely randomized design. Fifteen days after sowing, one group of plants (half of the total number) were temporarily removed from the room and treated with a spray containing 1 g l⁻¹ Confidor® systemic insecticide (Imidacloprid; Bayer CropScience AG) to prevent unwanted aphid infestation. The other half were sprayed with purified water. This latter group was infested with BGA 18 d after sowing, as described for the F2 population. Nineteen days after infestation, the infested plants were scored for aphid damage and aphids were removed, oven-dried, and weighed, as described for the F2 population. All plants in the experiment, including the non-infested controls, were excised at soil level, oven-dried, and weighed. The relative reduction in plant biomass due to infestation was calculated by subtracting the biomass of each infested replicate plant from the mean control (non-infested) biomass for that genotype, and dividing this difference by the mean control biomass.

DNA samples from the 80 F2 plants and the 93 F6 progenitor plants were analysed for several markers on the north arm of chromosome 3, a region known to harbour loci related to aphid defence (Klingler et al., 2005, 2007). Correlations between these markers and phenotypic traits related to BGA infestation were analysed using JMP 7.0 software.

Test of PA performance and plant tolerance on selected RILs: In order to test the relation between BGA resistance and PA resistance, RILs with known genotypes at the AIN locus were selected for infestation with PA. Twelve RILs homozygous for the A17 genotype of the AIN-linked SSR marker 34TC15, and 12 RILs homozygous for the A20 genotype of this marker were randomly selected from the 93 RILs tested in the previous experiment with BGA. Two replicate plants of each of the 24 RILs and eight replicate plants of each parent (A17 and A20) were grown in separate pots in a completely randomized design in a growth chamber. Fourteen days after sowing, each plant was infested with two PA apterae and covered with a whole-plant 'bottle' cage of the design described for initial experiment with the parental genotypes A17 and A20, and their F1. Fourteen days after infestation, the cages were removed, plants were scored for PA-induced damage, and aphids were brushed from the plants. Fresh weights of aphid colonies and above-ground plant fresh weights were then recorded; the mean of the two replicate plants for each RIL was used for analysis. Since parental line data were analysed based on individual plants, whereas RIL data were analysed based on means of two replicate plants per RIL, separate t tests that assume unequal variance were performed between the two parental lines, and between the two groups of RILs, to test for associations between the AIN genotype and phenotypic traits related to PA infestation, using JMP 7.0 software.

Results

Aphid performance in no-choice tests

Genetic analysis of BGA resistance in cv. Jester, which contains the dominant resistance gene AKR, was previously performed by crossing this cultivar with susceptible genotypes A17 and A20 (Klingler et al., 2005). This earlier study revealed striking differences in plant damage symptoms and levels of BGA populations in the two AKR genotypes. In the
present study, aphid performance was compared directly between these genotypes and their F$_1$ generation in no-choice tests using whole-plant cages. By the end of the 19 d infestation period, genotype A17 had developed damage symptoms consistent with earlier observations (Klingler et al., 2005): plants were stunted and many trifoliate leaves had necrotic or chlorotic lesions approximately 1–3 mm in diameter; the white, necrotic lesions were often surrounded by rings of dark red pigment (Fig. 1A). Experience with this plant–aphid interaction has shown that chlorotic lesions are an early response to the aphid, which may or may not be followed by the formation of necrosis at these sites. Some petioles of A17 were sharply bent with dark pigment at or near the region of bending. On some A17 plants, tissue death spread over entire leaflets or entire trifoliate leaves and their petioles. By contrast, genotype A20 showed no obvious symptoms of stunting or leaf tissue damage by aphids, even though aphid numbers were relatively high (Fig. 1B). F$_1$ plants showed symptoms of damage similar to those of A17, although they appeared to be expressed to a lesser degree, with relatively smaller and fewer necrotic lesions and no leaves that were completely dead. Aphid performance, as measured in grams of colony fresh weight per gram of plant fresh weight, was nearly twice as high in A20 as in A17; F$_1$ plants had a range of values overlapping those of the parental lines (Fig. 2). A one-way ANOVA showed a highly significant effect of plant genotype on this measure of aphid performance ($F=6.63; P=0.006$). Tukey–Kramer multiple comparisons indicated that the means for A20 and A17 were significantly different from each other ($P<0.05$), while the F$_1$ generation was intermediate in its level of resistance and not significantly different from either parent.

Leaves of A17 produce H$_2$O$_2$ coincident with BGA-induced leaf necrosis

The macroscopic chlorotic and necrotic lesions that are associated with BGA feeding on A17 resemble a HR to microbial pathogens (Klingler et al., 2005). Since a common feature of hypersensitivity in many plant species is an oxidative burst that includes the local production of H$_2$O$_2$, infested leaves of A17 and A20 were tested for the presence of this compound using DAB staining. A time point of 3 d after infestation was chosen for this analysis because this was generally the amount of time required for all infested A17 leaves to produce macroscopically visible lesions in response to BGA. Reddish brown staining in ethanol-cleared leaves, indicative of H$_2$O$_2$ production, was prominently associated with lesions caused by BGA feeding on all three leaves of A17 (Fig. 1C). By contrast, leaves of A20 that had been exposed to BGA feeding, and which had no visible damage symptoms, showed no such staining (Fig. 1D). These results show a clear association between the presence of H$_2$O$_2$ and macroscopic tissue death in response to BGA feeding in genotype A17. Since H$_2$O$_2$ production at the site of a lesion is one hallmark of the plant HR, these results indicate that the necrotic lesions in response to BGA is a form of hypersensitivity to this species.

Leaf damage in A17 occurs only as a local response to aphid feeding

Some aphid–plant interactions can lead to systemic as well as local feeding damage (for example, see Klingler et al., 2007). The spatial occurrence of BGA-induced lesions...
relative to aphid feeding sites on A17 was tested by protecting a single trifoliate leaf on each infested plant using a clear, plastic, ventilated leaf cage, while allowing aphids to roam freely and feed from the rest of the plant. By the end of the 8 d infestation period, BGA had clearly stunted the growth of infested plants, as indicated by comparing the total number of leaves on infested and control plants (Table 1). All protected leaves appeared to have nearly reached their maximal degree of expansion, and would have been available as strong sinks for possible aphid-derived elicitors during the infestation. No damage symptoms were produced on these caged leaves, nor was any damage observed on leaves of non-infested control plants. By contrast, an average of 86% of unprotected leaves of infested plants showed some form of BGA-associated damage (chlorotic or necrotic spots, or complete death). These results indicate that, while BGA significantly stunts the growth of genotype A17, aphid-induced lesions are produced locally, not systemically.

Mode of inheritance of BGA-induced necrosis trait

A combined total of 192 A17×A20 F2 plants were phenotyped for BGA-related traits in two separate rounds of greenhouse testing. The parental lines in each test showed pronounced differences in reaction to BGA: necrotic lesions and/or leaf death were visible on all plants of A17, while no such damage occurred on A20. Similarly, aphid density scores were substantially lower on A17 than on A20. A test for interaction between phenotyping experiment and aphid density score was non-significant for these parental lines; therefore, samples were pooled for analysis. The mean density score for A17 was 2.3±0.9 SE and for A20 was 8.4±1.7 SE; t test P <0.0001. When F2 individuals were classified as either susceptible to BGA damage (like A17) or resistant to damage (like A20), a ratio of 147 plants with one or more damaged leaves to 45 plants with no visible damage was observed. These results are consistent with a 3:1 ratio (χ²=0.25; df=1; P=0.62), suggesting that a single dominant gene in A17 controls the BGA-induced lesion response. However, several F2 plants had only one leaf with any damage resembling that caused by BGA, well outside the range of damage for parental line A17 (which had a minimum of seven leaves with damage). Moreover, the frequency distribution of damaged leaves of F2 plants appeared to be skewed toward 0, compared with the distribution of parental line A17 (data not shown). One further difference between BGA-damaged F2 plants and parental line A17 was that many F2 plants had lesions that were all relatively small (less than 1 mm diameter), whereas all A17 plants had necrotic lesions ranging from 1 mm to a size encompassing the entire leaf. These results suggested that, quantitatively, a single gene conditioning BGA-induced lesions may act with incomplete dominance. In addition, the results suggested that some plants might have been misclassified because of environmental ‘noise’ in the phenotyping experiments. For example, it is possible that some plants were scored as having no damage due to a stochastically determined ‘escape’ from sufficient aphid feeding pressure. Conversely, it is possible that a low level of leaf damage could have been caused by some other biotic or abiotic stress (such as a pathogen or fertilizer burn, respectively) rather than BGA.

Mapping of the BGA-induced damage trait

The 192 F2 plants were genotyped using a selection of PCR-based markers that had been placed on the A17×A20 reference map by the Medicago truncatula Consortium (maps and marker information available at http://medicago.org/genome). Since two aphid resistance loci had already been mapped to separate clusters of R gene-like sequences on chromosome 3 (Klingler et al., 2005, 2007), markers from this chromosome, including two that are tightly linked to the known aphid resistance loci, were immediately tested for linkage with the qualitative BGA-induced damage phenotype. These comprised the following, listed in order of centiMorgan position on the reference map: 004A05, 003G03, 003A03, 004H01, BE187590. Analysis by MapMaker software supported linkage between the BGA-induced necrosis trait and a chromosome region distal to marker 004H01 and proximal to marker 004A05; these markers are separated by 10.4 cM on the reference map (http://medicago.org/genome).

Since the phenotyping results suggested the potential for a low rate of error in the classification of F2 plants, a selection of F2:3 families was tested for BGA-induced necrosis as in the F2 generation, in order to determine with greater certainty the F2 genotype at the locus conditioning this trait. The selection of the particular families to phenotype was based upon the following criteria: it included families whose progenitor F2 plant had a recombination breakpoint somewhere between markers 004H01 and 004A05, and/or had been noted as simultaneously having necrotic lesions extremely small in diameter and relatively high aphid densities. These summed to a total of 14 families chosen for testing. In addition, four F2:3 families whose F2 progenitor phenotypes appeared unambiguous were chosen as controls (two families corresponding to each parental

Table 1. Determination of local versus systemic damage in response to BGA in the presence of an exclusion cage placed on a single leaf of each infested or control plant

|                     | A17 infested | A17 non-infested |
|---------------------|--------------|------------------|
| Caged leaves with damage | 0            | 0                |
| Unprotected leaves   | 8.8±0.9      | 20.1±1.8         |
| Leaves with necrosis | 4.9±0.9      | 0                |
| Leaves with chlorotic spots | 1.6±0.5     | 0                |
| Dead leaves          | 1.1±0.4      | 0                |
| Undamaged leaves     | 1.1±0.4      | 20.1±1.8         |
| Proportion with necrosis | 0.56±0.06   | 0                |
| Proportion damaged   | 0.86±0.05    | 0                |
phenotype). If F\textsubscript{2} progenitor plants had been originally scored as having no damage (the recessive phenotype), then 8–10 F\textsubscript{2:3} progeny were infested to determine their BGA-related phenotypes. If F\textsubscript{2} progenitor plants had originally been scored as having at least some damage (the dominant phenotype) then 16–18 F\textsubscript{2:3} progeny were tested with BGA. Nine of the 14 families in question were determined to be segregating for BGA-induced damage. Of these, three families were derived from F\textsubscript{2} progenitor plants that had originally been scored as having no damage and intermediate or high levels of aphid density. The remaining five of the 14 families were determined to be homozygous for the recessive phenotype of resistance to BGA-induced damage. Three of these came from F\textsubscript{2} progenitor plants that had originally been scored as having low numbers of necrotic lesions—noted as exceptionally small in diameter—along with relatively high aphid densities. These results indicate the potential for error in scoring the F\textsubscript{2} population for BGA-induced damage.

The results of F\textsubscript{2:3} progeny testing were combined with the unambiguous F\textsubscript{2} phenotyping data to confirm genetic segregation consistent with control by a single dominant gene for BGA-induced damage, based on a total of 192 F\textsubscript{2} progeny. Since PA (A. pisum) causes similar damage to genotype A17 (see results below), the proposed name for this gene is AIN (Acyrthosiphon-induced necrosis). The F\textsubscript{2} data were re-analysed to position the AIN locus between SSR markers 003G03 and 003A03 on chromosome 3. The genetic map produced from the 192 F\textsubscript{2} progeny shows a 4.3 cM interval between these markers. The physical mapping data for this region of the genome (http://medicago.org/ genome) allowed the design of a new SSR marker within this interval; this marker, 34TC15, was found to co-segregate with the AIN phenotype for the 192 F\textsubscript{2} plants.

A genetic map of the AIN locus is shown in Fig. 3.

**BGA-induced damage and BGA performance are correlated with AIN dosage**

The study involving aphids confined to individual plants of genotypes A17, A20, and their F\textsubscript{1} generation, along with the phenotypic analysis of F\textsubscript{2:3} families, suggested that the degree of aphid damage and aphid density may be dependent on the dosage of AIN. To address this question, a set of 80 randomly selected F\textsubscript{2} plants from A17×A20 were phenotyped quantitatively for BGA-induced leaf damage and for aphid colony dry weight as a function of plant dry weight. The F\textsubscript{2} plants segregated for AIN-linked SSR marker 34TC15 in a ratio of 18 A17-allele homozygotes:41 heterozygotes:21 A20-allele homozygotes, consistent with the 1:2:1 segregation pattern of a single co-dominant marker ($\chi^2$=0.28; df=2; $P$=0.87). Figure 4 shows that, when...
F₂ plants were classified according to their genotype for marker 34TC15, heterozygous plants averaged levels of both plant damage and BGA performance that were intermediate between plants with two copies and zero copies of the gene. One-way ANOVAs for these two variables in response to the AIN genotype included parental lines along with the three categories of F₂ plants. Both analyses showed highly significant differences among genotypic means. (For leaf damage, $F=86.6; df=4; P<0.0001$. For BGA performance, $F=79.1; df=4; P<0.0001$.) Tukey–Kramer multiple comparisons indicated that heterozygous plants were significantly different and intermediate between homozygotes for both aphid-related variables ($P<0.05$).

**AIN conditions BGA resistance**

To characterize BGA-induced necrosis and resistance to colonization in A17 further, a RIL population generated from A17×A20 was employed. This allowed the replication of genotypes as a means of controlling for environmental variables and the incomplete penetrance and variable expressivity of the necrosis phenotype. Ninety-three F₂:7 families, each considered a RIL, were analysed for interactions with BGA in a greenhouse. In conjunction with this experiment, DNA samples from the F₂:6 progenitors of the RILs were genotyped for several molecular markers on chromosome 3. These included three markers that are tightly linked to the three known loci mediating interactions with aphids (34TC15 at AIN; 004H01 at AKR: h2.1e24a at TTR) and markers 004A05 (located distal to 34TC15) and h2.6i7c (located between AKR and TTR). The genotyping results indicated a higher rate of residual heterozygosity than expected for a F₂:6 generation. Twelve of the 93 RILs (13%) were heterozygous for SSR marker 34TC15 (the expectation for F₂:6 generation=3.125%, or three heterozygous RILs, assuming no segregation distortion); RILs homozygous for the A17 and A20 genotypes at this locus numbered 38 and 43, respectively.

The relationship between damaged leaves per plant and aphid colony dry weight per plant dry weight is shown in Fig. 5. The parental lines and their F₁ generation showed relative degrees of BGA-induced damage and BGA performance that are consistent with other genetic experiments described above. The clustering of RILs according to genotype at the AIN locus is consistent with AIN controlling both BGA-induced leaf damage and BGA resistance. The residual heterozygosity detected in 12 of the F₂:6 progenitors of the RILs was reflected in the tendency for these lines to lie between the separate clusters for the homozygous lines, consistent with a model of semidominance of AIN in control of both plant damage level and aphid colony weight. A one-way ANOVA, using aphid weight per plant weight as the response variable and AIN gene dosage in the F₆ generation as the independent variable, shows that the locus explained 88% of the variance in this index of aphid resistance ($R^2=0.88; F=322.56; df=2; P<0.0001$). With this same analysis, SSR markers 004A05 and 004H01, which flank the AIN locus at distances of 5.9 cM and 8.1 cM, respectively, explained 43% and 54% of the variance in aphid resistance, respectively. These results are consistent with the AIN locus as the major determinant of BGA resistance in genotype A17.

**AIN-mediated resistance to BGA does not involve tolerance**

The inclusion of non-infested control plants allowed for a test of an interaction between RIL genotype at the AIN locus and the relative reduction in plant biomass due to aphid infestation. Among the control plants, A20 showed a higher dry weight ($±SE$) than A17 (0.52±0.03 g and 0.33±0.03 g, respectively; $t$ test $P=0.0008$). The dry weights of the non-infested F₁ plants were more variable, but the mean was intermediate between the two parental lines (0.40±0.11 g). As a measure of plant tolerance to infestation, the relative reduction in plant biomass was calculated using the non-infested plants as controls. By this method, A17 showed a trend toward greater tolerance to BGA than did A20, but there was no significant difference between these means (data not shown). All 93 RILs were grouped according to AIN genotype and analysed by one-way ANOVA for relative reduction in plant biomass due to infestation; no significant effect of AIN genotype was observed ($R^2=0.015; F=0.69; df=2; P=0.504$). These results suggest that tolerance is not a significant mode of BGA resistance in A17.

**Pea aphid interaction with AIN**

The pea aphid (PA), like its congener BGA, causes necrotic lesions on genotype A17 (Gao et al., 2008). Two sets of
RILs, with or without the AIN gene, were randomly selected and infested with PA to determine whether the presence of AIN is correlated with PA-induced necrosis and with altered aphid performance. PA were confined to individual plants and allowed to feed and reproduce for 14 d on 12 AIN+ RILs and 12 AIN− RILs (two replicate plants for each RIL), along with eight plants each of parental lines A17 and A20. Figure 6A shows the results for levels of leaf damage on each set of genotypes. As expected, necrotic and chlorotic lesions appeared on all A17 plants; neither damage symptom developed on any plants of A20. Abundant necrotic lesions appeared on AIN+ RILs, while no necrotic lesions were observed on AIN− RILs. However, some AIN− plants exhibited small chlorotic lesions, which were included in the mean number of damaged leaflets for each RIL as shown in Fig. 6A (mean numbers of damaged leaflets: AIN+ RILs=15.42±0.93; AIN− RILs=0.89±0.93; t test P<0.0001). A marked difference was also observed between the parental lines with respect to aphid performance. PA colony fresh weight per plant fresh weight was significantly higher on genotype A20 than on A17, as shown in Fig. 6B (0.095±0.006 and 0.056±0.006, respectively; t test P=0.0003). Interestingly, in contrast to PA-induced damage levels, Fig. 6B illustrates that PA performance (aphid fresh weight per plant fresh weight) was not associated with the AIN genotype of the RILs (0.082±0.008 for AIN+ RILs and 0.077±0.006 for AIN− RILs; t test P=0.66). These results suggest that, while A17 is more resistant to PA than is A20, some other locus or loci in the A17 genome, apart from AIN, act to reduce PA performance.

Discussion

Genotype A17 was shown to possess a significantly higher level of BGA resistance than A20. This trait is controlled by a semi-dominant gene, called AIN, that also mediates the induction of a HR upon BGA infestation. Interestingly, although A17 is also more resistant to PA than A20, the comparison of PA performance on AIN+ and AIN− RILs indicated that the AIN locus is not responsible for this trait, even though the locus does appear to control HR-associated necrotic lesions in response to PA feeding. Thus, the AIN locus conditions an aphid-induced phenotype, HR-associated lesion formation, that is common to both Acyrthosiphon species. The other AIN-mediated phenotype of this study, aphid resistance, is specific to BGA.

BGA performance was measured in two different experimental designs in this study; a comparison of results from these designs provides additional insight on the modes of BGA resistance in A17. In the no-choice test using individual plant cages with A17, A20, and their F1 generation, aphids were forced to feed and reproduce on a single host plant. BGA performance, as measured by colony weight per plant weight, was 1.84-fold higher on A20 than on A17. In the inheritance studies using A17×A20 F2 progeny and RILs, BGA could move freely among plants of different genotypes during the infestation period, such that differences in aphid preference could influence colonization outcomes. In these two genetic experiments, BGA performance was 4.98-fold higher on A20 than on A17 with the F2 population and 4.71-fold higher with the RILs. The greater fold-difference in aphid performance in free-choice, versus no-choice, experiments suggests that at least some of the resistance in A17 is due to antixenosis (non-preference) rather than antibiosis against BGA.

In addition to antibiosis and antixenosis, a third possible mode of aphid resistance is plant tolerance. The experiment involving BGA infestation of RILs failed to show evidence of enhanced tolerance in AIN+ plants, as measured by relative reduction in plant biomass by the aphid. This result contrasted with earlier, uncontrolled observations of A17 and A20 under heavy infestation in the greenhouse, in which A20 plants died while A17 remained alive but stunted. Based on these and other experiences with M. truncatula...
interactions with aphids, it appears likely that measures of tolerance are highly sensitive to the initial infestation conditions, for example, the developmental stage of the plant and the number and developmental stage of the aphids. Manipulation of these variables while measuring other aspects of plant fitness could shed more light on the role of tolerance in AIN-mediated BGA resistance.

Since A17 is relatively resistant to BGA and PA, compared with A20, and since the *Acyrthosiphon*-induced necrotic lesions bear some resemblance to a HR against pathogens, the DAB staining method was used to test for the presence of H$_2$O$_2$, which is a hallmark of an oxidative burst associated with the HR (Orozco-Cardenas and Ryan, 1999; Thordal-Christensen et al., 1997). The co-occurrence of H$_2$O$_2$ production with necrotic lesions in A17, and its absence in A20, indicates that BGA (and, presumably, PA) induce a HR in the relatively resistant genotype A17. Both H$_2$O$_2$ and NADPH-oxidase activity (associated with H$_2$O$_2$ production) are induced in response to RWA in resistant wheat, which is another plant-aphid interaction involving a HR (Moloi and van der Westhuizen, 2006). The aphid exclusion experiment in the present study demonstrated that this HR is produced locally, rather than systemically, by aphid infestation, since lesions never occurred on leaves that were protected from direct contact with BGA. The initial chlorotic lesions induced by *Acyrthosiphon* species bear resemblance to the general chlorosis observed during leaf senescence. This process of necrotic lesion formation may operate through a mechanism similar to the accelerated senescence phenotypes associated with TTR-mediated resistance to the spotted alfalfa aphid (*Theroaphis maculata Monell f. trifolii*) in *M. truncatula* cv. Mogul (Klingler et al., 2007) and PAD4-mediated resistance to the green peach aphid (*Myzus persicae Sulzer*) in *Arabidopsis thaliana* (Pegadaraju et al., 2005).

Genetic analysis using the A17×A20 F$_2$ and RIL populations elucidated the relationship between BGA-induced hypersensitivity and BGA performance. An initial, qualitative analysis of the BGA-induced HR (i.e. presence versus absence of necrotic lesions) in the F$_2$ generation supported a single dominant gene controlling this trait. Subsequently, quantitative measures of aphid-induced damage in both the F$_2$ and RIL populations showed the semi-dominant nature of the hypersensitivity trait; this closely paralleled the quantitative, gene dosage-dependence of resistance to BGA. The strong genetic correlation between the degree of HR symptoms and the degree of BGA resistance suggests that AIN conditions both phenotypes. Alternatively, and less likely, two separate genes that are tightly linked and both semi-dominant are independently controlling these traits.

Necrotic lesions are often associated with plant susceptibility, rather than resistance, to biotic or abiotic stress. Cultivar Jester is nearly isogenic with A17 and differs by possessing the dominant AKR resistance gene, which is lacking in A17 (Klingler et al., 2005). AKR makes Jester much more BGA-resistant than A17 and immune to the macroscopic lesions and stunting that are induced by BGA in A17, even though BGA can still feed and reproduce on Jester. In tomato, near-isogenic lines with or without the *Mi*-1 resistance gene also differ in this regard. In response to at least one potato aphid isolate, a near-isogenic line that possesses the *Mi*-1 gene suffers less chlorosis and necrosis compared to its counterpart that lacks *Mi*-1 (Hebert et al., 2007). Although hypersensitivity in tomato roots has been associated with *Mi*-1-mediated resistance against *Meloidogyne* spp. of root knot nematodes, a HR involving H$_2$O$_2$ production is not associated with *Mi*-1-mediated resistance against potato aphid in leaves (de Ilarduya et al., 2003). Thus, AIN-mediated BGA resistance in A17 (relative to the highly susceptible line A20) differs from *Mi*-1-mediated resistance in tomato, since HR is clearly associated with the AIN resistance phenotype.

**AIN** is the fourth aphid resistance gene reported in *M. truncatula*. The other genes are AKR, which is specific against BGA (Klingler et al., 2005; Gao et al., 2008), TTR, which protects against SAA (Klingler et al., 2007) and APR, which protects against PA (Guo et al., 2009). Interestingly, at least three of these genes, including AIN, reside at separate loci on the north arm of chromosome 3. The AIN locus of reference genotype A17 is tightly linked to SSR marker 34TC15, which resides on bacterial artificial chromosome (BAC) contig (contiguous sequence) 1065 of the current *M. truncatula* genome assembly (Mt2.0, released on 10 August 2007). Within 378 kbp spanning four adjoining BACs, this contig contains nine open reading frames predicted to encode members of the CC-NBS-LRR (or CNL) subfamily of NBS-LRR resistance proteins (http://medicago.org/genome/assembly_table.php?chr=3). The two mapped SSR markers flanking the AIN locus, 003A03 and 003G03, reside on separate BAC contigs that also contain this subfamily of genes. Thus, the AIN locus, like that of AKR and TTR, resides within a cluster of resistance gene-like sequences. The north arm of chromosome 3 is a genomic region that holds over 80 CNL genes, constituting a major portion (around 40%) of the genome’s total number for this subfamily (Ameline-Torregrosa et al., 2008). Since the only cloned aphid resistance genes, *Mi*-1 in tomato and *Vat* in melon (*Cucumis melo* L.), are both members of the CNL subfamily (Milligan et al., 1998; Rossi et al., 1998; Dogimont et al., 2007), it is quite plausible that some or all of the known aphid resistance genes in *M. truncatula* are also members of this group.

If AIN controls both hypersensitivity and BGA resistance, the gene’s function may be similar to that of known CNL family members. Some of the best studied plant resistance factors are CNL proteins that interact with one or more specific proteins (virulence factors or effectors) from the microbial pest, conditioning a form of resistance known as effector triggered immunity (Jones and Dangl, 2006). The specific interaction, either direct or indirect, between the plant R protein and a corresponding effector from the pest triggers Ca$^{2+}$ fluxes, changes in cell redox status, an oxidative burst, and a HR (De Gara et al., 2003; da Cunha et al., 2006). Aphid saliva is a likely source of effectors analogous to those of microbes. This saliva has been shown...
to contain Ca²⁺ binding proteins that could serve to suppress Ca²⁺-mediated defence within the phloem (Will et al., 2007). Oxidative enzymes are also present in aphid saliva, and may serve to detoxify plant defensive chemicals such as phenolics (Miles, 1999; Carolan et al., 2009). The ‘redox hypothesis’ of plant–aphid interactions proposes an interplay between defence-related plant phenolics, plant oxidases, reactive oxygen species (ROS), antioxidants, and oxidases within aphid saliva; the outcome of these interactions might trigger visible damage symptoms in the host (Miles and Oertli, 1993). It is possible that AIN alters the balance of redox reactions to an extent that leads to a HR. Further work will be necessary to determine whether hypersensitivity is a central mechanism for this mode of aphid resistance or merely a by-product of a defensive reaction elicited by the presence of the AIN gene product. Since aphids are highly mobile on the plant surface, relative to microbial pathogens, a HR may have less impact on these insects, given that an aphid can simply move and establish a more suitable feeding site as the HR lesion develops.

Previous studies in *M. truncatula* with three other aphid genera, represented by the spotted alfalfa aphid, green peach aphid, and cowpea aphid (*Aphis craccivora* Koch), have failed to show any evidence of necrotic lesions such as those observed in response to *Acyrthosiphon* spp. (Gao et al., 2007h; Klingler et al., 2007). If aphid saliva plays a role in the AIN-mediated HR, it is possible that the specificity observed with *Acyrthosiphon* spp. is based upon differences in salivary components among aphid genera. Alternatively, or in addition, it is possible that differences in aphid probing behaviour underlie the specificity of the AIN-mediated HR against *Acyrthosiphon* species. Future comparisons of aphid probing behaviour between species or between AIN+ and AIN− plants, using electronic monitoring, may help to explain the physiological basis of the AIN-mediated HR.

A17 and Jester are distinctive in their visible damage symptoms among several *M. truncatula* genotypes studied under infestation with BGA and PA. For example, cv. Borung, which lacks both AKR and AIN, has a phenotype very similar to that of A20 under BGA and PA infestation (Klingler et al., 2007; Gao et al., 2008). Although PA causes necrotic lesions on both A17 and (to a lesser extent) the near-isogenic cv. Jester, this phenotype is likely to be specific to the A17 background since Jester also contains the genomic region harbouring the AIN locus (Gao et al., 2007).

An intriguing question arising is whether an interaction between the AKR locus and AIN in Jester influences defence against *Acyrthosiphon* species. Gao et al. (2007) identified the jasmonate signalling pathway as important in AKR-mediated defence against BGA. By contrast, Gao et al. (2008) found no specific association between this pathway and AKR-mediated defense against PA. The salicylate signalling pathway is known to play a major role in the HR (Torres et al., 2005), and abundant evidence exists for cross-talk, primarily antagonistic, between the jasmonate and salicylate pathways (Thatcher et al., 2005). Gao et al. (2008) observed activation of the salicylate pathway by PA in both A17 and Jester, which is consistent with the presence of macroscopic, HR-like symptoms in both genotypes in response to this aphid species. It is possible that activation of the jasmonate pathway by BGA suppresses necrotic lesion formation by this aphid in Jester. The lack of induction of this pathway in response to PA might allow the macroscopic HR to occur in Jester. Genetic dissection of the separate roles of AKR and AIN in defence responses could shed light on the interactions among branches of defence hormone signalling networks.

AIN may modulate HR-associated defence pathways such that interactions with other plant pests are altered. In recent years, evidence has emerged that susceptibility to necrotrophic pathogens can be increased by experimental elicitation of the HR, and can be decreased by suppression of HR signal pathways (Kliebenstein and Rowe, 2008). In parallel with interactions between defence hormone signalling pathways, antagonism is known to exist between defences against necrotrophs and biotrophs (Glazebrook, 2005). It is possible that AIN controls signalling that can impinge on relationships between these defences. It may be worthwhile to test for interactions between *Acyrthosiphon* spp. and microbial pathogens, as mediated by AIN, since ‘tripartite’ interactions among plants, herbivores, and microbes are increasingly recognized as significant factors in managed and natural ecosystems (Stout et al., 2006).

Hypersensitivity is a striking plant response well-suited to genetic analysis and the elucidation of defence signalling. For this reason, the discovery of a plant defence gene that conditions a form of aphid resistance and a HR presents new opportunities for the study of plant resistance to phloem-feeding insects. This is particularly promising, given that *M. truncatula* is a model legume, that genotype A17 is a reference genotype for *M. truncatula* genome studies, and that PA is a model aphid species (Brisson and Stern, 2006; Tagu et al., 2008). A draft genome sequence for PA is nearly complete; the project is described at www.hgsc.bcm.tmc.edu/project-species-i-Peagene. PA genome sequence data are hosted www.aphidbase.com. Although AIN appears to condition only hypersensitivity in response to PA, not resistance, BGA is closely related to PA and is the target of AIN-mediated defence as well as an inducer of the HR. Thus, genome resources for *M. truncatula* and PA are likely to be useful for cross-species functional genomics of *M. truncatula* interacting with both *Acyrthosiphon* species. It is possible that genetic variation for aphid virulence exists within natural populations of *Acyrthosiphon* species. If so, this could make possible the identification of a gene-for-gene interaction in this plant–aphid model system. Since HR-inducing insects play important roles in agriculture and forest ecology, the results could have broad implications for sustainable plant protection, and for understanding the evolution of plant–pest interactions.

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