Involvement of the Octadecanoid Pathway in Bluegreen Aphid Resistance in *Medicago truncatula*

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Aphids are major insect pests of plants that feed directly from the phloem. We used the model legume *Medicago truncatula* Gaert. (barrel medic) to elucidate host resistance to aphids and identified a single dominant gene which confers resistance to *Acyrtosiphon kondoi* Shinji (bluegreen aphid). To understand how this gene conditions resistance to bluegreen aphid, transcription profiling of 23 defense-related genes representing various signaling pathways was undertaken using a pair of near-isogenic lines that are susceptible or resistant to bluegreen aphid. All salicylic acid- and ethylene-responsive genes tested were induced by bluegreen aphid in resistant and susceptible plants, although there were some differences in the magnitude and kinetics of the induction. In contrast, 10 of 13 genes associated with the octadecanoid pathway were induced exclusively in the resistant plants following bluegreen aphid infestation. These results are in contrast to plant–pathogen interactions where similar sets of defense genes typically are induced in compatible interactions, but to a lesser degree and later than in incompatible interactions. Treatment of susceptible plants with methyl jasmonate reduced bluegreen aphid infestation but not to the same levels as the resistant line. Together, these results strongly suggest that the octadecanoid pathway is important for this naturally derived aphid resistance trait.

Additional keywords: antibiosis, jasmonic acid, lipoxygenase, proteinase inhibitor, phloem-feeding insects.

Aphids are phloem-feeding insects that are ubiquitous and serious pests of agricultural and natural ecosystems. In temperate regions, approximately one in four plant species can be colonized by at least one species of aphid (Dixon 1998). Aphids feed specifically from the sieve element and cause damage by draining plant nutrients and transmitting pathogenic viruses; they account for transmission of 50% of insect-vectored viruses (Ng and Perry 2004). Our understanding of plant–aphid interactions, particularly the molecular mechanisms underlying plant resistance against aphids, is limited compared with plant defense against both pathogens and chewing insects (Edwards and Singh 2005; Ferry et al. 2004; Kessler and Baldwin 2002; Thompson and Goggin 2006; Walling 2000; Zhu-Salzman et

al. 2005). An important advance was the cloning of the *Mi* gene in tomato, which confers resistance to potato aphid (Rossi et al. 1998). This gene belongs to the nucleotide binding site leucine-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) and whiteflies (Nombela et al. 2003). 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 (Brotman et al. 2002; Cevik and King 2002; Klingler et al. 2005; Seah et al. 1998). However, the mechanisms by which *R* genes recognize and defend against aphids remain unknown.

There has been growing interest in studying the defense-signaling pathways that operate during plant–aphid interactions, and much of the progress to date has come from susceptible interactions. Analysis of gene expression profiling upon aphid infestation has shown upregulation of both salicylic acid (SA)- and jasmonic acid (JA)-responsive genes, as well as induction of other types of aphid-responsive genes (Thompson and Goggin 2006). For example, infestation of susceptible *Arabidopsis* with green peach aphid and susceptible sorghum with greenbug aphid resulted in the strong upregulation of SA-responsive genes but only a weak induction of JA-regulated genes (Moran et al. 2002; Moran and Thompson 2001; Zhu-Salzman et al. 2004). Similar results were obtained in tomato during both susceptible (potato aphid and green peach aphid) and resistant (potato aphid) interactions, with a shift to earlier induction of PR1 in the incompatible interaction (de Illarduya et al. 2003), whereas aphid feeding in tobacco elicited only a weak response in terms of both the number of differentially expressed genes and their fold induction (Voelckel et al. 2004). Recently, the SA signaling pathway was found to be important for *Mi-1*-mediated resistance to potato aphid (Li et al. 2006). The octadecanoid pathway leading to accumulation of JA and other octadecanoid-derived compounds has been shown to mediate resistance to a number of chewing insects (Halitschke and Baldwin 2003; Kessler et al. 2004; Li et al. 2004; McConn et al. 1997; Royo et al. 1999) and also to cell content-feeding mites and thrips in tomato (Li et al. 2002). It remains unknown whether naturally derived aphid resistance operates by a similar pathway.

Research with *Arabidopsis* has helped our understanding of resistance to a range of pathogens (Holt et al. 2003). However, naturally occurring and simply inherited aphid resistance has not been reported in this model plant species, although quantitative variation in aphid performance exists among ecotypes (Cabrera y Poch et al. 1998). The legume *Medicago truncatula*

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(barrel medic) is a host for several aphid species (Nair et al. 2003). Resistance to *Acyrtosiphon kondoi* (bluegreen aphid [BGA] or blue alfalfa aphid), an agronomically important pest, has been introgressed into *M. truncatula* cultivars to create near-isogenic lines differing in their susceptibility to aphids (Crawford et al. 1989). In one of these resistant lines, Jester, resistance to BGA is mediated by a single dominant gene, *AKR* (*Acyrtosiphon kondoi* resistance) that has been mapped to a region flanked by resistance gene analogs (Klingler et al. 2005). Jester is nearly isogenic with a BGA-susceptible cultivar, Jemalong, which was Jester's recurrent parent in a backcrossing program (Hill 2000). This pair of closely related genotypes provides an attractive system to study the differences in defense signaling between resistant and susceptible plant-aphid interactions. *M. truncatula* has developed rapidly into a valuable model system for plant functional genomics (Cook 1999; May and Dixon 2004; Oldroyd and Geurts 2001) and the BGA-susceptible line A17, a derivative of Jemalong, is the reference genotype for the development of genomic resources in *M. truncatula*.

Therefore, this article has focused on the A17 and Jester interactions with BGA to determine the signaling pathways through which *AKR* conditions resistance. Transcription profiling experiments have been performed using quantitative real-time polymerase chain reaction (RT-qPCR) to analyze the expression of 23 selected defense-related genes in both the susceptible and resistant plant-aphid interactions. These genes represented various signaling pathways or showed relatively high abundance in cDNA libraries from various abiotic or biotic stress treatments in *M. truncatula*. Interestingly, although we found that genes involved in SA- and ethylene-signaling pathways were induced in a similar fashion in the susceptible and resistant plants, most of the genes involved in the octadecanoid pathway were induced exclusively in the resistant plants following BGA infestation.

RESULTS

Initially, we used RT-qPCR to examine the expression of a few genes to determine tissue location and timing of maximal gene expression upon aphid infestation. We analyzed expression in two sites for BGA feeding, mature leaflets and the petioles, at 48 h after infestation. Genes linked to the SA-signaling pathway, such as β -1,3-glucanase (*BGL*), were induced in both susceptible and resistant plants, whereas members of the lipoxygenase (*LOX*) family, involved in JA biosynthesis, were induced only in resistant plants (Fig. 1). The results for the *LOX* genes revealed that a much larger fold induction occurred in petioles compared with leaves in Jester due to a lower basal level of expression in the petioles. Therefore, subsequent work focused on the petiole, which also was the preferred site of aphid feeding in the susceptible plants. The gene transcription profiling results described in this article have been obtained consistently in at least four independent experiments. However, results from only one experiment with extended timepoints involving two biological replicates are presented in detail.

SA-responsive genes are highly induced in both susceptible and resistant plants following BGA infestation.

The SA-signaling pathway regulates defense against many plant pathogens, particularly biotrophs (Thomma et al. 1998). The expression of candidate SA-responsive genes (*BGL*, *PR5*, and *PR10*) was studied over a range of timepoints from 6 to 72 h after BGA infestation. The transcript levels of these genes increased in both the susceptible and resistant plants following BGA infestation, although there were differences in the kinetics and magnitude of gene induction (Fig. 2A to C). For *BGL*,

the increase in transcript level was seen at 12 h and reached a peak at 48 h in resistant plants whereas, in susceptible plants, the increase was later (at 24 h) but higher than in the resistant Jester at both 24 and 36 h. For *PR5*, expression patterns were similar in both interactions, peaking at 48 h and decreasing at 72 h. In the case of *PR10*, there were large differences in transcript levels at early timepoints (12 and 24 h) between the BGA-infested resistant (Jester) and susceptible (A17) plants. However, at the later timepoints (36 and 48 h), *PR10* transcripts were induced to similar levels in the resistant and susceptible plants (Fig. 2C). We also tested the response of these genes to SA treatment. All three genes were induced by SA at 12 h (Fig. 2D to F), which was earlier than the induction by BGA (at 24 or 36 h). Interestingly, the SA induction of *BGL*, *PR5*, and *PR10* was transient and had declined or returned to basal levels by 24 h (Fig. 2D to F), in clear contrast to the much higher and more prolonged induction seen following aphid infestation, where high levels of expression still were observed at 72 h (Fig. 2A to C).

Genes involved in the octadecanoid pathway are predominantly or exclusively induced in resistant plants following BGA infestation.

Most of the biosynthetic genes tested from the octadecanoid pathway (8 of 11) were induced exclusively in resistant plants following BGA infestation (Fig. 3; Table 1). For the *LOX* gene family, six members were chosen based on their abundance in

Fig. 1. Differential expression of genes representing A, salicylic acid or B, jasmonic acid signaling pathways in petiole and leaflet tissues of noninfested control or bluegreen aphid-infested A17 and Jester plants, at 48 h after aphid infestation; β -1,3-glucanase = *BGL* and lipoxygenase = *LOX1*. The relative transcript abundance is shown as ratio of the relative gene expression for each sample to that in the noninfested petiole of A17. Values are the mean and standard error of three biological replicates.

the expressed sequence tag (EST) libraries from *M. truncatula* plants with biotic or abiotic stresses. With the exception of *LOX6*, which was upregulated but to a much lower degree in both susceptible and resistant plants (Fig. 3I), the other five members were induced only in resistant plants (Fig. 3A, B, C, G, and H). The increase in gene transcripts occurred as early as 12 h (*LOX1*, -2, and -4) or 24 h (*LOX3* and -5) and peaked at 36 h with approximately 25 to 1,200 times higher transcript abundance in infested plants. The lack of induction of these genes in susceptible plants following BGA infestation was striking. A17 and Jester plants were treated with methyl jasmonate (MeJA) to determine whether these *LOX* genes were JA responsive in either the aphid-susceptible or -resistant plant genotypes. All *LOX* genes except *LOX6* showed a strong MeJA induction (Fig. 3. D to F and J to L). The transcript levels of each gene did not differ substantially between A17 and Jester genotypes.

To investigate whether other JA-responsive genes also were induced following BGA infestation, we studied the expression of proteinase inhibitor (*PI*) and vegetative storage protein (*VSP*) genes. As with *LOX1* through *LOX5*, the *PI* and *VSP* genes were induced only in the resistant Jester plants following aphid infestation (Fig. 4A and B). For both genes, the induction was observed at 12 h and transcript levels peaked at 36 h, when they were 100 and 500 times higher in infested versus noninfested plants for *PI* and *VSP*, respectively. In contrast, the expression of these genes did not differ significantly between noninfested and infested A17 plants. Both the *PI* and *VSP* genes also were found to be JA responsive to similar degrees in A17 and Jester (Fig. 4C and D). We also tried to analyze the expression of *PDF1.2* orthologs following BGA infestation, but were unable to detect the transcript for these genes in petiole tissues.

Although a major output of the octadecanoid pathway (Fig. 5) is the production of JA and induction of downstream genes, there are also branch points that lead to the production of other compounds, some of which have been linked to plant defense (Howe and Schilmiller 2002). To further investigate the potential involvement of the octadecanoid pathway in BGA-resistant plants, the expression of genes encoding other enzymes involved in the biosynthesis of octadecanoid-derived molecules was analyzed. Genes putatively encoding allene oxide synthase (*AOS*), 12-OPDA reductase (*OPR*), divinyl ether synthase (*DES*), and hydroperoxide lyase (*HPL*) were examined. Although aphid infestation did not alter the transcript level of the *HPL* and *DES* genes in either genotype (Table 1), the *OPR* gene showed a small, 2.5- to fourfold induction in both A17 and Jester at 36 and 72 h (Table 1). Aphid infestation also resulted in a small induction of the *AOS* gene at 36 h, but only in resistant plants (Table 1).

Induction of genes involved in other defense pathways.

We also examined the involvement of ethylene-responsive genes and other defense-related genes following aphid infestation (Table 1). Genes involved in the ethylene pathway were induced in both A17 and Jester following BGA infestation. For example, a putative ACC oxidase gene was induced at 36 and 72 h, when the gene transcripts reached 6 and 13 times higher in infested A17 and Jester, respectively, compared with noninfested controls. We also examined a phenylalanine ammonia lyase (*PAL*) gene involved in the phenylpropanoid pathway, which leads to the biosynthesis of salicylic acid, flavonoids, and isoflavonoids. The *PAL* gene transcript levels were induced in both the susceptible and resistant plants following BGA

Fig. 2. Regulation of genes representing the salicylic acid signaling pathway. **A, B, and C,** Bluegreen aphid-infested or noninfested control petioles of A17 and Jester and **D, E, and F,** salicylic acid-treated (SA) or control plants of A17 at the timepoints indicated. **A and D,** β -1,3-Glucanase (*BGL*); **B and E,** *PR5*; **C and F,** *PR10*. The relative transcript abundance is shown as ratio of the relative gene expression for each sample to that in the noninfested petiole of A17, and values are the mean and standard error of two biological replicates.

infestation (Table 1). The transcript levels of a sugar transport gene also have been observed to increase in *Arabidopsis* following aphid infestation (Moran et al. 2002; Moran and Thompson 2001). Of two *M. truncatula* sugar transport (*STP*

homolog) genes examined, *STP1* and *STP2*, only *STP2* was induced following BGA infestation; however, this was not specific to aphid resistance because it occurred in both susceptible and resistant plants. The expression of catalase-3 (*CAT*) and

Fig. 3. Different transcription patterns of representative members of the lipoxigenase gene family (*LOX*) in **A, B, C, G, H, and I**, bluegreen aphid-infested and noninfested control petioles of A17 and Jester and in **D, E, F, J, K, and L**, the methyl jasmonate (MeJA)-treated or untreated control plants of A17 and Jester at the timepoints indicated. The relative transcript abundance is shown as ratio of the relative gene expression for each sample to that in the noninfested petiole of A17 and values are the mean and standard error of two biological replicates for the bluegreen aphid infestation and three biological replicates for the MeJA treatment.

glutathione S-transferase (*GST*) genes did not change in either A17 or Jester upon BGA infestation (Table 1).

Link between *AKR*-mediated aphid resistance and the activation of the JA pathway.

To further examine the link between *AKR*-mediated aphid resistance and the activation of the JA pathway, the transcript levels of JA-responsive genes were measured following BGA infestation in two additional genotypes, A17*AKR*⁺ and Jester*AKR*[−] (discussed below) (Fig. 6A). Line A17*AKR*⁺ contains *AKR* and differs from the susceptible line A17 by only approximately 1%, whereas Jester*AKR*[−] no longer contains *AKR* and differs from the resistant line Jester by only approximately 1%. A17 and Jester also were included to allow comparison of the results with those of the experiments described above. A17*AKR*⁺ had a degree of resistance to BGA similar to that of Jester, and Jester*AKR*[−] and A17 had a similar degree of susceptibility to BGA (data not shown). The relative transcript levels of each gene in the noninfested or BGA-infested A17 and Jester at both 12 and 36 h were consistent with the results shown in Figures 1 to 4. The results for two representative members of the lipoxygenase gene family (*LOX1* and *LOX2*) and two genes downstream of the JA-signaling pathway, *PI* and *VSP*, are shown in Figure 6B to E. There was no significant ($P > 0.05$) difference between transcript levels for these genes in BGA-infested susceptible A17 and Jester*AKR*[−]. Similarly, no significant difference in transcript levels was observed between BGA-infested resistant Jester and A17*AKR*⁺. These results confirm that *AKR*-mediated aphid resistance is tightly linked with the activation of the JA pathway by the BGA.

MeJA treatment reduced aphid infestation in susceptible plants.

To determine whether MeJA treatment had any effect on aphid resistance, susceptible and resistant plants were treated with MeJA for 24 h and subsequently infested with BGA. After 3 days, aphid performance was measured in terms of aphid population growth rate (PGR). Regardless of whether the plants were treated with MeJA, PGRs were significantly lower in Jester than A17 (Fig. 7), as previously reported (Klingler et

al. 2005). In the case of Jester, the difference between MeJA and mock treatments was not significant. However, on A17, the aphid PGR was significantly ($P < 0.05$) reduced following MeJA treatment but was still higher than Jester. The reduced aphid PGR on A17 following MeJA treatment suggests that activation of this pathway may be capable of conferring at least partial resistance to BGA.

DISCUSSION

We previously have reported the identification of a single dominant gene, *AKR*, in the model legume *M. truncatula*, which confers resistance to BGA (Klingler et al. 2005). To identify the signaling pathways through which *AKR* may mediate aphid resistance, the transcription profiles of genes representing a number of defense-signaling pathways were analyzed in near-isogenic lines which were susceptible or resistant to BGA. Because the petiole is the preferred site of BGA feeding in the susceptible plants, the expression of a few defense genes was analyzed in petioles and leaflets to determine whether transcripts could be detected in these tissues. The petioles showed a lower basal level of *LOX* gene transcripts and a much larger fold induction in petioles compared with leaves following aphid infestation of the resistant Jester. Therefore, the subsequent gene expression work was focused on the petiole. We found no difference in expression levels for any of these genes between the susceptible and resistant plants prior to BGA infestation. In contrast, BGA infestation activated an array of defense pathways in both interactions, consistent with an emerging view that plant responses to phloem-feeding insects partially overlap with plant responses to both pathogens and chewing insects (Edwards and Singh 2005; Kessler and Baldwin 2002; Walling 2000; Zhu-Salzman et al. 2005). Interestingly, most of the genes examined that are associated with the octadecanoid pathway were induced predominantly or exclusively in the resistant plants that carry the *AKR* gene, whereas the transcript levels of genes associated with other defense pathways were induced in both the susceptible and resistant interactions, albeit with interesting quantitative differences in some cases.

The similar induction of SA-responsive genes also has been seen in both the susceptible and resistant interactions of *M.*

Table 1. Expression profiling of known defense-related genes in response to bluegreen aphid in susceptible and resistant *M. truncatula*

Putative function, gene	Fold induction ^a					
	Susceptible A17			Resistant Jester		
	24 h	36 h	72 h	24 h	36 h	72 h
Octadecanoid pathway						
<i>AOS1</i> (allene oxide synthase)	0.7 (0.2)	1.3 (0.4)	1.9 (0.6)	1.4 (0.3)	8.6 (2.5)*#	1.7 (0.4)
<i>AOS2</i>	1.3 (0.3)	0.8 (0.3)	1.1 (0.2)	0.7 (0.4)	3.8 (1.2)	0.5 (0.3)
<i>OPR</i> (12-OPDA reductase)	1.3 (0.1)	3.1 (0.6)*	3.6 (0.8)*	1.2 (0.2)	4.3 (1.4)*	2.5 (0.8)*
<i>DES</i> (divinyl ether synthase)	0.5 (0.3)	1.0 (0.2)	0.9 (0.2)	1.3 (0.4)	2.8 (0.6)#	0.8 (0.2)
<i>HPL</i> (hydroperoxide lyase)	1.0 (0.1)	1.7 (0.4)	1.6 (0.5)	1.0 (0.2)	0.9 (0.3)	1.7 (0.4)
Ethylene pathway						
<i>ACC</i> (ACC oxidase)	2.2 (0.3)	3.6 (0.8)*	6.5 (1.2)*	1.8 (0.3)	4.9 (1.2)*	11.9 (2.5)*
<i>HEL</i> (hevein-like protein)	1.0 (0.2)	6.4 (2.1)*	4.3 (1.4)*	1.7 (0.5)	3.8 (0.9)*	4.3 (1.3)*
<i>ERF1</i> (ethylene responsive factor)	2.1 (0.3)	8.3 (2.8)*	3.1 (0.8)#	1.5 (0.6)	12.9 (3.7)*	1.0 (0.2)
Phenylpropanoid pathway						
<i>PAL</i> (phenylalanine ammonia lyase)	7.9 (0.8)*	67.0 (9.5)*	8.2 (1.6)*	12.0 (2.1)*#	45.8 (9.7)*	5.5 (1.2)*
Reactive oxygen related						
<i>CAT</i> (catalase-3 enzyme)	1.5 (0.3)	1.2 (0.2)	1.4 (0.5)	1.6 (0.2)	1.5 (0.4)	1.8 (0.6)
<i>GST</i> (glutathione S-transferase)	1.7 (0.3)	1.9 (0.8)	1.3 (0.2)	1.2 (0.4)	1.7 (0.3)	1.6 (0.5)
Carbohydrate translocation						
<i>STP1</i> (sugar transporter protein)	0.9 (0.2)	0.7 (0.4)	1.3 (0.5)	1.3 (0.3)	1.1 (0.2)	1.4 (0.3)
<i>STP2</i>	1.1	5.0 (1.2)*	1.7 (0.6)	2.8 (0.9)#	9.1 (2.1)*#	11.8 (3.4)*#

^a Fold induction was determined as ratio of mean relative expression in infested plants to noninfested plants. Relative expression was determined by real-time polymerase chain reaction and numbers are the mean and standard error (in parentheses) of two biological replicates. Values are in bold if upregulated greater than twofold. *Indicates that the transcript level was significantly different from that of the noninfested control plants of the same genotype ($P < 0.05$, $n = 2$), and # indicates that the fold induction was significantly different between the susceptible A17 and resistant Jester plants at a given time point ($P < 0.05$, $n = 2$).

truncatula with BGA. Similar results were found in other susceptible plants following aphid attack (de Ilarduya et al. 2003; Heidel and Baldwin 2004; Moran et al. 2002; Moran and Thompson 2001; Voelckel et al. 2004; Zhu-Salzman et al., 2004). Park and associates (2006) demonstrated higher levels of SA-responsive gene transcripts in susceptible than resistant sorghum plants following greenbug infestation. However, we found interesting differences in the timing of the induction between susceptible and resistant interactions with BGA that could be important, with the induction of all three SA-responsive genes being earlier in Jester than A17 following BGA infestation. de Ilarduya and associates (2003) also found that, in tomato infested with potato aphids, an earlier and stronger induction of SA-responsive genes occurred in the resistant cultivar carrying the *Mi-1.2* gene than in the susceptible parent. Moreover, Li and associates (2006) used the SA-metabolizing *NahG* as a transgene in the *Mi-1.2* background to demonstrate that SA is essential for potato aphid resistance. Aphid-induced methyl salicylate (MeSA) is a strong aphid repellent that may deter aphids from settling on plants with already high aphid densities (Bernasconi et al. 1998; Hardie et al. 1994; Preston et al. 1999; Shulaev et al. 1997); thus, activation of the SA pathway may be a general mechanism of antibiosis or aphid repellence with limited effectiveness in susceptible hosts. In Jester,

early and strong induction of specific SA-responsive genes may potentiate resistance against infestation by BGA.

The octadecanoid pathway has been associated with defense against chewing insects and mechanical wounding which, unlike aphid feeding, involve maceration of plant tissue (Howe et al. 1996a,b; McConn et al. 1997). More recently, the octadecanoid pathway has been shown to mediate resistance to cell content-feeding mites and thrips in tomato (Li et al. 2002). Several lines of evidence suggest that the octadecanoid pathway plays an important role in BGA resistance in Jester. First, genes encoding lipoxygenase and other enzymes in the biosynthesis of octadecanoid-derived signaling molecules, including JA, were induced predominantly or exclusively in the resistant plants, contrasting with no or little induction of these genes in the susceptible plants (Figs. 3A to C and 6B and C; Table 1). Second, two JA-inducible genes, *PI* and *VSP*, also were induced only in the resistant interaction (Figs. 4A and B and 6D and E), suggesting activation of defenses downstream of JA. Third, application of exogenous MeJA also reduced BGA infestation of the susceptible cv. A17 (Fig. 7).

Our gene expression results contrast with those obtained in tomato with *Mi*-mediated resistance to potato aphid (de Ilarduya et al. 2003). That study found that JA-response genes showed similar induction patterns in both susceptible and resistant

Fig. 4. Transcript accumulation of genes down stream of the jasmonic acid signaling pathway in response to **A** and **B**, bluegreen aphid and **C** and **D**, methyl jasmonate (MeJA) treatment. **A** and **C**, proteinase inhibitor (*PI*); **B** and **D**, vegetative storage protein (*VSP*) in the bluegreen aphid-infested and noninfested control petioles of A17 and Jester at the timepoints indicated. The relative transcript abundance is shown as ratio of the relative gene expression for each sample to that in the noninfested petiole of A17 and values are the mean and standard error of two biological replicates for the bluegreen aphid infestation and three biological replicates for the MeJA treatment.

plants following aphid infestation. In fact, a recent study suggested that the SA pathway plays an important role in *Mi1*-mediated aphid resistance (Li et al. 2006). Nevertheless, these different gene expression results indicate that multiple mechanisms for aphid resistance exist. There is other evidence that also supports a link between the octadecanoid pathway and defense against aphids. Pretreatment with MeJA reduced aphid colonization of susceptible tomato, whereas JA treatment had no effect on aphid resistance in tomato plants expressing the *Mi* gene (Cooper and Goggin 2005), similar to what we observed with Jester containing the *AKR* gene. JA treatment also reduced aphid reproduction in sorghum, although the transcript levels of JA-responsive genes were not altered following aphid infestation (Zhu-Salzman et al. 2004). In addition, lower green peach aphid reproduction was observed on susceptible *Arabidopsis* plants when JA signaling was constitutively increased by mutation of the *CEV1* gene, although the precise role of JA in reducing the aphid performance needs further study given the pleomorphic phenotypes associated with this mutant (Ellis et al. 2002). Our research significantly extends what has been seen in these artificial systems involving susceptible interactions, and strongly suggests that the octadecanoid pathway can be an important component in naturally derived resistance to aphids.

The specific role or roles of the octadecanoid pathway in BGA resistance in Jester is unknown. The high and rapid accumulation of gene transcripts in the octadecanoid biosynthetic pathway, such as *LOX* genes and downstream JA-responsive genes, *PI* and *VSP*, suggest the production of JA during aphid infestation of resistant plants, although how JA actually mediates defense against aphids is unknown. Proteinase inhibitors and polyphenol oxidases that act as antifeedants against Lepidopteran chewing insects also may be effective against piercing-sucking insects, such as Homoptera (aphids and whiteflies) and Acari (spider mites). In fact, members of the Bowman-Birk protease inhibitors from pea seed exhibited significant toxicity to pea aphid (Rahbé and Febvay 1993; Rahbé et al. 2003b). The cysteine protease inhibitor oryzasystatin I (OC-I), when administered in artificial diets, reduced the growth of pea aphid, cotton and melon aphid, and peach potato aphid; the transgenic oilseed rape expressing OC-I reduced the fecundity and biomass of the peach potato aphid (Rahbé et al. 2003a). Vegetative storage proteins also have been shown to contribute to plant defense against herbivore insects (Liu et al. 2005). The role of JA in aphid de-

fense also may involve JA-regulated secondary metabolites of *M. truncatula* (Broeckling et al. 2005; Suzuki et al. 2005), some of which may serve as phytoalexins against aphids, such as phenylpropanoids (Grayer et al. 1992) or triterpene saponins (Shinoda et al. 2002; Taylor et al. 2004).

Interestingly, although MeJA treatment reduced BGA performance on the susceptible plants, it had less impact than the presence of *AKR* in resistant plants. This could be explained by a more prolonged induction of JA defenses mediated by *AKR* during continuous aphid feeding compared with a single MeJA treatment. It also is possible that aphids and MeJA activate similar but not identical defense responses in the plants, as indicated by the different magnitudes or kinetics of gene transcription levels (Figs. 3 and 4). For example, the white pine weevil and MeJA induced a similar but not identical terpenoid defense response in Sitka spruce (Miller et al. 2005). Alternatively, octadecanoid-derived JA signaling may only partially contribute to the BGA resistance in Jester. Other oxylipins of the octadecanoid pathway may also be important, including lipid-derived volatiles, particularly hexenals, which can cause reductions in aphid and spider mite fecundity, and in caterpillar feeding (Avidushko et al. 1997; Hildebrand et al. 1993; Kasu et al. 1995). Furthermore, in potato, depletion of a specific hydroperoxidase lyase, a key enzyme in oxylipin production, resulted in an increase in green peach aphid performance (Vancanneyt et al. 2001). BGA infestation of *M. truncatula* did not alter the transcript level of the *HPL* gene in either susceptible or resistant plants; however, posttranscriptional changes could be occurring.

A striking feature of our results was the almost complete lack of induction of genes involved in the octadecanoid pathway in the susceptible plants. Application of exogenous MeJA induced *LOX*, *PI*, and *VSP* genes and also reduced BGA infestation in A17, suggesting that A17, which lacks a functional *AKR* gene, is not defective in JA responses. Moreover, treatment of Jester with exogenous MeJA resulted in accumulation of *LOX*, *PI*, and *VSP* gene transcripts to comparable levels in Jester and A17, suggesting that Jester is not more sensitive to JA. The lack of induction of genes involved in the octadecanoid pathway in the susceptible plants contrasts with plant-pathogen interactions during which the defense responses in susceptible interactions typically differ primarily in a quantitative manner, with delayed or lessened induction of genes induced in the resistant interaction

Fig. 5. Schematic illustration of octadecanoid pathway (modification of Howe and Schilmiller 2002). LOX = lipoxygenase, AOS = allene oxide synthase, OPR = 12-OPDA reductase, DES = divinyl ether synthase, and HPL = hydroperoxide lyase. The corresponding genes for these enzymes were analyzed and the results are shown in Table 1.

(Katagiri 2004). This opens up the intriguing possibility that the susceptible plants lacking *AKR* are not able to recruit the octadecanoid system against BGA, or that aphids have evolved strategies to avoid or suppress this part of the defense response, leading to the successful infestation of the susceptible plant. The latter is also in line with the “decoy” hypothesis that aphids manipulate plant defense through pathway

cross-talk, whereby aphid activation of the SA pathway leads to suppression of the more biologically effective JA pathway (Thompson and Goggin 2006; Zhu-Salzman et al. 2004, 2005). Similar mechanisms have been documented in several plant–microbial pathogen interactions (Chisholm et al. 2006; Mudgett 2005). An example of the suppression of plant defense against insects occurs with the caterpillar *Helicoverpa*

Fig. 6. A, Chromosome 3 haplotypes of four *Medicago truncatula* lines tested for bluegreen aphid (BGA)-induced gene expression. The position of the *AKR* locus is indicated by the heavy horizontal line on each of the four chromosomes. The shading in the Jester chromosome indicates the introgression into the A17 genetic background that is present in this BGA-resistant cultivar. No other molecular polymorphisms between A17 and Jester have been detected on other chromosomes (Klingler et al. 2005). A17 × Jester F₂ plants with recombinations close to the *AKR* locus (just below marker 004H01) were used to produce experimental lines with and without the *AKR* gene, represented by A17AKR+ and JesterAKR-, respectively. The position of shading indicates the recombination breakpoints and location of the remaining Jester introgression. At left is a genetic map of chromosome 3, with numbers representing position in centimorgans. Horizontal bars indicate molecular markers tested for polymorphism between A17 and Jester. Labeled molecular markers were tested in the recombinant lines A17AKR+ and JesterAKR- to confirm their haplotypes. **B**, through **E**, Comparison of transcript levels of representative members of the lipoxigenase gene family (*LOX*) and genes downstream of the jasmonic acid signaling pathway in petioles of A17, A17AKR+, Jester, and JesterAKR- at 36 h following BGA infestation. **B**, *LOX1*; **C**, *LOX2*; **D**, proteinase inhibitor (*PI*); **E**, vegetative storage protein (*VSP*). The relative transcript abundance is shown as ratio of the relative gene expression for each sample to that in the noninfested petiole of A17 and values are the mean and standard error of three biological replicates.

zea, whose saliva contains glucose oxidase, which can suppress the production of nicotine in tobacco plants, thereby preventing an effective defense (Musser et al. 2002). The near-isogenic nature of A17 and Jester, coupled with the rapidly growing resources in *M. truncatula*, should allow us to explore these intriguing questions. The fact that Jester is an agronomically important cultivar, based on its durable BGA resistance, provides confidence that progress with this system should generate valuable information in helping control aphids in other plant species.

MATERIALS AND METHODS

Plant growth and aphid infestation.

Two genotypes of *M. truncatula* were the primary focus of this study: BGA-susceptible genotype A17 and BGA-resistant cv. Jester. Resistance in Jester, conditioned by a dominant gene called *AKR* (Klingler et al. 2005), was derived from accession SA1499 and introgressed into susceptible cv. Jemalong by recurrent backcrossing (Hill 2000). Based on its breeding pedigree, Jester has 89% of its genome derived from Jemalong. Genotype A17 is derived from Jemalong and is the reference genotype for the *M. truncatula* model system. A17 and two other derivatives from Jemalong were monomorphic for 4,000 molecular markers (Thoquet et al. 2002), suggesting a very low level of heterogeneity in Jemalong. Thus, in our studies, the aphid-susceptible genotypes A17 and Jemalong are considered equivalent and near-isogenic with Jester.

In addition to A17 and Jester, two genotypes specifically developed for molecular-genetic analysis of BGA resistance were tested. These genotypes were descended from an A17 × Jester F₂ population (Klingler et al. 2005), both derived from plants with recombination events less than 1 centimorgan from the *AKR* locus. Genotype NCAF013-2-1 is an F_{2.5} line homozygous for resistance gene *AKR*; approximately 90% of Jester's total introgression from other genotypes has been removed in this line, as determined by its genotype for molecular markers on chromosome 3. Thus, NCAF013-2-1 is BGA resistant, but is approximately 99% identical to susceptible line A17. Hereafter, this line is designated as A17AKR+. Genotype NSAC094-1 is an F_{2.4} line lacking *AKR*, but with approximately 90% of Jester's total introgression from other genotypes still present, as determined by molecular markers. Thus, NSAC094-1 is BGA susceptible, but is approximately 99% identical to Jester. Hereafter, this line is designated as JesterAKR-. A schematic illustration of chromosome 3 haplotypes of four *M. truncatula* lines is shown in Figure 6A.

Fig. 7. Effects of methyl jasmonate treatment on bluegreen aphid population growth rate. Population growth rate was measured as described by Klingler and associates (2005). Values are the mean and standard error of six replicates. Means with the same letter are not significantly different ($P < 0.05$).

Seed of the four genotypes were scarified, bleached, and germinated in the dark on moist filter paper for 2 days before planting into soil. Plants were grown in individual 0.9-liter pots in growth cabinets with 16 h of light (22°C) and 8 h of dark (20°C) under high-pressure sodium and fluorescent lamps at 280 $\mu\text{E m}^{-2} \text{s}^{-1}$. Plants were watered with a half-strength Hoagland's solution every second day.

For quantification of gene transcripts, two mature, fully expanded trifoliolate leaves from the primary stem of individual plants (approximately 3 to 4 weeks old) were infested with 15 late instars or adults (mixture of 10 apterae, the wingless, sedentary morph, and 5 alatae, the winged, migratory morph) and caged in linen mesh supported by a bamboo stick. For the noninfested control plants, two trifoliolate leaves of similar developmental stage were caged without aphids. These cages were air and light transmittable and had no significant effect on defense gene transcript level when compared with comparable noncaged leaves (data not shown). Samples were collected at 0, 6, 12, 24, 36, 48, and 72 h after aphid infestation for both control and aphid-infested plants. After de-assembling the cage, aphids were brushed off using a fine paint brush; the noninfested control received a similar extent of brushing. Leaflets were separated from petioles at harvest. Leaflets or petioles from the two trifoliolate leaves per plant were combined. All the samples were frozen in liquid N₂ and stored at -80°C until RNA isolation.

RNA isolation and cDNA synthesis.

RNA isolation and cDNA synthesis were performed using the Purescript RNA isolation kit (Gentra Systems, Minneapolis, MN, U.S.A.) (Oñate-Sánchez and Singh 2002). The absence of genomic DNA contamination was confirmed by PCR using a primer pair flanking an intron region of a trehalase gene (Salzer et al. 2000). RNA integrity was checked on a 1.5% (wt/vol) agarose gel and subsequently repurified with the Purescript RNA isolation kit. Total RNA (1 to 2 μg) was used for first-strand cDNA synthesis.

PCR primer design.

Putative defense-related genes were identified in *M. truncatula* by BLASTN or TBLASTN searching of the TIGR Medicago Gene Index database, with query sequences derived from characterized *Arabidopsis* genes and EST tentative consensus annotations. Sequences of gene family members were aligned to enable design of specific primer sets using the Multiple Sequence Alignment application (AlignX) of the Vector NTI Suite program (InforMax, Inc., North Bethesda, MD, U.S.A.), based on the Clustal W algorithm. Tentative consensus sequences were chosen according to abundance in defense or stress-related libraries shown in the TIGR MtGI database. Primer 3 software was used with primer picking parameters modified to suit the real-time quantitative PCR conditions (bulletin 2593; Bio-Rad, Hercules, CA, U.S.A.). BLASTN analysis of TIGR MtGI was performed on the primer sequences to confirm specificity of amplification. The sequences of each primer pair are given in Table 2.

RT-qPCR conditions and analysis.

RT-qPCR was performed using an iCycler (Bio-Rad Inc.). Reactions were set up according to Klok and associates (2002) with the thermal profile 95°C for 2.5 min; 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s; followed by a melt curve program of 70 to 95°C, with 0.5°C increase per cycle. In order to compare data from different PCR runs or cDNA samples, threshold cycle (C_T) values for all selected genes were normalized to the C_T value of a tubulin gene, whose expression remained constant among various aphid-

infested and noninfested tissues (results not shown). Relative gene expression was derived from using $2^{-\Delta C_T}$, where ΔC_T represents C_T of the gene of interest minus C_T of tubulin. The significance in difference between ratios was analyzed by two-way analysis of variance (ANOVA) (genotype: A17 and Jester or A17, Jester, A17AKR+, and JesterAKR-; treatment: infested and noninfested control) and compared by the least significant difference (LSD) test at a 5% significance level using GenStat 6.2 (Lawes Agricultural Trust, Rothamsted Experimental Station, U.K.).

SA and MeJA treatment of plants and measurement of aphid performance.

SA treatment of 2-week-old A17 plants was performed by spraying with 1 mM sodium salicylate until moisture run off in two replicate experiments. Treatment of plants with MeJA for gene expression studies was performed by sealing the plants in a container with 10 μ M MeJA in 5 μ l of ethanol per liter of air. Leaves of 10 control or treated plants were harvested at 0, 12, and 36 h after treatment, pooled, frozen in liquid nitrogen, and stored at -80°C until RNA extraction. Three biological repli-

Table 2. Genes and primer sequences

Putative function, gene ^a	TC ^b	Primer sequence ^c
Salicylic acid pathway <i>BGL</i> (β -1,3-glucanase)	TC98780	F: 5'-CAAATTGGGTCCAAAAATATGTGAC-3' R: 5'-GCACCATCATTGGGTGGATATGAAG-3'
<i>PR5</i> (thaumatin-like protein)	TC100682	F: 5'-TGCCTTAGCTTTGCATTCT-3' R: 5'-AATTTCCGCTGAGTTCTGTTG-3'
<i>PR10</i> (ribonuclease-like)	TC76513	F: 5'-AGCATCACCTTTGGTAGCGGATT-3' R: 5'-CATCAATAACCTTCGGGATAAGG-3'
Octadecanoid pathway <i>LOX1</i> (lipoxygenase)	TC100514	F: 5'-TTCAGACAGCGAATGGGAACCTTTCTTCG-3' R: 5'-CAAAAGCAGCTTTAATCATTTGGTTCTCCTC-3'
<i>LOX2</i>	TC100141	F: 5'-ATGAAGAGATGGAGAAAGATGACC-3' R: 5'-GCAAAAGTGTAGGGCATCTTAACCG-3'
<i>LOX3</i>	TC100155	F: 5'-CGATGAGATGGTGAAAAGTCTCCTC-3' R: 5'-GCTTGAAGTGGCCTTGTATCAGA-3'
<i>LOX4</i>	TC100162	F: 5'-TGAGCCTGACTCGAAACCTACC-3' R: 5'-TTGTGTGTCCAAATGCCTCATC-3'
<i>LOX5</i>	TC100513	F: 5'-AAAGCTGTGGCTGTGAGTGATG-3' R: 5'-CCACTGATCTATGCGTTTGGTG-3'
<i>LOX6</i>	TC97760	F: 5'-CATCTTCTTCTTCTGTTCTCCTGCTGA-3' R: 5'-GGTCTTCTTTGCAGCGTTTGT-3'
<i>PI</i> (proteinase inhibitor)	TC100490	F: 5'-GCTGAACCAAGAGGAGTAGTAGACA-3' R: 5'-GAGAGTTGGAATGTAATCAAGACC-3'
<i>VSP</i> (vegetative storage protein)	TC93960	F: 5'-GACCTTTGGGTGTTTGACATTGA-3' R: 5'-TCCTTCTGTTTGAGTGGTCTTCTCCT-3'
<i>AOS1</i> (allene oxide synthase)	TC101097	F: 5'-GGACACCGAAGTGGACTTGAC-3' R: 5'-AAACATCCAAACGCTCTGCTTC-3'
<i>AOS2</i>	TC110784	F: 5'-GGCCCATTCATTTCTCTAACC-3' R: 5'-AGAAGGCGTTTGAGTTGGTCGT-3'
<i>OPR</i> (12-OPDA reductase)	TC94406	F: 5'-TGGCACCACCTACAAGACAAAG-3' R: 5'-GAACAGCATCCACAATGGGTTT-3'
<i>DES</i> (divinyl ether synthase)	TC107037	F: 5'-CTCAAACCAACATGCCACCAG-3' R: 5'-CCACCGAAGAAATCAGTGGAAG-3'
<i>HPL</i> (hydroperoxide lyase)	TC101310	F: 5'-CACCGAAAGCAAGGCAAC-3' R: 5'-GAAAGGGAACGTGGGTGGAA-3'
<i>ACC*</i> (ACC oxidase)	TC106655)	F: 5'-GTCATATCAAGCAAAGGCTTAGATGT-3' R: 5'-CTTCTGCTAGTTTCTCTAACCTCAGAGA-3'
<i>HEL</i> (hevein-like protein)	TC94004	F: 5'-GTAGCAAGTATGGTTGGACTGC-3' R: 5'-AAAGAGTGATTAAATTCGCAATTAT-3'
<i>ERF1</i> (ethylene response factor)	TC105911	F: 5'-GATCAACGACGCTAACAACCTC-3' R: 5'-CCATCTTCAGAACCATCATCAC-3'
Phenylpropanoid pathway <i>PAL</i> (phenylalanine ammonia lyase)	TC106667	F: 5'-GAATTGCTTCCCATGATAGTGGTGTTAG-3' R: 5'-GGCATTCAAAAACCTAATTAGCTCC-3'
Active oxygen related <i>CAT</i> (catalase-3 enzyme)	TC100988	F: 5'-TTCTTCTTCTCCACCGTCTCTCA-3' R: 5'-TCCAAGAGAATTGGACCTCTGG-3'
<i>GST</i> (glutathione S-transferase)	TC69623	F: 5'-CCAGCTTGGTCTGAAGTCCT-3' R: 5'-TGTCTTCGATCATCCGTCAG-3'
Carbohydrate translocation <i>STP1</i> (sugar transporter protein)	TC101088	F: 5'-GGTGATGGTCCACGAACAAACAA-3' R: 5'-TGCTGCCAAATAAGGTCACAAG-3'
<i>STP2</i>	TC94579	F: 5'-GGTGTTATGAGTGGAGCAGCAA-3' R: 5'-ATGGTGTAACGACGACCAATCC-3'
House-keeping <i>Tub</i> (β -tubulin)	TC106434	F: 5'-CCTGTTGCCGGTTCATAATC-3' R: 5'-CCCAAACATAGATTGCTGCTT-3'

^a Due to the changing nature of tentative consensus annotation, members of a gene family have not been annotated in detail; however, for the purpose of this article, gene family members have been numbered to differentiate individual genes. The putative function of a gene and the number were based on information in the TIGR Medicago Gene Index database and sequence homology.

^b TC = tentative consensus.

^c Primers were designed using Primer 3 software; F = forward and R = reverse.

cates of control and MeJA treatments were conducted. Control plants were mock treated with water for the sodium salicylate treatment or 5 µl of ethanol per liter of air for the MeJA treatment. Gene transcripts were quantified as described above from two replicate experiments.

The effect of MeJA on the performance of BGA on A17 and Jester was measured following treatment of the plants with 10 µM MeJA in 5 µl of ethanol per liter of air for 24 h. Control plants were mock treated with ethanol only. Eight preweighed, early-instar nymphs of BGA were placed on each treated and control plant. The aphid survival and weight were measured after 3 days. The PGR of surviving nymphs was calculated as the per diem difference between the logarithm of the initial mean weight of aphids placed on the plant (W_{orig}) and the logarithm of the final, total weight of living aphids removed per aphid originally placed on the plant (W_{total}), according to Edwards (2001) and Leather and Dixon (1984), where $\text{PGR} = (\log W_{\text{total}} - \log W_{\text{orig}})/\text{number of days}$.

This statistic combines effects of aphid growth and survival, providing an estimate of colonization potential on the host plant (Edwards 2001; Klingler et al. 2005). PGR were analyzed by two-way ANOVA (genotype: A17 and Jester; treatment: MeJA treatment and untreated control) and compared by the LSD test at a 5% significance level using GenStat 6.2.

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AUTHOR-RECOMMENDED INTERNET RESOURCES

The Institute for Genomic Research (TIGR) *Medicago* Gene Index database: www.tigr.org/tigr-scripts/tgi/T_index.cgi?species=medicago
 TIGR MtGI database: tigrblast.tigr.org/tgi
 Whitehead Institute for Biomedical Research website's Primer 3 software: frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi