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Abstract

Although soybean aphid (*Aphis glycines*) resistance is commercially available in the form of the *Rag1* gene, the mechanism of this resistance is not fully understood. Amino acids are a limiting factor for aphid growth, and there is evidence that plant amino acid composition is related to aphid resistance. Antibiotic resistance like that conferred by *Rag1* could be associated in part with both protein and nonprotein free amino acids reducing survival, growth, and fecundity of the target pest. We posed two hypotheses: (1) *A.glycines* resistance is related to host quality in terms of free amino acids composition in the leaf, and (2) aphids may enhance host quality by inducing changes in the free amino acids composition. To test these hypotheses we conducted a field experiment using a split plot design, with soybean lines (a susceptible line and a related line carrying *Rag1*) as whole plots and aphid density as subplots (insecticide treated or left exposed to natural infestations). We analyzed free amino acids in leaves at three soybean developmental stages in all subplots. We observed significant whole and subplot effects on the concentration of a subset of amino acids tested. Susceptible and resistant plants had constitutive (whole-plot) differences in amino acids composition in all developmental stages analyzed. In addition, aphid-induced (subplot) responses of the plant to aphid infestation were found. We propose that the reduced nutritional quality of the resistant line and its reduced susceptibility to aphid-induced changes may contribute to aphid resistance conferred by *Rag1*.

Keywords

Aphis glycines resistance, antibiosis, free amino acids

Disciplines

Agriculture | Entomology

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ABSTRACT   Although soybean aphid (*Aphis glycines*) resistance is commercially available in the form of the *Rag1* gene, the mechanism of this resistance is not fully understood. Amino acids are a limiting factor for aphid growth, and there is evidence that plant amino acid composition is related to aphid resistance. Antibiotic resistance like that conferred by *Rag1* could be associated in part with both protein and nonprotein free amino acids reducing survival, growth, and fecundity of the target pest. We posed two hypotheses: (1) *A. glycines* resistance is related to host quality in terms of free amino acids composition in the leaf, and (2) aphids may enhance host quality by inducing changes in the free amino acids composition. To test these hypotheses we conducted a field experiment using a split plot design, with soybean lines (a susceptible line and a related line carrying *Rag1*) as whole plots and aphid density as subplots (insecticide treated or left exposed to natural infestations). We analyzed free amino acids in leaves at three soybean developmental stages in all subplots. We observed significant whole and subplot effects on the concentration of a subset of amino acids tested. Susceptible and resistant plants had constitutive (whole-plot) differences in amino acids composition in all developmental stages analyzed. In addition, aphid-induced (subplot) responses of the plant to aphid infestation were found. We propose that the reduced nutritional quality of the resistant line and its reduced susceptibility to aphid-induced changes may contribute to aphid resistance conferred by *Rag1*.

KEY WORDS   *Aphis glycines* resistance, antibiosis, free amino acids

Antibiosis and antixenosis are two distinct plant defense mechanisms against insects. Antixenosis is often associated with morphological barriers, including plant trichomes, surface waxes, and tissue thickness, and with chemical defenses that involve repellents, feeding deterrents, and feeding inhibitors (Smith 2005). The final effect of any of these antixenotic defenses is the inhibition of insect settlement on the plant providing resistance to colonization (Van Emden 2007). However, antibiotic mechanisms are associated with the production of allelochemicals (toxins or growth inhibitors), nonprotein amino acids, reduced levels of nutrients (host quality), and the hypersensitive response of blocking phloem sieve pores during aphid feeding (Smith 2005). These antibiotic mechanisms allow the settlement of insect population in the host but reduce insect survival, growth, and fecundity (Van Emden 2007).

Aphids feed on the phloem that provides carbohydrates and nitrogenous compounds (Montllor 1991).

Nitrogen moves in the phloem primarily in the form of free amino acids (Wilkinson and Douglas 2003). The total amount of amino acids present in phloem sap is low; thus, amino acids are a limiting factor for aphid's growth (Dixon 1998). Most aphids have a symbiotic relationship with bacteria (*Buchnera sp.*) to aid in amino acid synthesis from this N-limited source (Baumann et al. 1995). The complete genome of the bacterium has been sequenced and, even though genes coding for many amino acid biosynthesis pathways have been found (Shigenobu et al. 2000), the bacteria are not able to provide aphids with all the amino acids required for survival. Thus, in addition to amino acids as a source of N, essential amino acids must to be taken from the plant. Therefore, free amino acids are a relevant measurement of nitrogen available for phloem-feeding insects (Montllor 1991).

Free amino acids found in phloem affecting nutritional quality for aphids include protein amino acids, those used as building blocks for protein synthesis. However, nonprotein amino acids are found in the phloem as well (Dixon 1998). Nonprotein amino acids may act as feeding deterrents or feeding stimulants (Srivastava et al. 1983, Montllor 1991). In addition, these compounds, could be precursors of defensive chemicals (e.g., neurotoxins) (Dixon 1998), or act as

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substrates analogues of protein amino acids during protein synthesis and inhibit this process (Bell 2003, Douglas and Van Emden 2007). In addition, studies on artificial diet intake by *Aphis fabae* showed the role of specific amino acids as phagostimulants (Leckstein and Llewellyn 1974); it is has been suggested that phagostimulatory amino acids may play a role in host selection by eliciting feeding and oviposition (Bernays and Chapman 1994). The amino acid composition of the phloem has also been related with aphid host plant resistance. Studies in cereal crops including oats, barley, rice, and wheat have shown that specific amino acids are correlated with resistance to aphids (Weibull 1988, 1994; Kazemi and Van Emden 1992). Studies in legumes like *Vicia faba L.* (Holt and Birch 1984) and *Pisum sativum* (Auclair 1976) suggest a potential role for total content of free amino acids and amino acids composition in aphid resistance. Resistance to *Acyrthosiphon pisum* in 22 *Vicia* species was positively correlated with the concentration of protein amino acids in the leaves while resistance to *Aphis fabae* and *Megoura viciae* was positively correlated with nonprotein amino acids (Holt and Birch 1984). However, a wheat cultivar resistant to *Sitobion avenae* was positively correlated with specific protein and nonprotein amino acids (Ciepiela and Sempruch 1999). A possible role of nitrogen on *A. glycines* performance was recently described in soybean (Walter and DiFonzo 2007) where a higher rate of *A. glycines* growth on potassium deficient plants was attributed to a higher concentration of asparagine in the phloem sap (Myers et al. 2005).

The amino acid composition of the phloem has been used not only to explore host plant suitability comparing different plant species or cultivars (Holt and Birch 1984, Wilkinson and Douglas 2003) but also to investigate how variations in plant phenology determine changes in amino acid composition within species affecting seasonal aphid performance (Weibull 1987, Karley et al. 2002). In addition, it has been shown that aphids may elicit changes in amino acid composition of the host to enhance the nutritional quality of their diet (Eleftherianos et al. 2006).

Aphis glycines is an important soybean pest first discovered in the United States in 2000. It rapidly colonized midwestern states causing dramatic yield losses (Ragsdale et al. 2007, Beckendorf et al. 2008). Development of resistant cultivars is one option to reduce aphid effects. There have been some advances in the identification of sources of resistance. Hill et al. (2004) identified three *Aphis glycines* resistant genotypes: Dowling, Jackson, and PI 71506. Of these three, antibiosis was observed in Dowling and Jackson. Resistance in Dowling (PI 548663) (Hill et al. 2006) is conferred by a single dominant gene (*Rag1*). However, it is not known what mechanisms contribute to aphid resistance in any of these sources.

We hypothesized that the free amino acid composition of the leaves differs between two genetically related soybean lines: a susceptible line and a resistant line carrying the *Rag1* gene. In addition, changes in amino acid composition in both lines in response to aphid infestations were evaluated. We analyzed differences in amino acids composition between lines regardless of aphid presence (constitutive differences) and in response to natural aphid infestation (aphid-induced responses) that occurred in both lines (general aphid-induced responses) or that were dependent on the plant genotype (specific aphid-induced responses).

Materials and Methods

***Aphis glycines* Infestation Levels in Resistance and Susceptible Soybeans.** Two genetically related soybean lines (LD05–16060 and SD01–76R provided by B. Diers, University of Illinois, Urbana-Champaign, IL), and two aphid treatments were used to determine differences in aphid infestation between soybean lines and how these lines respond, in terms of leaf free amino acid concentration, to the presence or absence of aphids. The field experiment was conducted at Iowa State University, Curtiss Research Farm (Story Co., IA), and planted on 7 June 2007 and on 20 June 2008. The resistant line was developed by crossing Loda with Dowling and then three crosses were made to SD01–76R [(Dowling × Loda) × SD01–76R(3)]. This is a BC2F2-derived line that carries the *Rag1*-resistant allele. The susceptible line was developed by crossing (Stride × ResnikRR) × Stride and does not contain the *Rag1*-resistant allele. Insecticides were applied to foliage only when needed based on the experimental design (see below). Beyond *A. glycines*, we did not observe significant populations (above an established economic threshold) for any other pest insect.

The split-plot design consisted of four blocks, each one containing the two soybean lines as whole plots and aphid exposure (manipulated with insecticide) as subplots for a total of four experimental units per block. Whole plots were planted at 30 seeds/m^2 with 12 rows each 0.76 m apart and 16 m long. Subplot (6 rows wide and 16 m long) were randomly assigned within each whole plot and were either not treated with insecticide or treated with lambda-cyhalothrin at 94.6 ml/acre when >50 aphids per plant were observed. Depending on the severity of the aphid outbreak, the aphid-free subplots received one to three applications during the growing season. Number of aphids (all morphologies and stages) was recorded once a week throughout the growing season on a variable number of plants per plot. The number of subsamples varied with the level of infestation. On each sampling date, we randomly selected a row and distance into the plot and at that location the number of aphids on a set of plants was counted. Twenty plants were selected in a plot when <50% of plants were infested with aphids, reduced to 10 plants per plot when infestations were found on >50%, and reduced further to 5 plants per plot when >80% of plants were aphid infested.

We reported the average number of aphids per plant throughout the season and the total seasonal exposure to aphids per plant by calculating cumulative aphid-days (CAD) from the sum of the average num-

ber of aphids per plant multiplied by the consecutive number of days between sampling bouts (Hanafi et al. 1989):

$$CAD = \Sigma_{n-1} \left[(x_{i-1} + x_i)/2 \right] t$$

where x is the mean number of aphids per plant on sampling day i, x_{i-1} is the mean number of aphids per plant on the previous sampling day, and t is the number of days between samples $i - 1$ and i. A logarithmic transformation was applied to CAD values to meet the analysis of variance (ANOVA) assumption of equal variances. Data were analyzed using proc GLM with time treated as a repeated measure (SAS Institute 2002–2004) to compare either number of aphids per plant or log-CAD between susceptible and resistant lines in the 2 yr of evaluation. Adult and immature stages of *A. glycines* were preserved and deposited in the Iowa State Collection at Iowa State University, Ames, IA.

Concentration of Free Amino Acids in Soybean Leaves. The concentration of free amino acids in the leaf may vary with *A. glycines* exposure and plant development (Weibull 1987, Karley et al. 2002, Eleftherianos et al. 2006). Therefore, we collected leaf tissue samples at three developmental stages from the experiment described in the previous section during three distinct periods of the plants development (Fehr and Caviness 1977): V6 (vegetative), R2 (full bloom), and R4–R5 (full pod-beginning seed).

At each developmental stage and from each subplot, the central leaflet of the uppermost fully developed leaf of three different plants was detached, wrapped in aluminum foil, and immediately submerged into liquid nitrogen. Aphids were carefully removed from the leaves before detaching them from the plant using a small brush. The three leaflets were ground together using liquid nitrogen. The ground tissue, 50 mg, was placed in 1.5-ml tube and stored at −80°C for further amino acid extraction. The amino acid extraction was conducted using EZ:faast kit for free amino acids analysis (Phenomenex, KGO-7165) by gas chromatography-flame ionization detector (GC-FID). The tissue was treated with 300 μl of 10% TCA plus 50 μl of Reagent no. 1 (internal standard). After vortexing, the sample was centrifuged 3 min at 18,000g, and the supernatant was collected in a new 1.5-ml tube. The supernatant was centrifuged again at 18,000g for 3 min and placed in a glass vial. Extraction and derivatization of amino acids was performed as described in steps 3–13 from the kit user's manual. Amino acids were derivatized with propyl chloroformate. After extraction, amino acid mixtures were analyzed by GC-FID on an Agilent 6890 GC equipped with an Agilent 7863 G2613A autosampler and flame ionization detector controlled by MSD Chemstation (D.00.01) software. Hydrogen and synthetic air were used as auxiliary gasses for the FID detector. Analytes were separated at a flow rate of 1.2 ml/min using He as carrier gas and using a thermal gradient that started at 110°C (1 min) and then ramped to 290°C at a rate of 30°C/min.

Standards were used as specified in the kit user's manual except that we used 50 μl of reagent 1 and 2 as standard solutions (SD1 and SD2). Standard solutions were derivatized in the same manner as the tissue samples and used to identify amino acids peaks and to estimate the concentration of amino acids in the samples. Amino acids were identified based on comparison of their retention time to standard mixtures. Quantification was obtained by using the TIC response of the standard mixture and peak areas normalized to tissue mass and the internal standard. We were able to detect 24 amino acids: AAA (α-aminoadipic acid), ABA (α-aminobutyric acid), ALA (alanine), ASN (asparagine), ASP (aspartic acid), BAIB (β-aminoisobutyric acid), GLN (glutamine), GLU (glutamic acid), GLY (glycine), HIS (histidine), HYP (4-hydroxyproline), LEU (leucine), LYS (lysine), MET (methionine), ORN (ornithine), PHE (phenylalanine), PRO (proline), SAR (sarcosine), SER (serine), TRP (tryptophan), VAL (valine), THR (threonine), ILE (isoleucine), and TYR (tyrosine). Data were analyzed using proc GLM (SAS Institute 2002–2004) using years and blocks as random factors and lines and insecticide treatment as fixed factors. We reported amino acids concentration that showed significant line (whole plot) main effect (constitutive differences), insecticide treatment (subplot) main effect (general aphid-induced responses), and line by treatment interaction (whole plot × subplot) (specific aphid-induced responses) at each soybean developmental stage. Here, we reported results of amino acids composition changes consistent across years. Therefore, amino acids that showed interactions between years and any of the other factors are not reported.

Results

***Aphis glycines* Infestation Levels in Resistance and Susceptible Soybeans.** We observed a significant difference in density of *A. glycines* between the resistant and susceptible lines (Fig. 1). Despite this difference, populations on both lines were higher than the economic injury level (EIL; 674 aphids per plant; Ragsdale et al. 2007) at the peak of infestation. Natural aphid infestations reached an average of 3,359 aphids per plant in the susceptible line and an average of 783 in the resistant line in 2007 (Fig. 1a). In 2008, natural aphid infestations were higher, reaching an average of 6,400 aphids per plant in the susceptible line and an average of 2,458 in the resistant line (Fig. 1b).

Our results showed that differences between the resistant and susceptible lines in the number of aphid per plant in both years, and the logCAD in 2007 varied depending on the time point considered (Table 1, line × date interaction). In 2008, differences between resistant and susceptible lines in terms of logCAD were independent of the time point considered (Table 1; line × date interaction, $P = 0.86$) as the logCAD in the resistant line was consistently lower than in the susceptible line (Table 1, line main effect). Least significant difference (LSD) analysis showed that the number of aphids per plant on susceptible and resis-
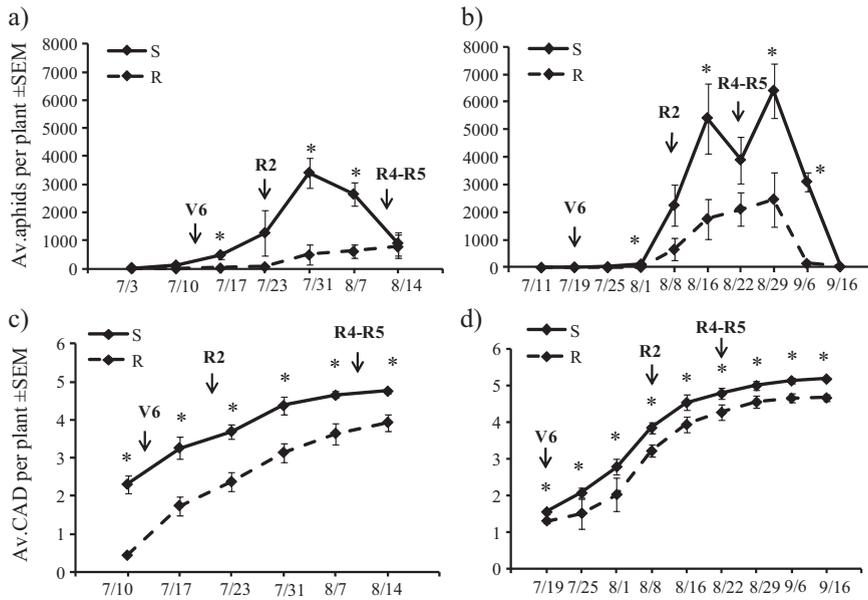
Fig. 1. Number of aphids per plant through the season in 2007 (a) and 2008 (b) and log-transformed cumulative aphid-day (logCAD) through the season in 2007 (c) and 2008 (d) in the resistant (full line) and susceptible (dashed line) lines. Arrows indicate soybean developmental stages for which tissue samples were collected and asterisk indicate significant differences between the lines (P < 0.05). LSD comparisons between susceptible and resistant lines in each time point were conducted using PROC GLM with time as a repeated measure.

tance lines was not statistically significant during the V6 stages in both years. (Fig. 1a and b). As the exposure of plants to *A. glycines* (logCAD) increased, differences between lines were observed almost immediately in 2007 and 2008 (Fig. 1c and d). After V6, the number of aphids per plant of *A. glycines* and the accumulated exposure was higher for the susceptible than for the resistant line.

Concentration of Free Amino Acids in Soybean Leaves. We detected differences in the concentration of leaf free amino acids between the susceptible (SD76R) and resistant (LD16060) lines. These differences are observed when the ANOVA was conducted for individual amino acids (the whole plot effect, reported in Table 2). We considered such differences as constitutive differences between lines because they were observed irrespective of the presence or absence of aphids and were consistent across years. The amino

acids that varied in concentration between lines varied depending on the soybean developmental stage. At V6 (Fig. 2a) and R2 (Fig. 2b) plant developmental stages, the aphid-resistant line had a lower concentrations of ABA, ASN, GLN, GLU, HIS, PRO, and SER than the aphid-susceptible line. At R4–R5 plant developmental stage, ABA, GLN, and HIS were in greater concentration in the aphid-resistant than the aphid-susceptible line (Fig. 2c). This is a reverse trend to that observed in the V6 stage. However, TRP and THR differed in concentration in the R4–R5 stage, but they did not change during the early stages. In the R4–R5 developmental stage, TRP was higher in aphid-resistant plants, whereas THR was higher in the aphid-susceptible line.

We also observed a significant subplot effect (Table 3) for some amino acids. Such an effect indicates that the concentration of leaf free amino acids differed

Table 1.   Differences in *A. glycines* exposure between soybean aphid–susceptible and resistant lines during the season in 2007 and 2008

Year	Source	Aphids per plant[a]			logCAD[b]		
		df	F value	P > F	df	F value	P > F
2007	Line[c]	1,6	26.1	0.002	1,6	28.9	0.0017
	Date	6,36	9.7	<0.0001	5,30	137.1	<0.0001
	Date × line	6,36	5.2	0.0006	5,30	3.6	0.01
2008	Line	1,6	51.1	0.0004	1,6	6.3	0.046
	Date	9,54	18.4	<0.0001	8,48	191.4	<0.0001
	Date × line	9,54	4.2	0.0004	8,48	0.5	0.86

[a] The average amount of aphids per plant was estimated once a week from July to September.
[b] LogCAD is an estimate of plant exposure to aphids between sampling dates. The equation for this estimate is provided in the text.
[c] Differences in aphid infestations between the lines were measured in the subplot untreated with insecticide.

Table 2. Constitutive differences in amino acid concn between resistant and susceptible lines

Stage	Constitutive differences[a]			
	AA	df[b]	F value[b]	P > F
V6	ABA	1,6	12.1	0.01
	ASN	1,6	27.4	0.002
	GLN	1,6	7.2	0.04
	GLU	1,6	12.1	0.01
	HIS	1,6	7.7	0.03
	PRO	1,6	8.0	0.03
	SER	1,6	10.5	0.02
R2	PRO	1,6	11.6	0.01
R4–R5	ABA	1,6	13.5	0.01
	GLN	1,6	26.9	0.002
	HIS	1,6	18.0	0.005
	TRP	1,6	29.4	0.002
	THR	1,6	22.6	0.003

[a] Whole plot main effect.
[b] Error term, df = 6 tested with whole plot × block (year).

Table 3. General aphid-induced responses in amino acids concentrations between plots infested with aphids and plots treated with insecticide

Stage	General aphid-induced responses[a]			
	AA	df[b]	F value	P > F
V6	ASP	1,6	8.75	0.02
R2	AAA	1,6	7.59	0.03
	ILE	1,6	17.29	0.006
	TRP	1,6	27.94	0.002
	TYR	1,6	13.54	0.01

[a] Subplot main effect.
[b] Error term, df = 6 tested with subplot × block (year).

between plants with naturally occurring aphid infestations and those kept aphid free with insecticide (i.e., uninfested). These differences were evident despite differences associated with the lines (i.e., whole plot effect) and years. We considered such differences as a general induced response of plants to aphid infestation. During the V6 stage, plants that were left untreated with insecticide had lower concentrations of ASP compared with insecticide treated plants irrespective of year and plant genotype (Fig. 3a). This difference was observed even though the density of aphids during the V6 stage was low. At R2, concentrations of AAA, ILE, TRP, and TYR were higher when

plants were infested with aphids than in plants that were uninfested (Fig. 3b).

Finally, a significant whole plot by subplot interaction (Table 4) was observed. This interaction appeared as a line-specific response to aphid infestations for some amino acids. At R2, the concentration of ALA, HIS, LEU, and ORN was higher when aphid-susceptible plants were infested with aphids (Fig. 4a) than kept free of aphids. However, no difference in the concentration of these amino acids was observed in the aphid-resistant line when it was infested with *A. glycines* or treated with insecticide (Fig. 4a). Even though we did not observe a general aphid-induced response (whole plot effect) for any amino acids at R4–R5 (data and analysis not shown), we detected line-specific responses for some amino acids at this stage. In the aphid-susceptible line, concentration of GLU and ASP were lower when plants were infested with aphids (Fig. 4b) than kept free of aphids. As in the
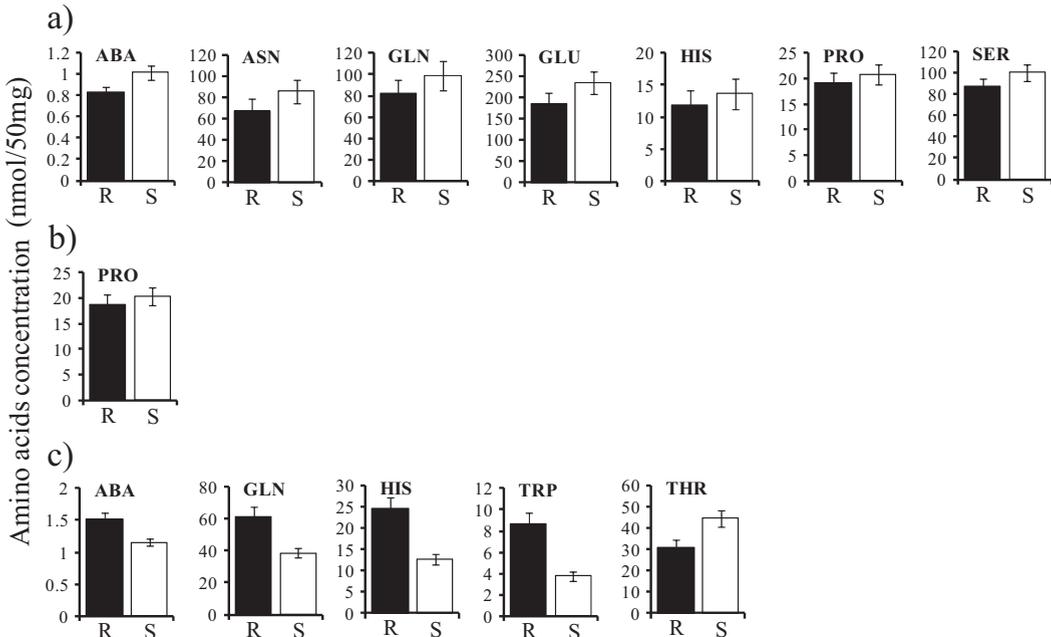
Fig. 2. Constitutive differences in amino acids concentration between resistant (R) and susceptible (S) lines (i.e., the whole plot main effect) at (a) V6, (b) R2, and (c) R4-R5 developmental stages. Values are mean ± SEM (*n* = 16).
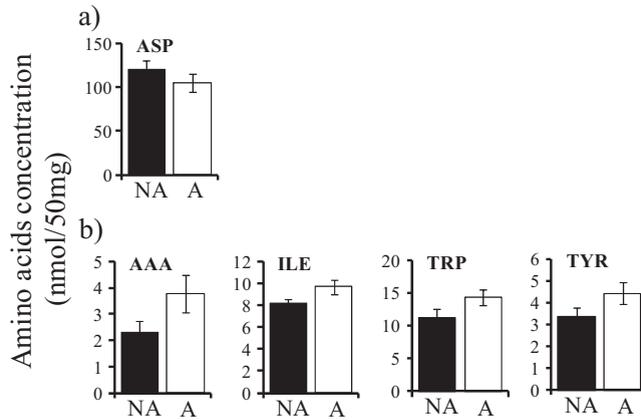
Fig. 3. General aphid-induced changes in amino acid concentrations (i.e., the subplot main effect) at (a) V6 and (b) R2 developmental stages. NA, no aphid; A, aphid treatments. Values are mean ± SEM ($n = 16$).

R2 stage, during R4–R5 differences in the concentration of GLU and ASP between aphid- infested and noninfested plants in the aphid-resistant line were not significant. Interestingly, for both ASP and GLU the concentrations in the susceptible line in the presence of aphids were comparable to that observed in resistant plants regardless of the presence of aphids in the later.

Discussion

In our field experiments, we consistently observed significant fewer aphids per plant on the resistant line than on the susceptible line. However, despite the presence of the *Rag1* gene, we found high aphid population levels on the resistant line in 2008. The cumulative number of aphids per plant (CAD) was also significantly different between the lines in both years, indicating that resistant and susceptible lines were differentially infested with aphids.

We detected consistent differences in leaf free amino acid composition between resistant and susceptible plants irrespective of aphid presence or absence (constitutive differences) and in response to aphids (aphid-induced response) at different soybean developmental stages. Aphid-induced changes in

Table 4. Line specific aphid-induced responses in amino acids concentrations between plots infested with aphids and plots treated with insecticide

Stage	Line specific aphid-induced responses[a]			
	AA	df[b]	F value	P > F
R2	ALA	1,6	14.85	0.008
	HIS	1,6	7.21	0.04
	LEU	1,6	6.32	0.04
	ORN	1,6	12.82	0.01
R4–R5	ASP	1,6	12.77	0.01
	GLU	1,6	10.34	0.02

[a] Whole plot × subplot interaction.
[b] Error term, df = 6 tested with whole plot × subplot × block (year).

amino acids concentration could be divided into general aphid-induced responses and line-specific aphid-induced responses. General aphid-induced responses are the result of plant response to aphids without distinction of the plant genotype. Line-specific aphid-induced responses could be defined as plant responses to aphid infestation that depends on the plant genotype, i.e., differential response between susceptible and resistant plants.

Constitutive differences in amino acids concentrations between susceptible and resistant plants showed that, at early stages (V6 and R2), the susceptible line had higher concentrations of specific amino acids. However, at the end of season some amino acids had higher concentration in the resistant line. Amino acids with higher concentration in the susceptible line early in the season, like ABA, ASN, GLN, GLU, HIS, PRO, and SER, could be associated with nutritional quality and could be important in determining host suitability. The lower concentration of these amino acids concomitant with a reduced aphid population in the resistant line is consistent with the antibiotic effect of *Rag1*. Therefore, nutritional differences between susceptible and resistant lines could explain in part a better aphid performance on the susceptible line than on the resistant line. It is important to note that we measured free amino acids from whole leaves, and amino acids can accumulated at different levels in different tissues, whereas aphids only feed from the phloem. However, it has been shown that there is a high correlation in amino acid composition between leaf and phloem exudates (Weibull et al. 1990), supporting the idea that some of the changes observed in our experiment could result in nutritional differences.

There is evidence that ASN, GLU, and PRO can support growth and prolonged survival of *A. pisum* reared on artificial diets, whereas TYR and ASP increased weight of this aphid species but did not prolong survival (Srivastava et al. 1983). In addition, omission of PRO in artificial diets reduced the size of adult alatae of *A. fabae* (Leckstein and Llewellyn 1974).
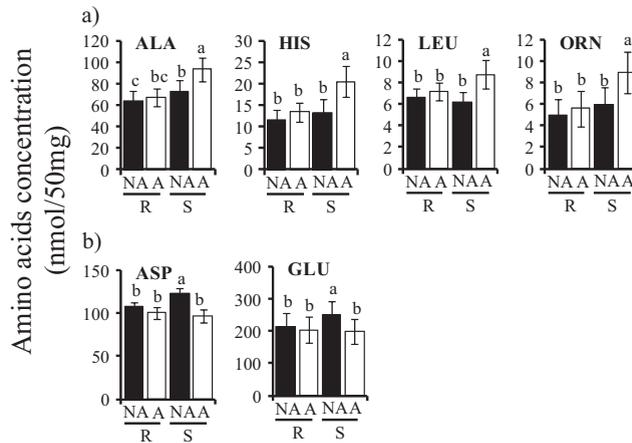
Fig. 4. Line-specific aphid-induced changes in amino acid concentrations (i.e., the whole plot by subplot interaction) at (a) R2 and (b) R4–R5 developmental stages. NA, no aphid treatment; A, aphid treatment; R, resistant line; S, susceptible line. Values are mean ± SEM ($n = 8$).

Previous studies showed that ASN was the only amino acid that positively correlated with *A. glycines* performance in soybean plants under potassium deficiency (Walter and DiFonzo 2007), supporting our hypothesis that lower levels of ASN in *Rag1* plants can contribute to the antibiotic mechanisms of resistance. In a recent study, the performance of *A. glycines* was evaluated in a set of different diets previously developed for the pea aphid and the green peach aphid (Wille and Hartman 2008). The performance of *A. glycines* was lower when the concentration of ASN, ASP, GLU, GLN, GLY, and VAL was reduced in the diet. In addition, GLU is the main amino acid that is imported into the symbiotic bacteria in aphids (Liadouze et al. 1995). Thus, reduction in this amino acid, even if it is not an essential amino acid for aphids, could have an important effect on aphid fitness. In accordance with our results, rice plants resistant to the brown planthopper (*Nilaparvata lugens*, a phloem sucking insect) had lower levels of ASN and GLU than susceptible plants (Sogawa and Pathak 1970). Previous research in other legume species also identified changes comparable to our results. For example, *A. fabae* performance was positively correlated with the concentration of protein and nonprotein amino acids in *Vicia* species (Holt and Birch 1984), and the concentration of the 10 essential amino acids were higher in susceptible *P. sativum* plants than in resistant varieties, with the difference for ASN and ASP being the greatest (Auclair 1976). However, and in contrast with our results, wheat cultivars resistant to *Rhopalosiphum padi* had high levels of ALA, HIS, and THR (Kazemi and Van Emden 1992), and GLU was negatively associated with *R. padi* performance (Weibull 1994). In addition, in a cucurbit species (melon, *Cucumis melo* L.), GLU was higher in the *Aphis gossypii*–resistant line than in the susceptible line (Chen et al. 1997). These contradictory results suggest that the relationship between amino acid concentration and resistance or aphid performance may be species specific or that

the effect of individual amino acids could be affected by the presence of other amino acids in the plant (Chapman 2003).

Insects have the ability to distinguish suitable hosts through chemical recognition that leads to increased feeding, and many amino acids can act as phagostimulants (Chapman 2003). Our results identified some amino acids having high concentration in the susceptible line (protein and nonprotein amino acids) that should be tested in further experiments for their role as feeding stimulants. There is evidence that PRO functions as phagostimulant for *A. fabae* (Leckstein and Llewellyn 1974). In contrast with our results of a positive association between susceptibility and increased amino acid concentration for nonprotein amino acids (ABA), Ciepiela and Sempruch (1999) found that ORN and GABA levels were negatively correlated with aphid performance in resistant and susceptible wheat cultivars to *S. avenae*, suggesting that, in this case, ORN and GABA may play a role other than phagostimulation.

At the end of the season (R4–R5), we could assume that the resistant line was more suitable for aphids because it had higher concentration of ABA, GLN, HIS, TRP, and THR than the susceptible line. This hypothesis is compatible with the observation that, whereas the population of aphids on susceptible plants declines at R4–R5 stage, it increases on resistant plants during the same period. Therefore, we propose an improvement in the nutritional quality or attractiveness of the resistant line late in the season. It is important to note that, whereas these lines are genetically related, they are not isogenic. Thus, some of the constitutive differences observed here could be the result of genetic variability unrelated to the *Rag1* gene.

The presence of aphids was also found to influence the amino acid composition of soybean leaves. We could distinguish general and line-specific responses of plants to aphid infestations. At V6 soybean stage, we only detected a general response in ASP with reduc-

tions of this amino acid in the presence of aphids. This reduction in ASP that occurred both in susceptible and resistant plants could be seen as part of a general plant defense response to aphid colonization early in the season when aphids are at low but of sufficient density to elicit a plant response. Similar results were observed in wheat plants susceptible to *D. noxia;* decreases of ASN, ASP, and GLU were observed at 12 d after infestation (Telang et al. 1999). However, this early general plant defense response could not be enough to totally impair aphid growth and reproduction, because aphid infestation was observed during the following developmental stage. It has been proposed that aphids are able to suppress effective defense responses through the elicitation of "decoy defense pathways" (Walling 2008), which not only reduce the ability of the plant to fight the pest but also produce changes in plant metabolism that result in an increase in nutritional value for the aphid (Goggin 2007). Following this idea, we hypothesize that these aphid-induced changes would suppress effective plant defenses and result in improved nutritional value of the plant for the aphid population. If aphids can suppress effective defenses, our hypothesis predicts that preinfestation should have a positive effect on aphid performance. In fact, this effect has been already reported (Prado and Tjallingii 1997, Nombela et al. 2009). Interestingly, at R2, all amino acids that responded to aphid infestation showed higher concentration when aphids were present, indicating a contrasting response compared with the one observed early in plant development. In addition, at this soybean stage, we found line-specific aphid-induced responses for the susceptible plants that follow the same trend, with increases in amino acid concentration in the presence of aphids while the resistant line was able to maintain lower levels even in the presence of aphids. Finally, line-specific response to aphid colonization was observed in the susceptible line at R4–R5. Reductions in the concentration of ASP and GLU in the susceptible line in the presence of aphids could indicate loss of plant quality because of prolonged exposure to aphids.

In conclusion, we identified amino acids that could be associated with the resistance to *A. glycines* in plants carrying the *Rag1* gene. In addition, we found changes in amino acids composition in response to aphid infestation. Aphid population growth along the season in the resistant and susceptible line followed the changes in nutritional quality of soybean leaves. A better nutritional quality early in the season allowed a rapid aphid population growth in the susceptible line. At the end of the season, a better nutritional quality of the resistant line (constitutive difference) and a reduced nutritional quality of the susceptible line because of aphid exposure (line specific aphid-induced response) could explain differences in aphid population growth between lines. Although in the resistant line the aphid population was maintained, in the susceptible line it was reduced. Based on these observations we hypothesize that (1) *A. glycines* induce physiological changes that result in changes in

the amino acids composition that improve the nutritional quality of the plants for a period of time. (2) *Rag1* gene confers a resistance to aphids that is in part because of constitutive reduction of available amino acids. To evaluate the role of the amino acids identified in this study as one of the elements determining aphid performance a more controlled experiment is required. The use of artificial diets was previously used to evaluate cause-effect relationships (Karley et al. 2002) in changes in amino acid concentrations and aphid performance. The knowledge of the soybean amino acid composition will help to develop artificial diets based on the actual source of food for *A. glycines* and will contribute to the previous finding by Wille and Hartman (2008) who used artificial diets previously developed for the pea aphid and the green peach aphid. Additionally, changes in the proportion of several amino acids could be carried out using mutants with deficient or silenced amino acid transporter genes, a strategy that was efficient in the Arabidopsis–*Myzus persicae* model (Hunt et al. 2006). The identification of amino acids involved in resistance is the first step required to further evaluate their possible mechanism of action.

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