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Plant Resistance

Feeding Behavior of Soybean Aphid (Hemiptera: Aphididae) Biotype 2 on Resistant and Susceptible Soybean

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Abstract

Host plant resistance to the soybean aphid, *Aphis glycines* Matsumura, is an effective means of controlling populations of this introduced pest species in the United States. *Rag* (Resistance to *Aphis glycines*) genes identified in soybean germplasm have been incorporated into commercial cultivars, but differential responses by soybean aphid biotypes to the *Rag* genes have made understanding mechanisms underlying resistance associated with *Rag* genes increasingly important. We compared the behavior of biotype 2 aphids on the resistant soybean line PI243540, which is a source of *Rag2*, and the susceptible cultivar Wyandot. Scanning electron microscopy revealed that the abaxial surface of leaves from resistant plants had a higher density of both long and glandular trichomes, which might repel aphids, on veins. Time-lapse animation also suggested a repellent effect of resistant plants on aphids. However, electropenatography (EPG) indicated that the time to first probe did not differ between aphids feeding on the resistant and susceptible lines. EPG also indicated that fewer aphids feeding on resistant plants reached the phloem, and the time before reaching the phloem was much longer relative to susceptible soybean. For aphids that reached the phloem, there was no difference in either number of feedings or their duration in phloem. However, aphids feeding on resistant soybean had fewer prolonged phases of active salivation (E1) and many more pathway activities and non-probing intervals. Together, the feeding behavior of aphids suggested that *Rag2* resistance has strong antixenosis effects, in addition to previously reported antibiosis, and was associated with epidermal and mesophyll tissues.

Key words: *Aphis glycines* biotype 2, soybean, *Rag2* resistance, electrical penetration graph

Since its discovery in the United States in 2000 (Hartman et al. 2001), the soybean aphid (*Aphis glycines* Matsumura) has spread rapidly to become a major agricultural pest that can cause more than 50% yield losses in soybean (*Glycine max* L. Merr.) (Ragsdale

et al. 2011). In addition to being a directly damaging pest, soybean aphids also transmit viruses to soybeans as well as many vegetable crops (Hill et al. 2001, Gildow et al. 2008). Resistance to soybean aphid associated with specific *Rag* (Resistance to *Aphis glycines*) genes has been identified in plant introductions from Asia (Hill et al. 2004, Hill et al. 2006, Kang et al. 2008, Mian et al. 2008b), and commercial cultivars with the *Rag* genes have been released (Hill et al. 2012).

Aphid resistance conferred by *Rag1* from soybean cv. 'Dowling', *Rag* from cv. 'Jackson', and *Rag2* from PI243540 was categorized as antibiosis (Hill et al. 2006, Mian et al. 2008a). Along with the discovery of resistant soybean lines, two aphid biotypes were identified by their differential responses to the *Rag1* and *Rag2* genes (Kim et al. 2008, Hill et al. 2010). *Rag2* resistance is highly effective against both biotype 1 and biotype 2 soybean aphids. When confined to plants, aphids suffered 92.5% mortality within the first 5 d of infestation (Mian et al. 2008b). In the same survey, the soybean cv. 'Wyandot' was found to be highly susceptible. Quickly colonized by the aphids, high levels of aphid infestation resulted in plants with stunted growth and curled yellow leaves within four weeks of aphid introduction. Interactions between insects and their host plants are complex, have varying degrees of specificity, and are subject to rapid change (Auclair 1989, Smith 1989). Biotype 2 aphids are quite virulent on *Rag1* plants (Kim et al. 2008), a feature that is used to maintain colony integrity.

An instrument for electronic monitoring of the stylet probing, or feeding, behaviors of piercing and sucking insects was originally developed by McLean and Kinsey (1964), enhanced by Tjallingii (1978), and recently updated (Backus and Bennett 2009). The electronic monitoring, originally termed electrical penetration graph, now re-named electropenetrography (EPG), involves a low-voltage signal applied to the plant or feeding substrate. Then, an insect to which a conductive (gold) wire is attached using a conductive (silver) adhesive is placed on the plant. The electrical circuit is completed when the insect inserts its stylet into the plant. The ensuing voltage fluctuations are amplified, digitized, and visualized graphically, then identified as waveforms that are consistent with specific feeding behaviors. Within hemipteran families, sub-families, and genera, including the Aphidea, waveforms can have strong similarities. Waveforms associated with aphid probing and feeding behaviors and stylet locations in plants were identified after microscopic examination of stylet tip and salivary sheath positions (McLean and Kinsey 1967, Mentink et al. 1984, Kimmins and Tjallingii 1985, Tjallingii 1987, Tjallingii and Esch 1993), measurement of insect honeydew pH, and other tests, including virus transmission (Walker 2000). The numbers and durations of various waveforms, and thus feeding behaviors, can be quantified for comparative analysis (Van Helden et al. 1993, Van Helden and Tjallingii 2000).

By comparing insect feeding behaviors on resistant and susceptible host plants, the location and characteristics of resistance can be identified (Van Helden et al. 1993, Prado and Tjallingii 1994, Alvarez et al. 2006, Prado and Tjallingii 2007). Previous EPG studies with soybean aphid compared the feeding behaviors of biotype 1 aphids on the resistant cv. Dowling and Jackson with those found for susceptible cultivars (Diaz-Montano et al. 2007, Crompton and Ode 2010). Resistance was suggested to be owing to sieve element factors associated with antibiosis, as aphids on these lines required longer probing time to reach the sieve elements and spent less time ingesting phloem sap.

To further characterize the resistance associated with *Rag2* to biotype 2 aphids, a time-lapse animation was generated to chronicle their arrival and departure times of aphids on PI243540 and Wyandot plants. Then, we used a scanning electron microscope (SEM) to examine abaxial leaf surface morphologies. Finally, EPG technology was utilized to study aphid feeding behaviors associated with the *Rag2* gene in soybean.

Materials and Methods

Aphids

Biotype 2 soybean aphids were collected in 2005 from soybean plots at the Ohio Agricultural Research and Development Center, Wooster, OH, and subsequently reared continuously on soybean cv. 'Williams 82' with a quarterly cycle on *Rag1*-containing (Jackson) plants to ensure that the colony was free from contamination with biotype 1 aphids, which are avirulent on *Rag1* (Kim et al. 2008). Aphids for the EPG study were descendants of a single aphid, reared on Jackson. They were kept in environmental chambers at 24°C with a photoperiod of 18:6 (L:D) h. Only adult apterae were used in experiments.

Soybeans

Seeds of the soybean cultivars Jackson and Wyandot, and PI243540 were obtained from field-grown plants. Single soybean seedlings were grown in 10-cm pots containing sterilized soil prepared from composted muck/Wooster silt loam mixed 4:1 with peat moss and agricultural lime for a pH of 6.8–7.0. Plants were grown in a greenhouse with a 30°C day, 18°C night temperature under natural light supplemented with high-pressure sodium lamps to extend the light period to at least 14 hr.

Scanning Electron Microscopy

First trifoliate leaves from resistant (PI243540) and susceptible (Wyandot) soybean were sampled for SEM analysis at 38 and 32 d after planting, respectively. Leaf sections (2 cm by 2 cm) were fixed (3% glutaraldehyde, 2% paraformaldehyde in 0.1 M potassium phosphate buffer, pH 7.4), then dehydrated by successive washes in an ethanol series, further dehydrated in a critical point dryer, and coated with palladium. They were viewed with a Hitachi-S4700 (Instruments, Hitachi, Ltd., Tokyo, Japan) SEM. The numbers of long, hairlike, and short, glandular trichomes on and between veins were counted within an area of the image corresponding to 2.5 by 1.6 mm^2 of the abaxial leaf surface. The number, type, and position with respect to veins of each trichome were counted. Data presented are the means for samples from five plants of each line; means were compared using the Kruskal–Wallis nonparametric, one-way analysis of variance function in JMP 9.0.2 (SAS Institute, Cary, NC).

Image Collection

Resistant (PI243540) and susceptible (Wyandot) soybean plants were grown to the V1 stage (Ritchie et al. 1985), removed from the greenhouse, and secured to the platform of an automated image collection system (Buenrostro-Nava et al. 2005) using laboratory labeling tape. Stems were chosen, rather than leaves, for video analysis because it was possible to maintain stems in a fixed position on the platform for the 2.6-d image collection period. A soybean stem infested with approximately 50 adult biotype 2 aphids was then placed on the petiole/stem junction of each secured plant. Plants and aphids were maintained on the image collection platform, at 25°C under a photoperiod of 16:8 (L:D) h. Images were collected for 2.6 d as one 0.2 s/frame every 30 m, under low magnification, using white light illumination. The frames were then assembled into the 24.8-s video using ImageReady (Adobe Systems, Mountain View, CA) with

manual image registration to correct for any imprecision in platform position between frames. To determine the number of aphids per frame and number of frames per aphid, the video was rendered into 124 individual jpeg images using Photoshop CS6 (Adobe Systems), and the number and position of aphids on the stems of the resistant and susceptible plants in each frame/image were determined. An aphid was counted as the same individual if it was found in the same position in successive frames. Thus, an individual aphid moving to a new site on the stem would be counted as a new individual. The numbers of aphids per frame and number of frames per aphid were compared between resistant and susceptible plants using the Kruskal–Wallis nonparametric, one-way analysis of variance function in JMP 9.0.2 (SAS Institute).

EPG and Experimental Design

An EPG monitor (2005 AC-DC 4-channel model, EPG Equipment Co., Otterville, MO; Backus and Bennett 2009) was used for monitoring aphid feeding and probing behaviors. A 25 mV DC substrate voltage was administered from the monitor to the soybean plants by inserting a copper probe (4 cm by 1.5 mm) into moist soil at the base of each potted plant. The abaxial surface of the first trifoliate was exposed by turning the leaf over and securing it to a plastic stake using Parafilm® strips. To convert aphids to an electrode, they were individually tethered to a 2-cm-long 25.4-µm-diameter gold wire (Sigmund Cohn Co., Mt. Vernon NY) using conductive glue made by mixing 1:1:1 (v/v/w) water, water-soluble school glue, and 8-10 µm silver flake (Inframat Advanced Materials, Manchester, CT). The free end of the gold wire was, in turn, attached to a length of copper wire soldered to a brass brad using conductive silver paint (Ladd Research Industries, Williston, VT). The tethered aphids were starved for 30 min before the brad was inserted into a head amplifier, set for 10^9 Ω input impedance (Ri). Recording began when the aphid was lowered to the leaf. Waveforms were acquired at 100 Hz, and the post-rectification/low-pass filtered signal output (labeled "AC out") was adjusted as needed by modulating gain and signal offset of the monitor. A Faraday cage made from aluminum-framed insect cages with steel screen door netting enclosed the four head amplifiers and monitor. Fluctuations in voltage owing to aphid feeding behaviors were digitally recorded using WinDaq Lite acquisition software and a DATAQ DI-720-USB analog-to-digital board (Dataq Instruments, Inc., Akron, OH).

Experiments were performed under natural light conditions, without supplemental lighting, at room temperature (24°C). For each replicate, two resistant and two susceptible plants were placed in random order inside the Faraday cage. Recordings began between 8:00 and 8:30 AM and ran overnight. If an aphid was not observed feeding or fell off the wire within the first 2 h of the test period, it was removed and replaced with a new aphid and plant. Files were discarded from analysis if an aphid became detached from the wire, fell off the plant, or too much electrical "noise" interfered with the recording.

Waveform Annotation and Data Assembly

Recordings from 17 aphids feeding on resistant (P1243540) and 13 aphids feeding on susceptible (Wyandot) plants were analyzed. For each insect, although the actual recordings continued overnight, the length of the file was truncated at 9 h. Acquired files were viewed and annotated using WinDaq Waveform Browser ver. 2.41 (Dataq Instruments, Inc.). Characteristic aphid waveforms were identified from previous correlation studies (Tjallingii 1978, Tjallingii 1985, Tjallingii 1987, Tjallingii 1990, Spiller et al. 1990, Van Helden and Tjallingii 2000, Reese et al. 2000, Tjallingii 2000) and manually annotated as Notepad (Microsoft, Redmond, WA) files before exporting to Excel (Microsoft, Redmond, WA). Files for each aphid were assembled individually, calculating a variety of response variables that were both non-sequential (e.g., duration and counts waveform events) and sequential (e.g., time to the first record of a waveform). The variables selected were those typically used to compare aphid feeding behaviors (Sarria et al. 2009). All data files for response variables were then assembled into a single Excel data set.

Descriptive statistics (means and standard errors) were calculated for each response variable for each aphid–plant pair. Differences between treatments (resistant and susceptible soybean) were identified using the Kruskal–Wallis nonparametric, one-way analysis of variance function in JMP 9.0.2 (SAS Institute).

Results

Leaf Morphology in Resistant and Susceptible Soybeans

Scanning electron micrographs of resistant (PI243540) and susceptible (Wyandot) leaves indicated that the abaxial surface of the first trifoliate leaf had long, hairlike trichomes, as well as short, glandular trichomes, both on and in between veins (Fig. 1). The density of both long and glandular trichomes on veins was greater on leaves from resistant than from susceptible plants (Table 1). No difference in the density of either trichome type was detected in the areas between the major veins.

Aphid Persistence on Resistant and Susceptible Soybean

A time-lapse video compiled from 124 individual frames (0.2 s each) taken every 30 min over a 2.6-d period was used to compare the response of soybean aphid biotype 2 with resistant (PI243540) and susceptible (Wyandot) soybean plants (Supp. Video S1). Analysis of aphid number and position in individual frames indicated that fewer aphids ($P < 0.01$) were present per frame on the stem of the resistant (0.53 ± 0.09, mean ± S.E.) than of the susceptible (1.77 ± 0.09) plant. Also, the mean number of successive frames that individual aphids were observed in was lower ($P < 0.05$) for aphids feeding on the resistant (7.33 ± 9.4) than the susceptible (43.80 ± 12.60) plant.

Monitoring Aphid-Feeding Behavior on Resistant and Susceptible Soybean

EPG was used to monitor aphid-feeding behavior on resistant (PI243540) and susceptible (Wyandot) soybean (Fig. 2). Waveforms recorded over a 9 h period were used to identify feeding behaviors as previously described by Reese and co-workers (2000). Examples of the waveforms recorded for aphids feeding on resistant and susceptible soybean and their interpretations are outlined in Fig. 2. Waveform E1, which is associated with active salivation from the insect's stylet positioned in the phloem, was identified as occurring at low voltage, with low amplitude, moderate frequency, and very regular waves (Fig. 2B panels a, d, f). E2 waveforms, associated with passive phloem ingestion (Tjallingii 1990), were identified as occurring after an E1, and with similar voltage level, amplitude, and regularity, but somewhat lower frequency (Fig. 2B, panels e, g). Waveforms A, B, and C, previously associated with stylet penetration activities Reese et al. 2000), were identified by their higher voltage levels than E1/E2 waveforms, varied amplitude, frequency, and regularity. In these experiments, the three waveforms were combined and designated "P" (for pathway) (Fig. 2B panels a, b, c, e, g).

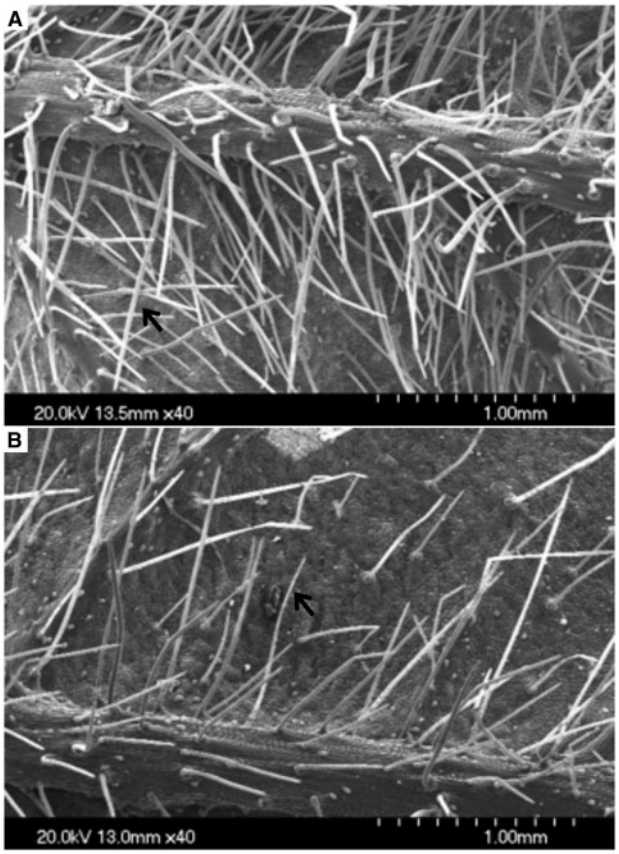

Fig. 1. Trichome density on resistant and susceptible soybean leaves. Scanning electron micrographs of the abaxial surface of the first trifoliate leaf of (A) resistant (PI243540) and (B) susceptible (Wyandot) soybean plants, showing long, hairlike trichomes (black arrows) and short glandular trichomes (white arrows) on and between veins.

Table 1. Trichome density on resistant and susceptible soybean

Trichome type	Location	Resistant[a]	Susceptible
Long trichomes	Veins	34.30 ± 7.96a	14.56 ± 3.33b
Long trichomes	Interveinal	13.72 ± 2.14a	9.26 ± 1.49a
Glandular trichomes	Veins	38.04 ± 8.20a	12.60 ± 2.19b
Glandular trichomes	Interveinal	3.66 ± 0.38a	4.44 ± 0.48a

[a] The mean density (hairs/mm^2 ± S.E.) for long and glandular trichromes on the abaxial surface of first trifoliate leaves was calculated from scanning electron micrographs. Means were compared using the Kruskal–Wallis nonparametric one-way test. Within a row, values followed by same letter are not different ($P < 0.05$).

Potential drops (pd) were identified as abrupt drops in voltage lasting 3–5 s (Fig. 2B panels a, b, c, e, g). These were previously associated with stylet penetration of a plant cell membrane (Kimmins and Tjallingii 1985, Tjallingii 1985). Z waveforms, associated with nonprobing behavior, were identified as flat lines bounded by P (Fig. 2B, panels c, e). G waveforms, associated with active ingestion from the xylem, were identified from their ragged fine structure and high frequency (Spiller et al. 1990) (Fig. 2B, panel d; Fig. 2C). F waveforms, associated with stylet work but not ingestion, were distinguished from G waveforms based on shape and the ratio of their amplitude to frequency (Fig. 2C).

Representative full-length EPG files showed a marked difference in aphid behaviors over time (Fig. 2A). The aphid on resistant soybean produced P, Z, and pd waveforms for the first 4 h, then G waveforms for about 1 h (Fig. 2A, top panel). This was followed by more P waveforms, then E1 waveforms for about 1 h, before P waveforms resumed. The aphid on susceptible soybean produced E1 waveforms within 0.5 h after initiating feeding that lasted for about 1.5 h (Fig. 2A, bottom panel). This was followed by about 1 h of G waveforms, then a series of P and pd preceding E1 then E2 waveforms from 4 to 8 h.

Only 65% aphids on resistant soybean recorded at least one E1 compared with 100% of aphids on susceptible soybean (data not shown). E1 always precedes E2, the waveform indicating phloem ingestion, so production of E1/E2 waveforms indicated successful feeding by the aphid. Because aphids that did not record E1 did not feed, data for these aphids were excluded from further analyses.

Non-Phloem Feeding Behaviors

Differences in aphids feeding on resistant and susceptible soybean were evident in the feeding behaviors that preceded E1/E2 waveforms. The mean interval between an aphid being placed on the leaf to the first stylet probe into the leaf, or time to first probe, was highly variable and not different between the treatments (Table 2). However, for those aphids that produced at least one E1 waveform, the time to the first E1 was more than twice as long for aphids on resistant soybean as for aphids on susceptible soybean. The numbers of probes and non-probing phases (Z waveforms), pathways (P waveforms), and potential drops (pd) were also significantly higher for aphids feeding on resistant soybean. The time that aphids spent in pathway activities over the 9-h assessment period was higher for those on resistant plants, as were the number of P waveforms before an E1. The number of P waveforms less than 3 min in length was higher for aphids on resistant soybean.

In contrast, no differences in production of waveforms associated with xylem ingestion (G waveforms) and mechanical stylet derailment (F waveforms) were observed. Most aphids on both resistant (9/11) and susceptible (10/13) soybean exhibited F waveforms, but no differences in the time to production of the first F waveform or duration of F waveforms were detected. Similarly, 6/11 and 8/13 aphids feeding on resistant and susceptible soybean, respectively, produced G waveforms, but differences in the time to first G or duration of G were not detected.

Phloem-Feeding Behaviors

For aphids exhibiting at least one E1 waveform, the number and duration of E1 and E2 were similar for aphids feeding on resistant and susceptible plants (Table 3). In addition, after excluding non-probing behaviors, proportion of time dedicated to each behavior was calculated as the E1 or E2 index, and these did not differ between aphids on resistant and susceptible soybean. However, marginally lower ($P < 0.1$) duration of E1 events and the number of prolonged (>10 min) E1 waveforms produced were detected for aphids feeding on resistant soybean.

Discussion

Rag2 gene is a dominant/co-dominant gene that confers antibiotic resistance to soybean aphid biotypes 1 and 2 (Kang et al. 2008; Mian et al. 2008a,b). Our goal was to further characterize the resistance associated with *Rag 2*. Differences in the morphology of trifoliate leaves from the resistant and susceptible soybeans might influence aphid ability to feed on plants. The abaxial leaf surface, the preferred feeding surface for soybean aphid (Tilmon et al. 2011),

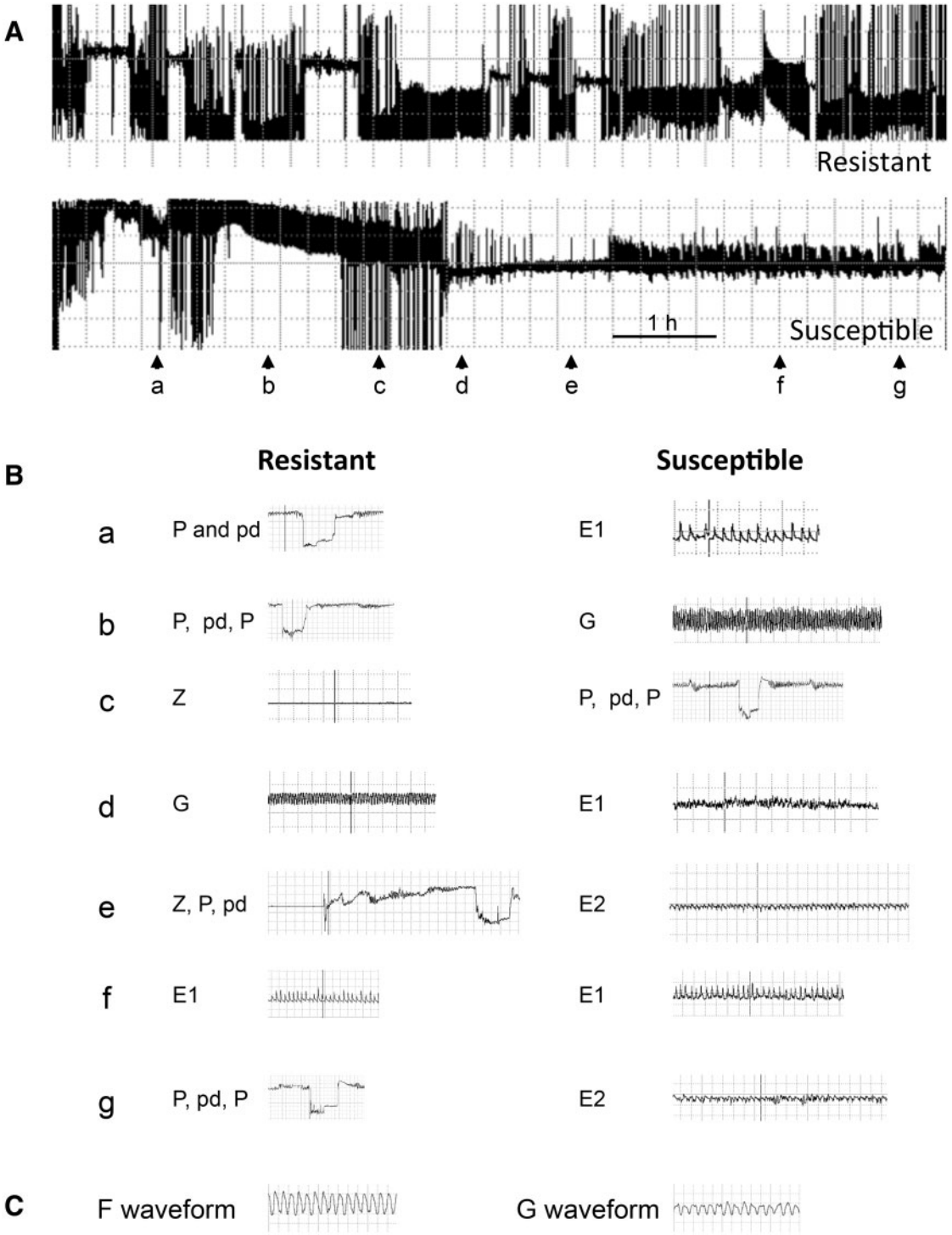

Fig. 2. Electrical penetration graphs (EPG) of soybean aphids feeding on resistant and susceptible soybean leaves. DC substrate (250 mV) was applied to the plants, and AC output voltage was registered. A: Feeding behavior of aphids on resistant (PI243540) and susceptible (Wyandot) soybean plants. Eight-hour segments from nine-hour graphs obtained for aphids feeding on resistant (top) and susceptible (bottom) are shown. The vertical divisions represent 1000 s, with the bar indicating 1 h. B: Waveforms associated with specific behaviors. Panels a–g show waveforms occurring at points a–g in each of the resistant and susceptible panels in A expanded to 1 s/division. Activities associated with specific waveforms were assigned according to Reese et al. (2000). Waveforms are: E1, active salivation; E2, phloem ingestion; G, xylem feeding; P, pathway; pd, potential drop; Z, stylet withdrawn from plant, non-probing. C: Examples of F and G waveforms exhibited by an aphid feeding on resistant soybean. Each longitudinal division represents 0.26 s.

of resistant genotype trifoliate leaves had higher densities of long, hairlike, and glandular trichomes on veins relative to those of susceptible plants (Fig. 1; Table 1). This morphological difference was limited to the veins, with no difference in trichome density in the interveinal areas. As soybean aphids prefer stems and young leaves for feeding, it was possible that a higher density of vein trichomes could delay feeding or encourage aphids to seek a new environment for feeding. However, from preliminary results for three

Table 2. Non-phloem feeding behaviors of aphids feeding on resistant and susceptible soybean

Parameter[a]	Resistant[b]	Susceptible	Pr > F
Time to 1st probe (min)	37.41 ± 21.26	11.25 ± 5.07	0.5820
Time to 1st E1 (min)	242.84 ± 39.51	109.94 ± 34.12	0.0257**
Time to 1st E2 (min)	302.26 ± 54.32	172.20 ± 49.97	0.0766*
Number of probes	32.82 ± 4.95	15.62 ± 4.74	0.0008**
Number of Z waveforms	32.82 ± 4.95	15.62 ± 4.74	0.0054**
Number of P waveforms	196.36 ± 19.95	118.31 ± 22.80	0.0138**
Number of pd	160.82 ± 15.12	96.92 ± 18.58	0.0162**
Number of P waveforms/probe	7.22 ± 9.26	24.78 ± 7.59	0.0637*
Number of P waveforms before 1st E1	91.36 ± 15.58	21.00 ± 5.40	0.0003**
Number of P <3 min before 1st E1	89.36 ± 15.50	19.85 ± 5.29	0.0003**
Number of pd before 1st E1	77.54 ± 12.80	17.08 ± 4.30	0.0002**
Time in P (min)	147.09 ± 14.49	96.49 ± 15.48	0.0257**
Time to 1st F waveform (min)	166.89 ± 56.65	259.26 ± 59.05	0.2960
Time in F (min)	109.29 ± 30.77	109.03 ± 29.38	0.7498
Time to 1st G waveform (min)	378.06 ± 53.73	308.09 ± 60.09	0.5321
Time in G (min)	22.27 ± 9.31	29.51 ± 10.66	0.7434

[a] Behaviors were assessed using the electron penetration graph technique, and waveforms were interpreted as outlined in Fig. 2.

[b] Data presented are the means ± S.E. for 11 aphid individuals on resistant soybean (PI243540) and 13 individuals on susceptible soybean (Wyandot) that produced at least one E1 waveform.

[c]Kruskal–Wallis one-way nonparametric test, df = 1, $**P < 0.05$; $*P < 0.10$.

near-isogenic lines in which aphid resistance was introgressed from PI2435540 into Wyandot, trichome density was similar to the susceptible control (data not shown), suggesting this morphological trait is not associated with *Rag2*. In addition, trichome type was previously shown not to influence aphid colonization of soybean, and aphid density was positively correlated with moderate to high trichome density (Dai et al. 2010). Further, in our experiments, no difference in the "Time to 1st probe" could be detected between aphids feeding on resistant and susceptible soybean (Table 1), an EPG parameter for which longer times are associated with aphid-feeding difficulties at the leaf surface (Van Helden et al. 1993, Alvarez et al. 2006). Together these results indicate that trichome density and other factors on the leaf surface are not likely to be important for resistance conferred by *Rag2*.

A video analysis of biotype 2 aphids allowed to feed on resistant and susceptible soybean suggested that there were differences in the responses of aphids to the two plants. Aphids were less frequently detected in individual frames of resistant plants from the video, and the number of successive frames, and therefore the time, aphids spent in a single position on the stem of resistant plants was less than for susceptible plants. These results indicated that differences in aphid behavior, and potentially aphid feeding behavior, might be associated with *Rag2*. Because EPG provides an excellent approach for characterizing parameters related to aphid probing and feeding, we used this technology to examine the behavior of aphids feeding on resistant and susceptible soybean.

Using EPG, no differences were detected in probing behaviors associated with either phloem or xylem ingestion for aphids feeding on resistant and susceptible soybean (Tables 2 and 3). Aphids feed on xylem for hydration, and the G waveform is correlated with xylem ingestion (Spiller et al. 1990). Of the aphids that exhibited an E1, a similar proportion recorded G waveforms, and no differences between host type in time to first feeding on xylem or duration of xylem ingestion were detected (Table 2). These results suggest xylem sap contents are not involved in *Rag 2* resistance.

For aphids that exhibited an E1 phase, indicating they had reached the phloem, behaviors associated with E1 and E2 waveforms were similar in number, duration, and quality (Table 3 and Fig. 2). Production of an E1 waveform indicates that the stylet is in the phloem tissue (McLean and Kinsey 1967), where E1 watery saliva, rich in enzymes, is injected into sieve elements to "condition" the phloem by interfering with plant wounding and defense responses (Tjallingii 1994, Tjallingii 2006, Will et al. 2007). Although marginally significant differences were detected in the duration of E1 events and number of prolonged E1 events for aphids feeding on resistant soybean, the overall duration of E1 events did not differ between the treatments. Shorter E1 could be associated either with resistance, if insects terminate feeding early because they cannot overcome plant defenses, or susceptibility, if insects quickly overcome plant defenses for successful and rapid feeding (Tjallingii 2006). A long interval between the first E2 relative to first E1 could indicate phloem resistance to the insect (Alvarez et al. 2006), but

Table 3. Phloem feeding behaviors of aphids on resistant and susceptible soybean

Parameter[a]	Resistant[b]	Susceptible	Pr > F^b
Number of E1 waveforms	8.63 ± 3.74	10.31 ± 3.46	0.3353
Number of E2 waveforms	7.27 ± 3.45	7.46 ± 2.86	0.3969
Time in E1 (min)	23.15 ± 12.87	90.11 ± 37.40	0.1396
Time in E2 (min)	24.20 ± 11.08	24.98 ± 10.65	0.6632
Mean E1 duration (min)	1.62 ± 3.29	9.33 ± 2.71	0.0874*
Mean E2 duration (min)	3.37 ± 1.57	3.13 ± 0.76	0.7903
Number of E1 > 10 min	0.18 ± 0.12	0.85 ± 0.32	0.0626*
Number of E2 > 10 min	0.54 ± 0.36	0.62 ± 0.24	0.4362
E1 index[d]	9.91 ± 6.54	18.48 ± 7.24	0.2129
E2 index	10.25 ± 4.27	5.97 ± 2.28	0.4831

[a] Behaviors were assessed using the electron penetration graph technique, and waveforms were interpreted as outlined in Fig. 2.

[b] Data presented are the means ± S.E. for 11 aphid individuals on resistant soybean (PI243540) and 13 individuals on susceptible soybean (Wyandot) that produced at least 1 E1 waveform.

[c] Kruskal–Wallis one-way nonparametric test, df = 1, $**P < 0.05$; $*P < 0.10$.

[d] E1 and E2 indices were calculated as the proportion of time in each waveform during the feeding period (Van Helden et al. 1993).

this was not observed in our experiments. The similar numbers of E1 and E2 waveforms for aphids feeding on either resistant or susceptible soybean also suggest that resistance is not associated with phloem. No differences were detected in the total number, duration, or length of E2 waveforms between aphids feeding on resistant and susceptible plants (Table 3), and conformations of E1/E2 waveforms were indistinguishable for the two types of host (Fig. 2). All of these factors indicate that if aphids succeeded in reaching the phloem, there was no difference in their feeding and phloem contents are not a factor in *Rag2* resistance.

Significant differences in a number of non-phloem-related feeding behaviors were identified for aphids feeding on resistant and susceptible soybean. Relative to aphids feeding on susceptible soybean, it took aphids feeding on resistant soybean more than three times as long (5.8 h vs. 1.8 h) to exhibit phloem-feeding behavior. Prior to reaching the phloem, aphids on resistant plants made more probes and made 4.5 times as many short-duration test probes of less than 3 min (Table 2). The delay in phloem ingestion was also associated with more potential drops and periods of non-feeding (Z waveforms) prior to the first E1. These behaviors are associated with the "pathway," a comprehensive term for behaviors that include the initial piercing of the leaf cuticle, and intercellular advancement of the stylet through the epidermis and mesophyll. The brief potential drops occur during pathway activities when the stylet pierces and ruptures a cell, as the aphid samples cell contents (Mclean and Kinsey 1967, Tjallingii 1978, Spiller et al. 1990, van Helden and Tjallingii 1993, Tjallingii and Esch 1993, Tjallingii 1995). In contrast to waveforms associated with pathway behaviors, no differences were identified in the initiation or duration of F waveforms, which are associated with stylet work not leading to ingestion of cell contents (Reese et al. 2000). These results suggest that factors in the plant associated with *Rag2* activity are present in the cuticle, epidermal, and/or mesophyll layers.

Differences among probing and pathway behaviors could provide some further definition of factors associated with *Rag2* resistance. During pathway phases, as the aphid also penetrates the plant by threading its stylet between epidermal and mesophyll cells, the stylet becomes encased in a sheath formed by hardened "sheath" saliva (Tjallingii 1978, Spiller et al. 1990, Tjallingii and Esch 1993, Tjallingii 1995). The sheath provides mechanical support for the stylet, and seals it off from the plant. When the stylet punctures a cell, factors in the salivary sheath seal the wound to diminish the loss of turgor pressure and plant wound responses (Tjallingii 2006, Will et al. 2007). During this time, a second watery saliva is secreted (McLean and Kinsey 1967, Will et al. 2007). The enzymatic compositions of sheath and watery saliva have been established (Miles 1985, Miles and Peng 1989, Ma et al. 1990), but the role of those enzymes (e.g., pectinases, cellulase, phenoloxidase, and peroxidase) in facilitating stylet entry has not been verified (Tjallingii and Esch 1993). Fewer pathway events per probe and/or fewer potential drops (intracellular punctures) per probe might be expected if aphid stylet was ineffective in intercellular and intracellular penetration (McLean and Kinsey 1967, Alvarez et al. 2006), and we observed fewer pathway waveforms per probe for aphids feeding on resistant soybean. Some egestion and ingestion occur during a potential drop (Kimmins and Tjallingii 1985), permitting the aphid to taste the cell contents. *Rag2* associated resistance is encountered soon after probing, and may indicate physical barriers to stylet penetration, or, after the brief tasting that a potential drop permits, recognizing that the cell contents are unacceptable. As probing continues, aphids might also continue in pathway because they fail to recognize that their stylets are approaching phloem, and therefore E1 is delayed.

One goal of this study was to determine whether the responses elicited by *Rag2* in aphids are different than those elicited by *Rag1* (Crompton and Ode 2010). The *Rag1*/biotype 1 system and the *Rag2*/biotype 2 systems differ, with biotype 1 being avirulent on both *Rag1*- and *Rag2*-containing soybean, and biotype 2 being avirulent on *Rag2* but virulent on *Rag1*. In addition, our results indicate that *Rag1* and *Rag2* activity elicit different aphid feeding behaviors. Crompton and Ode (2010) reported markedly increased xylem ingestion by biotype 1 aphids on the *Rag1*-containing line Dowling, but we identified no differences in xylem ingestion for biotype 2 aphids on the *Rag2*-containing PI243540. Aphids feeding on both lines exhibited longer time to first E1, but biotype 2 aphids on PI243540 recorded more probing and non-probing events in this interval than did biotype 1 aphids on Dowling. In addition, the duration of phloem ingestion was much shorter for aphids feeding on the *Rag1* line, but was unaffected in the *Rag2* system. Based on this shorter duration of phloem ingestion, the authors concluded that *Rag1* exerts antibiosis at the phloem level. No-choice feeding tests aphids on *Rag2*-containing soybean resulted in high mortality, suggesting that, like *Rag1* resistance, *Rag2* confers antibiotic resistance (Mian et al. 2008a). The observed antibiosis might also be consistent with our results indicating delays in or failure to achieve phloem ingestion observed for aphids feeding on resistant plants. Our EPG experiments indicate that *Rag2* also exerts a strong antixenosis component that appears to be associated with the epidermis/mesophyll. Perhaps the antibiosis agent is contained within these tissues. The detection of both antibiosis and antixenosis activity for *Rag2* highlights the utility and power of using EPG to deducing the location of potential resistance factors against aphids.

Supplementary Data

Supplementary data are available at *Journal of Economic Entomology* online.

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