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Evaluation of Greenbug and Yellow Sugarcane Aphid Feeding Behavior on Resistant and Susceptible Switchgrass Cultivars

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Abstract

Switchgrass (*Panicum virgatum* L.) is an emerging biofuel crop that serves as host for aphids. To discern the effects of plant age and possible resistance mechanisms, the feeding behavior of greenbugs (*Schizaphis graminum* Rondani.) and the yellow sugarcane aphid (*Sipha flava* Forbes.) was monitored on three diverse switchgrasses by the electrical penetration graph (EPG) technique. Callose deposition and genes associated with callose metabolism were also analyzed to discern their association with plant resistance. There was a strong host effect on greenbugs feeding on lowland cultivar Kanlow at the V3 stage of development, as compared to the greenbug-susceptible upland cultivar Summer and plants derived from Kanlow (♂) × Summer (♀) (K×S) crosses. These data confirmed that Kanlow at the V3 stage had antibiosis to greenbugs, which was absent in the Summer and K×S plants. In contrast, similar effects were not observed for yellow sugarcane aphids, excluding significant differences in the time to first probe on Kanlow plants at the V1 stage and reduction in time spent on pathway processes on Kanlow plants at the V3 stage. These data demonstrated that Kanlow plants may have multiple sources of resistance to the two aphids, and possibly some were phloem based. Microscopy of leaf sections stained with aniline blue for callose was suggestive of increased callose deposition in the sieve elements in Kanlow plants relative to Summer and K×S plants. RT-qPCR analysis of several genes associated with callose metabolism in infested plants was equivocal. Overall, these studies suggest the presence of multiple defense mechanisms against aphids in Kanlow plants, relative to Summer and K×S plants.

Keywords Switchgrass · Aphids · EPG · Feeding behavior · Callose · qPCR

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Introduction

Switchgrass, *Panicum virgatum* L., is a perennial, polyploid warm-season grass native to tallgrass prairies of North America, east of the Rocky Mountains [1–3] and has been recognized to have excellent potential as a biomass crop [4–6]. Limited attention has been given to potential pest issues in this nascent sector; however, it is anticipated that important pests will emerge with increases in production. Indeed, studies to date indicate that switchgrass will not be immune to pests [7–11]. Accordingly, the long-term sustainability of switchgrass as a biomass crop will require efforts directed at improved biomass yields under a variety of biotic and abiotic stressors.

One particularly attractive method for controlling insect pests is plant resistance [12, 13]. Differential resistance to two important cereal aphids, the greenbug, *Schizaphis*

graminum (Rondani), and the yellow sugarcane aphid, *Sipha flava* (Forbes), has been demonstrated in tetraploid switchgrasses, and resistance varied with plant age for individual aphids [7, 14, 15]. Furthermore, using the electrical penetration graph (EPG) technique [16], it was possible to directly evaluate differential feeding behavior of the greenbug on upland cultivar Summer and lowland cultivar Kanlow plants [15]. These studies indicated a potential for phloem-based resistance in Kanlow plants, and limited resistance to aphid feeding in Summer plants, consistent with previous evaluations of host suitability in tetraploid switchgrasses [7].

The EPG technique allows the recording of signal waveforms corresponding to different probing activities as well as the position of the aphid stylet tips within the plant tissues [16], which can provide valuable information on host acceptance and resistance mechanisms at the plant tissue level [17–19]. Both greenbugs and yellow sugarcane aphids are phloem feeders, and phloem-based resistance in other plants has been attributed to sieve tube occlusion by callose [20–23].

Prior work indicated that the yellow sugarcane aphid was far more successful in colonizing switchgrass compared to the greenbug, suggesting a greater potential for the yellow sugarcane aphid to use switchgrass as a host [7, 9, 14]. These studies also indicated that switchgrass resistance/susceptibility to aphids may change with plant age [9, 14]. In addition, Koch et al. (2014) indicated that a stabilized hybrid population of switchgrass initially derived by crossing Summer × Kanlow plants (hereafter referred to as K×S) served as hosts for greenbugs and yellow sugarcane aphids, and likely lacked the phloem-based resistance observed in the Kanlow parents.

The current study was undertaken to specifically determine (a) if the age of switchgrass plants affected aphid feeding as monitored by EPG and (b) if callose deposition and the genes contributing to callose biosynthesis and/or degradation were differentially regulated in diverse tetraploid switchgrasses in response to aphid herbivory. The expectations were that (1) plant age would affect resistance to aphids, especially greenbugs in Kanlow plants, and would not affect greenbug feeding on Summer or K×S plants; (2) there would be limited resistance to yellow sugarcane aphid feeding on any switchgrass, since these aphids colonized all three switchgrasses; and (3) callose and genes associated with callose biosynthesis and degradation would be differentially regulated in these switchgrasses.

Materials and Methods

Overview of Experimental Setup This study evaluated the effect of switchgrass plant age on the feeding behaviors of two aphids, namely greenbug and the yellow sugarcane aphid. Previous studies had documented that plants at the V1 stage [24] of lowland cultivar Kanlow likely contained phloem-

based resistance to greenbug feeding, whereas plants of the upland cultivar Summer did not. However, feeding behaviors of aphids on older plants of Kanlow, Summer, and K×S had not been evaluated [15]. No work has been reported on the feeding behaviors of the yellow sugarcane aphids on switchgrass.

The V1 stage of plant development (initial stage used for earlier EPG studies) was chosen to determine if callose was involved in response to aphid feeding, especially in Kanlow plants. Ectopic callose deposits in response to aphid feeding have been noted in other plant-aphid systems, and suggested to be an integral part of the plant defense response [25, 26]. For experiments reported here, changes in leaf callose were determined using clip cages to limit the feeding area available for aphids, and maximize the potential for detection of this polymer. For qPCR, expression of select genes annotated in the switchgrass genome (version 1.1) as callose synthases or β -1,3 glucanases (callose degradation) was studied as a proxy for possible changes in callose deposition. These experiments required more plant tissues, and aphids were allowed to feed on V1 stage plants for 3 days to provide a reasonable timeframe to detect plant defense responses.

Plant Material Seedlings of two switchgrass cultivars ‘Kanlow’ and ‘Summer,’ and one experimental strain, K×S (HP1 C1 High Yield strain), developed by Dr. Kenneth Vogel, USDA-ARS (retired), Lincoln, NE, were raised from seeds in Cone-Tainers (Ray Leach SC10; Stuewe & Sons, Inc., Tangent, OR) to the V1 or V3 stage [24] in a greenhouse under 400-W high-intensity lamps with a 16-h day and 8-h night photoperiod at a temperature of $23^{\circ} \pm 4^{\circ} \text{C}$.

Insect Colonies Colonies for *Schizaphis graminum* (greenbugs, biotype I) and *Sipha flava* (yellow sugarcane aphid) were obtained from Dr. John D. Burd, USDA-ARS in Stillwater, OK. Insect colonies were maintained on ‘BCK60’ sorghum plants as described earlier [14].

EPG Recording To assess the feeding behavior of *S. graminum* and *S. flava*, switchgrass plants were grown to the V3 developmental stage and selected for uniformity for all recordings. However, since no previous characterization of *S. flava* feeding behavior on switchgrass exists, a third study evaluated *S. flava* feeding behavior on plants in the V1 developmental stage. Before recordings, plants were transferred from the greenhouse to the laboratory ($23 \pm 5^{\circ} \text{C}$) and allowed to acclimate for approximately 24 h.

A Giga-8 EPG model (EPG Systems, Wageningen, The Netherlands) with a $10^9 \Omega$ resistance amplifier and an adjustable voltage was used to evaluate the feeding behavior of *S. graminum* and *S. flava* on switchgrass plants, essentially as described earlier [15]. Adult, apterous *S. graminum* and *S. flava* were held on a permissive host switchgrass (K×S and Summer, respectively) for 24 h prior to all recordings to

precondition them to their host. Immediately before a recording, the aphids were placed in a petri dish and denied food for 1 h to increase the likelihood of feeding and to allow resheathing of their stylets [27]. After the starvation period, a gold wire was attached to the dorsum of an aphid, and placed on switchgrass plants for EPG measurements [15].

Generally, EPG waveforms are grouped into three broad behavioral phases: pathway phase, xylem, and phloem or sieve element phase [16, 28, 29]. Recordings were scored as previously defined by [15] using the following waveform patterns: np (non-probing), C (pathway phase; general probing in all plant tissues), pd (potential drops corresponding to intracellular punctures by stylet tips), E (salivation secretions into sieve elements and ingestion of phloem sap), and G (xylem ingestion).

EPG feeding behavior parameters were selected from the Sarria Excel Notebook [30]. The calculated parameters included the mean time from start of recording to first probe (elapsed time of placement of aphid on the plant to insertion of mouthparts); time from the first aphid probe to first sieve element phase and first sustained ($E > 10$ min) sieve element phase; time to first sustained sieve element phase within a probe from the start of that probe; total number of potential drops, pathway phases, sieve element phases, sustained sieve element phases, xylem phases, and non-probing events; sum of duration of pathway phases, sieve element phases, xylem phases, non-probing events, and first sieve element phase; mean duration of sieve element phases; and potential phloem ingestion index (PPII) and percent of aphids with sustained phloem ingestion.

Statistical Analysis EPG files were annotated by waveform, and the duration of each was calculated in Microsoft Excel Workbook. Data were combined, separated by switchgrass population and aphid number (replication) for each experiment, and converted to comma-separated values (CSV). The combined data were checked for errors using a beta-program designed for SAS software [31]. Once errors in waveform labeling were corrected, the data were tested for significance by using analysis of variance (ANOVA), implemented in PROC GLIMMIX. When appropriate, means were separated using Fisher's least significant difference (LSD) test ($\alpha = 0.05$). Normality was assessed for all parameters using graphical analysis of the residuals and a Shapiro-Wilk test [32]. Parameters for waveform durations ranged widely and generally did not meet the assumptions of normality. Goodness-of-fit tests indicated that fitted lognormal or gamma distributions were good models for the distribution of duration parameters not meeting the assumptions of normality; therefore, data were analyzed with the appropriate probability distribution for each parameter.

Callose Histochemistry. Ten adult *S. graminum* or *S. flava* were confined within a custom aphid clip cage, constructed of two heavy-duty double-stick foam tape squares (25.4 by 25.4 by 1.5 mm; 3M Co., St. Paul, MN) and foam sheets.

Aphids were confined for 3 h, 6 h, 12 h, or 3 days on the newest, fully developed leaf of V1 switchgrasses. Control plants were similarly caged, but without aphids. At the end of the infestation period, leaf material within the clip cage was excised and immediately placed into a solution of ethanol/acetic acid (3:1 v/v). Samples were placed into a shaker and incubated at room temperature for at least 24 h, changing the solution several times, until all samples were cleared of pigments. Tissues were dehydrated in ethanol, embedded in paraffin, and sectioned to obtain 20- μ m sections (Veterinary Diagnostics Center, University of Nebraska-Lincoln). After removal of paraffin, slides were then stained with 0.01% (w/v) aniline blue in 0.01 M K_3PO_4 for 10 min, rinsed briefly in water, and observed using an Olympus BX-51 fluorescence microscope. Whereas earlier EPG measurements had suggested phloem-based resistance in Kanlow plants relative to Summer, and K \times S plants, especially at the V1 stage, it was not evident if these Summer and K \times S plants also produced callose in response to aphid herbivory, albeit at lower levels.

Quantitative Real-Time PCR Based on a previously published RNA-Seq dataset [11], the expression of three callose synthase-related and six β -1,3-glucanase-related genes was investigated using quantitative real-time PCR (RT-qPCR) (Table 1). Switchgrass plants were grown to the V1 developmental stage as described earlier before being infested with ten adult apterous greenbugs or yellow sugarcane aphids. The plants were arranged in a complete randomized design consisting of three treatments (greenbug-infested, yellow sugarcane aphid-infested, and control). Infested and control plants were individually caged with tubular plastic cages as described earlier. Plants were harvested and flash frozen 3 days after infestation, to mirror the last time point taken for callose measurements.

Four individual plants (biological replicates) were processed from each treatment. Total RNA was extracted from approximately 75 mg of frozen plant tissue as previously described [33, 34] using the TRIzol reagent (Invitrogen, Carlsbad, CA), and purified using the RNeasyTM MinEluteTM Cleanup Kit (Qiagen, Valencia, CA) according to the manufacturer's protocols. The integrity of RNA bands was confirmed via agarose gel electrophoresis, while quantification and purity of RNA were determined with a spectrophotometer (NanoDrop 1000, Wilmington, DE). First strand cDNA was synthesized using 2.5 μ g of total RNA with the ThermoScriptTM RT-PCR system (Life Technologies, Carlsbad, CA) utilizing manufacturer-suggested protocols.

RT-qPCR was performed on a 7500 Fast Real-time PCR (Applied Biosystems, Foster City, CA) using Bio-Rad SsoAdvancedTM SYBR[®] Green (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's protocol (95 $^{\circ}$ C for 30 s, followed by 40 cycles of 95 $^{\circ}$ C for 5 s and 60 $^{\circ}$ C for 30 s). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was included as the endogenous control gene. Gene ids and

Table 1 Gene ID, gene description, and gene primers (FWD and REV) used for RT-qPCR of callose-related genes in switchgrass plants

| Gene ID | Gene description | FWD primer | REV primer |
|---------------|---|-----------------------------|-------------------------------|
| Pavir.Ab00948 | Callose synthase 8-related | 5'-AAGAAGTGAT GCCCGAGAGA-3' | 5'-CAGTCCCCTACT GAGAAGAGCC-3' |
| Pavir.Bb02930 | 1,3 Beta-glucosidase precursor | 5'-GCATTGCCTC TGCTCTTCTT-3' | 5'-GCGTCGTAGA TCCTGACCAT-3' |
| Pavir.Ca01420 | Glycosyl hydrolase family 1 (glucanase) | 5'-TGGTCCAGGC TTATTCCAAG-3' | 5'-CAGGATCTGA GGGAAATCCA-3' |
| Pavir.Db00045 | 1,3-Beta-glucan synthase component | 5'-GCACTGGCTA CTGGAAGGAG-3' | 5'-TCTCCAGACC GATTTCCATC-3' |
| Pavir.Eb03869 | Glycosyl hydrolases family 17 (glucanase) | 5'-ACATTTGCAG CCATCCCTAC-3' | 5'-GTAGATGCGC ATGAGGTTGA-3' |
| Pavir.Ga01393 | Glycosyl hydrolase family 1 (glucanase) | 5'-AGGCAGATGT AGTGTGGGG-3' | 5'-GGGAGAAGGG AAGAAACCAG-3' |
| Pavir.Gb01472 | Beta-glucanase | 5'-ACCGAGTGAA AACTGGACC-3' | 5'-ACTTCCCTTT TGTACGGCCT-3' |
| Pavir.Ia04498 | Callose synthase 3 | 5'-GCTACTTCAC AACCGTGGGT-3' | 5'-GCCTTCCCAA ATCCTCTTTC-3' |
| Pavir.J17017 | 1,3 Beta-glucosidase precursor | 5'-CGTCAACAAC GTCATCAACC-3' | 5'-GTGGTGAAG TCGAAATCGT-3' |

primers used for amplification are given in Table 1. Calculations of $\Delta\Delta CT$ were performed with the values of cycle threshold (CT) for each primer and GAPDH as an endogenous control, according to Schmittgen, Livak [35], and the statistical significance of CT values was determined through generalized mixed model analysis (PROC GLIMMIX, SAS Institute 2008).

Results

EPG

Yellow sugarcane aphid V1 Analysis of variance determined that switchgrass effects were not significant for duration of major waveform patterns for yellow sugarcane aphids feeding on V1 switchgrasses (Online Resource 1). A significant difference was detected for the time from the start of the experiment to first probe due to a delay in probing on Summer (12.5 ± 6.8 min; $t_{26} = 2.14$; $P = 0.0422$) compared to Kanlow (1.3 ± 0.9 min). Additionally, the duration of the first sieve element phase was significantly lower on K×S ($24.4 \pm$

6.9 min) relative to both Summer (127.7 ± 50.0 min; $t_{57} = 3.60$; $P = 0.0007$) and Kanlow (66.8 ± 25.3 min; $t_{57} = 2.19$; $P = 0.0327$) (Table 2). No significant differences were found for mean number of stylet activities, potential phloem ingestion index (PIII), or the percentage of aphids with sustained ingestion (Online Resource 2).

Yellow sugarcane aphid V3 Analysis of variance detected significant differences for duration of two waveform patterns, specifically total duration of pathway and xylem phases, for yellow sugarcane aphids feeding on V3 developmental switchgrasses (Fig. 1). Yellow sugarcane aphids feeding on K×S (258.7 ± 32.4 min) spent significantly more time in pathway than aphids on Kanlow (187.5 ± 33.9 min; $t_{57} = 2.01$; $P = 0.0490$). Similarly, yellow sugarcane aphids also spent significantly more time in the xylem phase on K×S (115.4 ± 11.6 min) relative to Summer plants (75.6 ± 12.3 min; $t_{53} = 2.11$; $P = 0.0394$). However, analysis of variance did not detect significant differences for the total duration sieve element phases or non-probing. Likewise, there were no significant differences among any of the phloem-based parameters or for other aphid feeding parameters related to detailed time

Table 2 Comparison of EPG parameters (mean \pm SEM) for time and duration of pattern segments for 15 h of yellow sugarcane aphid feeding on switchgrass populations (V1 stage)

| Feeding variable | Mean \pm SEM ^a | | |
|--|-----------------------------|-------------------|-------------------|
| | Summer | K×S | Kanlow |
| Time to 1st probe ^b | 12.5 \pm 6.8a | 6.5 \pm 3.1ab | 1.3 \pm 0.9b |
| Time to 1st SEP ^c | 103.1 \pm 27.3a | 85.2 \pm 17.2a | 138.0 \pm 34.6a |
| Time to 1st sustained SEP ^d | 235.4 \pm 77.6a | 142.4 \pm 37.4a | 255.1 \pm 54.8a |
| Mean duration of SEP | 123.6 \pm 34.7a | 3.0 \pm 9.7a | 117.5 \pm 28.8a |
| Duration of 1st SEP | 127.7 \pm 50.0a | 24.4 \pm 6.9b | 66.8 \pm 25.3a |

SEP sieve element phase

^a Treatment means within the same row followed by the same letter indicate no significant differences ($P \leq 0.05$), LSD test

^b Time and duration calculated in minutes

^c Sieve element phase

^d Sustained sieve element phase ($E > 10$ min)

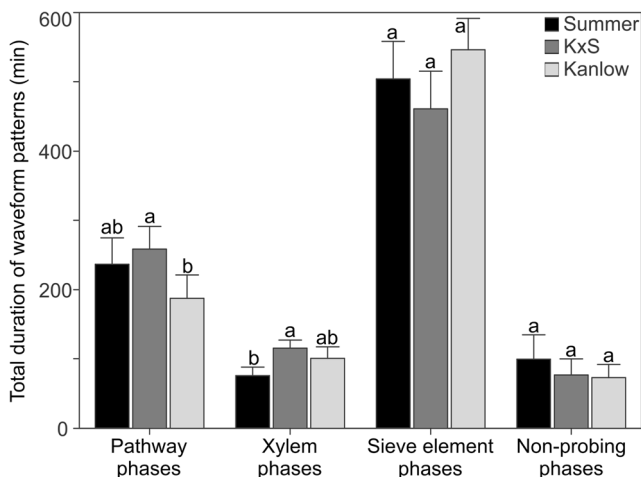


Fig. 1 Comparison of EPG parameters (mean \pm SEM) for duration of pathway, xylem, phloem, and non-probing phases for 15 h of yellow sugarcane aphid feeding on three switchgrass populations (V3 stage). Bars with the same letter within a column are not significantly different ($P > 0.05$), LSD test

and duration of pattern segments and for numerical parameters of aphid stylet activities among any of the switchgrasses (Online Resource 3, 4).

Greenbug V3 Analysis of variance detected significant differences for greenbug probing parameters linked to stylet pathway activities and sieve element phases on V3 switchgrasses. Greenbugs feeding on V3 switchgrass spent significantly less time in phloem sieve elements on Kanlow (66.8 ± 30.5 min) compared to KxS (239.4 ± 44.7 min; $t_{57} = 2.20$; $P = 0.0321$) (Fig. 2). While the duration of sieve element phases was higher on Summer (179.6 ± 45.6 min) relative to Kanlow, as well, this difference was not statistically significant. However, the duration for the first sieve element phase was significantly less on KxS (4.3 ± 1.2 min) relative to both Summer ($77.8 \pm$

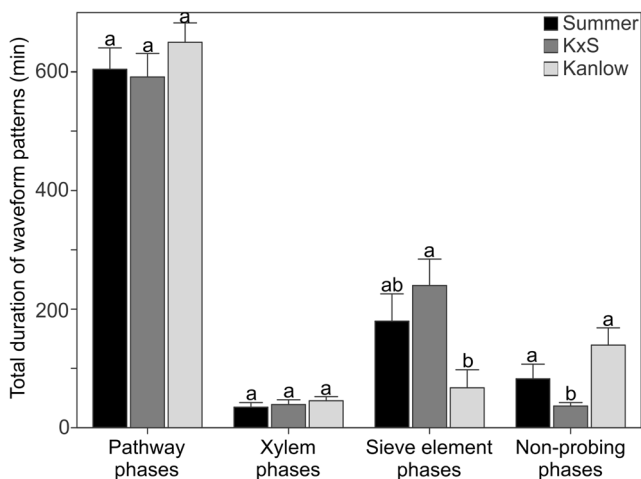


Fig. 2 Comparison of EPG parameters (mean \pm SEM) for duration of pathway, xylem, phloem, and non-probing phases for 15 h of greenbug feeding on three switchgrass populations (V1 stage). Bars with the same letter within a column are not significantly different ($P > 0.05$), LSD test

42.2 min; $t_{48} = 5.42$; $P < 0.0001$) and Kanlow (49.7 ± 32.5 min; $t_{48} = 3.86$; $P = 0.0003$) (Table 3). Significant differences were also discovered in the time that it took greenbugs to achieve a sustained sieve element phase from the first probe (Table 3) with aphids taking less time on KxS (557.7 ± 80.6 min) in comparison to Kanlow (830.6 ± 55.2 min; $t_{23} = 2.34$; $P = 0.0281$). Parameters for the mean number of sieve element phases and mean number of sustained sieve element phases also had significant differences (Table 4). Greenbugs had significantly fewer sieve element events on Kanlow (1.2 ± 0.4), relative to Summer (6.4 ± 1.1 ; $t_{48} = 4.46$; $P < 0.0001$) and KxS (5.7 ± 0.7 ; $t_{48} = 4.00$; $P = 0.0002$). Likewise, the aphids had fewer sustained sieve element events on Kanlow (0.3 ± 0.1) when compared to both Summer (0.8 ± 0.2 ; $t_{40} = 4.10$; $P = 0.0002$) and KxS (1.2 ± 0.2 ; $t_{40} = 4.96$; $P < 0.0001$). Accordingly, the percent of greenbugs with sustained sieve element ingestion was significantly lower for Kanlow (25) relative to Summer (60; $t_{57} = 2.18$; $P = 0.0332$) and KxS (85; $t_{57} = 3.49$; $P = 0.0009$) (Table 4).

Several significant differences were documented for non-phloem-based parameters as well. The total duration of time spent in non-probing (Fig. 2) was significantly lower for greenbugs on KxS (36.0 ± 5.7 min) compared to Summer (82.1 ± 24.8 min; $t_{57} = 2.28$; $P = 0.0263$) as well as Kanlow (139.0 ± 29.2 min; $t_{57} = 3.30$; $P = 0.0017$). In relation, the number of non-probing events (Table 4) was significantly greater for greenbugs on Kanlow (17.6 ± 1.5) relative to KxS (12.8 ± 1.6 ; $t_{57} = 2.48$; $P = 0.0160$). Finally, significantly more potential drops (Table 4) were recorded for aphids probing on Kanlow (262.7 ± 13.5) in comparison to KxS (220.9 ± 17.6 ; $t_{57} = 2.17$; $P = 0.0341$).

Callose

Histochemistry There were no obvious differences in callose deposition, regardless of treatment for 3-, 6-, or 12-h evaluations. Likewise, no conspicuous differences were observed between treatments at 3 days for KxS or Summer (Fig. 3). However, 3 days after infestation, callose deposits appeared to be relatively abundant on sieve plates and the cell walls of vascular tissue for Kanlow plants infested with greenbugs, relative to uninfested controls as well as the susceptible KxS (Figs. 3 and 4). Few callose deposits were also observed on Kanlow infested with yellow sugarcane aphids at 3 days. Similarly, results of staining leaves from V3 stage plants were equivocal and did not reveal any consistent evidence for callose deposition.

Quantitative real-time PCR Four β -1,3-glucosidase transcripts were significantly upregulated in switchgrasses fed on by greenbugs, relative to the uninfested controls (Fig. 5a). Specifically, the β -1,3-glucanases, Pavir.Gb01472 and Pavir.J17017, were significantly upregulated in Summer

Table 3 Comparison of EPG parameters (mean ± SEM) for time and duration of pattern segments for 15 h of greenbug feeding on switchgrass populations (V3 stage)

| Feeding variable | Mean ± SEM ^a | | |
|--|-------------------------|---------------|---------------|
| | Summer | K×S | Kanlow |
| Time to 1st probe ^b | 2.4 ± 1.0a | 1.4 ± 0.8a | 2.7 ± 2.0a |
| Time to 1st SEP ^c | 264.9 ± 45.2a | 302.8 ± 58.6a | 464.3 ± 94.7a |
| Time to 1st sustained SEP ^d | 666.0 ± 79.6ab | 557.7 ± 80.6b | 830.6 ± 55.2a |
| Mean duration of SEP | 82.6 ± 35.3a | 61.8 ± 15.4a | 72.9 ± 32.8a |
| Duration of 1st SEP | 77.8 ± 42.2a | 4.3 ± 1.2b | 49.7 ± 32.5a |

^a Treatment means within the same row followed by the same letter indicate no significant differences ($P \leq 0.05$), LSD test

^b Time and duration calculated in minutes

^c Sieve element phase

^d Sustained sieve element phase ($E > 10$ min)

plants, while Pavir.Eb03869 was significantly upregulated in both Summer and K×S, compared to their respective controls. Additionally, a fourth β-1,3-glucanase, Pavir.Ca01420, was also upregulated in K×S plants after 3 days of greenbug infestation. Differential expression between greenbug-infested and control plants was not significantly different for any of the callose synthase-related genes.

In response to yellow sugarcane aphid feeding, two callose-related genes were significantly upregulated (Fig. 5b). In Summer plants, the callose synthase 8-related gene, Pavir.Ab00948, was significantly upregulated after yellow sugarcane aphid feeding, compared to uninfested plants. The β-1,3-glucanase, Pavir.Eb03869, was the only gene to be significantly upregulated in yellow sugarcane aphid-infested K×S plants. Differential expression between aphid-infested and control plants was not significantly different for any of the genes examined in Kanlow plants with respect to either aphid species (Online Resource 5 and 6).

Discussion

Among tetraploid switchgrass evaluated for susceptibility to aphids, the lowland cultivar Kanlow was largely resistant to greenbug herbivory and moderately resistant to yellow sugarcane aphids. In contrast, the upland cultivar Summer and K×S plants were susceptible to injury by both aphids [14]. Plant resistance to aphids can occur via a number of mechanisms [36], and some ascribed to phloem-based mechanisms could involve callose [25, 37, 38]. The EPG technique is an effective way to document the feeding behavior of aphids. Waveforms documented through EPG are associated with different aspects of aphid probing and feeding on plant tissues. Based on the occurrence and duration of these waveforms, it is possible to infer plant resistance mechanisms [16, 39]. Previously, Koch et al. [15] documented significant differences in greenbug feeding behavior on the V1-stage Kanlow plants relative to Summer and K×S plants, and data suggested the presence

Table 4 Comparison of EPG parameters (mean ± SEM) for stylet activities for 15 h of greenbug feeding on switchgrass populations (V3 stage)

| Feeding variable | Mean ± SEM ^a | | |
|---|-------------------------|---------------|---------------|
| | Summer | K×S | Kanlow |
| Potential drops | 223.5 ± 12.1ab | 220.9 ± 17.6b | 262.7 ± 13.5a |
| Pathway phases | 22.9 ± 2.2a | 20.7 ± 2.0a | 22.0 ± 1.3a |
| Xylem phases | 2.7 ± 0.5a | 2.4 ± 0.4a | 3.1 ± 0.5a |
| SEP ^b events | 6.4 ± 1.1a | 5.7 ± 0.7a | 1.2 ± 0.4b |
| Sustained SEP ^c events | 0.8 ± 0.2a | 1.2 ± 0.2a | 0.3 ± 0.1b |
| NP ^d events | 13.7 ± 1.7ab | 12.8 ± 1.6b | 17.6 ± 1.5a |
| Potential phloem ingestion index (PPII) | 28.5 ± 7.4a | 40.6 ± 6.8a | 42.4 ± 13.1a |
| % of aphids showing sustained SEP ($E > 10$ min) | 60 (12/20)a | 85 (17/20)a | 25 (5/20)b |

^a Treatment means within the same row followed by the same letter indicate no significant differences ($P \leq 0.05$), LSD test

^b Sieve element phase

^c Sustained sieve element phase ($E > 10$ min)

^d Non-probing

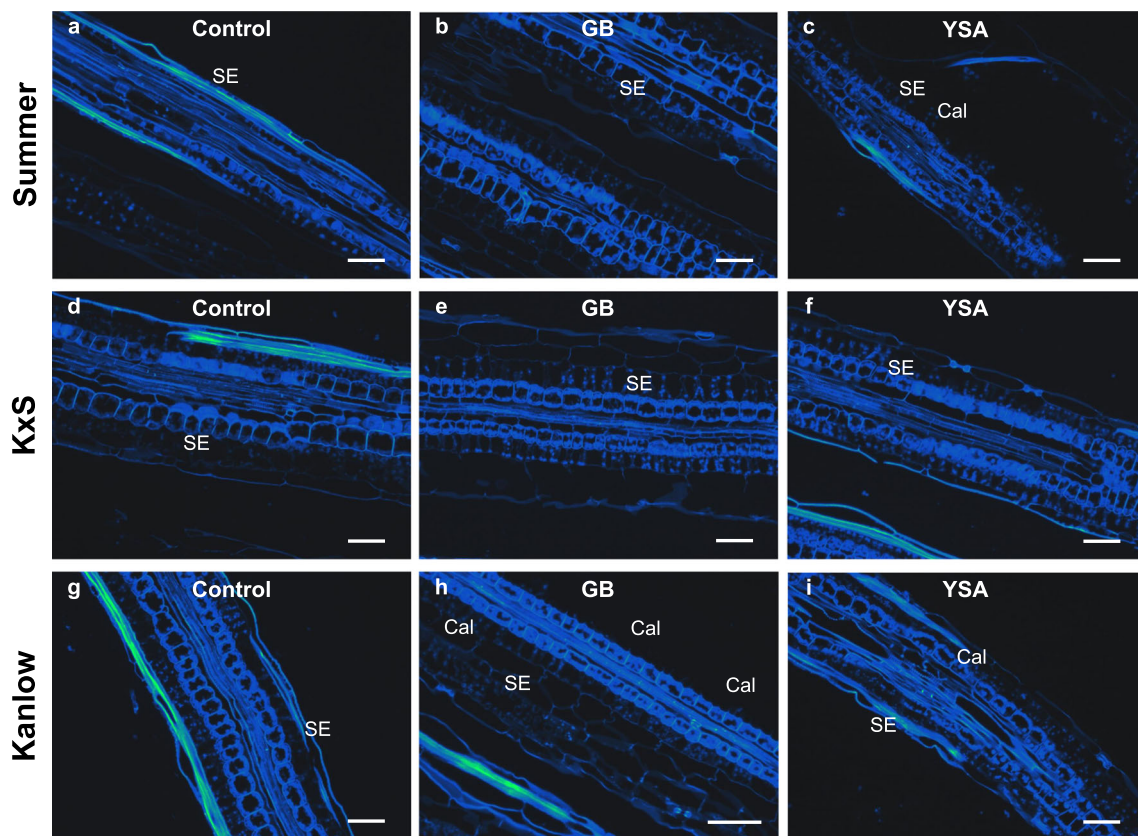


Fig. 3 Fluorescence micrographs of longitudinal leaf sections for switchgrass plants. **a–c** Summer, **d–f** K×S, and **g–i** Kanlow. Induced callose deposition (arrows) on the sieve plates (bluish-green fluorescence)

of phloem-based resistance to greenbugs in Kanlow plants, but not Summer or K×S plants.

Studies with switchgrass have indicated that plants become a less suitable host for several cereal aphids with increased age [9, 14]. Interestingly, results presented here indicate that greenbug phloem ingestion is reduced on more mature (V3) switchgrasses, compared to a previous report of greenbug feeding on V1 switchgrass. Koch et al. [15] demonstrated that in 15-h recordings, greenbugs spent more than one third of their time in sieve element phases on Summer and K×S plants (304.2 and 339.9 min, respectively). However, in this study, similar 15-h recordings on V3 switchgrass demonstrated that sieve element phases were reduced for greenbugs on Summer and K×S to 179.6 and 239.4 min, respectively. While this appears to support previous reports suggesting a compromise in successful aphid colonization on later developmental stages of switchgrass, it remains unclear if a reduction in phloem access contributes to the abated performance of greenbugs or is a consequence of other factors.

The lack of significant differences for yellow sugarcane aphid feeding behavior on both developmental stages of switchgrass is curious, given the greater levels of resistance in Kanlow, relative to Summer and K×S [7, 14]. Indeed, yellow sugarcane aphids appear to have little issue reaching sieve elements and sustaining ingestion on the resistant Kanlow. This would seem to suggest that resistance in Kanlow is truly

due to antibiosis, with no apparent contribution from antixenotic factors. Moreover, it also indicates that resistance is likely not a result of physical barriers during probing (e.g., callose or p-protein plugging of sieve pores). However, many other factors could be negatively affecting aphid fitness or demographics. For example, resistance could be conferred by the presence of plant secondary metabolites with toxicity to aphids (e.g., DIMBOA) [21, 40], growth inhibitors (e.g., quercetin) [41], or changes in plant metabolism to limit nutrient availability [12]. The benzoxazinoid, DIMBOA, confers toxicity to several cereal aphids, including greenbugs [42], and is an important element of *Rhopalosiphum maidis* resistance in maize [12, 21]. In maize, DIMBOA-Glc is activated by glucosidases to DIMBOA upon insect feeding, which then activates insect-deterrent metabolites [21, 43]. Crucially, benzoxazinoids are relatively ubiquitous in Gramineae and some evidence suggests that DIMBOA may be present in switchgrass [44].

Callose has been previously linked to resistance to piercing-sucking insects [20–23]. Kempema et al. [23] reported that *CALSI* mutant *Arabidopsis* plants upregulated callose synthase (*CALSI*) gene transcription in response to silverleaf whitefly, *Bemisia tabaci* (Gennadius), infestation. Moreover, the *CALSI* mutants also displayed significant callose deposition around whitefly feeding sites, indicating callose

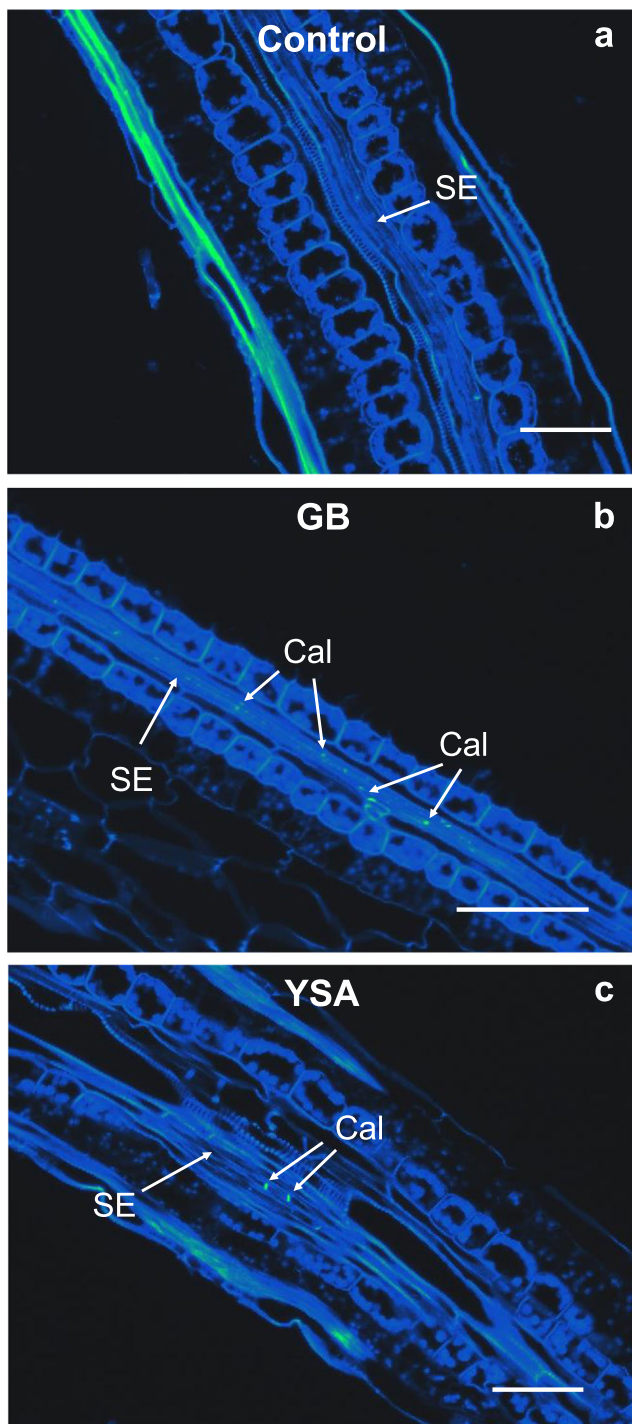


Fig. 4 Fluorescence micrographs of longitudinal leaf sections for Kanlow. **a** Control, **b** greenbug, and **c** yellow sugarcane aphid. Induced callose deposition (arrows) on the sieve plates

deposition may be an important part of *Arabidopsis*' induced defenses to whitefly feeding [23].

Despite multiple attempts aimed at elucidating the role of callose in switchgrass resistance, it remains unclear if sieve element occlusion via callose deposition is an important component of aphid resistance, based on our results. Generally,

significant callose deposits in switchgrass leaves were not observed, regardless of treatment, using histochemical studies. Although there appeared to be an increase in callose deposits on Kanlow plants after 3 days of greenbug infestation; however, more work is needed here to further clarify this response.

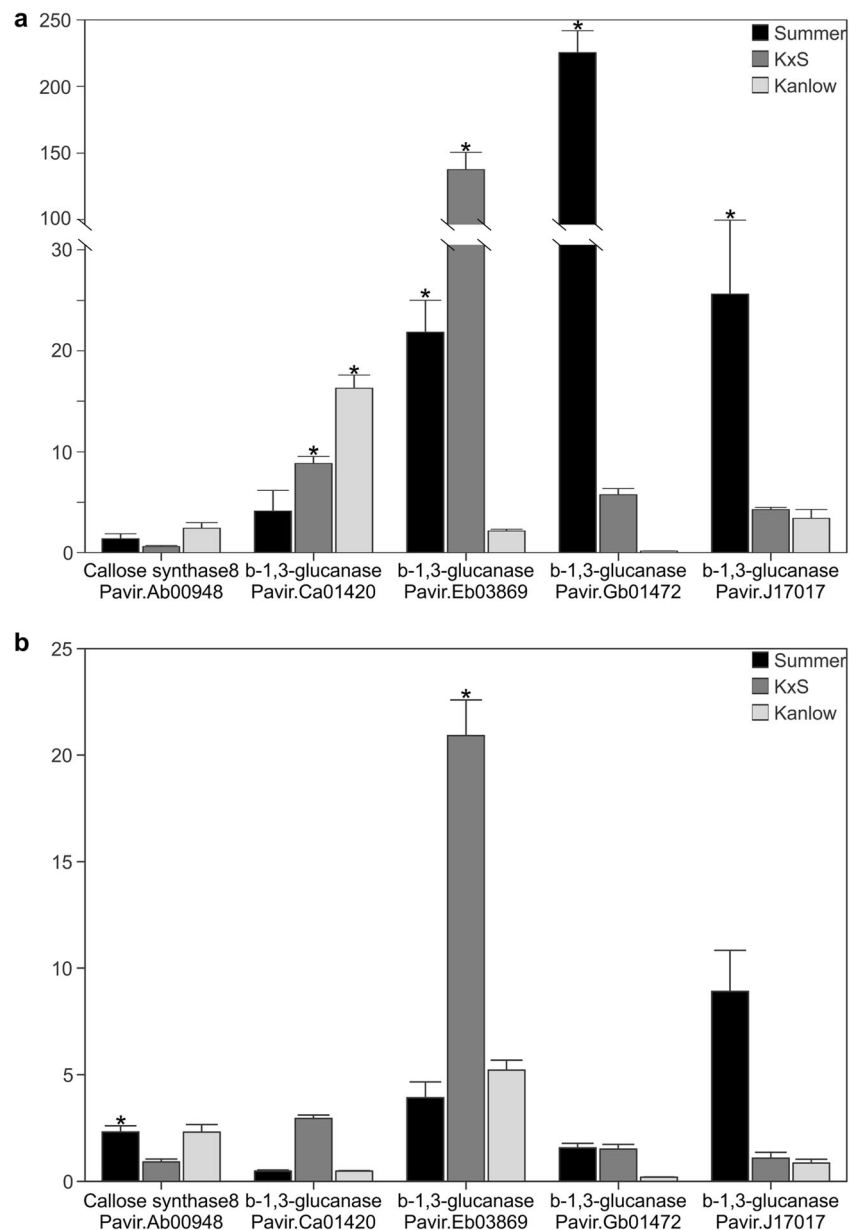
In response to greenbug feeding, none of the callose synthase genes evaluated were significantly upregulated. However, three β -1,3-glucosidase genes were upregulated in Summer while two were upregulated in K \times S in response to greenbugs. One possible explanation for this is that greenbugs could be inducing these glucanases to circumvent sieve element occlusion and create a more suitable feeding environment. For example, Du et al. [20] found that three callose synthase genes (*GSL1*, *GSL5*, and *GSL10*) in rice were upregulated by brown planthopper feeding in both resistant and susceptible plants. However, two glucanases (*GNS5* and *GNS9*) were downregulated on the resistant transgenic plants, suggesting that the reduction in the glucan hydrolyzing enzyme on resistant plants prevented callose from decomposing and leading to sieve element occlusion [20]. Similarly, Hao et al. [22] reported an upregulation of glucanases in the brown planthopper-susceptible rice plants, which may be responsible for unplugging of the sieve tubes, which otherwise remain plugged on resistant plants.

Conversely, Saheed et al. [45] reported that callose deposition appeared to be regulated at the protein level, rather than at the transcriptional level, in barley infested by cereal aphids. Saheed et al. [45] documented that none of the putative barley *GSL* sequences were regulated transcriptionally upon aphid attack, despite abundant callose deposition. Rather, it is possible that callose synthesis could also be activated by changes in the intracellular distribution of a glucoside activator as a regulatory mechanism [45, 46]. Moreover, Botha, Matsiliza [47] as well as van der Westhuizen et al. [48] have reported significant increases in glucanases on resistant plants following aphid feeding, suggesting that regulation of callose metabolism to prevent phloem transport cessation could be causally linked to resistance in some systems.

Intuitively, limiting phloem access would appear to be a particularly effective resistance strategy, since limiting the nutrient uptake by the aphids would not only preserve valuable resources in the host plant, but also negatively affect aphid demographics. However, although the EPG data pointed to phloem-based resistance in Kanlow plants, direct visualization of callose and qPCR of genes nominally associated with callose metabolism only provided equivocal supporting evidence. These data might indicate alternate sources of resistance in Kanlow, especially to greenbugs and limited resistance to the yellow sugarcane aphid in any of the tetraploid switchgrasses evaluated.

To our knowledge, this work provides the first detailed documentation of yellow sugarcane aphid feeding behavior. Previous work has documented a marked difference in

Fig. 5 Transcript abundance of callose-related genes in aphid-infested plants. Gene expression was detected by RT-qPCR in **a** greenbug-infested and **b** yellow sugarcane aphid-infested Summer, KxS, and Kanlow switchgrass. Expression of the indicated genes was compared to gene expression in uninfested control plants. A fold change > 1 represents higher transcript abundance in infested plants. Mean \pm SEM is shown. Statistical significance at $P < 0.05$ is indicated by an asterisk



greenbug feeding behavior on resistant and susceptible V1 switchgrasses. Here, it was possible to show a similar effect of Kanlow on greenbug feeding behavior at the V3 developmental stage as well. However, few differences were identified for yellow sugarcane aphid feeding behavior on resistant and susceptible switchgrass at both V1 and V3 developmental stages. Crucially, this suggests that multiple mechanisms of resistance may be present in Kanlow to cereal aphids, which could in turn provide more durable resistance to aphids.

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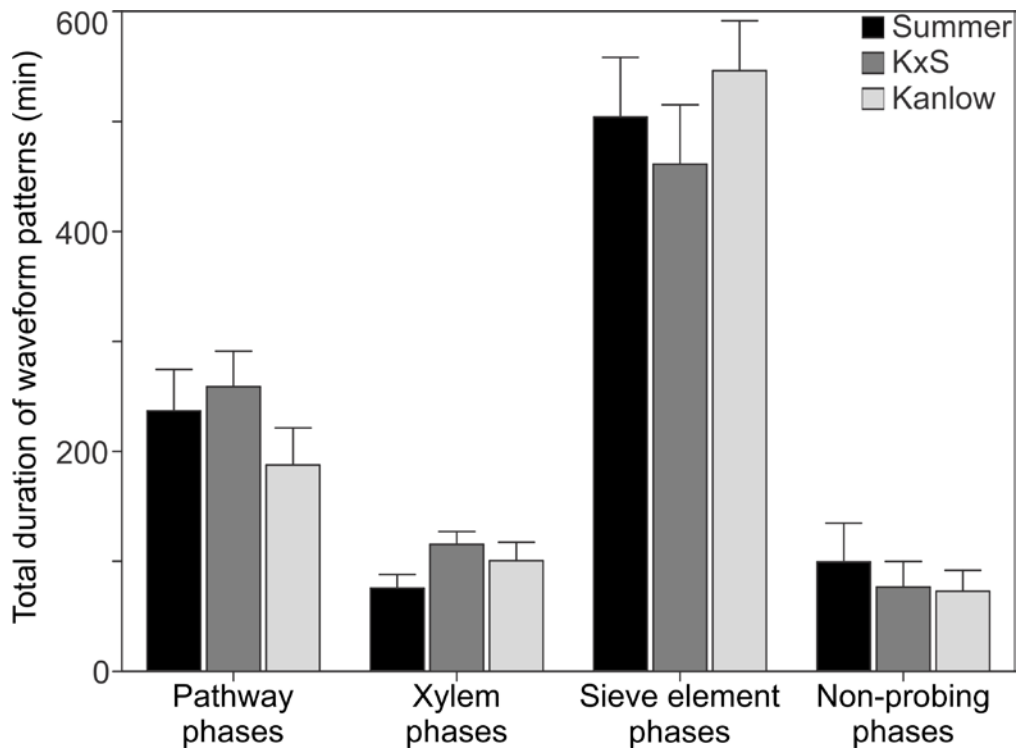
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Resource 1. Comparison of EPG parameters (mean \pm SEM) for duration of pathway, xylem, phloem and non-probing phases for 15 hr of yellow sugarcane aphid feeding on three switchgrass populations (V1 stage). No significant pairwise differences were detected between the means of duration variables of Summer, KxS and Kanlow ($P>0.05$), LSD test.

Online Resource 2. Comparison of EPG parameters (mean \pm SEM) for stylet activities for 15 hr of yellow sugarcane aphid feeding on switchgrass populations (V1 stage). No significant pairwise differences were detected between the means of probing variables of Summer, KxS and Kanlow ($P > 0.05$), LSD test.

| Feeding Variable | Mean \pm SEM | | |
|---|-----------------|-----------------|-----------------|
| | Summer | KxS | Kanlow |
| potential drops | 90.4 \pm 20.0 | 72.7 \pm 10.4 | 90.8 \pm 24.3 |
| pathway phases | 21.3 \pm 3.1 | 19.7 \pm 1.5 | 17.7 \pm 2.6 |
| xylem phases | 4.8 \pm 0.7 | 3.9 \pm 0.6 | 3.1 \pm 0.5 |
| SEP ¹ events | 7.5 \pm 1.0 | 7.7 \pm 0.7 | 7.5 \pm 1.1 |
| Sustained SEP ² events | 4.9 \pm 0.7 | 5.6 \pm 0.6 | 5.8 \pm 0.7 |
| NP ³ events | 9.6 \pm 2.2 | 8.4 \pm 1.1 | 7.4 \pm 2.1 |
| Potential phloem ingestion index (PPII) | 60.7 \pm 5.1 | 66.3 \pm 3.7 | 67.4 \pm 5.7 |
| % of aphids showing sustained SEP (E > 10 min.) | 95 (19/20) | 100 (20/20) | 95 (19/20) |

¹ Sieve element phase

² Sustained sieve element phase (E > 10 min)

³ Non-probing

Online Resource 3. Comparison of EPG parameters (mean \pm SEM) for time and duration of pattern segments for 15 hr of yellow sugarcane aphid feeding on switchgrass populations (V3 stage). No significant pairwise differences were detected between the means of probing variables of Summer, KxS and Kanlow ($P > 0.05$), LSD test.

| Feeding Variable | Mean \pm SEM | | |
|--|-------------------|-------------------|------------------|
| | Summer | KxS | Kanlow |
| Time to 1 st probe ^a | 1.2 \pm 0.5 | 1.1 \pm 0.6 | 1.2 \pm 0.6 |
| Time to 1 st SEP ¹ | 111.7 \pm 31.6 | 89.4 \pm 25.9 | 96.5 \pm 19.5 |
| Time to 1 st sustained SEP ² | 278.8 \pm 103.3 | 310.6 \pm 111.8 | 162.6 \pm 58.5 |
| Mean duration of SEP | 127.8 \pm 32.2 | 92.8 \pm 16.3 | 156.2 \pm 41.7 |
| Duration of 1 st SEP | 86.5 \pm 43.6 | 63.4 \pm 31.2 | 93.2 \pm 45.8 |

^a Time and duration calculated in minutes

¹ Sieve element phase

² Sustained sieve element phase ($E > 10$ min)

Online Resource 4. Comparison of EPG parameters (mean \pm SEM) for stylet activities for 15 hr of yellow sugarcane aphid feeding on switchgrass populations (V3 stage). No significant pairwise differences were detected between the means of probing variables of Summer, KxS and Kanlow ($P > 0.05$), LSD test.

| Feeding Variable | Mean \pm SEM | | |
|---|------------------|------------------|-----------------|
| | Summer | KxS | Kanlow |
| potential drops | 119.0 \pm 26.2 | 111.7 \pm 20.0 | 71.7 \pm 14.3 |
| pathway phases | 22.7 \pm 4.2 | 23.7 \pm 4.1 | 17.1 \pm 2.9 |
| xylem phases | 4.4 \pm 0.8 | 7.0 \pm 1.4 | 4.8 \pm 1.0 |
| SEP ¹ events | 6.6 \pm 1.0 | 7.0 \pm 0.9 | 5.2 \pm 0.6 |
| Sustained SEP ² events | 3.4 \pm 0.6 | 3.5 \pm 0.5 | 3.5 \pm 0.4 |
| NP ³ events | 12.2 \pm 3.4 | 10.0 \pm 2.7 | 7.4 \pm 1.9 |
| Potential phloem ingestion index (PPII) | 63.1 \pm 6.7 | 56.1 \pm 6.2 | 67.8 \pm 5.3 |
| % of aphids showing sustained SEP (E > 10 min.) | 90 (18/20) | 100 (20/20) | 95 (19/20) |

¹ Sieve element phase

² Sustained sieve element phase (E > 10 min)

³ Non-probing

Online Resource 5. Gene description, gene ID, and gene fold change for callose related genes when comparing greenbug infested and uninfested switchgrass. A fold change >1 represents higher transcript abundance in greenbug infested plants. A fold change <1 indicates higher transcript abundance in uninfested control plants. A fold change equal to 1 indicates no difference between transcript abundance for either treatment ($P < 0.05$).

| Transcript | Gene ID | Summer | | KxS | | Kanlow | |
|-------------------------------|---------------|-------------|-----------------------------|-------------|-----------------------------|-------------|-----------------------------|
| | | Fold change | <i>P-value</i> ^a | Fold change | <i>P-value</i> ^a | Fold change | <i>P-value</i> ^a |
| β-1,3-glucanase | Pavir.Bb02930 | 14.59 | 0.0799 | 6.83 | 0.1693 | 1.28 | 0.8775 |
| β-1,3-glucan synthase complex | Pavir.Db00045 | 8.64 | 0.3334 | 1.02 | 0.9436 | 2.03 | 0.4253 |
| β-1,3-glucanase | Pavir.Ga01393 | 0.94 | 0.9321 | 1.14 | 0.6508 | 0.89 | 0.6885 |
| Callose synthase 3 | Pavir.Ia04498 | 2.25 | 0.3627 | 0.50 | 0.2203 | 0.40 | 0.1054 |

^aStatistical significance at $P < 0.05$

Online Resource 6. Gene description, gene ID, and gene fold change for callose related genes when comparing yellow sugarcane aphid infested and uninfested switchgrass. A fold change >1 represents higher transcript abundance in infested plants. A fold change <1 indicates higher transcript abundance in uninfested control plants. A fold change equal to 1 indicates no difference between transcript abundance for either treatment ($P < 0.05$).

| Transcript | Gene ID | Summer | | KxS | | Kanlow | |
|-------------------------------|---------------|-------------|-----------------------------|-------------|-----------------------------|-------------|-----------------------------|
| | | Fold change | <i>P-value</i> ^a | Fold change | <i>P-value</i> ^a | Fold change | <i>P-value</i> ^a |
| β-1,3-glucosidase | Pavir.Bb02930 | 2.75 | 0.6345 | 4.21 | 0.9881 | 1.57 | 0.6679 |
| β-1,3-glucan synthase complex | Pavir.Db00045 | 5.10 | 0.0606 | 1.76 | 0.7953 | 4.13 | 0.1206 |
| β-1,3-glucosidase | Pavir.Ga01393 | 1.30 | 0.8752 | 1.44 | 0.9594 | 1.15 | 0.8081 |
| Callose synthase 3 | Pavir.Ia04498 | 2.74 | 0.1858 | 0.49 | 0.1841 | 0.80 | 0.8377 |

^aStatistical significance at $P < 0.05$