Chemical inhibition of Kir channels reduces salivary secretions and phloem feeding of the cotton aphid, *Aphis gossypii* (Glover)

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**Abstract**

**BACKGROUND:** The unique feeding biology of aphids suggests novel insecticide targets are likely to exist outside of the nervous system. We therefore aimed to directly test the hypothesis that pharmacological inhibition of inward rectifier potassium (Kir) channels would result in salivary gland failure and reduced sap ingestion by the cotton aphid, *Aphis gossypii*.

**RESULTS:** The Kir inhibitors VU041 and VU590 reduced the length of the salivary sheath in a concentration dependent manner, indicating that the secretory activity of the salivary gland is reduced by Kir inhibition. Next, we employed the electrical penetration graph (EPG) technique to measure the impact Kir inhibition has to aphid sap feeding and feeding biology. Data show that foliar application of VU041 eliminated the E1 and E2 phases (phloem feeding) in all aphids studied. Contact exposure to VU041 after foliar applications was found to be toxic to *A. gossypii* at 72 and 96 h post-infestation, indicating mortality is likely a result of starvation and not acute toxicity. Furthermore, VU041 exposure significantly altered the feeding behavior of aphids, which is toxicologically relevant for plant–virus interactions.

**CONCLUSION:** These data suggest Kir channels are critical for proper function of aphid salivary glands and the reduced plant feeding justifies future work in developing salivary gland Kir channels as novel mechanism aphicides. Furthermore, products like VU041 would add to a very minor arsenal of compounds that simultaneously reduce vector abundance and alter feeding behavior.

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**Keywords:** antifeedant; potassium ion channel; aphid control; plant pests

1 INTRODUCTION

Aphids are one of the most economically important plant-sucking pests in agriculture, inducing physical injury and transmitting plant viruses that result in economic losses exceeding US$1 billion dollars annually.1,2 For example, the cotton aphid, *Aphis gossypii* Glover (Hemiptera: Aphididae), is a polyphagous pest species that is considered to be one of the most destructive sucking pests worldwide due to the direct feeding damage to plant tissue3 as well as the transmission of viruses that reduce fruit development or palatability.4–6 Synthetic insecticides targeting the nervous system remain the primary method for controlling aphid populations in agricultural settings, yet the rate of evolution for the development of insecticide resistance is increased in aphids when compared to other insect pest species due to aphid parthenogenesis, which allows for dozens of generations within a single growing period.7 To this point, control failures are common with *A. gossypii* as they are highly resistant to the majority of approved chemical classes through increased metabolic detoxification8–10 and target site mechanisms.9,11 Currently, *A. gossypii* has been shown to be resistant to organophosphates and carbamates,12 neonicotinoids,13 pyrethroids,8 and Cry proteins.14 The high levels of resistance to the large majority of approved insecticidal classes has underscored the importance of characterizing underexplored targets for the development of novel mechanism insecticides that will compliment current aphid control measures.

Aphid feeding is dependent upon two major organ systems, the salivary gland and pharyngeal-cibarial pump complex, to enable proper feeding on plant tissue by secreting saliva that contains bioactive agents critical for feeding and generating a sucking action during feeding, respectively. Therefore, both of these organ systems represent a potential mechanism to induce feeding cessation and mortality that is distinctly different from commercialized neurotoxic insecticides. To support this notion, pymetrozine is a commercialized insecticide that has been shown to inhibit the musculature around the cibarial pump of sucking pests to prevent fluid ingestion.15 Interestingly, the salivary gland, which is the other organ essential for aphid feeding, has not been exploited as a target tissue for control.

In addition to being the site of pathogen transmission, the aphid salivary gland is the organ responsible for secreting ‘gel’ and watery saliva that form the stylet sheath and facilitate enzymatic food digestion, respectively.16 Importantly, genetic depletion of the aphid structural sheath protein through RNA interference...
significantly reduced the efficacy of phloem feeding, indicating that the gel saliva is critical for obtaining nutrients needed for reproduction and survivorship.\textsuperscript{16–18} Furthermore, aphid salivary secretions have been shown to prevent sieve tube plugging by suppressing a rise in free calcium through molecular interactions of salivary proteins and plant defenses.\textsuperscript{19} Therefore, the salivary gland is critical to the biological success of aphids and thus we hypothesize that reducing the secretory activity of the salivary gland will prevent feeding and reduce aphid-mediated crop loss. However, there is an incomplete understanding regarding the physiological machinery required for proper salivary gland function of aphids that limits the ability to develop products acting through novel mechanisms.

Inward rectifier potassium (Kir) channels conduct $K^+$ currents into cells at hyperpolarizing membrane potentials more readily than out of cells at depolarizing membrane potentials and have been found to play a critical role in multiple mammalian\textsuperscript{20,21} and arthropod\textsuperscript{22–28} physiological systems. In mammalian salivary glands, Kir channels drive anion efflux across the apical membrane of salivary exocrine acinar cells with minimum loss of $K^+$ from the cell as a means to support resting secretion.\textsuperscript{27–30} Similarly, several recent lines of genetic and pharmacological evidence suggest Kir channels play important physiological roles in exocrine systems of dipteran insects as well. For instance, genetic depletion or pharmacological inhibition of Kir channels have been shown to reduce the fluid and ion secretion of dipteran Malpighian tubules\textsuperscript{24,31–34} and previous work has indicated Kir channels are critical for the salivary gland function and feeding of arthropods pests.\textsuperscript{25,26,35}

The development of insect Kir channel pharmacology\textsuperscript{23,34,36,37} has enabled researchers to begin to characterize the physiological role of these channels in various arthropod tissue systems as well as determine the toxicological relevance to pest species. For instance, the insect-specific Kir inhibitor termed VU041 was shown to inhibit \textit{A. glycines} (soybean aphid) Kir1 and Kir2 currents in electrophysiological studies and topical application resulted in acute mortality,\textsuperscript{38} indicating Kir channels are important for aphid survivorship.

Considering (i) the importance of salivary secretions to aphid feeding and survivorship, (ii) aphid Kir1 and Kir2 channels are sensitive to previously developed insect Kir inhibitors (e.g. VU041), and (iii) the previously reported impact of Kir inhibition to salivary gland function and feeding efficacy to arthropods,\textsuperscript{25,26,35} we hypothesized that pharmacological inhibition of Kir channels would result in salivary gland failure and reduced sap ingestion by the cotton aphid, \textit{A. gossypii}. Therefore, the goals of the present study were to use novel pharmacology, electrophysiology, and biological assays to determine the relevance of Kir channels in \textit{A. gossypii} salivary gland function and feeding efficiency. Knowledge gained from this study may be used to broadly guide future development of novel synthetic insecticides, RNAi, or transgenic plants to mitigate economic losses that result from feeding of sap-sucking agricultural pests.

## 2 METHODS

### 2.1 Compounds and reagents

The Kir channel inhibitor VU041 and the significantly less potent analog VU937 were originally designed against the \textit{Anopheles gambiae} Kir1 channel.\textsuperscript{34} Both compounds were synthesized through custom synthesis by Molport (Riga, Latvia) and were purified by column chromatography and are >95% pure by $^1$H NMR analysis. VU625 was purchased by custom synthesis from Molport. VU590 was purchased from Tocris Bioscience (Bristol, UK). The solvents, dimethyl sulfoxide (DMSO) and absolute ethanol were purchased from Sigma-Aldrich (St. Louis, MO, USA). Molecular sieves OP type 3 Å were purchased from Sigma-Aldrich and were used to prevent water absorption within the DMSO stock. Fifty beads were added into a 100 mL stock solution. These sieves have a diameter of ~2 mm, a pore size of 3 Å, and a water absorbing capacity of ≥15%. Rhodamine B was purchased from Sigma-Aldrich. The chemical structures of the Kir channel modulators used in this study are shown in Fig. 1.

### 2.2 \textit{A. gossypii} stock and rearing conditions

The laboratory colony of cotton aphid, \textit{A. gossypii}, was developed from a single aptera collected from a cotton plant, \textit{Gossypium hirsutum} L., at Louisiana State University Agricultural Center Macon Ridge Research Station, Winnsboro, LA, in 2006. The cotton aphid colony was reared on cotton variety DP174RF (Deltapine, Monsanto Company, St. Louis, MO, USA) and maintained in screened cages (30 × 30 × 30 cm) assembled with Plexiglas and nylon mesh fabric under laboratory conditions at 20 to 22 ∘C and a photoperiod of 14:10 (L: D). All host plants were grown in growth chambers (Percival Scientific Inc., Perry, Iowa) at 25 ∘C and 50% RH, in 10-cm-diameter plastic pots filled with Miracle-Gro Potting Mix soil (Miracle-Gro Lawn Products Inc, Marysville, OH, USA) and Osmocote 14-14-14 (Scotts-Sierra Horticultural Products Company, Marysville, OH, USA) fertilizer. Fresh host plants were provided to the colonies every 2 to 3 weeks for colony maintenance.

### 2.3 Artificial host feeding assay

The construction of the feeding chamber was modified based on those previously described.\textsuperscript{35} The feeding chamber was modified to have a diameter of 9 mm and a cotton plug was inserted into the chamber to allow for approximately 4 mm of head space (Fig. 2(A)). Parafilm was then pulled across the top of the chamber to a thickness of approximately 15–20 μm and 20 μL of 15% sucrose\textsuperscript{40} was pipetted onto the Parafilm. A second piece of Parafilm was pulled across the top of the feeding chamber to prevent evaporation of the sucrose solution (Fig. 2(B)). The fluorophore, Rhodamine B, at a concentration of 200 ppm, was included in the sucrose solution to ensure the aphids included in analysis contained fluorescent mouthparts or bodies that indicated an attempt to feed. In addition, Rhodamine B was used to increase visualization of the salivary sheath. Individual aphids were put into each feeding chamber and were removed 24 h after insertion for analysis of survivorship or sheath measurements.

Kir modulators were solubilized in DMSO and then diluted into the 15% sucrose solution. Final DMSO concentrations did not exceed 0.1% and control solutions all contained 0.1% DMSO.

### 2.4 Salivary sheath measurements

The artificial membrane assay was used to collect the salivary sheath from individual aphids that were fed on sucrose solutions containing Kir inhibitors and methods were modified from those described in Begum and Wilkins\textsuperscript{41} and Ren \textit{et al.}\textsuperscript{45} Individual, yet synchronized apletic adult aphids were inserted into the artificial feeding system and given 24 h to feed on the sucrose with or without a discriminatory concentration of Kir modulators. The modulators were studied at concentrations of 700 μM (VU041), 700 μM (VU590), 1 mM (VU625), and 500 μM (VU937), which are at
Kir Inhibition reduces aphid salivary secretions and phloem feeding

Figure 1. Chemical structures of pharmacological agents used in this study.

or approaching solubility limits in sucrose solutions. After feeding, the aphids were removed and the Parafilm membrane was removed and mounted on a microscope slide. The membranes were observed under a Leica DM68 microscope at the Shared Instrumentation Facility (Louisiana State University, Baton Rouge, LA, USA) and localization of the sheaths was facilitated through Rhodamine B labeling that was included in the sucrose solution. All sheaths on the membrane were counted and measured using Leica LAS software (Buffalo Grove, IL, USA). Sheath lengths from each treatment group were averaged between 10 and 15 individuals, and statistically analyzed by a one-way analysis of variance (ANOVA) with multiple comparisons post test.

2.5 Electrical penetration graph recordings

Electrical penetration graph (EPG) studies were carried out to monitor the feeding behavior of apterous cotton aphid on treated and untreated leaves. Protocols followed those previously described by our group. Kir inhibitors were solubilized in DMSO, diluted into water, and 1 mL of solution was pipetted onto the leaf at a final concentration of 2 μg cm⁻² and dried for 24 h at room temperature. To prevent cytotoxicity, final DMSO concentrations did not exceed 0.1% and control treatments included 0.1% DMSO. EPG experiments were performed in a Faraday cage using a Giga8 DC amplifier (Wageningen Agricultural University, The Netherlands) with 1 gigaohm input resistance and an AD conversion rate of 100 Hz running only the first four channels. A DI-710 (DATAQ Instruments, Inc., Akron, OH, USA) acquisition card converted the analog signals to digital signals, which were recorded using WinDaq Serial Acquisition software (DATAQ Instruments, Inc.). An 18-μm gold wire (Semiconductor Packaging Material, Armonk, NY, USA) was attached to the dorsal tergum of an adult aphid with silver paint (Pelco Colloidal Silver Liquid no. 16034, Ted Pella, Inc., Redding, CA, USA), and then placed on the adaxial surface of the uppermost leaf that had been treated or left untreated. Four aphids were tested at a time with two aphids per plant per treatment and recorded for 4 h. The experiments were replicated six times for a total of 24 individual aphids per treatment. Six different behaviors were analyzed for each aphid per 4-h recording: total number of probes in 4 h, time to first probe, duration of each probe (min), percentage of 4 h probe in E1 (phloem salivation) and E2 (phloem ingestion), percentage of total probe duration, and percentage of 4 h probe in G phase (xylem ingestion). Identification and classification of EPG signals followed the nomenclature of the list of EPG variables previously used by our group and others. Waveform variables were calculated manually to increase the fidelity of the data when compared to automatic parameter calculation software.

2.6 Toxicity assessments

The toxicity of the Kir inhibitors was determined through the artificial host assay (described in section 2.3) and through foliar treatments of cotton leaves. For all toxicity assays, mortality was defined as no coordinated movement after the individual was exposed to mechanical stimuli. For the artificial host assay, 15 synchronized apterous adult aphids were inserted into the feeding chamber and allowed to feed on a 15% sucrose solution that included a discriminatory concentration (see section 2.3) of Kir channel inhibitors and Rhodamine B (200 ppm). A total of three individual replicates of 15 aphids each were performed and the percentage mortality for each replicate was averaged to result in the final percentage mortality for each chemical. For all time points, the aphids were removed and assed for Rhodamine B fluorescence to ensure consumption of the sucrose solution and mortality was tabulated. Aphids that were not fluorescent were not included in the analysis.

Contact and delayed mortality was assessed using live cotton plants by foliar applications of cotton leaves with Kir inhibitors. These methods mirrored those described for the EPG recordings (section 2.5) to enable comparison of EPG and toxicity data. Kir inhibitors were solubilized in DMSO, diluted into water, and 1 mL of solution was pipetted onto the leaf at a final concentration of 2 μg cm⁻² and dried for 24 h at room temperature. To prevent cytotoxicity, final DMSO concentrations did not exceed 0.1% and control treatments included 0.1% DMSO. The chemically treated...
Figure 2. In vivo assessment of the impact Kir inhibitors have on the secretory activity of A. gossypii salivary glands. Individual components and fully constructed membrane feeding chambers with Rhodamine B labeled sucrose solution is shown in panels (A)–(B). Representative images of salivary sheaths from aphids feeding on control (C) and VU041-treated sucrose solution (D) with measurement bars showing sheath length. Scale bars represent 10.0 μm. (E) Salivary sheath lengths of aphids exposed to a discriminatory concentration (see methods) of Kir channel inhibitors compared to vehicle control (black bar) where bars represent the mean (n = 10–30 individuals) length and error bars represent SEM. Length was measured 24 h post infestation of the membrane. Asterisks represent statistical significance at P < 0.01 as determined by an unpaired t-test to control.

leaf area was infested with 20 synchronized apterous adult aphids that were confined to the treated area by a modified clip-cage. The modified clip-cage had a length and width of 1.5 cm, and a height of 0.25 cm that was ringed with foam and covered with fine tulle mesh to prevent aphid escape. Aphids were monitored for 96 h and mortality was recorded every 24 h. Three replicates were performed and each replicate was performed with 20 individual aphids that were infested on a freshly treated adaxial surface of a unifoliate leaf. Experiments were performed in an environmental chamber with a light:dark setting at 14:10 h, 25 °C/50% RH.

3 RESULTS

3.1 Kir channel inhibitors reduce salivary sheath secretions of A. gossypii

Aphids are phloem-sap feeders that secrete gelatinous saliva from the salivary glands to form a sheath around the stylet mouthparts to facilitate phloem feeding and therefore the length of the salivary sheath is a direct measure of the secretory activity of the salivary gland. Here, we investigated the effects of structurally diverse Kir inhibitors (Fig. 1) to secretions of gelatinous saliva by measuring the length of salivary sheaths of A. gossypii. The length of the salivary sheath from control aphids was found to be 51 ± 6 μm. Inclusion of 700 μM VU041 or 700 μM VU590 in the sucrose solution reduced the secretory activity of the salivary gland by 2.9- and 1.9-fold, respectively, which are both statistically significant (P < 0.01) reductions when compared to the control (Fig. 2(E)). Representative images of salivary sheaths embedded into the Parafilm membrane for control and VU041 treated aphids are shown in Fig. 2(C) and (D), respectively. However, salivary sheath lengths of aphids exposed to 1 mM VU625 (solubility limit) were not different than control aphids (Fig. 2(E)). Importantly, VU937, the significantly less potent analog to VU041, did not alter the salivary sheath length and indicates that the reduction in sheath length in VU041 exposed aphids is indeed due to Kir inhibition (Fig. 2(E)).
of sheath length was found to occur at 700 μM inhibition (Fig. 3(B)). However, for VU041, maximum inhibition formation from 500 μM of control aphids to 35 ± 3 μm and 37 ± 4 μm, respectively. Exposure to 500 μM or sucrose significantly (P < 0.05) yielded salivary sheath lengths of 30 ± 3 μm and 28 ± 8 μm, respectively, which is a statistically significant (P < 0.05) reduction when compared to the control (Fig. 3(A), (B)). The 1.7-fold reduction in sheath length observed at 500 μM VU590 was maximal inhibition (Fig. 3(B)). However, for VU041, maximum inhibition of sheath length was found to occur at 700 μM as we observed 2.9- and 1.6-fold reductions when compared to control and 500 μM treatments, which are statistically significant (P < 0.05) reductions (Fig. 3(A)). For VU041, concentrations higher than 700 μM resulted in sheath lengths that were not different from the control, likely due to solubility limitations of VU041 in sucrose solutions.

3.3 Foliar applications of VU041 prevented phloem feeding by *A. gossypii*

Due to the influence of VU041 to salivary secretions and sheath formation, we measured the feeding efficiency of *A. gossypii* through electrical penetration graph recordings on leaves of cotton plants. Representative EPG recordings of aphids feeding on leaves treated with control and VU041 are shown in Fig. 4(A) and (B), respectively. Aphids feeding on control (water treatment only) leaves showed stereotypical feeding patterns that have been previously documented with clear probe events followed by C waves that represent the intercellular apoplastic stylopathway, E1 waves that defined as salivation into the phloem sieve elements, E2 waves that show phloem sap uptake, and G waves that are defined as active intake of water from xylem elements (Fig. 4(A) and a subset). Aphids that fed on leaves topically treated with VU041 displayed altered feeding patterns when compared to control aphid recordings. Probe events and xylem ingestion (G waves) were clearly distinguishable and were similar to that observed in control- and VU937-exposed aphids (Fig. 4B). Over the 4-h feeding period, VU041-exposed aphids fed on xylem 12 ± 4.5% of the total time, which were 1.3- and 1.5-fold longer xylem feeding times than observed control and VU937-exposed aphids, respectively (P < 0.07; Fig. 4C). However, VU041 inhibited phloem feeding as zero bouts of salivation into the phloem sieve elements (E1 waves) or ingestion of phloem sap (E2 waves) were observed in any aphid studied (n = 24; Fig. 4D). Importantly, aphids that fed on VU937-treated leaves spent 17 ± 5.8% of the 4-h feeding period in phloem as defined as E1 or E2 waves, which was a statistically significant (P < 0.001) increase when compared to VU041-exposed aphids (Fig. 4D).

3.4 Foliar applications of VU041 altered feeding behavior of *A. gossypii*

In addition to salivary gland function and phloem feeding efficacy, the feeding behavior of aphids has a significant impact on aphid survivorship and pathogen transmission, and thus the feeding behavior of aphids was studied using EPG. Surprisingly, the total number of probes that resulted in a potential drop, which indicates an intracellular puncture by aphid stylostets, was not significantly (P > 0.05) different among control-, VU041-, or VU937-exposed aphids (Fig. 5(A)). However, from the start of the EPG recording, the time to first probe for VU041-exposed aphids was found to be 56 ± 18 min, which is a significant (P < 0.05) increase of 3.8-fold in the time required for intracellular puncture of plant cells when compared to the control (Fig. 5(B)). In addition to the delayed probing, the time spent in each probe and total probe duration over the entire feeding period (4 h) were reduced from control aphids by 2.3- and 1.7-fold, respectively, a statistically significant reduction of feeding probe times (Fig. 5(C), (D)). Importantly, VU937 did not alter the time to first probe (Fig. 5(B)), the total time spent in each probe (Fig. 5(C)), or the total probe duration over the entire 4-h recording period, and these values were not significantly different to the control, which indicates that the altered feeding behavior shown in Fig. 4(D)–(D) is likely due to Kir channel inhibition.

3.5 Toxicity of Kir inhibitors to *Apis gossypii*

We employed an artificial host assay to measure aphid mortality after ingestion and found no significant difference in toxicity between VU041 or VU937 when compared to control aphids (Table 1). Aphids were given continuous access to chemically treated sucrose and toxicity was measured at 12, 24, and 48 h. The experiment was terminated after 48 h due to high control mortality. To assess contact and delayed toxicity of VU041, we treated cotton leaves of living plants with 2 μg cm⁻² and found no significant
difference in the toxicity of VU041 at 12, 24, or 48 h post infestation (Table 1). However, 3.5- and 4.5-fold increases in mortality were observed in VU041-treated leaves when compared to control (solvent only) treated leaves at 72 and 96 h post infestation, respectively, which is a statistically significant ($P < 0.05$) increase. Importantly, no significant difference in mortality was observed between VU937 and control treatment groups (Table 1). Molecules that are acutely toxic commonly result in diagnostic signs of intoxication after exposure, but interestingly contact exposure to VU041 after foliar applications did not result in any obvious signs of intoxication.

4 DISCUSSION

It is well established that aphid feeding results in a significant reduction of crop yield worldwide and thus reducing aphid populations through novel mechanisms has been of significant interest. Current aphid control programs rely heavily on the use of neurotoxic insecticides, and although neurotoxic insecticides are a validated approach for reducing aphid populations, it has been shown that some neurotoxic insecticides, such as pyrethroids, stimulate aphid probing and cause aphids to move from plant to plant, ultimately increasing virus spread. This plant-to-plant movement and increased probing behavior can increase the spread of nonpersistent plant viruses, thus the identification of molecules that induce mortality through a novel mechanism (e.g. starvation) while simultaneously altering feeding/probing behavior is likely to simultaneously reduce aphid populations and limit pathogen transmission. The unique feeding biology of aphids and their reliance on salivary secretions for successful feeding suggests that biochemical targets for novel mechanism aphicides that induce mortality and alter feeding behavior are likely to exist.

As with most other animals, the aphid salivary gland is one of the key organs that drives successful feeding events due to the multifaceted functions of the saliva. Aphids are known to produce two types of saliva that mediate interactions with the host plant. First, watery saliva is injected into adjacent plant cells to counteract plant defenses as the stylets follow an intracellular pathway toward the sieve tubes during cell penetration. Additionally, watery saliva is secreted after the initial salivation phase during fluid ingestion and is essential for continued fluid ingestion and mitigation of plant defenses. The second type of saliva is a gelatinous saliva that is secreted during styllet movement through the apoplast and is responsible for...
Kir Inhibition reduces aphid salivary secretions and phloem feeding

Figure 5. Probing and feeding behavior of *A. gossypii* after exposure to VU041. Representative EPG recordings from single aptera *A. gossypii* adults after foliar treatment of vehicle or 700 μM VU041. The influence of Kir inhibition to feeding behavior was assessed by quantifying changes in number of probes over entire feeding period (A), the time to first probe (B), the total time spent in each probe (C), and the total probe duration over the entire feeding period (D). In all panels, control treatments are represented by black bars, VU041 treatments are shown by red bars, and VU937 (less potent analog to VU041) treatments are shown by white bars. Asterisks represents statistical significance when compared to control, with * and ** representing $P < 0.05$ and $P < 0.01$, respectively. Statistical significance was determined by one-way ANOVA with a multiple comparisons post-test.

Table 1. Toxicity assessments of VU041 to *A. gossypii*

<table>
<thead>
<tr>
<th>% Mortality ± SD</th>
<th>Foliar application, 2 μg cm$^{-2}$</th>
<th>Artificial host, 700 μM</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Control</td>
<td>0 ± 0 A</td>
<td>0 ± 0 A</td>
</tr>
<tr>
<td>VU041</td>
<td>0 ± 0 A</td>
<td>0 ± 0 A</td>
</tr>
<tr>
<td>VU937</td>
<td>0 ± 0 A</td>
<td>0 ± 0 A</td>
</tr>
<tr>
<td></td>
<td>12 h</td>
<td>24 h</td>
</tr>
<tr>
<td>Control</td>
<td>14 ± 8 A</td>
<td>19 ± 10 A</td>
</tr>
<tr>
<td>VU041</td>
<td>17 ± 7 A</td>
<td>20 ± 13 A</td>
</tr>
<tr>
<td>VU937</td>
<td>20 ± 10 A</td>
<td>31 ± 9 A</td>
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Mortality is presented as mean percentage toxicity from $n = 3$ independent determinations. Each chemical treatment group not labeled by the same letter represents statistical significance ($P < 0.05$) within the same time point as determined by a one-way ANOVA with Tukey’s post test.

forming a continuous sheath that encases the full length of the stylet mouthparts to provide mechanical stability, lubrication, and protection against plant defenses during phloem feeding. $^{16,17,48}$ Importantly, disrupted sheath formation through inhibition of structural proteins or inhibition of salivary effectors that suppress plant defenses results in reduced aphid feeding from phloem sap through sieve tubes. $^{17}$ The importance of salivary secretions to aphid feeding and survivorship indicates that mechanisms to inhibit salivary gland function will prevent phloem feeding and survivorship, yet the physiological mechanisms driving the secretory activity of the aphid salivary gland are limited.

The realization that Kir channels constitute a critical conductance pathway in multiple arthropod systems $^{22,24–26,34,49}$ has driven the discovery of multiple structurally diverse and specific inhibitors of insect Kir channels. $^{23,31,36}$ The growing pharmacological library targeting insect Kir channels has allowed for toxicological characterizations of insect Kir channels and data suggest these ion channels represent promising molecular targets for inducing acute lethality through the development of small-molecule inhibitors. $^{34}$ Importantly, the mosquito-selective Kir channel inhibitor VU041 was shown to possess moderate inhibition potency to aphid Kir1 and Kir2 channels, $^{38}$ which enabled interrogation of the physiological role and toxicological potential of aphid Kir channels. Indeed, topical application of VU041 was shown to induce acute lethality to *Aphis glycines* Matsumura at a greater rate than the less potent analog VU937, $^{38}$ suggesting that Kir channels are important for aphid survivorship. Beyond acute lethality, Kir channels represent a putative molecular target to induce toxicity through antifeedant properties.
Kir channel mRNA is highly expressed in the salivary gland of the brown planthopper (Nilaparvata lugens) and the common fruit fly (Drosophila melanogaster) and these channels have been implicated in regulating the secretory activity of the salivary gland and subsequent feeding. For instance, chemical inhibition of Kir channels and salivary gland specific knockdown of Kir channel mRNA in D. melanogaster reduced the volume of sucrose ingestion and, similarly, chemical inhibition of Kir1 significantly reduced the salivary secretions and feeding behavior of N. lugens. Our data with A. gossypii support these previous studies by demonstrating that VU041 and VUS90 are capable of reducing salivary secretions during feeding (Figs 2(E) and contact exposure to VU041 prevented phloem feeding in EPG studies (Fig. 4). However, contact exposure to VU041 from leaf tissue did not result in mortality within 48 h and did not induce any obvious signs of intoxication. Furthermore, the lack of toxicity cannot be attributed to poor cuticular penetration of VU041 because ingestion of high concentrations of VU041 was found to be non-toxic to A. gossypii. The lack of poisoning symptomology after exposure to VU041 combined with the documented moderate inhibition potency of VU041 to aphid Kir channels suggests Kir channels may not be an acutely lethal target in aphids. Yet, although acute mortality is advantageous for aphid control, the lack of acute toxicity does not invalidate Kir channels as a novel target for aphicides because Kir channels have been shown to regulate insect systems that are likely to result in chronic mortality, such as reduced feeding efficacy.

A significant portion of plant damage and economic loss induced by aphids is through transmission of plant pathogens, thus molecules that alter feeding behavior to reduce pathogen transmission would be an advantageous addition to the aphicide arsenal. Our EPG data show that individual A. gossypii that fed on VU041-treated leaves were able to reach and feed in xylem and thus gain back sufficient fluids to replace water loss (Fig. 4(C)), yet VU041 prevented A. gossypii from sieve element salivation (E1) and phloem ingestion (E2; Fig. 4(D)). The preference for xylem is interesting and potentially due to dehydration of the aphid after exposure to VU041, which was previously noted. However, the inability to feed from phloem after exposure to VU041 is a unique and toxicologically relevant behavior, as it will eventually result in aphid death due to starvation because the phloem contains the extreme majority of macronutrients in the plant. To this point, we observed high mortality at 48- and 72-h time points after foliar applications of VU041 to living cotton plants, but no acute mortality was observed after ingestion or contact exposure to VU041. Previous reports have shown Kir channels are critical for proper function of the nervous system and osmoregulatory capacities of diptera. Thus mortality in these species after exposure to Kir inhibitors is likely due to altered neurological function, an inability to osmoregulate, or a combination of both. However, the lack of acute toxicity or signs of intoxication in A. gossypii after ingesting VU041 or after contact exposure, combined with the lack of phloem feeding, suggests the delayed mortality for this species is likely resultant of nutrient deficiencies stemming from the lack of phloem feeding (Fig. 4) and not from nervous system or Malpighian tubule failure. However, it is important to note that these data differ from previous reports that showed acute lethality to A. glycines after topical exposure, indicating species differences or a dependency on toxicological methods. In addition to toxicity, the lack of phloem feeding is likely to have a significant impact on plant–virus epidemiology. Acquisition and transmission of phloem-limited pathogens, such as luteoviruses, require long feeding times within the phloem for uptake and inoculation by their insect vector, respectively. Considering this, we speculate VU041 and other molecules that alter probe behavior will prevent acquisition and transmission of phloem-limited pathogens, but future research is required to test this hypothesis.

There has been a major shift in the field of insecticide science for the development of highly selective insecticides that mitigate toxicity to humans and beneficial insects. A phylogenetic analysis of A. glycines Kir channels shows significant differences between Kir2 encoding genes of aphids when compared to other hemipteran and dipteran insects, indicating that the development of molecules with high insect species selectivity is possible. Importantly, both Kir1 and Kir2 genes in A. glycines are evolutionarily divergent to the human Kir channel family that would enable the development of highly selective molecules against human Kir channels, as was done for VU041 and related analogs. Considering the potential for development of selective insecticides and VU041-inhibited aphid phloem feeding, investments in understanding the fundamental physiology of aphid salivary gland function and insecticide development campaigns targeting potassium channels in the salivary gland are likely to be well placed.

Although there is a close correlation between reduced phloem feeding and reduced sheath length (Figs 2 and 4), it is conceivable that the reduction in feeding could be due to altered Kir channel function at the chemosensory sensilla on the stylet tip or aphid tarsi, the feeding pump muscles, the salivary gland, or any combination of these systems. For instance, it is well documented that the ratio of watery and gel saliva in the aphid saliva is influenced by the variable environment detected by the chemosensory and mechanosensory sensilla in the precibarium and on the stylet tip, respectively. Thus, Kir channel inhibition with VU041 could alter the sensory sensilla to alter salivary composition and stylet navigation, ultimately preventing phloem feeding, as is observed in Fig. 4(D). Furthermore, the total number of probes was not altered but the duration of each probe event was significantly reduced after contact exposure to VU041 (Fig. 5), suggesting that the aphid was not able to feed after probe initiation due to salivary gland failure or the chemosensory sensilla were perturbed to cause cessation of feeding. Future work is needed to determine the role of Kir channels in salivary gland function, sensory sensilla activity, and to validate the functional linkage between Kir channels, salivary gland function, and phloem feeding.

This study provides the first insight into the functional role of Kir channels in the secretory activity of the aphid salivary gland and aphid feeding, and suggests that Kir channels expressed in the aphid salivary gland are critical for its proper function as evidenced by reduced salivary sheath length and the absence of phloem feeding. A significant amount of research remains to be performed to identify and characterize the major physiological pathways required for proper aphid salivary gland function and feeding, but this study indicates that aphid Kir channels are ‘druggable’ targets and justifies future work in developing Kir channels, and likely other ion channels and transporters, expressed in the salivary gland as novel mechanism aphicides. Furthermore, products like VU041 would add to a very minor arsenal of compounds that reduce vector abundance and alter feeding behavior simultaneously.

CONFLICTS OF INTEREST
The authors declare no conflicts of interest.
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