Real-time, *in vivo* intracellular recordings of caterpillar-induced depolarization waves in sieve elements using aphid electrodes

Vicenta Salvador-Recatalà¹,², W. Freddy Tjallingii³ and Edward E. Farmer¹

¹Department of Molecular Plant Biology, University of Lausanne, 1015 Lausanne, Switzerland; ²Institute for Molecular Plant Physiology and Biophysics, Julius-von-Sachs-Platz 2, 97082 Würzburg, Germany; ³EPG Systems, Dillenburg 12, 6703 CJ Wageningen, the Netherlands

**Summary**

- Plants propagate electrical signals in response to artificial wounding. However, little is known about the electrophysiological responses of the phloem to wounding, and whether natural damaging stimuli induce propagating electrical signals in this tissue.
- Here, we used living aphids and the direct current (DC) version of the electrical penetration graph (EPG) to detect changes in the membrane potential of *Arabidopsis* sieve elements (SEs) during caterpillar wounding.
- Feeding wounds in the lamina induced fast depolarization waves in the affected leaf, rising to maximum amplitude (c. 60 mV) within 2 s. Major damage to the midvein induced fast and slow depolarization waves in unwounded neighbor leaves, but only slow depolarization waves in non-neighbor leaves. The slow depolarization waves rose to maximum amplitude (c. 30 mV) within 14 s. Expression of a jasmonate-responsive gene was detected in leaves in which SEs displayed fast depolarization waves. No electrical signals were detected in SEs of unwounded neighbor leaves of plants with suppressed expression of *GLR3.3* and *GLR3.6*.
- EPG applied as a novel approach to plant electrophysiology allows cell-specific, robust, real-time monitoring of early electrophysiological responses in plant cells to damage, and is potentially applicable to a broad range of plant–herbivore interactions.

**Introduction**

Propagating electrical signals in plants that display fast movements, such as the flytrap, promote their survival (Burdon-Sanderson, 1873). However, the physiological relevance of electrical signals in non-sensitive, non-carnivorous plants, which represent most plant species, is not well established. Electrical signals in non-exotic plants are usually triggered by the application of drastic stimuli, such as burning or crushing (Rhodes *et al.*, 1996; Zimmermann *et al.*, 2009; Favre *et al.*, 2011), which may not represent natural wounding events, such as, for example, those inflicted by chewing herbivorous insects. However, a recent study has shown that exposure to butterfly larvae has a long-term impact on the membrane potential of cells of caterpillar-chewed leaves (Bricchi *et al.*, 2012). This suggests that small wounds inflicted by insect herbivores induce electrical responses in plants. Although the magnitude of these responses may be smaller than that of the responses induced by harsh stimuli, characterizing the responses to natural wounding events is necessary to understand how plant ion channels and other electrogenic membrane proteins are integrated in the defense responses of plants to damage.

Since the 1980s, evidence has accumulated for the phloem as a signal propagation pathway for electrical signals (Fromm & Eschrich, 1988; Fromm & Bauer, 1994; Rhodes *et al.*, 1996). More recent work has reinforced the role of the phloem as a major tissue for electrical propagation, and has also shown that Ca²⁺ entry from the apoplastic compartment plays a major role in these electrical signals (Furch *et al.*, 2007, 2009; Will *et al.*, 2007, 2009). The conventional procedure for the recording of electrophysiological activity from the deeply located sieve elements (SEs) that form the phloem vessels is highly invasive, as it uses glass electrodes, and requires extensive manipulations to achieve a mechanically stable preparation. In an alternative approach, which takes advantage of the minimally invasive manner in which aphids insert their stylets into plant tissues, stylectomy is performed on aphids – and other stylet-bearing insects – during the prolonged feeding phase, that is, when the stylet is stably inserted into an SE; a glass electrode is then connected to the stylet stump (Fromm & Eschrich, 1989). A disadvantage of using this technique in the context of plant defense is that stylectomy, with either a radiofrequency cauterizing or laser device, is likely to damage the plant, inducing unwanted defense responses. In addition, this preparation is still not sufficiently delicate or robust to allow for repetitive measurements of electrical signals induced by mechanically disturbing wounding events, such as by a chewing caterpillar on a relatively small *Arabidopsis* plant. Minor vibrations with amplitudes in the order of micrometers would be sufficient to separate the cut stylet from the connected glass electrode.
In contrast, living aphids are able to maintain their stylets stably inserted in SEs of plants that are in constant vibration, a trait that has probably been selected for during the long evolutionary relationship of c. 280 million years between plants and aphids (Grimaldi & Engel, 2005).

The robustness of the plant–aphid interaction has been demonstrated in experiments in which the aphid is made part of an electrical circuit, termed EPG (electrical penetration graph; McLean & Kinsey, 1964; Tjallingii, 1978). In EPG, the aphid is attached to a thin gold wire electrode (Fig. 1a), easily allowing for vibrations of the order of millimeters, up to 1 cm. In the direct current (DC) version of the EPG circuit (Tjallingii, 1978), the intracellular position of the stylet tips in an SE is displayed as a highly stable, long-lasting voltage drop that lasts for at least several minutes, and usually for several hours. These EPG recordings of the SE membrane potential do not contain signals from muscle, nerve and other internal potentials of the animal’s body, as these are completely short circuited in the body fluids (Tjallingii, 1985). The input resistance of the DC-EPG amplifier is lower ($10^{9}\Omega$) than that of amplifiers used for intracellular recordings ($10^{12}\Omega$), and therefore sensitive to aphid–plant resistance changes, which may interfere with accurate estimations of the membrane potential. However, once the stylet tips are in the stable intracellular position, changes in membrane potentials of SEs can be accurately and precisely determined by DC-EPG, and therefore we used this method to acquire the electrical signals that are propagated in the phloem in response to damage. Here, we characterize the biophysical features of the electrical signals acquired from SEs of wounded and unwounded leaves of Arabidopsis plants, which were induced by *ad libitum* caterpillar chewing. Moreover, our data indicate that the remote electrical signal in the phloem of Arabidopsis depends on at least one glutamate receptor-like (GLR) channel.

### Materials and Methods

#### Plants and growth conditions

Wild-type *Arabidopsis thaliana* L. plants, Columbia-0 accession, were used in all experiments. Plants were soil grown in growth chambers at 23°C, in 70% humidity, with a light intensity of 100 µmol m⁻² s⁻¹ in a 9 h : 15 h light : dark (L : D) photoperiod. Plants between 4 and 5 wk of age were used for experiments.

#### Insects and rearing conditions

**Aphids** Cabbage aphid clones, *Brevicoryne brassicae* L., originated from single virginiparous females collected in Villa del Prado, Madrid, Spain. Aphids were reared on turnip plants,
Brassica rapa L., cv Just-Right, in a growth chamber with controlled conditions: 23°C : 16°C (L : D), photoperiod 16 h : 8 h (L : D). For experiments, aphids were glasshouse reared on cabbage, Brassica oleracea L., without supplemental lighting, and temperatures of 20°C : 18°C (L : D).

**Butterfly caterpillars** Cabbage white butterflies (*Pieris brassicae* L.) were donated by P. Reymond (University of Lausanne, Switzerland). Fourth and fifth instar caterpillars used in feeding assays were collected from a colony of *P. brassicae* raised on cabbage (*B. oleracea*) under controlled glasshouse conditions: temperature 24°C : 20°C (L : D), photoperiod 16 h : 8 h (L : D).

**Electrical penetration graph (EPG)**

Probing (stylet penetration activity) by *B. brassicae* aphids was recorded with a Giga-4 EPG amplifier (four-channel DC system; EPG Systems, Wageningen, the Netherlands). The aphid–plant combination and the EPG pre-amplifier (probe) were set up inside a Faraday cage. The pre-amplifier (OpAmp CA3240) had an input bias current of <1 pA, a bandwidth of 0–3.7 MHz and 50× gain. The input resistance was externally modified to 1 GΩ. Aphids for EPG recording were attached to a gold wire (ø18 μm) using water-based glue with silver particles, as originally described (McLean & Kinsey, 1964; Tjallingii, 1978), and placed on the adaxial side of Arabidopsis leaves of potted plants, with a copper plant electrode inserted in the soil. Gold wire lengths were 10–15 mm, as shorter lengths were not tolerated well by aphids, and longer lengths did not allow control of the aphid’s stylet insertion site. Approximately 10 min after wiring, aphids were placed on the plant for EPG recording. Almost immediately after leaf contact, aphids inserted their stylets into the leaf, thus initiating the pathway phase waveforms, which lasted for at least 0.5 h before entering into the phloem phase. Three extracellular waveforms that show a large overlap have been described, termed A, B and C, together referred to as the ‘stylet pathway phase’. Waveform A represents the first electrical contact, waveform B represents sheath salivation, and mechanical stylet penetration occurs during waveform C. During the pathway phase, many brief (typically c. 7 s) intracellular punctures are shown (Fig. 1b). In most cases, the phloem phase was achieved after several hours in the pathway phase. The phloem phase appears after an abrupt voltage drop of c. −100 mV caused by the intracellular penetration by the stylet tips. The phloem phase contains two EPG waveforms with low-amplitude fluctuations: E1, corresponding to an initial period of watery salivation into an SE, and E2, corresponding to SE sap ingestion with concurrent secretion of watery saliva (Tjallingii, 1978). In combination with stylectomy, electron microscopy and various other techniques, the main EPG waveforms have been correlated experimentally with specific insect feeding activities, as well as with the positions of the stylet tips in specific cell types (Kimmins & Tjallingii, 1985; Prado & Tjallingii, 1994). Typically, aphids make several probes with long pathway phases before a probe with phloem feeding. Phloem phases always start with E1 salivation into the SE for at least 1 min, and are then followed by E2 ingestion from the same SE. In most EPG recordings, the phloem phase was stable, that is, without significant voltage fluctuations. Whenever the phloem phase contained fluctuations exceeding 20 mV, they were not used for experiments. In all cases, the EPG output was adjusted during the first probe with respect to plant voltage (DC offset) and gain for optimal display of waveforms. Signals were acquired and analyzed using Stylet+ software (EPG Systems, Wageningen, the Netherlands) on a PC at a sampling rate of 100 Hz using a DI-710 A/D converter (Dataq, Akron, OH, USA).

The 10⁹ Ω input resistance (Ri) of the DC-EPG amplifier, although much higher than in the original AC system (McLean & Kinsey, 1964), is relatively low in comparison with the Ri (10¹²–10¹⁵ Ω) in conventional pre-amplifiers used in plant and animal electrophysiology. Consequently, all electromotive (emf)-originated voltages in the measuring circuit, of insect and plant origin, will be attenuated in accordance with the actual aphid–plant Ri resistance ratio. In order to infer the real voltage from the EPG-recorded value, a calibration pulse unit was added to the DC-EPG system. In this manner, the known voltage of this pulse (−81 mV here) will be attenuated to exactly the same extent as the values for the emf signal components, including the membrane potential values. As the resistance differs between the extra- and intracellular electrode position, a pulse response should be measured before and after the cell puncture. In practice, an accurate measurement of the voltage level during the extracellular stylet tip position (i.e. pathway phase) is hampered by the large voltage fluctuations during this phase (Fig. 1b). However, during the intracellular phloem SE phase, the voltage level is fairly stable and the small amplitudes of aphid-originated signals (mainly streaming potentials arising from salivation and ingestion) can easily be ignored. Using the pulse response calibration (Fig. 1b), the EPG-recorded aphid allows for accurate estimations of the SE voltage fluctuations. The data acquisition rate of 100 Hz is also adequate for sampling of the relatively slow signals in plants, whose kinetics are of the order of seconds. The electrical signals that originate in the insect arise either within the stylet canals or in the valves and pumps that are located on top of the stylet and connect it to the rest of the insect’s body. Signals arising from the insect nervous and muscular systems have never been observed in the EPG, as they are short circuited in the haemolymph-filled body space. When metal electrodes were inserted at different places into the aphid’s body during EPG recording, no signals other than the relatively slow EPG waveforms (maximum frequency of 40 Hz) were recorded (Tjallingii, 1985). The emf components contributing to the EPG are caused by streaming potentials arising from the flow of saliva and plant sap within the salivary and food canal, respectively. The rhythmic movements of the cibarial and salivary pumps during SE salivation and ingestion give rise to these small-amplitude (c. 5 mV) rhythmic streaming potentials.
Caterpillar handling for EPG experiments

Fourth and fifth instar caterpillars were starved for 4 h before the EPG experiments. A single caterpillar per leaf per plant was introduced while the aphid was in SE ingestion phase (EPG waveform E2). Caterpillars were gently placed on selected leaves with a fine brush, and left to feed undisturbed (ad libitum). Feeding usually started either immediately after placement or after a few seconds (<10 s). To study local electrophysiological responses to feeding, caterpillars were placed on the same leaf as the recording aphid. For consistency, all local responses were studied in leaf 8. The electrophysiological and genetic responses to distal wounding events were studied for the directly connected leaf pair 8–13, the less directly connected leaf pair 8–10 and the distally connected leaf pair 8–9. The anatomical model of the Arabidopsis vasculature (Dengler, 2006) was used to determine the level of connectivity between selected pairs of leaves.

Gene expression

Total RNA was extracted from selected leaves 1 h after single feeding bouts or wounding (i.e. leaf cutting) by caterpillars, and 1 μg was copied into cDNA using the SuperScript II first-strand synthesis system (Invitrogen) and oligo (dT) primers, according to the manufacturer's instructions. Real-time quantitative PCR analysis was performed on a 5-μl sample from the 20× diluted cDNA, in a final volume of 20 μl, according to the instructions in the FullVelocity SYBR Green instruction manual (Stratagene, La Jolla, CA, USA). Real-time quantitative PCR experiments were performed in an Applied Biosystems (Darmstadt, Germany) 7900HT cycler with the following conditions: 50°C for 2 min, initial denaturation at 95°C for 2 min, followed by 40 cycles of 10 s at 95°C and 30 s at 60°C, and 30 s at 72°C. Transcript levels were standardized to ubiquitin-conjugating enzyme. At least three biological replicates were used in each experiment. Quantitative PCR primers were: ubiquitin-conjugating enzyme (AT5G25760), 5′-CAGTCTGTTGTGATAGCCTATCATAG CAT 5′-AGAAAGATTCCCTGAGTCGAGTT; JAZ10.3 (At5G13220.3), 5′-AAGGAGAGTATGATTCTTCAACA AT and 5′-AGTAGGTACGTAATCTC.

Data analyses

All statistical analyses were performed with SPSS software v. 22 (IBM Corporation, Armonk, NY, USA). Value ranges are given as mean ± standard error. The Kolmogorov–Smirnov test and the Shapiro–Wilk test were run simultaneously to test for normality of the datasets, and the results were verified visually with Q–Q plots. If data were normally distributed, Student’s t-tests or one-way analysis of variance (ANOVA) tests were applied to assess the differences between the means. If data were not normally distributed, the non-parametric Mann–Whitney U-test or the Kruskal–Wallis one-way ANOVA test was used instead. The level of statistical significance for all tests was set at P<0.05.

Results

Intracellular recordings from SEs of Arabidopsis by DC-EPG

Using the DC-EPG technique (Fig. 1a), the feeding behavior of B. brassicae aphids on A. thaliana leaves was monitored. Recordings contained a pathway phase, corresponding to the intercellular navigation of the aphid stylet, with brief (c. 7 s) punctures into cells, and a phloem phase, corresponding to stable stylet tip position in the phloem. The phloem phase is easily identified in DC-EPG recordings, as it appears as a long-lasting voltage drop (Fig. 1b). Although the voltage drop recorded by this aphid electrode probably does not reflect the real membrane potential because of the amplifier sensitivity to intra-/extracellular resistance differences, this technique provides an accurate estimation of the absolute magnitude of the changes in membrane potential that may occur after wounding. For this purpose, we applied a calibration pulse during the phloem phase in order to account for the resistance of that particular SE. Figure 1(b) shows a typical calibration pulse that corresponds to -81 mV, which was used to derive intracellular voltage scales.

The first 1–2 min of the phloem phase contains small-amplitude, rhythmic, short upward waves (E1 waveform) that correspond to salivation bouts; the second, longer part of the phloem phase contains small-amplitude, rhythmic, short downward waves (E2 waveform) that correspond to sap ingestion bouts with concurrent salivation. These two waveforms during the phloem phase are clearly distinct, and are caused by streaming potentials in the narrow capillary stylet canals. In this study, we were primarily interested in the phloem phase of the DC-EPG as, in this phase, the four stylets of the living aphid are assembled into what can be effectively regarded as a living intracellular electrode, stably inserted in an SE. Since the feeding phase of B. brassicae aphids on A. thaliana was robust and stable, we set to test whether caterpillar wounding induces electrical signals in the wounded leaf, as well as in unwounded leaves. For this purpose, caterpillars of P. brassicae were used. Their feeding behavior on Arabidopsis is described in the next section.

Feeding behavior of P. brassicae caterpillars on A. thaliana rosettes

In order to characterize the feeding behavior of P. brassicae caterpillars on Arabidopsis rosettes, fourth and fifth instar caterpillars were placed in the center of each rosette, and left to feed ad libitum for 30 min. Typically, one caterpillar per rosette and 10 rosettes were used per feeding assay. P. brassicae caterpillars did not feed in a continuous manner, but in discrete feeding bouts. After carving a wound or cutting a leaf, caterpillars invariably moved away from the wound site. On average, caterpillars inflicted 15 wounds per plant. Wounds could be easily classified into: (1) feeding wounds at the leaf base, which removed up to one-quarter of the lamina, leaving the midvein largely intact, as if avoiding it; these are referred to as type I wounds, representing 40% of all wounds; (2)
leaf cutting, referred to as type II wounds hereafter; (3) holes in the lamina (type III wounds); (4) feeding wounds on the lamina around the middle or apex of the leaf, sometimes including the distal part of the midvein (type IV wounds); and (5) small bites in the petioles (type V wounds). This feeding pattern of *P. brassicae* caterpillars on Arabidopsis was highly reproducible. A representative outcome of this assay is shown in Fig. 2.

Feeding bouts on the basal lamina of Arabidopsis leaves trigger action potential-like depolarizations in nearby midvein SEs

Wired aphids connected to the DC-EPG circuit (‘aphid electrodes’) were placed along the midvein of the selected leaf, at either c. 5 mm or c. 10 mm from the leaf base, one aphid per test plant. Caterpillars that had been starved for 3–4 h fed immediately from the base of the leaf if placed on the petiole with their heads oriented towards the plant periphery (Fig. 3a, left). Caterpillars were only placed after the aphid had committed to phloem feeding. Figure 3(a, right) shows a typical depolarization wave acquired through an aphid electrode that had been inserted in the

![Fig. 2 Typical outcome of an ad libitum feeding assay for *Pieris brassicae* caterpillars on Arabidopsis thaliana rosettes. *P. brassicae* caterpillars that had been starved for 4 h were placed on the center of 5-wk-old Arabidopsis rosettes, at one caterpillar per plant, and left to feed for 30 min.](image)

![Fig. 3 Type I feeding wounds consistently induce depolarization waves in sieve elements of Arabidopsis thaliana leaves. (a) Left: diagram showing the localizations of the electrical penetration graph (EPG)-recorded aphid (aphid electrode) and the chewing caterpillar. Aphids were placed along the midvein at either c. 5 mm or c. 10 mm from the lamina base. Right: typical fast depolarization wave with the kinetic phases annotated: depolarization phase, from start to peak; first repolarization phase (Rfast), from peak to a deflection that marks the start of the second repolarization phase (Rslow). Box: the initial phase of the depolarization is shown enlarged. The precise start of the depolarization phase was approximated by eye as the time when the signal trace departs from the baseline. (b) Repolarization time data from depolarization waves acquired at both c. 5 mm and 10 mm from the leaf base were pooled, and are displayed in boxplot format (thick line, median; box, 25–75% quartiles; error bar, data within 1.5× the quartile range; circle, outlier). ***, *P* = 0.0000004; Mann–Whitney *U*-test. *N* values are indicated in parentheses. Although the combined dataset for Rfast follows a normal distribution according to the Shapiro–Wilk test, the combined dataset for Rslow data does not (*W* = 0.86, df = 15, *P* = 0.028). (c) Time intervals between the start of the feeding bout and the start of the fast depolarization wave for depolarization waves acquired at 5 and 10 mm from the leaf base. *N* values are indicated in parentheses. (d) Aphid electrodes on a marginal vein recorded relatively smaller depolarizations induced by type I wounds.](image)
same SE before, during and after the feeding bout. These depolarization waves reached maximum amplitudes of c. 60 mV within c. 2 s of initiation. Then, a repolarization phase followed, consisting of two subphases: a relatively fast first subphase that lasted for 2.2 ± 0.2 s, followed by a second subphase, which lasted for 14.4 ± 2.9 s (mean ± standard error) (Fig. 3b). The ingestion waveform E2 was carried over the upstroke and the first repolarization phase, and a switch to the salivation waveform E1 was always detected either during the second repolarization phase or at the end of it. The average time interval between the start of the feeding bout and signal acquisition was significantly smaller for aphid electrodes placed c. 5 mm from the leaf base than for aphid electrodes placed c. 10 mm from the leaf base (Table 1). Nevertheless, the time intervals between the start of feeding and signal display showed significant variability (Fig. 3c). Factors that probably contributed to this variability are: (1) different latent periods between the placement of caterpillars on the leaf and the start of the feeding bout, usually no more than a few seconds; (2) differing starting points of the feeding bout: the farther from the midvein, the longer the lag for the signal to appear; this suggests that damage to the midvein is the event that triggers the depolarization waves; and (3) the distance between the aphid electrode and the lamina base, which was also variable, as aphids often moved 1–2 mm from the site of placement before stylet insertion. For aphids placed c. 5 mm from the lamina base, the minimum value was 13 s; for aphids placed c. 10 mm from the lamina base, the minimum value was 30 s. This suggests an apparent velocity for the fast depolarization wave of c. 0.3 mm s⁻¹. The biophysical phenotypes of fast depolarization waves acquired from midvein SEs near (c. 5 mm) and far (c. 10 mm) from the wound in the affected leaf were indistinguishable; see Table 1 for a summary of the values of the maximum (peak) amplitude, and depolarization and repolarization times. Depolarization waves acquired by aphid electrodes in marginal veins on the leaf edge, following type I feeding bouts, were relatively slow and small (Fig. 3d). Type I wounds did not induce electrical signals in the SEs of neighbor leaves (Supporting Information Fig. S1), which are more directly connected than non-neighbor leaves, according to the model for the vascular architecture of Arabidopsis (Dengler, 2006).

Deep midvein wounds induce slow and fast depolarization waves in SEs of intact neighbor leaves

To test whether type II wounds (Fig. 2) induce electrical signals in unwounded leaves, three pairs of leaves with increasing distance between them were chosen. For each leaf pair, transmission of the signal in both directions was tested (i.e. mirror experiments). Most (90%) of the electrical signals transmitted between leaves 8 and 13, which are directly connected (Dengler, 2006), consisted of two depolarization waves: a first slow-rising depolarization and a faster-paced second depolarization wave, action potential-like in shape, superimposed on the first (Fig. 4b). The waveform of the electrical signals transmitted between leaves 8 and 10 was, in 67% of cases, indistinguishable from that of the signal transmitted between leaves 8 and 13, as they consisted of a slow depolarization wave with a superimposed fast depolarization wave (Fig. 4c). The action potential-like depolarization transmitted between the leaf pairs 8–13 and 8–10 appeared during the depolarization phase of the slow wave or in the early repolarization phase. The slow depolarization wave was the only electrical signal transmitted between non-neighbor leaves 8 and 9 in all cases (Fig. 4d), in 10% of cases between leaves 8 and 13, and in 33% of cases between leaves 8 and 10. The time intervals between the start of caterpillar cutting and signal display were (mean ± standard error): 48 ± 5 s for the 8–13 leaf pair, 49 ± 4 s for the 8–10 leaf pair and 47 ± 6 s for the 8–9 leaf pair (Fig. 4e). No significant differences between the time intervals for the three leaf pairs were detected. Since the minimum time interval is c. 20 s and petioles measure c. 1 cm, the approximate apparent velocity of the long-distance signal is c. 1 mm s⁻¹.

In order to determine whether the mechanical aspect of caterpillar wounding alone is sufficient to trigger depolarization waves, additional experiments were performed in which leaves were cut at the lamina–petiole junction with scissors, as this was the site chosen by the caterpillars to inflict type II wounds (see Fig. 2). For consistency with the experiments with caterpillars, the leaf pair 8–13 was used. A typical recording of a distally EPG-acquired signal induced by artificial cutting is shown in Fig. 4(f). These artificially induced distal depolarization waves were indistinguishable from those induced by caterpillar cutting. Since the average time interval between scissor cutting of leaf 13 and signal display in leaf 8 was 8 ± 1 s, the estimated velocity of the signal induced by scissor cutting is c. 2.5 mm s⁻¹. Table 2 summarizes the values of the main biophysical variables for the distally transmitted slow and fast depolarization waves.

### Table 1 Quantification of biophysical variables of fast depolarization waves acquired by direct current (DC) electrical penetration graph (EPG) from sieve elements of wounded Arabidopsis leaves during feeding bouts of *Pieris brassicae* caterpillars

<table>
<thead>
<tr>
<th>Biophysical variable</th>
<th>Location of the aphid electrode</th>
<th>5 mm (n = 7)</th>
<th>10 mm (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peak amplitude (mV)</td>
<td></td>
<td>56.3 ± 10.3</td>
<td>63.3 ± 12.7</td>
</tr>
<tr>
<td>Depolarization phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Rise time (s)</td>
<td></td>
<td>2.2 ± 0.5</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Repolarization phase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>First subphase or Rfast (s)</td>
<td></td>
<td>1.8 ± 0.3</td>
<td>2.5 ± 0.3</td>
</tr>
<tr>
<td>Second subphase or Rslow (s)</td>
<td></td>
<td>16.9 ± 4.9</td>
<td>12.6 ± 3.7</td>
</tr>
<tr>
<td>Lag between stimulus and response (s)</td>
<td></td>
<td>24.4 ± 2.9</td>
<td>56.8 ± 7.8*</td>
</tr>
</tbody>
</table>

*See Fig. 3(a) for description of the biophysical variables.

*Refers to the approximate distance between the EPG-recorded aphid, placed on the midvein, and the base of the lamina.

*Significantly different between 5 and 10 mm, *P* = 0.002 (Student’s *t*-test). All variables were normally distributed.

Values represent mean ± standard error.

Single caterpillar wounds induce significant expression of a jasmonate (JA) -dependent gene in both wounded and unwounded leaves

To test whether single wounds inflicted by caterpillars induce JA-mediated signaling, we measured the relative expression of a
well-established marker gene, JAZ10, by quantitative PCR. Our data show that type I wounds increased JAZ10 expression by c. 130-fold in the wounded leaf after 1 h, but did not induce the expression of this gene in a directly connected leaf (Fig. 5a). In contrast, leaf cutting or severe wounds to the midvein induced remote expression of JAZ10 in neighbor leaves, but not in non-neighbor ones. Thus, the signal that induces JAZ10 expression is transmitted between leaves 8 and 13, and between leaves 8 and 10, but not between leaves 8 and 9 (Fig. 5b).

The SEs of the double mutant glr3.3a glr3.6a do not produce depolarization waves in response to remote wounding. In order to explore whether the phloem plays a role in GLR channel-dependent leaf-to-leaf transmission of wound-induced electrical signals, DC-EPG recordings were acquired from the glr3.3a glr3.6a plants used in Mousavi et al. (2013). For consistency with the previous experiments, the leaf pair 8–13 was chosen; EPG-recorded aphids were placed on the midvein, at the base of leaf 8, and leaf 13 was cut with scissors at the petiole–lamina junction, in order to mimic caterpillar type II wounds.

**Discussion**

**DC-EPG as a tool for intracellular recordings of SEs induced by biotic stresses**

Here, we present DC-EPG as a suitable technique to acquire propagating electrical signals in SEs with sufficient detail and accuracy for quantitative analyses of biophysical features. In particular, we applied DC-EPG to monitor and record SE membrane potential changes induced by plant feeding by a chewing herbivore insect, a major source of biotic stress in plants. A significant advantage of using the EPG-recorded aphid over glass or styletectomy electrodes is its flexibility, given by the robust mechanical stability of the plant during stylet penetration. Aphids...
Table 2 Quantification of the main biophysical features of depolarization waves acquired from Arabidopsis sieve elements in unwounded leaves by direct current (DC) electrical penetration graph (EPG), induced by leaf cutting by Pieris brassicae caterpillars

<table>
<thead>
<tr>
<th>Leaf pair</th>
<th>n</th>
<th>% cases with fast wave</th>
<th>Duration (s)</th>
<th>Slow depolarization wave</th>
<th>Fast depolarization wave</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Amplitude (mV)</td>
<td>Rise time (s)</td>
</tr>
<tr>
<td>Caterpillar wounding</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>8–9</td>
<td>18</td>
<td>0</td>
<td>119 ± 10</td>
<td>35 ± 3</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>9 ⇒ 8</td>
<td>10</td>
<td></td>
<td>134 ± 13</td>
<td>36 ± 4</td>
<td>15 ± 1</td>
</tr>
<tr>
<td>8 ⇒ 9</td>
<td>8</td>
<td></td>
<td>101 ± 13</td>
<td>35 ± 5</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>8–10</td>
<td>12</td>
<td>67</td>
<td>114 ± 10</td>
<td>29 ± 4</td>
<td>13 ± 1</td>
</tr>
<tr>
<td>10 ⇒ 8</td>
<td>7</td>
<td></td>
<td>135 ± 10</td>
<td>27 ± 4</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>8 ⇒ 10</td>
<td>5</td>
<td></td>
<td>85 ± 12</td>
<td>31 ± 5</td>
<td>13 ± 2</td>
</tr>
<tr>
<td>8–13</td>
<td>10</td>
<td>90</td>
<td>100 ± 9</td>
<td>30 ± 5</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>13 ⇒ 8</td>
<td>5</td>
<td></td>
<td>100 ± 17</td>
<td>31 ± 9</td>
<td>12 ± 1</td>
</tr>
<tr>
<td>8 ⇒ 13</td>
<td>5</td>
<td></td>
<td>100 ± 11</td>
<td>29 ± 4</td>
<td>13 ± 4</td>
</tr>
<tr>
<td>Artificial* wounding</td>
<td>13–8</td>
<td>8</td>
<td>100</td>
<td>111 ± 24</td>
<td>40 ± 6</td>
</tr>
</tbody>
</table>

*Artificial wounding was inflicted by cutting with scissors at the junction between lamina and petiole.

Grouped data (in bold) for each leaf pair were compared statistically to test for differences in the biophysical parameters of the electrical signals transmitted between the different leaf pairs. All biophysical variables were normally distributed, except for the amplitude of the fast depolarization wave elicited by artificial wounding, according to the Shapiro–Wilk test (W = 0.791, df = 8, P = 0.023). The total duration and the biophysical variables of the slow and fast depolarization waves transmitted between the three pairs of leaves, following natural or artificial wounding, were not significantly different according to one-way analysis of variance (ANOVA) tests or, in the case of the amplitude of the fast depolarization wave, according to the Kruskal–Wallis ANOVA test. Values represent mean ± standard error.

![Graph](Image)

**Fig. 5** Caterpillar wounding induces the expression of a jasmonate-responsive gene, JAZ10.3, in both wounded and unwounded leaves of Arabidopsis thaliana. (a) Type I wounds induce significant expression of JAZ10.3 in the affected leaf (leaf 8), 1 h after wounding, but not in an unwounded but directly connected leaf (leaf 13). (b) For leaf pairs 8–13 and 8–10, leaf cutting of a leaf in the pair induces significant expression of JAZ10.3 in the other leaf. However, the signal that induces remote JAZ10.3 expression was not transmitted between leaves 8 and 9. For each independent experiment, three to four plants were tested, and each experiment was repeated at least once. Bars represent mean ± standard error. All independent experiments were combined for each pair of leaves for each mirror experiment. The number of plants is indicated in parentheses.

are well adapted for phloem SE feeding in open environments whilst plants are moved and vibrated by wind and rain. The aphid electrode needs no instrumentation for fixing plant parts or electrode manipulation. Each plant used in this study was exposed to a single aphid, usually during <12 h. A single aphid would have only a minimal impact on the expression of defence genes of the whole plant as the tissue damage is very small (Tjallingii & Hogen Esch, 1993). Stylet tracks follow an intercellular route and cells that are punctured by the aphid stylets survive very well, as shown by the inoculation success of plant viruses injected during such punctures (Fereres & Collar, 2001).

The information on ingestion/salivation included in EPG recordings may be useful for determining the ionic basis of depolarization waves, as it has been shown that the switch from ingestion (E1 waveform) to salivation (E2 waveform) is provoked by the entry of Ca²⁺ into the SE (Knoblauch et al., 2001; Will & van Bel, 2006; Furch et al., 2010). By chelating free intracellular Ca²⁺, the aphid’s saliva could, in theory, affect the shape and/or duration of the repolarization phase. In the fast depolarization waves, the E2–E1 switch appears in the middle of or near the completion of the repolarization phase (Figs 3a, 4b). Therefore, aphid salivation is not likely to significantly affect the duration/kinetics of the early repolarization phase, but could alter the duration of the late repolarization phase.

**Depolarization waves in SEs of A. thaliana induced by insect wounding**

The immediate and highly predictable feeding behavior by large white cabbage butterfly larvae permitted testing whether chewing induces electrical signals in the phloem, using the aphid electrode...
Research

Type I wounds (Fig. 2) induced a significant depolarization wave in midvein SEs of the affected leaves, but only relatively small depolarization waves of ≤ 20 mV in the marginal SEs of these leaves (Fig. 3), and no signals in directly connected leaves (Fig. S1) were detected. Since the botanical use of action potential refers to electrical signals that are transmitted without attenuation (Trebecz et al., 2006), we hesitate to classify these fast depolarization waves as action potentials. Nevertheless, given the extensive gaps in our knowledge of propagating electrical signals in plants, one cannot discard the possibility that different types of action potential exist in the plant kingdom, with differing biophysical properties and functions. The repolarization phase of the feeding-induced fast depolarization waves consists of two subphases (Fig. 3) that differ in their kinetics. What are their underlying mechanisms? One explanation is that there are two sets of repolarizing ion channels that activate on depolarization at different rates. Another possibility is that there is only one repolarization phase that is slowed down by a second, delayed depolarization wave. This second hypothetical depolarization could be caused by Ca\(^{2+}\) release from intracellular stores (e.g. the endoplasmic reticulum). In animals, Ca\(^{2+}\)- or depolarization-induced Ca\(^{2+}\) release from intracellular stores is a well-established phenomenon in both excitable and non-excitable cells (Fabriato, 1983; Islam et al., 1992).

To our knowledge, the intriguing leaf cutting behavior of caterpillars (Fig. 2) has not been described for Pieris, although it has been observed in another genus of the Pierid family (Dussourd, 2009). Leaf cutting induced large electrical signals in unwounded leaves that followed a consistent spatial pattern. Electrical signals transmitted between directly connected neighbor leaves (according to Dengler, 2006) contained two depolarization waves: a first, slow wave, and a second, faster paced wave similar to that induced by type I wounds. In contrast, only the slow depolarization wave was transmitted between non-neighbor leaves, suggesting that the fast wave cannot reach them. The rise time of the type I wound-induced fast wave is \(c. 2\) s, whereas the rise time of the type II wound-induced fast wave is \(c. 1\) s. An explanation for this disparity is that, in the unwounded leaf, the first part of the depolarization of the fast signal is masked by the slow wave (Fig. 4), which is absent in the electrical signal induced locally by feeding (Fig. 3). It seems likely that the display of the fast depolarization wave recorded from unwounded leaves does not depend on the occurrence of the slow depolarization wave; otherwise, the fast wave would be transmitted between non-neighbor leaves 8 and 9, which always transmit between them the slow wave.

The slow depolarization wave might correspond to a variation potential (VP), a plant-specific electrical signal characterized by its relatively slow kinetics and longer duration (Van Sambeek & Pickard, 1976; Stahlberg & Cosgrove, 1997). VPs are thought to be induced by hydraulic waves of pressure that propagate through the xylem (Malone, 1993; Stahlberg & Cosgrove, 1996, 1997; Stankovi & Davies, 1996). It is likely that leaf cutting at the leaf base, where the vascular bundle is thicker, induces a sudden loss of turgor, which is transmitted systemically through the vasculature as a hydrostatic pressure wave. In fact, small feeding bouts of Spodoptera caterpillars at the base of tomato leaflets induced a significant hydraulic signal, measured as an increase in thickness of distal leaflets (Alarcon & Malone, 1994).

Here, we show that distal electrical signals elicited by caterpillar cutting are indistinguishable from those elicited by a purely mechanical stimulus (Fig. 4, Table 2). Therefore, the mechanical aspect of insect chewing is sufficient to induce the full electrophysiological response in the SEs of unwounded leaves. These results are not incompatible with the notion that the caterpillar saliva contains factors that induce defensive responses. These factors have a number of biological activities, including the activation of indirect defenses (Bonaventure et al., 2011).

The remote wound-induced depolarization waves in the phloem depend on GLR channels

A recent study has shown that the remote, wound-induced electrical signals in Arabidopsis are completely abolished in mutant plants that lack expression of GLR3.3 and GLR3.6 (Mousavi et al., 2013). However, electrophysiological recordings were acquired in that study with non-specific surface electrodes that do not inform of the cellular substrates for these signals. Here, we tested whether the phloem of the same double mutant plants produces electrical signals in response to wounding, using the EPG technique.
approach to plant electrophysiology which uses aphid electrodes, presented in this article. Interestingly, no remote electrical signals were recorded from the SEs of ghr3.3a ghr3.6a plants, which indicates that GLR3.3, GLR3.6 or both are necessary for the production of both the slow and fast (action potential-like) remote depolarization waves. Therefore, it is conceivable that the depolarization waves produced by SEs in response to remote wounding contributed to the global wound-activated surface potentials (WASPs) detected with external electrodes by Mousavi et al. (2013).

Roles for fast and slow wound-induced depolarization waves in SEs

Wound-induced electrical signals have been functionally related to SE occlusion (legumes, Furch et al., 2007; cucurbits, Furch et al., 2010; Brassica, Will et al., 2009) and to Ca\(^{2+}\) hot spots in the phloem (Hafke et al., 2009). Wound-induced electrical activity and expression of proteinase inhibitor (PIN) genes in remote, unwounded leaves occur sequentially, suggesting a functional relationship between these two events (Wildon et al., 1992; Stanković & Davies, 1996; Davies et al., 1997). Here, we detected significant expression of the marker gene JAZ10 1 h after caterpillar wounding, in wounded and unwounded neighbor leaves, but not in unwounded non-neighbor leaves, consistent with previous studies (Yan et al., 2007; Glauser et al., 2009; Mousavi et al., 2013). Only the leaves whose SEs respond with fast depolarization waves to caterpillar-mediated leaf cutting show activation of the JA pathway, consistent with a role for the phloem vasculature in the activation of the JA pathway via its fast electrical signals. The findings that mild feeding wounds induce the expression of the JA pathway marker JAZ10 in the affected leaf only, and fast depolarization waves only in its SEs, and that these feeding wounds did not induce electrical signals or expression of JAZ10 in neighbor leaves (Figs 5, S1), together reinforce this hypothesis. Therefore, there is the possibility that the fast electrical signals in the phloem, or the molecular effectors underlying them, are integral components of the JA signaling pathway, or are relevant to the activation of this pathway in vascular and/or non-vascular cells. Here, it is important to note that non-electrical signals may also play a role in the remote activation of the JA pathway, because, although the remote electrical signal is completely suppressed in the ghr3.3ghr3.6 mutant, the reduction in the relative expression of JAZ10 was not completely abolished in this mutant (by c. 80% with respect to wild-type plants, 1 h after wounding; Mousavi et al., 2013). Our data do not provide clues regarding the role of the wound-induced slow depolarization wave in SEs. However, since this slow signal was never detected in wounded leaves (Fig. 3), which nevertheless show significant expression of JAZ10 upon wounding (Fig. 5), it is unlikely to play a role in the activation of the JA pathway.

Conclusion

The data presented here answer questions regarding biophysical, transmission, and molecular aspects of wound-induced electrical signals in the phloem vasculature. However, many aspects of these propagating electrical signals remain unknown, such as their molecular basis, and their cellular and physiological roles. The induction of fast depolarization waves, slow depolarization waves, or both, appears to depend exclusively on the type of wound (type I feeding vs type II cutting) and on the anatomical connection between the leaf of the responding SE and the wounded leaf. This is consistent with the hypothesis that all SEs are capable of producing these three different types of wound-induced electrical signal. This electrophysiological plasticity may represent a strategy for localizing the wound, and perhaps inducing the expression of an appropriate defense program in and near the SE/companion cell system.

Acknowledgements

We gratefully acknowledge the thoughtful comments of Aart van Bel (Justus Liebig University, Germany) on the manuscript. We also thank Elisa Garzo and Alberto Fereres (Instituto de Ciencias Agrarias, CSIC, Madrid, Spain) for aphids, and Philippe Reymond (University of Lausanne, Switzerland) for caterpillars. We also thank Professor Rainer Hedrich (University of Würzburg, Germany) for kindly providing space and materials to perform some of the experiments for this study. This research was supported by a Marie Curie IIF Grant to V.S.-R., and funding from SNSF (Grant 31003A–138235) and SystemsX.ch to E.E.F.

References


Fabiot A. 1983. Calcium-induced calcium release from the cardiac sarcoplasmic reticulum. American Journal of Physiology 245: C1–C14.


Hafke JB, Furch AC, Fricker MD, Van Bel AJ. 2009. Forisome dispersion in Vicia faba is triggered by Ca\textsuperscript{2+} hotspots created by concerted action of diverse Ca\textsuperscript{2+} channels in sieve elements. Plant Signalling and Behaviour 4: 968–972.


**Supporting Information**

Additional supporting information may be found in the online version of this article.

**Fig. S1** Feeding wounds by caterpillars do not induce electrical activity in the sieve elements of unwounded leaves.

Please note: Wiley Blackwell are not responsible for the content or functionality of any supporting information supplied by the authors. Any queries (other than missing material) should be directed to the New Phytologist Central Office.