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Measuring surface potential changes on leaves

Seyed A. R. Mousavi[a], Chi Tam Nguyen[a], Edward E. Farmer[a] & Stephan Kellenberger[b,*]

[a], Department of Plant Molecular Biology, University of Lausanne, Biophore, CH-1015 Lausanne, Switzerland

[b], Department of Pharmacology and Toxicology, University of Lausanne, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland

*, to whom correspondence should be addressed, Tel. ++4121 692 5422 Fax ++4121 692 5355, Stephan.Kellenberger@unil.ch

Summary

We provide here a detailed protocol to study changes in electrical surface potential of leaves with an emphasis on *Arabidopsis*. This method has been developed over the years by plant physiologists and is currently used in different variants in many laboratories. We record surface potential changes to measure long distance electrical signals induced by diverse stimuli such as leaf wounding or current injection. This technique can be used to determine signalling speeds, the connectivity between different plant organs and – by exploiting mutant plants – to identify transporters and ion channels involved in electrical signalling. Recently, the measurement of surface potential changes has allowed the identification of genes required for long distance jasmonate signalling after wounding. This approach can be combined with the analysis of mRNA expression and of metabolite concentrations to correlate electrical signalling to specific physiological events. Following wound infliction, surface potential recording takes ~15 min per plant.

INTRODUCTION

Electrical activity has important and established roles in rapid signalling in animals. Action potentials (APs) were, however, also observed early on in plants whose organs undergo rapid movements, such as *Dionaea muscipula* and *Mimosa pudica*[1-3]. In 1873 Burdon-Sanderson described the propagation of APs through the leaf of *Dionaea*. Bose and other researchers observed APs in *Mimosa* after wounding of a leaflet (reviewed in[1]). Electrical signalling was however also investigated in other plants and in 1926 Bose measured electrical signals in isolated vascular bundles of fern, to show that the electrical signals travel along these structures. In 1930, Umrath was able to record APs from *Nitella* with intracellular KCl-filled electrodes[1-3]. Several reviews on electrical signalling in plants written in the 70's indicated that all higher plants may use electrical signals to regulate various physiological functions [2,4].

The most commonly described types of potential changes are action potentials (APs) and variation potentials (VPs)[3,5,6]. APs are rapidly propagated depolarisations of the membrane potential, induced in an all-or-nothing manner and travelling with constant amplitude over distance. In many cases they also show a refractory period, meaning that during a certain interval after the passage of an AP, no further AP can be generated at a given location[3]. VPs or slow wave potentials consist of a transient depolarization followed by a long, delayed repolarization[7,8]. In comparison to APs, they display a longer repolarization and they show a large degree of variation. VPs can be induced by wounding, flaming or organ excision. Their amplitude is stimulus intensity-dependent and VPs are not self-perpetuating. The amplitude and speed of a VP decreases therefore with increasing distance from the injury site. Whereas long distance signalling by APs occurs mostly in the phloem, the VP is able to pass through dead xylem[7,9]. It is thought that VPs are caused by a transient shutdown of a H^+-ATPase in the plasma membrane[10]. In addition to APs and VPs a further type of potential change known as a system potential has been described. These latter signals are induced by wounding and involve a plasma membrane hyperpolarisation that is self-propagating and lasts several minutes[11]. Pharmacological approaches suggest that system potentials are due to an activation of the plasma membrane H^+-ATPase. Two basically different approaches for measuring electrical potentials in plants are mainly used, intracellular and extracellular recordings.

Extracellular recording

Types of extracellular recording. Extracellular recordings are widely used in animal electrophysiology. They measure the sum of the electrical activity of a large number of cells. Examples of such measurements that are much used in medical practice are electroencephalograms (EEG) and electrocardiograms (ECG). As described above, electrophysiological recordings were carried out early on in plants, and for many years, extracellular recordings were the only available technique for measuring potentials. In higher plants, two variants of extracellular recordings can be performed, 1) measurements using inserted metal electrodes or 2) surface recordings. In order to measure extracellular potentials there must be a complete circuit; therefore an additional electrode is needed. This may be a ground electrode put in the soil (as we do in our experiments) or a reference electrode at some other part of the plant[12]. Measurements using inserted (extracellular) electrodes were, for example, made with various tree species showing daily and yearly rhythms[13]. However, the insertion of electrodes inevitably causes wound reactions. By contrast, surface recordings are non-invasive. Electrodes for surface recordings are usually Ag/AgCl wires in contact with a KCl solution that is made viscous with agar[3,14]. The basic techniques of extracellular recordings were developed by early plant electrophysiologists and evolved in several directions[1,2]. A typical approach to surface recordings from *A. thaliana* is described in a recent article on AP generation in this plant[15]. Differences between laboratories consist mainly in the positioning of the ground electrode and the way by which the KCl solution, in which the Ag/AgCl electrodes are bathed, contacts the plant surface. In a variation of surface potential recording, glass microelectrodes are placed in the sub-stomatal cavity of open stomata in a way that the electrode tip makes contact with the aqueous phase of the apoplast[12]. Surface potential changes have been associated with wound-response gene expression. It was shown that changes in surface potential induced by mechanical wounding correlated strongly with proteinase inhibitor gene expression[16]. In the years that followed, there have been many other studies of wound-activated surface potential changes in a wide variety of plants and in some cases these studies have simultaneously investigated surface potentials and intracellular electrical activity[11,17].

Study of wound-induced electrical signalling in *Arabidopsis*. We carried out surface potential measurements on *Arabidopsis* to test whether long distance wound signalling is electrical[18]. Leaf wounding either used insect (caterpillar) feeding or was carried out manually with plastic forceps. Additionally, we used surface potential measurements on different leaves to investigate the leaf-to-leaf signalling. To mimic the arrival of a long distance signal in a distal leaf we injected electrical current between two platinum (Pt) electrodes placed in the leaf. Current injection was carried out with a stabilized current source controlled from the acquisition software. The surface potential recording determined the speed of propagation of the electrical signal and identified distant leaves that were electrically connected to the wounded leaf. Its combination with RT-PCR and RNA expression analysis showed that the speed and connectivity of the electrical signal correlated with that of jasmonate signalling and that current injection and wounding induced overlapping changes in expression of jasmonate-related genes. Finally, by screening mutant plants we identified *GLUTAMATE RECEPTOR-LIKE* genes (GLRs) as critical elements of long distance wound signalling. The electrophysiological methods used in that study are, however, broadly applicable to other plants including monocotyledons. Moreover, the method of surface potential recording can obviously be applied to other stimuli and to investigate other physiological responses.

Comparison with other methods

Measured changes in surface potential are indirectly related to electrical signalling events occurring in internal structures of the leaf. The comparison with chilling-induced surface potential changes, known to induce membrane depolarization, indicates that a negative deflection in surface potential corresponds to a membrane depolarization [18-20]. This approach does however not provide absolute values of membrane potentials, constituting a real limitation of the technique. The membrane potential of cells can be measured by intracellular recordings. To this end, glass microelectrodes with a fine tip (< 1μm in diameter) filled with a KCl solution contacting an Ag/AgCl wire or pellet are carefully inserted in the cytoplasm or the vacuole of the cell. A second electrode, the reference electrode, is placed in the bath surrounding the cell or the leaf containing the cell, and the potential difference between the two electrodes is measured, yielding an absolute value of the transmembrane potential

difference[21]. These intracellular measurements are invasive and are normally possible only for short periods of time (at most 1-2 h). Although surface potential recordings are only indirect measurements of electrical signalling, they have the advantage over intracellular recordings of being relatively easy to learn and rapid – which is an advantage for screening of mutant plants – and non-invasive, which is important for the study of wound signalling.

Electrical activity depends on ion transport and local ion concentrations can be measured with ion-specific microelectrodes, providing information on ion movements underlying the electrical signals[22]. Since changes in cytoplasmic Ca^{2+} concentrations may contribute to signalling, methods monitoring intracellular Ca^{2+} concentrations have contributed to the understanding of wound signalling mechanisms in plants[23]. Additionally, H^+ fluxes are important to determine membrane resting potentials[11] and also appear important in wound signalling[24]. For this reason, intra- and extracellular pH measurements are also used to analyse signalling.

Experimental design

Plant growth. Optimal growth conditions are more important for leaf-to-leaf signalling than for other types of experiments[18,25], therefore it is of crucial importance to grow the plants according to the conditions indicated below and to keep them strictly pest-free. We use 5-week-old *Arabidopsis thaliana* grown in soil, one plant per pot of 7 cm diameter, grown in light (100 µE s^{-1} m^{-2}) at 22 °C, 70 % humidity for 10 h and dark at 18 °C, 70 % humidity for 14 h and are watered gently. At 5 weeks the plants should look like the one shown in Fig. 2c. The plant should not have started to produce a flower stem.

Environment for recordings. Experiments are conducted in an air-conditioned room at 22 °C. The light is provided by a growth lamp positioned behind or beside the Faraday cage. The light intensity reaching the plant should be 100 µE s^{-1} m^{-2}. The plants are placed in the Faraday cage at least 10 min before the immobilization of the leaves.

Fixing the leaf in wounding experiments. If the leaf is to be wounded with forceps, it needs to be properly immobilized to reduce the wounding-induced artefacts to a minimum. This is done 5 min before the recording. In many experiments, larger leaves will be wounded. These leaves will grow beyond the rim of the pot (Fig. 1) and are therefore easily accessible to the forceps. Cut a support in the shape of a half-circle from soft plastic (e.g. from plastic document folders) as shown in Fig. 1, place it carefully under the leaves and attach it to the pot by adhesive tape. Place a smaller, rectangular piece of plastic over the leaf and fix it to the plastic support, as indicated.

Wounding the leaf with forceps. For wounding, press the leaf briefly between the forceps which are oriented in a way that the ridges are parallel to the long axis of the leaf. Inflict the first wound at the leaf tip, the second adjacent to it, and so on, until 40-50% of the leaf is wounded. The wounding procedure should be completed within ~10 s.

Wounding with insects. We routinely use 4th instar *Spodoptera littoralis* larvae for insect wounding. The insects are reared on cabbage and starved for 2 h prior to applying to *Arabidopsis* plants.

Preparation of current injection experiments. One day before the recordings, number the leaves if applicable[25], then place the injection electrodes in the appropriate leaf. Make sure that damage to the leaf is limited and that the Pt wires do not touch the soil. The plant will have one day to recover from the wound inflicted by the electrode insertion before the recording.

Single use of plants. Each plant is only used once per wound stimulation. For non-destructive stimulations such as current injection, several stimulations should be possible but would require prior analysis of the refractory period to determine the time the system requires for complete recovery.

MATERIALS

REAGENTS

Potassium chloride (KCl) (Sigma, www.sigmaaldrich.com , cat. no. P9541)

Agar (Sigma, cat. no A5306)

EQUIPMENT

- Solid table with a surface of ~80 cm x 60 cm (Vibration isolation tables are not necessary)

- Faraday cage of suitable dimensions to accommodate the measuring setup and the amplifier (homemade or from TMC, www.techmfg.com)

- A metal plate or breadboard as the basis of the measuring setup (TMC, 75 series or Newport, www.newport.com, SG series)

- Manual 3-axis micromanipulator with base and a clamp to hold the headstages; travel range of each axis of 20-40 mm (e.g. World Precision Instruments, www.wpiinc.com , cat. no. M3301). For recording at 4 positions, 4 micromanipulators are needed, if possible two left- and two right-handed. In some laboratories, homemade, cheaper positioners are used instead of micromanipulators.

- High impedance amplifier, e.g. FD223A (World Precision Instruments). For recording at 4 positions, two such dual channel amplifiers are required.

- Data acquisition interface and software, e.g. LabTrax-4/16 with Datatrax2 (or Labscribe) software (World Precision Instruments) or InstruTECH LIH 8+8 with ChartMaster software (HEKA Electronics, www.heka.com).

- Stabilized current source with the possibility of control by data acquisition software (homemade or DS3 Isolated Constant Current Stimulator/Stimulus Isolator from Digitimer Research Instruments, www.digitimer.com).

- BNC cables (BNC-to-BNC cable, World Precision Instruments, e.g. cat. no. 2851)

- Silver wire 0.5 mm diameter for Ag/AgCl electrodes (World Precision Instruments, cat. no. AGW2010) and adaptor metal piece (from local electronics supplier; Fig. 2a) fitting on the 2 mm jack of the headstages.

- Pt wire 0.1 mm diameter for current injection experiments (Advent Research Materials, www.advent-rm.com, cat. no. PT5401). Other materials used for making current injection electrode: isolated copper wire, tooth picks and adhesive tape.

- Plastic non-locking thumb forceps for leaf wounding (acrylonitrile butadiene styrene straight tip forceps, Sigma-Aldrich, cat. no. Z708356), whose tips are modified before use (see in EQUIPMENT SETUP).

- Lamp (halogen lamp, 230 VAC, 400 W, on stand, from hardware supplier)

REAGENT SETUP

Potassium chloride 10 mM / agar 0.5% (w/v) solution should be autoclaved or dissolved under heating and cooled down gently with agitation (magnet stirrer) to prepare a homogenous viscous solution. The solution is stored at RT and can be used for up to two months.

EQUIPMENT SETUP

Ag/AgCl recording electrodes Cut a 4 cm long piece of silver wire, bend one end by 180° to obtain a half-circle with a diameter of ~1 mm (Fig. 2a). Solder the other end to an adaptor piece matching the 2 mm jack of the headstage. Chloridize the curved end of the wire on 1-2 cm as described below, to coat it with a layer of AgCl. After a few uses the wires need to be re-chloridized. For chloridation, connect the cathode of a 1.5 V battery to a regular wire and insert its end in a HCl 0.1 M solution. Connect the Ag/AgCl electrode to the anode of this battery and dip its curved end 1-2 cm deep into the solution for a few tens of seconds. Ag atoms in the silver wire give up their electrons and combine with Cl^- ions in the solution to make insoluble AgCl, visible as a dark coating.

Ground electrode Prepare a straight Ag/AgCl electrode of 3 cm length, solder it to a wire that is connected to the amplifier ground and chloridize the electrode as described above. Cut the last millimeter of the fine end of a pipette tip, fill it with the KCl/agar solution and place the Ag/AgCl electrode in the tip (Fig. 2b). This ground electrode will then be stuck into the soil of the plant from which you record. After a few hours of recording, replace the KCl solution in the ground electrode.

Forceps Modify the tip of disposable forceps by gluing two straight 0.5 x 2 cm pieces cut from a mineral water bottle lid on the tips (Fig. 1b).

Current injection electrodes Solder a 0.5-1 cm long piece of Pt wire on a 15-cm insulated copper wire (you need two per electrode). Fix two 6.5 cm long tooth picks to each other with adhesive tape that they are aligned parallel to each other at a distance of slightly more than 1 cm. Fix the wires with tape and bend them in a way that the Pt wires are slightly out of the plane constituted by the two tooth

picks and that the distance between the two Pt wires is 1 cm (Fig. 3a). The electrode needs to be made in a way that when the electrode is fixed by the two tooth picks to the soil along the petiole, the two Pt wires are placed in the petiole at 1 cm distance from each other (Fig. 3b).

Arranging the recording setup Cut the upper half of a plastic plant pot and fix the lower half to the ground plate or to an elevated platform in the recording rig to be used as a support for the pots of the plants from which you measure. Place the amplifier headstages on the micromanipulators and attach a bent Ag/AgCl recording electrode to the tip jack of each headstage. Place the micromanipulators around the pot in a way that each of them can be easily manipulated and that the Ag/AgCl electrodes are in close proximity of the pot carrying the plant during the recording (Fig. 2c). Many amplifiers produce less electrical noise if they are placed on a shelf inside the Faraday cage.

Electrical connections and signal flow Connect the headstages to the amplifiers; use BNC cables to connect the signal output of the amplifiers to the input (A/D) jacks of the interface (1 cable per channel). If current injection is made under the control of the acquisition program (this is not possible with some interfaces such as the LabTrax-4/16), connect the chosen channel output (D/A) on the interface with a BNC cable to the current source. The interface in turn is connected to the computer via a USB cable.

Electrical grounding To minimize radiative electrical pickup (mostly frequency noise from light and power sockets), the measuring setup is placed in a Faraday cage and all metallic parts (cage, ground plate, micromanipulators) are connected to the signal ground of the amplifier. To avoid ground loops, connect all the wires used for grounding at one single point, e.g. a hub connected to the signal ground of the amplifier. This signal ground is in turn connected at only one place to the power ground that is provided by a wall socket.

Acquisition software Make sure that the channels of the interface that are physically connected by BNC cables to the amplifiers are correctly attributed in the software and that their gain is correctly entered (some amplifiers have a 10-fold or other gain on the output, this needs to be considered). Prepare a continuous recording protocol for durations of several minutes. The sampling frequency needs to be adapted to the speed of voltage changes and needs to be high for plants such as *Dionaea* and *Mimosa* whose organs undergo rapid movements involving rapid signalling, but can be lower with

plants that lack such fast signals, where sample frequencies of 40-100 Hz (corresponding to 40-100 samples per s) are typically used[15,18]. If the sampling frequency is too low, voltage changes may not be well resolved or may be distorted due to aliasing. For initial experiments we suggest therefore to use a relatively high sampling frequency, e.g. 1-10 kHz. Once the speed of the observed signals is known, the sampling frequency can be lowered in experiments of the same type to a rate that still allows resolution of the observed events, but makes smaller files.

Current injection It is important to control that under the chosen conditions of current injection the plant is not damaged at or between the sites of current injection. We have e.g. used injection of 40 µA for 10 s. For a distance between the two current injection wires of 1 cm this corresponded to a voltage of 12.7 ± 0.9 V [18].

Amplifier The amplifier needs to be turned on at least 30 min before recording to guarantee stable recordings. Before and between recordings the amplifier should be in standby mode. This clamps the voltage at the probe input near zero volts to protect the input.

PROCEDURE

Recording of surface potential changes **Timing: 15 min per plant (1 stimulation per plant)**

1. This step can be performed using option A if a leaf is wounded with forceps, option B if the leaf is wounded with insects or option C if current is injected into the leaf.

Option A – wounding with forceps

If applicable, number the leaves of the plant from which you will record[25], choose the leaf to be wounded and the leaves from which you want to record. Immobilize the leaf to be wounded as described (Experimental design, Fig. 1), wait for 5 min, then place the pot containing the plant in the support and position it in a way that this leaf is easily accessible from the front and that the leaves from which you want to record are accessible to the micromanipulators.

Option B – wounding with insects

Prepare the plant exactly as for option A. Then place a ring of 1-2 cm diameter (a section of a 50 ml Falcon tube, 2 cm in height) on the leaf to avoid insects escaping to other leaves during the experiment.

Option C - current injection

Place a pot containing a healthy plant with correctly placed Pt electrodes (see Experimental design, Fig. 3) in the support. Turn the pot in a way that the leaf in which the Pt electrodes were inserted faces the front. Be careful not to move this leaf to avoid any wounding around the Pt electrodes. Connect the injection electrode to the current source.

2. Place the ground electrode in the soil. Make sure that the ground electrode does not touch any leaves.

3. By gently controlling the micromanipulator, place the recording electrodes in the chosen positions on the leaves with their curved ends facing the leaves (Fig. 2c). The recording electrode should not touch the leaf surface to avoid any damage of the cuticle.

4. CRITICAL STEP Pipette 10 µl of KCl/agar solution into the space between recording electrode and the surface of the leaf. The KCl drop should have a round shape with a diameter of ~2 mm. The curved tip of the electrode should be immersed into the KCl/agar drop (Fig. 2c).

TROUBLESHOOTING

5. Switch the recording channels from “standby” to “operate” (or “record”). Adjust the voltage offset on the amplifier for each channel to 0. Observe during 1-3 min the stability of the baseline. Re-adjust the offset if necessary.

TROUBLESHOOTING

6. If the voltage readings on the amplifiers are stable, start the recording protocol and record the baseline.

7. This step can be performed using option A if a leaf is wounded with forceps, option B if the leaf is wounded with insects or option C if current is injected into the leaf.

Option A – wounding with forceps

After having recorded several tens of seconds of baseline, apply the wound to the leaf by plastic forceps (as described in Experimental design).

Option B - wounding with insects

After having recorded several tens of seconds of baseline put one or several insects (e.g. caterpillars) in the ring on the leaf and cover its top.

Option C - current injection

After a few tens of seconds of baseline recording induce the current injection from the acquisition software for the desired duration and amplitude.

TROUBLESHOOTING

8. Stop the recording when the potential has recovered close to the initial baseline, switch the recording channels to “standby” and discard the plant. This is 2-3 min for current injection and for wound-induced signals in distal leaves, and substantially longer (8-10 min) for wound-induced signals on the wounded leaf itself.

TIMING

The growing of the plants takes 5 weeks. The daily preparation of the setup, once the solutions and electrodes have been made, takes ~30 min, and recording of the wound-induced electrical surface potential change takes ~15 min per plant.

TROUBLESHOOTING

Troubleshooting advice can be found in Table 1.

To obtain reproducible recordings, careful attention has to be given to the following aspects. The same light and temperature conditions as in the growth room need to be provided during the recordings. The plants need to be grown in insect-free rooms and must be in good health. Water the plants on the day before the experiment; during the experiment the soil should be humid but the leaves must not be wet. Fix the leaves without injuring them. The curved tip of the Ag/AgCl electrode needs to be inserted well in the KCl drop, without however touching the leaf surface. Verify frequently the chloridation status of the electrodes and re-chloridize if necessary. Reproducible wounding by the experimenter is also critical. These skills develop with practice.

ANTICIPATED RESULTS

Once the recording is started, the surface potential is measured in real time on the computer screen by the acquisition software. In addition, the values are shown on the displays of the amplifiers. Figure 3c-

d illustrates a typical current injection experiment. Current (40 μA) was injected during 10 s between two Pt electrodes inserted in the petiole as indicated, and the surface potential was recorded from the leaf. Figure 4 illustrates a typical recording of a wound-induced surface potential change. The data were recorded with two electrodes that were placed on the leaves of an *Arabidopsis* plant as indicated. After ~1 min of baseline recording, leaf 8 was wounded with plastic forceps. After a short lag, the surface potential dropped by more than 50 mV. This amplitude depends on the extent of wounding and the position of the electrode[18]. Electrode e2, placed on the distal leaf 13 recorded a decrease in electrical surface potential that arrived later and reversed more rapidly than the signal in the wounded leaf. Figure 5 illustrates experiments with excessive electrical noise and with artefacts due to touching or approaching by the experimenter and by moving the leaf during wounding due to insufficient leaf immobilization or harsh wounding.

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AUTHOR CONTRIBUTIONS

S.A.R.M. developed the technique together with S.K and E.E.F and provided some figures; C.T.N. applied the technique, drafted the manuscript and provided most figures; S.K. and E.E.F. wrote the manuscript. All authors contributed to the final manuscript.

COMPETING FINANCIAL INTERESTS

The authors declare that they have no competing financial interests.

FIGURE LEGENDS

Figure 1 Immobilization of an *Arabidopsis* leaf for wounding with forceps. (a) Schematic illustration of the leaf fixation, top view. Only a part of the plant is shown with the leaf to be wounded drawn with

solid lines and the neighbouring leaves that would cover part of the support drawn with dashed lines. (b) Plastic non-locking thumb forceps used for wounding. Note that the tips are modified as described in EQUIPMENT SETUP.

Figure 2 Ground and recording electrodes. (a) Each recording electrode consists of a Ag/AgCl electrode with bent tip, soldered at its back end to an adaptor. (b) The ground electrode consists of an Ag/AgCl electrode inserted in a pipette tip filled with KCl/Agar solution. The scale bar in a and b represents a distance of 1 cm. (c) Illustration of the recording setup.

Figure 3 Current injection into leaves. (a) The current injection electrode contains a pair of Pt wires in a distance of 1cm, each soldered to insulated copper wire which is connected during the experiment to the current source. The pair of Pt electrodes are mounted on a scaffold made from tooth picks and adhesive tape. (b) The electrode is fixed in the soil in such a way that the two Pt electrodes are inserted into the petiole at a distance of 1cm, without touching the soil. The two arrowheads highlight the two insertion points. (c) Scheme illustrating the positioning of the Pt electrodes and the measuring electrode (e). (d) Surface potential change after 10 s / 40 μA current injection from a typical experiment. The timing of the current injection is indicated by a horizontal bar (C.I.) and arrows point to the artefacts in the signal due to current injection.

Figure 4 Traces from a typical recording of wound-induced surface potential changes. (a) Schematic indication of the site of wounding on leaf 8 and of the positions of the two recording electrodes on leaves 8 and 13. (b) Traces plotting the surface potential as a function of time are shown for the two electrode positions. The bar above the traces indicates the period of baseline recording (grey) and of wounding by forceps (black).

Figure 5 Traces illustrating technical problems. (a) Schematic indication of the site of wounding and the position of the electrodes. (b) Traces plotting the surface potential as a function of time are shown for the two electrode positions. The bar above the traces indicates the period of baseline recording

(grey) and of wounding by forceps (black). The trace recorded with electrode e2 showed electrical noise and an unstable signal due to insufficient chloridation. Artefacts induced by the experimenter (touching, approaching, wounding) are indicated by red arrowheads.

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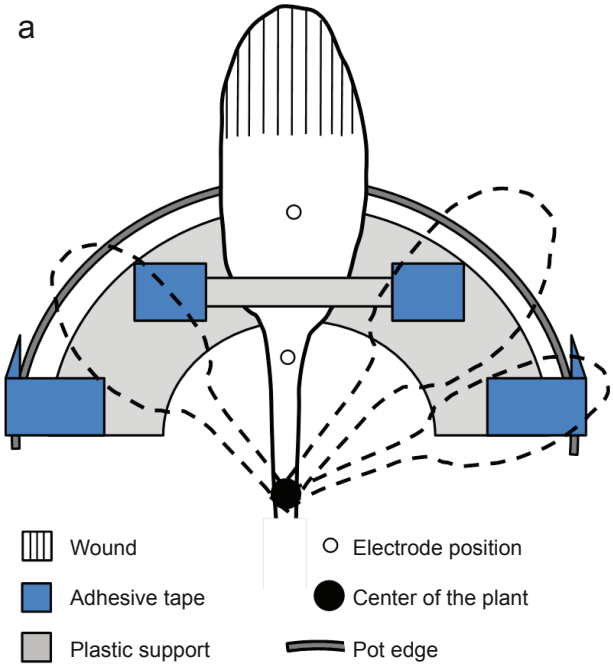
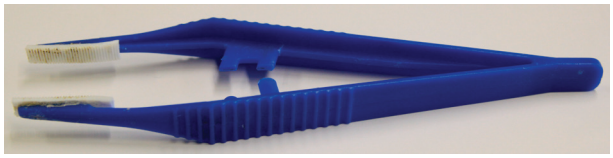
Table 1 Troubleshooting

Step	Problem	Possible cause	Solution
7	Abrupt electrical signalling artefact during wounding	Leaf moves while it is wounded with plastic forceps	Immobilize the leaf as shown in Fig. 1a Wound gently
5	Electrical signalling offset value is too high (not possible to zero the electrode potential at the beginning of the recording)	Soil is too dry	Water the plant adequately the day before doing experiments
4	The size of the KCl drop is too large	Leaf is not completely dry or KCl solution is not viscous enough	Water the plant the last time at least 12 h before doing the experiment; if the KCl solution is not viscous, prepare it exactly according to the instructions, if necessary increase Agar concentration
5, 7	Electrical line frequency noise (50 Hz)	Insufficient grounding of setup and/or presence of a source of noise in close proximity	Check and restore if necessary the correct connection to ground of the cage, all metal devices and amplifiers. Test whether switching off or moving of nearby possible noise sources affects the signal. Placing the amplifiers in the Faraday cage may also help reduce the noise.
5, 7	Unstable electrical signal	Ag/AgCl electrodes are dechloridized; or bad connections	Re-chloridize Ag/AgCl electrodes; check connections with Ohm meter and clean them if necessary
5, 7	Artefacts on the surface potential trace during the recording (Fig. 5)	Electrical signal disturbed by experimenter	Touch ground or Faraday cage when approaching or touching the platform
7	No change in electrical signal after wounding or current injection	One of the recording electrodes or KCl drop is connected to ground	Make sure that the leaf is well separated from the soil and the electrodes are not in contact with the soil

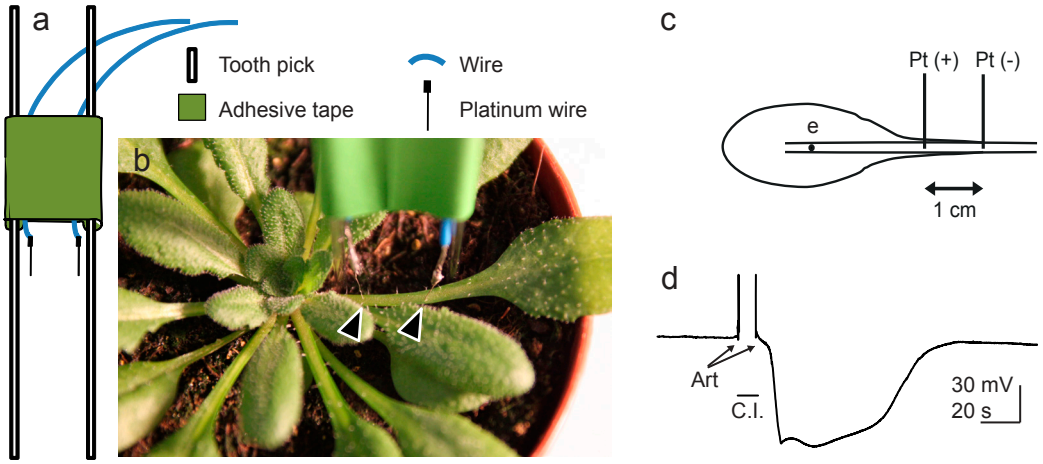
a



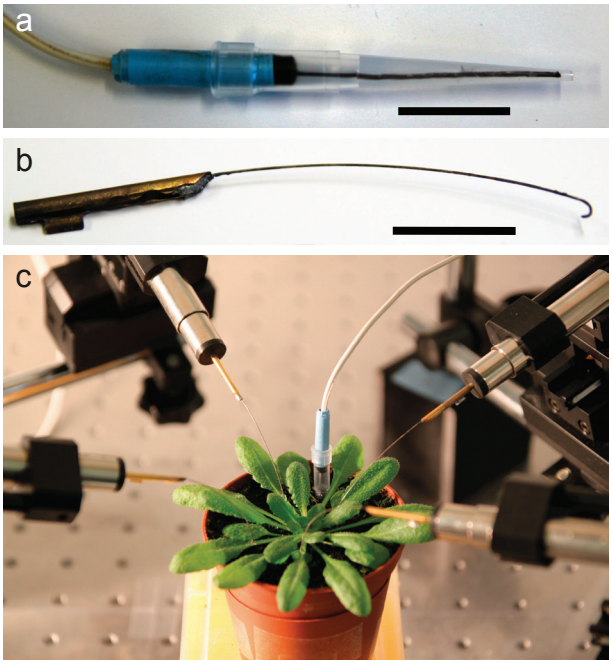
b



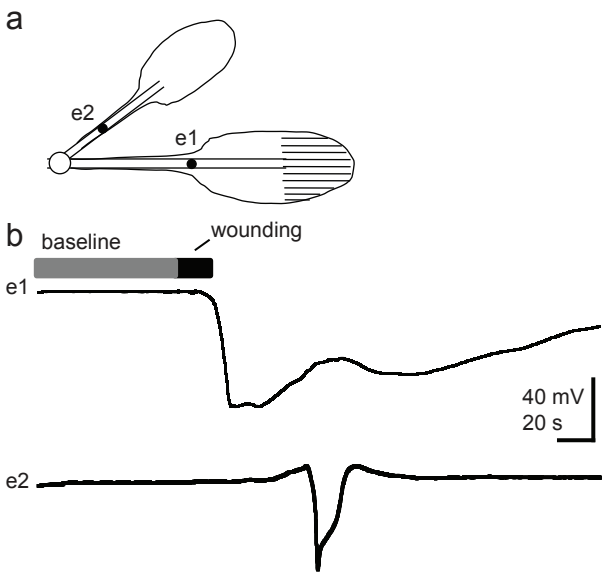
Mousavi et al., Figure 1



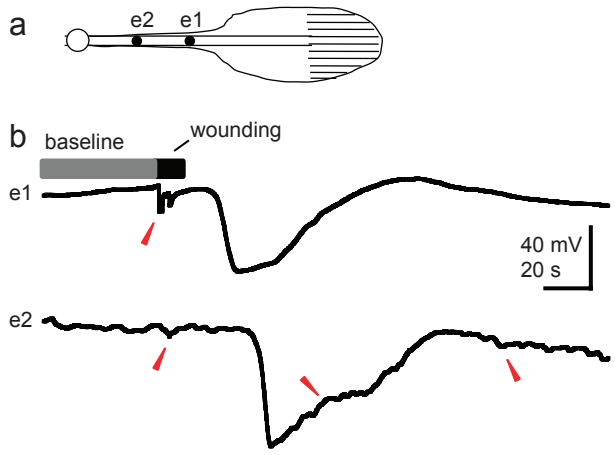
Mousavi et al., Figure 2



Mousavi et al., Figure 3



Mousavi et al., Figure 4



Mousavi et al., Figure 5