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# Touch-induced action potentials in *Arabidopsis thaliana*

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## Abstract

*Arabidopsis thaliana* (col accession) leaves were touched with a brush (30 mm<sup>2</sup>) in the middle of the leaf (equiv. weight ~1-2 g, for ~2 s). This soft stress generated reversible depolarizations that had most of the characteristics of so-called action potentials (APs). These electrical events moved away from the stressed zone at a speed of 1.3 mm s<sup>-1</sup>. The AP duration was ~10-20 s. We measured the extracellular electric potential with electrodes that were very thin silver wires inserted into the plant tissues at different distances from the touched zone. The APs could be clearly distinguished from the eventual artifacts (e.g., due to a moving electrode) because the latter were recorded simultaneously with the touching, whereas genuine APs were always delayed. The recorded APs had characteristics (amplitude, duration and propagation speed) that were very similar to previously described APs induced in the same plant tissue by electrical stimulation or touching or by cold-induced APs. Although the signals appear to be uniform (peak shape), we encountered a significant proportion of measurements that had more complex dynamics in which the signal moved away from the leaf until it was near the end of the petiole, near the center of the rosette, and then returned back to the leaf. We named these signals going-coming APs (GCAPs). We suggest that such complexity might arise from the vascular organization of the plant (i.e., many vascular bundles are present at locations where the electrodes were inserted in the plant) and from the extracellular nature of the measurements. This work confirms the high sensitivity of *A. thaliana* (Columbia accession) plants to another abiotic stress: touch. Moreover, this light mechanical stimulation can elicit APs without the simultaneous presence of other electrophysiological signals such as variation potentials.

**Keywords:** Arabidopsis, Action potential, electrophysiological signals, mechano-stimulation, touch, GCAP

## Introduction

### General introduction

Plants are sessile organisms. Because their environment (abiotic and biotic) changes dramatically over (long and short) time and space scales, they must coordinate (both locally and between distant parts) their internally induced responses, to allow for optimal growth and successful reproduction: plants obviously need a way to “sense” accurately their environment.

The question of plant “sensitivity” has been addressed for a long time with varying amounts of success. The “Sensitive plant” (i.e., *Mimosa pudica*) al-

ready described in Hooke’s (1665) book is emblematic in this respect. When touched, this plant reacts with a movement – folding its leaflets within few seconds – which is sufficiently rapid to be perceived by the human eye. Movements are a type of defense reaction to mechanical stress that is very understandable to us. They are usually interpreted as a reflex and/or as the symbol of will and are commonly considered to be a sign of life in a body. Early considerations about the concept of plant “sensitivity” were reported during the 17<sup>th</sup> century (see Webster, 1966). In animals, sensitivity turned out to be based on specialized excitable cells (neurons) and was mediated by a transient electrical signal, which led to the discovery of the nervous system (animal electrophysiology and neurosciences since the Galvani-Volta era at the 18<sup>th</sup> and early 19<sup>th</sup> century). At a molecular

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level, that signal is linked to membrane and ionic diffusion/transport and has protein transporters and carriers that can generate a self-propagating electrical transient signal along the neuron membrane called an action potential (AP). APs shift along the cell and convey information to the muscles (e.g., to move) and other neurons and cells (e.g., Dale et al. 2004).

In the middle of the 19<sup>th</sup> century, some botanists (e.g., Fée 1858<sup>1</sup>) did suggest that other plants, without visible movements, could also have a kind of “sensitivity”. It was only at the end of the 19<sup>th</sup> century, with the work of Charles Darwin, that this topic was advanced. After his book “*On the Origin of species*” (Darwin 1859), he published 3 books, among others, that were linked to movements and “sensitivity” in plants: “*Insectivorous plants*” (Darwin 1875a), “*The movements and Habits of climbing plants*” (in 1875b) and, in collaboration with F. Darwin, “*The power of movements in plants*” (Darwin and Darwin, 1881). In his “*Insectivorous plants*”, Darwin reestablished, among other findings, another forgotten but very remarkable organism: *Dionaea muscipula*<sup>2</sup> as a truly carnivorous plant. This plant traps small (animal) prey within two special leaves (lobes) and starts a real process of digestion. The prey itself triggers a fatal and very fast movement by touching twice, within a limited time interval; small hairs inside the open trap (see a recent review by Krol et al. 2011). The two lobes close together sufficiently fast (tenth of a second) to capture the prey, even a flying insect! Interestingly, the movement is dependent on a plant AP that travels from the sensor hairs to the tissues that are responsible for the trap closure. This AP was the first AP recorded in plants (Burdon-Sanderson 1873, 1888). It should be mentioned that Darwin directly suggested to Burdon-Sanderson the use of this plant for this purpose.

Upon contact, other plants revealed their “sensitivity” with movements that were visible to the naked eye, such as *Biophytum sensitivum* (Sibaoka 1973) and *Aldrovanda vesiculosa* (Iijima and Sibaoka 1982), and some that are even faster than can be seen (*Utricularia*, Vincent et al. 2011). It is interesting and not without significance that reviews that address “electrical signals” in plants have been linked primarily to these types of plants and their move-

ments (e.g., Sibaoka 1969). However, as can be easily observed, fast perceptible movements are not the rule in plants.

## ■ Distant and systemic signals in plants

Sanderson experiments in *Dionaea* and other studies on the sensitive (e.g., Houwink 1935, 1938 and Roblin 1979) showed that effects could also be distant and propagating, e.g., touching part of a sensitive leaflet can fold all of the leaves of the plants entirely in a type of visible propagation wave. This arrangement is reminiscent of the nerve - muscle information that is conveyed by APs in animals.

A recent result in *Arabidopsis* plants has added serious support to the involvement of plant electrophysiological signals in conveying information from one part of a plant to another distant part (Mousavi et al. 2013), in the absence of movement. Complex electrophysiological signals (Wound Activated Surface Potential: WASP) were elicited by caterpillars eating a leaf blade. Single short electrical peaks, longer depolarizations and even damping oscillations of the membrane potential have been described (Mousavi et al 2013; Extended data Fig. 1). Other abiotic stresses (wounding, electrical stimulation, but not touching) applied to the leaves of a plant also resulted in distantly elicited production of jasmonate, a phytohormone that is implicated in plant defense and communication (Farmer and Ryan, 1990; Okada et al., 2014) in distant unstressed leaves. Distant signaling in plants is also called systemic signaling, which is not so new *per se*. Indeed, in vascular plants, the circulation of water and mineral nutrients from the soil to the shoot takes place in the xylem tissue and from sources (photosynthetic leaves or reserve tissues) to sinks via the phloem. Both of these tissues comprise the vascular plant system that interconnects the different plant parts (see, e.g., Taiz and Zeiger 2010). In fact, many molecules, namely phytohormones, can circulate in these ways or via other cell-to-cell interconnections in other tissues (e.g., Auxin) and even some in the form of gaseous substances (e.g., ethylene) (see Taiz and Zeiger 2010). Although measurements are missing for *Arabidopsis*, the velocity of phloem sap in plants is in the range of 0.05 to 0.4 mm s<sup>-1</sup> (Lüttge and Higginbotham, 1979; Windt et al. 2006; Mullendore et al. 2010; Savage et al. 2013) and for xylem in *Ricinus* 0.2-0.4 mm s<sup>-1</sup> (Peuke et al. 2001). The term systemic signaling, however, concerns more fast signals, which likely move faster than the usual xylem or phloem transport rates (Baluška 2013) and/or are not directly transported within these tissues in the sap fluxes. See also, for a slightly different definition, Baxter et al. (2014).

<sup>1</sup> In: Fée (1858): «Aussi pensons-nous qu'il existe des plantes, à tissus tout aussi excitables que ceux de la-Sensitive, qui cependant ne peuvent se mouvoir, faute d'organes appropriés au mouvement. Ce n'est pas assez que d'avoir-la faculté, il faut encore avoir l'instrument.» p. 460.

<sup>2</sup> In: Darwin (1875)a: «This plant, commonly called Venus' flytrap, from the rapidity and force of its movements, is one of the most wonderful in the world.» p. 286.



Systemic signaling has been found to involve a significant number of physiological/developmental processes after abiotic and biotic stresses according to Baxter et al. (2014), as follows:

- (1) Systemic acquired resistance (SAR), in response to viruses, bacteria and fungi.
- (2) Systemic wound response, in response to insect attacks or mechanical injury.
- (3) Systemic acquired acclimation (SAA), in response to local high light, UV, heat, cold, or salinity.
- (4) Systemic metabolic response, in response to local changes in sugars, phosphates, and other metabolites.
- (5) Systemic developmental response, in response to light conditions, CO<sub>2</sub>.

For this fast systemic signaling, the local initiation process involves changes in Ca<sup>2+</sup> or ROS (reactive oxygen species) concentrations and/or membrane electrical polarization (membrane potential). In fact, three types of «waves», Ca<sup>2+</sup>, ROS and electrical, apparently propagate at similar speeds (in the range of a few mm.s<sup>-1</sup>, which suggests a not yet completely elucidated coordination between them. The propagation of the fast signals also includes these elements (and perhaps others) in an autocatalytic way (Baxter et al. 2014). Interestingly, in *Arabidopsis*, fast moving signals have been observed for Ca<sup>2+</sup> (see Xiong et al. 2014), ROS (Miller et al. 2009, Gilroy et al. 2014) and extracellular electrical potentials (Favre and Degli Agosti, 2007, Mousavi et al. 2013), the front of which move at a speed of ~mm s<sup>-1</sup>.

Recent contributions have also shown Ca<sup>2+</sup> waves moving in plants, with a systemic root-to-shoot signaling control:

- (6) Systemic salt signaling, in response to root salt stress (Stephan and Shroeder 2014; Choi et al. 2014).
- (7) Systemic flowering protein FT, in response to a photoperiod or flowering inducing conditions (Shalit et al. 2009; Liu et al. 2013; and see also Marti and Webb (2014) and Endo et al. (2014) Fig 4.).
- (8) Systemic rapid carbon allocation in relation to apical auxin dominance, in response to decapitation (Mason et al. 2014; Van den Ende 2014).

Moreover, the distribution of nutrients in a plant (i.e., photosynthesis products) through the phloem is also rapidly affected by stress (cold, prick, cutting, burn). This arrangement is realized by transient occlusion by the forisome complex (Thorpe et al. 2010) along the tissue (i.e., distant from the locally wounded site). It has been recently proposed that these fast responses (seconds to minutes) are controlled by Ca<sup>2+</sup> and that electrophysiological signals move along the phloem tissues (Van Bel et al. 2014):

- (9) Systemic phloem translocation rate control, in response to cooling, pricking, cutting, burning.
- (10) Systemic glucose content regulation during photoperiodic changes, in response to pricking, cutting, ozone stress (Degli Agosti, 1985; Degli Agosti and Greppin, 1998; for the importance of glucose as a regulatory molecule in plants, see Sheen, 2014).
- (11) Systemic peroxidase activity, in response to Red and Far-Red light changes (Karege et al., 1982).
- (12) Systemic suppression of nodulation via root-to-shoot long distance signaling (Soyano et al., 2014).

Clearly, little doubt remains that plants coordinate activities in space and time, with more subtle signals and in a more rapid and complex way than commonly thought. For many of these processes (1-6, 9), electrophysiological signals are directly implicated or proposed to participate in the systemic signaling network (e.g., Baxter et al., 2014; Gilroy et al., 2014; Steinhorst and Kudla, 2014; Van Bel et al., 2014).

## ■ Electrophysiological signals in plants

The history of electrophysiological signals research in plants is rather long and somehow too complex to be detailed here. Indeed, electrophysiological signals in vascular plants are more versatile (see Pickard, 1973), variable and diverse than in animals. Moreover, their naming/definition and classification could be confusing. For introductory reviews on this topic, the interested reader can consult recent reviews (e.g., Fromm and Lautner, 2007; Król et al., 2010; Zimmerman and Mithöfer, 2013). At least three types of electrical signals can be recorded in plants: action potentials (APs, which are similar to those recorded in animals), variation potentials (VPs) and system potentials (SPs) (Zimmerman and Mithöfer, 2013). These three types of signals are often mixed in recordings that are obtained in plant tissues, which unfortunately make deciphering them, a very complex task.

Plant APs appear to share common properties with animal APs, although these properties have not always been tested. They are (1) the characteristic depolarization and repolarization phases, (2) the all-or-nothing law (i.e., the stimulation level must be above a threshold to yield a response), (3) the self-propagation property and (4) a refractory period (i.e., a minimum interval of time that separates two excitations before a full AP can again be elicited). In contrast to animal APs, in which the amplitude is constant along the path of propagation, it is often observed that the amplitude of plant APs changes slightly. For example, in *Arabidopsis*, the amplitude of APs that are measured with extracellular electrodes appears to increase from the leaf to the petiole



(Favre and Degli Agosti 2007, and present work). In different plants (Zimmerman and Mithöfer, 2013), the AP duration is heterogeneous (median 60-90 s), and the propagation median speed is 2-5 mm s<sup>-1</sup>. So-called variation potentials (VPs) are another type of propagation of electrical events in plants. Their median duration is significantly longer (900-1050 s) and their amplitude decreases with increasing distance from the stimulated zone, but their median speed of propagation is similar to APs (0.9-1.3 mm s<sup>-1</sup>). They can pass dead tissue (Roblin 1985; Roblin and Bonnemain 1985). VPs are often superimposed with putative APs and sometimes other oscillations of the membrane potential (Favre et al. 2011, Mousavi et al. 2013). For comparison, the AP time frame duration in animals is of the order of 10<sup>-3</sup> s, and the propagation speed is 10 000 mm s<sup>-1</sup> (Dale et al. 2004), which is thus several thousand times faster.

The basis of these electrophysiological signals is ionic, which is the same as for animals (Hille 2001). Mathematical models have been built to obtain simulations of APs and VPs in plants. These models are based on the present knowledge of the ion distribution within plant tissues and the kinetics properties of the membrane transporters (pumps, channels and carriers) of Ca<sup>2+</sup>, Cl<sup>-</sup>, K<sup>+</sup>, H<sup>+</sup> ions (for APs: Sukhov and Vodeneev 2009, for VPs: Sukhov et al. 2013). Present knowledge of the succession of ionic events that underlie the electrical signals of plants is essentially based on such models.

Physiological effects of these signals (AP, VP and SP) are very diverse. Some of these effects have been presented in Favre and Degli Agosti (2007); Fromm and Lautner (2007); Król et al. (2010); Zimmerman and Mithöfer (2013), Van Bel et al. (2014). In addition to their effects on movement and their implications to the distant systemic signaling already described below, it is important to mention that electrophysiological signals are implicated in the control of the most characteristic significant physiological process of plants, which is photosynthesis (see Sukhov et al (2014) and the references cited therein).

Currently, it is considered that in plants, these signals (AP) are conveyed mainly via the phloem tissues, with a participation of nearby cells/tissues (see, e.g., Fromm and Lautner, 2007; Van Bel et al., 2014). However, in some cases, e.g., as *D. muscipula*, the AP is transmitted in whole lobes, apparently in tissues that are devoid of conducting tissue (Stănescu et al., 2008; see also Dziubinska, 2003). In *Conocephalum conicum*, a non-vascular plant, APs are elicited locally and transmitted to the remainder of the tissue (Paszewski et al., 1982). In *Phycomistrella patens*, a bryophyte that is also devoid of conducting tissue, APs have also been reported

(Koselski et al. 2008). Finally, in a single-celled unicellular algae *Thalassiosira pseudonana* (diatom phytoplankton), APs have been observed (Taylor 2009). Cellular electrical excitability has been found in very primitive animals, e.g., sponge, (see Mackie 1970), and the conduction of excitability appears to be an ancient life property because it is reported also in animal organisms that are devoid of nerve cells (Mackie 1970). In plants, Goldsworthy (1983) suggested that electrophysiological signals could be the remnant signs of very primitive initial evolutionary cellular activity for repairing membrane integrity. We can mention here studies on artificial membrane ("proto cells") in which electrical activity was observed (Przybylski et al. 1982). Indeed, one can imagine that changes in the membrane potential (i.e., the electric potential between interior and exterior compartments due to the ionic differences between them) is among the earliest signals that an ancient protocell in early biological evolution could have had as a sign of change in the environment and/or the cell's integrity. Using the membrane potential as a source of cellular and intercellular signaling of environmental changes should thus be obvious for plants, also. However, plant electrophysiology is not fully inserted into mainstream basic plant science (with the exception of Characeae; see, e.g., Beilby 2007). This lack is surprising when one considers the actual state of advancement in plant cell and single channel electrophysiology. Indeed, a very complex set of ionic membrane transporters, channels and carriers has been discovered and characterized since approximately 40 years ago and has recently been reviewed by Hedrich (2012) for plants. Clearly, all of the molecular elements/equipment are present for electrophysiological signaling to occur at a whole, inter-organ, tissue and/or cellular (e.g., stomatas) integration level.

## Touch and mechanosensing in plants

A primary interaction of a living organism with its environment lies in mechanical (i.e., touch) contact. In fact, at the molecular level, touch sensing is known to rely on specific protein sensors (e.g., stretch-activated channels) in all living cells, from bacteria to animals, including plants (e.g., Gillespie and Walker 2001; Vogel and Sheetz 2006; Sachs 2010; Haswell et al. 2011; Iida et al. 2014). Common sense should tell us that between the force exerted by a huge herbivore such as the elephant, when it snatches part of the plant to eat it, and the tiny force exerted by the pathogenic fungi appressoria when penetrating a single plant's epidermal plant cell (~17 μN; Bechinger et al. 1999), a vast number of abiotic and biotic touch interactions can exist at time, space and intensity scales.



Plant researchers were aware of touch “sensitivity”, as already illustrated by the works on *Dionaea*, *Mimosa* and *Drosera* (see also Darwin, 1875a, Darwin and Darwin, 1881). However, these plants were considered to be some types of specific systems. Thus, “sense” has been relatively underestimated in other plants in mainstream plant science until recently. However, some researchers were aware of the effect of touch on other plants in general; they even created a new term for it; namely, thigmomorphogenesis (see Jaffe, 1973; Jaffe et al., 2002; Chehab et al., 2009). The physiological and morphological effects of touch, mainly a reduction in growth, were observed by Boyer (1967) and Turgeon and Webb (1971). In 1990, a key paper was published by Braam and Davis (1990) that showed the control of specific genes that were linked to  $\text{Ca}^{2+}$  cellular control after touch in *Arabidopsis* plants (see also Braam 2005). This treatment has also been shown to rapidly (seconds) affect intracellular  $\text{Ca}^{2+}$  in seedlings of this plant (Knight et al. 1991, 1992). A review was published by Telewski (2006) that proposed that the cascade  $\text{Ca}^{2+}$  – plasma membrane voltage (i.e., electrophysiological signals) changes with the initial rapid events in the touch effects. It is worthwhile mentioning that electrophysiological signals (APs) after touch were suggested to be involved early in research studies with *Characeae* (Shimmen 1966; Kishimoto 1968) and that mechanosensory channels in the membrane are present (Shepherd et al 2002; Kaneko et al 2005). Currently, mechanosensing at the cell level is thought to participate in the cell and, finally, the whole plant architecture (growth and development) (Hamant 2008, 2013; Monshausen and Gilroy 2009; Monshausen and Haswell 2013). Moreover, mechanical interactions are thought to participate in early events between pathogenic fungi and fungi that are symbiotic with plants (Jayaraman et al., 2014). Cross abiotic – biotic protection (i.e., enhanced protection by a previous innocuous mechanical – touch stress) has been shown (Wick et al., 2003; Chehab et al., 2012). Benikhlef et al. (2013) have obtained evidence for the initial implications of intracellular  $\text{Ca}^{2+}$  and ROS signals in the enhancement of this plant resistance (see also Kurusu et al., 2013).

### ■ Aim of this paper

Although we have regularly used wet electrodes for a long time now, they are somehow large relative to the plant size and not easy to position. They might exert a small (equivalent ~3 g) mechanically interfering pressure. Furthermore, the electrode solution flows from the electrode to the extracellular liquid (apoplast) and could potentially modify locally the membrane equilibrium. We thus tested directly if

mechanostimulation by touch could also elicit APs that could be measured with a different method, i.e., by thin silver wires (dry and non-chloridized) inserted extracellularly near the touched place. Preliminary experiments of rubbing (Favre, 2004) and touching (Thouroude 2011) have shown that *Arabidopsis thaliana* could elicit APs when the leaf was stressed in these manners. We undertook here to test and report whether a relatively soft, rapid and localized touch to the leaf by a brush could reproducibly elicit APs in *A. thaliana* with similar characteristics to «genuine» electrically induced APs (Favre and Degli Agosti 2007), here recorded with a different measurement method.

## ■ Materials and methods

### Plant material

*Arabidopsis thaliana* (L.) Heynh accession Columbia (Col = Col-0) – wild type plants – seeds were sown in potting compost under a L:D (light:dark) photoperiod (8 h:16 h) for 3 weeks. Seedlings were individually transplanted into new pots (L×W×H=9cm×9cm×10 cm) and cultivated under L:D (8 h:16 h, Sylvania 36W Luxline-Plus, 75  $\mu\text{mole}^{-2} \text{s}^{-1}$  PAR). Once they reached 42- to 60-day old, the plants were transferred to a thermo- and hygro-regulated room (22±1 °C and 73±2% rH) under L:D (12 h:12 h) for measurements in the experimental plant chamber (fluorescent tubes, Sylvania, 18W Standard, 115  $\mu\text{mole}^{-2} \text{s}^{-1}$  PAR).

### Electrophysiological measurements, dry implanted silver wire electrodes

The characteristics of the electrometer (impedance:  $10^{15} \Omega$ ) and the A/D D/A card have been described in previous papers (Favre et al. 2001; Favre and Degli Agosti 2007). The plants, the measuring installation and the touch stimulation zone are shown in Fig. 1. The electrical potential recorded is the difference between a measurement (E1 for leaf and E2 for petiole) and the reference electrode (Eref) with respect to the electrical earth. In the present paper, we used another type of extracellular electric potential measurement: silver wires (ca. 1 cm long, diameter 0.125 or 0.25 mm, WPI Inc, US). These wires were soldered to a very thin, flexible and insulated copper wire (0.1-mm diameter, DISTRELEC, CH), which allowed the plant movements to occur with negligible mechanical constraints from the electrodes. These electrodes were connected to the electrometer as described in Fig. 1. The insertion was performed one day or more before the experiments. Dry (non-chlo-



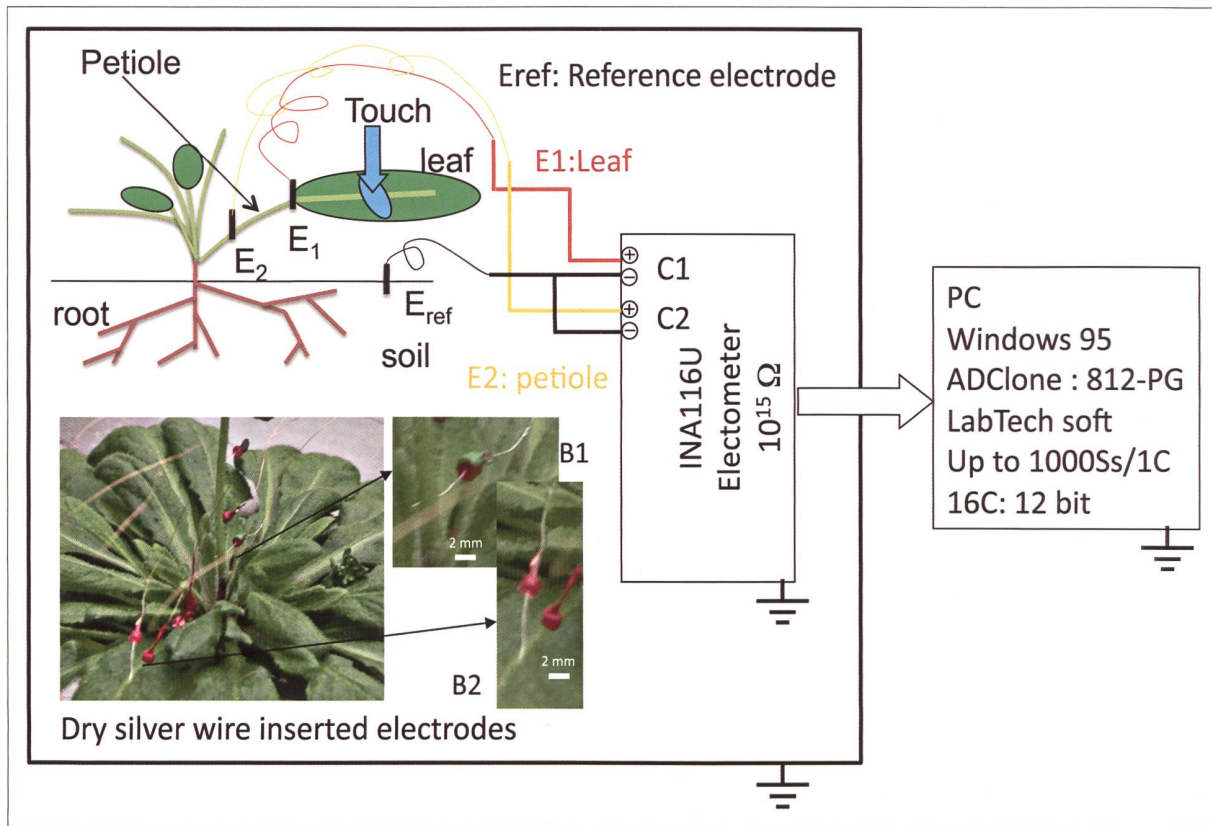


Fig. 1. Diagram of the measuring set-up of electrophysiological signals in *Arabidopsis thaliana*. Plants and electrometer (16 differential channels, INA116,  $10^{15} \Omega$ ) are within a Faraday cage. The acquisition is via an A/D board (for more details, see Favre and Degli Agosti, 2011; Favre et al. 2007). Dry silver wire extracellular electrodes are positioned at the end of the leaf (E1: Leaf) and at a second position on the petiole (E2: petiole). Inserted electrodes are connected to the electrometer with a very thin copper wire. The insertion in the floral stem (B1) and in the leaf vein midrib (B2) is shown in more detail.

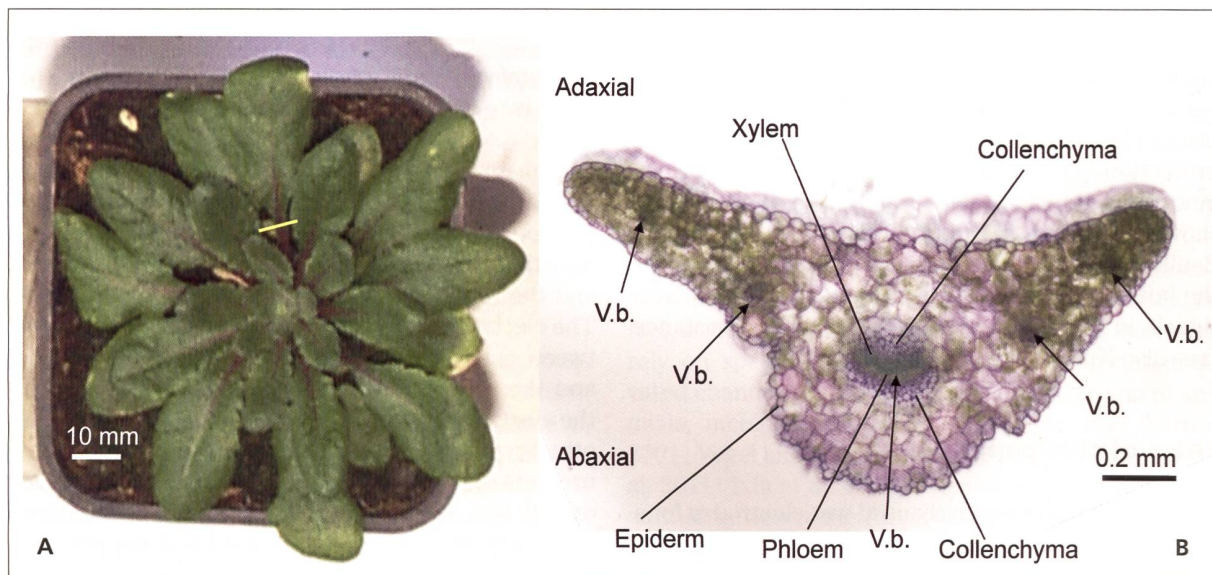


Fig. 2. A. 6 weeks old *A. thaliana* rosette in SD (8:16 h L: D). The yellow line shows the freehand cut - where the petiole E2 electrode is usually positioned. B. Free hand cut of fresh material, colored with toluidine blue, rinsed and mounted in water. Cuts were made by A. Utz of the plant imaging unit (Dr. S. Loubery of the University of Geneva, Plant Botany and Physiology department). V.b: vascular bundles. A main V.b. is visible in the centre of the petiole with xylem, phloem and collenchyma tissues surround it.

ridized silver) electrodes were inserted in the petiole, the principal leaf midrib (Fig. 1). They were unsuitable for contact with the leaf mesophyll because adjacent tissues dried and strongly affected the electrical contact. Because of their small size, up to 4 electrodes could be inserted along the petiole of the same leaf. In Fig. 2 we present a rosette of *A. thaliana* (col) with the distribution of vascular bundles (Fig. 2 V.b.) in the petiole at the location of the E2 (petiole) electrode (see yellow line in Fig 2. A).

The user interface has been developed with LABTECH NOTEBOOK software (v. 8.02, Laboratory Technologies Corp., Wilmington, MA). The sampling rate was 50 Hz (i.e., 50 Samples per s or 50 Ss). We show in an accompanying paper (Parisot and Degli Agosti, 2014) that this sampling rate is fast enough to fully capture APs in *Arabidopsis thaliana* Columbia accessions elicited by touch and likely other treatments (e. g., electrical stimulations).

### Touch stimulation

Touch was realized with a painting pencil (Royal Talens 229, no 8, contact surface:  $30 \pm 9 \text{ mm}^2$ ). To minimize the electrical interference within the Faraday cage, the stimulation was performed by hand, with the experimenter's wrist electrically connected to ground. The stimulation zone was in the middle of the leaf blade. To quantify the intensity and duration of the touch treatment, *A. thaliana* leaves were mounted on a Mettler-Toledo AG204 Delta Range balance (Greifensee, CH), and 50 touch treatments were realized on them. The time course and extent of change in the weight was recorded at  $\sim 10$  Hz via a serial acquisition from the precision balance

interfaced to a computer. Examination of these data showed that the stimulations have a Gaussian shape in time. The data were thus fitted with the Sigmaplot program (nonlinear regression) to obtain the maximum (= maximum weight). The mean equivalent weight was  $1.6 \pm 0.7 \text{ g}$ , and the duration (time at  $-3\sigma$  to  $+3\sigma$ ) was  $2.6 \pm 0.2 \text{ s}$ . The touch was exerted perpendicular to the midrib plane and only once. Whole leaf movement was avoided as much as possible by inserting previously under the leaf and petiole a rigid electrically isolating plastic, as presented in Favre and Degli Agosti (2007)

Statistics were performed with Sigmaplot (Systat software Inc, Us, V. 11.0)

### Results

A slight touch in the middle of an adult *Arabidopsis* plant leaf reproducibly yielded a relatively fast and transient electrical depolarization of the AP type, of which the ability to travel away from the excited zone was assessed by recordings made by distant electrodes. This AP was first detected at the leaf (E1) and then at a more distant petiole electrode (E2). A typical recording is shown in Fig. 3A. Successive data treatments are also illustrated in panels B and C of this figure. The original data in Fig. 3A was filtered with a moving average over 51 points (i.e., 1 s). Then, the values were subtracted from the reference electrical potential that was recorded after the end of the touch treatment (Fig. 3B). In the last panel, the derivatives of the electrical potentials in both E1 and E2 are displayed. It can be observed that during touch, artifacts (due to the introduction of the hand of the experimenter in the Faraday cage) are present

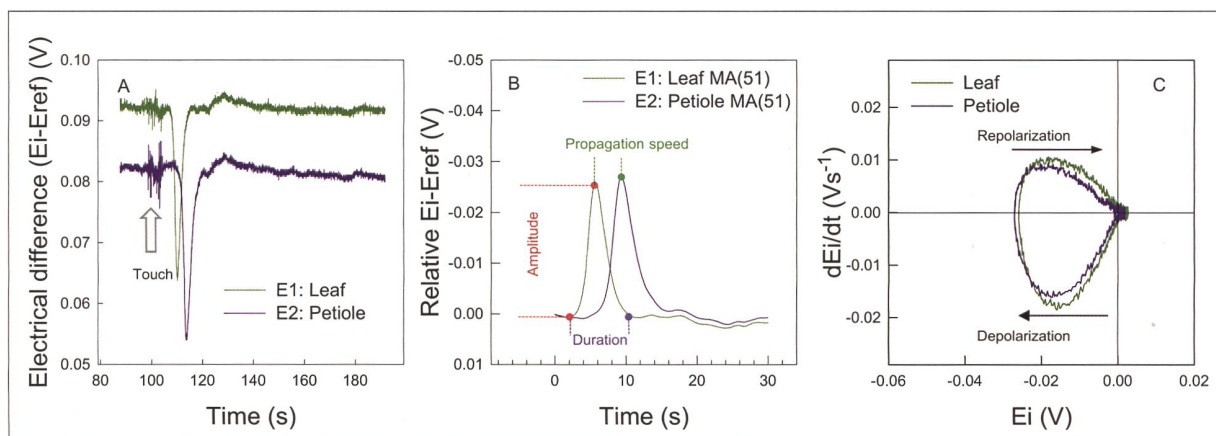


Fig. 3. A: original electrical difference potential. After touching the leaf (T), a transient depolarization is first detected at the leaf electrode and then at the petiole electrode (data are sampled at 50 Ss). B: data have been filtered with a moving average of 51 samples (MA(51)), and values are subtracted (zeroed) with respect to the sample immediately after the end of the touch period. Characteristic points for the determination of the Amplitude, Duration and propagation speed are shown. C: time derivative of the MA(51) electrical values vs. the electrical values.



Table 1. Characteristics of Action potentials that are elicited after a soft touch on an *Arabidopsis thaliana* (Columbia accession) leaf. Measurements are made with inserted non-chloridized silver wire electrodes. In parenthesis, following the medians are the 1st and last quartiles (25%; 75%).

Type of electrode	Inserted silver wire
Number of experiments	21
Number of APs elicited in the leaf (% of experiments)	21 (100%)
Number of APs transmitted to the petiole (% of AP elicited)	20 (95%)
Median AP Amplitude, leaf (mV)	-33.9 (-39.8; -26.8)
Median AP Amplitude, petiole (mV)	-37.7 (-45.1; -26.7)
Median AP Duration, Leaf (s)	10.74 (9.23; 11.51)
Median AP Duration, petiole (s)	11.3 (9.86; 16.74)
Median AP transmission speed (mm s <sup>-1</sup> )	1.32 (1.10; 1.57)

on both electrodes (Touch, in Fig. 3A), and clearly, later an AP signal is detected on the leaf; the AP signal moves away to the distant electrode petiole. The transients' electrical potential shape are typically (but not always, see later in the article) represented by simple peaks, as can be seen by the inspection of the derivative of the potential (Bean 2007) (Fig. 3C). A depolarization occurs first, followed by a repolarization. In Fig. 3B, the quantification method for these touch-induced potential transients in *A. thaliana* is shown; this method allows for the determination of amplitude, duration and speed of transmission of the AP.

Quantitative characterization of these transient electric signals is presented in Table 1. They correspond

to a depolarization, a potential difference with respect to soil that becomes less polarized by decades of millivolts. Their duration is for a decade of seconds, with a propagation speed of approximately 1 mm s<sup>-1</sup>.

**Touch artifacts**

Although the touch was short and relatively soft, it was realized by hand. We observed in some experiments that this treatment could slightly move the first measuring electrode (i.e., the electrode that is nearest to the touch stress). In these situations, a fast change in the electric potential was immediately observed (see Fig. 4). When present, these effects started always simultaneously during the touch period (grey bar in Fig. 4) and then decayed rapidly (Fig. 4). The effects were, in almost all cases, clearly distinguishable from the genuine AP that reached the proximal and distant electrodes and that occurred after the touch stimulation (compare Figs. 3 and 4).

The directly implanted dry silver wire electrodes allowed us to position 4 electrodes (E1 to E4) on a single *A. thaliana* petiole, with a last electrode (E5) easily inserted into the hypocotyl. The setup is presented in Fig. 5A. A clear AP was generated and successively transmitted along the petiole but not through the hypocotyl (Fig. 5B). In many instances, as in the example shown in Figure 5B, the amplitude of the AP was found to increase upon propagation away from the stimulated zone.

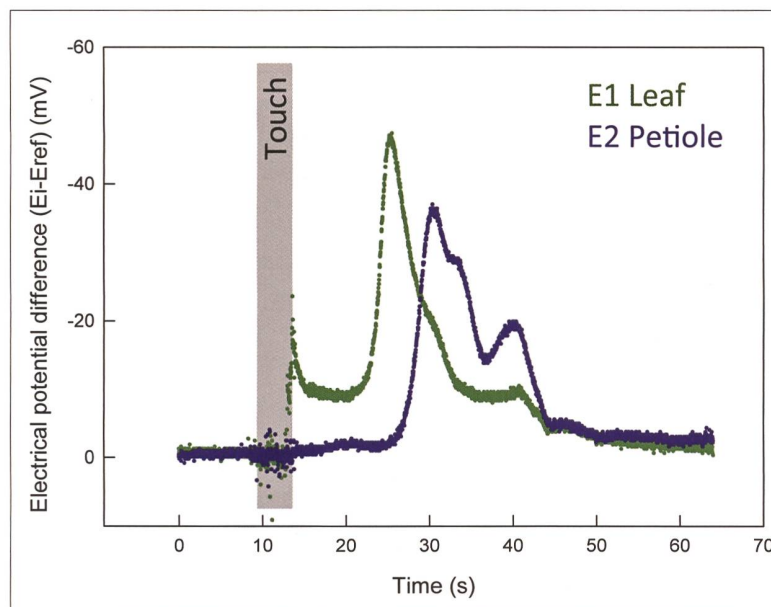


Fig. 4. An example of an electrical artifact that can occur during the touch treatment. During the touch treatment (grey rectangle), a very fast change in the electric potential takes place, followed by a decay. This change is due to the slight movement of the electrode that is nearest to the touch treatment. The Leaf AP is clearly distinguishable ~7 s later followed by an undisturbed (no artifact) AP at the petiole position approximately 4-5 s later.

### Acro- and basi-petal propagation of touch-generated APs

In a significant number of experiments (8/21), we observed, after the touch stress treatment, the movement of the AP from the leaf to the petiole and its return back to the leaf. We used small implantable dry electrodes to capture this event in detail (Fig. 6A). The sequence of AP after touch is observed first at E1, then E2, E3 and not at E4, and back to E3, E2 and E1 (Fig. 6B). The amplitudes appear to increase from the E1 to E3 positions and then decrease on the way back. Apparently,

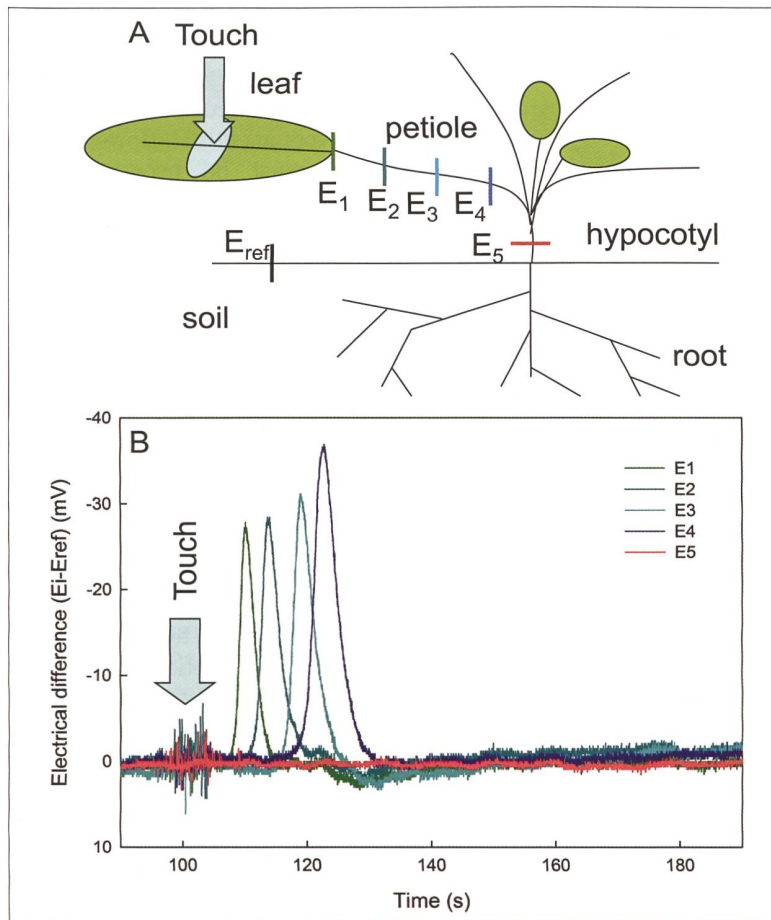


Fig. 5. Measurements with dry (non-chloridized) silver wire inserted electrodes. A: the diagram of the experimental set-up shows the placements of 5 such electrodes on an *A.s thaliana* plant. The stimulation zone is indicated in light blue with an arrow. B: Electrical potential difference of each electrode with respect to a symmetrical (silver wire) reference electrode ( $E_{ref}$ ) inserted in the soil compost. After the touch stimulation, a transient depolarization is transmitted progressively from E1 to E4 but not E5.

the extracellular AP amplitudes do not systematically increase with an increase in the total amount of distance travelled by the AP because they decrease again once they approach again the E1 (leaf) electrode region (see also Fig. 6). This aspect requires further investigation.

## Discussion

Under our conditions, softly and rapidly touching *A. thaliana* leaves induced a transient depolarization in the extracellular voltage that moved away from the touched zone until it reached the end of the petiole near the center of the rosette, which is a typical plant AP. The characteristics of these signals are extremely

<sup>3</sup> In Mousavi (2013): "Leaf 8 was touched gently 3-4 times by moving it up and down with plastic forceps." p. 44, and result p. 45.

similar to touch-induced APs, as measured by wet electrodes, and to the APs elicited by a short (~2 s) and low electrical potential (~3 V), as presented in Favre and Degli Agosti (2007) (Table 2). In the latter paper, almost all of the "classical" properties of animal APs were shown for these plant electrical signals, and they were called APs as were those recorded here. It is worthwhile noting that after the elicited AP, from either the touch or electrical treatment, no further or other signals were recorded, unless we repeated the stimulating treatment after a minimum critical time interval. This finding was not the case when there was a more injurious treatment, such as a wound and a 10  $\mu$ L KCl 1 M drop (see Favre et al, 2001, and Favre et al, 2011). In the latter situation, peaks, short and longer waves (VP?), and oscillations could be observed. We can suggest that different signals can be generated according to the quality and intensity of the applied stress signal and the location of the stressed zone.

Touching the leaf can slightly move the first nearest electrode, creating measurement artifacts in the form of a rapid voltage decrease followed by a more or less rapid return to the baseline (Fig. 4). When they occur, these events were systematically occurring during the touch treatments.

A genuine AP at the leaf electrode did take place some seconds afterward, once the experimenter's hand was outside the measuring Faraday cage. The second transmitted AP to the petiole was detected later. Therefore, artifacts were not to be confounded with genuine plant-generated APs.

The present APs differ somehow from recent published WASP signals, although the front wave is moving at a very similar speed (Mousavi et al., 2013). The authors suggested that touch treatment was ineffective (see Fig. 1c in Mousavi et al., 2013). A careful reading of the original Mousavi's (2013) work makes it clear that their touch treatment was different from ours<sup>3</sup>. Wounding (a more strong event, see Mousavi, 2013; Mousavi et al., 2013) work did elicit WASPs, of which the durations were notably longer than the APs reported here (Table 1) and in Favre and Degli Agosti (2007). Indeed, an electrically induced WASP signal is approximately 100 s long (Mousavi et al., 2013). When



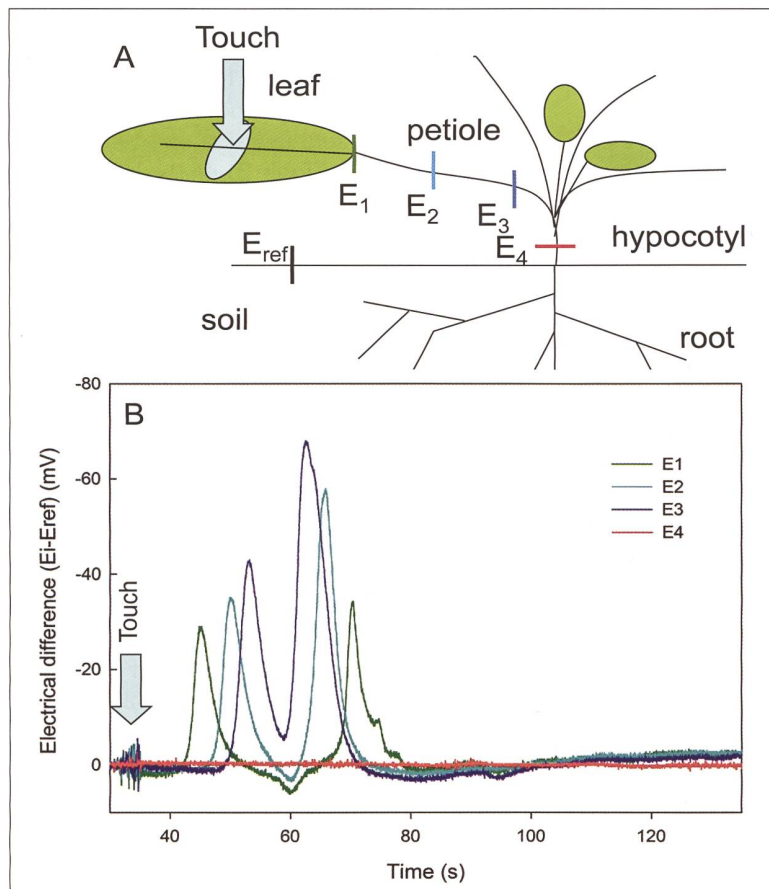


Fig. 6. A. Setup with 3 directly inserted silver wire electrodes. B: A Going-coming AP (GCAP) in *A. thaliana* induced by touch treatment. The AP depolarization is observed first on E1, then E2, and then E3 and not at E4. The AP returns along the petiole to the leaf via E3, then E2, and then E1. This figure can be compared with Fig. 5B.

applying a biotic stress (caterpillars), the signal is far more complex over time and could reflect the progressive dynamics of the caterpillar when it eats the leaf. The signal can contain single peaks that are 10-20 s long (also obtained with cold water only, see extended Fig. 1 in Mousavi et al. 2013) and slow waves with or without electric potential oscillations. With biotic interaction, WASPs appear, thus, to be a complex mixture of putative APs and VPs, at least. This finding is extremely interesting. Designing a reproducible and standardized device that will allow for controlled (time, size, force and position) mechanical stress would be a step forward in the deciphering of mechanoperception and electrophysiological signaling in plants. Moreover, the findings suggest that different parts and different stress on an *Arabidopsis* leaf could elicit specific signals.

Some problems with plant electrophysiological measurements are linked to the method of extracellular electrode measurement, itself being attributable until now to the absence of easily accessible individual cell/tissue within an intact plant that is isolated from

the surrounding tissues (except in Charophytes). In this respect, it is significant that the extracellular method is still currently employed (Mousavi et al., 2013). However, this method must be used carefully. Indeed, it is well documented that the extracellular measurement of an AP from even a single neuron modifies the amplitude and shape of the original signal (Gold et al., 2006). An even more complex situation is present when more cells are measured simultaneously, as is the case with vascular intact plants! In particular, an inserted dry or touching wet electrode measures the firing of a potentially large number of cells. It is currently admitted that these signals (APs) are conveyed within the phloem and/or nearby cells; in fact, in an *Arabidopsis* petiole, there are at least 5 vascular bundles (see Fig. 2), containing many phloem cells. Of interest would be that some are activated whereas others not, which creates a nice complexity of communication coding possibilities.

Using wet electrodes could have disadvantages: a small weight (pressure) can be exerted at the contact zone, and the fluid of the electrode could diffuse into the apoplast of the region to be measured, affecting the extracellular ionic equilibrium. This possibility has been tested in this study by the direct insertion of thin silver wires, and little difference, if any, has been found (see Table 2). Our result validates our preceding results. Certainly, this method of measurement suffers from the drawback of having an initial strong wounding during the silver wire insertion. It is hopeful that this problem appears to be circumvented by measuring signals after a recovery period of one day.

Acro- and then basi-petal propagation APs (GCPAs) are slightly puzzling. They have been regularly observed (Favre, 2004) but not yet described in the literature. Different hypotheses can be formulated. The main hypothesis is that extracellular methods are measuring the results of many tissues. It is believed (see introduction) that APs move along phloem and/or in/with their immediate vicinity cells/tissues. However, in an *Arabidopsis* petiole, there are 5 such vascular (xylem-phloem) complexes with many phloem cells distributed in it and separated by parenchyma (Fig. 2). We can hypothesize that near the center of the rosette connections with other parts



Table 2. Comparison of electrophysiological signals (APs) that were obtained after different stimulations and measurement methods in *Arabidopsis thaliana* (Columbia).

	Touch Dry inserted silver electrodes <sup>a</sup>	Touch Wet contact electrodes <sup>b</sup>	Electrical Wet contact electrodes <sup>c</sup>
Amplitude (leaf) [mV]	-33.9 (-39.8; -26.8)	-24.9 (-35.1 - -18.2)	-37.1 (-65 - -25)
Amplitude (petiole) [mV]	-37.7 (-45.1; -26.7)	-44.6 (-75.9 - 31.4)	-63.3 (-98 - 24.3)
Duration (leaf) [s]	10.7 (9.2; 11.5)	11.8 (10.4 - 14.0)	14 (9.5 - 18.3)
Duration (petiole) [s]	11.3 (9.9; 16.7)	13.3 (11.4 - 16.5)	15.3 (10 - 20.1)
Transmission speed [mm s <sup>-1</sup> ]	1.32 (1.1; 1.6)	1.33 (1.1; 1.7)	1.15 ± 0.26

<sup>a</sup> AP: Present work

<sup>b</sup> AP: Recalculated from Thouroude (2011)

<sup>c</sup> AP: From: Favre and Degli Agosti (2007)

x (y; z): median (25%; 75%); x (y - z): mean (min - max); x ± y : mean ± SD

of the vascular system, bundles of the same petiole are established, which allows for a “return” of the AP via phloem anastomoses (e.g., Aloni and Barnett 1996). Alternatively, special cells or tissues exist near the center of the rosette, which can “restart” excitation and allow its return in the leaves. The significance of these GCPAs requires further investigation. Interestingly, recent results with whole intact plant measurements of Ca<sup>2+</sup> waves (Xiong et al., 2014) also showed complex waves patterns, among which were going-coming patterns. It has been known for a long time now (see Pickard 1973) that different types of electrophysiological signals can be measured in plants. The reader can retain APs and VPs, the nature of which is also known to be different (e.g., Zimmermann and Mithöfer 2013). It is also known that these signals can occur simultaneously, creating complex signals. We can suggest here that due to the extracellular method, even more complex signals can occur, linked also to the underlying fine structure of the plant vascularization, which needs to be described in more detail in adult *Arabidopsis* plants.

To precisely define which signal is doing what in *Arabidopsis*, a better simple description of truly **unmixed** (e.g., PA without PV, PA separated from PV, PV alone) electrophysiological signals must be urgently undertaken with an appropriate controlled, calibrated and reproducible stress stimulation protocol at a minimum. Evidence that electrophysiological signal (rapid and slow depolarization waves) transmission and effects could depend on their nature has been obtained in *Arabidopsis* by Salvador-Recata et al. (2014).

Of great interest is the similar propagation speeds of touch and electrically generated APs and systemic signals such as Ca<sup>2+</sup> waves (Xiong et al., 2014) and ROS (Baxter et al., 2014; Gilroy et al., 2014). Hence the possible actual non-invasive measurements of these signals in whole intact *Arabidopsis* plants by simultaneous imaging and electrophysiological measurements would certainly be a very smart move. Putting together this puzzle with the information gathered on the molecular and biophysical single-channel properties (e.g., mutants) for the understanding of whole plant distant signaling in *Arabidopsis* is becoming an achievable goal.

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