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Action potentials and variation potentials in sunflower: An analysis of their relationships and distinguishing characteristics

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Sunflower plants (*Helianthus annuus* L.) were given an electrical stimulus to the stem or a heat (flame)-wound to a single leaf or a cotyledon. The resulting electrical activity was monitored with extracellular electrodes. An electrical stimulus applied to the stem frequently evoked an action potential (AP), but never a variation potential (VP). In contrast, a heat-wound applied to a leaf virtually always elicited a VP, which was often accompanied by one or more superimposed spikes (putative APs). The kinetic parameters of the AP and the VP were investigated. The AP appears to propagate without decrement in velocity or magnitude, whereas the VP parameters decrease significantly with distance. The heat stimulus triggered rapid alterations in stem elongation/contraction, which preceded changes in electrical potential, indicating the transmission of a hydraulic signal. Light-off and light-on stimuli evoked negative- and positive-going changes in extracellular electrical potential, respectively, corresponding to de- and hyper-polarization of the plasma membrane. Membrane depolarization (extracellularly manifested as a VP) evoked by both the light-off and heat-wounding stimuli was able to trigger one or more APs. We interpret these results to suggest that APs are "genuine" electrical signals involving voltage-gated ion channels or pumps, which can be evoked directly by electrical stimulation or indirectly by changes in membrane potential occurring during the VP or after the light-off stimulus. In contrast, VPs appear to be a local (non-transmissible) electrical consequence of the passage of a rapidly transmitted hydraulic signal in the xylem, presumably acting on mechanosensitive ion channels or pumps in adjacent living cells.

Key words – Action potential, *Helianthus annuus*, hydraulic signal, sunflower, variation potential, wounding.

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Introduction

Numerous wound stimuli of varying intensity, including cutting, chewing, crushing, and heating, applied to one part of the plant have been shown to elicit gene expression in distant regions, thus implying the existence of a long-distance wound signal (Wildon et al. 1992, Malone 1996, Stanković and Davies 1997). Conventional wisdom suggests that the main intercellular communication signal operating in such cases is chemical (hormonal). However, interest in alternatives such as physical (electrical and hydraulic) signals is rapidly increasing (Malone and Stanković 1991, Stahlberg and Cosgrove 1992, 1997, Davies 1993) at least in part as a result of recent findings suggesting that electrical signals are the elicitors of wound-induced gene expression in tomatoes (Wildon et al. 1992, Herde et al. 1995,

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Stanković and Davies 1996, 1997) and in *Bidens* (Vian et al. 1996). Unfortunately, the array of physical signals is far from being understood. While electrical stimulus-generated action potentials (APs) have clearly defined properties, both hydraulic and hormonal signals can evoke local electrical consequences, which may be incorrectly interpreted as long-distance signals (Davies 1993, Stanković et al. 1997). Thus, confirmation of the role of genuine electrical signals awaits our ability to distinguish them clearly from physical or hormonal signals exhibiting electrical aftermaths.

In previously published work using sunflower plants, we described the characteristics of the electrical stimulus-generated AP (Zawadzki et al. 1991), spontaneously generated APs (Zawadzki et al. 1995), and the heat stimulus- and pressure-induced variation potentials (VPs) (Stanković et al. 1997). We also investigated some long-term electrical and growth (stem elongation/contraction) phenomena in externally stimulated sunflowers (Davies et al. 1991). However, direct comparison of the characteristic parameters and possible relationships between the AP and the VP in sunflower have not been reported. In light of the recent interest associated with the possible role of electrical signals in the wound signaling in planta (Wildon et al. 1992, Peña-Cortes et al. 1995, Vian et al. 1996, Stanković and Davies 1996, 1997), the major purpose of this work was to develop protocols which would enable us to distinguish between these signals (AP and VP) as a prerequisite to determining which, if any, are the signals evoking systemic transcriptional and translational responses. By examining changes in electrical potential and in tissue deformation immediately following application of stimuli, we can more fully describe the distinguishing characteristics of the AP and the VP, and we might be able to establish their possible interrelations.

Abbreviations – AP, action potential; VP, variation potential.

Materials and methods

Plant material and growth conditions

Sunflower (*Helianthus annuus* L. cv. Big Russian) plants were grown in a greenhouse for 20–24 days at 20–30°C, and those of similar height (about 30 cm) and appearance were selected for the experiments and transferred into the laboratory. The laboratories in Lincoln, NE, USA, and Lublin, Poland, in which the experiments were carried out over a period of 4 years were windowless air-conditioned rooms, where the temperatures were kept at 21–23°C, and the humidity was 40–60%. The plants were illuminated with white fluorescent lights furnishing about 30 μmol m^{-2} s^{-1} photosynthetically active radiation at plant level.

Measurement of electric potential and stem elongation/contraction

Two types of electrodes were used for measurement of the extracellular apoplastic electrical potential differences. Surface-contact felt-tip calomel electrodes, which are non-damaging to the plant, but tend to dry out and can only be used for short-term (<6 h) recordings, were attached to the plant through 1 m*M* KCl bridges (Zawadzki et al. 1991). Alternatively, silver wires (0.2 mm diameter) which can be used for long-term (>4 days) monitoring, directly pierced the plant to a depth of about 2 mm (Zawadzki et al. 1995). The reference electrode was located in the soil or was attached to the plant. Stem length (longitudinal extension and contraction) was monitored continuously by Metripak angular position sensing transducers (Brush Instruments, Cleveland, OH, USA). The transducer needle was attached to the stem with a drop of Elmer's glue (Borden Inc., Columbus, OH, USA). The voltage outputs from the electrodes were passed through a custom-made high impedance (10^{12} Ω) operational amplifier used as a voltage follower and the results were either directly plotted using a chart recorder or were acquired and displayed through an IBM-compatible PC (Comtrade 486DX/33) containing a 16-channel A/D converter (AT-MIO-16L, National Instruments, Austin, TX, USA), using custom-made software.

Application of stimuli

Non-damaging electrical stimulus was given with a custom-made generator (voltage divider) furnishing a squared DC pulse applied for about 3 s between a pair of inserted silver electrodes spaced about 1 cm apart. The stimulating voltage ranged from 2 to 15 V, as indicated in the text. Damaging heat stimuli were performed by passing a lit match for about 3 s underneath the tip region (about 3 cm^2) of a chosen leaf. This is a stimulus used in plant electrophysiology research for almost a century and recently employed for proteinase inhibitor gene expression studies, since it almost invariably evokes major systemic responses in tomato (Wildon et al. 1992, Stanković and Davies 1996, 1997). Control experiments verified that the electrical and heat stimuli evoked measurable electrical responses only when they were directly applied to plant tissue. For light-off and light-on experiments, a surface-contact electrode was attached to an upper leaf.

Results

Induction of VPs and APs by heat-wounding and electrical stimulation

When sunflower plants were treated with different stimuli, various electrical responses were manifested. Sun-

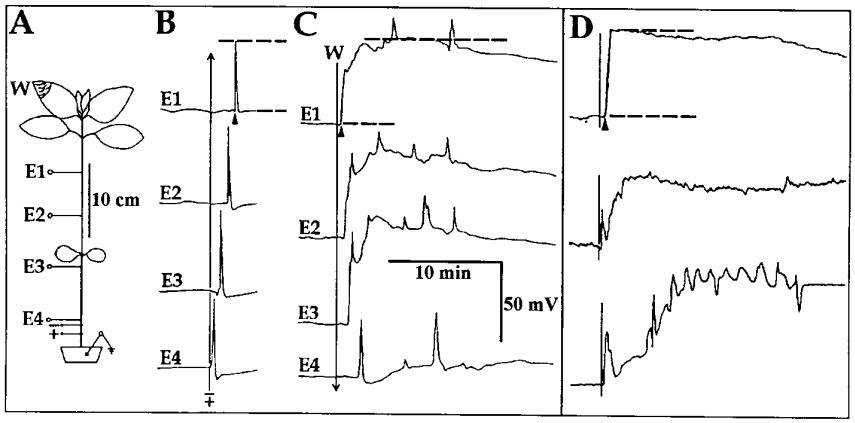

Fig. 1. Action potentials and variation potentials evoked in *Helianthus annuus*. A. Diagram of the sunflower plant. E1, E2, E3, and E4; surface contact measuring electrodes. An identical calomel electrode was inserted in the soil (ground symbol). ±, Electrical stimulation. Hatched area on an upper leaf denotes wounding by heat, i.e. flame (W). B. At the time point indicated with the vertical line, the plant was stimulated electrically at the base (2 V, 2 s). An AP evoked by electrical stimulation appeared successively at electrodes E4, E3, E2, and E1. Propagation velocity between electrodes: E4–E3 = 10.2; E3–E2 = 9.7; E2–E1 = 11.4 cm min^{-1}. C. At the time point indicated with the vertical line, the tip of an upper leaf (W, hatched leaf area in A) was stimulated by heat-wounding for about 2 s. The resulting VP and accompanying APs appeared successively at electrodes E1, E2, E3, and E4. Propagation velocity: W–E1 = 43.0; E1–E2 = 20.3; E2–E3 = 17.8; E3–E4 = 2.3 cm min^{-1}. D. Different types of changes in electrical potential triggered by heat wounding. Examples from three individual experiments when application of a stimulus yielded either a "pure" variation potential (top tracing), or a complex electrical response consisting of both a VP and one (middle tracing) or multiple APs (bottom tracing). The black arrowheads in the top tracings in B, C and D indicate the approximation of the time of signal arrival in a given electrode (for measurement of propagation velocity). The horizontal dashed lines in the top tracings in B, C and D indicate the approximation for measurement of the amplitude of the electrical signal.

flower plants (Fig. 1A) stimulated by non-damaging electrical impulses exceeding threshold levels (Zawadzki et al. 1991) generated reasonably simple electrical responses. These were action potentials which were transmitted at a velocity of 7–10 cm min^{-1} and had a magnitude of about 40–50 mV (Figs 1B and 2). Sunflower plants stimulated by heat (a mild flame wound which causes massive irreversible tissue damage) generated complex electrical responses. These have been called variation potentials by some (van Sambeek and Pickard 1976, Roblin 1985, Malone and Stanković 1991) or slow waves by others (Julien et al. 1991, Stahlberg and Cosgrove 1997). The VPs were often accompanied by one or more spikes, putative APs (Fig. 1C,D). The conditions for generating the train of putative APs following heat wounding have not been determined, since their evocation is extremely variable. As others have shown in tomato (van Sambeek and Pickard 1976), it is very difficult to correlate the stimulus strength or the VP magnitude with the occurrence or amplitude of the spikes.

These data are summarized quantitatively in Fig. 2, where it can be seen that the amplitude and propagation velocity of the voltage-generated AP were fairly constant throughout the stem. In contrast, the respective parameters of the VP decreased significantly with increased distance from the injured site. The average initial magnitude of the VP was about 45 mV, as measured in a proximal electrode located ca 10 cm from the injured site. Its average initial velocity (about 30 cm min^{-1}) was markedly greater than that of the electrically induced AP (ca 10 cm min^{-1}). However, further from the wounded region, the VP decreased significantly, to 10–20% of its initial values, both in terms of amplitude (down to about 10 mV) and in terms of propagation velocity (ca 5 cm min^{-1}). Thus it is possible to distinguish between APs and VPs by using several electrodes on the same plant and monitoring the velocity and magnitude of the electrical change (Figs 1 and 2).

Heat-wounding and electrical stimulus-induced changes in elongation/contraction

It was shown recently (Malone and Stanković 1991) that the heat wound-induced VP which spreads throughout wheat plants is associated with a rapidly propagated hydraulic pressure wave. Boari and Malone (1993) later demonstrated that systemic wound-induced leaf swelling (reflecting a propagated hydraulic signal) is a more general phenomenon, occurring in various species including sunflower. Stahlberg and Cosgrove (1992) provided evidence for wound-induced alterations in growth rate in pea stems. Here we investigated whether the electrically or heat-wound-induced changes in electrical properties were associated with changes in stem length. As can be seen in Fig. 3, heat-wounding of an upper leaf evoked a typical VP transmitted with

decreasing velocity and magnitude down the stem, as monitored with the electrodes (E1 and E2). However, a position-sensing transducer (T) placed between the electrodes, just above the cotyledon (about 20 cm from the wounded leaf area), monitored an almost immediate, small, but highly reproducible surge in stem length. It was followed by a slower, more marked, and long-lasting reversible stem contraction, typically lasting 40–60 min (Stanković et al. 1997). The very rapid, transient increase in tissue length and even the beginning of the slower tissue contraction, clearly preceded the changes in electrical activity.

In contrast to the results with heat-wounding, electrical stimulation caused slight decrease in elongation rate

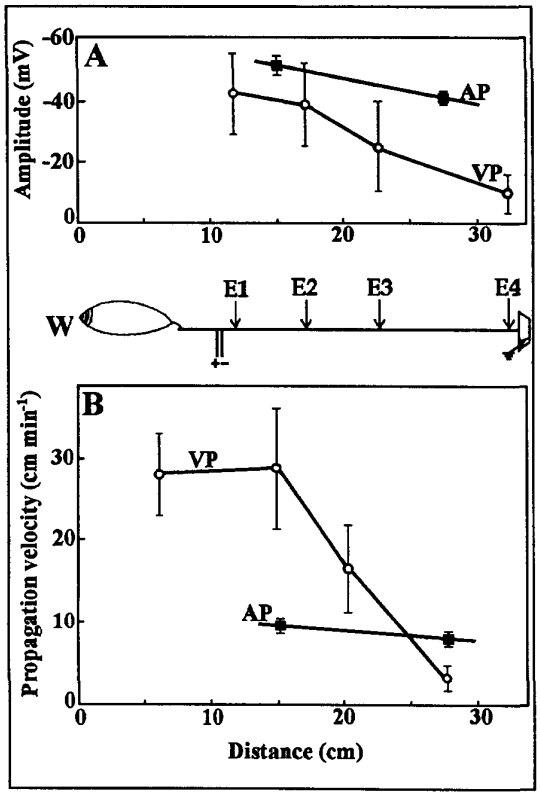

Fig. 2. Amplitudes (A) and propagation velocities (B) of action potentials and variation potentials. The data for electrical stimulus-induced APs (■) are calculated from the results obtained on upper and lower half of the stem (compare with Tab. I from Zawadzki et al. 1991), only additional samples were included. The amplitude of the AP was calculated from n = 59 stimulations on 43 plants. The propagation velocity of the AP was calculated from n = 32 stimulations on 24 plants. The results for VPs are denoted with open circles as means ± SEM at n = 30 stimulations on 15 plants (two applied stimuli per plant – one stimulation per upper leaf). W, heat-wounded region; ±, electrically-stimulated region. The diagram of the plant including the distance between electrodes is drawn to scale for comparison.

concomitant with, or slightly following, the AP (Fig. 4). Such electrical stimulation never evoked tissue elongation, nor did the inhibition of growth ever precede the AP. In those cases when the electrical stimulation did not evoke an AP, no tissue deformation was seen.

Induction of VPs and APs by light/dark stimuli

In spite of the fact that APs and VPs are distinguishable (Figs 1–4), they are, nevertheless, interrelated. For instance, when VPs are generated with heat stimulus, they are often accompanied by one or more APs (Fig. 1). We were interested in determining whether it was the wound-induced hydraulic signal or the change in electrical potential following the hydraulic signal (i.e. the VP itself) that elicits the AP. To do this we needed to use methods which would evoke non-injurious changes in electrical potential, i.e. a VP. We chose to use the light-off stimulus, which we have demonstrated earlier evokes depolarization (Davies et al. 1991). As shown in Fig. 5, when sunflower plants were set up for recording in the electrophysiology room, turning off the lights in the room triggered a wave of electrical potential (reflecting membrane depolarization) starting 40–50 s after the stimulus. Once this change in electrical potential exceeded a value of ca 10 mV, an AP was triggered (Fig. 5A).

A more extensive analysis employing alternating light and dark periods, i.e light-on and light-off stimuli, in combination with application of electrical stimuli, provides further support for the change in electrical potential (VP) influencing the triggering of an AP (Fig. 5B). During the period in which depolarization had been induced by turning the light off, a 2-V voltage stimulus was sufficient to overcome the threshold necessary for triggering an AP. Following the refractory period, which is 10–12 min in sunflower (Zawadzki et al. 1991), a mild voltage stimulation (3 V) again was sufficient to evoke an AP. However, during the refractory period, even when a strong voltage stimulus (15 V) was given, an AP could not (by definition) be triggered, regardless of the obviously depolarized membrane (Fig. 5B).

A quantitative analysis of the correlation between the degree of depolarization (as manifested by a variation potential wave) and the capacity of sunflowers to trigger an action potential was performed in a series of experiments (Fig. 6). The plants were kept under alternating depolarizing conditions (complete darkness in the electrophysiology room), or hyperpolarizing conditions (light turned on in the electrophysiology room). During both of these periods, electrical stimuli of varying intensities were given for a period of 3 s. One to 2 min after turning the lights off, voltage pulses in increments of 1 V were applied at 30-s intervals under depolarizing conditions, until an AP was triggered. Under depolarizing conditions (+values), application of very low voltage stimuli (2–3 V) was generally

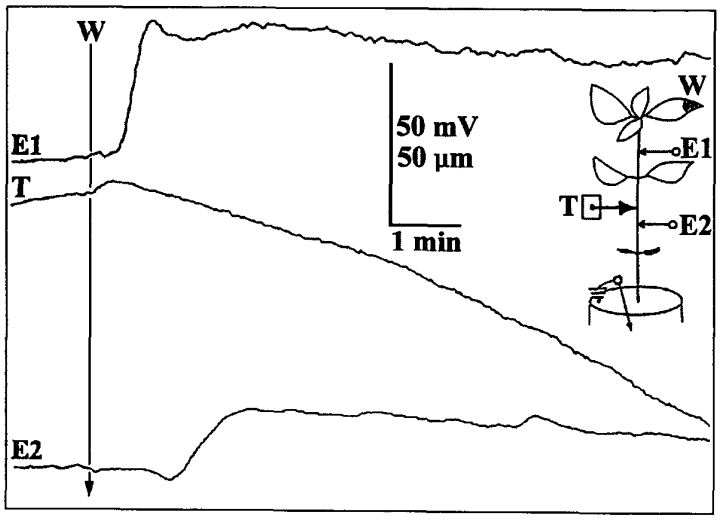

Fig. 3. Changes in electrical potential and stem length induced by heat-wounding. At the time point indicated with a vertical line the plant shown schematically was heat-wounded on an upper leaf (W, hatched area). The extracellular electrical potential was measured with electrodes (E1, E2) located 12 and 22 cm away from the injured region, respectively. Stem elongation/contraction was monitored with a transducer (T) located between the electrodes, approximately 20 cm distant from the wounded region and about 12 cm above the ground level.

sufficient to generate an AP. However, under hyperpolarizing conditions (light on; −values), stronger electrical stimuli (10–13 V) were needed to trigger an AP. The data obtained imply that there is a strong correlation between the degree of depolarization (i.e. the offset from resting, steady-state value, as manifested in the form of a VP), and the capacity for triggering an AP.

To understand more fully the role of light (dark) in signal generation, we made a comparison of the electrically induced APs of plants kept in the dark vs ones kept in the light. The AP propagation rate in the dark was 30–45 cm min^{-1}, and they propagated through the entire plant in 97% (32/33) of the cases examined. In contrast, in the light, the AP propagation velocity was 8–14 cm min^{-1}, and in only 67% (20/30) of the experiments did the AP propagate through the entire plant.

Discussion

General context

In light of the recent findings associated with electrical signals and their putative role in gene expression (Wildon et al. 1992, Peña-Cortes et al. 1995, Stanković and Davies 1996, 1997, Vian et al. 1996), there is a pressing need to understand more fully the array of electrical signals that plants are capable of generating and transmitting, as well as to find out how these signals may integrate plant activity. The primary purpose of the present work was to define methods to distinguish between the two main types of electrical signal, the AP and the VP, as a prerequisite to determining which evokes a response at the cellular or molecular level (e.g. wound-induced transient calmodulin expression in distant leaves – data not shown). Since our earlier work has involved the use of sunflower plants to catalogue the major parameters of the AP (Zawadzki et al. 1991, 1995) and of the VP (Stanković et al. 1997), we chose to use the same species to define the parameters for distinguishing the AP from the VP. We monitored changes in extracellular potential, which mirror and correspond to the plasma membrane (de)polarization (Zawadzki and Trebacz 1985, Stahlberg and Cosgrove 1997).

Distinguishing features of the AP and the VP

As can be seen from the data in Figs 1–5, it is quite easy to distinguish between the AP and the VP in sunflower plants. The AP is transmitted at an almost constant rate and magnitude (Figs 1 and 2) and has an "all-or-none" character (Zawadzki et al. 1991). In contrast, the VP decreases in magnitude with distance from the site of generation (Figs 1 and 2) and its properties vary with the intensity and distance from the site of the stimulus (hence the name "variation" potential).

Not only can these signals be distinguished by their electrical properties, they also differ in respect to their relationship with deformation (elongation/contraction) of the stem (Figs 3 and 4). The VP is correlated with a very rapid, but transient elongation (relaxation of xylem tension?) followed by a massive and prolonged contraction (Fig. 3; see also Stanković et al. 1997), perhaps reflecting water loss. The magnitude of this transient contraction varied among individual plants. It was dependent on the position of the transducer along the stem, and on the plant water status. Even though the contraction generally ranged between 10 and 100 µm, sometimes it exceeded 150 µm (data not shown). These changes in tissue dimension, i.e. the hydraulic component, precede changes in electrical potential, i.e. the electrical component; thus the hydraulic component may elicit the electrical response in the form

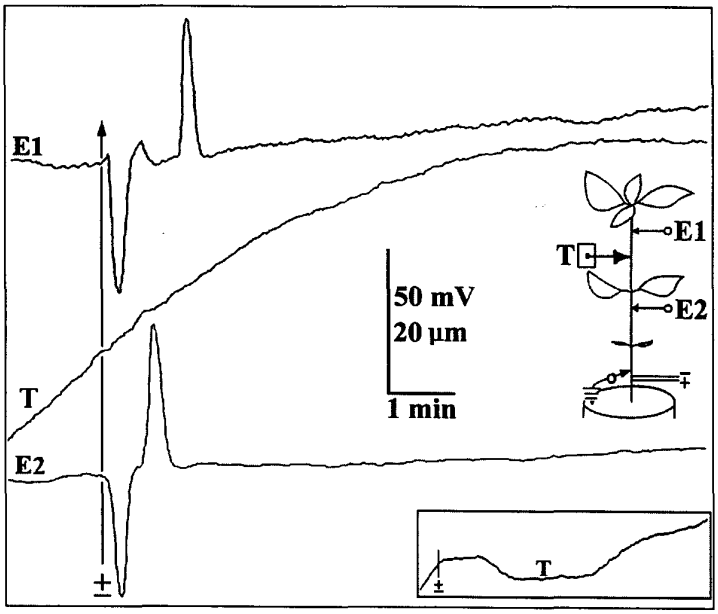

Fig. 4. Changes in electrical potential and elongation/contraction induced by electrical stimulus. At the time point indicated with a vertical line the plant was electrically stimulated (4 V, 3 s) near the base of the stem (±). The electrical potential was measured with inserted electrodes (E1, E2) located 11 and 6 cm away from the stimulated region, respectively. Stem elongation was monitored with a transducer (T) located between the electrodes, about 9 cm away from the site of electrical stimulus and about 20 cm above the ground level. Note that the reference electrode is inserted in the stem proximal to the stimulating electrodes, so the AP passes through the reference before passing through the distant measuring electrodes. Note also that the time of passage of the AP can be measured with great accuracy, but it is difficult to determine the exact timing of the change in elongation. The inset represents a transducer trace showing the effect of electrical stimulus on long-term transient inhibition of growth (monitored over the period of 1 h).

of a VP. The variation potential reflects membrane depolarization, which per se can trigger an action potential, once the threshold is exceeded. In contrast, during an AP, the decrease in stem elongation never precedes the change in electrical potential, but either occurs concomitantly with the electrical change, or follows it closely (Fig. 4). Thus the electrical signal (AP) might be the cause of decreased stem elongation. However, the growth inhibition accompanying the AP is smaller compared to the tissue deformation observed during a VP, and it also lacks the initial transient surge in elongation (cf. transducer tracings T in Fig. 3 and Fig. 4). In our hands, then, and contrary to recent claims (Malone 1996), not only do both the AP and the VP exist in plants such as sunflower, but they can be clearly distinguished on the basis of both electrophysiological parameters and tissue deformation. It should also be noted that the AP was never seen to evoke a VP, whereas a VP frequently evokes one or more APs (Figs 1, 2, 5 and 6).

What is the (electro)physiological basis for the AP and the VP?

Since these signals involve changes in membrane potential which is governed by ions passing through channels, and since the AP and the VP differ in many aspects, it is almost mandatory that they depend on different kinds of ion channels or pumps. What might these ion channels be? The self-propagating AP is mediated through voltage-gated channels or pumps (Gradmann 1976, Wayne 1994). Unfortunately, one cannot, a priori, ascribe a particular channel or pump to the mechanism underlying the VP, but two models have been proposed to explain how a heat (flame)-induced signal can evoke a VP. The first model (van Sambeek and Pickard 1976, Malone 1996) suggests that, after tissue wounding, ions and other chemicals such as wound hormones are dispersed with the xylem fluid and these elicit changes in membrane potential either directly (ions) or indirectly through hormone-modulated ion channels. The second model (Malone and

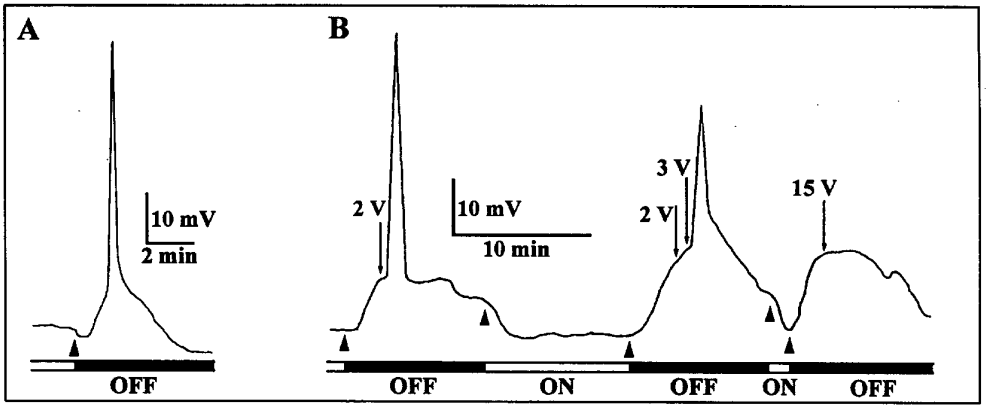

Fig. 5. Triggering of action potentials following depolarization as a consequence of light/dark transition. A. Darkness-induced AP which is preceded by a VP. A surface-contact electrode was placed on the lamina of a leaf. The initial depolarization (VP) began about 45 s after the lights were turned out ("OFF"). After a depolarization of approximately 10 mV, an AP was triggered, with an amplitude of about 50 mV. B. Generation of APs with electrical stimulation under depolarizing conditions. Successive switching "OFF" and "ON" of the laboratory lights is shown, as is the applied voltage. A surface-contact electrode was used to measure the electrical potential in the lamina, when the plant was subjected to alternating light/dark treatments ("ON"/"OFF"). Turning the light off evoked a VP corresponding to membrane depolarization. Conversely, switching the light on caused hyperpolarization. Under depolarizing conditions, and when the plant was not in the refractory period, mild voltage pulses were sufficient to trigger an AP (cf. Fig. 6).

Stanković 1991, Davies 1993, Stanković et al. 1997) is based on the demonstration that simple application of pressure in the absence of any tissue damage (and release of putative wound hormones) will evoke rapid changes in electrical potential resembling a VP. It suggests that the loss of tension in the xylem (the hydraulic signal) lessens the tension in living cells surrounding the xylem, thereby modulating plasma membrane-located stretch-(in)activated ion channels or pumps. Both of these models imply that the electrical component of the VP is a local (non-moving) change in membrane potential evoked by a hydraulic signal. Based upon experiments with wounded tomato and *Mimosa* plants, Malone (1996) showed that the hydraulic signal spreads throughout the plant with a velocity exceeding 15–30 mm s^{-1}.

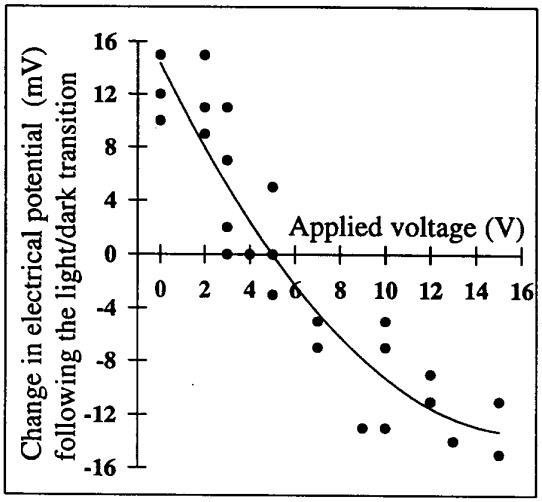

Fig. 6. Triggering of action potentials as a function of darkness-induced depolarization. The data represent the best-fit line for all applied voltages required to induce an AP under depolarizing (+1 to +15 mV) and hyperpolarizing (−1 to −15 mV) conditions, brought about by noninjurious light/dark transitions. The data were obtained using a series of voltage stimuli of increasing intensity on 15 plants (1–2 data points per plant). Note in several instances under depolarized membrane conditions (light "OFF"), spontaneous APs were observed (cf. Zawadzki et al. 1995).

To further establish a correlation between the VP and the AP, we addressed the somewhat related question "How can a VP elicit an AP?" The results in Figs 5 and 6 show both qualitatively and quantitatively that changes in electrical potential (a VP) can modify the tissue's capacity to generate an AP. In some instances, just switching the light off (which causes depolarization, i.e. a VP) can trigger an AP, if the critical threshold for generation of an AP is reached (Fig. 5A). Following depolarization of 10–15 mV (shortly after the light-off stimulus), an AP can readily be triggered with voltage stimuli of very low intensity (Figs 5B and 6). Thus it appears that following dark-triggered depolarization exceeding the necessary threshold, an action potential can be induced. In some instances, turning the light off induced a whole train of APs superimposed on a VP wave (not shown).

The observed difference between the occurrence and the propagation rates of APs in plants kept in the light vs ones kept in the dark implies that sunflowers kept in the dark are electrically poised to generate APs. This is

not altogether surprising, since Greppin et al. (1996) recently showed that at night (i.e. dark period) the resting membrane potentials in spinach and beans are less negative, i.e. the membrane is in a relatively depolarized state compared to daytime (i.e. light period). Such a depolarized membrane could be prone to generating APs more readily. Perhaps it is to the plant's advantage to have alternative signaling mechanisms. One could speculate that in the light, when xylem tension is higher, plants mainly use VPs, but at night, when xylem tension is low, they employ the "genuine" electrical signal, the AP.

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