Plant Physiol. (1997) 115: 1083–1088

Characterization of the Variation Potential in Sunflower[1]

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A major candidate for intercellular signaling in higher plants is the stimulus-induced systemic change in membrane potential known as variation potential (VP). We investigated the mechanism of occurrence and long-distance propagation of VP in sunflower (*Helianthus annuus* L.) plants. Here we present evidence of the relationship among injury-induced changes in xylem tension, turgor pressure, and electrical potential. Although locally applied wounding did trigger a change in membrane potential, it evoked even faster changes in tissue deformation, apparently resulting from pressure surges rapidly transmitted through the xylem and experienced throughout the plant. Externally applied pressure mimicked flame wounding by triggering an electrical response resembling VP. Our findings suggest that VP in sunflower is not a propagating change in electrical potential and not the consequence of chemicals transmitted via the xylem, affecting ligand-modulated ion channels. Instead, VP appears to result from the surge in pressure in the xylem causing a change in activity of mechanosensitive, stretch-responsive ion channels or pumps in adjacent, living cells. The ensuing ion flux evokes local plasma membrane depolarization, which is monitored extracellularly as VP.

The existence of electrical potential differences along the plant apoplast is a phenomenon that has been documented for more than a century (for refs., see Stern, 1924). Stimulus-induced extracellular electrical phenomena in plants are complex and consist of (a) transient APs considered to be "genuine" electrical signals, self-propagated through living cells such as the phloem (Pickard, 1973; Paszewski and Zawadzki, 1976; Opritov and Retivin, 1982), and (b) more gradual changes or slow oscillations in apoplastic electrical potential termed VP, graded potential, or slow wave. VPs have been reported in a variety of plant species in response to various stimuli, including light (Hartman, 1975), gravistimulation (Imagawa et al., 1991), wounding (Van Sambeek and Pickard, 1976; Roblin, 1985; Frachisse and Desbiez, 1989; Julien et al., 1991; Malone and Stanković, 1991; Stahlberg and Cosgrove, 1995; Stanković and Davies, 1997), osmotic stress and irrigation (Gensler and Diaz-Muñoz, 1983; Ishikawa et al., 1983), localized increase in xylem pressure (Malone and Stanković, 1991; Stahlberg and Cosgrove, 1997), chilling (Woodley et al., 1976), and auxin treatment (Newman, 1963; Morath and Hertel, 1978).

The propagation rate of VP ranges from 0.1 to 10 mm s^{-1} (for refs., see Stahlberg and Cosgrove, 1997). It can be transmitted through regions of tissue that have been locally cooled (2°C) (Roblin and Bonnemain, 1985; Wildon et al., 1992), heat-killed (Roblin, 1985), bark-stripped (Paszewski and Zawadzki, 1976), and potassium cyanide-poisoned (Stahlberg and Cosgrove, 1997). All of this evidence strongly implies transmission through the xylem, and yet the bulk of the xylem is dead and has no membrane potential. Therefore, there must be a strong connection between the xylem-transmitted signal and changes in membrane potential taking place in (presumably) adjacent, living cells. Two major hypotheses have been put forward to explain this connection.

One hypothesis holds that hormones released from damaged cells leak into, move through, and then pass out of the xylem into adjacent living cells, where they activate ligand-modulated ion channels/pumps (Houwink, 1935; Sibaoka, 1969; Pickard, 1973; Van Sambeek and Pickard, 1976; Malone, 1996). The nature of the electrogenic substance(s) is unknown, although it has been suggested that it could be a depolarizing compound that moves from the injured site in the transpiration stream through a mechanism of hydraulic dispersal (Malone et al., 1994).

The second hypothesis holds that the injury-induced loss of tension in the xylem allows water to move into adjacent living cells, where increased turgor activates pressure-sensitive channels and/or pumps (Stahlberg and Cosgrove, 1997; Stanković and Davies, 1997). It was recently suggested that systemic changes in extracellular electric potential that follow localized wounding are triggered by a hydraulic signal, i.e. a rapidly propagated pressure wave (Malone and Stanković, 1991; Stahlberg and Cosgrove, 1992, 1995). Recent work (Stahlberg and Cosgrove, 1997) established a dose-response correlation between the magnitude of applied pressure and the degree of depolarization of pea (*Pisum sativum* L.) epicotyl cells. This physical mode of long-distance signaling is rendered possible based on the following observations: (a) several different processes that either directly or indirectly involve the plasmalemma are turgor-sensitive (Zimmermann and Beckers, 1978); (b) mechanosensitive ion channels can be activated by applied pressures within the physiological range (Morris, 1990); (c) ion channels in guard cells of *Commelina* sp.

[1] This work was supported by National Science Foundation grant no. IBN-93-10508 to E.D. and B.S., the University of Nebraska, Lincoln (UNL), Research Council (E.D.), the UNL Center for Biotechnology (B.S.), and the State Committee for Scientific Research (grant no. KBN NO P04C072 10) (T.Z.).

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

can be activated within seconds by pressure applied to the plasma membrane (Schroeder and Hedrich, 1989); and (d) externally applied hydrostatic pressure triggers systemic physiological responses, e.g. remote stomatal closure in *Zea mays* L. within 30 s following a stimulus (Raschke, 1970). The existence of rapid, intercellular, hydraulic-signaling mechanisms may be ubiquitous in plants, since their presence has been demonstrated in numerous species (Boari and Malone, 1993). Coupled with the stretch-activated ion channels (Ramahaleo et al., 1996), physical (hydraulic/electrical) signals could provide an efficient signal transmission/transduction mechanism in planta upon osmotic stress, mechanical stimulation, and wounding.

The major thrust of this study was to determine which of these hypotheses can best explain the heat-stimulus-induced VP in sunflower (*Helianthus annuus* L.). To establish whether VP results from the action of wound-induced release of hormones or a wound-induced release of tension in the xylem, we used a nondamaging stimulus, direct application of low pressure, which has been shown to evoke pressure surges and changes in electrical potential in wheat leaves (Malone and Stanković, 1991) and in pea epicotyls (Stahlberg and Cosgrove, 1997).

MATERIALS AND METHODS

Plant Material and Growth Conditions

Sunflower (*Helianthus annuus* L. cv Big Russian) plants were grown in a greenhouse for 20 to 24 d at 20 to 30°C, and those of similar height (about 30–35 cm) and appearance were selected for the experiments and transferred into the laboratory. Experiments were carried out in a windowless air-conditioned room, where the temperature was kept at 21 to 23°C and the RH was 40 to 60%. The plants were illuminated with white fluorescent lights furnishing about 30 μmol m^{-2} s^{-1} PAR at plant level.

Measurement of Electric Potential and Growth (Stem Elongation/Contraction)

Two types of electrodes were used for measurement of the extracellular apoplastic electrical potential. Surface-contact felt-tip calomel electrodes were attached to the plant through 1 mM KCl ionic bridges (Zawadzki et al., 1991). Alternatively, silver wires (0.2 mm in diameter) directly pierced the plant (Zawadzki et al., 1995). Stem length (longitudinal extension and contraction) was monitored continuously using Metripak angular position-sensing transducers (Brush Instruments, Cleveland, OH). The transducer needle was attached to the stem using a drop of Elmer's glue (Borden, Columbus, OH). In the experiments in which volume changes were examined following stimulus application, a pair of angular transducers was placed laterally side by side to measure changes in stem diameter, and a third transducer measured the stem elongation/contraction. The voltage output from the electrodes was passed through a custom-made high-impedance (10^{12} Ω) operational amplifier that was used as a voltage follower, and the results 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) using custom-made software.

Application of Stimuli

A local heat stimulus was applied by burning the tip of a chosen leaf or cotyledon (about 3–4 cm^2) with a lit match for about 3 s. Attempts to mimic the putative pressure changes through the plant were performed through local application of external air pressure. For that purpose the tip of the stem, including the uppermost two pairs of leaves, was placed and gently clamped in the chamber of a pressure bomb (Scholander 1000, PMS Instruments, Corvallis, OR). Following recovery of the plant as monitored by resumption of the growth (stem elongation) and stabilization of the electrical potential, pressure was applied in the pressure chamber. It consisted of about 0.3 MPa compressed air obtained through the laboratory air supply system, applied either in the form of a pressure pulse (duration 3–4 s) or as continuous pressure, typically applied for several minutes. Since pressure increase in a closed chamber may result in a temperature increase and a possible wound response, the temperature increase in the chamber during the time of applied pressure was examined with a thermocouple. It did not exceed 3°C (data not shown) and is unlikely to induce a wound response.

RESULTS

Kinetic Parameters of VP

The results shown in Figures 1 and 2 indicate that both a flame stimulus (Fig. 1B) and the local application of exter-

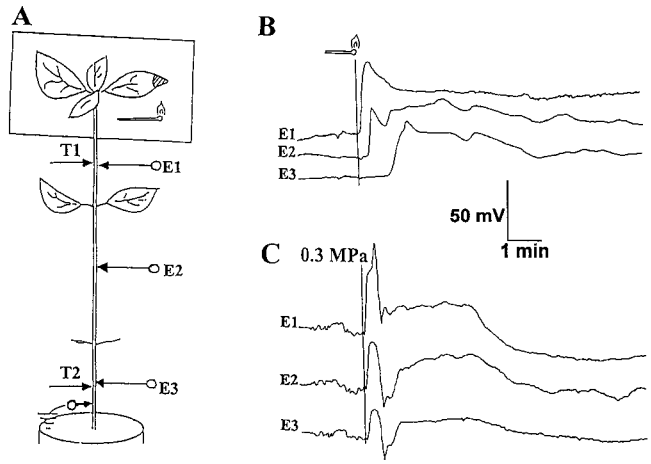

Figure 1. Plants similar to that shown in A were stimulated with a mild flame (hatched area) on an upper leaf (B). The tips of similar plants were placed in a pressure bomb chamber and subjected to a 4-s pressure pulse of about 0.3 MPa (C). The vertical line indicates the time of treatment. Measuring electrodes 1, 2, and 3 were placed approximately 5, 15, and 25 cm from the stimulated site (tip of the plant). Position-sensing transducers were located at the top and the base of the stem (T1 and T2). The electrical responses to flame stimulus are shown in B and to the pressure pulse in C.

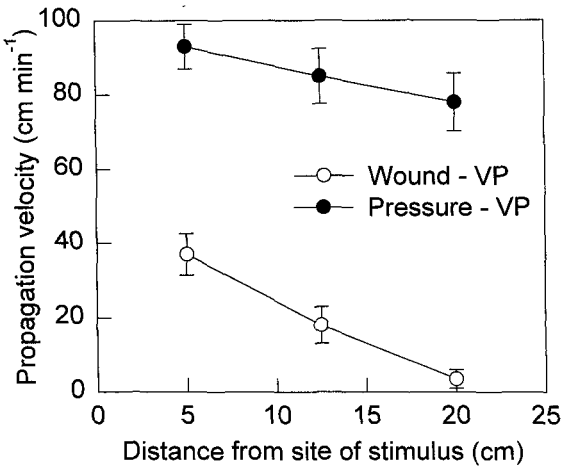

Figure 2. The values of "apparent velocities" of the flame-wound- and pressure pulse-induced VPs were obtained for a series of plants similar to those shown in Figure 1A. The data for the flame-induced VPs were obtained from the responses induced by singeing (for about 3 s) of the tip of an upper leaf with a match, and apparent signal velocities are denoted with open circles as mean values ± SE; $n = 7$. The data for the pressure-induced VPs were obtained from the responses induced by application of a 0.3-MPa pressure pulse for about 4 s, applied to the tip of the plant. The velocities are denoted with filled circles as mean values ± SE; $n = 5$. The scale of the abscissa corresponds to the real distances between electrodes (1–3) on the stem in the plant shown in Figure 1.

nal pressure (Fig. 1C) are capable of evoking VP. In both cases the magnitude of VP was about 40 to 50 mV when measured 5 cm away from the region stimulated and declined to about 10 to 15 mV at 20 cm. The apparent velocity of VP evoked by a flame stimulus also declined with distance from the region stimulated, being about 40 to 50 cm min^{-1} at 5 cm and 5 to 15 cm min^{-1} at 20 cm (Fig. 2). In contrast, the apparent velocity of the pressure-induced VP was exceedingly rapid and appeared almost simultaneously at all three electrodes (Figs. 1C and 2). The flame treatment is undoubtedly a severely damaging stimulus. However, since the pressure pulse lasted only 4 s at a pressure of only about 0.3 MPa, which is about 50% of the turgor pressure values present in sunflower cells under resting conditions (Kutschera and Köhler, 1993), and since the plants showed no permanent damage as a result of this treatment, we considered it a nondamaging stimulus and thus highly unlikely to cause the release of wound hormones into the xylem.

If the flame-induced VP is the result of a hydraulic signal (loss of tension) passing through the xylem, then the change in xylem tension (stem length) must precede the electrical change. Simultaneous measurement of electrical potential and length along a sunflower stem did, indeed, reveal marked and rapid stem lengthening preceding an electrical potential change, regardless of the direction of propagation of the signal (Fig. 3). This is most easily seen when comparing the expanded tracing for the electrode and transducers (T1 and T2) for downward (Fig. 3B) and both upward and downward transmission (Fig. 3C). Heat wounding of a leaf or cotyledon triggered very rapid and transient stem elongation, with a rise half-life of about 30 s. It was followed by massive, long-lasting (generally 40–60 min) stem contraction. The magnitude of stem elongation was about 5 to 15 μm and it was followed by a long-lasting stem contraction of many micrometers, sometimes exceeding 150 μm, which varied depending on the individual plant, its water status, and the positioning of the transducer along the stem. The younger, rapidly growing region of the stem showed larger tissue deformations regardless of the direction of the hydraulic signal (Fig. 3, compare T1 and T2). This can be explained by the fact that tissue deformations (height of the plant) in young tissue include deformations in old tissue but not vice-versa. Moreover, it also probably reflects different elastic properties of the cells in the two regions.

Since rapid and massive changes in stem length were observed in response to localized wounding, it was important to discover whether these occur because of a redistribution of water in the xylem conduits (i.e. the stem getting

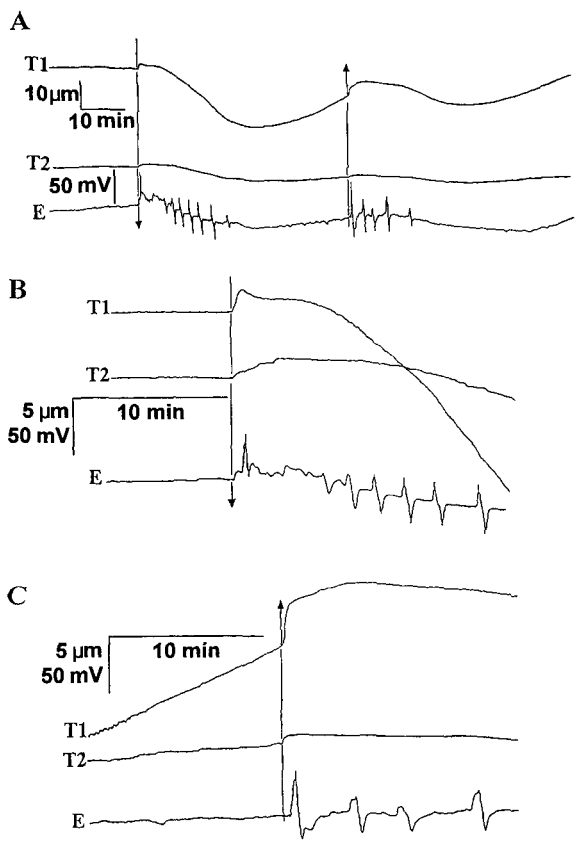

Figure 3. Stem length (elongation/contraction) was simultaneously monitored at two places along the stem as indicated with transducers in Figure 1A (T1, top; T2, bottom). Upward deflection of the transducers corresponds to stem lengthening. At the time indicated with the first vertical line a top leaf was wounded using a match flame for about 3 s. At the time indicated with the second vertical line one cotyledon was flame wounded for about 3 s. A, Long-term electrical activity and alterations of stem length. B, Expanded time scale of the application of the first stimulus shown in A. C, Expanded time scale of the application of the second stimulus shown in A. E, Electrode located by T2.

thinner while elongating) or to an increase of pressure (loss of tension), which would be associated with a transient increase in stem volume. Experiments were conducted using three transducers simultaneously to measure stem volume, with one transducer placed vertically to measure stem length (Fig. 4, T) and a pair of transducers placed horizontally to monitor changes in stem diameter (Fig. 4, R1 and R2). There was a transient increase in both stem length (Fig. 4, upward deflection of T) and diameter (Fig. 4, divergence of the curves R1 and R2), and so there must have been a transient increase in volume. Immediately after this period of volume increase lasting about 30 to 60 s (Fig. 4), there was a prolonged period of shortening (Fig. 4, T) and thinning (Fig. 4, R1 and R2), indicating a rather marked decrease in stem/tissue volume. The data show that the initial outcome of flame wounding is a rapid, but transient, increase in volume immediately following the stimulus, followed by a massive, long-term volume decrease (Fig. 4). The volume increase obviously precedes, whereas the volume decrease more-or-less coincides with, the change in electrical activity (compare Figs. 1–3).

A calculation was performed to determine the magnitude of tissue deformation taking place during VP. A typical sunflower plant has a diameter of 3 to 3.5 mm, and the observed stem diameter increase was 10 to 30 μm, i.e. a maximum increase in diameter of 1%, whereas a plant with a stem 300-mm long experienced transient elongation of 5 to 15 μm, i.e. a maximum increase of 5×10^{-3}% in length. This corresponds to a maximum increase in volume of 1 to 1.5%. This value is well within physiological range considering the elastic properties of the cell walls.

Pressure-Induced Electrical Activity

Since a locally applied, brief pressure pulse is capable of triggering an electrical response throughout the entire sunflower stem (Fig. 1C), and the flame-induced VP is preceded by volume changes in the stem (Fig. 4), we investigated the consequences of maintaining the plant under increased pressure for an extended period. As shown in Figure 5, when pressure was applied for several minutes, the pattern of pressure-induced stem elongation was mirrored by the change in electrical potential. There was a rapid increase in length during the first few seconds, followed by a slower elongation during the next few minutes, until the stem was about 400 to 500 μm longer than at the beginning, but when the pressure was released, the stem contracted almost immediately to close to its original length (Fig. 5, T). Similarly, upon application of pressure, there was a rapid depolarization during the first few seconds, followed by a slower depolarization wave during the next few minutes, reaching a maximum of about 60 mV, but when the pressure was released, the electrical potential did not return all the way to its original level (Fig. 5, E).

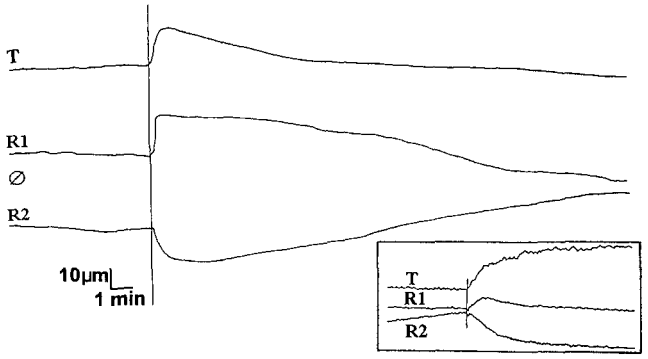

Figure 4. Sunflower plants were flame-wounded as in Figure 1, and position-sensing transducers were placed in the upper half of the stem (T1 in Fig. 1A), oriented in both the vertical position (T) and the horizontal position (R1 and R2) to measure both stem length and diameter, respectively. Upward deflection of the transducer T corresponds to stem lengthening. Increased divergence of the curves for the transducers measuring stem diameter (R1 and R2) indicates increased stem thickening. At the time indicated with a vertical line a top leaf was flame-wounded for about 3 s. The inset shows the results from a similar plant that was left unwatered for 4 d and then watered, i.e. irrigation-induced volume changes.

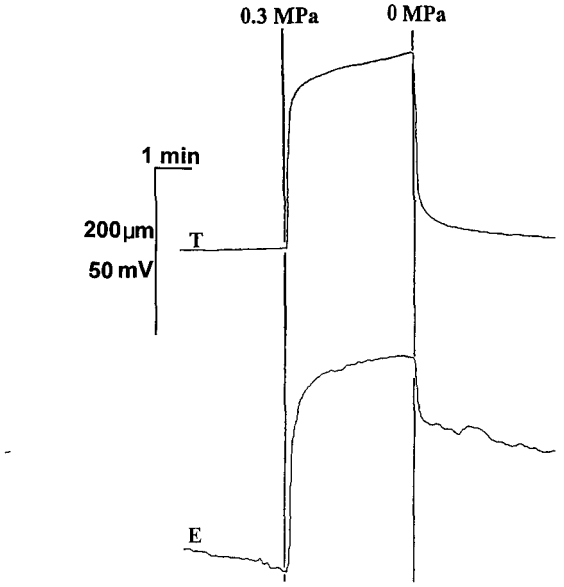

Figure 5. The tip of a plant similar to that in Figure 1 was subjected to a continuous pressure of 0.3 MPa during the period between the first and second vertical lines. T, Transducer (T1 in Fig. 1A) recording; E, electrode (E1 in Fig. 1A) recording.

DISCUSSION

The Importance of Electrical Signals

Recent findings suggest that signals other than (or in addition to) hormonal signals transported through the phloem are involved in wound-induced gene expression in tomato (Wildon et al., 1992; Peña-Cortes et al., 1995; Malone, 1996; Stanković and Davies, 1996, 1997) and in *Bidens pilosa* (Vian et al., 1996). Thus, interest has begun to focus on the role of two primary alternative signals: the genuine electrical signal, AP, and the hydraulic signal with its electrical aftermath, VP (Davies, 1993). AP is modulated through voltage-gated channels or pumps (Gradmann, 1976; Davies, 1993; Wayne, 1994), and several aspects of

the AP in sunflower have already been characterized (Zawadzki et al., 1991, 1995). There are two competing theories put forward to explain VP, and the main purpose of this work was to distinguish between these alternative theories.

Heat Wounding and Pressure-Induced Changes in Electrical Potential

If flame-wound-induced VPs result from activation of mechanosensory pumps or channels, then application of pressure should mimic flame wounding. The effects of the nondamaging (pressure) stimulus were almost identical to those of the damaging (wound) stimulus, insofar as potential differences of about 50 mV were evoked in tissue close to the region stimulated, and the magnitude declined with distance (Fig. 1). This strongly implies that the change in electrical potential is mediated through mechanosensitive rather than ligand-modulated ion channels or pumps. The only major difference between the flame-induced VP and the pressure-induced VP is that the former declined in "apparent" velocity, whereas the latter did not (Figs. 1 and 2). We presume that this is a reflection of the fact that the relief of tension evoked by wounding will have lessened impact further from the wounded site, i.e. because of xylem capacitance the loss of tension will be dampened in distant regions. Since water is not easily compressible, with externally applied pressure the mechanosensory effect is essentially simultaneous through the whole plant.

A salient feature of VP is the accompanying rapid change in stem length. Here we show that these rapid, flame-wound-induced increases in stem length precede the electrical response (Fig. 3), and they are matched by a similar transient increase in stem diameter, resulting in a transient increase in stem volume, followed by a massive, long-lasting decline in volume (Fig. 4).

A close correlation exists between the pressure-induced tissue deformation and the electrical potential monitored along the sunflower stem (Fig. 5). These pressure-induced rapid "growth" responses are not, however, followed by growth reduction (Fig. 5), indicating the nondamaging effect of localized pressure application. They do, however, imply a causal relationship between the xylem water potential and the plant's electrical activity.

The Nature of the VP

Taken together, the results presented here support the hypothesis that VP results from a hydraulic pressure surge transmitted rapidly in the xylem and sensed by living cells, triggering change in the activity of mechanosensitive channels or pumps and yielding an altered ion flux across the plasma membrane, which is monitored as a change in apoplastic potential. Therefore, VP is not a long-distance, self-propagating electrical signal, and it does not appear to be a consequence of wound hormones released from the xylem into adjacent living cells. Instead, it appears to be a local consequence of a transmitted hydraulic signal, which elicits local electrical changes along its pathway. As the hydraulic signal declines in intensity with distance from the wound, so does the electrical response.

Received April 14, 1997; accepted July 15, 1997.

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