Both action potentials and variation potentials induce proteinase inhibitor gene expression in tomato

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Abstract Tomato plants (*Lycopersicon esculentum*) accumulate proteinase inhibitor 2 (*pin2*) mRNA in response to insect attack, crushing and flaming in leaves distant from those treated. Most earlier work suggests that the systemic wound signals are chemical; here we try to determine whether electrical or physical (hydraulic) signals can also evoke *pin* expression. We used a mild flame to evoke a systemic hydraulic signal and its local electrical aftermath, the variation potential (VP), and we used an electric stimulus to evoke a systemic electrical signal, the action potential (AP). We determined the kinetic parameters of both the VP and AP. Flame-wounded plants essentially always exhibited major electrical responses throughout the plant and a several-fold increase in *pin2* mRNA within 1 h. Electrically stimulated plants that generated and transmitted a signal (AP) into the analyzed leaf exhibited similarly large, rapid increases in *pin2* mRNA levels. Plants which generated no signal, or signals of just a few microvolts, had unchanged levels of *pin2* mRNA. Since the AP and VP both arrived in the receiving leaf before accumulation of *pin2* mRNA began, we conclude that, in addition to the previously shown chemical signals, both hydraulically induced VPs and electrically induced APs are capable of evoking *pin2* gene expression.

Key words: Action potential; *Lycopersicon esculentum*; Proteinase inhibitor; Tomato; Variation potential

1. Introduction

Tomato plants respond to insect attack and to abiotic stresses such as wounding (crushing, flaming) by rapidly elevating the levels of transcripts for proteinase inhibitors 1 (*pin1*) and 2 (*pin2*) in tissue both adjacent to and distant from the site of wounding [1]. Controversy reigns as to whether the wound signal evoking transcription in distant tissue is a hormone transported in the phloem or xylem, a hydraulic (tension or pressure) surge, an electrical signal, or possibly some combination [2–4], although the vast bulk of evidence supports a role for chemical signals carried in the phloem [1]. A major effort to test for alternatives [5] used cold-girdling to prevent transport of a chemical signal through the phloem. This work provided support for a major role for electrical signals or action potentials (AP) but did not, however, clearly rule out wound-induced hydraulic signals [6,7] and their electrical aftermath, the variation potential (VP), nor did it rule out substances transmitted via the xylem [3,7,8].

This pioneering paper [5] has stimulated others to more fully decipher the role of electrical signals in gene expression. First, using tomato, Fisahn and co-workers [9] showed that electrical stimulation did, indeed, evoke an AP followed by the accumulation of *pin2* mRNA some 5–6 h later. Unfortunately, this time period is sufficiently long to allow transport of a wound hormone from the wounded site to the region analyzed; thus the crucial role of the of the AP could only be inferred. Second, recent work has confirmed that a heat-induced VP can evoke gene expression (calmodulin in *Bidens*) as can another electrical-like signal, which was tentatively described as a 'non-propagated AP' [10]. However, a 'non-propagated AP' is almost a contradiction in terms and the electrical response seen [10] was most likely a local hydraulically induced change in membrane potential.

Here, by flame wounding or electrically stimulating individual plants and monitoring for the passage of an electrical signal, either a VP or an AP, through the petiole or the blade of the leaf analyzed or into the lamina proper, we directly tested whether passage of either of these electrical signals is followed shortly thereafter by transcript accumulation. The results show unequivocally that both the systemic electrical signal (AP) and the systemic hydraulic signal and its local electrical aftermath (VP) precede and may thus evoke the expression of *pin2*.

2. Materials and methods

2.1. Plant material and growth conditions

Tomato plants (*Lycopersicon esculentum* cv Heinz 1350) were grown in a greenhouse for 4–5 weeks and then transferred into the laboratory where they were left undisturbed in a Faraday cage for a minimum of 24 h at 22–24°C, and 40–60% relative humidity, under white fluorescent lights furnishing 30 μmol m^{-2} s^{-1} photosynthetically active radiation at plant height.

2.2. Measurement of electrical signals

Two types of electrodes were used for measuring the extracellular electrical potential. Surface-contact, felt-tip, calomel electrodes were appressed to the plant through 1 mM KCl ionic bridges, while inserted silver wires (0.2 mm diameter) directly pierced the plant [11,12]. After attaching the electrodes, plants were left undisturbed for several hours, except for rewetting the surface contact electrodes as necessary. Continuous measurement of electrical activity showed that steady-state electrical potential values were attained within 0.5–1 h following attachment of electrodes (data not shown). Electrode outputs were passed through a custom-made high-impedance (10^{12} Ω) operational amplifier used as a voltage follower. The resultant voltage outputs were acquired, stored and processed through an IBM-compatible PC (Comtrade 486DX/33MHz) containing an A/D converter (AT MIO 16 L-25, National Instruments, Austin, TX) using custom-made software.

2.3. Application of stimuli

Plants were locally wounded on leaf 3 (next-to-youngest) by passing a lit match for about 3 s underneath a region about 3–4 cm^2. This is a wound recently employed for *pin* gene expression studies, since it al-

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Abbreviations: AP, action potential; *pin*, proteinase inhibitor gene; VP, variation potential

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most invariably evokes major responses [5] and, in our hands, evokes a VP in over 95% of the plants monitored (data not shown). Electrical stimulus was given to the petiole of the same leaf (leaf 3) using a voltage generator furnishing a 9 V squared DC pulse applied for 3–4 s through a pair of inserted silver electrodes spaced about 1 cm apart. This stimulus evokes an AP in about 20–25% of the plants monitored (data not shown). A typical plant is depicted in Fig. 1A showing the region stimulated and the location of measuring electrodes. For the experiments depicted in Figs. 2 and 3 an identical reference electrode was positioned in the petiole of leaf 2 (second oldest leaf).

2.4. Gene expression analysis

RNA was extracted from the youngest leaf (leaf 4) by phenol/guanidine thiocyanate using TRIzol Reagent (Life Technologies Inc., Grand Island, NY) essentially as described [13]. The method involved grinding up to 0.4–0.6 g frozen tissue in an Omni homogenizer in 10 vol. TRIzol, removal of polysaccharides by pelleting for 10 min at $12000 \times g$, extraction of the organic phase with 0.2 vol. of chloroform, and precipitation of the RNA-containing aqueous phase with isopropanol. After centrifugation for 10 min at $12000 \times g$ at room temperature, the RNA pellet was rinsed with 70% RNAse-free ethanol and repelleted. The pellets were resuspended in DEPC-treated ddH_2O and RNA concentration determined spectrophotometrically, assuming that 1 A_{260} unit equals 40 μg ml^{-1} RNA. RNA was electrophoresed in denaturing 1.2% agarose formaldehyde gels according to [14], blotted overnight using 10× SSC and UV-immobilized with 160 mJ cm^{-2} using a UV cross-linker (UV 2400 Stratalinker, Stratagene, La Jolla, CA) onto a nylon membrane (Hybond N, Amersham, UK). RNA transfer and integrity were checked using methylene blue staining [15]. DNA probes for hybridization were made with about 40 ng denatured template according to [16] using a DNA labeling kit based on random hexamer primers (Rediprime, Amersham) and with α-^{32}P-labeled dCTP at 3000 Ci $mmol^{-1}$ (Redivue, Amersham) according to the manufacturer's instructions. The *pin2* cDNA clone was kindly supplied by Clarence A. Ryan and the 18S ribosomal DNA (rDNA) clone by John Osterman.

Membranes bearing immobilized RNA were prehybridized for 4 h at 42°C in a solution containing 50% formamide, 5× SSC, 50 mM sodium phosphate, pH 7.2, 50 mg ml^{-1} salmon sperm DNA, 1× Denhardt's solution (i.e., 0.02% each of Ficoll, polyvinylpyrrolidone, BSA fraction V), 1% SDS (w/v), 10% dextran sulphate (w/v). Hybridization was carried out in a hybridization oven (Hybaid, Midwest Scientific, St. Louis, MO) at 42°C, typically for 16–18 h, with about $3–4 \times 10^6$ cpm probe per milliliter of hybridization solution. Following hybridization, non-specifically bound probe was removed by a low stringency wash in 2× SSC, 0.2% SDS, at room temperature, for 15 min, twice; and a high stringency wash in 0.2× SSC, 0.2% SDS, at 42°C, for 15 min, twice. Membranes wrapped in SaranWrap were exposed to X-ray film (Kodak X-Omat AR, New Haven, CT) for 1–24 h, depending on signal intensity. The radioactivity bound to membranes was quantitated with a radioactive imaging and analysis system, AMBIS 1000 MK2 (Ambis Inc., San Diego, CA) and normalized to rRNA values for possible unequal loading.

3. Results

3.1. Generation of propagated action potentials and variation potentials

In order to determine whether genuine electrical signals (AP) or hydraulic signals and their electrical aftermath, the VP, can elicit expression of genes in remote regions, we needed to establish conditions which generate such signals in tomato. Electrical stimulation of many higher plants evokes the generation and propagation of APs [17,18] and in plants such as sunflower these APs can be generated routinely [19], while flame wounding of similar plants evokes a VP [11].

Four-week-old tomato plants similar to those depicted in Fig. 1A were placed in the Faraday cage and surface contact or inserted electrodes were placed on strategic locations throughout the plant. After several hours of equilibration,

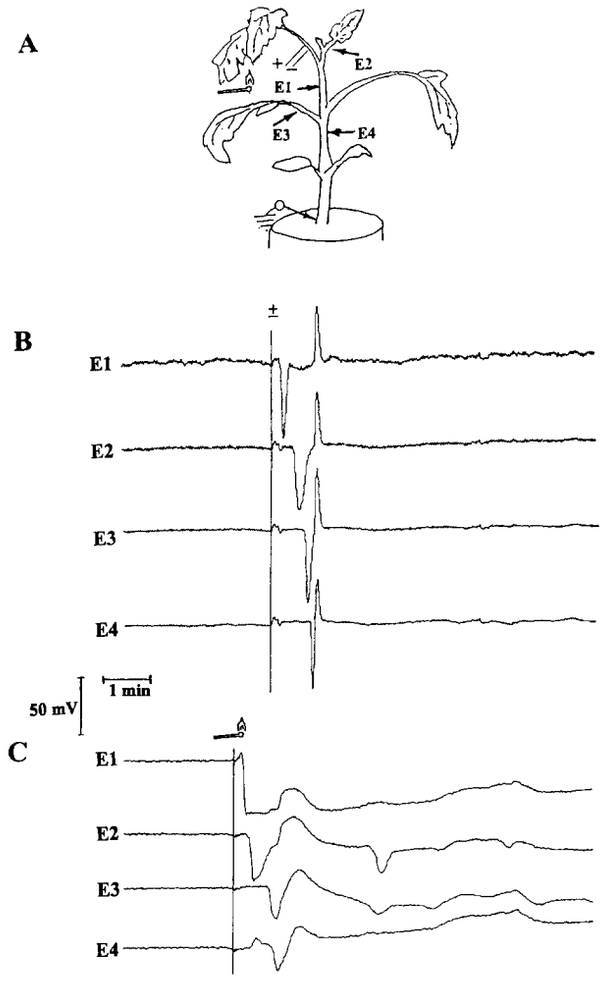

Fig. 1. Generation of AP and VP in tomato. (A) Typical tomato plant used in the experiment. (B) Electrical stimulus-induced AP. Electrical potential was measured in the petioles of a 4-week tomato plant using inserted electrodes. At the time point indicated with a vertical line a squared DC electrical pulse (9 V, 4 s) was given using a pair of silver electrodes (+ −; 0.2 mm diameter) spaced 1 cm apart inserted in the petiole of leaf 3 as diagramed. E1–E4, electrodes. (C) Flame-wounding evoked VP. At the time point indicated with a vertical line, the lamina of leaf 3 was wounded using match flame for about 3 s.

an electrical stimulus was given between a pair of inserted stimulating electrodes, spaced 1 cm apart. When a propagated AP was seen, it consisted of a sharp signal of about 40 mV traveling at a velocity of about 3.5–4.5 mm s^{-1} (Fig. 1B). The propagation velocity was slightly (10–15%) higher in the acropetal versus basipetal direction. The magnitude and propagation velocity are in accordance with the kinetic parameters of APs characterized in other plants [18]. The downward, negative deflections of the electrode tracings E1–E4 in Fig. 1B reflect membrane depolarization (propagated AP) passing through successive electrodes. The upward deflection monitored simultaneously in all four electrodes indicates arrival of the propagated AP into the reference electrode. It was thus established that evocation of 'pure' electrical signals in tomatoes is possible, as a prelude to determination of their involvement in systemic gene expression.

Similar plants (Fig. 1A) were given a mild flame treatment and the hydraulic signal and its electrical aftermath, the VP

[3], were measured. Over 95% of the plants so treated generated a VP (Fig. 1C) sometimes accompanied by superimposed spike(s), putative APs. These changes in electrical activity were preceded by surges in tissue volume, indicating the prior transmission of a hydraulic (tension/pressure) wave in the xylem (data not shown), thus the VP itself was not actually transmitted. Nevertheless, the apparent rate of transmission of the VP declined from about 25 cm min^{-1} at the closest electrode to about 10 cm min^{-1} at the most distant, while its initial magnitude almost always exceeded 40 mV.

3.2. *Direct electrical induction of systemic proteinase inhibitor expression*

Tomato plants exhibit highly variable responses to electrical stimulation: some plants generate APs, while the same voltage evokes no AP in similar plants; in some instances low amplitude APs were evoked that only partially propagated through the stem, or propagated unidirectionally and were not measured in all the petioles and/or the stem (data not shown). We took advantage of this variability by subjecting individual plants to identical electrical stimuli, and then monitoring propagation of the electrical signals generated. Accordingly, four weeks old tomato plants similar to those depicted in Fig. 1A were placed in the Faraday cage and left untreated (control), or their third leaf was flame wounded and the fourth (youngest) leaf analyzed for *pin2* mRNA levels (Figs. 2 and 3). To minimize wound effects due to electrode attachment, only a single measuring electrode and a reference electrode were used.

In the first experiment (Fig. 2), we used inserted electrodes with the measuring electrode impaled in the petiole of leaf 4 (cf., Fig. 1), and pairs of plants with similar electrical tracings were pooled and *pin2* mRNA levels were assayed in the pooled tissue. Monitoring for the appearance of an electrical signal in the petiole of the youngest leaf, and analyzing for subsequent *pin2* expression in pooled (paired) plants, indicates that: (1) when no stimulus was given, no electrical response and no *pin2* expression ensued (Fig. 2a); (2) when the electrical stimulus evoked a typical AP in one plant, but not in the other, intermediate level of *pin2* expression was observed in the pooled sample (Fig. 2b); (3) when the electrical stimulus triggered an AP in both measured plants, *pin2* levels increased several-fold within 1 h (Fig. 2c); (4) when the electrical stimulus did not result in a detectable AP in the petiole of the leaf assayed, no *pin2* transcriptional activation was observed (Fig. 2d,e); (5) when the plant was flame wounded, a typical VP was observed followed by systemic *pin2* accumulation (Fig. 2f).

In every case where no electrical signal (or only a very small signal of less than 8 mV) was detected in the petiole of the leaf analyzed (Fig. 2a,d,e), *pin2* mRNA level remained low. However, when electrical stimulation evoked an AP (Fig. 2b,c) or flame wounding evoked a VP (Fig. 2f), the level of *pin2* mRNA was greatly increased.

There were 2 possible short-comings in this experimental set-up. First, it was not always possible to pool samples with identical electrical signals, thus we decided to isolate RNA from individual leaves. Note: in the case of sample (b) above, where one plant gave a big AP and the other a small one, the value for *pin2* mRNA levels was intermediate between the control and the samples with large APs. Second, the fact that an AP was evident in the petiole does not necessarily mean that it was propagated into the lamina; thus we needed to monitor electrical activity in the lamina itself.

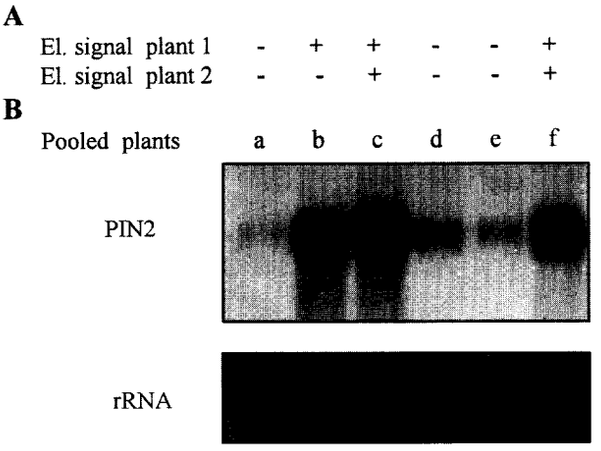

Fig. 2. Direct electrical induction of *pin2* expression. I. Two different electrode arrangements were used for flame wounding and electrical stimulation (Figs. 2 and 3) of tomato seedlings. Plants used for results in Fig. 2 were stimulated electrically with a squared DC pulse (9 V, 3–4 s) as described in Fig. 1. To minimize possible wound effects due to electrode attachment, only a single measuring electrode was used. It was inserted in the petiole of leaf 4 (the leaf analyzed). An identical reference electrode was located in the petiole of leaf 2. Tomato plants were stimulated on leaf 3 either electrically or with a match flame, and electrical signals generated by the plant were monitored in the leaf analyzed (youngest leaf, leaf 4). One hour after stimulation, leaf 4 was harvested, frozen in liquid N_2, tissue with similar electrode tracings pooled (detection of a signal for both plants shown) and analyzed for *pin2* transcript levels. (A) Detection of a measurable el. signal in the petiole of the youngest leaf (leaf 4). The plus sign indicates passing of a signal through the measuring electrode. (B) Autoradiographs showing *pin2* mRNA and 18S rRNA content. In (B), lanes correspond to: (a) control plants with electrodes attached, but no stimulus given (thus no signal generated); (b) plants stimulated electrically, one plant had a major AP, the other a smaller AP (about 8 mV); (c) plants as in (b), but both generated a major AP; (d) plants as in (b), an AP was seen only in the reference electrode, not in the petiole of the leaf analyzed; (e) electrical stimulus, no AP; (f) plants flame wounded as in Fig. 3, and a major VP generated. In both cases where an AP (lanes b,c) or a VP (lane f) passed through the petiole of leaf 4, *pin2* mRNA levels were high. In all cases where no AP entered the analyzed leaf (lanes a,d,e), *pin2* mRNA levels remained low.

Accordingly, additional experiments were conducted in which surface contact electrodes were used to monitor electrical activity directly in the leaf analyzed, and individual plants were analyzed for *pin2* mRNA levels (Fig. 3). Plants (a) and (b) were without electrodes, thus no measurements were made, while in plant (c) there was no stimulus given. However, plant (d) had a typical flame-induced VP; plant (e) had an insignificant (only few mV) deflection in leaf 4; plants (f) and (g) had a typical, massive AP both in leaf 4 and in the reference electrode; while plant (g) gave no electrical response.

As with the pooled tissue (Fig. 2), in every case where no electrical signal (AP or VP) was detected in the blade of the leaf analyzed (Fig. 3a,c,h), *pin2* mRNA levels remained low. Similarly, in all but one case where electrical stimulus evoked an AP in the leaf blade (Fig. 3f,g) and in all cases of a flame-evoked VP (Fig. 3b,d), the level of *pin2* mRNA was massively increased. Note that the only exception seen to this AP(VP)→ *pin2* expression rule was in Fig. 3e, where a small electrical signal (AP) of about 7–8 mV was detected in the analyzed

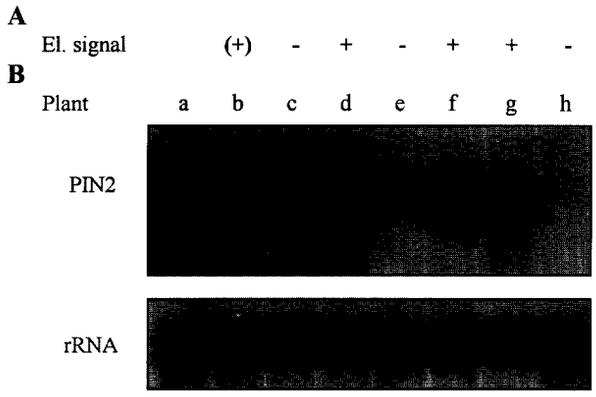

Fig. 3. Direct electrical induction of *pin2* expression. II. The experiment was conducted as in Fig. 2, except that surface contact electrodes were used with the measuring electrode directly on the lamina of the leaf analyzed. Tomato plants were stimulated as in Fig. 2, electrical responses monitored, RNA extracted and assayed for *pin2* mRNA and rRNA. (A) Detection of measurable electrical signal in the lamina of leaf 4. The plus sign indicates passing of an electrical signal through the measuring electrode. (B) autoradiographs showing *pin2* mRNA and 18S rRNA content. Lanes in (B) correspond to: (a) untreated control without electrodes attached; (b) flame wounded, no electrodes (+). Note: in over 95% of the plants monitored, flaming evokes a VP; (c) control with electrodes attached, no stimulus, thus no AP or VP; (d) flame wounded as in (b), electrodes attached, major VP seen; (e) electrical stimulus, minor AP ($\approx$8 mV) in leaf assayed, none in reference; (f) electrical stimulus, large AP in leaf assayed and in reference; (g) electrical stimulus, large AP in leaf assayed and in reference; (h) electrical stimulus, no AP. In all cases where an AP (or VP) greater than 8 mV reached the analyzed leaf, pin mRNA levels increased massively over the controls (lanes a,c). In all cases lacking an AP (or VP) greater than 8 mV, little or no increase in *pin2* mRNA was evident.

leaf, but not in the reference. Such a small AP may not be transmitted from the main vein where the electrode was located to the tissue which synthesizes the bulk of the *pin2* mRNA. Alternatively, signals below a certain magnitude (e.g., amount of ion flux) may elicit little or no response.

4. Discussion

The raison d' être for this research was to contribute towards understanding the mechanisms of signal transduction in higher plants, focusing on a likely candidate for rapid intercellular signaling (changes in electrical potential), whilst employing a convenient systemic molecular marker (wound-induced *pin2* expression). Association of VPs and *pin2* transcript accumulation has previously been shown [5] as has an association between VPs (and 'putative APs') with *calmodulin* gene expression [10]. Here we show that in tomato a 'genuine' electrical signal (AP) is triggered by application of a local electrical stimulus, while a hydraulic signal followed by a change in membrane potential is triggered by flaming. We also determined the kinetic parameters of both APs and VPs to determine if there is a cause–effect relationship between either APs or VPs (or both) and systemic *pin* expression.

The main question we were trying to answer is "What is the nature of this rapidly generated signal that evokes *pin2* mRNA accumulation?". The results here (Figs. 1C, 2f and 3d) confirm those reported earlier [5] in showing that the long-distance (intercellular) signal evoking *pin2* expression in tomatoes in response to flame wounding is the hydraulically induced local change in membrane potential (VP). Of paramount importance, however, is our finding that the propagated electrical signal (AP), generated in response to electrical stimulus (Fig. 1B), is equally effective in elevating *pin2* mRNA levels in distant tissue (Figs. 2b,c and 3f,g).

The results reported here differ significantly in scope from other work on the role of electrical signals in gene expression. Here we show that 5-fold or greater increase in *pin2* mRNA levels occurs within 1 h of both electrical stimulation and flame wounding (Figs. 2 and 3) and 2–3-fold increases are seen within 15 min (data not shown). Thus the time interval in our work between the arrival of the AP/VP and in increase in systemic *pin2* mRNA is sufficiently short to essentially exclude a chemical from being the initial long-distance wound signal. Other workers [9] showed that it takes 5 h after electrical stimulation to evoke a systemic increase in *pin2* mRNA. This time lapse is sufficiently long that it cannot rule out a primary role for chemical signals. Our results agree with findings with *Bidens* [10] that heating evokes a VP which induces gene expression. However, the 'non-propagated AP' they [10] described after placing a water drop on the stem is more easily interpreted as a local hydraulic surge leading to a localized change in membrane potential (i.e., a local VP).

It must be pointed out that even though these results provide persuasive evidence for both electrical signals (AP) and hydraulic signals (VP) being major intercellular signals involved in systemic induction of *pin* expression in young tomato plants, they do not disprove a role for chemicals (hormones). Indeed, recent work provides very strong evidence for systemin playing a major role as a systemic signal [20,21], although it may not be transported rapidly enough [22] to evoke the responses seen here. However, the opposite is also true; evidence to support a role for chemical signals does not disprove a role for APs or VPs. One might imagine that it would be to the plant's advantage to have a choice (or back-up) in signaling mechanisms, so that in the winter, for instance, when the voltage-gated channels may be inactive [11], alternative mechanisms could come into play as suggested earlier [3]. It is also likely to be to the plant's advantage to be able to distinguish between insect chewing, heat, cold, electrical stimulus, etc., so that appropriate responses can be manifested. There may be important interactions between the various systemic signals, insofar as it has recently been shown that chemical signals (oligogalacturonides) can evoke electrical responses [23], and it has been known for some time that the systemic signal evokes changes in plasma membranes in distant leaves in tomato [24,25]. The ability for one region of a plant to communicate rapidly with other regions would appear to be of major importance in environmental adaptation.

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