Is membrane potential involved in calmodulin gene expression after external stimulation in plants?

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Abstract **In *Bidens pilosa* (cv. *radiata*), a non-injurious stimulus induces a local and transient change in membrane potential, and an injurious stimulus induces a transmitted electrical signal described as the combination of an action potential and a slow wave. We have studied calmodulin gene expression after these stimuli. When the stimulus is non-injurious, calmodulin mRNA accumulation is only increased in the stimulated region. In contrast, when the stimulus is injurious, mRNA accumulation takes place in both wounded and distant, unwounded tissue. We propose that the slow wave plays a role in the long-distance transmission of a wound-induced information in plants.**

Key words: Action potential; Calmodulin; Gene expression; Variation potential; Wounding; *Bidens pilosa*

1. Introduction

It is now well-accepted that plants continually sense environmental conditions and transduce these external stimuli into physiological responses. These physiological responses can be evoked by a great variety of external stimuli. In some cases, the physiological response has been observed at a distance from the site where the external stimulus was applied implying that a signal was transmitted through the plant [1–3].

Three different hypothesis have been proposed to explain long-distance information transfer: (i) it has been suggested that a mobile chemical signal is the causal agent and candidates such as oligosaccharides [4], abscisic acid [5] and systemin [6] have been proposed; (ii) it has also been shown that one response to external stimuli in higher plants is the elicitation and the spreading of a variation in the transmembrane electrical potential and several authors suggested that these electrical signals could be the 'information' carrier [1,3,7,8]; (iii) it has been demonstrated that hydraulic signals are transmitted from damaged tissue [9,10] and it has been proposed that hydraulic signals could form part of a widespread mechanism for coordination of plant responses [11].

With regard to the second hypothesis described above, it is worth noting that there is disagreement in the literature about the nature of the electrical signal which would act as the 'information' carrier [12]. Nevertheless, it has been shown previously that the electrical responses of plants differ according to the type of stimulus, namely injurious or non-injurious [1,13–18]. When the stimulus is not injurious (electrical stimulation, cold shock, light–dark transition, etc.) plant cells generate an action potential (AP) according to an all-or-none reaction, which is propagated at approximately 1 cm s^{-1} [17,19–21], and when the stimulus is injurious (wounding, burning, crushing, etc.) an electrical potential variation that is composed of an AP followed by a slow wave (SW), but also called a variation potential (VP), is triggered and propagated with a velocity of 1 mm s^{-1} [12,17,22,23].

The major aim of this work was to determine whether changes in membrane potential (or in their underlying ion fluxes) would be directly related to changes in mRNA accumulation. In addition, we studied the delayed effect of the signal on growth responses in the elongation region.

2. Materials and methods

2.1. Plant material and treatment

Achenes of *Bidens pilosa* (cv. *radiata*) were germinated for 5 days on the ion-rich nutrient medium Cera III [24]. On day 5, the seedlings were transferred to deionized water for 24 h. On day 6, plants were stimulated either by burning (injurious stimulation) or by putting a water drop on the hypocotyl (non-injurious stimulation). The upper first centimeter of the hypocotyl, which is the growing region, or the basal part of the hypocotyl were harvested at different times after stimulation, and immediately frozen in liquid nitrogen.

For some experiments, a mark was made 1 cm below the node of the cotyledon with India ink, before stimulation. The plants ($n > 30$) were allowed to grow for 24 h on deionized water and the length of the hypocotyl was measured. The growth inhibition was measuring as $[(DL-dl)/DL] \times 100$, where DL and dl are the hypocotyl length between the cotyledon node and the India ink mark for untreated (control) and stimulated plants, respectively.

2.2. Experimental design for electrophysiology

Intracellular measurements were performed using standard electrophysiological techniques. The micropipets were prepared as previously described [25]. Briefly, pipets were pulled from glass capillaries containing glass fibers (Hilgenberg) on a David Kopf vertical instrument and backfilled with 0.5 M KCl. The Ag/AgCl wires were connected to a high-impedance amplifier (World Precision Instrument, FD 223).

The plantlet was put horizontally (Fig. 1) so that a 5-mm length hypocotyl was inside a Plexiglas chamber with a volume of 0.3 cm^3 containing deionized water. The reference electrode, made of a glass capillary and filled with a mixture of 0.5 M KCl and 1% (w/v) agar, was located in the Plexiglas chamber. The microelectrode was impaled in a hypocotyl cell approximately 8–10 mm from the Plexiglas chamber (Fig. 1).

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Abbreviations:: AP, action potential; CAL, calmodulin; I, injurious; NI, non-injurious; St, stimulation; SW, slow wave; VP, variation potential.

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The non-injurious stimulation (NI St) was given by placing a room temperature water drop or a drop of cold water (4°C) with a pipet tip either near the microelectrode (1–4 mm) or at a distance of 3–4 cm. The injurious stimulation (I St) was given by heating the hypocotyl, for 2 s, with a small resistor surrounding the hypocotyl 1.0 cm away from the plant (Fig. 1). The incandescence temperature approached 150°C and such a stimulation injured about a 2-mm region of the hypocotyl.

2.3. RNA isolation and hybridization

Total RNA was isolated from 100 hypocotyls (200–300 mg fresh weight) using the hot borate method described earlier [26]. Equal amounts of RNA (10 μg) were separated on a 1.2% (w/v) agarose gel and transferred onto Nylon membranes (Amersham Hybond N$^+$). The RNA was cross-linked on the membrane by exposure to UV in a Spectrolinker UV-1500 (Spectronics Corporation, Westbury, USA) using the standard program.

The membrane was incubated 3 h at 42°C in prehybridization cocktail (50% (v/v) formamide, 6 × SSPE, 0.1% (w/v) SDS, 100 μg ml^{-1} of denatured herring sperm DNA (Sigma), 0.5% (v/v) Denhardt's solution). The *Bidens* calmodulin cDNA probe (EMBL access number X89890) was labeled with [^{32}P]dCTP (Amersham, specific activity >3000 Ci mol^{-1}) using the Ready-to-Go DNA-labeling kit as described by the manufacturer (Pharmacia). The probe was purified on a Qiagen tip 5 mini-column, heat-denaturated and added to fresh hybridization solution. The hybridization was performed overnight at 42°C. The membrane was rinsed twice at room temperature in 2 × SSPE, 0.1% (w/v) SDS for 15 min and twice in 0.2 × SSPE, 0.1% (w/v) SDS at 42°C and 50°C for 15 min each. The membrane was then allowed to dry and autoradiographed (Biomax MR Film, Kodak) with an amplification screen (DuPont Cronex quanta fast).

3. Results

3.1. Non-injurious stimulation

The application of a cold (4°C) water drop at the base of the hypocotyl has no effect on the membrane potential of the growing part of the plantlet (Fig. 2A, dashed line). When a similar cold water drop is applied in the growing part near the impaled microelectrode (0.1–1 cm from it), it elicits a transient change in membrane potential in the adjacent microelectrode (Fig. 2A, solid line). Similar responses were obtained after the application of a room temperature water drop, thus, it is not merely a response to cold.

Several batches of plants were stimulated as described above and the growth of each plant was measured 24 h later and compared with the growth of control plants. As shown in Table 1, the growth modification is only about −1.5% when the plants were stimulated in the basal zone with a room temperature water drop (NI stimulation type A) and 2.5% with a cold water drop (NI stimulation type C). When the growing part is stimulated, the growth modification is also quite low (−11% and −3% NI stimulations types B and D, respectively).

Total RNA of control and stimulated plants was extracted and hybridized with *Bidens* calmodulin cDNA. The steady-state level of calmodulin transcripts in control plants was low (Fig. 3, lane 1) and was used as the reference. Twenty minutes min after the application of a room temperature water drop (Fig. 3, lane 2) or of a cold water drop (4°C, Fig. 3, lane 4) in the growing part of the plantlet, a 3-fold increase in the amount of calmodulin mRNA was observed. In contrast, when a room temperature water drop was applied to the basal zone of the hypocotyl, no significant accumulation of calmodulin mRNA (1.2-fold) was seen in the apical region (Fig. 3, lane 3).

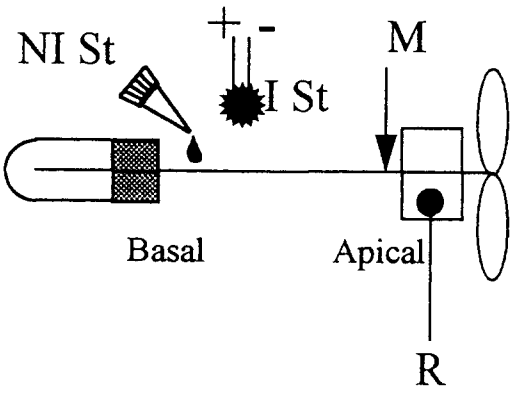

Fig. 1. Electrophysiology design for injurious and non-injurious stimulations. Non-injurious (NI St, drop of cold or room temperature water) or injurious (I St, heat burning) stimulations. The treatments are given to the plants at a distance from (basal region) or close to (apical region) the inserted electrode (M) for the non-injurious stimulation, and only in the distant (basal) location for the heat stimulation. The reference electrode R is placed in the perfusing chamber.

3.2. Injurious stimulation

The injurious stimulation was given by heating the hypocotyl 1 cm above the plant and the transmembrane potential was measured in the growing part (3–4 cm away from the site of heating) of the hypocotyl. The heat stimulation elicits, at a distance from the stimulation point, a transmembrane potential change (Fig. 2B) called a slow wave (SW) or a variation potential (VP). The SW (or VP) is composed of a fast depolarization phase (15–20 s) followed by a period where the membrane potential was quite stable (3–5 min), and by a slow repolarization phase (15–20 min).

The heat stimulation was applied to several batches of plants and the growth of each plant was measured 24 h later and compared with the growth of control plants. The root (nutrient source) and the region where the heat stimulation was applied was removed 20 s after the injurious treatment, in heated as well as in control plants. Hence the length of the control for the

Table 1
Hypocotyl growth response to non-injurious stimulation (drop of water applied on the hypocotyl)

	Control	NI stimulation type A	NI stimulation type B	NI stimulation type C	NI stimulation type D
Increase in length in 24 h (mm)	3.38 ± 0.16	3.42 ± 0.12	3.74 ± 0.13	3.30 ± 0.07	3.47 ± 0.08
Number of plants	47	50	49	133	130
Modification of growth 24 h after the stimulation (%)	–	−1.5	−11	2.5	−3

The plants were stimulated in different ways and the elongation of the hypocotyl was measured 24 h later. Stimulation type (A) room temperature water drop applied at the base, (B) same, but applied in the growing region, (C) cold water drop applied at the base, (D) same, but applied to the growing region. Values are mean ± S.E.

injurious treatment is so much less than for the non-injurious treatment (Tables 1 and 2). The heat stimulation induced a significant growth inhibition of 35% (Table 2) compared with the control.

Total RNA was extracted at different times after treatment either from the first centimeter above the heated zone of the plant or 3–4 cm away in the growing part. In all cases, RNA was extracted from non-injured tissue and hybridized with *Bidens* calmodulin cDNA. Three minutes after heat stimulation, no significant accumulation (1.5-fold) of calmodulin mRNA was observed either in the region 1 cm above the heated zone (Fig. 3, lane 5) and in the region 3 cm distant (Fig. 3, lane 7). Twenty minutes after the heat stimulation, the accumulation of calmodulin mRNA was obvious (4-fold) both in the region directly above the heated zone (Fig. 3, lane 6) and in the growing part of the plant (Fig. 3, lane 8).

4. Discussion

The main aim of this research was to determine whether electrical signals (i.e. changes in membrane potential based on transmembrane ions fluxes), generated in response to injurious and non-injurious treatments, were able to evoke physiological responses in adjacent and distant tissue.

The choice of calmodulin cDNA as a probe to monitor changes in gene expression in relation to variations in membrane potential was made because several reports indicate rapid accumulation of this transcript in response to different kinds of stress [27–29].

Under conditions where a local change in membrane potential was evoked in adjacent tissue by the non-injurious treatment of a drop of water, a transient change in membrane potential (Fig. 2A, solid lane) was followed by a substantial increase in the amount of calmodulin mRNA (Fig. 3, lane 2). Distant tissue, which showed no change in membrane potential (Fig. 2A, dashed line), showed no change in calmodulin mRNA accumulation (Fig. 3, lane 3). In contrast, when a change in membrane potential (Fig. 2B) was evoked in distant tissue by the injurious treatment of burning, an accumulation in calmodulin mRNA occurred 20 min later (Fig. 3, lanes 6 and 8). These data agree with those of Braam and Davis [27] who showed an increase of the *tch* mRNA levels in response to non-injurious (water spraying) or injurious (wounding) stimulations.

A separate, yet related, aim of this study was to determine whether changes in membrane potential evoked by a genuine electrical signal such as action potentials (AP), by an electrical response both called either SW [1,23,25] or a VP [12,14,17] or both [12] were the signal-evoking gene expression.

Table 2
Hypocotyl growth responses to injurious (heat) stimulation

	Control	Injurious stimulation
Increase in length in 24 h (mm)	1.82 ± 0.05	1.18 ± 0.16
Number of plants	149	173
Modification of growth 24 h after the stimulation (%)	–	−35

Elongation of the hypocotyl was measured 24 h after the application of the treatment. The heat-stimulated region and the roots were removed 20 s after the stimulation in both the treated plants and the controls (non-stimulated plants). Values are mean ± S.E.

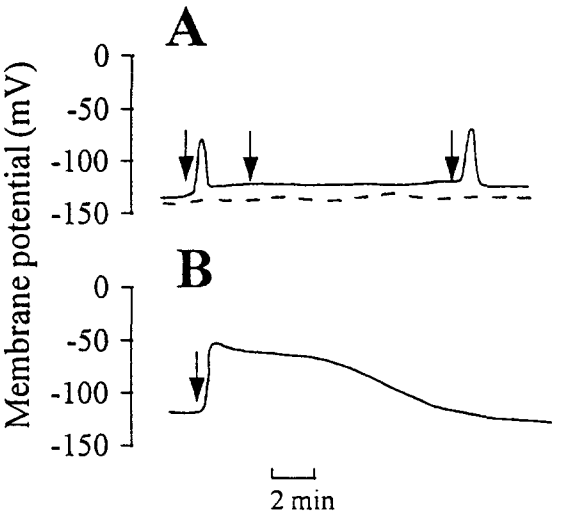

Fig. 2. Electrical response of *B. pilosa* to non-injurious and injurious stimulations. (A) Electrical response to a non-injurious stimulation. Each arrow indicates the placing of a water drop on the plant. A transient depolarization is obtained when the drop of water is put close to the measuring electrode (apical NI St, solid line) and none is observed when the deposit is far from the measuring electrode (basal NI St, dashed line). No response was observed if the drop came too soon (10–15 min) after the previous one. (B) Electrical response to an injurious stimulation: the arrow indicates the application of the heat stimulation, made in the basal part of the hypocotyl.

Our results agree with those of Wildon et al. [3] in so far as burning the plants in one location does evoke a distant electrical response followed by the accumulation of specific transcripts. Our interpretation, however, differs. In our system (*Bidens* plantlets), and in most other systems studied, burning evoked a VP (also called a SW) rather than an AP. This VP (or SW) is not a genuine electrical signal in so far as its method of propagation is still unknown. It appears to be the consequence of the prior passage of a hydraulic signal [9,12,30]. Thus, it is a VP rather than an AP that evokes calmodulin transcript accumulation at a distance followed by the inhibition of growth in *Bidens*.

Nevertheless, two major questions remain unanswered. First, what is the nature of the ion fluxes accompanying the changes in membrane potential during a VP (SW)? Our previous work [25,31] indicates the involvement of proton pumps and Ca^{2+} in the SW: treatments with 1 mM EGTA or 1 mM lanthanum reduced the amplitude of the heat-induced SW. However, the actual influx of calcium accompanying the SW has never been directly measured. The accumulation of calmodulin transcript following the SW (Fig. 3, lanes 6 and 8) may indicate that cytosolic Ca^{2+} is increased in response to an injurious stimulation. Second, what is the nature of the electrical signal evoked by a drop of water? The change in membrane potential evoked by a drop of water is either a non-propagated AP or a local electrical response to water application. Some evidences would suggest it is an AP; (i) the same response is obtained with a drop of cold water or by a temperature transition (from 22 to 16°C) in a perfusing chamber (data not shown); (ii) the majority of workers describe it as an AP [32,33] and the presence of a refractory period of about 10 min are reminiscent of an AP [19,32]. However, its lack of transmission argues against it being a genuine AP. Thus, one may speculate either that it is

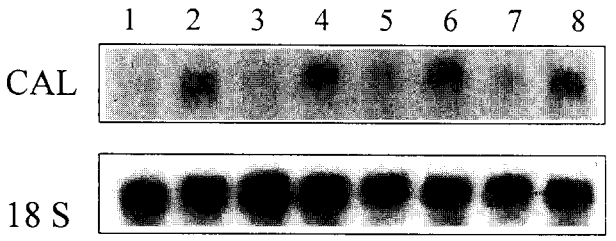

Fig. 3. Accumulation of calmodulin messenger after non-injurious or injurious stimulation. RNA was extracted from control plants (lane 1), from the basal region 20 min after basal NI stimulation (lane 2), from upper growing distant region 20 min after basal NI stimulation (lane 3), from upper growing region 20 min after apical NI stimulation (lane 4), from the first centimeter above the heated zone since 3 min (lane 5) and 20 min (lane 6) after the stimulation, and from the growing distant region, 3 min (lane 7) and 20 min (lane 8) after the heat stimulation. 18S: standardization of RNA loading with 18S cDNA probe.

not an AP or that the lack of transmission could be due to the age of the plantlets. Zawadzki [34] and Shiina and Tazawa [35] proposed that the structures responsible for excitation develop with maturity.

The hypocotyl growth inhibition (30%) induced by cotyledonary pricks has been described by Desbiez et al. [36]. The authors reported that SW (or VP) was elicited by this injurious treatment. The data presented in Table 2 show that an other kind of injurious stimulation (heat) induces a similar growth inhibition (35%). In contrast, a non-injurious stimulation given to the basal part of the hypoctyl or to the growing region caused no significant modification of growth as measured 24 h later (Table 1).

In conclusion, the data presented in this paper showed that an increase in CAL transcripts is only observed in tissues displaying a change in membrane potential. The long-distance change in membrane potential resulted in a SW or VP induced by an injurious stimulation rather than in an AP induced by a non-injurious stimulation. There was a correlation between the SW and the hypocotyl growth inhibition, suggesting that the SW carries the growth inhibition message as previously reported by Frachisse et al. [23] in the induction of correlation between the cotyledonary buds in *B. pilosa*. However, even though changes in membrane potential appear to be part of this long-distance signaling process, we do not know yet how these changes are transduced at the cellular level to evoke CAL mRNA accumulation and later to cause hypocotyl growth inhibition.

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