

# Analysis of the Transient Increase in Cytosolic Ca<sup>2+</sup> during the Action Potential of Higher Plants with High Temporal Resolution: Requirement of Ca<sup>2+</sup> Transients for Induction of Jasmonic Acid Biosynthesis and PINII Gene Expression

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Plants respond to various abiotic stimuli by activation and propagation of fast electrical signals, action potentials. To resolve the temporal increase in cytosolic Ca<sup>2+</sup> during the action potentials of higher plants, we regenerated transgenic potato plants that expressed the Ca<sup>2+</sup> photoprotein apoaequorin. These genetically engineered potato plants were used for simultaneous measurements of transient changes in the membrane potential and the Ca<sup>2+</sup> luminescence triggered by heat-induced action potentials. High temporal resolution for recording of the fast transient electrical and light signals was accomplished by a sampling rate of 1 kHz. Upon elicitation by heat the membrane potential depolarization preceded the rise of cytosolic Ca<sup>2+</sup> by 50–100 ms. Several Ca<sup>2+</sup> channel blockers were tested to inhibit the rise in cytosolic Ca<sup>2+</sup>. Treatment of plants with Ruthenium Red blocked the elevation in cytosolic Ca<sup>2+</sup> that was associated with heat-stimulated action potentials. Furthermore, action potentials have been demonstrated to stimulate jasmonic acid biosynthesis and PINII gene expression. Therefore, we measured jasmonic acid and PINII gene expression levels subsequent to action potential initiation by a short heating pulse. As expected, jasmonic acid biosynthesis and PINII gene expression were induced by action potentials. Pretreatment of potato plants with Ruthenium Red inhibited induction of jasmonic acid biosynthesis and PINII gene expression that was generally triggered by heat-activated action potentials.

**Keywords:** Aequorin — Calcium — Jasmonic acid — PINII — Signal transduction—*Solanum tuberosum*.

## Introduction

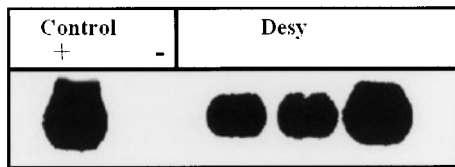
Plants sense and respond to abiotic stresses by activation of various signal transduction cascades that are often associated with an increase of cytosolic free Ca<sup>2+</sup> (Knight et al. 1997). A variety of signals has been identified as being of chemical origin, including oligosaccharides (Bishop et al. 1981), abscisic acid (Peña-Cortés et al. 1991), jasmonates (Farmer and

Ryan 1990), the peptide systemin (Pearce et al. 1991) and salicylic acid (Malamy et al. 1990). In addition to these chemical signals, fast signal transduction mechanisms have evolved within plants. These action potentials are short pulses of electric (ionic) current which rapidly travel through the entire plant (Wildon et al. 1992, Herde et al. 1995). Action potentials generally appear as a positively (intracellular electrodes) or negatively (extracellular electrodes) fast component peaking at –100 mV to 0 mV, returning to the baseline, frequently followed by a shoulder or separate peak, the recovery. It has been reported that action potentials in plants are not only triggered by insect attacks but also by mechanical damage of leaves, cold/heat treatment, and electric current application (Wildon et al. 1992, Herde et al. 1995).

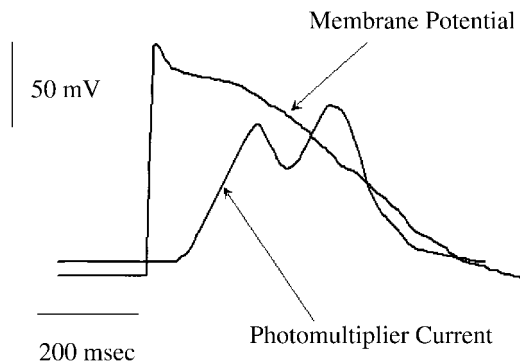
Previous studies indicated that an increase in the cytosolic free Ca<sup>2+</sup> concentration occurred during the depolarization phase of the action potential of characean algae (Williamson and Ashley 1982, Kikuyama et al. 1993). However, controversial results for the sequence of ionic fluxes during the action potential in algal cells have been reported (Thiel et al. 1997, Kikuyama and Tazawa 1998, Tazawa and Kikuyama 2003). Kikuyama and Tazawa (1998) demonstrated that the action potential and the Ca<sup>2+</sup> transient began around 100 ms after the onset of electric stimulation in characean algal cells. There was no significant difference in the starting time between the membrane potential depolarization and the luminescence initial. In contrast, Thiel et al. (1997) measured the rise in Ca<sup>2+</sup> during an action potential of *Chara* in distilled water and showed that the rise began with a delay of 0.8 s after the end of electric stimulation.

Action potentials in higher plants have been demonstrated to induce jasmonic acid (JA) biosynthesis and proteinase inhibitor gene expression (Wildon et al. 1992, Peña-Cortés et al. 1995). However, the signal transduction cascade that links ionic fluxes associated with the action potentials to induction of gene expression or JA biosynthesis has not been resolved up to now. Therefore, the nature of the ion involved in triggering the signal transduction cascade that mediates induction of gene expression needs further investigation. Here we will provide evidence that Ca<sup>2+</sup> ions could function as second messengers between

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**Fig. 1** Northern blot of the transformed lines. Potato plants (*Solanum tuberosum* L, cv. Desiree) were transformed with cytosolic apoaquorin. The expression of this gene was identified by Northern blot analysis. Three independently generated transformed lines were selected for further analysis. Desy (cv Desiree) denotes transgenic potato lines expressing the apoaquorin gene in the Desiree background.



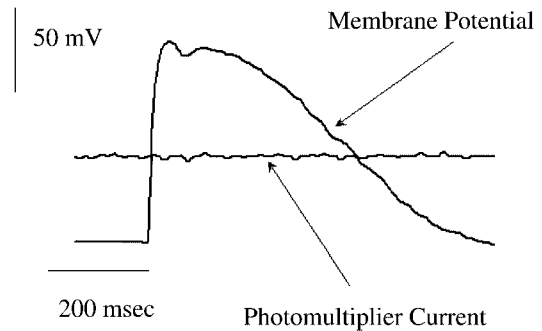
**Fig. 2** Simultaneous recording of luminescence and membrane potential. Prior to the luminescence measurements the roots of these plants were incubated in coelantherrazine. Typical responses are shown; the number of repeats of each experiment was  $n = 20$ . Luminescence and electrical membrane potential responses were recorded by a microcomputer with high temporal resolution. To elicit the action potential a heated metal cube ( $1 \text{ cm}^3$ ) was brought in contact with the tip of a leaf under dark conditions.

action potential-induced changes in cytoplasmic ionic composition and induction of PINII gene expression or JA biosynthesis.

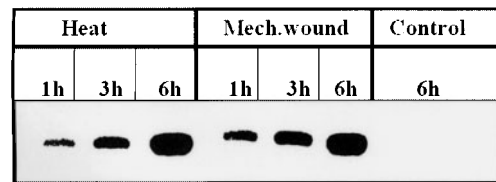
In the present study we demonstrate that action potentials of higher plants are accompanied by transient increases of cytosolic Ca<sup>2+</sup>. The time course of elevation in cytosolic Ca<sup>2+</sup> in relation to the plasma membrane depolarization will be analysed with high temporal resolution. Increases in cytosolic Ca<sup>2+</sup> could be blocked by Ruthenium Red. The effect of Ruthenium Red pretreatment for the activation of JA biosynthesis and PINII gene expression was investigated.

## Results

Expression of apoaquorin mRNA in regenerated potato plants was confirmed by Northern blot analysis (Fig. 1). Several lines of transformants were obtained and tested for luminescence emission upon elicitation. If not otherwise noted heat treatment was applied to evoke action potentials and to induce Ca<sup>2+</sup>-dependent luminescence. Finally, three lines of transformants were selected for further investigation.



**Fig. 3** Inhibition of the luminescence response by Ruthenium Red. Leaves were pretreated with Ruthenium Red (0.1 mM). The action potential was triggered by heating of the tip of the leaf by a hot metal cube ( $n = 20$ ).

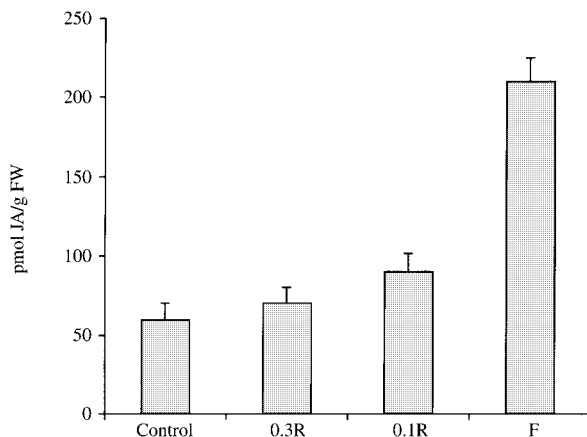


**Fig. 4** PINII gene expression induced by heat or by mechanical wounding. Samples for Northern blot analysis were taken at 1 h, 3 h, and 6 h after stimulus application.

To elucidate the time course of increasing levels of cytosolic Ca<sup>2+</sup> during the action potential, we performed simultaneous recordings of the membrane potential and the Ca<sup>2+</sup>-dependent luminescence (Fig. 2). Subsequent to insertion of a microcapillary into an epidermal cell of a potato leaf, elicitation by heat was used to trigger the action potential. The high sampling rate of our measuring system (1 kHz) provided sufficient temporal resolution to discriminate between transients in the membrane potential and the luminescence response. Obviously, the membrane depolarization preceded the rise in cytosolic Ca<sup>2+</sup> by 20–50 ms (Fig. 2). Ca<sup>2+</sup>-induced luminescence reached a first maximum when the membrane potential had already begun to repolarize. During the repolarization of the membrane potential a second peak in Ca<sup>2+</sup> induced luminescence was detected.

Pretreatment of plants with Ruthenium Red, a known Ca<sup>2+</sup> channel blocker, completely inhibited the increase in cytosolic calcium during membrane potential depolarization (Fig. 3). In addition, further Ca<sup>2+</sup> channel blockers were tested (LaCl<sub>3</sub>, Nifedipine, Verapamil). However, none was successful in inhibiting the rise in cytosolic Ca<sup>2+</sup> during the action potential.

To confirm induction of PINII gene expression by action potentials we used a PINII probe for Northern blot hybridization (Fig. 4; Wildon et al. 1992). Triggering the action potential by heat treatment or mechanical wounding (Peña-Cortés et al. 1995) gave rise to the local and systemic induction of PINII gene expression. Highest levels of transcripts were detected 6 h



**Fig. 5** Induction of JA biosynthesis was inhibited by pretreatment of the leaves with Ruthenium Red. Untreated leaves (F) resulted in a strong increase in JA biosynthesis upon stimulation by heat. 0.3 R: pretreatment with 0.3 mM Ruthenium Red; 0.1 R pretreatment with 0.1 mM Ruthenium Red ( $n = 20$ ).

after stimulus application. However, PINII transcripts could already be detected 20 min after elicitation.

Previous studies demonstrated that PINII gene expression is correlated with induction of JA biosynthesis. Therefore, we measured JA levels after stimulation of potato leaves by heat (Fig. 5). As expected JA biosynthesis was induced by heat stimulation. However, induction of JA biosynthesis was absent in potato plants that were pretreated with Ruthenium Red (Fig. 5). Similarly, the induction of PINII gene expression was suppressed by Ruthenium Red pretreatment (data not shown).

## Discussion

To elucidate the sequence of ionic currents mediating action potential signaling of higher plants we introduced the Ca<sup>2+</sup>-sensitive photoprotein aequorin into transgenic potato plants. Simultaneous measurements of the electrical membrane potential and the Ca<sup>2+</sup> luminescence with high temporal resolution demonstrated that increases in cytosolic Ca<sup>2+</sup> levels emerged in a very early phase in the time course of the action potential (Fig. 2). In particular, a rapid elevation in cytosolic Ca<sup>2+</sup> within the first 100 ms after stimulation was indicated by the luminescence signal. Similar time courses for spikes in cytosolic Ca<sup>2+</sup> were described by Kikuyama and Tazawa (1998). These authors investigated action potential mechanisms of characean algae cells and suggested that influx of exogenous Ca<sup>2+</sup> into the cytoplasm could activate Cl<sup>-</sup> channels. Evidence in support of this hypothesis was provided by replacement of the external Ca<sup>2+</sup> with Mg<sup>2+</sup> or Mn<sup>2+</sup>. Under these conditions the transient rise in cytosolic Ca<sup>2+</sup> was abolished; however, action potentials were generated. In contrast, Thiel et al. (1997) reported the emergence of Ca<sup>2+</sup> currents in significantly later phases of the time course of the action poten-

tial. Therefore, our measurements provide support to the hypothesis of Kikuyama and Tazawa (1998) in regard to the time course of Ca<sup>2+</sup> transients associated with action potentials in plant cells.

Action potentials in potato plants were able to propagate without elevation in cytosolic Ca<sup>2+</sup> (Fig. 3). Similar results were reported for the action potential in characean algae cells (Shiina and Tazawa 1987). However, these action potentials were elicited in tonoplast free cells of the freshwater alga *Chara*. Ca<sup>2+</sup> levels inside these algal cells were kept constant due to addition of the strong Ca<sup>2+</sup> chelator EGTA (ethyl-ene-glycol-bis-( $\beta$ -aminoethylether)-*N,N,N,N*-tetraacetic acid; Kikuyama et al. 1984). In addition to absence of a cytosolic Ca<sup>2+</sup> transient, these action potentials were deficient in Cl<sup>-</sup> currents. Action potentials in higher plants that proceeded without elevation in cytosolic Ca<sup>2+</sup> failed to induce JA biosynthesis (Fig. 5). This finding suggests that action potentials with a complete Ca<sup>2+</sup> signature are required for activation of plant defense reactions against pathogens or abiotic stress conditions. Plasma membrane ion channels are rapidly activated by pathogen infection or elicitor treatment of plant cells. In particular, extracellular alkalization, Ca<sup>2+</sup> influx and effluxes of K<sup>+</sup> and Cl<sup>-</sup> led to depolarization of the plasma membrane in parsley cells. When these cells were pretreated with Ruthenium Red they failed to activate the fast Ca<sup>2+</sup> spike upon treatment with Pep-13, an oligopeptide fragment of a 42-kDa *Phytophthora sojae* cell wall glycoprotein (Blume et al. 2000). However, elicitation by Pep-13 is on a slower time scale than the described action potential observed in the present study.

The action potential mechanism of higher plants that we have investigated by using transgenic potato plants thus resembled action potentials in characean algae. In future work the additional ionic components will have to be identified that precede the rise in cytosolic Ca<sup>2+</sup>. However, it can be hypothesized, that these initial ionic currents are mediated by K<sup>+</sup> and Cl<sup>-</sup> ions. Similar to mammalian tissue the action potential of higher plants provides the fastest mechanism for long-distance signaling between roots and shoots or among leaves. Previous work has demonstrated that the occurrence of action potentials within plants induces expression of pathogen response genes (Wildon et al. 1992). A putative link between ionic currents and induction of defense genes could be provided by the JA biosynthetic pathway which has been demonstrated to induce PINII gene expression.

## Materials and Methods

### Plant material

The cytoplasmic-targeting apoaequorin expression vector A-6793 (MoBiTec, Göttingen, Germany) was transferred to *Solanum tuberosum* Mill.cv.Desiree plants, using the *Agrobacterium tumefaciens* pBIN19 binary vector system to provide constitutive expression (Smith et al. 1988). Plants were grown in axenic culture at 22°C, 50% rel. humidity, 3,000 Lux and a 16 h light photoperiod. Expression was detected at the RNA level and by checking the functionality of the

gene product. The aequorin reconstitution from apoaequorin in whole plants was performed by incubating the plants in coelenterazine solution (5 µM Coelenterazine hcp, 8 h incubation). Experimental plants were 2–4 weeks old and sizes differed between 10 and 20 cm in height.

#### Measurement of Ca<sup>2+</sup>

Luminescence signals generated by heat treatment were detected in a specially prepared darkroom by a photomultiplier (Nikon P 102, Japan). Luminescence and electrical membrane potential responses were recorded simultaneously by an analog to digital converter connected to a microcomputer. Sampling rates of 1,000 Hz were used to provide sufficient temporal resolution.

#### Membrane potential recordings

Quartz glass capillaries with a solid filament (Hilgenberg, Malsfeld, Germany) were pulled on a special laser heated pulling device (P2000, Sutter Instruments, U.S.A.). These capillaries were filled with 0.1 M KCl (Fisahn et al. 1986). For recordings of the membrane potential these capillaries were mounted on the headstage of a gene clamp amplifier HS 2A (Axon Instruments, Foster City, CA, U.S.A.). The ground reference consisted of an Ag/AgCl silver wire electrode that was attached to the stem of the experimental plant as described by Fromm et al. (1995).

The entire setup was placed in a specially prepared dark room that enabled detection of luminescence by a photomultiplier. Luminescence and electrical membrane potential responses were recorded simultaneously by a microcomputer.

#### Mechanical wounding and heat treatment

To elicit the action potential a cube of hot metal (1 cm<sup>3</sup>, 80°C) was brought in contact with the experimental leaf for 1 s. For wounding the plants mechanically, dialysis clamps were applied as described by Sanchez-Serrano et al. (1986).

#### Gel blot analysis of RNA

Plant total RNA was isolated and subjected to electrophoresis (10 µg of RNA per slot) in agarose-formaldehyde gels as described by Logemann et al. (1987). Blotting and hybridization conditions were as described by Amasino (1986). Probes used for radioactive labeling consisted of potato PINII (cDNA; Sanchez-Serrano et al. 1986) and small subunit of Rubisco cDNA (rbcS; Eckes et al. 1985; Fig. 1, control plus and minus rbcS).

#### Application of Ca<sup>2+</sup> channel blockers and JA analysis

Potato leaves were detached from the entire plant and petioles were incubated in a solution of the inhibitor for 1 h (Herde et al. 1997). Immediately after incubation the heat stimulus was applied and petioles were kept in the solution for 4 h. JA isolation and analysis was as detailed in Herde et al. (1996).

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