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Electric Signaling and *Pin2* Gene Expression on Different Abiotic Stimuli Depend on a Distinct Threshold Level of Endogenous Abscisic Acid in Several Abscisic Acid-Deficient Tomato Mutants[1]

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Experiments were performed on three abscisic acid (ABA)-deficient tomato (*Lycopersicon esculentum* Mill.) mutants, *notabilis*, *flacca*, and *sitiens*, to investigate the role of ABA and jasmonic acid (JA) in the generation of electrical signals and *Pin2* (proteinase inhibitor II) gene expression. We selected these mutants because they contain different levels of endogenous ABA. ABA levels in the mutant *sitiens* were reduced to 8% of the wild type, in *notabilis* they were reduced to 47%, and in *flacca* they were reduced to 21%. In wild-type and *notabilis* tomato plants the induction of *Pin2* gene expression could be elicited by heat treatment, current application, or mechanical wounding. In *flacca* and *sitiens* only heat stimulation induced *Pin2* gene expression. JA levels in *flacca* and *sitiens* plants also accumulated strongly upon heat stimulation but not upon mechanical wounding or current application. Characteristic electrical signals evolved in the wild type and in the *notabilis* and *flacca* mutants consisting of a fast action potential and a slow variation potential. However, in *sitiens* only heat evoked electrical signals; mechanical wounding and current application did not change the membrane potential. In addition, exogenous application of ABA to wild-type tomato plants induced transient changes in membrane potentials, indicating the involvement of ABA in the generation of electrical signals. Our data strongly suggest the presence of a minimum threshold value of ABA within the plant that is essential for the early events in electrical signaling and mediation of *Pin2* gene expression upon wounding. In contrast, heat-induced *Pin2* gene expression and membrane potential changes were not dependent on the ABA level but, rather, on the accumulation of JA.

The plant hormones ABA and JA play a predominant role in the conversion of environmental signals into changes in plant gene expression. An increase in endogenous ABA and JA levels precedes and is involved in *Pin2* (proteinase inhibitor II) gene expression upon wounding (Peña-Cortés et al., 1989, 1991, 1995, 1996; Farmer and Ryan, 1992; Farmer et al., 1992). This increase in ABA and JA is not restricted to the tissue damaged directly but can also be detected in the nonwounded, systemically induced tissue (Peña-Cortés et al., 1989; Peña-Cortés and Willmitzer, 1995). The accumulation of ABA and JA have been described for several plant species, including potato, tomato, and tobacco (Sanchez-Serrano et al., 1991; Peña-Cortés and Willmitzer, 1995).

Further evidence for the involvement of ABA and JA in wound-induced *Pin2* gene expression was provided by a series of experiments in which potato plants were sprayed with ABA or JA and *Pin2* mRNA accumulated in the absence of any wounding (Peña-Cortés et al., 1989; Hildmann et al., 1992). Both nonsprayed leaves and leaves that were sprayed directly showed increased *Pin2* mRNA levels with a pattern identical to the one described for wounded plants (Peña-Cortés et al., 1988; Peña-Cortés and Willmitzer, 1995). Conclusive evidence for the involvement of ABA in wound-induced *Pin2* activation was obtained from mutants impaired in ABA biosynthesis. Consequently, wound induction of *Pin2* was not observed in the *droopy* mutant of potato or the *sitiens* mutant of tomato (Peña-Cortés et al., 1989). However, in these mutants treatment with ABA caused a return of the accumulation of *Pin2* mRNA to levels normally found in wild-type plants upon wounding (Peña-Cortés et al., 1991).

Like wounding, the application of electrical current was able to initiate ABA and JA accumulation in wild-type plants but not in ABA-deficient plants (Herde et al., 1996). These results suggested that, like wounding, electrical current requires the presence of ABA for the induction of *Pin2* gene expression (Herde et al., 1996). In contrast to wounding and electrical current, burning of leaves activated *Pin2* gene expression in *sitiens* mutants by directly triggering the biosynthesis of JA via an alternative pathway that is independent of endogenous ABA levels (Herde et al., 1996).

To determine the endogenous levels of ABA that are sufficient to mediate electrical current-, heat-, and wound-induced *Pin2* gene expression via electrical signals, we used several tomato mutants containing progressively reduced levels of ABA. The effects of these attenuated ABA levels on JA content and membrane potentials and the expression pattern of *Pin2* genes were analyzed. Analysis of JA content was conducted to confirm the existence of an

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Abbreviation: JA, jasmonic acid.

alternative pathway that is independent of endogenous ABA levels in the different ABA-deficient mutants.

MATERIALS AND METHODS

Plant Material and Growth Conditions

Wild-type and the ABA-deficient mutants *flacca*, *notabilis*, and *sitiens* (generously donated by Dr. Maarten Koornneef, Wageningen, The Netherlands) of tomato (*Lycopersicon esculentum* Mill. cv Moneymaker [MM]) were grown in the greenhouse at 26°C day/20°C night temperatures, 70% to 80% RH, and 14 h of light. Characterization of the mutants and determination of the endogenous levels of ABA were published previously (Taylor, 1987). Two weeks after germination, the mutants were wrapped in plastic to provide 100% humidity. All measurements were performed on 21- to 28-d-old plants at 100% humidity.

Mechanical Wounding, Current Application, Heat Treatment, and Elicitation by ABA

Mechanical damage to plants was due to dialysis clamps applied as described by Sanchez-Serrano et al. (1986). For current application a direct-current power supply provided 10 V for 30 s, as described by Herde et al. (1995). Current was supplied extracellularly through Ag/AgCl surface electrodes. Heat treatment was performed on the tip of a leaf. Approximately 1 cm^2 was burned as described by Malone and Stankovic (1991) and Herde et al. (1996).

In all experiments stimuli were applied on leaf 4, which we refer to as "local." Leaf 2 is referred to as "systemic" (not treated). Treatment of plants was at 4 PM; at 10 PM tissue samples were taken for analysis of *Pin2* gene expression and ABA and JA contents.

Application of ABA was via a microincision in the major vein of a leaf 2 cm from the point of measurement. Cutting was with a high-frequency microcauter (hf SURG, Meyer-Haake, Oberursel, Germany), which provided a surgical tip 1 μm in diameter. No alterations in the membrane potential upon cutting could be detected. The experimental solution was water buffered with Tris-HCl at pH 7.5, with a final concentration of 10 μM ABA. A drop containing 10 μL of this solution was introduced in the microincisions.

ABA and JA Quantification

Endogenous ABA and JA levels were determined as described by Lehmann et al. (1995). In this ELISA an antibody highly specific for (−)-JA and L-amino acid conjugates of (−)-JA, as specified by Knöfel et al. (1990), was used. ABA/JA sample quantitation values given are averages ± SD (n = 5).

Gel-Blot Analysis of RNA

Total plant 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 *Pin2* (cDNA 1; Sanchez-Serrano et al., 1986). Each experiment was repeated independently at least five times. The northern blots and ABA/JA quantification shown in the figures are representatives of the average.

Membrane Potential Recordings

Quartz glass capillaries with a solid filament (Hilgenberg, Malsfeld, Germany) were pulled on a laser-heated pulling device (P2000, Sutter Instruments, Navaro, CA), resulting in a tip diameter of 150 nm. These capillaries were filled with 0.1 M KCl, as described by Fisahn et al. (1986). For recordings of the membrane potential, these capillaries were mounted on the headstage of a gene-clamp amplifier (model HS 2A, Axon Instruments, Foster City, CA). The ground reference consisted of an Ag/AgCl wire electrode that was attached to the stem as described by Fromm et al. (1995). For a detailed description of the protocol for membrane potential recordings, see Herde et al. (1998).

RESULTS

Pin2 Gene Expression Occurs in *flacca and sitiens* Only after Heat Treatment

Whole plants were mechanically wounded, treated with electrical current, or gently burned at the tip of a leaf. After 6 h, the time at which wound-induced JA accumulation reached its highest levels, treated and nontreated systemic leaves were harvested and analyzed for *Pin2* gene expression by northern analysis (Fig. 1). As expected, mechanical damage and electrical current application led to a local and systemic accumulation of *Pin2* mRNA in wild-type plants (Fig. 1). *notabilis* exhibited the same pattern of *Pin2* gene expression as the wild type with all three stimuli. In contrast, the *flacca* and *sitiens* mutants activated *Pin2* gene expression only with heat treatment (Fig. 1). Further extended exposure of the autoradiograms did not reveal accumulation of *Pin2* mRNA in *sitiens* plants following either wounding or electrical current application (data not shown).

Differential Accumulation of ABA and JA

Endogenous levels of ABA and JA in the mutants *notabilis*, *flacca*, and *sitiens* were determined with all three stimuli. In particular, hormone concentration was measured 6 h after treatment in both treated leaves (local) and leaves located distal (systemic) of the treated ones. As expected, ABA and JA accumulated in wild-type tomato plants following wounding, current application, and heat treatment (Figs. 2 and 3). Because of inhibition in ABA biosynthesis, the mutants *flacca* and *notabilis* produced less ABA than the wild type (Fig. 2). Furthermore, these mutants exhibited reduced levels of ABA compared with the wild type upon mechanical wounding, current application, or heat treatment (Fig. 2). The *sitiens* mutants were unable to substantially increase their ABA levels upon elicitation (Fig. 2).

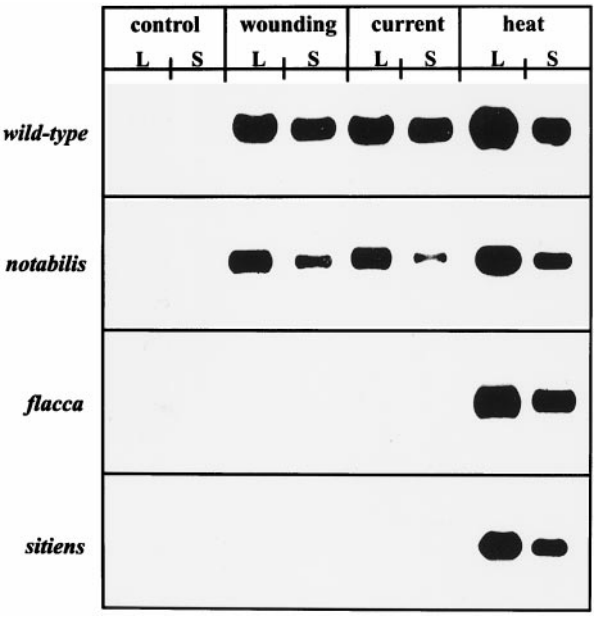

Figure 1. Mechanical wounding, electrical current, and heat treatments initiate the local and systemic accumulation of *Pin2* mRNA. Wild-type (MM) and ABA-deficient (*notabilis*, *flacca*, and *sitiens*) mutant tomato plants were wounded or treated with electrical current or heat. Six hours after treatment, total RNA was isolated from the treated leaves (L) and from leaves located distal (S) to the treated ones. The autoradiogram shows the result of a gel-blot hybridization of total RNA (10 μg per slot) against radioactive *Pin2* (Sanchez-Serrano et al., 1986).

JA was measured to confirm the existence of an alternative pathway that is independent of endogenous ABA levels and was found to accumulate to a minor extent after elicitation by mechanical wounding or current application in *notabilis*, *flacca*, and *sitiens* mutants compared with wild-type plants (Fig. 3). This reduction in JA levels resembled the decrease in endogenous ABA in *notabilis* and *flacca* mutants upon mechanical wounding or current application (Figs. 2 and 3). Conversely, JA accumulation upon heat treatment was not influenced by endogenous levels of ABA. All of the mutants exhibited JA contents comparable to those observed in wild-type plants in the absence of elicitation (Fig. 3).

Membrane Potential Recordings

In the signal transduction cascade mediating elicitor-induced *Pin2* gene expression, membrane transport events precede hormone biosynthesis and changes in gene expression patterns. To measure relaxation kinetics of membrane potentials, a microelectrode was targeted to the region of the most negative membrane potential within the major vein of a tomato leaf (Herde et al., 1998). Upon arrival of the microelectrode in the target region and establishment of a steady-state recording of the membrane potential, one of the three stimuli was applied to a remote leaf and the electrical signal was recorded (Herde et al., 1998). In wild-type tomato plants all three stimuli resulted in an initial rapid depolarization that was followed by a second time constant (Herde et al., 1998). A prolonged depolarization was observed during this second phase.

The relaxation kinetics of the membrane potential that were generated in *notabilis* and *flacca* plants upon wounding, current application, and heat treatment resembled those of wild-type plants (Fig. 4; Herde et al., 1998). In addition to this similarity, two differences became most obvious: (a) the depolarization of the slow component within the ABA-deficient plants was not as pronounced as in the wild type, in accordance with the endogenous levels of ABA; and (b) the absolute values of the membrane potential were more negative in the ABA-deficient plants, in accordance with decreasing levels of endogenous ABA within the mutants (Fig. 4; Herde et al., 1998). No changes in the membrane potential upon mechanical wounding or current application occurred in the mutant with the lowest endogenous ABA content (*sitiens*). In contrast, application of a heat stimulus to *sitiens* resulted in relaxation kinetics of the membrane potential that consisted of a fast initial depolarization followed by a second, slow depolarization (Herde et al., 1998). Similar to the mechanisms of the heat-induced *Pin2* gene expression, generation of electrical signals upon heat stimulation also seemed to be independent of endogenous ABA levels.

Application of exogenous ABA to microincisions of the major vein of wild-type tomato leaves induced character-

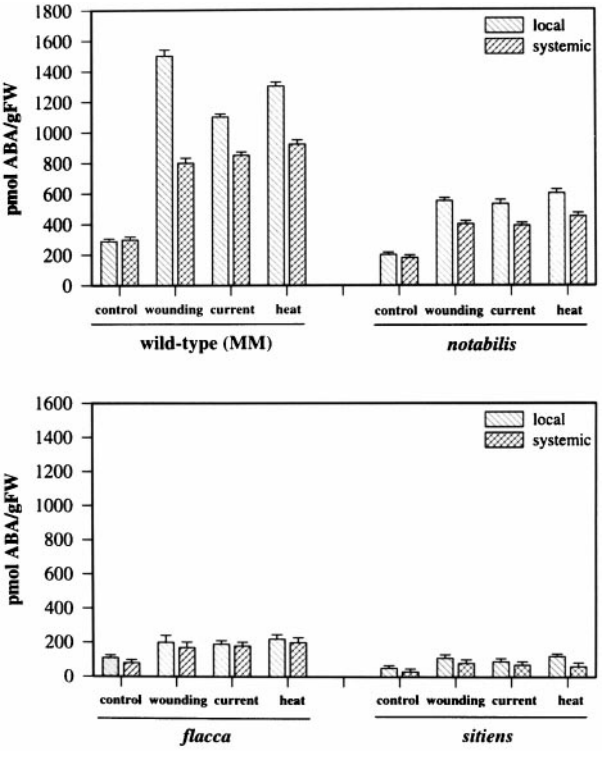

Figure 2. ABA content in tomato plants. Wild-type (MM) and ABA-deficient (*notabilis*, *flacca*, and *sitiens*) mutant tomato plants were wounded or treated with electrical current or heat. Both directly treated (local) and nontreated distal leaves (systemic) were harvested after 6 h. Endogenous levels of ABA were determined as described in "Materials and Methods" and are indicated as means ± SE (n = 5). FW, Fresh weight.

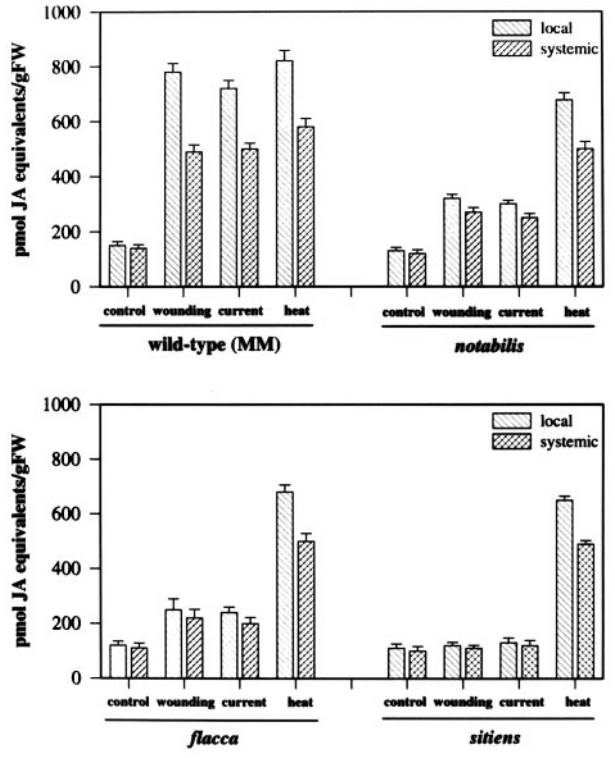

Figure 3. Effect of wounding, electrical current, and heat treatment on endogenous levels of JA. Wild-type (MM) and ABA-deficient (*notabilis*, *flacca*, and *sitiens*) mutant tomato plants were wounded or treated with electrical current or heat. Both directly treated (local) and nontreated distal leaves (systemic) were harvested after 6 h. Endogenous levels of JA were determined as described in "Materials and Methods" and are indicated as means ± SE (n = 5). FW, Fresh weight.

istic changes of the membrane potential (Fig. 5). In particular, ABA treatment resulted in a rapid depolarization followed by a period of damped oscillations lasting for 10 to 15 min. In contrast, application of the experimental solution without ABA had no effect on the membrane potential.

DISCUSSION

Previous studies revealed local and systemic induction of *Pin2* gene expression and JA accumulation upon mechanical wounding and electrical current in wild-type tomato plants (Fig. 1). In contrast, *sitiens* mutants were unable to respond to mechanical damage or electrical stimulation in terms of JA accumulation and *Pin2* gene expression. However, burning leaves of *sitiens* mutants gave rise to local and systemic accumulation of JA (Herde et al., 1996). Our findings suggest that (a) wound- or electrically induced JA accumulation might depend on distinct levels of ABA that exist in wild-type plants but not in *sitiens* plants and (b) heat-induced JA accumulation does not require elevated levels of ABA.

Since exogenous application of JA did not evoke an elevation in ABA (Peña-Cortés et al., 1996), the accumulation of this hormone in wild-type plants upon heat treatment demonstrated that elicitation by heat has the capacity to activate two biosynthetic pathways independently (i.e. ABA and JA biosynthesis). Furthermore, the accumulation of JA in *sitiens* plants indicated that heat treatment was able to activate JA biosynthesis independently of the internal levels of ABA (Fig. 3). Convincing evidence has been reported showing the involvement of JA in several stress responses of higher plants (Sembdner and Parthier, 1993). A model was proposed by Farmer and Ryan (1992) in which the release of the peptide systemin as a result of tissue damage may lead to an activation of a lipase in the plasma membrane. The subsequent release of linolenic acid would then produce a rapid accumulation of JA, which in turn may trigger the activation of wound-responsive genes. Based on this proposed model, we investigated the role of JA in wound-, current-, and heat-induced *Pin2* gene expression and in the process of generating electrical signals.

By analyzing tomato mutants that were progressively impaired in ABA biosynthesis, we obtained additional information concerning a minimal content of ABA required for stress-induced *Pin2* gene expression (Fig. 1). In *notabilis* tomato mutants, which have an ABA content that is 47% of the wild type, all three stimuli induced *Pin2* gene expression. In contrast, *flacca* tomato mutants, which have an ABA content that is 21% of the wild type, resembled the *Pin2* gene expression pattern found in *sitiens* tomato mutants, which have an ABA content that is 8% of the wild type. Only heat treatment led to the accumulation of *Pin2* mRNA in *sitiens* mutants; mechanical wounding and current application did not (Fig. 1). Since *Pin2* mRNA was not detected upon wounding or current application in mutants with progressive attenuation in ABA content, it could be suggested that a certain threshold value of ABA exists, functioning as an on/off switch for wound-induced *Pin2*

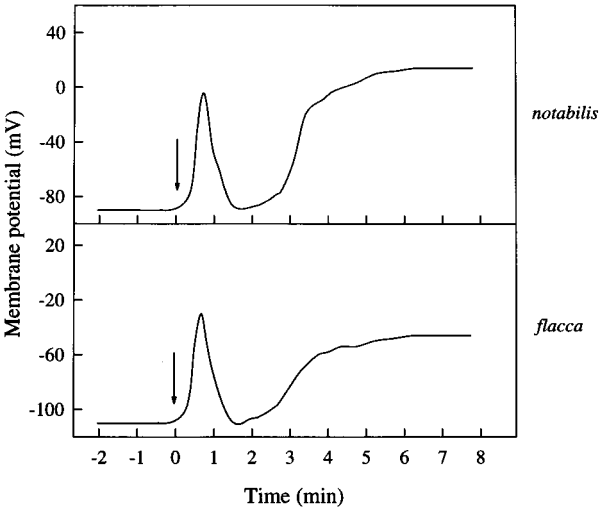

Figure 4. Characteristic membrane potential relaxation kinetics in the main vein of a leaf of ABA-deficient tomato mutants caused by mechanical wounding (indicated by an arrow). Membrane potential recordings caused by current application and heat treatment resembled those after mechanical wounding (data not shown). The results shown are characteristic examples of a pool of 30 measurements per plant.

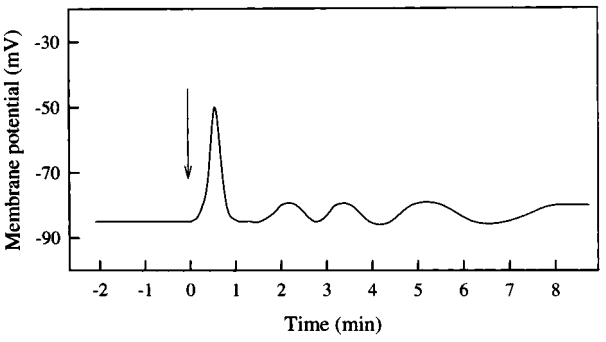

Figure 5. Characteristic membrane potential relaxation kinetics in the main vein of a wild-type tomato plant caused by exogenous application of ABA (indicated by an arrow) . The results shown are characteristic examples of a pool of 30 measurements per plant.

gene expression (Fig. 1). Therefore, the ABA level functioning as the threshold value for the ABA-dependent pathway that leads to *Pin2* gene expression upon wounding and current application was between 21% and 47% of that found in wild-type plants.

The ABA level that accumulated in wounded and current-treated plants was roughly reflected by the respective JA content of wild-type and mutant plants after these treatments. These findings are in line with the results of Peña-Cortés et al. (1995), who demonstrated that the application of ABA is able to induce JA biosynthesis and, furthermore, that JA is located downstream of ABA in the signal transduction cascade leading to *Pin2* gene expression. Conversely, unwounded control plants of the wild type and the *notabilis*, *flacca*, and *sitiens* mutants had almost identical levels of JA, suggesting that JA biosynthesis is not influenced by endogenous levels of ABA. Harms et al. (1995) reported that high endogenous levels of JA in transgenic plants overexpressing a flax allene oxide synthase cDNA did not induce constitutive *Pin2* gene expression. One possible explanation for these phenomena is that endogenous ABA and JA are stored in cellular compartments different from those where ABA and JA accumulate upon wounding. JA biosynthesis is suggested to occur in the chloroplast (Harms et al., 1995), which implies that accumulation of JA in this organelle is not sufficient to lead to changes in the expression of JA-responsive genes such as *Pin2*.

The results presented here indicate an involvement of ABA in the early events of the signal transduction cascade mediating wound-induced *Pin2* gene expression. In particular, wild-type tomato plants generated an electrical signal upon all three stimuli, mechanical wounding, current application, and heat treatment. Similarly, the relaxation kinetics of the membrane potential that were generated in *notabilis* and *flacca* plants upon these stimuli resembled those of wild-type plants (Fig. 4; Herde et al., 1998). Exogenous application of ABA to wild-type tomato plants induced transient changes in membrane potential as well. These changes in the membrane potential consisted of a fast, transient depolarization followed by a period of damped oscillations lasting for 10 to 15 min (Fig. 4). Like the initial fast depolarization, these oscillations could have been due to an influx of Ca^{2+} into the cytosol. Oscillations in Ca^{2+} are often observed in animal cells, especially when phosphoinositide metabolism is stimulated (Berridge and Galione, 1988; Berridge et al., 1988).

In the mutant with the lowest endogenous ABA content, *sitiens*, there were no changes in the membrane potential upon mechanical wounding or current application (Herde et al., 1998). In contrast, application of a heat stimulus resulted in relaxation kinetics of the membrane potential consisting of a fast depolarization followed by a second time constant. The results of the *Pin2* gene expression analysis suggested that the generation of electrical signals upon heat treatment is independent of endogenous ABA levels. Moreover, the depolarization of the slow component within the ABA-deficient plants was not as pronounced as in the wild type, which is in agreement with a progressive reduction in endogenous levels of ABA within the different mutants. Like wound-induced *Pin2* gene expression, there was a distinct threshold value required for propagation of electrical signals within the plant. Compared with the role of ABA in wound-induced *Pin2* gene expression, two differences are obvious: (a) the variation potential generated upon mechanical damage or current application became less pronounced in correlation with progressive attenuation in endogenous ABA levels, indicating a graduated response to different levels of ABA; and (b) the ABA threshold value required for a change in the membrane potential upon wounding and current application (between 8% and 21% of the wild type) was below that required for an induction of *Pin2* gene expression (between 21% and 47% of the wild type). Resting membrane potential is also dependent on ABA levels. In parallel to progressive decreases in ABA content, the resting membrane potentials became more negative (Fig. 4; Herde et al., 1998).

The results presented in this report suggest that the ABA-dependent pathway leading to *Pin2* gene expression upon wounding and current application requires ABA levels ranging between 21% and 47% of those found in wild-type tomato plants. Within these ranges, a distinct threshold value seems to exist, functioning as an on/off switch for the subsequent *Pin2* gene activation. In contrast, heat-induced *Pin2* mRNA accumulation is regulated by triggering directly the biosynthesis of JA by an alternative pathway that is independent of normal levels of ABA. In terms of the membrane potential, two major differences emerged between wild-type and ABA-deficient tomato plants. First, ABA-deficient mutants were not able to generate electrical signals upon wounding and current application below a certain threshold value of endogenous ABA. Conversely, stimulation by heat induces the generation of electrical signals independently of ABA. Unlike the threshold level of ABA required for *Pin2* gene expression, the threshold level of ABA for membrane transporter activation ranged between 8% and 21% of the wild type. Second, resting membrane potential was more negative in ABA- deficient mutants, in accordance with decreasing levels of endogenous ABA. Exogenous application of ABA to wild-type tomato plants induced transient changes in membrane potentials, indicating the essential role of ABA in the generation of electrical signals within plants.

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