Electrophysiological Characterization of the Arabidopsis
avrRpt2-Specific Hypersensitive Response in the
Absence of Other Bacterial Signals

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The hypersensitive response (HR) is defined as rapid cell collapse at the infection site and often accompanies plant resistance. The physiological processes leading to HR are not well understood. Here, we report an electrophysiological characterization of bacterial HR caused by a single avirulence gene in the absence of other bacterial signals. We used dexamethasone (dex)-inducible transgenic Arabidopsis (Arabidopsis thaliana) plants containing the avrRpt2 gene from Pseudomonas syringae pv tomato. Membrane depolarization in these plants began 1 to 1.5 h after dex application, hours before electrolyte leakage. Progressive depolarization was a sensitive early indicator of HR that occurred only in Arabidopsis leaf cells expressing both avrRpt2 and a functional RPS2 gene. Hyperpolarization of fully depolarized membranes by fusicoccin, a fungal toxin that activates the H^+ATPase, indicates that depolarization did not result from a nonfunctional pump or leaky membranes. Depolarization and electrolyte leakage were inhibited in RPS2 plants by the calcium channel blocker LaCl_3, highly correlating these events and suggesting that Ca^2+ entry into cells is required for both. Also correlated were inhibition of depolarization, electrolyte leakage, and HR following salicylic acid pretreatment. In salicylic acid-pretreated RPS2 seedlings, avrRpt2 transcript was produced after dex treatment. However, AvrRpt2 protein accumulation was greatly reduced, suggesting a possible mechanism for inhibition of HR in plants with induced resistance. This experimental system is a very sensitive assay that lends itself to the dissection of physiological processes leading to HR in plants, and provides a baseline for future research within a genetic framework.

Plants are resistant to infection by many pathogens. Plants expressing a resistance (R) gene rapidly initiate defense responses following contact with a pathogen that expresses a corresponding avirulence (avr) gene (Flor, 1971). Isogenic plants lacking the R gene fall prey to the pathogen. The hypersensitive response (HR), rapid cell collapse at the site of infection, is often dramatic evidence of resistance, particularly in bacteria-induced HR (Goodman and Novacky, 1994; Heath, 2000).

Many defense responses associated with bacteria-induced HR have been described since Klement et al. (1964) first demonstrated the total collapse of non-host plant leaves following infiltration of pathogenic bacteria. They include ion fluxes, increased salicylic acid (SA) production, membrane depolarization, and pathogenesis-related (PR) gene induction (Goodman and Novacky, 1994; Dangl et al., 1996; Hammond-Kosack and Jones, 1996; Dangl and Jones, 2001). Yet, the mechanism of HR induction is not well understood.

The functional significance of bacteria-induced HR also is not clear because defense responses and resistance can occur without HR. For example, systemically acquired resistance, resistance to a broad spectrum of pathogens, can be induced in normally susceptible plants by pathogen infection or application of SA (Ryals et al., 1996; Hammerschmidt, 1999; Alvarez, 2000; Heath, 2000). These now resistant plants limit the multiplication of avirulent bacteria without undergoing HR. Similarly, some mutant plants such as Arabidopsis (Arabidopsis thaliana) dnd1 and hrl1 are resistant and do not undergo HR (Yu et al., 1998; Devadas and Raina, 2002).

In the past decade, the isolation of bacterial elicitors, e.g. harpin, and advances in molecular techniques have made it possible to investigate signaling and related plant membrane responses during HR. Ion fluxes were observed immediately after harpin from Erwinia amylovora (harpin_Ea) was applied to tobacco (Nicotiana tabacum) suspension cells (Baker et al., 1993), and the membrane potential depolarized just minutes after harpin_Ea was added to the bath solution surrounding suspension cells (Popham et al., 1995) or leaf segments (Pike et al., 1998). Depolarization patterns distinguished between leaves from plants that underwent HR and those that did not collapse (Pike et al., 1998). Yet, the pathway between harpin perception, depolarization, and HR-like cell death is unknown.

The signaling pathway from the interaction between a bacterial avr gene product and a specific plant R gene product that leads to HR is also largely unknown. It

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may differ from the pathway(s) used by externally sensed elicitors because avr gene products are injected into the plant cell through a bacterial type III secretion system (Alfano and Collmer, 1997; Mudgett and Staskawicz, 1998), obviating the need for transmembrane signaling. Significant sustained increases in intracellular calcium were found during HR caused by AvrB or AvrRpm1 in a study of transformed Arabidopsis expressing the Ca^{2+}-sensitive luminescent protein aequorin (Grant et al., 2000). No sustained Ca^{2+} increase was found in corresponding susceptible interactions. However, when bacteria initiate the HR, they produce both nonspecific elicitors that induce plant defense responses, for example, flagellin (Felix et al., 1999), and bacterial effectors that suppress plant defense responses (Abramovitch et al., 2003; Espinosa et al., 2003; Jamir et al., 2004), raising other possible explanations for these differences in observed Ca^{2+} signal. To eliminate these confounding effects, the effect(s) of an avr gene needs to be studied in isolation.

In this report, we used transgenic Columbia-0 (Col-0) containing the avrRpt2 gene from Pseudomonas syringae pv tomato under the control of a glucocorticoid-inducible promoter (McNellis et al., 1998). In these plants, a chimeric glucocorticoid-regulated transcription factor is expressed constitutively and remains in the cytoplasm in an inactivated state by interacting with heat shock proteins. This tripartite fusion protein (GVG) consists of the DNA-binding domain from the yeast (Saccharomyces cerevisiae) GAL4, the transactivating domain from the herpes simplex virus VP16, and the hormone-binding domain of the rat glucocorticoid receptor protein. When the steroid hormone analog dexamethasone (dex) is applied to these plants or infiltrated into leaves, the GVG protein is converted into an active state, enters the nucleus, and binds to the target promoter. Within a half hour, avrRpt2 message was detectable in seedlings treated with 30 μM dex; protein was detectable within 2 h (McNellis et al., 1998). Leaves from plants that also expressed the cognate resistance gene exhibited HR collapse in as little as 6 h and leaked electrolytes by 12 h; leaves that expressed the nonfunctional rps2-101C allele did not collapse (McNellis et al., 1998).

An inducible system makes it possible to observe the earliest membrane response of cells undergoing HR after induction of an avr gene. However, in patch-clamp experiments, protoplasts were nonresponsive to the induction of avrRpt2 by dex (W. Gassmann, R.L. Jones, and B.J. Staskawicz, unpublished data). We therefore studied the membrane responses of mesophyll cells in intact Arabidopsis leaves with glass microelectrodes. The membrane potential is a negative charge differential (the sum of relatively stable charged molecules within interconnected cells and ion fluxes from or into cells) that changes in response to external stimuli or internal processes (Sze et al., 1999). In plants, a negative potential is maintained by the plasma membrane H^+-ATPase, an enzyme that hydrolyzes ATP and pumps protons out of the plant cell.

We report here an electrophysiological characterization of bacterial avrRpt2 gene-specific HR in the dex-inducible system. We demonstrate that membrane potential measurement is an extremely sensitive assay to explore the mechanisms of gene-specific HR without the confounding effects of other bacterial products. We show that: (1) rapid irreversible depolarization occurs only in dex-induced leaves that are capable of undergoing HR, (2) that the depolarization occurs much earlier than electrolyte leakage or visible symptoms, (3) that the depolarization is not the result of a nonfunctional pump or leaky membranes, (4) that the depolarization and electrolyte leakage may be calcium dependent, and (5) that SA pretreatment prevents depolarization as well as electrolyte loss and HR collapse in dex-induced RPS2 plants. With seedlings grown in liquid culture, we show that SA-induced resistance limits AvrRpt2 protein accumulation, suggesting a mechanism that may prevent HR symptoms and depolarization.

**RESULTS**

**Rapid Depolarization Occurs Exclusively in Plants That Mount an HR**

We established that the dex-inducible system functioned under our conditions as previously reported by McNellis et al. (1998). Leaves from transgenic Col-0 containing the dex-inducible avrRpt2 gene and expressing wild-type RPS2 (RPS2 plants) showed wilting that began 4 to 6 h after infiltration of 1 μM dex, with HR collapse by 24 h (Fig. 1); leaves from transgenic plants expressing the rps2-101C allele (rps2 plants) did not collapse from dex infiltration 24 or 48 h after infiltration (Fig. 1) as shown previously (McNellis et al., 1998).

We hypothesized that SA pretreatment would eliminate HR symptoms in dex-treated RPS2 leaves as was shown when avrRpt2-expressing bacteria were infiltrated into Col-0 Arabidopsis leaves following SA pretreatment (Devadas and Raina, 2002). Indeed, 17 of 27 SA-pretreated transgenic RPS2 leaves infiltrated with dex did not exhibit any HR symptoms (Fig. 1), but they yellowed by 48 h. Seven leaves showed only slight loss of turgor. Two leaves were partially

**Figure 1.** avrRpt2-induced HR only occurs in RPS2 plants and is abrogated by prior SA treatment. SA-pretreated leaves were sprayed with 1.5 mM SA 24 h prior to the experiment. Photographs were taken 24 h after infiltration with 0.1% ethanol or 1 μM dex.
collapsed, and one showed collapse indistinguishable from RPS2 leaves that were not pretreated. SA-pretreated RPS2 and rps2 leaves infiltrated with 0.1% ethanol (solvent for dex) remained green up to 4 d after infiltration when observation was terminated. Thus, HR occurs only in leaves that express AvrRpt2 in an RPS2 background and is counteracted by prior SA treatment.

We examined the relationship of membrane function and HR in dex-induced RPS2, rps2, and SA-pretreated RPS2 leaf cells. Average resting potentials of RPS2, rps2, and SA-pretreated RPS2 leaf cells were similar prior to dex application (Fig. 2, A–C; Table I). Membranes of rps2 plant cells expressing AvrRpt2 did not depolarize and often slowly hyperpolarized during the first 4 h after dex application, suggesting that the membrane sealed more tightly around the electrode over time (Fig. 2A; Table I). By contrast, RPS2 cells began to depolarize 1 to 1.5 h after 1 μM dex was applied for only 15 min (Fig. 2B). By 3 to 4 h after dex application, depolarization of RPS2 membranes slowed and leveled (Fig. 2B; Table I). The average membrane potential at 4 h was near the diffusion potential (Table I). Pretreatment with SA largely prevented RPS2 depolarization during the first 4 h after dex application in more than half of the experiments (Fig. 2C). In the others, cells were depolarized between 2 and 3 h, but by 4 h repolarized cells were measured (Table I). Thus, by 3 h after dex, hours before HR symptoms appeared on infiltrated leaves, it was clear that rapid membrane depolarization occurred only in leaves that underwent HR (Table I).

**Electrolyte Leakage Occurs after Depolarization and Is Limited to Plants Undergoing HR**

Electrolyte leakage, measured as an increase in conductivity of the solution bathing treated leaf discs, is often used to assay the time course of membrane damage during HR (Goodman and Novacky, 1994). We measured the conductivity of leaf discs from RPS2 or rps2 plants with or without SA pretreatment and after addition of 1 μM dex or ethanol. No differences in conductivity were measured during the first 2 h of incubation with ethanol or dex (Fig. 3), although by then RPS2 segments treated for only 15 min with dex had depolarized 40 mV on average (Table I). There was little difference in electrolyte loss for discs from dex-treated rps2 leaves and ethanol-treated RPS2 or rps2 leaves throughout the experiment (Fig. 3). At 12 h the conductivity of these treatments in three experiments ranged from 2.4 ± 0.2 to 4.0 ± 0.2 μS cm⁻². Differences between treatments appeared 4 to 6 h after dex addition (Fig. 3), about the time that the first wilting was observed in RPS2 plant leaves infiltrated with dex but after depolarization was complete. From 6 to 12 h after dex addition, the electrolyte leakage of dex-treated RPS2 discs was much greater than that of all other plants and treatments (Fig. 3). By 12 h, conductivity for dex-treated RPS2 samples in the three experiments ranged from 15.8 ± 0.4 to 22.4 ± 2.5 μS cm⁻².

SA pretreatment substantially blocked dex-induced electrolyte leakage from RPS2 discs (Fig. 3). In the three experiments, the conductivity at 12 h after dex averaged 4.9 ± 0.4 to 6.6 ± 1.6 μS cm⁻². SA pretreatment of RPS2 leaves without dex did not increase the electrolyte leakage above background (data not shown). Similarly, SA pretreatment did not increase leakage from dex-treated rps2 discs above background (Fig. 3). In summary, depolarization, macroscopic HR, and electrolyte leakage were highly correlated.

**Is Calcium Influx Needed for Depolarization and Electrolyte Leakage?**

We investigated whether the calcium channel blocker LaCl₃ or other means to inhibit Ca²⁺ uptake prevent membrane depolarization and electrolyte leakage.
leakage in the avrRpt2 dex-inducible system. When 1 mM LaCl3 was added to the bathing solution after dex-induced depolarization had commenced in RPS2 cells, it caused an immediate transient increase in rate of depolarization (Fig. 4A). This was nonspecific because LaCl3 also immediately transiently depolarized the membrane potential in dex-treated rps2 controls by 6 mV (data not shown). The potential subsequently repolarized and when depolarization resumed 30 to 40 min after addition of LaCl3, it was at a much slower rate than it had been initially (Fig. 4A; Table II). When added prior to dex-induced depolarization, 1 mM LaCl3 prevented depolarization completely (data not shown).

Average potentials at the time of dex addition were similar in RPS2 plants that were never treated with LaCl3 (Table I) and those that were used in the LaCl3 experiments (data not shown). By contrast, the average membrane potential 60 to 90 min after LaCl3 addition was measured a little more than 3 h after dex addition was significantly more negative than the 3-h average potential of dex-treated RPS2 leaf segments without LaCl3 (Table I; P ≤ 0.05). This is a strong inhibition, considering that 1 mM LaCl3 was not in excess of the 1 mM CaCl2 that was present at all times.

The requirement for external Ca2+ could not be tested in other electrophysiological experiments. Measurements without Ca2+ in the bathing medium were very unstable. The Ca2+ chelator EGTA caused major depolarization by itself. Cinnarizine, a Ca2+ channel inhibitor in the piperazine family that has been reported to reduce Ca2+ uptake by elicitor-stimulated parsley (Petroselinum crispum) cells (Nürnberger et al., 1994), produced multiple transient depolarizations and instability when applied together with dex.

We therefore tested the effect of LaCl3 on HR in electrolyte leakage experiments, modified with rinsing steps so that CaCl2 and LaCl3 could be added and their effect on HR could be tested without interfering with conductivity measurements (see “Materials and Methods”). One millimolar LaCl3 reduced electrolyte leakage to 50% to 30% of that without LaCl3 (Fig. 4B). Thus, a mere 1.5-h treatment with 1 mM LaCl3 prevented later leakage even when 1 mM external Ca2+ was present together with the LaCl3.

### How Does SA Impact HR-Associated Depolarization and Electrolyte Leakage?

The simplest explanation for inhibited depolarization, electrolyte leakage, and collapse in SA-pretreated dex-induced RPS2 plants would be reduced accumulation of avrRpt2 transcript or AvrRpt2 protein. To test these hypotheses, we initially attempted immunoblotting using leaves as in the above experiments. However, we were not able to detect AvrRpt2 protein within the time scale of our experiments, perhaps because AvrRpt2 accumulation induces rapid cell death in RPS2 leaves. For this reason, we used Col-0 and RPS2 seedlings grown in liquid culture (McNellis et al., 1998; see “Materials and Methods” and “Discussion”). Pretreatment with SA induces the defense gene PR1 in leaves (Kwon et al., 2004) and liquid-cultured

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**Table 1. Average membrane potentials of Arabidopsis leaf cells and HR phenotype after avrRpt2 induction**

<table>
<thead>
<tr>
<th>Plant</th>
<th>HR</th>
<th>0 h</th>
<th>1 h</th>
<th>2 h</th>
<th>3 h</th>
<th>4 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>rps2</td>
<td>–</td>
<td>–166 ± 12 (12)</td>
<td>–175 ± 12 (11)</td>
<td>–184 ± 15 (12)</td>
<td>–189 ± 18 (9)</td>
<td>–183 ± 13 (8)</td>
</tr>
<tr>
<td>RPS2</td>
<td>+</td>
<td>–171 ± 12 (15)</td>
<td>–179 ± 13 (14)</td>
<td>–139 ± 22 (14)</td>
<td>–99 ± 27 (10)</td>
<td>–88 ± 22 (10)</td>
</tr>
<tr>
<td>RPS2 + SA</td>
<td>–</td>
<td>–178 ± 10 (9)</td>
<td>–177 ± 10 (8)</td>
<td>–167 ± 22 (8)</td>
<td>–166 ± 22 (6)</td>
<td>–176 ± 11 (8)</td>
</tr>
</tbody>
</table>

*HR was evaluated 24 h after infiltration of 1 µM dex into remaining leaves of plants that were used in electrophysiological experiments.

5-Hours are time points after replacement of the original bath solution with one containing 1 mM dex (see “Materials and Methods”). Each time course is from one leaf segment but not necessarily from the same cell. The value at a given time point was only tabulated when a stable membrane potential from a living cell was available. *RPS2 leaves were sprayed with 1.5 mM SA 24 or 48 h prior to the experiment to induce systemic resistance.

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**Figure 3.** Conductivity increases in cells competent to undergo an HR. Conductivity measurements of the solution bathing RPS2 and rps2 leaf discs after 1 µM dex or 0.1% ethanol (EtOH) was added. Twenty-four hours prior to the experiment, a subset of plants was sprayed with 1.5 mM SA. Points are means of three replicates ± SD. Two other experiments were performed with similar results.
seedlings (see “Materials and Methods”) under our conditions.

The avrRpt2 mRNA was detected only in dex-treated seedlings containing the inducible transgene (Fig. 5, top). SA pretreatment caused a slight decrease in the avrRpt2 mRNA level that was not significant when normalized using profilin mRNA as a control. This indicated that SA-induced resistance did not block the transcription of avrRpt2.

Total protein was subjected to immunoblotting using polyclonal rabbit antibody against AvrRpt2 protein. As shown in the bottom section in Figure 5, no AvrRpt2 was detected in wild-type Col-0 plants, and the very small amount seen in RPS2 transgenic seedlings prior to dex treatment was approximately the same with or without SA pretreatment. As expected, dex-treated transgenic RPS2 seedlings produced a large amount of AvrRpt2, in contrast to solvent ethanol-treated seedlings. The mature AvrRpt2 protein is predicted to be 28.2 kD in size (Innes et al., 1993). However, AvrRpt2 is processed in planta to a 22-kD form, and an additional smaller band of approximately 21 kD is sometimes detected (McNellis et al., 1998; Mudgett and Staskawicz, 1999; Jin et al., 2003). Surprisingly, SA pretreatment dramatically reduced AvrRpt2 levels in RPS2 seedlings 4 and 8 h after dex was applied (Fig. 5). In a repetition of the experiment, even less AvrRpt2 protein was detected in the SA-pretreated RPS2 seedlings after dex treatment (data not shown). Thus, SA-mediated reduction of AvrRpt2 protein is a posttranscriptional event.

DISCUSSION

In this study, we used the very sensitive technique of membrane potential measurement to study the physiology of bacterial avr gene-specific HR in a biological system that is free from the confounding effects of other bacterial elicitors. Our results show that membrane depolarization is a reliable early indicator of HR in leaves. Progressive irreversible depolarization occurred only in Arabidopsis expressing both avrRpt2 and a functional RPS2 gene, plants that were destined to leak electrolytes several hours later and to exhibit tissue collapse by the following day. Our results with the rps2 line also indicate that the virulence function(s) of AvrRpt2 does not affect the membrane potential at early time points. We show here that depolarization and HR symptoms are highly correlated. Lanthanum or pretreatment with SA prevented depolarization and electrolyte leakage, and SA pretreatment also prevented HR collapse of dex-induced RPS2 leaves.

Similarly, in previous work, irreversible depolarization was an early characteristic of both HR that is associated with species level resistance to a broad range of pathogens (Kepler and Novacky, 1986; Pavlovkin and Novacky, 1986; Pike et al., 1998) and HR-like necrosis that was caused by harpin Ea (Pike et al., 1998). In the first case, multiple external and internal bacterial effectors likely led to electrolyte leakage and confluent necrosis. In the second, these symptoms resulted from a single external bacterial effector. In both cases, depolarization could have been part of a transmembrane signal for the host to undergo HR. Our results show that similarly early progressive irreversible depolarization occurs when HR is caused by an internally produced protein. Thus, our results not only provide a genetic framework for linking depolarization and avr gene-specific HR, but also show that depolarization is not part of a transmembrane signal in the cell being measured, although it may function as a membrane-associated signal to surrounding cells that is propagated via plasmodesmata.

The Significance of Membrane Depolarization

Membrane potential measurement is one of the few ways to study membrane function in leaf cells during
HR triggered by avrRpt2 and similar bacterial avr genes. Although elegant patch-clamp experiments have characterized ion fluxes and changes in channel regulation following extracellular elicitor application (Zimmermann et al., 1997), they were not useful for characterizing dex-inducible HR in RPS2 plants. Application of dex to isolated protoplasts induced avrRpt2 transcript only after 18 h (compared to 30 min in whole tissue; T.W. McNellis, M.B. Mudgett, and B.J. Staskawicz, personal communication), which precluded their use for patch-clamp experiments. Protoplasts derived from dex-treated rps2 and RPS2 leaves at various time points on the other hand were not different in patch-clamp experiments (W. Gassmann, R.L. Jones, and B.J. Staskawicz, unpublished data), perhaps because both had been elicited by cell wall fragments during the protoplasting process, or because cells that had initiated the HR pathway did not survive the isolation procedure. By contrast, cutting leaf segments minimally perturbs the natural state of cells in whole tissue. Consequently, we measured healthy responsive potentials for hours in mesophyll cells several layers below the cut surface. Because changes in membrane potential reflect the sum of ion fluxes from or into cells, pharmacological tools were used to tease apart underlying processes.

The observed inhibition of membrane potential depolarization induced by the calcium channel blocker LaCl₃ was very strong. A mere 1 mM LaCl₃ treatment for 1.5 h also greatly inhibited electrolyte leakage for 12 h (Fig. 4) and even for 24 h (data not shown). In previous work, LaCl₃ prevented tissue collapse, electrolyte leakage, and depolarization caused by harpinEa (He et al., 1994; Pike et al., 1998). LaCl₃ also prevented an increase in cytosolic calcium and H₂O₂, and HR in Arabidopsis leaves when coinfiltrated with bacteria expressing avrRpm1 (Grant et al., 2000). However, in our experiments, it did not delay or prevent dex-induced HR in leaves (data not shown). Possibly, LaCl₃ may be removed from the apoplastic space and is no longer available to counteract a greater and longer production of AvrRpt2 in the dex-infiltrated leaves.

Experiments varying the timing of LaCl₃ and dex exposure could establish the conditions for La³⁺ effectiveness.

Calcium has been reported to play a major role in initiating HR; for example, after a nonspecific transient increase, cytosolic Ca²⁺ levels were elevated in a second, long-lasting transient increase during HR caused by infiltrated bacteria expressing avrRpm1 or avrB (Grant et al., 2000). The long sigmoidal depolarization that we observed in dex-induced RPS2 leaf cells would be consistent with the sustained Ca²⁺ increase produced by AvrRpm1 and AvrB. However, in these experiments, AvrRpt2 did not produce a comparable second Ca²⁺ increase, and it was suggested that the much smaller Ca²⁺ increase correlated with the slower timing and less severe HR induced by AvrRpt2 as compared to AvrRpm1 and AvrB (Grant et al., 2000).

The dex-induced HR in RPS2 plants was previously shown to be much faster and stronger than the HR

### Table II. Membrane potential and rate of potential change before and after addition of 1 mM LaCl₃ to the bath solution of depolarizing dex-induced RPS2 leaf cells

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Membrane Potential</th>
<th>Rate of Potential Change</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 min</td>
<td>30 min</td>
</tr>
<tr>
<td>1</td>
<td>-156</td>
<td>-166</td>
</tr>
<tr>
<td>2</td>
<td>-147</td>
<td>-137</td>
</tr>
<tr>
<td>3</td>
<td>-146</td>
<td>-135</td>
</tr>
<tr>
<td>4</td>
<td>-143</td>
<td>-139</td>
</tr>
<tr>
<td>5</td>
<td>-145</td>
<td>-157</td>
</tr>
<tr>
<td>Average (±SD)</td>
<td>-147 ± 5</td>
<td>-146 ± 15</td>
</tr>
</tbody>
</table>

*Each experiment is the time course of one leaf cell as shown in Figure 4A. +, Rates of depolarization; −, repolarization. The 1 mM LaCl₃ addition point is the point at which LaCl₃ first reached the measuring chamber, before complete bath exchange. End potentials occurred 3.2 ± 0.8 h after dex addition.*

Figure 5. SA pretreatment reduces AvrRpt2 accumulation in RPS2 seedlings. Accumulation of avrRpt2 transcript (top) and AvrRpt2 protein (bottom) in Col-0 and RPS2 seedlings after treatment with and without SA and 30 μM dex at designated time points. Reverse transcription was performed in the presence (+) or absence (−) of reverse transcriptase, and PCR reactions contained avrRpt2- and profilin-specific primers. Top, Number at left denotes DNA size standard in bp. Bottom, 100 μg of total protein was loaded in each lane. The positions of the 31 kD and 17 kD molecular mass standards are shown. The expected position of the processed form of AvrRpt2 (22 kD) and a second smaller band of approximately 21 kD (McNellis et al., 1998) is indicated. No bands smaller than 17 kD were detected in this blot.
induced by bacteria expressing avrRpt2 (McNellis et al., 1998). Thus, it is likely that the robust depolarization measured in dex-induced RPS2 cells results from overexpression of the avr gene and that a strong Ca\(^{2+}\) influx would be expected in this system. Alternatively, the strong depolarization triggered by AvrRpt2 may suggest that membrane potential measurements are a more sensitive indicator of Ca\(^{2+}\) fluxes during HR than Ca\(^{2+}\) measurements with aequorin.

It should be noted that our LaCl\(_3\) data do not rule out fluxes from ions other than calcium; channels blocked by LaCl\(_3\) include anion channels and outwardly-rectifying K\(^+\) channels (Lewis and Spalding, 1998). The ion fluxes during HR are likely to be complex; simultaneous transient extracellular alkalization, Ca\(^{2+}\) uptake, and K\(^+\) efflux are characteristic of bacterial HR in suspension cells (Atkinson et al., 1985, 1990). Because all these ion fluxes are down the chemical gradient, they would occur as a direct consequence of membrane depolarization caused by membrane damage or H\(^+\)-ATPase down-regulation, and do not necessarily constitute a specific signal. Membrane damage or unregulated H\(^+\)-ATPase malfunction can probably be ruled out as the source of early membrane depolarization because the fungal toxin fusicoccin always hyperpolarized even very depolarized membranes (data not shown), as found previously with bacterial HR (Pavlovkin and Novacky, 1986) and harpin (Popham et al., 1995; Pike et al., 1998). Fusicoccin locks the H\(^+\)-ATPase in the active position, resulting in hyperpolarization when the H\(^+\)-ATPase is functional and the membrane is capable of separating charge (Sze et al., 1999; Bunney et al., 2002). The negligible amount of electrolyte leakage measured at 4 h, after depolarization was complete, further supports this idea.

Regulated depolarization could consist of direct down-regulation of the H\(^+\)-ATPase or be an indirect result of limited ATP availability caused by another HR process, for example, increased alternative oxidation (Simons et al., 1999) or the oxidative burst (Keppeler et al., 1989). Direct down-regulation of the H\(^+\)-ATPase could serve multiple functions. It would permit diversion of energy from maintenance of an ATP-consuming pump to execution of the cell death process and manufacture of defense gene products. Alternatively, depolarization and the resulting K\(^+\) and anion release into the apoplast could serve to limit bacterial multiplication (Wright and Beattie, 2004).

### Induced Resistance Limits AvrRpt2 Accumulation in Seedlings

The hrl1 mutants produce high levels of SA constitutively and, when crossed with the dex-inducible RPS2 plants, do not undergo HR when infiltrated with dex (Devadas and Raina, 2002). Our original intent in inducing resistance with SA was to determine if dex-inducible HR and depolarization are highly correlated as in our LaCl\(_3\) experiments, which indeed was the case. We anticipated that avrRpt2 mRNA would be produced as was reported for hrl1 mutants crossed with the dex-inducible RPS2 plants (Devadas and Raina, 2002), although protein levels were not determined in those experiments. We used the lowest concentration of dex that reproducibly caused HR collapse and depolarization so that subtle effects by agonists or antagonists were more likely to be observed. However, AvrRpt2 protein had not reached a detectable concentration in leaves 4 and 8 h after they were infiltrated with 1 \(\mu\)M dex (data not shown).

To compare AvrRpt2 levels in untreated and SA-treated RPS2 tissue, we therefore assayed liquid seedling cultures. The dosage dependency and time course of avrRpt2 transcript and AvrRpt2 protein accumulation in this system were found to be very consistent with the observed HR phenotype in dextreated leaf tissue (McNellis et al., 1998). Unexpectedly, our reverse transcription (RT)-PCR and protein gel-blot results from liquid-cultured seedlings suggested that the AvrRpt2 protein does not accumulate in SA-induced seedlings. This may be consistent with the suggestion that plants with induced resistance react more quickly or strongly to subsequent pathogen attack because they have been primed (Alvarez, 2000; Conrath et al., 2002), perhaps to degrade active foreign proteins. In the case of AvrRpt2, regulation of protein stability during induced resistance may be mediated by RIN4, a host protein that interacts with both AvrRpt2 and RPS2 (Axtell and Staskawicz, 2003; Mackey et al., 2003), or other host targets of AvrRpt2. Further research is needed to address this question.

In summary, we have developed an experimental system that lends itself to the dissection of the physiological processes leading to HR in plants within a genetic framework. We have shown that the extreme sensitivity of this system permits measurement of early differences between plants undergoing or not undergoing HR hours before electrolyte leakage or tissue collapse occur. We have presented an electrophysiological characterization of gene-for-gene HR that will be a valuable starting point for future research.

### MATERIALS AND METHODS

#### Plant Growth and Infiltration

Transgenic Arabidopsis (*Arabidopsis thaliana*) contained the avrRpt2 gene from *Pseudomonas syringae pv* tomato under control of the glucocorticoid-inducible promoter and either a functional RPS2 resistance gene or the mutant allele rps2-101C that encodes a truncated protein (McNellis et al., 1998). Plants were grown in individual pots of soil (Pro-Mix BX; Premier Horticulture, Quakertown, PA) in an environmental growth chamber (TC-30 or E-15; Conviron, Winnipeg, Manitoba, Canada). Temperature was 24°C and humidity 70% with an 8-h-light/16-h-dark cycle and a photosynthetic flux of 90 to 130 \(\text{\textmu}\text{mol}\) photons m\(^{-2}\) s\(^{-1}\). Plants were grown 4.5 to 6 weeks after planting. Reagent-grade chemicals were purchased from Sigma (St. Louis) or Fisher Scientific (Pittsburgh) unless otherwise noted. SA at a final concentration of 1.5 mM was sprayed until run off 24 or 48 h before experiments with induced resistance. Plants were returned to the growth chamber and covered with clear plastic for the first 4 h to minimize localized high concentrations of SA resulting from evaporation (Devadas and Raina, 2002); however, in rare cases,
SA itself caused small necrotic spots or partial wilting. Leaves affected by SA were not used in experiments. Dex stock solution was maintained as described (McNellis et al., 1998). For observation of symptoms, 1 μM dex, the lowest concentration that consistently collapsed leaves 24 h later, was infiltrated into remaining leaves of plants used in electrophysiological or conductivity measurements with a needless syringe placed over a needle hole. Plants remained at room temperature under fluorescent light and were evaluated 24 h later.

Electrophysiology

The tip and petiole portions of fully expanded, mid-age Arabidopsis leaves were excised with a sharp razor blade, leaving central 7-mm segments that were mounted on Plexiglas holders with elastic bands. They were placed in an aerated bathing solution consisting of 1 mM KCl, 1 mM CaCl2, 2 mM MgCl2, buffered with 5 mM MES and adjusted to pH 5.5 with a final concentration of 1.25 mM KOH. They were equilibrated 2 h under fluorescent light before measurement.

Micropipettes were pulled from borosilicate glass capillaries (1.0-mm diameter) containing fiberglass filament on a vertical pipette puller (David Kopf Instruments, Tujunga, CA). Each segment was placed in a Plexiglas measurement chamber and was perfused continuously with perfused solution by gravity flow at about 2.5 mL min⁻¹. The bath electrode was connected to the measuring chamber via an agar bridge containing 3 mM KCl. Spongy mesophyll cells were impaled through the cut edge. When a stable potential was reached, the perfusion solution was exchanged for one containing 1 μM dex. Ten minutes after one solution exchange, the dex-containing bathing solution was again exchanged for the dex-free solution. Including solution exchange time, total exposure to dex was approximately 15 min. This exposure time resulted in consistent irreversible, maximum depolarization under our conditions. In leaf tissue, when a cell died, a potential of similar value usually is measured in a nearby cell because cells are connected through plasmodesmata, a feature that permits continuous measurement of cells in one leaf segment for 2 to 6 h. It was not unusual for individual impaled cells to be viable for more than an hour. When a cell died, another cell in the same segment was impaled. Measurements were performed in white light from a goose neck fiber optic lamp with a photosynthetic flux of 170 μmol photons m⁻² s⁻¹. Measurements were recorded and analyzed with a digital recording device (Digidata 1322A; Axon Instruments, Union City, CA). For tabulations of individual points, noise was eliminated by manual readings ± 1 s.

For LaCl₃ measurements, 1 μM dex was added and removed as described above. After dex-induced depolarization was consistent, the original bathing solution was exchanged for one with added 1 mM LaCl₃. Rates of potential change were calculated for the 10-min period prior to LaCl₃ addition and the 10-min period beginning at 30 min after LaCl₃ addition. To rule out the possibility that the reduced rate of depolarization after LaCl₃ addition was independent of LaCl₃, we calculated rates of depolarization over 10-min time periods at comparable membrane potentials for these cells but not LaCl₃ (initial rates 10 min prior to potentials of ~130 and ~135 mV and final rates 30–40 min after these values). The average 30- to 40-min rate of depolarization for these cells was +1.03 ± 0.64 mV min⁻¹ (n = 20), significantly more than that of the LaCl₃-treated cells (+0.14 ± 0.24, Table II; P ≤ 0.01). Fusococcin, a gift from Dr. E. Marre (Milan), was used at a final concentration of 15 μM. It was added at various times (0.5 h, 2.3 h, or 4.5 h) after the dex treatment.

Conductivity Measurements

Electrolyte leakage was determined by conductance measurement of the bathing solution with a conductivity bridge (Yellow Springs Instruments, Yellow Springs, OH). Twelve discs were punched from leaves with a 5-mm cork borer and floated on 7 mL of double-deionized sterile water in 30-mL beakers. The bathing solution used for electrophysiology was not used in these experiments because the background conductivity was then too high. One to two discs were punched per leaf; one to two plants were used for each of the three replicates. The beakers were placed on a reciprocal shaker under fluorescent light at room temperature. After 1 h of equilibration, dex or ethanol at a final concentration of 1 μM and 0.1%, respectively, was added to the water and remained throughout the experiment. Three experiments were performed. Data were expressed as the change in conductivity from 0 time per cm².

In experiments with LaCl₃, to measure electrolyte leakage in conditions comparable to those in the electrophysiology experiments, the solution was removed 15 min after dex addition and 5 mL of fresh water was added. Fifteen minutes later, LaCl₃ and CaCl₂ were added, final concentrations 1 mM. To reduce background conductivity, 1.5 h later the solutions were again removed, the discs rinsed once, and 7 mL of water added to each beaker. Immediately after the final water addition and at 2-h intervals, the conductivity was measured as described above. Removing solution and rinsing steps caused little change in timing or magnitude of electrolyte leakage for RPS2 dex-induced discs but increased variability (Fig. 4B). Electrolyte leakage was similar from dex-treated RPS2 discs with and without 1 mM external Ca²⁺ present for 1.5 h (data not shown).

Immunoblotting and RT-PCR

For immunoblotting and RT-PCR, wild-type Col-0 and transgenic RPS2 seedlings were grown in liquid medium as described previously (McNellis et al., 1998). The plants were grown at 24°C with gentle shaking at a speed of 80 rpm and a 16-h-light/8-h-dark photoperiod. After 6 d, SA was added. The final concentration, 150 μM, 10-fold less than that applied on leaves, was selected to account for the continuous exposure of liquid-cultured seedlings to SA. RT-PCR was used to verify that PR1 is induced by SA treatment in this system (data not shown). Dexamethasone was added 24 h after SA. The final concentration, 30 μM, was the concentration that produced detectable arrrpt2 message and Arrrpt2 protein in liquid-cultured transgenic seedlings 4 and 8 h after dex (McNellis et al., 1998). The seedlings were removed at designated time points, rinsed in deionized water, and immediately frozen in liquid nitrogen. Two aliquots of each treatment were stored at −80°C for protein and RNA isolation, respectively.

Total protein was extracted by grinding tissues in liquid nitrogen and mixing with 150 μL of extraction buffer (20 μM Tris-HCl, pH 7.5, 150 mM NaCl, 1 μM EDTA, 1% Triton X-100, 0.1% SDS, 5 mM dithiothreitol, and plant protease inhibitor cocktail [Sigma]). Soluble protein was collected by centrifugation at 20,000g for 15 min at 4°C. Protein concentration was determined using the Bradford assay. Protein samples were separated on a 15% SDS-PAGE gel and transferred to a nitrocellulose membrane (Osmonics, Westborough, MA). Immunoblotting was carried out with a rabbit polyclonal antibody at a dilution of 1:1,000 and the ECL detection kit (Amersham, Piscataway, NJ).

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