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Modulation of Plasma Membrane H^+-ATPase Activity Differentially Activates Wound and Pathogen Defense Responses in Tomato Plants

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Systemin is an important mediator of wound-induced defense gene activation in tomato plants, and it elicits a rapid alkalinization of the growth medium of cultured *Lycopersicon peruvianum* cells. A possible mechanistic link between proton fluxes across the plasma membrane and the induction of defense genes was investigated by modulating plasma membrane H^+-ATPase activity. Inhibitors of H^+-ATPase (erythrosin B, diethyl stilbestrol, and vanadate) were found to alkalinize the growth medium of *L. peruvianum* cell cultures and to induce wound response genes in whole tomato plants. Conversely, an activator of the H^+-ATPase (fusicoccin) acidified the growth medium of *L. peruvianum* cell cultures and suppressed systemin-induced medium alkalinization. Likewise, in fusicoccin-treated tomato plants, the wound- and systemin-triggered accumulation of wound-responsive mRNAs was found to be suppressed. However, fusicoccin treatment of tomato plants led to the accumulation of salicylic acid and the expression of pathogenesis-related genes. Apparently, the wound and pathogen defense signaling pathways are differentially regulated by changes in the proton electrochemical gradient across the plasma membrane. In addition, alkalinization of the *L. peruvianum* cell culture medium was found to depend on the influx of Ca^{2+} and the activity of a protein kinase. Reversible protein phosphorylation was also shown to be involved in the induction of wound response genes. The plasma membrane H^+-ATPase as a possible target of a Ca^{2+}-activated protein kinase and its role in defense signaling are discussed.

INTRODUCTION

Plants respond to local injury with rapid changes in gene expression and the concomitant accumulation of defense proteins (Green and Ryan, 1972; Graham et al., 1986). In tomato plants, these proteins are collectively called SWRPs (for systemic wound response proteins) and include proteases, inhibitors of serine, cysteine, and aspartic proteases, and components of the wound signaling pathway (Schaller et al., 1995; Bergey et al., 1996). Much work has focused on the dissection of the signal transduction pathway leading to the induction of defense genes and on the identification of the systemically transmittable wound signal. Molecules involved in the wound response include jasmonic acid (Farmer and Ryan, 1990), abscisic acid (Peña-Cortés et al., 1989, 1996), ethylene (O'Donnell et al., 1996), oligosaccharides (Walker-Simmons et al., 1983), and systemin (Pearce et al., 1991).

Several lines of evidence indicate that the oligopeptide systemin is a primary systemic signal. These include its extremely high activity, the suppression of the wound response by antisense inhibition of the systemin precursor, induction of the wound response in plants overexpressing prosystemin, as well as systemin's mobility within the phloem, which is compatible with timing and propagation of the endogenous wound signal throughout the plant (reviewed in Schaller and Ryan, 1995). Nevertheless, controversy exists as to whether the long-distance transport of systemin or the propagation of an electrical signal is responsible for triggering the systemic wound response (Wildon et al., 1992; Malone, 1996). In tomato plants, it has been shown that action potentials as well as variation potentials, which are the consequence of a hydraulic pressure surge caused by wounding, lead to the induction of proteinase inhibitor genes (Herde et al., 1995; Stankovic and Davies, 1996, 1997). Because there is evidence for both chemical and electrical signaling, an interaction between the two mechanisms seems likely. Indeed, systemin as well as oligouronides have been reported to evoke electrical responses at the the plasma membrane (Thain et al., 1995; Moyen and Johannes, 1996).

In cultured cells of *Lycopersicon peruvianum*, the addition of systemin causes rapid alkalinization of the growth medium, H^+ influx paralleled by the efflux of K^+, induction of phenylalanine ammonia–lyase activity, and ethylene production (Felix and Boller, 1995). A correlation has been made between the extent of medium alkalinization of the cell

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culture and the SWRP-inducing activity of systemin analogs in whole tomato plants. This correlation suggests a link between systemin-triggered proton fluxes and the induction of defense genes (Felix and Boller, 1995; Schaller, 1998). Our goal was to determine whether there is a causal relationship between the two phenomena by modulating the proton gradient across the plasma membrane, which is built up and maintained by the activity of the plasma membrane H^+-ATPase (Palmgren, 1991; Michelet and Boutry, 1995). Whereas activation of the H^+-ATPase causes a hyperpolarization of the plasma membrane and extracellular acidification, its inhibition leads to rapid membrane depolarization and alkalinization of the cell exterior. We used inhibitors (orthovanadate [VO_4^{3-}], erythrosin B, and diethyl stilbestrol) as well as an activator (fusicoccin [FC]) of the proton pump to investigate the possible involvement of this enzyme and of the proton electrochemical gradient in wound signaling. The results show that wound and pathogen defense signaling pathways in tomato plants are differentially activated by the modulation of the proton gradient across the plasma membrane.

RESULTS

Suppression of Systemin-Induced Medium Alkalinization by FC

Cell cultures of *L. peruvianum* respond to the addition of systemin with a rapid alkalinization of the growth medium (Felix and Boller, 1995). We used inhibitors and an activator of the plasma membrane H^+-ATPase to investigate the role of the proton pump in medium alkalinization. Erythrosin B, diethyl stilbestrol, and VO_4^{3-} caused medium alkalinization after they were added to *L. peruvianum* cell cultures, as shown in Figure 1. Although none of these inhibitors is entirely specific for the plasma membrane H^+-ATPase (Cocucci, 1986; Serrano, 1990), the use of inhibitors with different structures but similar effects argues for the involvement of the plasma membrane–located ATPase. Medium alkalinization, as a result of proton pump inhibition, was comparable in speed and magnitude to systemin-triggered medium alkalinization (cf. Figures 1 and 2). Conversely, as shown in Figure 2, treatment of *L. peruvianum* cells with FC, a fungal toxin that is an activator of the proton pump, resulted in rapid acidification of the growth medium. Furthermore, pretreatment with FC rendered the cells insensitive to the subsequent addition of systemin (Figure 2).

The suppression of systemin-triggered medium alkalinization may be explained by the mechanism of action of FC. FC has been proposed to lock the H^+-ATPase into an activated state (Jahn et al., 1997; Oecking et al., 1997). Alternatively, not the activation state of the proton pump but the decrease in external pH may preclude systemin activity. To test the latter possibility, we adjusted the pH of the culture medium to 4.2 by adding HCl. The cultured cells were found to be fully responsive to systemin at this pH (Figure 2). The suppression of the systemin response after FC treatment could not be alleviated by the addition of KCl (2 mM). These findings are consistent with the hypothesis that medium alkalinization in response to systemin treatment is a result of H^+-ATPase inhibition.

Modulation of H^+-ATPase Activity Affects Defense Signaling in Tomato Plants

Tomato plants respond to wounding with the accumulation of SWRPs (Green and Ryan, 1972; Schaller et al., 1995; Bergey et al., 1996). The effect of modulators of H^+-ATPase activity on SWRP transcript levels was investigated. When supplied to young tomato plants, H^+-ATPase inhibitors (erythrosin B, diethyl stilbestrol, and VO_4^{3-}) induced the accumulation of SWRP mRNAs. As shown in Figure 3, the maximum induction levels were close to those observed after systemin treatment. Therefore, proton pump inhibitors mimic systemin activity with respect to their ability to alkalinize the medium of cultured *L. peruvianum* cells (Figure 1) and the induction of SWRP gene expression in whole tomato plants (Figure 3). However, we could not directly corre-

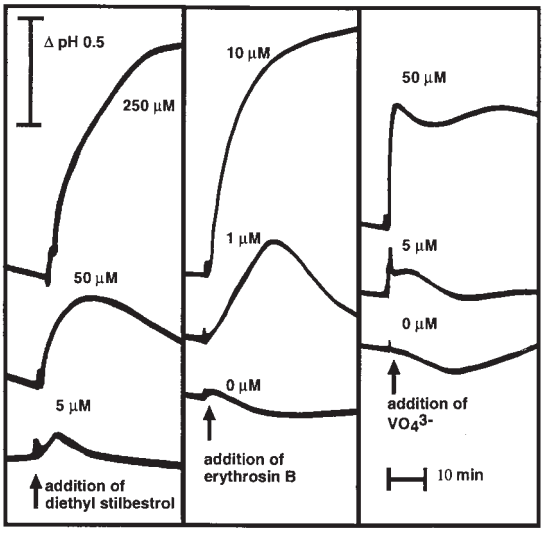

Figure 1. pH Responses Elicited by Inhibitors of H^+-ATPase.

The pH of the growth medium was monitored over time. The initial pH of the culture medium ranged from 4.9 to 5.0. Arrows indicate the addition of proton pump inhibitors. Each experiment was repeated at least three times with two independent batches of cells, and similar results were obtained.

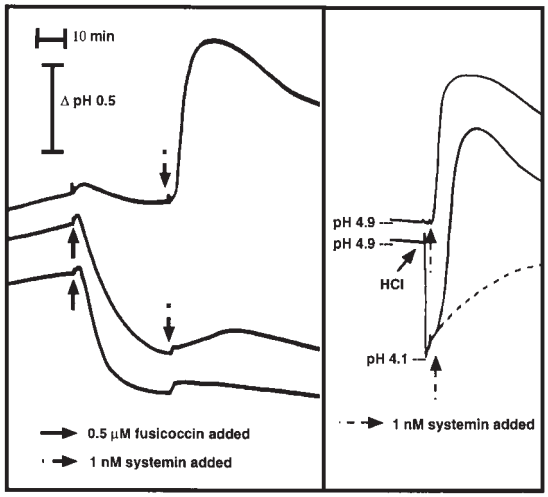

Figure 2. Systemin- and FC-Elicited pH Responses.

The pH of the growth medium of cultured *L. peruvianum* cells was monitored continuously. The initial pH of the culture medium ranged from 4.9 to 5.0. Arrows indicate the addition of FC, systemin, or HCl. The broken line at right shows the pH trace that was obtained when no systemin was added. The results shown are representative of at least three independent experiments with two different batches of cells.

late levels of SWRP mRNA induction and the extent of medium alkalinization. In particular, we observed that erythrosin B concentrations causing a pronounced alkalinization of the culture medium were less effective in SWRP mRNA induction than was the addition of VO_4^{3-}, which at low concentrations caused only a slight alkalinization response (cf. Figures 1 and 3). This may well be due to differences between whole-plant and cell culture assay systems. It is not clear whether the three inhibitors are transported equally well to their target locations when supplied to excised tomato plants through their cut stems.

Our data suggest that proton fluxes across the plasma membrane as a result of the inhibition of the plasma membrane proton pump are involved in wound signal transduction. This conclusion is supported by the observation that pretreatment of tomato plants with FC, that is, the activation of the plasma membrane H^+-ATPase, suppressed the accumulation of SWRP transcripts after wounding, after treatment with systemin and jasmonic acid, and also after treatment with bestatin, as demonstrated by RNA gel blot analyses shown in Figure 4. Quantification of the data (data not shown) by using a PhosphorImager and ImageQuant software revealed that FC-mediated inhibition of mRNA accumulation ranged from nearly complete inhibition (92%) in the case of systemin-treated plants to only partial inhibition (57%) for bestatin-treated plants. The substantial accumulation of SWRP mRNAs in bestatin- and jasmonic acid–treated plants (Figure 4) indicated that the plants were still able to respond to wound stimuli and that the inhibitory effect of FC was not due to general FC toxicity.

Treatment of tomato plants with FC not only inhibited the activation of SWRP genes but also led to the induction of pathogenesis-related (PR) genes. The mRNAs for basic as well as acidic PR proteins accumulated in leaves of tomato plants after treatment with FC (Figure 4). The expression of P69, an extracellular PR protein in tomato plants (Tornero et al., 1996), was regulated in a more complex way than were the other PR proteins tested. Induction of P69 by FC was suppressed in plants that were treated with jasmonic acid or bestatin. Induction of PR genes indicated that FC activated the signaling pathway leading to pathogen defense responses. The development of a hypersensitive response and of systemic acquired resistance after pathogen infection is known to be associated with the accumulation of salicylic acid (Durner et al., 1997). Therefore, the levels of salicylic acid in leaves of control plants were analyzed and compared with those in plants treated with FC. As shown in Figure 5, there was a rapid increase in leaf salicylic acid concentrations 4 hr after FC treatment of tomato plants.

In conclusion, our data suggest a role for proton fluxes across the plasma membrane in defense signaling in tomato plants. These proton fluxes may result from changes in

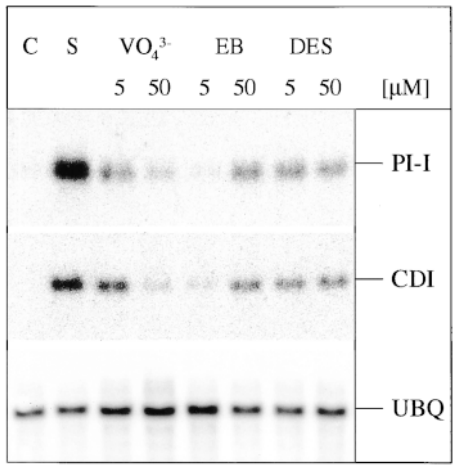

Figure 3. Induction of SWRP mRNAs by H^+-ATPase Inhibitors.

Excised young tomato plants were treated with buffer alone (C), systemin (S; 50 nM), and buffered solutions of orthovanadate (VO_4^{3-}), erythrosin B (EB), or diethyl stilbestrol (DES) at the indicated concentrations. After 1 hr, plants were transferred to water. RNA was isolated after 8 hr. Five micrograms of total RNA was analyzed by RNA gel blotting and probed for the presence of proteinase inhibitor I (PI-I), cathepsin D inhibitor (CDI), and ubiquitin (UBQ) mRNAs.

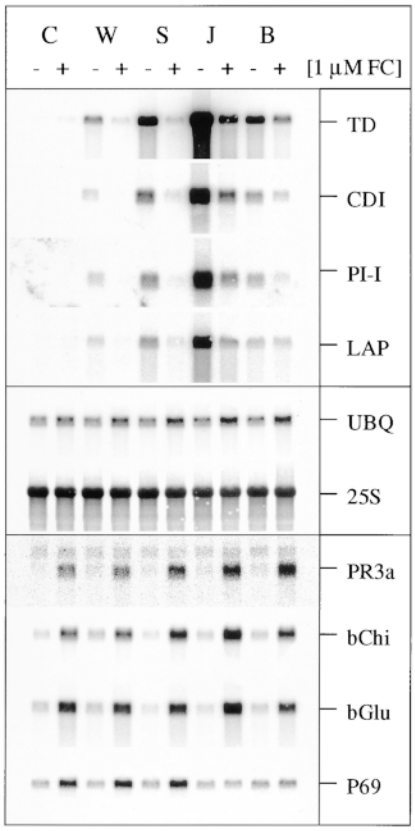

Figure 4. Effects of FC on the Induction of the mRNAs for SWRPs and PR Proteins.

Excised young tomato plants were pretreated with water (−) or 1 μM FC (+) for 45 min. Subsequently, plants were treated with buffered solutions of bestatin (B; 1 mM), jasmonic acid (J; 400 μM), systemin (S; 50 nM), and buffer alone (C; W) or were wounded at the terminal leaflet (W). RNA was isolated after 8 hr, and 5 μg of total RNA was analyzed by RNA gel blotting. Blots were hybridized with probes for SWRPs (threonine deaminase [TD], the cathepsin D inhibitor [CDI], proteinase inhibitor I [PI-I], and leucine aminopeptidase [LAP]), PR protein (tobacco acidic chitinase [PR3a], potato basic chitinase [bChi], potato basic glucanase [bGlu], and tomato PR-P69 [P69]), and control RNAs (ubiquitin [UBQ] and 25S rRNA [25S]).

H^+-ATPase activity. Apparently, energization of the plasma membrane by action of the H^+-ATPase not only provides the driving force for nutrient uptake and cellular growth but also provides information about environmental cues, such as pathogen attack and wounding. Treatment with FC, which leads to activation of the proton pump and to extracellular acidification, results in the induction of PR protein expression. On the other hand, inhibition of the H^+-ATPase causing extracellular alkalinization results in the induction of SWRP gene expression.

The Influx of Ca^{2+} and the Activity of a Protein Kinase Are Necessary for Systemin-Mediated Medium Alkalinization

The following experiments aimed at the identification of other cellular factors necessary for systemin-triggered medium alkalinization of *L. peruvianum* cells. As shown in Figure 6A, a Ca^{2+} channel inhibitor (La^{3+}) was found to block systemin-induced medium alkalinization. Furthermore, chelation of extracellular Ca^{2+} by 1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N*′,*N*′-tetraacetic acid (BAPTA) also abolished the response to systemin in *L. peruvianum* cells (Figure 6A). Therefore, systemin-mediated medium alkalinization is preceded by the influx of Ca^{2+}. However, the response to systemin could not be mimicked by the addition of the Ca^{2+} ionophore A23187 (data not shown), indicating either that Ca^+ influx is not sufficient to initiate downstream responses or that a general increase in cytosolic Ca^{2+} leads to the activation of several opposing responses.

To investigate a possible involvement of reversible protein phosphorylation in systemin-mediated medium alkaliniza-

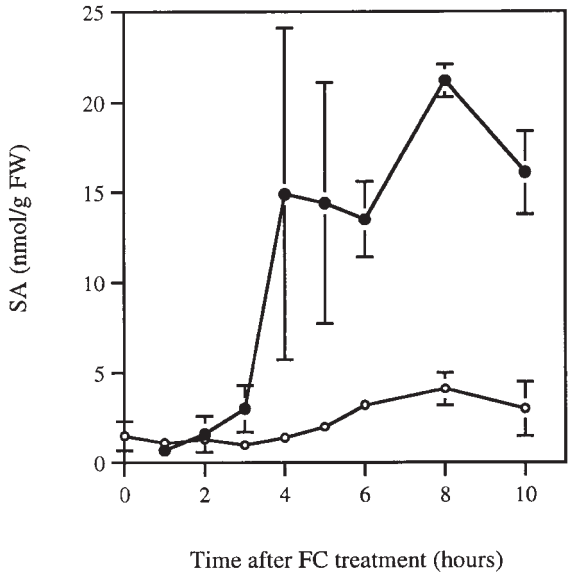

Figure 5. Accumulation of Salicylic Acid in FC-Treated Tomato Plants.

FC (1 μM) was supplied to young tomato plants (filled symbols) through their cut stems for 1 hr, after which they were transferred to water. Control plants (open symbols) were treated with water alone. Leaves were harvested for analysis of salicylic acid content (SA; in nanomoles per gram fresh weight [FW]) at the time points indicated after the beginning of FC treatment. The results shown are the average of three independent experiments. Missing error bars indicate that the standard error of the mean was within the size of the symbols.

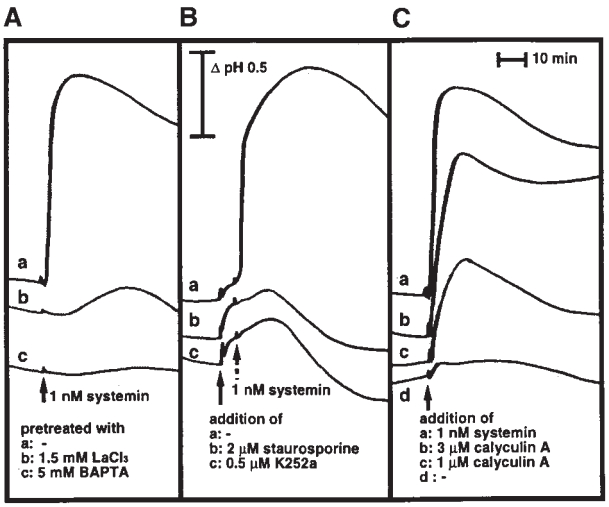

Figure 6. pH Responses of Cultured *L. peruvianum* Cells.

The pH of the growth medium was monitored over time. The initial pH of the cultures ranged from 4.9 to 5.0. Arrows indicate the addition of the chemicals to be tested. Each experiment was repeated at least three times with two independent batches of cells, and representative results are shown.

(A) The effects of pretreatment of cell cultures with a calcium channel blocker ($LaCl_3$; trace b) and a calcium chelator (BAPTA; trace c) on the subsequent addition of systemin (1 nM; arrow) were analyzed and compared with trace a (no pretreatment).

(B) The effect of protein kinase inhibitors (trace a, solvent control; trace b, staurosporine; trace c, K252a) on the subsequent addition of systemin (1 nM; arrows) was analyzed.

(C) A comparison of the pH response to systemin (trace a, 1 nM) with that to calyculin A (trace b, 3 μM; trace c, 1 μM; trace d, solvent control).

tion, we analyzed the effect of several inhibitors of protein kinases. K252a and staurosporine were found to inhibit systemin-induced medium alkalinization (Figure 6B). The inhibitory effect of K252a had been observed previously by Felix and Boller (1995). Bisindolylmaleimide and KN-62, two highly selective inhibitors of protein kinase C and Ca^{2+}/calmodulin–dependent kinase II, respectively, which have been reported to inhibit phosphorylation of the plasma membrane H^+-ATPase in tomato cells (Xing et al., 1996), had no effect on medium alkalinization (data not shown), indicating that the kinase involved is of a different type. On the other hand, the addition of the protein phosphatase inhibitor calyculin A to cultured cells of *L. peruvianum* elicits a dose-dependent alkalinization of the growth medium similar to the response elicited by systemin (Figure 6C). Alkalinization of the growth medium after the addition of calyculin A also had been observed by Felix and Boller (1995).

In whole tomato plants, calyculin A was found to induce SWRP gene expression. As shown in Figure 7, SWRP transcripts accumulated in a concentration-dependent manner in the leaves of calyculin A–treated tomato plants, with maximum induction at 1 μM of the protein phosphatase inhibitor. Taken together, these data are consistent with the hypothesis that systemin exerts its effects via Ca^{2+}-dependent protein phosphorylation.

DISCUSSION

Relevance of the Plasma Membrane H^+-ATPase for Defense Signaling

The signaling peptide systemin mediates the systemic induction of SWRPs in leaves of tomato plants in response to wounding (Pearce et al., 1991). In cultured cells of *L. peruvianum*, systemin elicits H^+ influx/K^+ efflux, the induction of phenylalanine ammonia–lyase activity, and ethylene production (Felix and Boller, 1995). SWRP genes, however, are not activated in *L. peruvianum* cells by systemin, jasmonic acid, or by bestatin, probably because of a downstream block in the signaling pathway (A. Schaller, unpublished results).

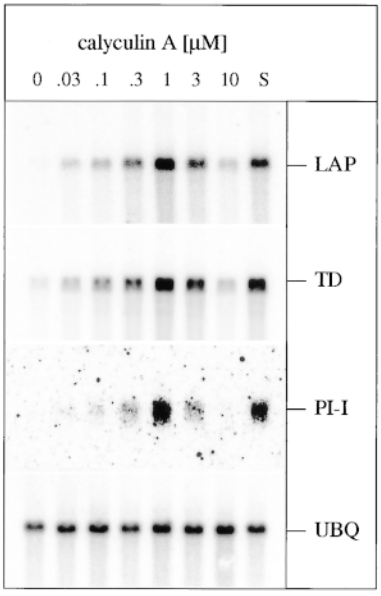

Figure 7. SWRP Transcript Induction by Calyculin A.

Excised young tomato plants were treated with increasing concentrations of calyculin A or systemin (S; 50 nM). After 1 hr, plants were transferred to water. RNA was isolated after 8 hr. Five micrograms of total RNA was analyzed by RNA gel blotting and probed for the presence of leucine aminopeptidase (LAP), threonine deaminase (TD), proteinase inhibitor I (PI-I), and ubiquitin (UBQ) mRNAs.

Several lines of evidence indicate that SWRP induction in whole plants and medium alkalinization in the cell culture are mediated by the same perception system. (1) A systemin derivative harboring a single amino acid substitution (Ala for Thr-17) acts as a systemin antagonist in both systems (Pearce et al., 1993; Felix and Boller, 1995). (2) The C-terminal part of systemin was found to be most relevant for its inducing activity in both systems (Pearce et al., 1993; Felix and Boller, 1995; Meindl et al., 1998). (3) A correlation has been observed between the activity of metabolically stabilized systemin derivatives in intact plants and in the cell culture (Schaller, 1998). (4) A binding site for radiolabeled systemin has been identified in membrane preparations of *L. peruvianum* cells (Meindl et al., 1998) exhibiting characteristics that were predicted for the systemin receptor from an analysis of the structure/activity relationship of systemin in whole tomato plants (Pearce et al., 1993). (5) Systemin as well as oligogalacturonides have been found to cause a depolarization of the plasma membrane in tomato leaf mesophyll cells (Doherty and Bowles, 1990; Thain et al., 1995; Moyen and Johannes, 1996), possibly mediated by inhibition of the plasma membrane H^+-ATPase.

These observations suggest a link between proton fluxes across the plasma membrane and the induction of defense genes. However, attempts to link H^+-ATPase activity to SWRP gene induction have been inconclusive thus far, because both inhibition as well as activation of the proton pump appeared to induce a chymotrypsin inhibitory activity (i.e., the induction of a SWRP) in tomato leaves (Doherty and Bowles, 1990). In contrast, we have shown here that the activation of the plasma membrane H^+-ATPase in the cell culture system suppressed systemin-induced medium alkalinization. In addition, in whole tomato plants, activation of the proton pump by FC prevented the accumulation of SWRP transcripts after treatment with systemin and other elicitors of the wound response. Conversely, inhibitors of H^+-ATPase activity were shown to cause medium alkalinization in the cell culture and SWRP transcript accumulation in tomato plants. These data extend the correlation observed previously between the pH response and the induction of SWRP gene expression. Furthermore, they show that the pH response is necessary for downstream gene activation, suggesting the involvement of H^+-ATPase inhibition in wound signaling.

Although SWRP genes were induced after inhibition of the H^+-ATPase, FC-mediated activation of the proton pump, concomitant with the inhibition of SWRP gene induction, caused salicylic acid accumulation and PR gene expression, which are known to be associated with the establishment of systemic acquired resistance (Delaney et al., 1994; Ryals et al., 1995; Bol et al., 1996; Durner et al., 1997). This finding is consistent with data reporting the activation of the H^+-ATPase in tomato plasma membranes by treatment with elicitor preparations from an avirulent, but not from a virulent, pathogen (Vera-Estrella et al., 1994; Xing et al., 1996). Apparently, the two defense signaling pathways leading to the induction of SWRPs on the one side and to the induction of PR proteins on the other side are differentially regulated by the inhibition and activation of the plasma membrane H^+-ATPase, respectively. These data suggest that the plasma membrane H^+-ATPase may serve as a switch between the wound and the pathogen defense signaling pathways.

Not only was the wound signaling pathway found to be activated by inhibition of the H^+-ATPase, but it also appeared to be inhibited when the proton pump was activated, that is, under conditions that activated pathogen defense signaling. The downregulation of the wound signaling pathway may be mediated by salicylic acid, which was found to accumulate after FC treatment (Figure 5). In fact, salicylic acid has previously been shown to downregulate the wound response (Doherty et al., 1988; Peña-Cortés et al., 1993; Doares et al., 1995). Alternatively, it may result from the activated state of the H^+-ATPase after FC treatment, which is supported by the observation that systemin-mediated medium alkalinization was also suppressed after activation of the proton pump by FC (Figure 2).

Relevance of Protein Phosphorylation/Dephosphorylation and Ca^{2+} for Wound Signaling

Systemin-induced alkalinization of *L. peruvianum* cell culture medium could be mimicked by addition of the protein phosphatase inhibitor calyculin A (cf. Figure 6C; Felix and Boller, 1995). In tomato plants, calyculin A caused the accumulation of SWRP transcripts (Figure 7). Conversely, the protein kinase inhibitors K252A and staurosporine were found to suppress medium alkalinization in response to systemin (Figure 6B). Apparently, the pH response and the subsequent induction of SWRP genes are regulated by reversible protein phosphorylation. Reversible protein phosphorylation has previously been shown to regulate wound signal transduction in Arabidopsis (Rojo et al., 1998). Two independent wound signaling pathways exist in Arabidopsis, both of which appear to be regulated by a protein phosphorylation switch (Titarenko et al., 1997; Rojo et al., 1998). One pathway, which is dependent on jasmonic acid, was found to be negatively regulated by a staurosporine-sensitive protein kinase and to be positively regulated by an okadaic acid–sensitive protein phosphatase (Rojo et al., 1998). This inhibitor sensitivity is in contrast to the one observed here. Therefore, wound signaling appears to be regulated by reversible protein phosphorylation at multiple sites.

The systemin response in *L. peruvianum* resembles in many ways the nonhost resistance response of parsley to the fungus *Phytophthora sojae* (Hahlbrock et al., 1995). Cultured parsley cells respond to a peptide elicitor derived from a fungal glycoprotein with medium alkalinization paralleled by the efflux of K^+ (Nürnberger et al., 1994; Jabs et al., 1997). In this system, an initial influx of Ca^{2+} is necessary but not sufficient to initiate downstream reactions. A Ca^{2+} channel has been identified in parsley protoplasts that is activated when treated with the peptide elicitor (Zimmermann et al., 1997).

This finding prompted us to analyze a possible involvement of Ca^{2+} fluxes in systemin-triggered medium alkalinization of the *L. peruvianum* cell culture. Like protein kinase inhibitors, a Ca^{2+} channel inhibitor (La^{3+}) and a chelator of extracellular Ca^{2+} (BAPTA) were found to suppress medium alkalinization in response to systemin (Figures 6A and 6B). Apparently, the alkalinization response depends on both the influx of Ca^{2+} and the activation of a protein kinase. These data are consistent with the hypothesis that systemin exerts its effect via Ca^{2+}-dependent protein phosphorylation.

Interestingly, the plasma membrane H^+-ATPase is known to be phosphorylated in a Ca^{2+}-dependent manner (Schaller and Sussman, 1988; Schaller et al., 1992). In beet roots, calcium-dependent phosphorylation was shown to inhibit H^+-ATPase activity (Lino et al., 1998). Also, in fava bean, calcium has been shown to inhibit the enzyme's proton pumping activity (Kinoshita et al., 1995). In contrast, dephosphorylation in response to fungal avirulence factors resulted in the activation of the proton pump in tomato plasma membranes (Vera-Estrella et al., 1994). Furthermore, protein kinases have been identified in tomato (Stratmann and Ryan, 1997) and tobacco plants (Seo et al., 1995; Usami et al., 1995). These kinases are rapidly activated upon wounding and appear to function early in the signal transduction pathway. These findings are consistent with the hypothesis that systemin responses are mediated by inhibition of the plasma membrane H^+-ATPase via Ca^{2+}-dependent phosphorylation.

We suggest a model in which systemin may activate a Ca^{2+} channel in tomato plasma membranes either by direct interaction or after binding to a putative systemin receptor. A Ca^{2+} channel activated by a peptide elicitor has in fact been identified in parsley protoplasts (Zimmermann et al., 1997). A Ca^{2+}-dependent protein kinase, activated by increased cytosolic Ca^{2+}, may then evoke ion fluxes that lead to extracellular alkalinization and depolarization of the plasma membrane. Our data suggest the plasma membrane H^+-ATPase as a possible target of this protein kinase. These data do not exclude the possibility, however, that the observed ion fluxes are carried by channels directly regulated by phosphorylation. The question of how these events might activate the octadecanoid pathway remains to be answered. The activation of a phospholipase mediated by a mitogen-activated protein kinase has been suggested to be involved in this process (Stratmann and Ryan, 1997). Finally, octadecanoid biosynthesis results in the formation of jasmonic acid and ultimately in the induction of SWRP gene expression (Farmer and Ryan, 1992).

The proposed signaling scheme has the potential to reconcile hitherto conflicting results concerning chemical signaling by the translocation of systemin on the one hand versus electrical signaling by depolarization of the plasma membrane on the other hand. The scheme provides a useful paradigm for directing future experiments that address wound signal transduction. For example, it will be interesting to confirm, by using pulse labeling experiments, the phosphorylation of the plasma membrane H^+-ATPase after systemin treatment. Recently, it has been shown that plant homologs of eukaryotic 14-3-3 proteins are important regulators of the H^+-ATPase (Jahn et al., 1997; Oecking et al., 1997). Future experiments will address the question of whether these regulatory proteins are involved in the transduction of the wound signal.

METHODS

Cell Culture and pH Response Measurements

A suspension cell culture from a wild tomato species (*Lycopersicon peruvianum*; kindly provided by T. Boller, Universität Basel, Basel, Switzerland) was maintained as described previously (Felix and Boller, 1995). Continuous measurements of extracellular pH were performed in 5 mL of cultured cells 6 to 8 days after subculture. Fusicoccin (FC), systemin, $LaCl_3$, 1,2-*bis*-(2-aminophenoxy)ethane-*N*,*N*,*N′*,*N′*-tetraacetic acid (BAPTA; Sigma), and orthovanadate (VO_4^{3-}) were added from 100-fold concentrated aqueous stock solutions. A23187, K252a, staurosporine, and calyculin A (Alexis Corp., Läufelingen, Switzerland) were added from 1000-fold concentrated stock solutions in DMSO.

Growth of Plants and Bioassay

Tomato (*L. esculentum* cv Castlemart II) plants were grown as described previously (Pearce et al., 1993). Experimental plants were 12 to 14 days old and had two fully developed leaves. For bioassays, tomato plants were excised at the base of their stems and transferred to 10 mM phosphate buffer, pH 6.5, containing the compound to be tested at the concentrations indicated. The plants were allowed to imbibe ~100 μL of the test solution for 1 hr, after which they were transferred to water. Leaf material was harvested for RNA extraction after 8 hr.

RNA Gel Blot Analyses

RNA was isolated from tomato leaves ground in liquid N_2 by using a phenol-based extraction procedure (Sambrook et al., 1989). Five micrograms of total RNA was separated on formaldehyde–agarose gels, transferred to nitrocellulose membranes, and hybridized with radiolabeled probes by using standard laboratory procedures (Sambrook et al., 1989). The probes for tomato SWRP (for systemic wound response protein) and tobacco PR-3a mRNAs have been described previously (Schaller et al., 1995). The probe for tomato PR-P69 corresponded to nucleotides 89 to 631 of the P69A cDNA (Tornero et al., 1996) and was amplified by polymerase chain reaction. The probes for potato basic chitinase and 1,3-β-glucanase have been described previously (Beerhues and Kombrink, 1994). For tomato DNA probes, prehybridization was in 50% formamide, 5 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate), 0.5% SDS, 2 × Denhardt's solution (1 × Denhardt's solution is 0.02% Ficoll, 0.02% PVP, and 0.02% BSA), and 200 μg/mL of denatured salmon sperm DNA at

42°C for 4 hr. Hybridization was performed under the same conditions for 15 hr. Blots were washed twice for 30 min in 0.2 × SSC and 0.5% SDS at 60°C. For tobacco and potato DNA probes, the conditions were identical except that (pre)hybridization solutions contained 40% formamide and washing was performed in 0.5 × SSC. Blots were evaluated by using a PhosphorImager and ImageQuant software (Molecular Dynamics, Sunnyvale, CA).

Assay of Salicylic Acid Concentration

Salicylic acid was extracted from leaves of FC-treated tomato plants and water-treated controls, as described by Raskin et al. (1989), with the modifications described by Malamy et al. (1992). Briefly, 1 g of tissue was extracted in 90% methanol. The solvent was evaporated, and the residue was resuspended in 5% trichloroacetic acid and partitioned with ethyl acetate–cyclopentane–isopropanol (50:50:1). After evaporation of the organic solvent, the residue was resuspended in 20% methanol/10% acetic acid and analyzed by reversed-phase HPLC on a C18 column (LiChroCART 250-4, RP-18; Merck, Dietikon, Switzerland). The HPLC system (Beckman System Gold; Beckman Instruments, San Ramon, CA) was equipped with a diode array (Beckman Instruments) and a fluorescence detector (Kratos Analytical, Ramsey, NJ) for quantification of salicylic acid (excitation at 313 nm and emission cutoff at 418 nm). The rate of recovery was determined by spiking duplicate samples with salicylic acid.

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Modulation of Plasma Membrane H^+-ATPase Activity Differentially Activates Wound and Pathogen Defense Responses in Tomato Plants

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