Planta (2000) 210: 599–606

Planta

Salicylic acid-independent induction of pathogenesis-related gene expression by fusicoccin

Andreas Schaller, Philippe Roy, Nikolaus Amrhein

Institute of Plant Sciences, ETH-Zürich, Universitätstrasse 2, 8092 Zürich, Switzerland

Received: 26 May 1999 / Accepted: 3 September 1999

Abstract. Treatment of tomato plants (*Lycopersicon esculentum* Mill.) with fusicoccin (FC), an activator of the plasma-membrane H^+-ATPase which maintains an electrochemical gradient across the plasma membrane, resulted in a dose-dependent accumulation of transcripts for intra- and extracellular pathogenesis-related (PR) proteins. The accumulation of PR protein transcripts was paralleled by an increase in leaf salicylic acid (SA) content. Transcripts of PR proteins and SA started to accumulate 3 h after FC treatment. 2-Aminoindan-2-phosphonic acid, an inhibitor of SA synthesis, was used to assess the role of SA in FC-mediated induction of PR gene expression. 2-Aminoindan-2-phosphonic acid was found to suppress the accumulation of SA but not the induction of PR gene expression in response to FC treatment. Furthermore, in transgenic tobacco plants overexpressing a bacterial salicylate hydroxylase gene (*nahG*-tobacco), PR transcripts accumulated after FC treatment to levels similar to those observed in control tobacco plants. The data indicate a role for the proton gradient across the plasma membrane in the SA-independent induction of PR gene expression.

Key words: Fusicoccin – *Lycopersicon* – *nahG*-tobacco – Plasma-membrane H^+-ATPase – Pathogenesis-related protein – Salicylic acid

Introduction

The P-type H^+-ATPase is the major ion pump in the plasma membrane of plant cells. It builds up electrical and pH potentials across the plasma membrane by translocation of protons into the apoplast. Nutrient uptake and maintenance of turgor depend on the proton-motive force maintained by the H^+-ATPase. Furthermore, acidification of the cell wall in response to auxin is a prerequisite for elongation growth, and alkalinization of the cytoplasm may be involved in triggering cell division (Palmgren 1991; Michelet and Boutry 1995). In view of the physiological importance of the H^+-ATPase it is not surprising that the activity of the pump is affected by numerous factors including hormones, light, and fungal toxins (Palmgren 1991; Michelet and Boutry 1995). Fusicoccin (FC), a toxin produced by the fungus *Fusicoccum amygdali*, the causal agent of peach and almond canker, has been reported to cause the displacement of a C-terminal autoinhibitory domain from the catalytic site of the H^+-ATPase, resulting in an activation of the enzyme (Johansson et al. 1993; Lanfermeijer and Prins 1994). It has been shown recently that FC stabilizes in vivo a pre-existing complex between the H^+-ATPase and 14-3-3 proteins representing an activated state of the enzyme, thereby locking the pump in its activated form (Jahn et al. 1997; Oecking et al. 1997; Baunsgaard et al. 1998; Olivari et al. 1998; Piotrowski et al. 1998). All effects of FC in vivo, including the opening of stomata, the stimulation of elongation growth, and the promotion of seed germination, appear to be consequences of its primary effect, i.e. the activation of the plasma-membrane H^+-ATPase (Turner and Graniti 1969; Ballio et al. 1981; Kutschera and Schopfer 1985). In *Rhynchosporium secalis*, a fungal pathogen of barley, a family of necrosis-inducing peptides (NIPs) has been identified, the appearance of which has been correlated with the development of necrotic lesions, indicating a function in the killing of host cells. Both NIP1 and NIP3 have been shown to stimulate the activity of the plasma-membrane H^+-ATPase in barley and other species (Wevelsiep et al. 1993). Furthermore, activation of the H^+-ATPase has been shown to be one of the early events in the incompatible interaction between *Cladosporium fulvum* and tomato (Hammond-Kosack et al. 1996).

We have recently shown that modulation of plasma-membrane H^+-ATPase activity in tomato plants differentially activates wound and pathogen defense responses,

Abbreviations: AIP = 2-aminoindan-2-phosphonic acid; FC = fusicoccin; PAL = phenylalanine ammonia-lyase; PR = pathogenesis related; SA = salicylic acid

Correspondence to: A. Schaller;
E-mail: andreas.schaller@ipw.biol.ethz.ch; Fax: +41-1-632-1084

respectively. Treatment with FC inhibited the induction of wound-response genes but, on the other hand, induced the expression of pathogenesis-related (PR) genes and the accumulation of salicylic acid (SA; Schaller and Oecking 1999). Induction of PR gene expression in tomato leaves in response to FC treatment has also been reported by Roberts and Bowles (1999). Here we present a detailed analysis of FC-induced PR gene expression and SA accumulation. By use of an inhibitor of SA biosynthesis (2-aminoindan-2-phosphonic acid, AIP) and of transgenic plants unable to accumulate significant amounts of SA (*nahG*-tobacco plants) we show that SA is not required for FC-mediated PR gene induction. Our results rather indicate that FC induces PR gene induction via an SA-independent mechanism.

Materials and methods

Treatment of plants. Tomato plants (*Lycopersicon esculentum* Mill. cv. Castlemart II; Ochoa Seed Company, Gilroy, Calif., USA) were grown at a 16.5 h, 25 °C/7.5 h, 18 °C day/night cycle with 300 μmol photons m^{-2} s^{-1} of white incandescent light and 75% relative humidity. Plants used in the experiments were 12–14 d old and had two fully developed leaves. For treatment with FC, plants were excised at the base of their stem and transferred to 10 mM phosphate buffer (pH 6.5) containing FC at the concentrations indicated. The plants were allowed to imbibe 100 μl of the feeding solution for 1 h after which they were transferred to water. Leaf material from six plants was harvested for extraction of RNA or SA 8 h after the onset of FC treatment or at the time points indicated. *Nicotiana tabacum* L. cv. Xanthi plants (wild type and *mahG* transgenic) were grown under the same conditions and were 4–5 weeks old when used in feeding experiments. Treatment of tobacco plants with FC was performed as described for tomato plants. To analyze the SA inducibility of PR gene expression, tobacco plants were sprayed with 15 μl cm^{-2} of 5 mM SA in 10 mM phosphate buffer (pH 6.5) containing 0.05% Tween 80 and were harvested for RNA extraction after 24 h.

Analyses of RNA, and probes used in this study. The extraction of RNA, RNA gel blotting, and hybridization with radiolabelled DNA probes were performed as described by Schaller and Oecking (1999). The probes used were labeled to similar specific activities and therefore, band intensities reflect the abundance of the respective RNAs. Partial cDNAs of tomato PR proteins were cloned by reverse transcriptase polymerase chain reaction (PCR) using RNA from FC-treated tomato plants as the template. Three micrograms of total RNA was reversely transcribed for 1 h at 37 °C using Moloney Murine Leukemia Virus reverse transcriptase (Promega, Madison, Wis., USA) and oligo(dT) as the primer. After heat inactivation (10 min, 95 °C) and ethanol precipitation, 10% of the reaction products were used as template in the PCR using oligonucleotides corresponding to published sequences of tomato PR proteins (Van Kaan et al. 1992; Danhash et al. 1993) as 5′ and 3′-primers (see below). The oligonucleotides comprised *Eco*RI restriction sites to facilitate the cloning of PCR products. Thirty-eight cycles of amplification (95 °C/30 s; 58 °C/45 s; 72 °C/1 min) were performed in a thermal cycler (Cetus; Perkin Elmer, Foster City, Calif., USA) using 1.25 U *Taq* polymerase (TaqBead; Promega). Reaction products were gel-purified and cloned into bluescript pSK(-) (Stratagene, La Jolla, Calif., USA). The identity of the clones was confirmed by sequence analysis. For PR-1a (formerly PR-P4; Van Kaan et al. 1992), a 437-bp fragment was amplified using the oligonucleotides GCGAATTCGGGGT-TGTTCAACATCTCATTG and GCGAATTCTAGTTGCAA GAAATGAACCACC as the 5′ and 3′-primers, respectively. The respective 5′ and 3′-oligonucleotide primers were for PR-2a [acidic β-1,3-glucanase (Van Kaan et al. 1992), 451-bp fragment] GCGA ATTCGGCTAAACGATGCAGGATATCA and GCGAATTCG GAACACAAAGAGGCCATACA, for PR-2b [basic β-1,3-glucanase (Van Kaan et al. 1992), 383-bp fragment] GCGAATTCCCG-TATGCTCTTTTTACAGCAC and GCGAATTCACTCTTTCA GACACCCCAAAGT, for PR-3a [acidic chitinase (Danhash et al. 1993), 351-bp fragment] GCGAATTCCCATCATGCCACAACG-TTATCA and GCGAATTCGGGCATACAGAATCCCCTTA-TT, and for PR-3b [basic chitinase (Danhash et al. 1993), 383-bp fragment] GCGAATTCGGACCTTTTGAACAATCCCGA and GCGAATTCGGGGCCTTATATGATGATGAAGTCG. The probe used for PR-7a (also known as PR-P69; Tornero et al. 1996) has been described previously (Meichtry et al. 1999). Clones for tobacco PR-1a (acidic PR-1), PR-2a (acidic β-1,3-glucanase), PR-3b (acidic class II chitinase), and PR-8a (acidic class III chitinase) as well as their basic isoforms were obtained from R. Dietrich, L. Friedrich and U. Conrath. The nomenclature for PR proteins used throughout this study follows the recommendations of Van Loon et al. (1994).

Assay of SA concentration. Leaf SA concentrations were determined as described by Raskin et al. (1989) with minor modifications (Malamy et al. 1992; Schaller and Oecking 1999).

Assay of phenylalanine concentration. The levels of phenylalanine in tomato leaf tissue were analyzed as described by Görlach et al. (1995). Briefly, amino acids in 80% ethanolic extracts were derivatized with *o*-phthaldialdehyde in the presence of *N*- isobutyryl-D-cysteine and separated by reversed-phase HPLC. For identification and quantification, derivatized standard amino acids (Pierce Chemical Co., Rockford, Ill., USA) were used.

Results

Accumulation of PR trancripts and SA in response to FC treatment. Treatment of tomato plants with FC resulted in the induction of PR gene expression. As shown in Fig. 1B, the transcripts of intracellular (PR-2b, PR-3b) as well as extracellular PR proteins (PR-1a, PR-3a, PR-7a) accumulated in the leaves of FC-treated plants as a function of FC concentration. A slight induction of transcript levels was observed after treatment with 0.3 μM FC, while the response did not seem to be saturated at 10 μM FC (Fig. 1B). At a given FC concentration, the mRNAs of extracellular PR-1a and PR-3a accumulated to higher levels than those of intracellular PR proteins. Likewise, SA accumulated in tomato leaves in response to FC treatment. A substantial increase in leaf SA levels was observed in response to 0.3 μM FC and increasing FC concentrations led to further SA accumulation (Fig. 1A). Thus, the induction of PR gene expression and of SA accumulation showed the same dependence on the FC concentration.

The accumulation of SA and of PR transcripts as a function of the time after FC treatment is shown in Fig. 2. The PR transcript levels were significantly increased after 3 h and continued to rise during the 8-h experiment (Fig. 2B). With the exception of PR-7a, transcripts of extracellular PR proteins appeared to accumulate to higher levels than those of intracellular ones. A concomitant increase in SA concentration in leaf tissue was observed. Salicylic acid started to accumulate between the second and third hours after FC treatment as compared with water-treated controls (Fig. 2A).

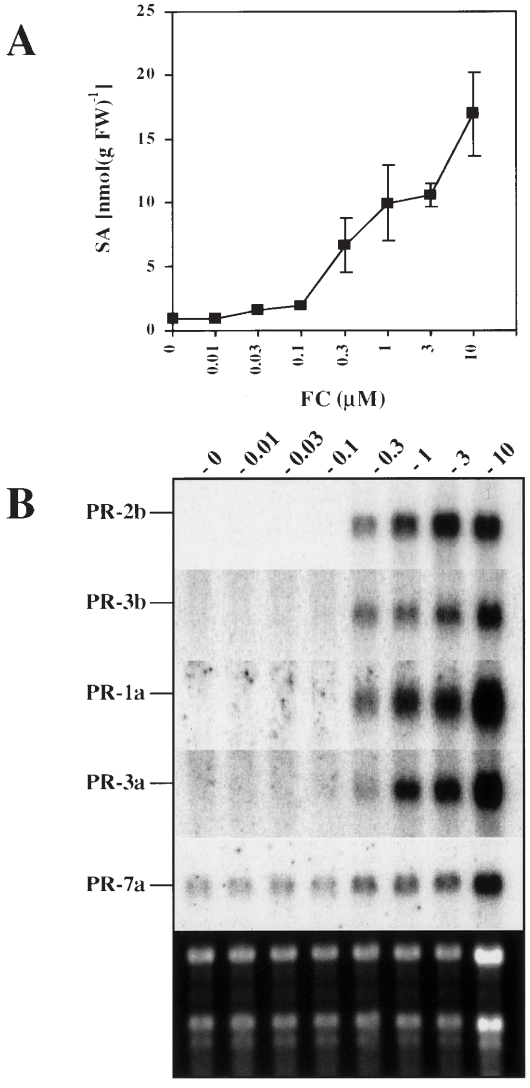

Fig. 1A,B. Accumulation of SA and PR transcripts as a function of FC concentration. Excised tomato plants were treated with the indicated concentrations of FC for 1 h. Leaf tissue was harvested after 5 and 8 h for extraction of SA and RNA, respectively. **A** The concentration of SA in leaf tissue is plotted against increasing concentrations of FC. The data are derived from four independent experiments. The standard error of the mean is indicated. **B** Five micrograms of total RNA isolated from tomato leaf tissue was analyzed by RNA gel blotting and probed for the presence of intracellular (*PR-2b*, *PR-3b*) and extracellular (*PR-1a*, *PR-3a*, *PR-7a*) PR-protein transcripts. A duplicate gel was stained with ethidium bromide as a control for RNA loading

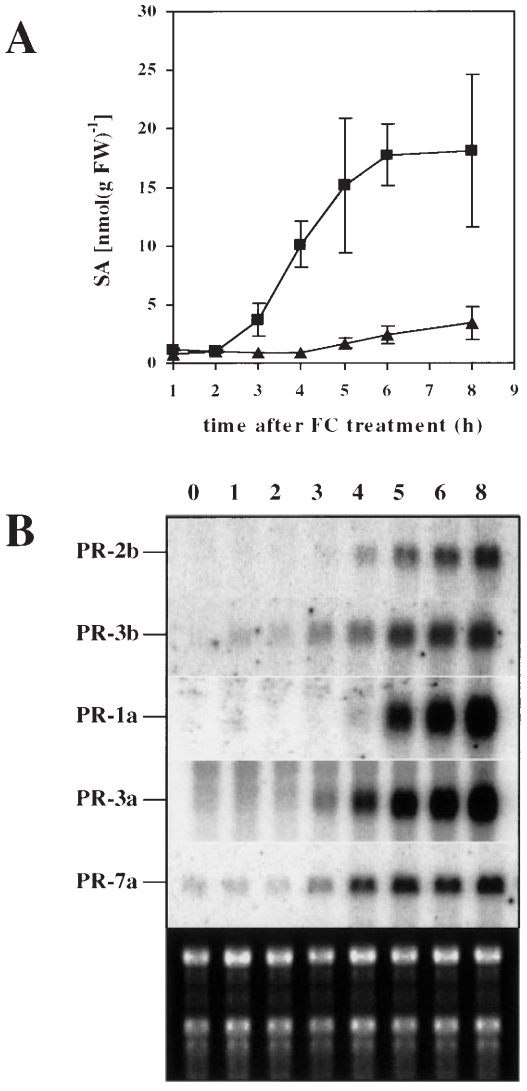

Fig. 2A,B. Time course of SA and PR transcript induction after FC treatment. Excised tomato plants were treated with 3 μM FC for 1 h and leaf tissue was harvested at the time points indicated. **A** The concentration of SA in the leaves of FC-treated plants (*squares*) was compared with that in control leaves (*triangles*). The data are derived from three independent experiments. The standard error of the mean is indicated. **B** Five micrograms of total RNA isolated from tomato leaf tissue was analyzed by RNA gel blotting and probed for the presence of the transcripts of intracellular (*PR-2b*, *PR-3b*) and extracellular (*PR-1a*, *PR-3a*, *PR-7a*) PR-proteins. A duplicate gel was stained with ethidium bromide as a control for RNA loading

Fusicoccin-induced PR gene induction in tomato plants does not require the accumulation of SA. The SA and PR transcripts appeared to accumulate concomitantly rather than consecutively in response to FC treatment (Fig. 2), prompting us to investigate whether SA accumulation is a prerequisite for PR gene induction. The biosynthesis of SA was blocked by use of the inhibitor of phenylalanine ammonia-lyase (PAL) activity, AIP (Zon and Amrhein 1992). As expected, SA levels were reduced in the leaves of AIP-treated tomato plants compared with buffer-treated controls (Fig. 3A). Likewise, the accumulation of SA in response to FC was suppressed in plants pretreated with AIP. However, suppression of SA accumulation by AIP did not abolish the induction of PR gene expression by FC (Fig. 3B). Pretreatment with AIP rather potentiated the FC-triggered accumulation of intracellular PR protein transcripts and only partially inhibited the induction of transcripts for extracellular PR proteins. These results indicate that SA is not required for FC-mediated induction of intracellular PR transcripts, while SA-dependent and SA-independent components may contribute to the induction of extracellular PR transcripts in response to FC. The observation that AIP effectively suppresses FC-induced SA

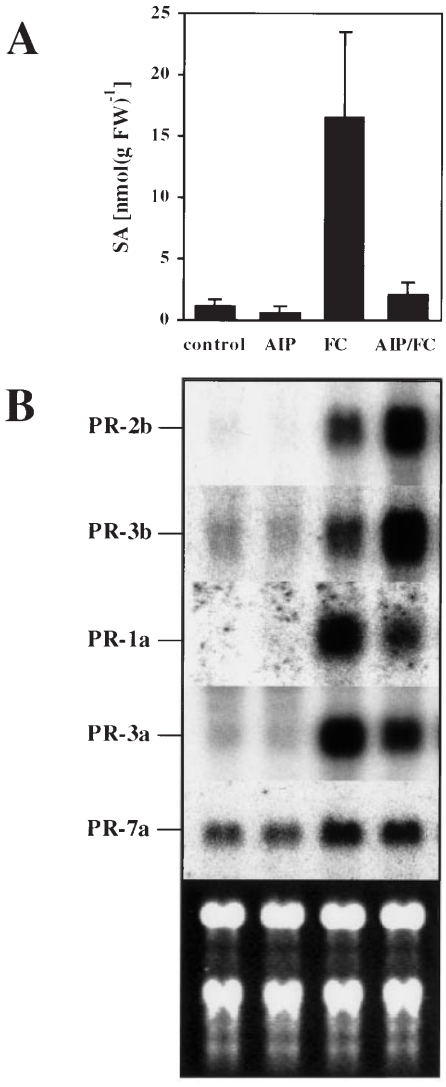

Fig. 3A,B. The effect of AIP on the FC-mediated induction of SA and PR transcripts. Excised tomato plants were treated with either 10 mM phosphate buffer, pH 6.0 (*control*, *FC*) or else with 30 μM AIP in the same buffer (*AIP*, *AIP*/*FC*). After 45 min, 3 μM FC was added (*FC*, *AIP*/*FC*). After another hour, plants were transferred to phosphate buffer (*control*, *FC*) and to buffered 30 μM AIP (*AIP*, *AIP*/*FC*), respectively. Leaf tissue was harvested for SA and RNA extractions 8 h after onset of the FC treatment. **A** The SA concentration shown is the average of three independent experiments. The standard error of the mean is indicated. **B** Five micrograms of total RNA isolated from tomato leaf tissue was analyzed by RNA gel blotting and probed for the presence of intracellular (*PR-2b*, *PR-3b*) and extracellular (*PR-1a*, *PR-3a*, *PR-7a*) PR-protein transcripts. A duplicate gel was stained with ethidium bromide as a control for RNA loading

accumulation suggests that SA accumulation is due to its de-novo biosynthesis and not to release from internal stores, i.e. to the hydrolysis of SA conjugates. This conclusion is supported by the analysis of phenylalanine concentrations in tomato leaves (Fig. 4). Inhibition of PAL activity by AIP resulted in a dramatic increase in leaf phenylalanine content. When the SA-biosynthetic pathway was blocked as a consequence PAL inhibition, phenylalanine accumulation was further enhanced after treatment with FC as compared with plants that were treated with AIP alone. The data indicate an increased flux through the shikimic acid pathway for enhanced SA biosynthesis as a consequence of FC treatment.

Salicylic acid-independent induction of PR gene expression in nahG tobacco plants. Transgenic tobacco plants expressing the bacterial salicylate hydroxylase from *Pseudomonas putida* (*nahG*-tobacco) are unable to accumulate significant amounts of SA and have been widely used to analyze the involvement of SA in the induction of systemic acquired resistance (reviewed by Ryals et al. 1996). We used *nahG*-tobacco plants to investigate whether SA is required for FC-mediated induction of PR gene expression. Consistent with the expression of salicylate hydroxylase, spraying of plants with 5 mM SA induced the expression of PR-1a, -2a, -3b, and -8a in wild-type (Xanthi) but not in *nahG*-tobacco plants (Fig. 5). In contrast, FC treatment resulted in the accumulation of transcripts for extracellular PR-3b and PR-8a in control plants and, to a somewhat lower extent, in *nahG*-tobacco plants (Fig. 5). Likewise, intracellular PR-2e, PR-3c, and PR-8b were induced by FC in both control and *nahG*-tobacco plants (Fig. 5). For PR-1a, PR-2a, and PR-1 g, no induction of gene expression was observed in response to FC treatment in either control or *nahG*-tobacco plants. With the exception of PR-1a and -2a, transcript levels of extracellular PR proteins were induced to similar steady-state levels after FC and SA treatments. The data support our conclusion that FC-mediated induction of PR transcripts is in part SA-independent.

Discussion

Induction of SA and PR transcripts by FC. We previously observed that FC-treatment of tomato plants leads to enhanced expression of PR genes and to the accumulation of SA in leaf tissue (Schaller and Oecking 1999). Here we present a kinetic analysis and the dose response of these two phenomena. Levels of transcripts of intra- and extracellular PR proteins as well as SA levels were found to increase 3 h after FC treatment, and 0.3 μ FC was the minimal dose required for induction of SA and PR transcript accumulation. The data are in good agreement with those reported by Roberts and Bowles (1999) who also observed FC-induced accumulation of PR protein transcripts. The expression of PR-1, -2, and -3 has been analyzed in detail in compatible and incompatible interactions of tomato with the fungus *Cladosporium fulvum* by De Wit and co-workers (Van Kaan et al. 1992, 1995; Danhash et al. 1993). The intra- and extracellular forms of these proteins were found to be induced in both the compatible and the incompatible interactions. However, the induction of extracellular PR proteins was found to be much faster in the incompatible than in the compatible interaction which may be a prerequisite for the establishment of an effective host resistance (Van Kaan et al. 1992, 1995). In response to FC, transcripts of extracellular PR-1a and PR-3a appeared to accumulate to higher levels, but induction

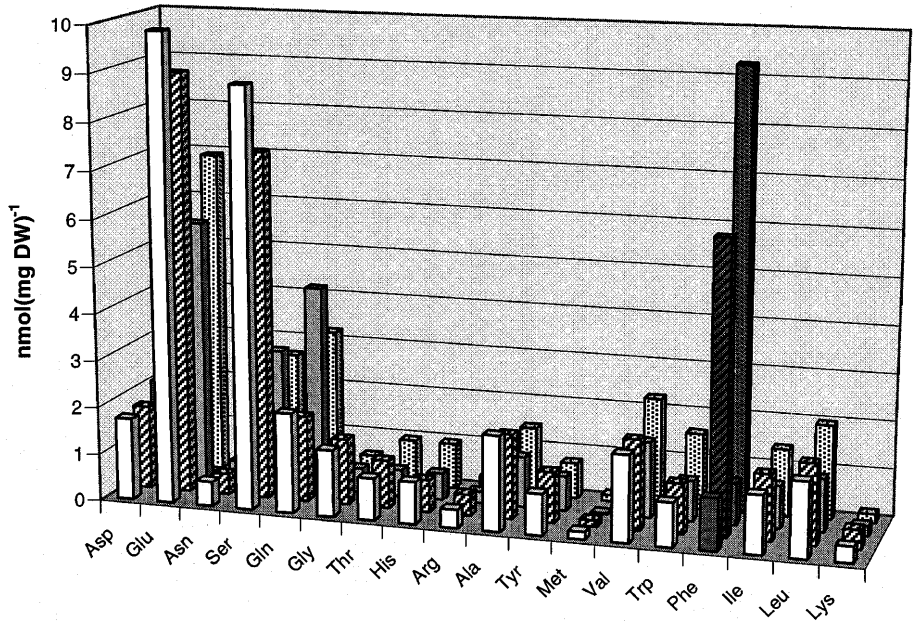

Fig. 4. Effects of AIP and FC on the levels of free amino acids in tomato leaves. The concentration of free amino acids [in nmol(mg DW)$^{-1}$] was analyzed in leaves of buffer-treated control plants (*white bars*), AIP-treated plants (*hatched bars*), FC-treated plants (*gray bars*) and plants that were treated with AIP prior to and during FC treatment (*dotted bars*). Phenylalanine concentrations are indicated in a *dark gray*. The concentrations of AIP and FC used, and the duration of treatments are indicated in the legend to Fig. 3. For each extraction, the pooled leaf material of six plants was used. The experiment was repeated twice yielding similar results

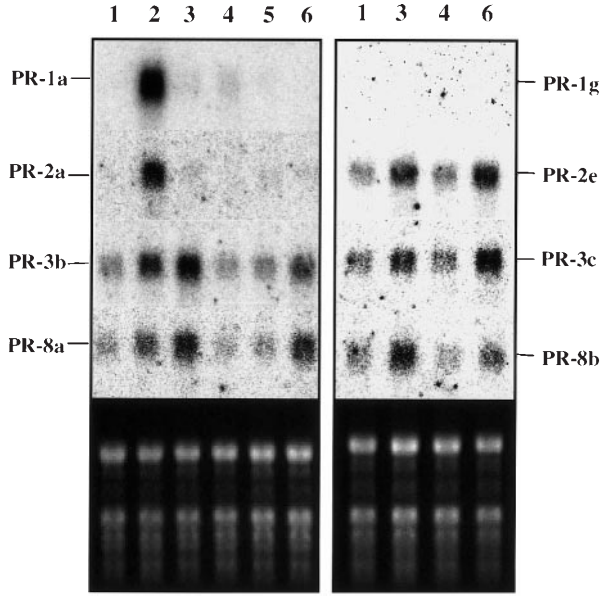

Fig. 5. Induction of PR transcript accumulation in *nahG*- and control (Xanthi) tobacco plants. Total RNA was extracted from control tobacco plants (*lanes 1–3*) and from *nahG*-tobacco (*lanes 4–6*) 8 h after treatment with 10 mM phosphate buffer, pH 6.5 (*lanes 1, 4*) or 3 μM FC in the same buffer (*lanes 3, 6*). Plants in *lanes 2* and *5* were treated with 5 mM SA for 24 h prior to RNA extraction. Five micrograms of total RNA was analyzed by RNA gel blotting and probed for the presence of extracellular (PR-1a, PR-2a, PR-3b, and PR-8a; *left panel*) and intracellular (PR-1g, PR-2e, PR-3c, and PR-8b; *right panel*) transcripts. A duplicate gel was stained with ethidium bromide as a control of RNA loading

was not faster than that of intracellular PR protein transcripts. The same observation was also made by Roberts and Bowles (1999). However, the induction of both, the intra- and extracellular forms of PR-3 was faster than that of PR-1, -2, and -7. De Wit and co-workers also analyzed the effects of SA and its functional analogue 2,6-dichloroisonicotinic acid (INA) on the expression of tomato PR proteins (Van Kaan et al. 1995). They observed induction by SA and INA for most PR proteins that are induced after pathogen infection. However, intracellular chitinase (PR-3b) was induced by neither SA nor by INA, and for PR-1, which is one of the first PR proteins detectable in the incompatible interaction between tomato and *C. fulvum*, slow and low-level induction was observed after treatment with SA. The authors concluded that SA may be involved in signaling PR gene induction in tomato as it is in tobacco, but that additional signals may be required for fast and full-level induction. Our data suggest that activation of the plasma-membrane H^+-ATPase by FC, or by an alternative signal, may be the stimulus that is required for full induction of PR gene expression in tomato plants. Consistent with this hypothesis, activation of the plasma-membrane H^+-ATPase has been reported in tomato cells in response to elicitor preparations from *C. fulvum* (Vera-Estrella et al. 1994). These observations and our own data, according to which there is no increase in leaf SA levels prior to the accumulation of PR transcripts, led us to further investigate the role of SA in FC-mediated PR gene induction.

Effects of AIP on PR transcript levels. In the biosynthetic pathway to SA (reviewed by Ryals et al. 1996), which proceeds from phenylalanine via cinnamic and benzoic acids to SA, the conversion of phenyalanine to cinnamic acid catalyzed by PAL seems to be the rate-limiting step in potato plants (Coquoz et al. 1998). 2-Amidoindan-2-phosphonic acid, a specific inhibitor of PAL activity in vivo (Zon and Amrhein 1992) has previously been used to assess the role of SA in the induction of pathogen defense responses (Mauch-Mani and Slusarenko 1996). In *Arabidopsis*, inhibition of PAL by AIP suppressed the resistance response to *Peronospora parasitica*, and the AIP effect was reversed

by application of SA, indicating that PAL has an important function in disease resistance in providing the precursors for SA biosynthesis (Mauch-Mani and Slusarenko 1996). We therefore used AIP to investigate the role of SA in FC-mediated induction of PR gene expression. In AIP-treated tomato plants, leaf SA concentrations were found to be below control levels and, furthermore, the accumulation of SA in response to FC was suppressed. The data indicate that SA accumulation in response to FC treatment is due to de-novo biosynthesis and not to the hydrolysis of conjugates of either benzoic acid or SA which could contribute to the increase in free SA (Malamy et al. 1992; Henning et al. 1993). Inhibition of SA biosynthesis by AIP enabled us to assess the involvement of SA in FC-mediated induction of PR gene expression. For intracellular PR proteins, FC-induced accumulation of transcripts was found to be greatly enhanced in the presence of AIP. This indicates that SA is not only not necessary for the expression of intracellular PR proteins, but, on the contrary, it appears to inhibit the accumulation of these transcripts, as has been observed in wounded tobacco leaves for basic PR protein isoforms (Niki et al. 1998). Alternatively, inhibition of PAL by AIP may promote the production of an unidentified signal that induces the expression of intracellular PR genes. The FC-mediated induction of extracellular PR gene expression was reduced to varying degrees for PR1a, PR-3a, and PR-7a when SA accumulation was suppressed. The SA-dependent as well as SA-independent signaling pathways thus seem to contribute to the induction of extracellular PR protein expression. The electrochemical gradient across the plasma membrane appears to be involved in the SA-independent pathway. These data are in agreement with those of De Wit and co-workers who proposed the existence of additional signals that contribute to the induction of extracellular PR protein expression (Van Kaan et al. 1995).

Induction of PR transcripts in nahG-tobacco. Fusicoccin was found to induce the accumulation of transcripts for some PR proteins in tobacco plants (Fig. 5). The induction by FC was not as strong as that observed in tomato plants but it was comparable to that observed after spraying tobacco plants with 5 mM SA. Pathogenesis-related transcripts were also found to be FC-inducible in transgenic tobacco plants overexpressing the bacterial *nahG* gene for salicylate hydroxylase. These plants are unable to accumulate significant amounts of SA and have been instrumental in establishing the generally accepted, central role for SA in controlling the expression of PR genes in the development of systemic aquired resistance (Gaffney et al. 1993; Delaney et al. 1994; Vernooij et al. 1994; Bi et al. 1995; Friedrich et al. 1995; Du and Klessig 1997). The FC-mediated induction of PR gene expression which we observed in *nahG*-tobacco provides evidence for SA-independent signaling of PR gene expression by modulation of the H^+-gradient across the plasma membrane also in tobacco plants. This conclusion is in agreement with our finding of FC-induced PR gene expression in tomato even when SA biosynthesis was inhibited by AIP. However, the fact that only a subset of the SA-inducible PR transcripts accumulated after FC treatment may indicate a more limited role for the proton electrochemical gradient in the regulation of PR gene expression in tobacco as compared to tomato.

The role of the plasma-membrane H^+-ATPase in plant defense. Activation of the plasma-membrane H^+-ATPase has been observed in several plant pathogen interactions including the incompatible interaction between *C. fulvum* and tomato (Hammond-Kosack et al. 1996). In this system, activation of the H^+-ATPase by dephosphorylation in response to *C. fulvum* avirulence factors (*avr5*) was observed only in cells and membrane preparations from tomato carrying the corresponding resistance gene (*Cf-5*). Furthermore, two different Ca^{2+}-dependent protein kinases were shown to be involved in the elicitor-dependent rephosphorylation of the proton pump (Vera-Estrella et al. 1994; Xing et al. 1996). These data support a role for the plasma-membrane H^+-ATPase in the development of resistance in the incompatible tomato/*C. fulvum* interaction. Interestingly, tomato plants expressing the *nahG* transgene under control of the cauliflower mosaic virus 35S promoter were reported to be fully resistant to *C. fulvum* (Hammond-Kosack and Jones 1996). This observation is in full agreement with our conclusion that activation of the plasma-membrane H^+-ATPase leads to SA-independent induction of PR gene expression. Increased activity of the proton pump may thus constitute a signal for attenuation of pathogen defense responses. In conflict with this hypothesis, however, PR transcript induction has been observed in suspension-cultured tomato cells after treatment with non-specific elicitors. These elicitors also triggered an alkalinization of the culture medium which may be the result of inhibition of the plasma-membrane H^+-ATPase. Presently, we are not able to explain this discrepancy. However, the cell culture used in this study was altered in several aspects of defense gene regulation (lack of response to a race-specific elicitor; constitutive expression of PR-3b) likely due to differences in physiological condition and developmental stage as compared with intact plants (Honée et al. 1998). This led Honée et al. (1998) to argue that conclusions drawn from studies on suspension-cultured cells may not be directly applicable to the defense response in intact plants.

We have previously shown that activation of the proton pump, in addition to inducing PR gene expression, causes a downregulation of wound-induced defense gene expression. Inhibition of the H^+-ATPase, on the other hand, induced the accumulation of wound-responsive mRNAs (Schaller and Oecking 1999). Apparently, the wound and pathogen defense signaling pathways are differentially affected by changes in the proton electrochemical gradient. Therefore, the plasma-membrane H^+-ATPase has the potential to act as a switch activating either wound or pathogen defense responses. While there is evidence for a direct involvement of the plasma-membrane H^+-ATPase

(Vera-Estrella et al. 1994; Xing et al. 1996), it seems likely that other factors affecting the proton electrochemical gradient across the plasma membrane will also have an effect on defense gene expression. Consistent with this hypothesis, it was found that transgenic tobacco plants overexpressing a proton pump from *Halobacterium halobium* (bacterio-opsin) developed a lesion-mimic phenotype. Multiple defense responses were activated and systemic resistance was induced in plants expressing the active proton pump but not in transgenics expressing a mutated, inactive protein (Mittler et al. 1995). Expression of bacterio-opsin was also shown to confer a lesion-mimic phenotype and resistance to fungal pathogens to transgenic potato (Abad et al. 1997).

Salicylic acid-independent activation of disease resistance responses. There are several SA-independent defense signaling pathways in plants, like the ones leading to rhizobia-mediated induced systemic resistance (ISR), to the expression of defensins and thionins, and the wound signaling pathway (reviewed by Pieterse and Van Loon 1999). Recently, evidence was obtained also for SA-independent induction of those PR proteins associated with SAR by use of *nahG*-transgenic plants, and of *Arabidopsis* mutants that are SA insensitive (*npr1*, *nim1*, *sai1*). Limited expression of PR genes was observed in *npr1-, nim1-,* and *sai1*-mutants as well as in *nahG*-overexpressing plants after pathogen infection, indicating the existence of SA-dependent and SA-independent modes of induction (Lawton et al. 1995; Glazebrook et al. 1996; Shah et al. 1997). The SA-independent induction of PR gene expression has also been reported by Heo et al. (1999). We propose here a role for the proton electrochemical gradient across the plasma membrane as a regulatory factor in SA-independent activation of pathogen defense responses.

We thank Drs. R. Dietrich and L. Friedrich (Novartis Agribusiness, Inc., Research Triangle Park, N.C., USA) for the kind gift of *nahG* tobacco seeds, Drs. R. Dietrich, L. Friedrich and U. Conrath (Universität Kaiserslautern, Kaiserslautern, Germany) for clones of tobacco PR proteins, and Dr. C. Oecking (Ruhr-Universität Bochum, Bochum, Germany) for critical reading of the manuscript, helpful discussions, and a sample of fusicoccin. This work was supported in part by a grant (No. 31.46818.96) of the Swiss National Science Foundation to AS.

References

Abad MS, Hakimi SM, Kaniewski WK, Rommens CMT, Shulaev V, Lam E, Shah DM (1997) Characterization of acquired resistance in lesion-mimic transgenic potato expressing bacterio-opsin. Mol Plant-Microbe Interact 10: 635–645

Ballio A, de Michelis MI, Lado P, Randazzo G (1981) Fusicoccin structure-activity relationships. Stimulation of cell enlargement and promotion of seed germination. Physiol Plant 52: 471–475

Baunsgaard L, Fuglsang AT, Jahn T, Korthout HAAJ, De Boer AH, Palmgren MG (1998) The 14-3-3 proteins associate with the plant plasma membrane H^+-ATPase to generate a fusicoccin binding complex and a fusicoccin responsive system. Plant J 13: 661–667

Bi Y-M, Kenton P, Mur L, Darby R, Draper J (1995) Hydrogen peroxide does not function downstream of salicylic acid in the induction of PR protein expression. Plant J 8: 235–245

Coquoz JL, Buchala A, Métraux J-P (1998) The biosynthesis of salicylic acid in potato plants. Plant Physiol 117: 1095–1101

Danhash N, Wagemakers CAM, Van Kaan JAL, De Wit PJGM (1993) Molecular characterization of four chitinase cDNAs obtained from *Cladosporium fulvum*-infected tomato. Plant Mol Biol 22: 1017–1029

Delaney TP, Uknes S, Vernooij B, Friedrich L, Weymann K, Negrotto D, Gaffney T, Gut-Rella M, Kessmann H, Ward E, Ryals J (1994) A central role of salicylic acid in plant disease resistance. Science 266: 1247–1250

Du H, Klessig F (1997) Role for salicylic acid in the activation of defense responses in catalase-deficient transgenic tobacco. Mol Plant-Microbe Interact 7: 922–925

Friedrich L, Vernooij B, Gaffney T, Morse A, Ryals J (1995) Characterization of tobacco plants expressing a bacterial salicylate hydroxylase gene. Plant Mol Biol 29: 959–968

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of salicylic acid for the induction of systemic acquired resistance. Science 261: 754–756

Glazebrook J, Rogers EE, Ausubel FM (1996) Isolation of *Arabidopsis* mutants with enhanced disease susceptibility by direct screening. Genetics 143: 973–982

Görlach J, Raesecke H-R, Rentsch D, Regenass M, Roy P, Zala M, Keel C, Boller T, Amrhein N, Schmid J (1995) Temporally distinct accumulation of transcripts encoding enzymes of the prechorismate pathway in elicitor-treated, cultured tomato cells. Proc Natl Acad Sci USA 92: 3166–3170

Hammond-Kosack KE, Jones JD (1996) Resistance gene-dependent plant defense responses. Plant Cell 8: 1773–1791

Hammond-Kosack KE, Silverman P, Raskin I, Jones JD (1996) Race-specific elicitors of *Cladosporium fulvum* induce changes in cell morphology and the synthesis of ethylene and salicylic acid in tomato plants carrying the corresponding *Cf* disease resistance gene. Plant Physiol 110: 1381–1394

Henning J, Malamy J, Grynkiewicz G, Indulski J, Klessig DF (1993) Interconversion of the salicylic acid signal and its glucoside in tobacco. Plant J 4: 593–600

Heo WD, Lee SH, Kim JC, Chung WS, Chun HJ, Lee KJ, Park CY, Park HC, Choi IY, Cho MJ (1999) Involvement of specific calmodulin isoforms in salicylic acid-independent activation of plant disease resistance responses. Proc Natl Acad Sci USA 96: 766–771

Honée G, Buitink J, Jabs T, De Kloe J, Sijbolts F, Apotheker M, Weide R, Sijen T, Stuiver M, De Wit PJGM (1998) Induction of defense-related responses in Cf9 tomato cells by the AVR9 elicitor peptide of *Cladosporium fulvum* is developmentally regulated. Plant Physiol 117: 809–820

Jahn T, Fuglsang AT, Olsson A, Brüntrup IM, Collinge DB, Volkmann D, Sommarin M, Palmgrem MG, Larsson C (1997) The 14-3-3 protein interacts directly with the C-terminal region of the plant plasma membrane H^+-ATPase. Plant Cell 9: 1805–1814

Johansson F, Sommarin M, Larsson C (1993) Fusicoccin activates the plasma membrane H^+-ATPase by a mechanism involving the C-terminal inhibitory domain. Plant Cell 5: 321–327

Kutschera U, Schopfer P (1985) Evidence for the acid-growth theory of fusicoccin action. Planta 163: 494–499

Lanfermeijer FC, Prins HBA (1994) Modulation of H^+-ATPase activity by fusicoccin in plasma membrane vesicles from oat roots. Plant Physiol 104: 1277–1285

Lawton K, Weymann K, Friedrich L, Vernooij B, Uknes S, Ryals J (1995) Systemic acquired resistance in *Arabidopsis* requires salicylic acid but not ethylene. Mol Plant-Microbe Interact 8: 863–870

Malamy J, Henning J, Klessig D (1992) Temperature-dependent induction of salicylic acid and its conjugates during the

resistance response to tobacco mosaic virus infection. Plant Cell 4: 359–366

Mauch-Mani B, Slusarenko AJ (1996) Production of salicylic acid precursors is a major function of phenylalanine ammonia-lyase in the resistance of *Arabidopsis* to *Peronospora parasitica*. Plant Cell 8: 203–212

Meichtry J, Amrhein N, Schaller A (1999) Characterization of the subtilase gene family in tomato (*Lycopersicon esculentum* Mill.). Plant Mol Biol 39: 749–760

Michelet B, Boutry M (1995) The plasma membrane H^+-ATPase. A highly regulated enzyme with multiple physiological functions. Plant Physiol 108: 1–6

Mittler R, Shulaev V, Lam E (1995) Coordinated activation of programmed cell death and defense mechanisms in transgenic tobacco plants expressing a bacterial proton pump. Plant Cell 7: 29–42

Niki T, Mitsuhara I, Seo S, Ohtsubo N, Ohashi Y (1998) Antagonistic effect of salicylic acid and jasmonic acid on the expression of pathogenesis-related (PR) protein genes in wounded mature tobacco leaves. Plant Cell Physiol 39: 500–507

Oecking C, Piotrowski M, Hagemeier J, Hagemann K (1997) Topology and target interaction of the fusicoccin-binding 14-3-3 homologs of *Commelina communis*. Plant J 12: 441–453

Olivari C, Meanti C, De Michelis MI, Rasi-Caldogno F (1998) Fusicoccin binding to its plasma membrane receptor and the activation of the plasma membrane H^+-ATPase. Plant Physiol 116: 529–537

Palmgren MG (1991) Regulation of plant plasma membrane H^+-ATPase activity. Physiol Plant 83: 314–323

Pieterse CMJ, Van Loon LC (1999) Salicylic acid-independent plant defence pathways. Trends Plant Sci 4: 52–58

Piotrowski M, Morsomme P, Boutry M, Oecking C (1998) Complementation of the *Saccharomyces cerevisiae* plasma membrane H^+-ATPase by a plant H^+-ATPase generates a highly abundant fusicoccin binding site. J Biol Chem 273: 30018–30023

Raskin I, Turner IM, Melander WR (1989) Regulation of heat production in the inflorescence of an Arum lily by endogenous salicylic acid. Proc Natl Acad Sci USA 86: 2214–2218

Roberts MR, Bowles DJ (1999) Fusicoccin, 14-3-3 proteins, and defense responses in tomato plants. Plant Physiol 119: 1243–1250

Ryals JA, Neuenschwander UH, Willits MG, Molina A, Steiner H-Y, Hunt MD (1996) Systematic acquired resistance. Plant Cell 8: 1809–1819

Schaller A, Oecking C (1999) Modulation of plasma membrane H^+-ATPase activity differentially activates wound and pathogen defense responses in tomato plants. Plant Cell 11: 263–272

Shah J, Tsui F, Klessig DF (1997) Characterization of a salicylic acid-insensitive mutant (*sai1*) of *Arabidopsis thaliana*, identified in a selective screen utilizing the SA-inducible expression of the *tms2* gene. Mol Plant-Microbe Interact 10: 69–78

Tornero P, Conejero V, Vera P (1996) Primary structure and expression of a pathogen-induced protease (PR-P69) in tomato plants: Similarity of functional domains to subtilisin-like endoproteases. Proc Natl Acad Sci USA 93: 6332–6337

Turner NC, Graniti A (1969) Fusicoccin. A fungal toxin that opens stomata. Nature 223: 1070–1071

Van Kaan JAL, Joosten MHAJ, Wagemakers CAM, Van den Berg-Velthuis GCM, De Wit PJGM (1992) Differential accumulation of mRNAs encoding extracellular and intracellular PR proteins in tomato induced by virulent and avirulent races of *Cladosporium fulvum*. Plant Mol Biol 20: 513–527

Van Kaan JAL, Cozijnsen T, Danhash N, De Wit PJGM (1995) Induction of tomato stress protein mRNAs by ethephon, 2,6-dichloroisonicotinic acid and salicylate. Plant Mol Biol 27: 1205–1213

Van Loon LC, Pierpoint WS, Boller T, Conejero V (1994) Recommendations for naming plant pathogenesis-related proteins. Plant Mol Biol Rep 12: 245–264

Vera-Estrella R, Barkla BJ, Higgins VJ, Blumwald E (1994) Plant defense to fungal pathogens. Activation of host-plasma membrane H^+-ATPase by elicitor-induced enzyme dephosphorylation. Plant Physiol 104: 209–215

Vernooij B, Friedrich L, Morse A, Reist R, Kolditz-Jawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic acid is not translocated signal responsible for inducing systemic acquired resistance but is required in signal transduction. Plant Cell 6: 959–965

Wevelsiep L, Rüpping E, Knogge W (1993) Stimulation of barley plasmalemma H^+-ATPase by phytotoxic pepetides from the fungal pathogen *Rhynchosporium secalis*. Plant Physiol 101: 297–301

Xing T, Higgins VJ, Blumwald E (1996) Regulation of plant defense response to fungal pathogens: two types of protein kinases in the reversible phosphorylation of the host plasma membrane H^+-ATPase. Plant Cell 8: 555–564

Zon J, Amrhein N (1992) Inhibitors of phenylalanine ammonia-lyase: 2-aminoindan-2-phosphonic acid and related compounds. Liebigs Ann Chem: 625–628