Fusicoccin activates pathogen-responsive gene expression independently of common resistance signalling pathways, but increases disease symptoms in *Pseudomonas syringae*-infected tomato plants.

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Abstract

Fusicoccin (FC), an activator of the plant plasma membrane H^+-ATPase, induces several components of plant pathogen resistance responses, including defence hormone biosynthesis and pathogenesis-related (PR) gene expression. The mechanism by which these responses occur, and the effect they have on plant pathogen interactions is unknown. Here, we show that PR gene expression in response to FC in tomato (*Lycopersicon esculentum* Mill) plants does not strictly require the common defence hormones, salicylic acid (SA), jasmonic acid (JA) and ethylene. We also show that FC-induced PR gene expression requires neither Ca^{2+} nor reactive oxygen species (ROS), typical early pathogen resistance response signals. The possibility that PR gene expression is related to FC-induced dehydration stress is also discounted. Finally, we show that the defence responses elicited by FC in tomato are not sufficient to confer resistance to the bacterial pathogen *Pseudomonas syringae*. Rather, FC increases the rate and severity of disease symptom formation in an ethylene-dependent manner.

Key words

Defence response, fusicoccin, *Lycopersicon*, pathogenesis-related gene, signalling.

Abbreviations

DPI, diphenylene iodonium: EGTA, ethyleneglycol-bis(b-amino-ethyl ether)-N,N′-tetraacetic acid: FC, fusicoccin: HR, hypersensitive response: INA, 2,6-dichloroisonicotinic acid: SA, salicylic acid: JA, jasmonic acid: PR, pathogenesis-related: ROS, reactive oxygen species: PM, plasma membrane: SWRP, systemic wound response protein

Introduction

Plants respond to pathogens or their elicitors by activating multiple defence responses. These include the hypersensitive response (HR), cell wall strengthening, phytoaxlein synthesis and synthesis of pathogenesis-related (PR) proteins (for review see Hammond-Kosack and Jones 1996). Such responses can occur systemically, at distant uninfected sites, as well as in the local, challenged leaves. A small number of core signalling mechanisms are important for the responses leading to resistance against a great many plant pathogens. In particular, many resistance mechanisms are based on either a salicylic acid (SA)-dependent pathway (Dempsey et al. 1999) or a jasmonic acid (JA) and ethylene-dependent pathway (Pieterse and van Loon 1999). Salicylic acid is required for systemic acquired resistance (SAR), whilst JA and ethylene are typically associated with induced systemic resistance (ISR) and certain other SA-independent resistance responses. Other signalling events commonly observed during plant disease resistance responses are ion fluxes, including the movement of protons, anions and calcium and potassium ions across the plasma membrane (PM), changes in protein phosphorylation and the production of reactive oxygen species (ROS) (Hammond-Kosack and Jones 1996; Jabs 1997; Ebel and Mithöfer 1998; Blumwald 1998).

Amongst the earliest observed signalling events in plant-pathogen interactions are ion fluxes and changes in PM electrical potential (Ebel and Mithöfer 1998; Blumwald et al. 1998; Heath 2000). Most commonly, pathogen elicitors cause depolarisation of the PM and alkalinisation of the extracellular space. Several mechanisms could cause PM depolarisation, such as inhibition of the proton pumping H^+-ATPase, the activation of Ca^{2+}, K^+ or non-selective cation channels or activation of anion efflux channels (Ward

et al. 1995). Several authors have provided evidence that elicitor-induced PM depolarisation is a direct consequence of inhibition of the H^+-ATPase (e.g. Ladyzhenskaya et al. 1991; Popham et al. 1995; Simon-Plas et al. 1997). The PM H^+-ATPase generates a transmembrane electrochemical gradient via the extrusion of H^+ ions from the cell, using energy from ATP hydrolysis. Physiologically, this electrochemical gradient is utilised by the plant for nutrient uptake, phloem loading, stomatal opening and maintenance of cellular pH (Palmgren 2001). Despite these key physiological functions of the H^+-ATPase, because it provides a force for ion transport and can regulate the activity of voltage-gated ion channels, pathogen-induced changes in the PM electrical potential constitute a possible signalling role for the H^+-ATPase.

Several studies have suggested a direct involvement of the H^+-ATPase in plant pathogen defence. Vera-Estrella et al. (1994) investigated defence responses in tomato cells activated by the *Cladosporium fulvum* race-specific elicitor Avr5. Treatment of suspension culture cells containing the corresponding *Cf5* resistance gene caused a rapid, four-fold increase in H^+-ATPase activity, membrane hyperpolarisation and acidification of the extracelluar medium. In contrast to this, however, elicitation of transgenic *Cf9* tobacco cells with the *C. fulvum* Avr9 elicitor results in an extracellular alkalinisation, consistent with H^+-ATPase inhibition (Piedras et al. 1998). Interestingly, over-expression of a bacterial proton pump in transgenic tobacco and potato plants caused an HR-like programmed cell death and increased pathogen resistance (Mittler et al. 1995; Abad et al. 1997, Rizhsky and Mittler 2001). More direct links between H^+-ATPase activity and pathogen defence have been made in monocotyledonous plants. A 2.5-fold increase in H^+-ATPase activity and concomitant acidification of the

extracellular space was demonstrated in resistant pearl millet seedlings inoculated with the downy mildew *Sclerospora graminicola*, whilst there was no change in H^+-ATPase activity in susceptible varieties (Madhu et al. 2001). H^+-ATPase activity levels correlated strongly with the level of resistance to *S. graminicola* across a range of cultivars. In barley, activation of the H^+-ATPase is associated with HR during resistance to powdery mildew (Zhou et al. 2000).

By manipulating the activity of the H^+-ATPase *in vivo* using pharmacological agents, several groups have provided evidence for a role in the induction of defence responses. For example, treatment of leaves with the phytotoxin fusicoccin (FC), which activates the PM H^+-ATPase, caused an increase in HR lesion density in incompatible barley-powdery mildew interactions (Zhou et al. 2000). In tomato and tobacco plants, application of FC leads to increased SA biosynthesis and expression of PR genes, and regulates a range of other genes associated with plant defence responses (Roberts and Bowles 1999; Schaller and Oecking 1999; Schaller et al. 2000; Frick and Schaller 2002). Interestingly, activation of the H^+-ATPase by FC also inhibits accumulation of systemic wound response proteins (SWRPs) and their transcripts (Doherty and Bowles 1990; Schaller and Oecking 1999). Conversely, H^+-ATPase inhibition by vanadate causes SWRP transcript induction. Schaller and Oecking (1999) suggested that the H^+-ATPase may therefore serve as a switch between wound and pathogen defence signalling.

The signalling mechanisms for FC mediated induction of PR genes and other defence responses remain unknown. The accumulation of SA in FC treated tobacco plants and

the ability of SA to induce PR gene expression suggested one possible causal link. However, SA is apparently not required for the induction of PR genes by FC in tomato or tobacco plants. Schaller et al. (2000), used 2-aminoindan-2-phosphonic acid, an inhibitor of phenylalanine ammonia-lyase activity, to prevent FC-induced SA biosynthesis in tomato plants, but observed no affect on PR gene expression following this treatment. Consistent with this observation, FC-induced PR gene expression was unaffected in SA-deficient *nahG* transgenic tobacco plants (Schaller et al. 2000). Therefore, in FC treated tomato and tobacco plants, PR gene expression is SA-independent. We were interested to extend this line of investigation, and report here on experiments to determine whether other well-characterised pathogen-induced defence signalling pathways are required for FC-mediated PR gene expression, and whether FC promotes resistance to pathogens.

Materials and Methods

Plant material and bioassays

Tomato (*Lycopersicon esculentum* Mill) plants used in these experiments were the wild-type cultivars Moneymaker and RioGrande, the ethylene-insensitive mutant *Never-ripe* in the Pearson background (C.M. Rick Tomato Genetics Resource Center), the JA-deficient JL5(*def1*) mutant (Lightner et al. 1993) and transgenic plants containing a bacterial salicylate hydroxylase gene, *nahG* (Brading et al. 2000). All plants were grown in glasshouses or controlled environment growth chambers at 25°C under a 16 h day light regime. Plants were used for experiments at 3-4 weeks old. Unless otherwise stated, FC and pharmacological agents were applied through the transpiration stream. Plants were excised by cutting the stem with a razor blade, just above soil level, and

placed in water or sodium phosphate buffer pH 6.0 (SPB) containing the appropriate chemical for 30 minutes, with a 30 minute pre-treatment where necessary. Reagents were applied at the following concentrations: 1 μM FC, 0.1 or 0.5 M gadolinium chloride, 5 mM EGTA and 5 mM DPI. Wounding was performed by crushing the leaflets with blunt forceps. After treatments, plants where transferred to water and returned to the growth room. Leaf material was harvested by flash freezing in liquid nitrogen 24 hours, or 12 hours (for wounding treatment), later.

For experiments at different humidity levels, plants were either incubated in growth chambers set at 70% relative humidity (normal growth conditions), or in the same chambers but enclosed in sealed plastic boxes lined with water-saturated paper towels to produce a high humidity environment. For the experiment on wilted plants, water was withheld from 3-week old plants for 7 days, until plants exhibited severe wilting. At this point, plants were either harvested for RNA extraction, or cut at the base and subjected to FC application through the transpiration stream or placed into water alone.

RNA northern gel blot gel blot analysis.

RNA was extracted from harvested tissue using scaled-up version of the method of Verwoerd et al., (1989). RNA was separated on agarose gels before the transfer onto Magna nylon membranes (Micron Separations, Westborough, MA). ^{32}P-labelled probes were prepared by random-primed labelling and purified on Sephadex G-50 columns before overnight hybridisation with filters in 0.25 M sodium phosphate, pH 7.0 and 7% (w/v) SDS at 65^{o}C. Filters were washed in 0.02 M sodium phosphate, pH 7.0, and 1% (w/v) SDS at 65^{o}C. Bands were visualised by autoradiography.

P. syringae inoculation of whole leaflets

Colonies of *P. syringae* pathovar *tomato*, strain T1 were cultured overnight in 50 mL of King's B medium containing 50 µg/L rifampicicin at 28°C with shaking at 250 rpm. Cells were harvested by centrifugation for 10 mins at 1500 x *g*. The supernatant was removed and the cell pellet resuspended in 25 mL of 10 mM of $MgCl_2$. Cells were centrifuged again and resuspended in 10 mL of 10 mM $MgCl_2$. Cell density was estimated by the optical density at 600 nm and an inoculum prepared containing 2.3 x 10^7 cfu/mL of cells in 0.05% Silwet L-77 in 10 mM $MgCl_2$. Leaves of whole plants were inoculated by dipping them into this bacterial cell suspension for 15-30 seconds. Excess inoculum was blotted away using paper towels. Disease symptoms were scored over the following six days. Mock inoculations were carried out using 0.05% Silwet L-77 in 10 mM $MgCl_2$. Pre-treatments were performed by infiltrating either 10 mM $MgCl_2$, 1 µM FC or 0.1 M INA into the leaf space using a plastic 1 mL syringe.

Reproducibility

All experiments involved a minimum of 3 plants per treatment and were carried out on at least three independent occasions.

Results

PR gene induction by FC is independent of SA, JA and ethylene signalling

Using two independent approaches – transgenic tobacco plants unable to accumulate SA and pharmacological inhibition of SA biosynthesis in tomato – Schaller et al. (2000), showed that SA is not required for the induction of PR genes by FC. Beyond this,

nothing is known of the mechanisms by which FC induces PR gene expression. Since SA, JA and ethylene are key hormones in pathogen induced defence signalling, we investigated further the roles of these hormones in FC-mediated PR gene induction in tomato. The JA-deficient mutant, JL5, the ethylene-insensitive mutant, *Never ripe* (NR), and a transgenic *nahG* tomato line that accumulates low levels of SA, were tested along with wild type (WT) tomato plants for their ability to express the PR genes PR3A and PR3B in response to FC. PR3A and PR3B mRNA levels were measured by northern gel blot analysis 24 hours after treatment. Figure 1 shows that FC caused induction of both PR3A and PR3B genes in WT, JL5, NR and *nahG* plants. In the case of PR3A, expression levels were similar in all lines, whereas for PR3B, expression was higher in the *nahG* plants than in the other lines. These data suggest that neither JA, ethylene nor SA are essential for FC signalling in tomato, and further, that SA negatively regulates PR3B gene expression during the FC response.

PR gene induction by FC does not require the generation of reactive oxygen species

Since FC-mediated PR gene induction does not appear to operate via typical 'late' pathogen-induced defence signalling pathways, we decided to investigate 'early' signalling events. ROS are key signals for several defence responses in plants, including pathogen responses (Low and Merida, 1996; Lamb and Dixon 1997; Wojtaszek 1997). The PM NADPH-dependent oxidase, which generates superoxide, is regarded a major source of ROS production in many systems (Low and Merida, 1996; Lamb and Dixon 1997; Wojtaszek 1997). Chemical inhibitors of the NADPH oxidase, such as diphenylene iodonium (DPI), have been show to significantly reduce defence responses induced by pathogens and wounding (e.g. Alvarez et al. 1998; Orozco-Cárdenas et al.

2001). DPI inhibits generation of other ROS besides superoxide, including H_2O_2 and nitric oxide (Bestwick et al. 1999). We therefore tested the ability of DPI to block FC signalling. FC was able to induce normal levels of expression of PR3A and PR3B following DPI pre-treatment (Fig. 2). In contrast, in plants that were wounded after the DPI pre-treatment, expression of the wound-inducible proteinase inhibitor II (PIN II) gene was significantly reduced compared to controls (Fig. 2), as reported previously (Orozco-Cárdenas et al. 2001). This confirms that DPI was successfully taken up through the transpiration stream and blocked the production of ROS under our experimental conditions.

FC induction of PR genes in the presence of inhibitors of calcium signalling

We next investigated whether calcium is involved in mediating FC-induced PR3A and PR3B expression. Ca^{2+} is an important second messenger involved in many biotic and abiotic stress responses (Knight and Knight 2001). Increases in cytosolic free Ca^{2+} can be reduced using the non-specific calcium channel blocker gadolinium, or by using EGTA, an extracellular Ca^{2+} chelator. We pre-treated plants for 30 minutes with either 0.1 M or 0.5 M gadolinium chloride, or 5 mM EGTA, before application of FC. Figure 3 shows that PR3A and PR3B expression levels were very low in control plants treated with inhibitors of calcium signalling or water alone, but that FC was able to induce expression to high levels in the presence of Gd^{3+} and EGTA.

FC-induced PR gene expression is not regulated by water loss

One of the main physiological consequences of FC treatment of plants is wilting, caused by H^+-ATPase-driven stomatal opening. In our experiments, we often observed wilting

of the leaves during FC treatment that was reversed on transfer to water. Some PR genes such as osmotin (Grillo et al., 1995) are up-regulated by osmotic stress. We therefore wanted to discount the possibility that the response we were measuring was a general dehydration effect caused by FC. To prevent water loss from plants during FC treatment, we conducted experiments in sealed containers with a water-saturated atmosphere, and applied FC to the cut stem in our typical transpiration stream application assay, or infiltrated FC directly into the leaf space using a plastic syringe. When applied through the transpiration stream, FC was ineffective in inducing gene expression at high humidity compared with the response observed under normal growth conditions (Fig. 4A). This could mean either that water loss is a pre-requisite for gene expression, or that the high humidity environment prevents uptake of FC. When FC was infiltrated directly into leaves, however, gene expression was induced in plants at both normal and high levels of humidity. Together, these results suggest that high humidity prevents transpiration (and uptake of FC) but not PR gene expression in response to directly applied FC. Hence, in this experiment, FC-induced PR gene expression is apparently not mediated by dehydration.

To confirm this suggestion, we also tested whether PR3A was expressed in wilted plants and whether FC was able to act as an inductive signal in wilted plants. RNA was extracted from untreated, severely wilted WT tomato plants, whilst other wilted plants were subjected to FC application through the transpiration stream or placed into water. In both cases, plants rapidly regained turgor. The results shown in Figure 4B demonstrate that PR3A is not expressed in intact, dehydrated plants, but is induced by FC in similarly wilted plants that are taking up water and gaining turgor.

Effect of FC on a plant-pathogen interaction

Since FC is able to promote a number of responses typical of disease resistance, including SA biosynthesis (Schaller et al. 2000), ethylene biosynthesis (Chen and Kao 1993; Malerba et al. 1995; Malerba and Bianchetti 1996) and defence gene expression, we investigated the ability of FC to increase resistance against the plant pathogenic bacterium *Pseudomonas syringae* pathovar tomato, the causal agent of bacterial speck disease in tomato.

3-4 week old WT tomato leaves were infiltrated with 1 μM FC, 10 mM $MgCl_2$ (negative control) or 0.1 M INA, a molecule that induces the acquired resistance response (Vernooij et al. 1995). These plants were then either mock-inoculated or inoculated with a virulent, disease-causing strain of *P. syringae*, and symptoms monitored over the following six days (Figure 5 and Table 1). In mock-inoculated control and FC-treated leaves, no symptoms were observed (Fig 5A & B). As expected, inoculated INA-treated leaves showed no disease symptoms either (data not shown). In control leaves inoculated with *P. syringae*, mild disease symptoms, visible as leaf collapse and necrosis, occurred on day two. The severity of necrosis then increased over the next four days (Table 1), with characteristic bacterial specks becoming visible after five to six days (Fig. 5C). However, tomato leaves infiltrated with FC and then inoculated with *P. syringae*, exhibited extensive necrosis and leaf collapse within one day of treatment. Necrosis increased over the next five days (Table 1) and bacterial speck occurred at greater density than in inoculated control leaves (Fig. 5D). These symptoms were more rapid (Table 1) and severe (compare Fig. 5C & D) than in

controls. Since virulent strains of *P. syringae* can suppress PR gene expression by activation of JA signalling pathways (Zhao et al., 2003), we also performed an experiment in which FC treatment was carried out five days prior to inoculation with bacteria, to allow PR protein accumulation and establishment of any resistance before pathogen challenge. Severe symptoms were also observed in these leaves, similar to concurrent treatments (Fig 5E).

Contrary to expectation, FC clearly increases the speed and severity of disease symptoms caused by *P. syringae* in tomato leaves. We formed two hypotheses to account for this observation. Firstly, FC treatment might increase entry of *P. syringae* into the leaf, since they infect the plant through the stomata, which would be expected to have larger apertures in FC-treated leaves. Secondly, FC stimulates ethylene production (Chen and Kao 1993; Malerba et al. 1995; Malerba and Bianchetti 1996) and ethylene promotes necrosis during compatible interactions (Lund et al. 1998; O'Donnell et al. 2001, 2003). Hence, precocious ethylene production induced by FC might account for the increased necrosis that we observed. To discriminate between these two possibilities, we tested the effect of FC on the interaction between *P. syringae* and the ethylene insensitive NR tomato mutant. Ethylene does not appear to play a significant role in stomatal behaviour in tomato (Hussain et al. 1999). In plants with no pre-treatment, development of disease symptoms was similar in NR plants to that in WT plants (Table 1). However, unlike in WT plants, in FC-treated leaves of NR plants, there was no increase in severity or rate of disease symptom formation (Table 1). Therefore, as NR tomato plants are insensitive to ethylene, we conclude that ethylene plays a direct role in the FC-mediated increase in necrosis in *P. syringae*-infected WT tomato plants.

Discussion

Numerous defence-related genes are expressed following treatment of tomato plants with FC. These include "late" defence response genes, such as the PR protein genes, and "early" defence response genes, such as those encoding ACC oxidase, MAPKs, CDPKs, calmodulin and NADPH oxidase (Roberts and Bowles 1999; Frick and Schaller 2002). Significantly, the timing of induction of these genes by FC is consistent with their patterns of induction in pathogen-induced defence responses, in that the early and late pathogen-induced genes maintain the same early and late transcription patterns in the FC response. Since the only recognised target of FC in plants is the PM H^+-ATPase, it is likely that these gene expression patterns are mediated by increased H^+-ATPase activity following FC treatment. In this regard, it is interesting to note that increased H^+-ATPase activity is associated with resistance in several plant-pathogen interactions (Vera-Estrella et al. 1994; Madhu et al. 2001; Zhou et al. 2000). The aims of this study were to investigate the mechanism of FC induced PR gene expression and to test whether the FC-induced defence responses provide protection against pathogen infection.

We first tested whether the common defence signalling hormones SA, JA and ethylene are required for FC-induced PR gene expression. These hormones are involved in the vast majority of characterised resistance responses (Dempsey et al. 1999; Pieterse and van Loon 1999). Expression of the marker genes PR3A and PR3B was not inhibited in transgenic *nahG* plants unable to accumulate SA, in the JA-deficient mutant JL5, nor in

the ethylene-insensitive mutant, *Never ripe*. We therefore conclude that the response to FC is mediated by an SA, JA and ethylene-independent pathway. In agreement with the findings of Schaller et al. (2000), who found increased PR2B and PR3B gene expression in the presence of the SA biosynthesis inhibitor, AIP, we noted that the expression of PR3B was elevated in the *nahG* plants compared to the other lines tested. These results indicate a negative affect of SA on FC-induced expression of genes encoding basic PR proteins.

We also investigated the roles of two early defence response signalling components, ROS and Ca^{2+}. An oxidative burst that generates ROS is a common feature of many plant-pathogen interactions, and is linked with the initiation of several defence responses (Low and Merida, 1996; Lamb and Dixon 1997; Wojtaszek 1997). Whilst DPI, an inhibitor of the production of various ROS (Bestwick et al. 1999), prevented wound-induced PIN II gene expression as predicted, no effect on FC-induced PR gene expression was observed. FC signalling does not therefore require the generation of ROS of the type commonly associated with plant defence responses. The activation of plasma membrane Ca^{2+} channels is also a common early defence response signalling mechanism (Ebel and Mithöfer 1998; Blumwald et al. 1998). We used both EGTA, an extracellular Ca^{2+} chelator, and Gd^{3+}, which blocks PM Ca^{2+} channels, to inhibit Ca^{2+} signalling in tomato. Both these inhibitors have been shown previously to be effectively taken up and transported within intact plants (Chung and Ferl, 1999). Neither treatment affected the expression of PR genes in response to FC, eliminating a requirement for Ca^{2+} too. Hence we have as yet been unable to identify a recognised defence signalling pathway through which FC might act.

Since FC has a major physiological impact on the plant by causing stomatal opening and increased water loss by transpiration, we wanted to test whether or not this stress response might be linked to the expression of PR genes. We conclusively show here that water loss from the leaf is not a causal factor in the induction of defence responses by FC. Firstly, FC is able to act in leaves in which transpiration is prevented by manipulating humidity, and second, PR genes are not expressed in severely wilted plants, but their transcription can be activated in wilted plants by the application of FC during rehydration.

Despite the failure to identify a known pathogen response pathway that FC might act on, several responses to FC (defence gene expression and SA and ethylene biosynthesis) are typical pathogen-induced responses. One explanation for our results might be that FC acts downstream of SA, JA and/or ethylene in activating gene transcription and other responses. This is unlikely, however, given that the available evidence indicates that FC specifically activates the PM H^+-ATPase. This evidence includes the observation that all physiological effects of FC (apart from those described above) can be interpreted in terms of H^+-ATPase activation (Marrè 1979), and the recent solution of the 3-dimensional crystal structure of the complex between FC, the H^+-ATPase and the activating 14-3-3 protein (Wurtele et al. 2003). Interestingly, it has recently been found that the elicitor protein NIP1 from the barley pathogen *Rhynchosporium secalis*, which also activates the PM H^+-ATPase, induces necrosis and activates expression of several PR genes (Fiegen and Knogge 2003; Steiner-Lange et al. 2003). Targets for FC in plants other than the H^+-ATPase may yet exist, however. There is recent evidence to

show that FC can dramatically impact on *Xenopus* embryogenesis via a mechanism involving 14-3-3 proteins, but in the absence of homologues of plant H^+-ATPases (Bunney et al. 2003). An alternative explanation is that FC activates an as yet uncharacterised defence pathway. Several pathogen resistance mechanisms that are independent of SA, JA and ethylene have been reported previously. For example, in Arabidopsis, resistance to *Phytophthora porri* was maintained in plants insensitive to ethylene, JA and SA (Roetschi et al. 2001), and resistance to *Peronospora parasitica* mediated by the RPP7 resistance gene is also SA, JA and ethylene-independent (McDowell et al. 2000). Similarly, resistance to *Phytophthora infestans* is not affected in NR, JL5 or *nahG* tomato plants (Smart et al. 2003). Perhaps FC might activate a pathway in tomato similar to that responsible for resistance to *P. infestans*?

We tested the ability of FC to generate resistance to the casual agent of bacterial speck disease, *Pseudomonas syringae*. Unexpectedly, we found no increase in resistance. It has recently been shown that virulent strains of *P. syringae* suppress PR gene expression in tomato by activating jasmonate signalling pathways with the bacterially produced JA analogue, coronatine (Zhao et al., 2003). If coronatine also suppressed FC-induced PR gene expression, this could explain the lack of FC-induced resistance to P. syringae. However, we found that *P. syringae* was unable to suppress resistance induced by the chemical elicitor, INA, and more importantly, treatment of plants with FC prior to pathogen challenge, to allow accumulation of PR proteins, did not increase resistance. We do not, therefore, belive that coronatine-dependent suppression of PR genes is a major cause of the lack of FC-induced resistance in our experiments.

Rather than promote resistance, FC in fact accelerates and exaggerates the development of disease symptoms. Since this effect was not observed in the NR mutant, we conclude that the increased necrosis seen in FC-treated *P. syringae*-infected leaves is a consequence of abnormally high levels of ethylene synthesis during the infection process. Ethylene has been shown to promote the expansion of necrotic lesions in a number of compatible plant-pathogen interactions (e.g. Lund et al. 1998; O'Donnell et al. 2001, 2003) as well as in some incompatible interactions (Knoester et al. 2001). Lund et al. (1998) demonstrated that foliar disease development in tomato plants can be separated into two stages with regard to endogenous ethylene. The early stage involves pathogen infection and primarily lesion formation and occurs independently of ethylene, whereas the later stage requires ethylene for the expansion of necrosis surrounding primary lesions. Perhaps FC-stimulated ethylene production mimics the later stage of disease development, promoting early and massive leaf necrosis. It is also possible that the over-production of ethylene caused by FC masks an underlying FC-induced resistance mechanism suggested by defence gene expression, or that a real FC-induced resistance response exists that is specific to pathogens other than *P. syringae*.

The link between increased H^+-ATPase activity and defence gene expression and/or pathogen resistance is now well established in several systems (Roberts and Bowles 1999; Zhou et al. 2000; Madhu et al. 2001; Frick and Schaller 2002). What is unclear, however, is whether these responses generate effective resistance against some or all pathogens, and whether some plant species use H^+-ATPase signalling as part of their defence against pathogens more than others. At least in tomato, FC-induced defence gene expression cannot be linked with known defence signalling pathways such as the

JA, ethylene or SA-dependent pathways, nor with the generation of ROS or Ca^{2+} signalling. Whilst FC application can provide resistance against powdery mildew in barley (Zhou et al. 2000), it remains to be seen whether activation of the H^+-ATPase, by FC or otherwise, is sufficient to generate *bona fide* pathogen resistance in plants such as tomato.

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Table 1

Development of necrotic lesions in wild-type and *Never ripe* tomato plants infected with *P. syringae*.

	Day 0	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
WT + *P. syringae*	-	-	+	++	++++	++++	++++
WT + FC + *P. syringae*	-	+++	+++	+++	++++	++++	++++
NR + *P. syringae*	-	-	+	++	++++	++++	++++
NR + FC + *P. syringae*	-	-	+	++	++++	++++	++++

Figure 1. FC-induced PR gene expression in wild-type and mutant tomato plants. Wild-type (WT), JL5, NR and *nahG* plants were treated via the transpiration stream with either buffer alone (SPB) or SPB containing 1 μM FC, and expression of the PR3A and PR3B genes 24 h later assayed by northern blotting. The lower panel shows an ethidium bromide-stained gel before transfer to illustrate RNA loading.

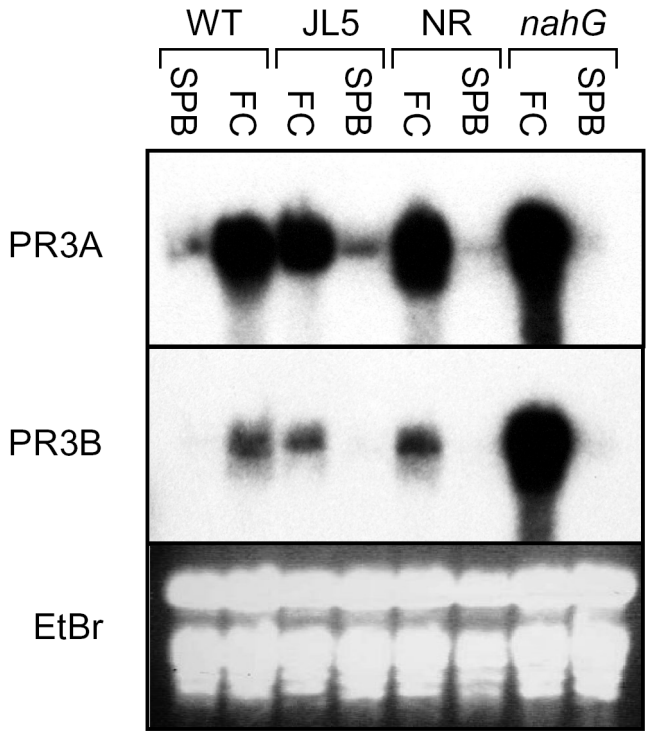


Figure 2. FC-induced PR gene expression is not dependent on the generation of ROS. Wild-type tomato plants were treated via the transpiration stream with buffer alone (SPB), or with SPB containing 1 μM FC, 5 mM DPI, or both. RNA was extracted 24 h later. Additional plants were wounded after application of SPB or SPB containing 5 mM DPI and harvested for RNA extraction 12 h later. Expression of the PR3A, PR3B and PinII genes was assayed by northern blotting. The lower panel shows an ethidium bromide-stained gel before transfer to illustrate RNA loading.

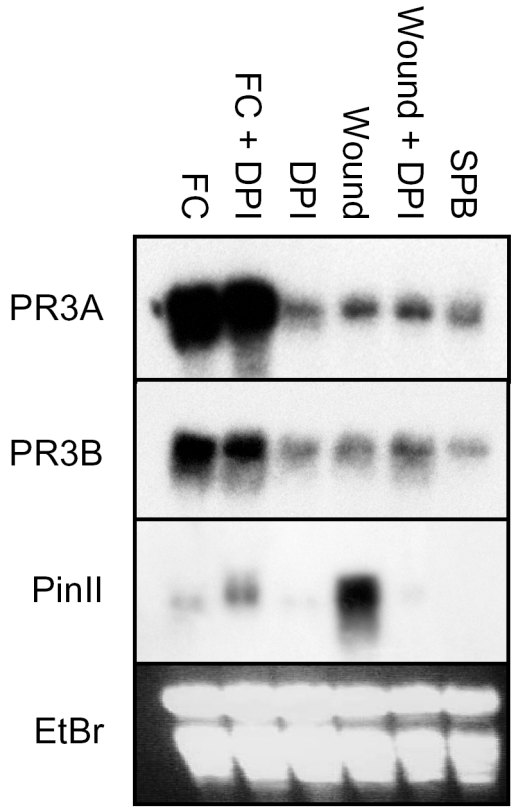


Figure 3. FC-induced PR gene expression is not dependent on Ca^{2+} influx

Effect of inhibitors of calcium signalling on FC-induced PR gene expression in tomato. Wild-type tomato plants were treated via the transpiration stream with buffer alone (SPB), or with SPB containing 1 μM FC, following pre-treatment with either 0.1 or 0.5 M gadolinium chloride (Gd^{3+}) or 5 mM EGTA. Expression of the PR3A and PR3B genes 24 h later was assayed by northern blotting. The lower panel shows an ethidium bromide-stained gel before transfer to illustrate RNA loading.

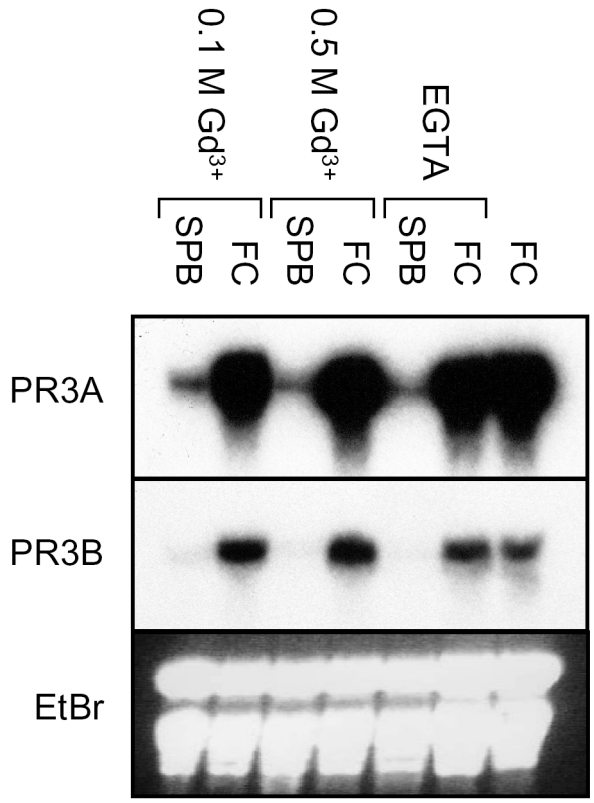
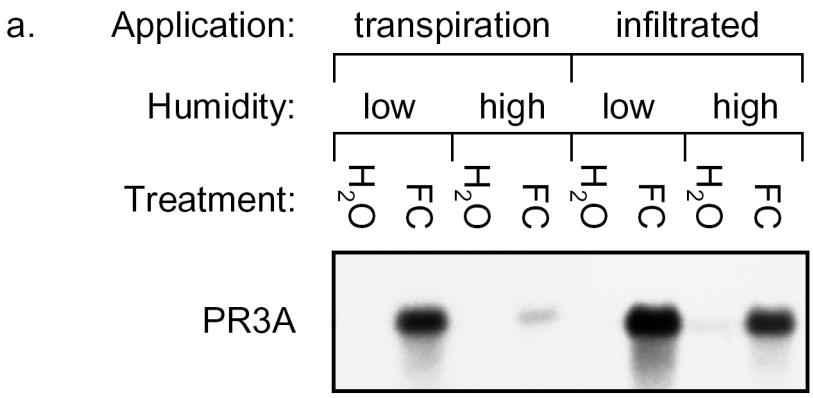


Figure 4. FC-induced PR gene expression is independent of transpirational water loss.

a. Wild-type tomato plants contained in normal growth conditions (low humidity) or in water-saturated boxes (high humidity) were treated with 1 µM FC either via the transpiration stream or via direct infiltration into the leaf space. Expression of the PR3A gene 24 h later was assayed by northern blotting.

b. Expression of PR3A in wilted plants and in wilted plants that were treated via the transpiration stream with water or 1 µM FC, assayed by northern blotting.



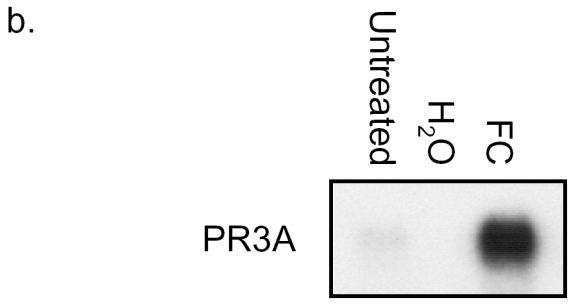


Figure 5. Disease symptom formation in tomato leaves infected with *P. syringae* is accelerated by FC.

Leaves were either mock-inoculated or inoculated with *P. syringae* and imaged 7 days later.

a. Mock-inoculated control leaf.

b. Mock-inoculated FC-treated leaf. The small areas of dead tissue are the sites of infiltration of FC.

c. *P. syringae*-inoculated control leaves.

d. *P. syringae*-inoculated FC-treated leaves.

e. Appearance of disease symptoms in leaves pre-treated with FC five days prior to inoculation with *P. syringae*.