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K^+ channels of *Cf-9* transgenic tobacco guard cells as targets for *Cladosporium fulvum Avr9* elicitor-dependent signal transduction

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Summary

The *Cf-9* gene encodes an extracytosolic leucine-rich repeat (LRR) protein that is membrane anchored near its C-terminus. The protein confers resistance in tomato to races of the fungus *Cladosporium fulvum* expressing the corresponding avirulence gene *Avr9.* In *Nicotiana tabacum* the *Cf-9* transgene confers sensitivity to the Avr9 elicitor, and leads on elicitation to a subset of defence responses qualitatively similar to those normally seen in the tomato host. One of the earliest responses, both in the native and transgenic hosts, results in K^+ salt loss from the infected tissues. However, the mechanism(s) underlying this solute flux and its control is poorly understood. We have explored the actions of Avr9 on *Cf-9* transgenic *Nicotiana* using guard cells as a model. Much detail of guard cell ion channels and their regulation is already known. Measurements were carried out on intact guard cells in epidermal peels, and the currents carried by inward- ($I_{K,in}$) and outward-rectifying ($I_{K,out}$) K^+ channels were characterized under voltage clamp. Exposures to Avr9-containing extracts resulted in a 2.5- to 3-fold stimulation of $I_{K,out}$ and almost complete suppression of $I_{K,in}$ within 3–5 min. The K^+ channel responses were irreversible. They were specific for the Avr9 elicitor, were not observed in guard cells of *Nicotiana* lacking the *Cf-9* transgene and, from kinetic analyses, could be ascribed to changes in channel gating. Both K^+ channel responses were found to be saturable functions of Avr9 concentration and were completely blocked in the presence of 0.5 μM staurosporine and 100 μM H7, both broad-range protein kinase antagonists. These results demonstrate the ability of the *Cf-9* transgene to couple Avr9 elicitation specifically to a concerted action on two discrete K^+ channels and they indicate a role for protein phosphorylation in Avr9/*Cf-9* signal transduction leading to transport control.

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Introduction

Resistance (*R*) genes in plants play a crucial role in preventing disease. Many *R* genes are dominant, or incompletely dominant, and require specific dominant avirulence (*Avr*) genes in the pathogen for their function (Flor, 1946). An *R* gene enables plants to recognize the pathogen, provided that the pathogen carries the corresponding *Avr* gene. Numerous *R* genes have now been cloned, and it seems increasingly likely that *Avr* genes specify the production of ligands that are recognized by *R* gene-dependent receptors (Hammond-Kosack and Jones, 1996). Upon recognition, a variety of defence responses are initiated which include generation of reactive oxygen species (ROS), alterations in bulk solute (K^+ and Cl^-) flux, synthesis of salicylic acid and cell wall cross-linking. These, and related downstream events, act as antimicrobial and/or as signalling intermediates to co-ordinate induction of other defence mechanisms leading to controlled cell death (Hammond-Kosack and Jones, 1997).

The association of the leaf mould fungus *Cladosporium fulvum* with tomato is well characterized as an agronomically important plant–pathogen interaction. The *Cf-2*, *Cf-4*, *Cf-5* and *Cf-9* genes confer resistance to distinct races of *C. fulvum*, and these genes have now all been cloned and sequenced (Dixon *et al.*, 1996; Hammond-Kosack and Jones, 1997; Jones *et al.*, 1994) and, thus, provide excellent models to study avirulence stimulus-response coupling. The *Avr4* and *Avr9* genes of the fungus have also been cloned, and encode secreted cysteine-rich peptides that elicit a defence response on *Cf-4*- and *Cf-9*-containing tomato plants, respectively (Hammond-Kosack and Jones, 1997; Jones and Jones, 1997; Joosten *et al.*, 1994; Van den Ackerveken *et al.*, 1992). In *Cf-9*-containing tomato, ROS can be detected within 2 h of infiltrating Avr9 into leaves (May *et al.*, 1996). Lipid peroxidation and glutathione production are also initiated, and a loss of solute is evident within this time period or shortly thereafter, with cell death becoming marked only some hours later.

At present, comparatively little information is to hand bearing on the early signalling events downstream from elicitor recognition, although parallels to defence re-

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sponses in animals are noteworthy (Low and Merida, 1996; Mehdy *et al.*, 1996; Mehdy, 1994; Segal and Abo, 1993). Ca^{2+} fluxes and changes in protein phosphorylation have been correlated with defence-related cellular signalling in plants (Conrath *et al.*, 1997; Du and Klessig, 1997; Felix *et al.*, 1994; Sessa *et al.*, 1996; Zhang and Klessig, 1997). Likewise, activation of phospholipase C and synthesis of inositol-1,4,5-trisphosphate, a second messenger mediating intracellular Ca^{2+} release, have been associated with the oxidative burst in elicited plant cell cultures (Legendre *et al.*, 1993), and nitric oxide has been implicated as a downstream intermediate in defence responses (Delledonne *et al.*, 1998). Recently *Cf-5*-carrying tomato protoplasts and *Phytophthora megasperma* Pep13-sensitive parsley cell cultures have been shown to harbour Ca^{2+}-permeable channels at the plasma membrane that respond, albeit transiently, to elicitation (Gelli *et al.*, 1997; Zimmermann *et al.*, 1997). However, the relationship of these channels to any changes in cytosolic-free $[Ca^{2+}]$ ($[Ca^{2+}]_i$) remains to be ascertained. Furthermore, although plausible, the actions on any specific transport pathways for K^+ and Cl^- flux of elicitor-evoked protein (de-)phosphorylation events have remained undefined.

The *Cf-9* gene was recently introduced into tobacco, where it confers a set of responses specific for the Avr9 elicitor similar to those observed in *Cf-9* tomato (Hammond-Kosack *et al.*, 1994). In a novel approach to analysing the role of ion channels in resistance gene function, we used guard cells of *Cf-9* transgenic tobacco plants to explore the action of Avr9 elicitation on plasma membrane K^+ channels. These channels mediate bulk solute flux for stomatal movements. Stomatal behaviour is known to be affected by Avr9 elicitation in tomato and in *Cf-9* transgenic tobacco (Hammond-Kosack *et al.*, 1994), although the underlying transport characteristics have yet to be examined in detail. We report here that Avr9 evokes a profound shift in the balance of K^+ channel activities, biasing the membrane for a net loss of solute. Furthermore, the action of Avr9 on the K^+ channels of *Cf-9* transgenic tobacco guard cells is prevented by antagonists of serine/threonine protein kinases.

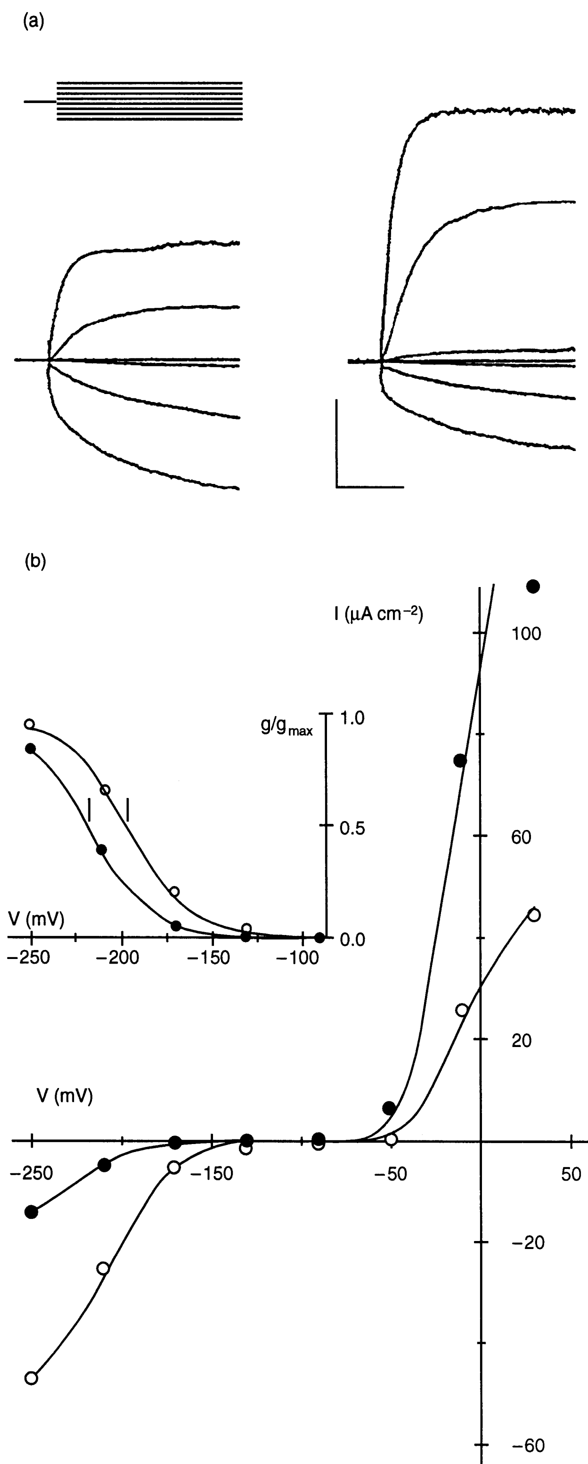
Results

Avr9 *elicitation biases the guard cell K^+ channels for K^+ efflux*

The guard cell plasma membrane is normally dominated by two major K^+ currents with distinct kinetic and regulatory properties (Blatt and Grabov, 1997; Thiel and Wolf, 1997): currents carried by one class of K^+ channels ($I_{K,out}$) are activated with sigmoid kinetics over 200–500 ms by depolarization to voltages positive to the prevailing K^+ equilibrium potential (E_K); currents carried by a second class of K^+ channels ($I_{K,in}$) activate at a fixed voltage, usually negative of −120 mV, with roughly exponential kinetics and over periods of 100–300 ms. We found that both $I_{K,in}$ and $I_{K,out}$ in *Cf-9* transgenic tobacco guard cells were sensitive to Avr9 treatment, compared with the results of control experiments (below). The Avr9 reagent was prepared as an apoplastic filtrate from *Avr9* transgenic *Nicotiana* engineered to give high expression of the elicitor, which, in a typical preparation, gave a full hypersensitive response in tomato at a hundred-fold dilution (= 0.01 U Avr9) (Hammond-Kosack *et al.*, 1994, 1996, 1998). Figure 1 shows the cumulative effect on the currents of a 21-min exposure to 0.001 U Avr9 from one guard cell bathed in 5 mM Ca^{2+}-MES buffer, pH 6.1, with 10 mM KCl. In this experiment, membrane voltage was clamped sequentially from a holding potential of −100 mV to test voltage steps between +50 mV and −250 mV (Figure 1a, top). Prior to Avr9 exposure, steps to test voltages positive of E_K (≅−70 mV in 10 mM $[K^+]_o$) evoked the characteristic time- and voltage-dependent activation of $I_{K,out}$, while steps to voltages negative of −120 mV led to the more rapid activation and inward (= negative) current of $I_{K,in}$ (Figure 1a, bottom left). After exposure to the elicitor, the balance between these two currents was profoundly shifted to favour the K^+ efflux pathway: $I_{K,out}$ increased by approximately threefold, while $I_{K,in}$ was reduced to less than 25% of the control (Figure 1a, bottom right). This effect of Avr9 treatment is clearly seen in the steady-state current–voltage (I–V) relations of Figure 1b, obtained by subtracting the instantaneous (background) currents at the start of each test voltage step from the corresponding steady-state currents recorded at the end of the step. Furthermore, while the increase in $I_{K,out}$ was essentially voltage independent, elicitor-evoked suppression of $I_{K,in}$ was most pronounced at more positive voltages, suggesting a displacement of the voltage sensitivity for activation of $I_{K,in}$. Indeed, the steady-state conductance–voltage curve for $I_{K,in}$ was found to shift negative-going by approximately 25 mV (Figure 1b, inset).

Equivalent voltage clamp measurements were carried out at intervals before and throughout the course of treatment with the elicitor in this experiment, and steady-state values for $I_{K,in}$ (at −200 mV) and $I_{K,out}$ (at 0 mV) were used to construct the response time–course shown in Figure 2. Similar results were obtained in 17 independent experiments (17 guard cells) with *Cf-9* transgenic tobacco and are pooled in Figure 2 after normalizing to the corresponding steady-state currents before elicitor additions. $I_{K,out}$ mean values rose to 2.7 ± 0.2-fold above the control (range 2.4–3.5-fold). Concurrently, the inactivation of $I_{K,in}$ gave a mean decrease in the current to 24 ± 5% of the control before Avr9 treatment (range 15–32%). No significant lag in response to elicitor additions was evident in either of the K^+ currents. However, delays in response of

up to 1 min could have gone undetected in these measurements. Voltage clamp recordings were carried out after washing the elicitor out of the bath in four experiments (data not shown) and indicated that Avr9 action was not significantly reversed, at least over periods of up to 24 min



K^+ channel response to Avr9 *requires the Cf-9 gene*

Voltage clamp recordings like those in Figure 1 were used to determine the specificity of the K^+ channel responses to Avr9 treatments and their relative sensitivities to the elicitor. Measurements were carried out with Avr9 on guard cells of non-transformed *Nicotiana* and of plants transformed with the empty binary transformation vector alone. Because the elicitor was prepared as an apoplastic filtrate from leaves from *Avr9*-transformed *Nicotiana*, experiments were also included with filtrate obtained from non-transformed plants. Data from the resulting two-by-two interaction matrix are summarized in Figure 3. Additions with the filtrate obtained from non-transformed *Nicotiana* were in every case without effect on $I_{K,in}$ and $I_{K,out}$. The currents also failed to respond to Avr9 treatments in *Nicotiana*, except in guard cells of *Cf-9*-transformed *Nicotiana*. Thus, the K^+ channel responses were specific to, and required both, the Avr9 and the *Cf-9* elements.

Voltage clamp recordings were also used to determine the efficacy of Avr9 action after serial dilutions of the elicitor. Measurements were carried out in experiments either with single exposures to 0.00001, 0.0001, 0.001 and 0.01 U Avr9 or with single guard cells exposed to increasing concentrations of the elicitor. Similar results were obtained in each case and the results have been pooled in

Figure 1. Avr9 elicitor promotes current through outward-rectifying K^+ channels ($I_{K,out}$) and inactivates current through inward-rectifying K^+ channels ($I_{K,in}$) in *Cf-9* transgenic *Nicotiana* guard cells.

Data from one guard cell bathed in 5 mM Ca^{2+}-MES, pH 6.1, with 10 mM KCl.

(a) Whole-cell currents recorded during the final 500 ms of 1-sec conditioning steps at −100 mV followed by 3-sec steps to test voltages between +30 and −250 mV (8 cycles, voltage steps top left). Scale: horizontal 1 sec; vertical, 40 µA cm^{-2} or 350 mV. Current traces were recorded immediately before (left) and 21 min after (right) adding 0.001 U Avr9.

(b) Current–voltage curves determined from the currents recorded at the end of the test voltage steps in (a) after subtracting the instantaneous current recorded at the start of each voltage step (Grabov and Blatt (1997). Symbols: ○, before; ●, after Avr9 addition. Inset: Relative conductance-voltage curves for $I_{K,in}$ determined from data points in (b) negative of −100 mV and additional voltage clamp scans (data not shown). Solid lines in the main figure and inset are results of fittings to a Boltzman function (Grabov and Blatt (1997) separately for $I_{K,out}$ (points positive of −100 mV) and $I_{K,in}$ (points negative of −100 mV) with these data and additional voltage clamp scans (data not shown). Fitted parameters (± SE) for maximum conductance (g_{max}), voltage sensitivity coefficient (δ) and voltage giving half-maximal activation ($V_{1/2}$) were:

	$I_{K,out}$		$I_{K,in}$	
	−Avr9	+Avr9	−Avr9	+Avr9
g_{max} (µS cm^{-2})	448 ± 28	1470 ± 60	266 ± 18	78 ± 8
δ	2.02 ± 0.06	2.0 ± 0.1	1.47 ± 0.04	1.45 ± 0.02
$V_{1/2}$ (mV)	−28 ± 2	−28 ± 3	−194 ± 5	−218 ± 4

Figure 4. Stimulation of $I_{K,out}$ and suppression of $I_{K,in}$ were both well-fitted to a hyperbolic function of the form:

$$I = \frac{I_{max}[\text{Avr9}]}{[\text{Avr9}] + K_{1/2}} \quad (1)$$

where for $I_{K,in}$, I and I_{max} represent the relative and maximum relative inactivation, $1-I_{Avr9}/I_o$, and for $I_{K,out}$ these parameters represents the relative and maximum relative activation, I_{Avr9}/I_o. For $I_{K,out}$ non-linear least-squares analysis (Marquardt, 1963) gave a predicted maximum rise of 2.76 ± 0.05-fold over the control, with an apparent $K_{1/2}$ of $4.1 \pm 0.8 \times 10^{-5}$ U; for $I_{K,in}$ the analysis indicated a maximum inactivation of $83 \pm 1\%$ and a similar $K_{1/2}$ of $4.2 \pm 0.3 \times 10^{-5}$ U.

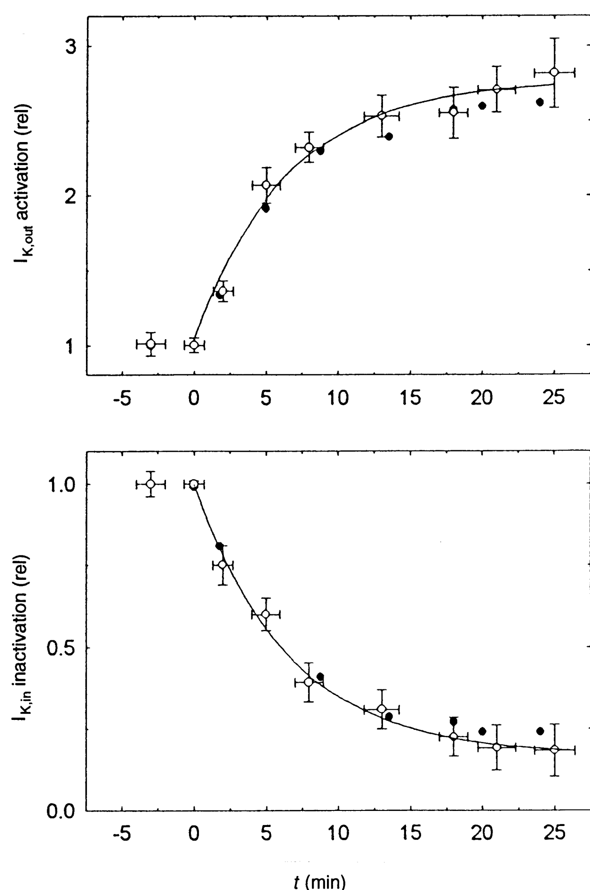


Figure 2. Avr9 elicitor affects guard cell K^+ channels with a common time–course in *Cf-9* transgenic Nicotiana.
Data for $I_{K,out}$ and $I_{K,in}$ from one guard cell in Figure 1 (●) and pooled means ± SE (○) from all 17 cells exposed to 0.001 U Avr9 in 5 mM Ca^{2+}-MES, pH 6.1, with 10 mM KCl. Currents were normalized to measurements carried out immediately before Avr9 additions (at $t = 0$). Solid lines are results of fittings of the means to single exponential functions. Fitted parameters, for $I_{K,out}$: τ, 6.3 ± 0.4 min, maximum current rise relative to the control, 2.8 ± 0.2-fold; for $I_{K,in}$: τ, 6.5 ± 0.2 min, maximum current rise relative to the control, 0.24 ± 0.05-fold.

Avr9 *elicitation affects K⁺ channel gating*

Could the effects of Avr9 on $I_{K,in}$ and $I_{K,out}$ arise from changes in the number of functional channels in the membrane or their individual conductances? In principle, such actions would be expected to affect the steady-state current amplitudes in scalar fashion without altering the voltage sensitivities of the currents or their relaxation kinetics in response to voltage changes. Hence, the change in voltage sensitivity for $I_{K,in}$ (Figure 1b, inset) implied a more subtle impact on the characteristics of K^+ channel gating. To assess elicitor action on gating, the activation kinetics for $I_{K,in}$ and $I_{K,out}$ were determined as a function of voltage from measurements such as shown in Figure 1. Figure 5 shows the cumulative means ± SE of all 17 experiments with data from voltage clamp scans obtained before and 18–21 min after adding 0.001 U Avr9. It was evident that elicitor treatments dramatically slowed the activation of $I_{K,in}$ on negative-going voltage steps, in

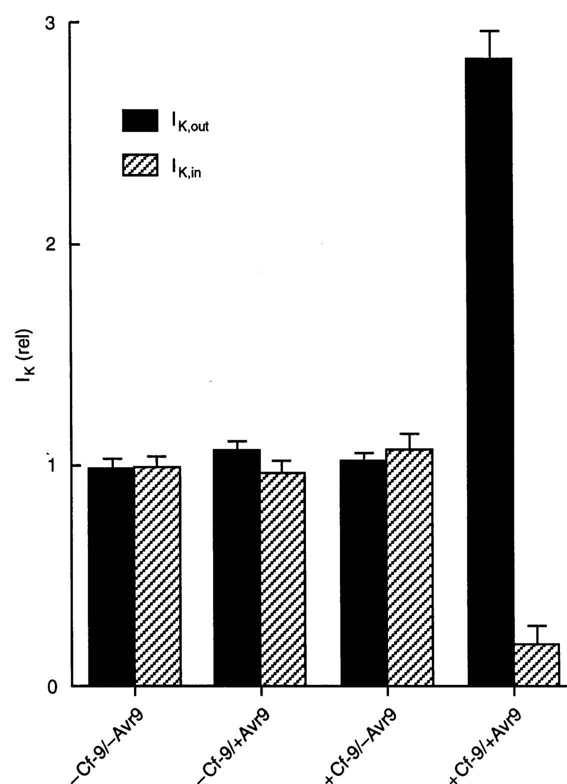


Figure 3. Avr9 elicitor action is specific to *Nicotiana* carrying the *Cf-9* transgene.
Data recorded at 18–20 min after treatments normalized to measurements taken immediately before elicitor (or -Avr9 dummy) additions, for $I_{K,out}$ at 0 mV (solid bars) and for $I_{K,in}$ at –200 mV (diagonal-hatched bars). Measurements were carried out on non-transformed plants and plants carrying the null vector (–*Cf-9*) and on plants carrying the *Cf-9* transgene (+*Cf-9*) with 0.001 U leaf perfusates either without (–Avr9) or with (+Avr9) the elicitor. Data are means ± SE of five (–*Cf-9*/–Avr9), eight (–*Cf-9*/+Avr9), seven (+*Cf-9*/–Avr9) and 17 (+Cf-9/+Avr9) independent experiments.

addition to altering the voltage dependence of the steady-state current (Figure 1b). Interestingly, elicitor treatments also slowed the activation of $I_{K,out}$ on positive-going voltage steps by roughly a factor of 2. Thus, the gating of both K^+ currents was affected by the Avr9 elicitor.

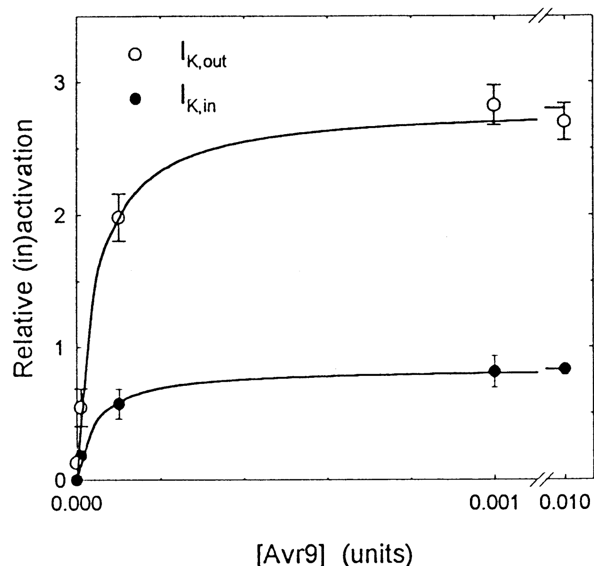


Figure 4. Both guard cell K^+ channel responses are saturable functions of the Avr9 elicitor and show similar concentration dependencies.
Currents determined at 0 mV ($I_{K,out}$) and –200 mV ($I_{K,in}$) as in Figure 3. Each data point represent means $\pm$ SE of at least five independent experiments. Solid lines are non-linear least-squares fittings to a hyperbolic function. Fitted parameters, for $I_{K,out}$: $K_{1/2}$, $4.1 \pm 0.8 \times 10^{-5}$ U, maximum current relative to the control, 2.76 ± 0.05; for $I_{K,in}$: $K_{1/2}$, $4.2 \pm 0.3 \times 10^{-5}$ U, maximum current inactivation relative to the control, 0.83 ± 0.01.

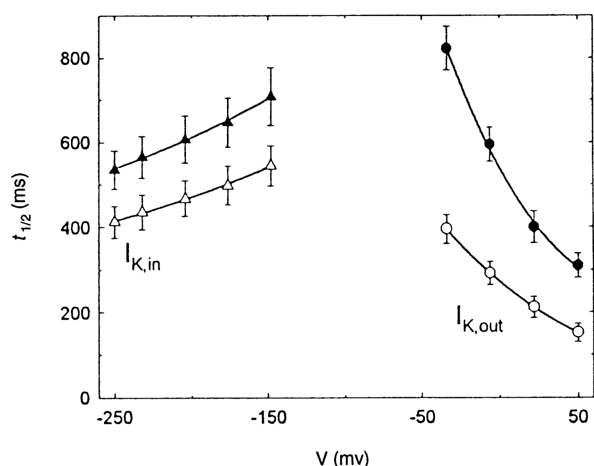


Figure 5. Avr9 elicitor slows the activation of both inward- and outward-rectifying K^+ channel currents.
Mean halftimes $\pm$ SE for activation of $I_{K,in}$ (left) and $I_{K,out}$ (right) as a function of clamp voltage before (○,△) and 18–20 min after (●,▲) Avr9 additions. Data of 17 guard cells from *Cf-9* transgenic *Nicotiana*. Activation halftimes determined directly from current traces (see Figure 1) or, as required, after estimation of the current maximum by non-linear least-squares fitting to a sum of two exponential components ($I_{K,in}$).

Avr9-evoked control of K^+ channels requires protein phosphorylation

In guard cells both $I_{K,in}$ and $I_{K,out}$ are subject to protein (de)phosphorylation (Luan *et al.*, 1993; Thiel and Blatt, 1994), best characterized by the effects of the (dominant negative) mutant abi1 protein phosphatase on the channels in transgenic tobacco (Armstrong *et al.*, 1995). Previous studies have shown that at micromolar concentrations neither H7 nor staurosporine, both broad-range serine/threonine protein kinase antagonists, affect membrane current, either of $I_{K,in}$ and $I_{K,out}$ (Armstrong *et al.*, 1995) or of the anion channel currents that are promoted by abscisic acid (Grabov *et al.*, 1997). However, the kinase antagonists were able to rescue the ABA sensitivities of the K^+ channels in *abi1* transgenic tobacco (Armstrong *et al.*, 1995). Because Avr9 action on the K^+ channels might be mediated through protein phosphorylation, we tested the effects of these antagonists on Avr9 signal coupling to the channels. Both $I_{K,out}$ and $I_{K,in}$ were recorded under voltage clamp, first in the absence, then in the presence of the protein kinase antagonist, and finally in the presence of the antagonist with the addition of 0.001 U Avr9. In each of five independent experiments, prior exposure for 10 min to 0.5 μM staurosporine completely prevented the actions of subsequent treatment with Avr9 in stimulating $I_{K,out}$ and in inactivating $I_{K,in}$ (Figure 6). Similar results were obtained in three other experiments with 100 μM H7 (data not shown).

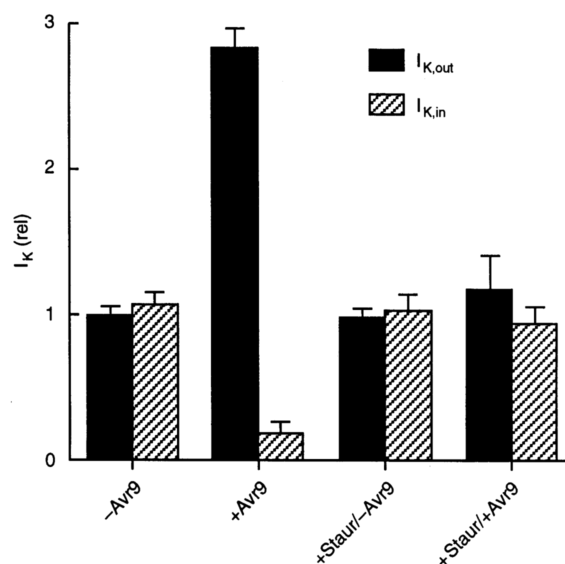


Figure 6. Response of guard cell K^+ channels to the Avr9 elicitor is blocked by protein kinase antagonists.
Data from guard cells of *Cf-9* transgenic *Nicotiana*. Currents determined at 0 mV ($I_{K,out}$, solid bars) and –200 mV ($I_{K,in}$, diagonal-hatched bars) as in Figure 3. Each data point represent means $\pm$ SE of at least five independent experiments recorded over 18–20 min without (–Avr9) and with (+Avr9) elicitor, either without or with (+Staur/–Avr9; +Staur/+Avr9) 10-min pretreatment in 0.5 μM staurosporine.

Adding 0.5 μM staurosporine on its own had no appreciable effect on either of the K^+ channel currents, but in the presence of the protein kinase antagonist subsequent stimulation of $I_{K,out}$ by Avr9 was virtually blocked (Figure 6, solid bars). Furthermore, the pretreatment with staurosporine also protected $I_{K,in}$ from suppression in the presence of Avr9 (Figure 6, diagonal-hatched bars). These results point to a fundamental role for protein phosphorylation, not only as a component in $I_{K,out}$ response to the elicitor, but also as a prerequisite for the concurrent reduction in $I_{K,in}$. The data also suggest that Avr9- and ABA-evoked (de-)phosphorylation events mediate control of the K^+ channels through different pathways, despite the similarities in response to the two stimuli.

Discussion

Changes in ion fluxes are known to be among the earliest responses to elicitors and pathogen attack in plant cells, and commonly include the rapid stimulation of K^+ as well as Cl^- efflux (Atkinson *et al.*, 1990; Chen and Howlett, 1996; Hammond-Kosack and Jones, 1997; Jabs *et al.*, 1997; Woods *et al.*, 1988; Zimmermann *et al.*, 1997). In itself, this loss of inorganic solutes is probably not the cause of cell death associated with pathogen-induced hypersensitive reactions. None the less, wholesale solute efflux, and its consequence for osmotic balance and turgor pressure in the plant cell, could contribute to some of the gross characteristics of diseased tissues.

Despite its obvious importance to pathogenesis, very little detail has been available of the mechanisms underlying the changes in osmotic solute flux or their link to elicitor-evoked signal cascades. Our data now demonstrate an unambiguous effect of *Cf-9*-dependent recognition of Avr9 on the concerted modulation of two distinct K^+ channel currents. Furthermore, this report provides the first example of ion channel control in a transgenic plant cell where the only difference is the presence or absence of the resistance gene. Three lines of evidence link an Avr9/*Cf-9*-evoked signal cascade to specific control of the K^+ channels in *Cf-9* transgenic *Nicotiana* guard cells: (i) Avr9 treatments led to a stimulation of $I_{K,out}$ and a complementary inhibition of $I_{K,in}$ (Figures 1 and 5), giving an overall bias of the membrane towards net K^+ efflux and with a time–course roughly equivalent to previous reports of stimulated solute efflux; (ii) both K^+ channel responses were strictly dependent on the joint presence of Avr9 and the *Cf-9* gene (Figure 3), and both showed saturable dependence on elicitor concentration with very similar apparent values for $K_{1/2}$ (Figure 4); finally (iii) Avr9 action on both K^+ channel currents was suppressed by the protein kinase antagonists H7 and staurosporine (Figure 6).

Avr9/Cf-9 coupling affects discrete actions on K^+ channels

Of the three, the first line of evidence, that *Cf-9*-dependent Avr9 recognition selectively augments $I_{K,out}$ while reducing current through $I_{K,in}$, discounts the idea that solute flux stimulation might result from non-specific membrane damage in the presence of the elicitor, possibly through the action of reactive oxygen species. Such a simple 'leakage' would be evident as an overall (and predominantly voltage independent) rise in membrane conductance under voltage clamp. Although we cannot rule out the possibility of a small conductance of this type in response to Avr9 treatments, it is clear that such an effect could not give rise to either of the K^+ current responses (cf. Figures 1, 2 and 5). Indeed, any non-specific action of the elicitor might account at most for about 1.6% of the change in membrane conductance near 0 mV. This calculation assumes that all of the change in background conductance in Avr9 was attributable to non-specific solute leakage and therefore is almost certainly an overestimate.

In contrast, the consequences of *Cf-9*-dependent Avr9 recognition on both K^+ channel currents indicates profound and discrete alterations in the control of gating in each case. Ensemble current in the whole cell is related to the corresponding single-channel current as:

$$I_x = i_x N P_o = \gamma_x (V - E_x) N P_o \qquad (2)$$

where I_x and i_x are the ensemble and single-channel currents, respectively, V is the membrane voltage, E_x is the equilibrium voltage for ion x, γ_x is the single-channel conductance, N the number of channels, and P_o the mean open probability of the channel gate ($0 \leqslant P_o \leqslant 1$). The parameters N and γ_x are normally constants for any given set of ionic conditions; however, P_o may be sensitive to membrane voltage and intracellular second messengers, among other factors. So, whereas an effect of *Cf-9*-dependent recognition of Avr9 on N and γ_x can result only in scalar changes to current relaxation and to the ensemble steady-state I–V relations, an effect on P_o in many cases will alter the voltage dependence and kinetic characteristics of the current in the whole cell. For $I_{K,in}$, the proportionately larger decrease in steady-state current at more positive voltages (Figure 1b, inset) clearly indicates such an action, as do the changes to $I_{K,in}$ and $I_{K,out}$ activation kinetics (Figure 5).

It is evident, too, that both the suppression of $I_{K,in}$ and $I_{K,out}$ stimulation are tightly coupled to *Cf-9*-dependent Avr9 recognition. Both K^+ channel responses were strictly dependent on the joint presence of Avr9 and the *Cf-9* gene (Figure 3), and titrations demonstrated a high and saturable dependence of each current on Avr9 concentration, with roughly equivalent $K_{1/2}$ values near 4×10^{-5} U. This degree of sensitivity is similar to the results of Piedras *et al.* (1998) obtained for generation of reactive oxygen species by the

elicitor in *Cf-9* transgenic *Nicotiana* cell suspensions. However, much higher concentrations of Avr9 are required for necrosis of leaf tissues from these plants (Hammond-Kosack *et al.*, 1998). These observations support the view that inorganic solute loss is probably not a primary factor in cell death and that other mechanisms must be activated that lead to apoptosis during the plant defence responses.

The fact that alterations to the K^+ channel activities occur in response to *Cf-9*-dependent Avr9 recognition does not rule out additional actions of the elicitor-response gene pair. Extracellular pH changes have been reported in other elicitor-treated plant tissues (Bolwell *et al.*, 1995; Jabs *et al.*, 1997). Vera-Estrella *et al.* (1994) observed acidification of the media on elicitor treatments with *Cf5* tomato cells, a response that they ascribed to an increase in H^+-ATPase activity at the plasma membrane. In contrast, Piedras *et al.* (1998) reported Avr9-dependent alkalinization in *Cf-9* transgenic tobacco cell cultures. In either case, the consequences for the K^+ currents could be important, as both are known to be sensitive to pH (Blatt, 1992; Grabov and Blatt, 1997; Hoshi, 1995; Ilan *et al.*, 1996; Miedema and Assmann, 1996; Roelfsema and Prins, 1997). Furthermore, our own measurements (data not shown) point to other actions on one or more elements of the background conductance in the *Cf-9* transgenic *Nicotiana*. The latter observation is consistent with the activation of an inward current, possibly mediated by anion channels. These observations and any role(s) for pH changes will require further attention.

Protein phosphorylation control of $I_{K,in}$ and $I_{K,out}$

An elicitor-inducible influx of Ca^{2+} has been thought to be a prerequisite to protein kinase activation and oxidative burst in several plant tissues (Hammond-Kosack and Jones, 1996, 1997), and some evidence of Ca^{2+} channels that could mediate such an influx is now available (Gelli *et al.*, 1997; Zimmermann *et al.*, 1997). Assuming that such Ca^{2+} movements lead to in an increase in $[Ca^{2+}]_I$, and this has yet to be demonstrated, the combined actions of both Ca^{2+} and protein phosphorylation could explain the effects seen in the K^+ channels (Grabov and Blatt, 1999; Thiel and Wolf, 1997). However, the effects of $[Ca^{2+}]_i$ do not extend to $I_{K,out}$ (Blatt and Grabov, 1997; Thiel and Wolf, 1997). So Avr9 action on the K^+ channels is most easily understood as a consequence of its effect on protein (de-)phosphorylation.

In fact, the action of protein (de-)phosphorylation in K^+ channel control is well-defined (Blatt and Grabov, 1997; Thiel and Wolf, 1997). Key evidence for its physiological importance has come from work with the (dominant negative) *abi1* mutant of *Arabidopsis* that is insensitive to the water-stress hormone abscisic acid (Koornneef *et al.*, 1984). This gene is now known to encode a 2C-type protein phosphatase (Leung *et al.*, 1994; Meyer *et al.*, 1994), and renders both $I_{K,in}$ and $I_{K,out}$ insensitive to abscisic acid and alkaline cytosolic pH (Armstrong *et al.*, 1995; Grabov and Blatt, 1997), which also bias plasma membrane transport for net solute efflux and drive stomatal closure (Blatt and Grabov, 1997). Furthermore, protein kinase antagonists have been shown to rescue the abscisic acid- and cytosolic pH-sensitivities of the guard cell K^+ channels in *abi1* transgenic *Nicotiana* (Armstrong *et al.*, 1995; Blatt and Grabov, 1997).

Several lines of evidence have implicated protein phosphorylation in transducing elicitor-evoked signals and activating plant defence responses (Yang *et al.*, 1997). Rapid changes in protein phosphorylation patterns and protein kinase activation have been detected in elicitor-treated cells (Felix *et al.*, 1991). Furthermore the *Pto* and *Xa21* resistance genes carry protein kinase catalytic domains (Martin *et al.*, 1993; Song *et al.*, 1995). There are also indications of requirements for protein phosphorylation in elicitor-evoked generation of active oxygen species from studies using protein kinase antagonists, and in some instances protein phosphatase antagonists can mimic the actions of elicitor treatments (Droge-Laser *et al.*, 1997; Felix *et al.*, 1994; Levine *et al.*, 1994; Piedras *et al.*, 1998; Shirasu *et al.*, 1997). Considerable evidence now establishes that various elicitors, and also the *Cf-9*/Avr9 interaction in tobacco (Romeis *et al.*, 1999) stimulate a MAP kinase pathway (Ligterink *et al.*, 1997; Zhang and Klessig, 1998; Zhang *et al.*, 1998).

Considering the probable role for protein phosphorylation in elicitor-evoked signalling on one hand, and its known action in K^+ channel control on the other, we anticipated that broad-range protein kinase antagonists might protect at least $I_{K,out}$ from the effects of *Cf-9*-dependent Avr9 recognition. The supposition proved correct, and the results (Figure 6) thus implicate protein phosphorylation as an intermediate event linking *Cf-9*-dependent Avr9 recognition to the control of $I_{K,out}$. The fact that staurosporine and H7 also prevented the suppression of $I_{K,in}$ under these circumstances suggests that protein kinase activity is also a prerequisite for Avr9-and *Cf-9*-dependent control of these K^+ channels, independent of any other action on $[Ca^{2+}]_i$. However, our results at present do not distinguish between the alternative possibilities for protein kinases functioning either directly in transmitting the elicitor signal, or indirectly in sensitizing the K^+ channels to other *Cf-9*-dependent signal cascades. Furthermore, the relationship between these observations, and any $[Ca^{2+}]_i$ rise evoked by Avr9, now needs to be explored in detail.

Are guard cells targets for Avr9/*Cf-9-dependent signalling?*

Finally, it is of interest that the guard cell K^+ channels do respond to Avr9 in *Cf-9* transgenic *Nicotiana* and, further-

more, that the pattern of response should bias the membrane for K^+ efflux similar to that observed in abscisic acid (Blatt and Grabov, 1997; Thiel and Wolf, 1997). Previous studies of the *Cf*-9 tomato have suggested that the guard cells are less affected than adjacent epidermal or mesophyll cells by treatment with Avr9 at the light-microscopic level. Hammond-Kosack *et al*. (1996) noted that the pathogen response led to supraoptimal stomatal opening and that the elevation of transpirational water loss accounted, at least in part, for the grey necrosis of the underlying mesophyll tissue. A simple interpretation, then, might suggest that the elicitor should promote solute uptake by the guard cells rather than favouring its loss. In fact, stomatal aperture is not only a function of guard cell turgor but also of the balancing back-pressure from the surrounding epidermis (Willmer and Fricker, 1996). Loss of turgor in the epidermal cell layer favours stomatal opening, as is commonly seen in epidermal peels when the neighbouring epidermal cells are damaged. So, because the leaf *Cf-9*- and Avr9-dependent response is accompanied by a collapse of epidermal as well as mesophyll tissues, it seems most likely that an early loss of turgor in the epidermis accounts for the initial stomatal opening (Hammond-Kosack *et al*., 1994, 1996). Thus we conclude that the *Cf-9*- and Avr9-dependent ion channel changes in *Cf-9* transgenic *Nicotiana* guard cells reflect processes that occur in other epidermal and in mesophyll cells, and that this system provides an excellent opportunity to investigate the role and mechanism of changes in ion channel regulation during resistance gene-dependent defence responses.

Experimental procedures

Plant material and Avr9 *extracts*

Nicotiana tabacum var. Petite Havana transformed with either the tomato *Cf-9* resistance gene or with the *Cladosporium Avr9* gene were grown and Avr9 leaf apoplast filtrates prepared as described previously (Hammond-Kosack *et al*., 1998; Piedras *et al*., 1998). Epidermal strips were prepared from newly expanded leaves of plants 4–6 weeks old (Armstrong *et al*., 1995). All operations were carried out on a Zeiss Axiovert microscope (Zeiss, Oberkochen, Germany) fitted with Nomarski DIC optics with strips bathed in rapidly flowing solutions (10 ml min^{-1} ~20 chamber volumes min^{-1}) at 20–22°C. The standard medium was prepared with 5 mM 2-(N-morpholino)propane sulphonic acid (MES) titrated to its pK_a (=6.1) with $Ca(OH)_2$ (final $[Ca^{2+}]$~ 1 mM). KCl and other compounds were included as required and Avr9 filtrates were diluted directly in this medium. Buffers and salts were from Sigma Chemicals (Poole, UK). Staurosporine and H7 were from Calbiochem (Cambridge, UK) and were prepared as stocks in ethanol before diluting ⩾1000-fold in the perfusion medium. At this concentration, ethanol on its own had no measurable effect on the K^+ channels (Armstrong *et al*., 1995).

Electrophysiology

Electrical recordings were achieved with double-barrelled microelectrodes coated with paraffin to reduce electrode capacitance, and filled with 200 mM KOAc to minimize salt leakage and salt-loading artefacts associated with the Cl^- anion (Blatt and Armstrong, 1993). Connection to the amplifier headstage was via a 1 M KCl|Ag-AgCl halfcell, and a matching halfcell and 1 M KCl-agar bridge served as the reference (bath) electrode. Membrane currents were measured by voltage clamp under microprocessor control (μLAB/μLAN; WyeScience, Wye, UK) using 3-pulse protocols (sampling frequency, 2 kHz) and bipolar staircase duty cycles (Blatt and Armstrong, 1993).

Numerical analysis

Data analysis was carried out by non-linear least-squares (Marquardt, 1963) and, where appropriate, results are reported as the mean ± standard error of (*n*) observations.

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