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Cholera toxin elevates pathogen resistance and induces pathogenesis-related gene expression in tobacco

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In animals, plants and fungi, cholera toxin (CTX) can activate signalling pathways dependent on heterotrimeric GTP binding proteins (G-proteins). We transformed tobacco plants with a chimeric gene encoding the A1 subunit of CTX regulated by a light-inducible wheat *Cab-1* promoter. Tissues of transgenic plants expressing CTX showed greatly reduced susceptibility to the bacterial pathogen *Pseudomonas tabaci*, accumulated high levels of salicylic acid (SA) and constitutively expressed pathogenesis-related (PR) protein genes encoding PR-1 and the class II isoforms of PR-2 and PR-3. In contrast, the class I isoforms of PR-2 and PR-3 known to be induced in tobacco by stress, by ethylene treatment and as part of the hypersensitive response to infection, were not induced and displayed normal regulation. In good agreement with these results, microinjection experiments demonstrated that CTX or GTP-γ-S induced the expression of a *PR1–GUS* reporter gene but not that of a *GLB–GUS* reporter gene containing the promoter region of a gene encoding the class I isoform of PR-2. Microinjection and grafting experiments strongly suggest that CTX-sensitive G-proteins are important in inducing the expression of a subset of *PR* genes and that these G-proteins act locally rather than systemically upstream of SA induction.

Keywords: cholera toxin/G-proteins/pathogenesis-related genes/plant pathogens/*Pseudomonas tabaci*

Introduction

Infection of resistant plants with pathogens or potential pathogens triggers a complex series of biochemical and cellular events at or near the infection site which is called the hypersensitive response (HR; for a review, see Collinge and Slusarenko, 1987). The HR is characterized by rapid cell death leading to the formation of necrotic lesions, the production of cytotoxic compounds such as the phytoalexins, the deposition of lignin and callose in cell walls and the production of pathogenesis-related (PR) proteins, which are believed to help defend plants by limiting the spread of infection. In many plant species, including tobacco, the primary infection can also trigger an enhanced, systemic resistance of the plant to subsequent infection by a variety of pathogens. This non-specific form of immunity is known as systemic acquired resistance (SAR; Ross, 1961; Ryals *et al.*, 1994).

Specific PR proteins including PR-1 and the class I and class II isoforms of PR-2 (β-1,3-glucanase) and PR-3 (chitinase) are locally induced near the site of infection as part of the HR. Only a subset of these proteins, PR-1 and class II isoforms of PR-2 and PR-3 are systemically induced in plants showing SAR (for a review, see Ryals *et al.*, 1994). In SAR, a signal is translocated from the site of infection to distal tissues where it acts to induce pathogen resistance and production of the specific pattern of PR proteins (Ward *et al.*, 1991). Free and bound salicylic acid (SA) accumulate in tobacco plants with the onset of pathogen resistance in SAR (Enyedi *et al.*, 1992; Hennig *et al.*, 1993b) and free SA is necessary for SAR induction (Gaffney *et al.*, 1993). Although SA is required for the biochemical responses in distal tissues, it is not the primary systemic signal (Vernooij *et al.*, 1994). Several components of signal transduction pathways related to plant defence have been identified (for reviews, see Godiard *et al.*, 1994; Boller, 1995). In contrast, apart from evidence suggestive of active O_2 species being involved in SA action, no other components of the SAR-specific pathways are known (for a review, see Godiard *et al.*, 1994).

In a variety of maize, barley and *Arabidopsis* mutants (e.g. Walbot *et al.*, 1983), at least some of these host defence responses are clearly activated in the absence of pathogen infection. The majority of these mutants develop lesions that are similar to pathogen-elicited necrotic lesions and, in some *Arabidopsis* 'lesion mimic' mutants, the appearance of HR-like lesions is accompanied by induction of PR proteins, SA and phytoalexin accumulation (Greenberg and Ausubel, 1993; Dietrich *et al.*, 1994). In contrast, Bowling *et al.* (1994) reported the isolation of another class of *Arabidopsis* mutants, which do not show necrotic lesions but constitutively express PR-1, contain elevated levels of SA and display increased resistance to pathogen attack.

In animals and fungi, membrane-associated heterotrimeric GTP-binding proteins (G-proteins) mediate the transduction of signals from receptors at the cell surface to downstream effector proteins (for reviews, see Hepler and Gilman, 1992; Simon *et al.*, 1994). The G-proteins appear to have a similar function in plants in responses

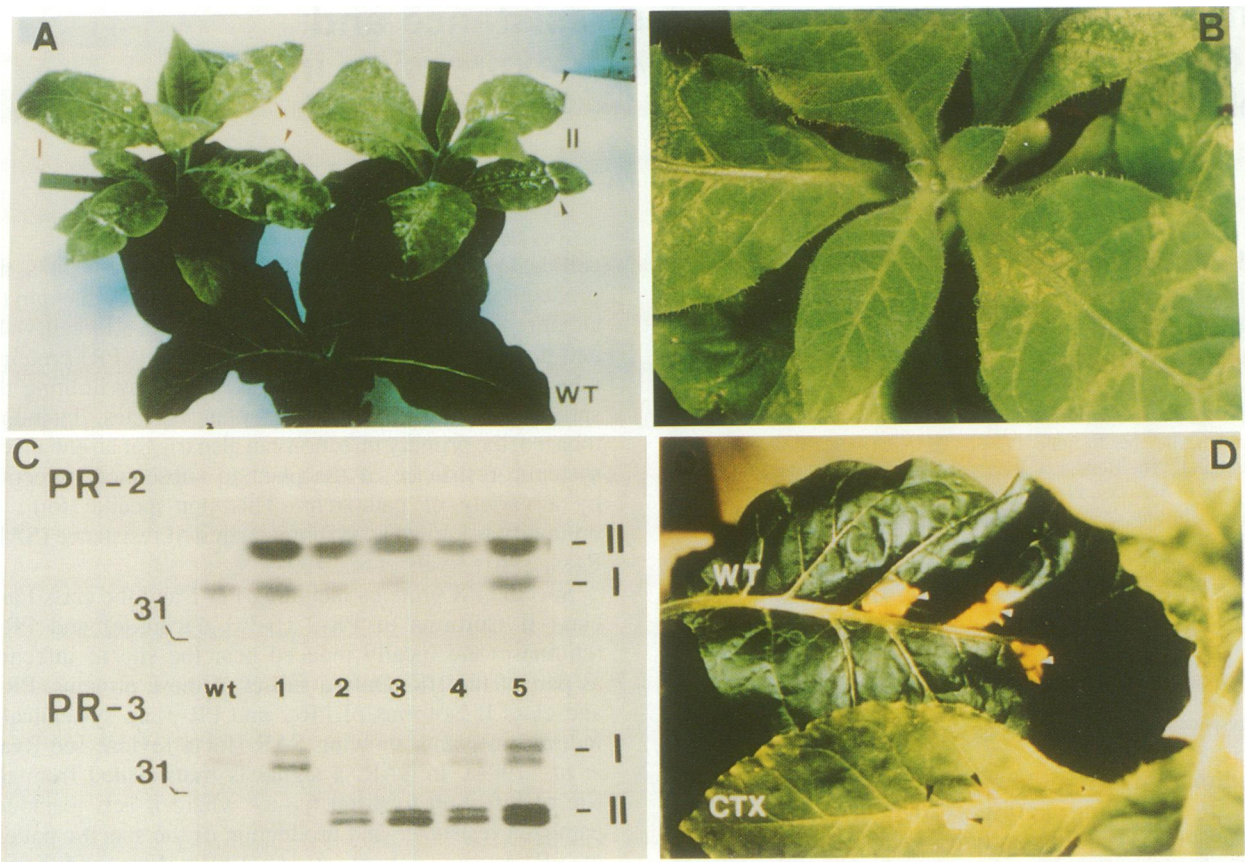

Fig. 1. The phenotype of uninfected and *P.tabaci*-infected CTX transformants. (**A**) Two independent, homozygous CTX transformants (I and II) and a wild-type plant (WT) photographed ~10 weeks after germination. Note the pale colouration and diffuse chlorotic lesions on leaves of CTX plants and the occasional formation of transverse necrotic stripes on older leaves (arrows). (**B**) Close up view of a CTX-expressing transgenic plant showing that the upper three leaves formed at the shoot apex do not exhibit macroscopic lesions. (**C**) Immunoblot analyses showing the variation in class I and II PR-2 and PR-3 accumulation in leaves sampled from the middle of a wild-type (WT) plant and independent lines of CTX transformants (1–5). Each lane was loaded with 5 μg of protein. Symbols: I and II positions of class I and II isoforms, respectively; 31, position of 31 kDa mol. wt marker. (**D**) Comparison of lesions induced on wild-type (WT) and homozygous CTX plants 7 days after infection with *P.tabaci* at an inoculum concentration of 10^7 colony-forming units (c.f.u.)/ml. The necrotic lesions resulting from infection (arrows) are clearly distinguishable from the diffuse spontaneous lesions forming on the CTX plants.

to auxin and light and in the regulation of potassium ion channels and phosphoinositide metabolism (for a review, see Verhey and Lomax, 1993). Cholera toxin (CTX) is a multimeric protein consisting of A1, A2 and five B subunits (Mekalanos *et al.*, 1983). The A1 subunit catalyses the ADP-ribosylation of G_α, which irreversibly blocks the GTPase activity of G-proteins leading to the sustained activation of the downstream signalling pathway (for reviews, see Hepler and Gilman, 1992; Simon *et al.*, 1994). Recent studies show that CTX is also effective in plants; CTX can mimic the effects of red light on Cab-1 gene expression which is mediated by phytochrome (Neuhaus *et al.*, 1993; Bowler *et al.*, 1994).

In the present work, tobacco plants were transformed with a chimeric gene encoding the A1 subunit of CTX driven by the light-inducible wheat *Cab-1* promoter. Tissues of transgenic plants expressing CTX mimic the SAR state in the absence of infection and show greatly reduced susceptibility to the bacterial pathogen *Pseudomonas tabaci*.

We show that CTX expression in transgenic plants selectively induces a subset of PR proteins and that CTX microinjected into cells induces the transcription of genes encoding these proteins. We also provide evidence from grafting and microinjection experiments that G-proteins modified by CTX generate a non-systemic signal which acts upstream of SA induction.

Results

Susceptibility of wild-type plants and CTX transformants to P.tabaci infection

Tobacco plants were transformed with a chimeric gene encoding the A1 subunit of CTX regulated by the wheat *Cab-1* gene promoter. Eighteen of 25 plants expressing the *CTX* gene were pale-green in colour and developed more slowly than wild-type plants. Of these, 13 formed large, widespread, spontaneous lesions (Figure 1A and B) and five transgenic lines showed somewhat smaller, less frequent lesions. RNA-blot hybridization confirmed that expression of the *CTX* gene, although variable in different transformants, was correlated with the size and incidence of lesions and showed the light-induced and tissue-specific pattern of the *Cab-1* promoter in tobacco (Nagy *et al.*, 1987; Fejes *et al.*, 1990) (data not shown). Independent of their size, the lesions differed from necrotic lesions associated with HR in tobacco, i.e. they were usually localized in the palisade layer, did not stain for callose

Table I. Susceptibility of 10-week-old wild-type plants and CTX transformants to *P.tabaci* infection

Genotype	Leaf	Disease symptoms[a]			Bacterial growth[b]		
		10^6 c.f.u./ml	10^7 c.f.u./ml	10^8 c.f.u./ml	Day 0	Day 2	Day 5
Wild-type	Upper	100[b]	54 ± 3	90 ± 5	$4.3 \pm 0.4\times10^8$	$8.4 \pm 1.0\times10^7$	$5.3 \pm 0.4\times10^8$
	Lower	100[b]	62 ± 5	92 ± 8	$4.4 \pm 0.5\times10^3$	$11.4 \pm 0.79\times10^7$	$4.7 \pm 0.3\times10^8$
CTX	Upper	0	13 ± 1	36 ± 3	$3.5 \pm 0.3\times10^3$	$1.8 \pm 0.2\times10^6$	$9.7 \pm 0.6\times10^3$
	Lower	16 ± 3[b]	44 ± 2	60 ± 7	$3.5 \pm 0.3\times10^3$	$8.4 \pm 0.8\times10^6$	$2.4 \pm 0.2\times10^6$

[a]Mean % infected leaf area necrotic ± SEM for 14 replica leaves scored 7 days after inoculation for the indicated inoculation density in c.f.u./ml.
[b]Chlorotic lesions indicative of an early stage of infection, but no necrosis.
Mean c.f.u./8 mm diameter disk cut from inoculated regions ± SEM for eight replica leaves and the indicated day after inoculation with 5×10^6 c.f.u./ml of bacteria.

(data not shown) and were distributed randomly over the leaf surface. The lesions usually appeared on the third or fourth leaf from the top and persisted as the leaf aged (Figure 1A and B). In a few cases, leaves showed a characteristic, transverse stripe of necrotic tissue (Figure 1A, arrows). Lesions formed in both soil-grown and axenically cultured plants, indicating that they were not due to microbial infection. No lesions were found in plants grown for 7 days in the dark, conditions in which the *Cab-1* promoter is not active. Unless indicated otherwise, experiments were performed using leaves showing large spontaneous lesions on CTX transformants homozygous for the transgene.

We compared wild-type and CTX plants for susceptibility to the bacterial leaf pathogen *P.tabaci*, which is the causal agent of tobacco wildfire disease (Lucas, 1975). Leaves of wild-type and CTX plants were infiltrated with a bacterial suspension and scored for disease symptoms after 7 days. Wild-type plants showed typical symptoms of disease in the infiltrated regions (Figure 1D, arrows). In this and subsequent experiments with *P.tabaci*, no difference was found between uninfected and mock-infected plants. For the two highest doses of bacteria tested (10^7 and 10^8 c.f.u./ml), essentially the entire infected area of wild-type plants was necrotic or no differences in the extent of necrosis were noted in upper and lower leaves (Table I). In contrast, CTX plants, as judged by the percentage of the infected region which was necrotic, showed a pronounced reduction of disease symptoms, which was consistently greater in upper leaves than in lower leaves. The infiltrated regions were also scored for bacterial growth (Table I). The bacterial titre of upper and lower leaves of wild-type plants increased with time after infection to the same extent, i.e. ~10^5-fold. In upper leaves of CTX plants, the bacterial titre increased on day 2 then fell to values close to those in freshly inoculated leaves; in lower leaves this effect was less pronounced. This dramatic effect—reduction in bacterial titres of up to 10^5-fold relative to controls—as well as the effect on disease symptoms was correlated with the established pattern of *CTX* gene expression, i.e. high in young, upper leaves and low in leaves near the bottom of the plant. CTX treatment did not inhibit the growth of *P.tabaci* in culture, indicating that disease resistance resulted from the action of CTX on the plant host (data not shown).

Induction of PR proteins

We compared the patterns of PR-2 and PR-3 induction in wild-type and CTX plants in response to ethylene treatment and infection with *P.tabaci* (Figure 2). As judged from immunoblot analyses, untreated, wild-type plants showed a gradient of class I PR-2 and PR-3 expression: high in older leaves near the bottom of the plant and low in young leaves near the top of the plant (Figure 2A). This gradient was more pronounced for PR-2 than for PR-3, was somewhat variable, and depended on the age and physiological state of the individual plant, as reported earlier (Meins *et al.*, 1992). Class II isoforms of PR-2 and PR-3 did not accumulate in leaves of untreated wild-type plants.

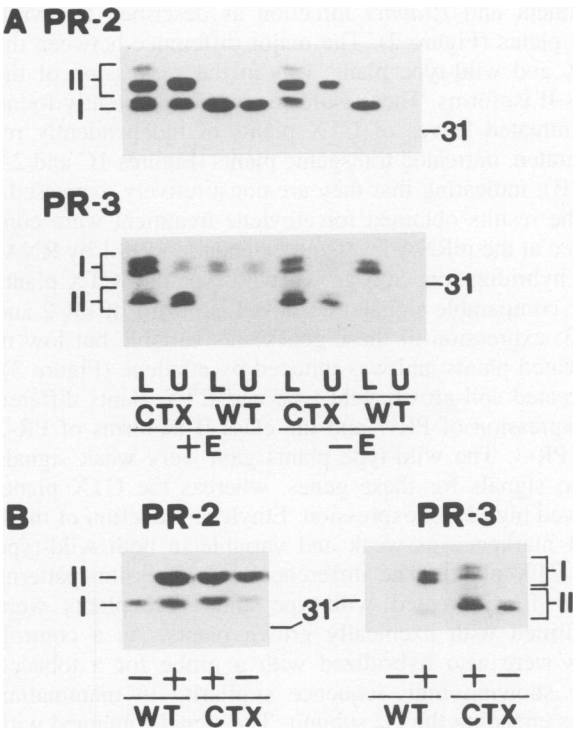

Fig. 2. Immunoblot analyses of class I and class II isoforms of PR-2 (β-1,3-glucanase) and PR-3 (chitinase) in wild-type (WT) and CTX plants. (**A**) Effect of ethylene treatment. Soil-grown plants were incubated for 4 days in chambers with an ethylene adsorbent (–E) or with 20 p.p.m. ethylene (+E). Lanes were loaded with extracts prepared from upper leaves (U) and lower leaves (L) containing 15 μg of protein. (**B**) Effect of *P.tabaci* infection. Leaves of soil-grown plants were mock infected (–) or infected with 10^7 c.f.u./ml of *P.tabaci* (+) and then sampled after 7 days. Each lane was loaded with 5 μg of protein. Symbols: I and II positions of class I and II isoforms, respectively; 31, position of 31 kDa mol. wt marker. Note that, in the case of PR-2, the class II isoforms are higher in molecular weight than the class I isoforms, whereas in the case of PR-3 the class II isoforms are lower in molecular weight than the class I isoforms.

The occasional weak signal found probably results from cryptic microbial infection because signals were never detected with leaves of axenically grown plants.

In wild-type plants, class I isoforms of PR-2 and PR-3 were induced in leaves by treatment with 20 p.p.m. of ethylene for 4 days (Figure 2A). Induction was variable in older leaves and most pronounced in young leaves. No induction of the class II isoforms was found. In contrast, both the class I and class II isoforms of PR-2 and PR-3 were induced in leaves of wild-type plants 7 days after infection with *P.tabaci* (Figure 2B).

In CTX plants, the class I isoforms of PR-2 and PR-3 showed variable expression (Figure 1C), but accumulated preferentially in lower leaves and were induced by ethylene treatment and *P.tabaci* infection as described for wild-type plants (Figure 2). The major difference between the CTX and wild-type plants was in the expression of the class II isoforms. These isoforms were consistently found in untreated leaves of CTX plants of independently regenerated, untreated transgenic plants (Figures 1C and 2A and B), indicating that they are constitutively expressed.

The results obtained for ethylene treatment were confirmed at the mRNA level and extended to PR-1 by RNA-blot hybridization. Soil-grown wild-type and CTX plants gave comparable signals for class I isoforms of PR-2 and PR-3: expression of these genes was variable but low in untreated plants and was induced by ethylene (Figure 3). Untreated soil-grown wild-type and CTX plants differed in expression of PR-1 and the class II isoforms of PR-2 and PR-3. The wild-type plants gave very weak signals or no signals for these genes, whereas the CTX plants showed high level expression. Ethylene induction of these SAR markers was weak and variable in both wild-type and CTX plants. The differences in expression patterns found for untreated wild-type and CTX plants were confirmed with axenically grown plants. As a control, blots were also hybridized with a probe for a tobacco gene showing high sequence similarity to mammalian genes encoding the G_α subunit. The signals obtained with this probe were comparable in wild-type and CTX plants and were not affected by ethylene treatment.

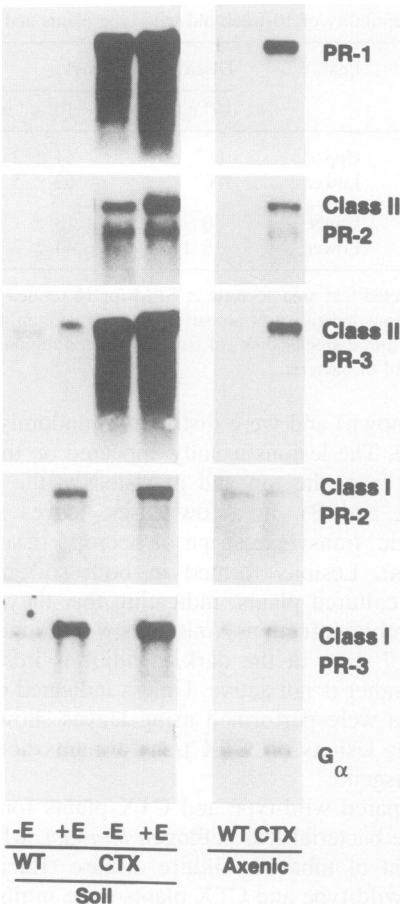

Fig. 3. RNA-blot analysis of PR-1, PR-2 and PR-3 mRNAs in leaves of wild-type (WT) and CTX plants. Total RNA was prepared from leaves of soil-grown plants (Soil) after 4 days incubation in chambers with an ethylene adsorbent (–E) or 20 p.p.m. ethylene (+E). Leaves from plants raised under axenic conditions were used as a control for microbial contamination. Lanes were loaded with 10 μg of RNA. Blots were hybridized with probes for genes encoding PR-1, class I and class II PR-2 and PR-3, and a tobacco protein showing high levels of sequence similarity to mammalian G_α.

Accumulation of free and bound SA in uninfected plants

We measured the content of free and bound SA in wild-type and CTX plants. The representative results in Table II show that leaves and roots of wild-type plants accumulate low levels of SA. No conspicuous differences were found between upper and lower leaves. In striking contrast, CTX plants showed a pronounced developmental gradient of SA accumulation—very high in young leaves near the top of the plant decreasing toward the bottom of the plants. Comparable upper leaves accumulated up to ~1500-fold higher levels of free SA and ~25 000-fold higher levels of bound SA in CTX plants than in wild-type plants. These high levels were similar to those reported for uninfected tobacco hybrids showing constitutive PR protein expression and tobacco mosaic virus (TMV) resistance (Yalpani *et al.*, 1993). In contrast, only low levels of SA, similar to wild-type, accumulated in roots of CTX plants (Table II) where the *Cab-1* promoter is not active and class II PR proteins are not expressed (data not shown). Taken together, these data show that CTX transformation results in a marked increase in SA accumulation which is roughly correlated with the spatial pattern of *Cab-1* promoter activity.

Grafting experiments

We performed reciprocal grafting experiments using wild-type and transgenic tobacco plants to characterize the nature of the signal generated by CTX. Three weeks after grafting, when the plants had recovered from the grafting procedure and the scions had formed additional expanded leaves, leaves of the scion and root stock adjacent to the graft site were assayed for free and bound SA, content of class I and II isoforms of PR-2 and PR-3 and content of PR-1 mRNA.

Independently of whether the scion or root stock were assayed, CTX-expressing leaves always showed elevated levels of free and bound SA (Table III) and a high level of PR-1 mRNA (Figure 4B) and accumulated class II PR-2 and PR-3 (Figure 4C). In contrast, leaves sampled from the scion and root stock of wild-type tobacco consistently showed very low levels of free and bound SA, a low level of PR-1 mRNA, and did not accumulate

Table II. Developmental gradient in the salicylic acid content of wild-type and CTX plants

Organ[a]	Salicylic acid (μg/g fresh weight)					
	CTX1		CTX2		Wild-type	
	Free	Bound	Free	Bound	Free	Bound
Roots	0.05	0.22	0.01	0.15	0.05	0.18
Leaf 1	0.22	10.4	0.04	6.0	0.02	0.0025
Leaf 2	0.10	10.1	0.70	7.7	0.02	0.0019
Leaf 3	0.11	12.0	0.11	9.4	0.05	0.0024
Leaf 4	0.15	17.2	0.28	27.8	0.01	nd[b]
Leaf 5	0.15	21.3	0.46	13.7	0.01	0.0073
Leaf 6	0.39	23.3	6.19	35.9	0.02	0.0008
Leaf 7	2.10	21.6	8.79	19.7	0.05	0.0028
Leaf 8	9.15	34.5	6.37	8.20	0.03	0.0038
Leaf 9	14.4	43.9	–	–	0.01	0.0016

[a]Leaves counting from the bottom of the plant from two independent transformants (CTX1 and CTX2) and a wild-type plant of comparable height. CTX2 was assayed at the onset of flowering and had one leaf less than the other plants.
[b]Not detectable.

Table III. Concentration of free and bound salicylic acid in leaves from the scion and root stock of plants 3 weeks after grafting

Source of graft partners		Salicylic acid (μg/ g fresh weight)			
Scion	Root stock	Scion[a]		Root stock[b]	
		Free	Bound	Free	Bound
Wild-type	CTX	0.0	0.51	0.11	7.00
Wild-type	CTX	0.0	0.07	0.10	4.19
CTX	Wild-type	0.12	7.70	0.04	0.12
CTX	Wild-type	0.20	16.1	0.04	0.10
Wild-type	Wild-type	0.0	0.34	0.0	0.15

[a]First leaf of scion above the graft site.
[b]First leaf of root stock below the graft site.
The accumulation of PR-1 mRNA and class I and class II isoforms of PR-2 and PR-3 were determined in the same leaves is shown in Figure 4.

appreciable amounts of the class II PR-2 and PR-3 (Figure 4). Expression levels of the class I isoforms of PR-2 and PR-3, on the other hand, were comparable in all the wild-type and CTX tissues analysed (Figure 4). Taken together, these data indicate that the CTX-generated signal is not graft-transmissible, suggesting that it acts locally in cells containing the *CTX* gene rather than systemically.

Microinjection experiments

Earlier studies had established the utility of microinjection as a means of analysing signal transduction pathways in higher plants (Neuhaus *et al.*, 1993). This approach was used to confirm the results with CTX transformants and to establish a system for studying regulation of PR protein gene expression in greater detail. The tobacco *PR-1* gene (Uknes *et al.*, 1993) and the *GLB* gene encoding a class I PR-2 isoform are regulated at the level of transcription (Vögeli-Lange *et al.*, 1994). We microinjected transgenic plants shown to express the *GUS* reporter gene constructs *PR1–GUS* (Uknes *et al.*, 1993) or *GLB–GUS* (Vögeli-Lange *et al.*, 1994) in a regulated fashion to characterize the effect of different compounds on the expression of these reporter genes. The results obtained are summarized in Table IV.

CTX cannot activate G-proteins directly. It acts to maintain the active state of G-proteins with bound GTP. GTP-γ-S, a non-hydrolysable homologue of GTP, is known to potentiate CTX effects (Neuhaus *et al.*, 1993). We found that co-microinjection of CTX and 1 μM GTP-γ-S resulted in the induction of the *PR1–GUS* transgene (Figure 5A). Microinjection of 1 μM GTP-γ-S alone was ineffective, but at 50-fold higher concentrations led to a markedly increased level of GUS enzyme activity (Figure 5B). In contrast, microinjection of either CTX or GTP-γ-S did not induce the expression of the *GLB–GUS* transgene (Figure 5D and E). As a positive control, we microinjected 1-aminocyclopropane-1-carboxylic acid (ACC), which induces ethylene-responsive gene expression, presumably because it is converted rapidly to ethylene (Kende, 1989). ACC efficiently induced the expression of the *GLB–GUS* transgene (Figure 5F) but not that of the *PR1–GUS* transgene (Figure 5C). Furthermore, microinjection of GDP-β-S and bovine serum albumin (BSA) did not induce expression either of the *PR1–GUS* or the *GLB–GUS* transgenes (Table IV).

The induction of the *PR1–GUS* and *GLB–GUS* transgenes was always localized to the microinjected cells and, at most, to three to four surrounding cells (Figure 5A–F). This induction never spread systemically to distal regions of injected cotyledons or hypocotyls (data not shown). As judged from appearance under the microscope, cells remained viable for at least 1 week after microinjection with the compounds tested. Taken together, the results of the microinjection experiments and studies with the CTX transformants indicate that CTX acts by modifying one or more heterotrimeric GTP binding protein(s) and that it

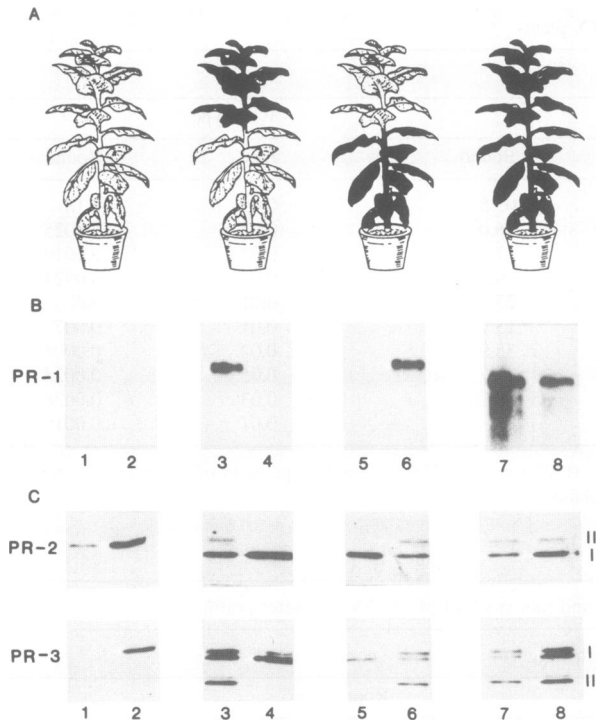

Fig. 4. Expression levels of genes coding for PR1, class I and II isoforms of PR-2 and PR-3 proteins in root stock and scion leaves of grafted CTX and wild-type plants. (**A**) Schematic illustration of the reciprocal grafting of wild-type (unshaded) and CTX-expressing (shaded) plants. RNA-blot analyses of *PR-1* mRNA (**B**) and immunoblot analyses of the class I and II PR-2 and PR-3 (**C**) in leaves of the scion (lanes 1, 3, 5 and 7) and root stock (lanes 2, 4, 6 and 8) sampled as indicated in Table III.

selectively induces expression of the *PR1* gene, but not of the *GLB* gene.

Discussion

Tissues of transgenic tobacco plants expressing a CTX transgene exhibited increased *P.tabaci* resistance, constitutively expressed genes encoding PR-1 and class II isoforms of PR-2 and PR-3 known to be induced as part of SAR (for a review, see Collinge and Slusarenko, 1987) and accumulated very high levels of SA characteristic of SAR (Enyedi *et al.*, 1992; Gaffney *et al.*, 1993; Hennig *et al.*, 1993a). Genes encoding the class I PR-2 and PR-3 isoforms are induced in tobacco by ethylene or by other stress (Neale *et al.*, 1990; Cutt and Klessig, 1992; Schraudner *et al.*, 1992), and locally as part of the HR, but they are not induced systemically in SAR (Ward *et al.*, 1991; Cutt and Klessig, 1992; Meins *et al.*, 1992). These genes were not induced and showed normal regulation, suggesting that CTX expression does not trigger non-specific stress reactions. Microinjection experiments corroborated these data and showed that CTX induces the expression of the *PR1–GUS* transgene but not that of the *GLB–GUS* transgene containing the promoter region of the tobacco *GLB* gene encoding a class I isoform of the PR-2. Ethylene treatment of the CTX-expressing transgenic plants and microinjection of ACC had reciprocal effects: they induced the expression of genes encoding class I PR isoforms, but not PR-1 and the class II isoforms.

These results and the finding that SAR signal transduction is ethylene independent in *Arabidopsis* (Lawton *et al.*, 1994) support the hypothesis that there are at least two classes of signal transduction pathways involved in plant defence reactions. One class, which is insensitive to CTX, is associated with HR and is required for induction of class I PR-2 and PR-3 proteins by ethylene. The second class, which is activated by CTX, is required for SAR-associated induction of PR-1 and class II PR-2 and PR-3 proteins. Apart from SA and active O_2 species (for a review, see Godiard *et al.*, 1994), no other molecules have been identified as components of the signal transduction chain(s) in SAR. Although free SA is unlikely to be the primary systemic signal for SAR, it is translocated in plants and is required for induction of pathogen resistance and the SAR-specific subset of PR protein-encoding genes in distal tissues (Rasmussen *et al.*, 1991; Vernooij *et al.*, 1994). Expression of the wheat *Cab* promoter used to drive the *CTX* gene, pathogen resistance, *PR* gene induction and SA accumulation were correlated in upper and lower leaves. Grafting experiments indicated that CTX-expressing tissues, despite the elevated levels of free and bound SA, do not contain a graft-transmissible signal for the systemic induction of *PR* genes known to be induced by SAR. Furthermore, microinjection of CTX never led to the systemic induction of the *PR1–GUS* transgene. Finally, microinjection of GTP-γ-S, which like CTX is known to activate G-protein-mediated signal transduction, also led to a markedly elevated, but local induction of the *PR1–GUS* transgene. Therefore, we propose that CTX acts locally rather than systemically to generate a SAR-type response and that one or more CTX-sensitive G-proteins are involved in the cellular response of the target tissue to systemic signals in SAR.

Several mutants and transgenic plants have been described that exhibit partially overlapping phenotypes with features of SAR. The *lsd* mutants of *Arabidopsis thaliana*, which spontaneously form necrotic lesions, show increased resistance to the fungal pathogen *Peronospora parasitica*, and induced expression of *PR-1*, *PR-2* and *PR-5* genes (Dietrich *et al.*, 1994). Expression of a structural variant of ubiquitin leads to spontaneous lesion formation in transgenic tobacco susceptible to TMV infection and to increased resistance to TMV infection and increased TMV-induced accumulation of PR-1, but not constitutive PR-1 expression in lines resistant to TMV (Becker *et al.*, 1993). Transgenic tobacco plants over-expressing a small GTP binding protein show elevated levels of the growth hormone cytokinin, increased resistance to TMV infection, constitutive expression of the *PR-1* gene and increased SA levels in response to wounding (Sano *et al.*, 1994). In contrast to the CTX transformants, these transgenic plants did not develop spontaneous lesions and did not show high, constitutive levels of SA accumulation prior to wounding. In this regard, it is of interest that lesions arising on leaves of CTX transformants differ from HR necrotic lesions and microinjection of compounds inducing *PR-1* gene expression did not result in cell death. There is also evidence that both HR-like cell death and SAR can be triggered alone or in combination by mutations affecting cellular processes not exclusively involved in pathogenesis (e.g. Becker *et al.*, 1993; Mittler *et al.*, 1995). These observations suggest that induction of HR-

Table IV. Summary of microinjection experiments

Seedlings injected	Substances injected	No. of cells injected	Injected cells showing GUS activation (%)
Wild-type	Injection buffer	121	0
	BSA (10 000)	76	0
	GTP-γ-S (50 μM)	84	0
	GDP-β-S (50 μM)	74	0
	CTX (1000) + GTP-γ-S (1 μM)	67	0
PR1–GUS	Injection buffer	110	0
	BSA (10 000)	123	0
	GTP-γ-S (1 μM)	122	0
	GTP-γ-S (50 μM)	115	13.0
	GDP-β-S (50 μM)	126	0
	CTX (1000) + GTP-γ-S (1 μM)	100	12.0
GLB–GUS	Injection buffer	118	0
	BSA (10 000)	70	0
	GTP-γ-S (1 μM)	111	0
	GTP-γ-S (50 μM)	75	0
	GDP-β-S (50 μM)	115	0
	CTX (1000) + GTP-γ-S (1 μM)	108	0
	ACC (25 000)	75	17.2

Numbers in parentheses indicate the number of molecules injected or the concentrations microinjected per cell in a volume of 5 pl calculated as described by Neuhaus *et al.* (1993).

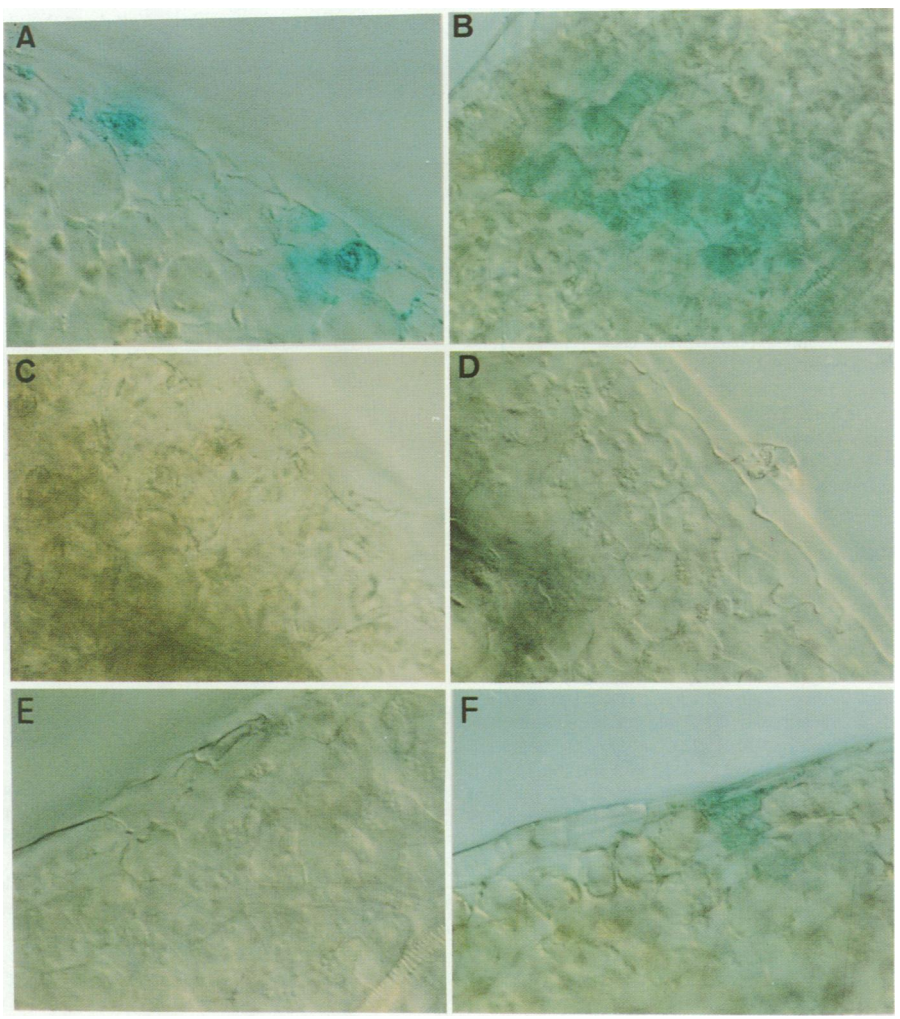

Fig. 5. Selective induction of the *PR1–GUS* and *GLB2–GUS* reporter genes in transgenic seedlings by microinjection assays. *PR1–GUS* cells injected with CTX + 1 μM GTP-γ-S (**A**), 50 μM GTP-γ-S (**B**) and ACC (**C**). *GLB–GUS* cells injected with CTX + 1 μM GTP-γ-S (**D**), 50 μM GTP-γ-S (**E**), and ACC (**F**). The concentrations of compounds injected and efficiency of *GUS* reporter gene expression are summarized in Table IV.

like cell death is not necessarily required for induction of SAR and that the complete SAR response appears to involve multiple signal transduction pathways. This hypothesis is further supported by the finding that expression of a SAR marker gene, *PR-1*, is mediated by at least two signalling pathways, e.g. UV-B-induced expression of this gene requires active O_2 species, but is independent of SA action (Green and Fluhr, 1995).

CTX might activate various as yet unidentified G-proteins functioning in SAR and in other regulatory pathways. Therefore, we speculate that lesion formation might be a secondary effect of the activation of these pathways and is not a prerequisite for the induction of pathogen resistance. We hope that further microinjection experiments and isolation of mutants will help to elucidate these pathways and lead to novel approaches for obtaining disease-resistant plants.

Materials and methods

Plant materials, infection with bacteria and ethylene treatment

SR-1 tobacco (*Nicotiana tabacum* L. cv. 'Petit Havana SR-1') was raised in soil and axenically in glass containers in a growth chamber (25°C, 16 h light, 8 h dark). Plants were mock infected and infected with *P.tabaci* using the methods and bacterial strain described (Meins and Ahl, 1989). Where indicated, plants were incubated in chambers for 4 days with 20 p.p.m. of ethylene or the ethylene-adsorbent $KMnO_4$ (Felix and Meins, 1987).

DNA transformation of plants

CTX transformants were obtained by Ti plasmid-mediated leaf disk transformation of SR-1 tobacco plants and selection for kanamycin-resistant shoots as described (Horsch *et al.*, 1988). The modified pMON 501 vector used contained: (i) the promoter region from −357 to +31 of the wheat *Cab-1* gene fused to the coding sequence of the A1 subunit of the *CTX* gene (Burton *et al.*, 1991) and the NOS transcription terminator; (ii) the *NOS–NPTII–NOS* chimeric gene to provide a plant-selectable marker; and (iii) the *35S–GUS–NOS* reporter gene to facilitate the rapid identification of transgenic plants (Fejes *et al.*, 1990). Transgenic plants resistant to kanamycin and expressing the *GUS* reporter gene were grown to maturity in a growth chamber and then selfed. All genetic studies described were performed at the Biological Research Centre of the Hungarian Academy of Sciences, Szeged, Hungary. Selected lines homozygous for the transgene were propagated vegetatively in axenic culture and transferred to soil in a growth chamber for infection and ethylene induction experiments. All transgenic material was used before flowering and was sterilized by autoclaving prior to disposal.

Immunoblot and RNA-blot hybridization analyses

Sampling of leaves, extraction of proteins and immunoblot analyses were as described (Beffa *et al.*, 1993). PR-2 isoforms were detected by probing with anti-tobacco class I β-1,3-glucanase antibody which cross-reacts with the class II isoform (Beffa *et al.*, 1993). PR-3 isoforms were detected by probing with a mixture of antibody directed against tobacco class I chitinase and class II PR-3 (Shinshi *et al.*, 1987; Payne *et al.*, 1990). Total RNA was extracted and RNA-blot hybridization was performed as described (Dallmann *et al.*, 1992). The probes used were tobacco cDNA inserts encoding: PR-1 and class I PR-2 and PR-3 (Ward *et al.*, 1991); class II PR-2 and PR-3 (Shinshi *et al.*, 1987); and a protein homologous to the G_α subunit of mammalian heterotrimeric GTP binding protein (Haizel *et al.*, unpublished).

Measurement of free and bound salicylic acid

Tissue extracts were prepared and assayed for free and bound SA with *o*-anisic acid as internal standard as described (Meuwly and Métraux, 1993), except that a LC-SAL column (Supelco Inc.) was eluted with a gradient of 17.5–80% (v/v) acetonitrile in 10 mM KH_2PO_4, pH 3.5 at 35°C.

Grafting experiments

Four-week-old plants with about seven to nine expanded leaves were raised in a greenhouse under long-day conditions. Reciprocal grafts were made by joining V-shaped cuts made in the internodes between leaf 4 and leaf 5 (counting from the bottom) and were covered with Stericrepe tape (Beacon and Janis, UK). Samples were collected 3 weeks later, when the scions had formed additional fully expanded leaves.

Microinjection assays

The seedlings used were Xanthi variety tobacco, Xanthi tobacco transformed with a *PR1–GUS* reporter gene construct (Uknes *et al.*, 1993), and Havana 425 tobacco transformed with a *GLB–GUS* reporter gene construct (Vögeli-Lange *et al.*, 1994). Seedlings, 7–10 days after germination on MS medium, were prepared and microinjected as previously described (Neuhaus *et al.*, 1993). Seedlings were analysed after 48 h for cellular responses in the injected cells. GUS staining was performed by the method of Jefferson *et al.* (1987).

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