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Modification of competence for in vitro response to *Fusarium oxysporum* in tomato cells. II. Effect of the integration of *Agrobacterium tumefaciens* genes for auxin and cytokinin synthesis

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Abstract We have studied the effect of a change in the endogenous hormone equilibria on the competence of tomato (*Lycopersicon esculentum*) cells to defend themselves against the fungal pathogen *Fusarium oxysporum* f. sp. *lycopersici*. Calluses from cvs 'Davis' and 'Red River', respectively resistant and susceptible to *Fusarium* and transgenic for an auxin- or cytokinin-synthesizing gene from *Agrobacterium tumefaciens*, were used. The integration of *Agrobacterium* hormone-related genes into susceptible cv 'Red River' can bring the activation of defense processes to a stable competence as assessed by the inhibition of mycelial growth in dual culture and gem-tube elongation of *Fusarium* conidia, the determination of callose contents, peroxidase induction and ion leakage in the presence of fusaric acid. This is particularly true when the transformation results in a change of phytohormone equilibria towards an higher cytokin in concentration. On the contrary, in resistant cv 'Davis' the inhibition of both fungal growth in dual culture and conidia germination is higher when the hormone balance is modified in favour of the auxins. No significant effect was observed for ion leakage and peroxidase induction, probably because of a constitutive overproduction of cytokinins in 'Davis' cells.

Key words *Lycopersicon esculentum* · *Fusarium oxysporum* f. sp. *lycopersici* · *Agrobacterium* hormone synthetic genes · Defense response

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Introduction

Host-pathogen interactions in plants are controlled by a complex network of processes, probably induced by recognition events, whose quantitative modulation is critical for the choice between susceptibility and resistance. In other words, although a gene-for-gene system (Flor 1942) probably has a trigger effect on the timing and the intensity of active defense responses to the presence of the pathogen, modulation by exogenous and endogenous factors may sensibly affect the outcome of the interaction. This would explain the dependence of resistance levels on environmental conditions (Beckman 1987) as well as the observed differences in the response between parts of plants with the same genotype. A better knowledge of the physiology of resistance is therefore required for a more effective planning of protective measures, including the possible creation of cultivars with an appreciable level of "horizontal" resistance as these will presumably be more competitive in the evolutionary plant-pathogen race than genotypes whose competence for defense only relies on the presence of single genes.

Encouraging data relevant to this hypothesis come from the growing body of evidence concerning acquired resistance (Madamanchi and Kùc 1991), i.e. a process through which plants challenged with one pathogen become "horizontally" and systemically resistant (tolerant) to a broad spectrum of other biotic agents. Such an aspecific competence for defense, moreover, can also be induced by abiotic stresses like treatment with $HgCl_2$ or salycilic acid and in a few cases transmitted to regenerants in tissue culture (Tuzun and Kùc 1987, 1989) or even to the progeny of induced plants (Wieringa-Brants and Dekker 1987). Within this frame plant phytohormones, due to their key role in the overall regulation of development, are obvious candidates as major control agents in host-parasite interactions. Data favouring such an hypothesis include the effect of exogenously added phytohormones both on biochemical parameters indicative of a hypersensitive response to bacterial and fungal pathogens and on the extent of lesions determined in vivo by infections (Plich 1976; Faccioli et al. 1984; Mills et al. 1986; Kaplan et al. 1988), the

differences in phytohormone contents in resistant and susceptible plants (Dua et al. 1978; Matsumoto et al. 1980; Eidel'nant et al. 1983; Vizarova and Vozar 1984; Vizarova 1985), the changes in the endogenous concentrations of auxin and cytokinin during the induction of systemic acquired resistance or during pathogen challenge (Balazs et al. 1977; Kratka and Kudela 1981; Sarhan et al. 1991). In all of these cases, however, the evidence collected seems to be circumstantial and, in some cases, contradictory mainly due to differences in the endogenous concentrations of free phytohormones, biosynthetic capacity and modulation mechanisms. These factors may sharply increase the difficulties in comparing data and interpretations based on experiments carried out in different systems. Moreover, with one exception (Zakhar'ev et al. 1988), no attempts have been made to our knowledge to exploit possible beneficial effects of changes in phytohormone equilibria through the integration of genes involved in hormone synthesis and/or utilization in susceptible genotypes.

The aim of the investigation presented here has been to study the effect of the integration of an auxin- and cytokinin-synthesizing gene cluster from *Agrobacterium tumefaciens* (i.e. the genes *iaaH*, *iaaM*, *ipt*) in which either *iaaM* (gene 1) or *ipt* (gene 4) had been inactivated by insertion (Inzé et al. 1984) on the defense competence of tomato cells from resistant and susceptible cultivars challenged with *Fusarium oxysporum* f. sp. *lycopersici*. For this purpose, we have used an in vitro dual culture system developed in our laboratory (Buiatti et al. 1987; Storti et al. 1990, 1992). The parameters used as indicators of the induction of defense processes were those previously shown to discriminate between resistant and susceptible cells in vitro (Storti et al. 1992), i.e. inhibition of fungal growth and conidia germination, polysaccharide content, cell browning and toxin tolerance as judged by ion leakage from the treated cells.

Materials and methods

Plants and fungus cultures

The commercial tomato cvs 'Red River' and 'Davis' are respectively susceptible and resistant to *Fusarium oxysporum* f. sp. *lycopersici*. Establishment and growth of the callus cultures have been described in a previous paper (Buiatti et al. 1987). *F. oxysporum* f. sp. *lycopersici* race 1 was maintained on Czapek Dox Broth medium (Difco).

L. esculentum transformation

Tumours were induced on *L. esculentum* cotyledons by the leaf disk transformation technique described in Horsch et al. (1985). The *A. tumefaciens* strains used were C58C1Clm^R (pGV2282, gene 1$^-$ pTiB6S3 mutant) and C58C1Clm^R (pGV2250, gene 4$^-$ pTiB6S3 mutant) (Inzé et al. 1984). Tumours were excised from the explants after 20 days and maintained on hormone-free medium.

DNA isolation, digestion and Southern hybridization

DNA was isolated from tumour and control tissue according to Rogers and Bendich (1985). Total DNA (10 µg/lane) was digested overnight with 5 u/µg *Bam*HI (Boehringer), fractionated in 0.8% agarose gels and blotted onto Hybond N$^+$ membranes (Amersham) by means of an alkaline blotting procedure (Reed and Mann 1985). The probes used were two 0.93- and 0.45-kb *Bam*HI internal fragments of T_L-DNA (Hirsch and Beggs 1984) isolated by elution from agarose gels and labelled with [α-^{32}P]CTP (Amersham) using the random primed labelling method of Feinberg and Vogelstein (1983, 1984). Membranes were prehybridized for at least 6 h in 2 × SSPE, 1% SDS at 45 °C and hybridized overnight in 4 × SSPE, 1% SDS, 5 × Denhardt's solution, 200 µg/ml salmon sperm DNA and 50% formamide at 42 °C. After hybridization, the filters were washed twice with 2 × SSC, 0.5% SDS at room temperature for 20 min and twice with 0.1% SSC, 0.1% SDS at 42 °C for 20 min and exposed to Kodak XOMAT-AR films.

Dual culture

Dual culture experiments were carried out by sowing *F. oxysporum* conidia on filter paper disks ($3 \cdot 10^5$ conidia/disk). The disks were then plated on A_1 petri dishes at the same distance from one explant of each of two clones to be tested. Inhibition of fungal growth was evaluated as the difference of mycelial radius towards the callus piece (R1 in Fig. 3) or not (R2 in Fig. 3).

Peroxidase extraction and assay

Soluble, ionically bound and covalently bound peroxidases were extracted from calluses of the different clones as previously described (Storti et al. 1989). Protein content was determined by the Bradford reagent method (Bradford 1976). The spectrophotometrical assay for peroxidase activity was based on that developed by Haskins (1955). The peroxidase visual assay by guaiacol in vivo staining was as previously described (Storti et al. 1989).

Preparation of heat-released mycelial cell-wall components (elicitor) and callus treatment

F. oxysporum f. sp. *lycopersici* was surface cultured in 150 ml Czapek Dox Broth (Difco) in 500-cc conical flasks at 24 °C for 21 days. Heat-released cell wall components (elicitor) were isolated as described (Buiatti et al. 1985). The concentration of elicitor was measured by the phenol-sulphuric acid method (Hodge and Hofreiter 1962) and expressed as glucose equivalents. Ten-day-old callus pieces (about 1 g f.w.) of the different clones were treated with 100 µl of elicitor solution (0.85 mg/ml glucose equivalents); controls were treated with distilled water in the same manner. The cultures were incubated in a growth chamber at 24° ± 1 °C under continuous light, and browning caused by elicitor treatment was visually evaluated after 24 h.

Callose extraction and determination

Callose was extracted from the cells of the different clones according to Kohle (1985) with some minor modifications. Cells were collected by vacuum filtration on scintered glass filters and washed with about 50 ml distilled water. To remove autofluorescent soluble materials, the cells were soaked for at least 2 min in 20 ml ethanol. The dried cells were then homogenized in 3 ml 1 *N* NaOH, and the resulting suspension was incubated at 80 °C for 15 min to solubilize the callose and centrifuged for 5 min at 380 *g*. Aliquots of 100 µl of the supernatant were used for the callose assay.

Callose determination was carried out according to Kohle (1985). Samples supplemented with 1 *N* NaOH to make a final volume of 200 µl were mixed with 400 µl 0.1% aniline blue in water; 210 µl 1 *N* HCl was then added, and the final pH was adjusted with 590 µl 1 *M* glycine/NaOH buffer, pH 9.5, followed by vigorous mixing. Samples were further incubated for 20 min at 50 °C and 30 min at room temperature, and the

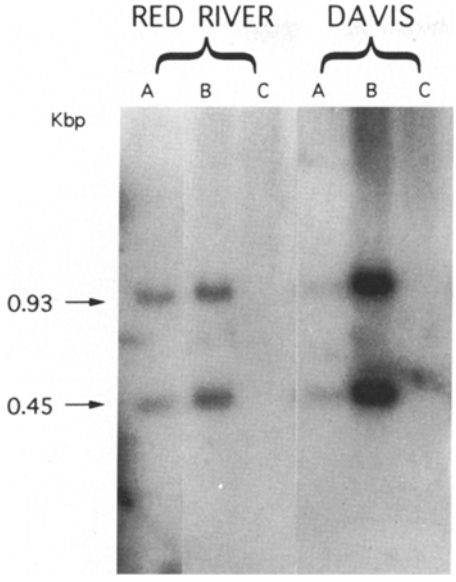

Fig. 1 Southern blot analysis of DNA from tomato calluses transgenic for *Agrobacterium* oncogenes. *Lanes A*, *B* and *C* represent, respectively, *Bam*HI digests of DNA from pGV2250 (4^-, deletion of the *ipt* gene), pGV2282 (1^-, deletion of the *iaaM* gene) tumorous lines and untransformed control

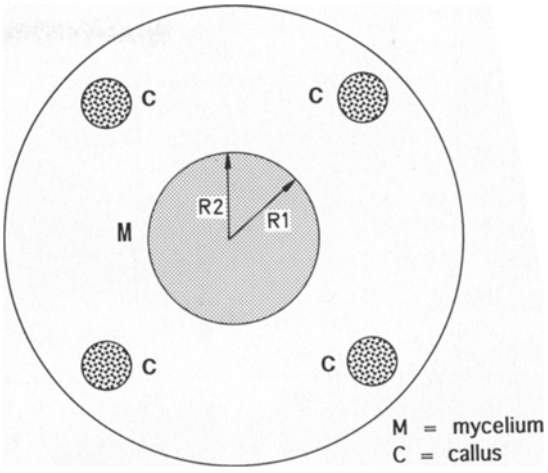

Fig. 3 Diagrammatic representation of a dual culture experiment. Four pieces of callus tissue (*C*) are placed in a petri dish (diameter 90 mm) equidistant from a filter paper disk sown with *Fusarium* conidia. After 3 days of incubation, the diameter of the growing mycelium (*M*) is measured in the direction of the callus pieces (*R1*) and not in this direction (*R2*). The inhibition of fungal growth is indicated when the R2 value is higher than the value of R1

fluorescence was read in a Perkin Elmer 650-10S fluorescence spectrophotometer (excitation 400 nm, emission 510 nm, slit 10 nm, PM gain normal). Calibration curves were established using a freshly prepared solution of 1,3-β-glucan from baker's yeast (Boehringer Mannheim) in NaOH.

Electrolyte leakage

Ion leakage measurements in the presence of 3 m*M* fusaric acid were performed as already described (Storti et al. 1992) with a Top Tronic X74174 digital conductivity meter.

Extraction and bioassay of phytoalexins

Antimicrobial compounds were extracted as described in a previous paper (Scala et al. 1983) from transformed 'RedRiver' and 'Davis' and control callus treated or untreated with *Fusarium* elicitor. The presence of phytoalexins in ethanolic extracts was assayed by determining the percentage inhibition of the length of the germ-tubes of growing conidia of *F. oxysporum* f. sp. *lycopersici* race 1 on hanging drop glass slides. Incubation mixtures contained 170 µl Czapek Dox Broth, 5 µl conidia suspension ($2 \cdot 10^5$ spores/ml) and 10 µl ethanolic extracts. The slides were first incubated in the dark for 12–18 h at 24 °C, and then the germ tube length was determined.

Results

In order to assess the presence of the *A. tumefaciens* genes involved in auxin and cytokinin synthesis (*iaaH*, *iaaM*, *ipt*) in transformed callus tissue from the susceptible cv 'Red River' and resistant cv 'Davis', an hybridization experiment was carried out using the 0.93- and 0.45-kb *Bam*HI internal frag-

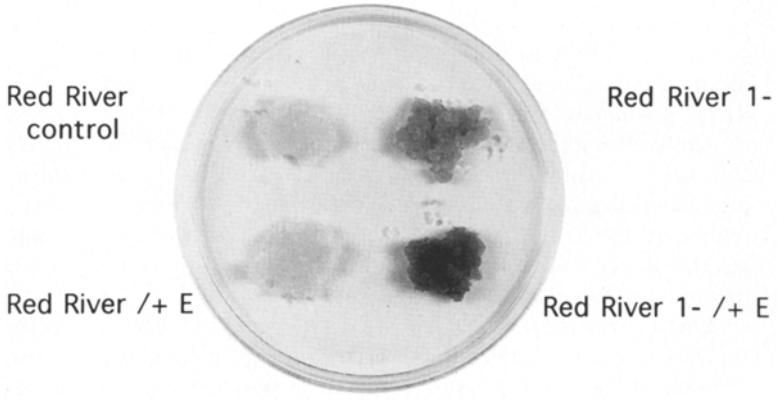

Fig. 2 Cell browning after treatment with *Fusarium* elicitor (+E) of 'Red River' control callus and of 'Red River 1^-' transgenic for the *iaaH* and *ipt* genes (2^+, 4^+)

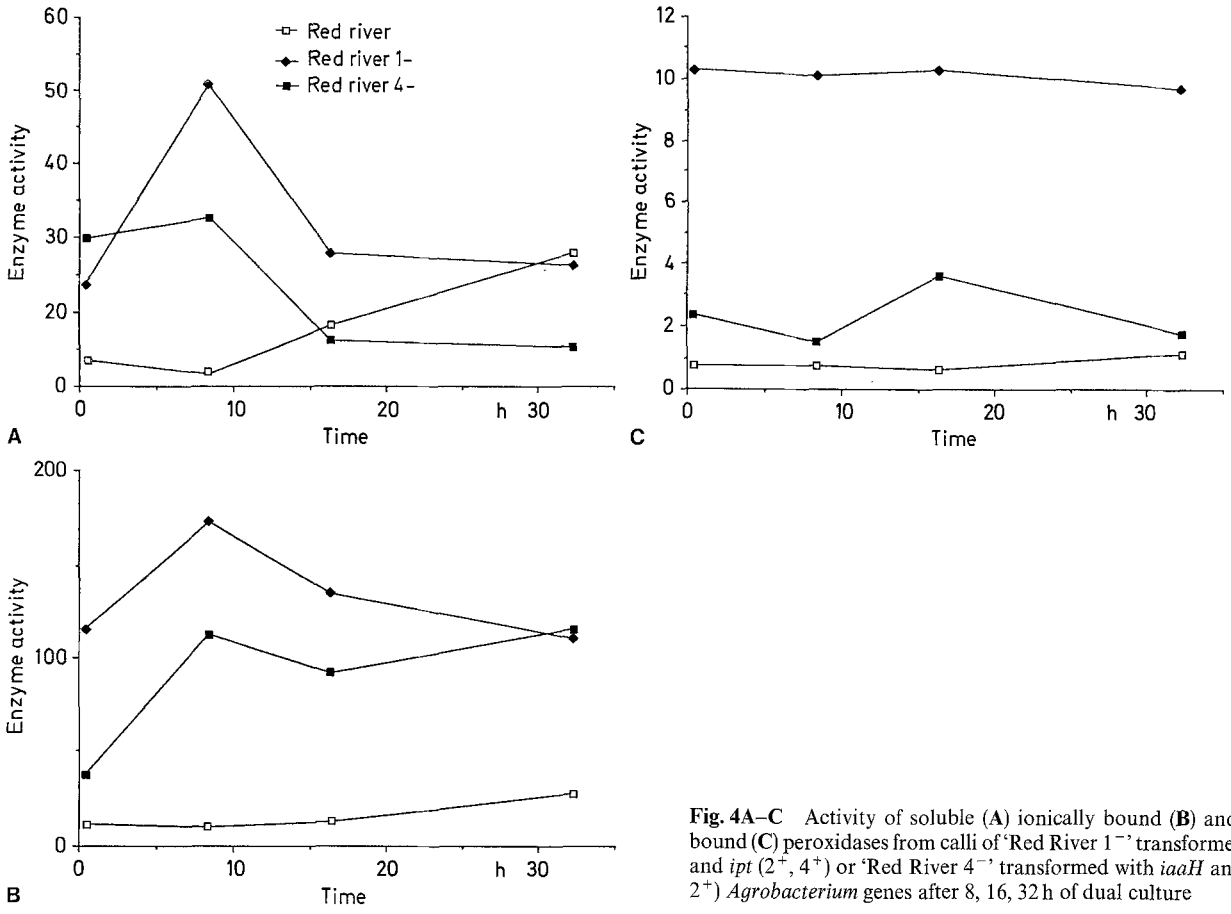

Fig. 4A–C Activity of soluble (**A**) ionically bound (**B**) and covalently bound (**C**) peroxidases from calli of 'Red River 1^-' transformed with *iaaH* and *ipt* (2^+, 4^+) or 'Red River 4^-' transformed with *iaaH* and *iaaM* (1^+, 2^+) *Agrobacterium* genes after 8, 16, 32 h of dual culture

Table 1 Inhibition of *F. oxysporum* growth in dual culture experiments by 'Red River' and 'Davis' cell clones transformed or untransformed with the *iaaH* and *ipt* (1^-, 2^+, 4^+) and *iaaM* and *iaaH* (4^-, 1^+, 2^+) genes. Fungal growth inhibition was evaluated by measuring the diameter of the growing mycelium (M) both in the direction of the callus pieces (R1) and not in this direction (R2) (see Fig. 3)

Clones	R1	R2
Red River	1.57	1.57
Red River 1^- (2^+, 4^+)	1.57	1.75
Red River 4^-, (1^+, 2^+)	1.70	2.02
Davis	1.62	1.85
Davis 1^- (2^+, 4^+)	1.75	1.75
Davis 4^- (1^+, 2^+)	1.90	2.12

Table 2 Inhibition of germ-tube elongation of *Fusarium* conidia by ethanolic extracts of 'Red River' and 'Davis' transgenic and control callus treated or untreated with fungal cell-wall components

Clones	Germ-tube length (mm)	Germ-tube length (% of control)
Red River control	2.28 ± 0.12	100
Red River elicitor	0.81 ± 0.08	35.08
Red River 1^- (2^+, 4^+) control	2.15 ± 0.15	100
Red River 1^- (2^+, 4^+) elicitor	0.12 ± 0.02	5.56
Red River 4^- (1^+, 2^+) control	1.09 ± 0.16	100
Red River 4^- (1^+, 2^+) elicitor	0.75 ± 0.13	68.82
Davis Control	0.92 ± 0.10	100
Davis elicitor	0.28 ± 0.02	30.43
Davis 1^- (2^+, 4^+) control	0.91 ± 0.07	100
Davis 1^- (2^+, 4^+) elicitor	0.67 ± 0.07	73.62
Davis 4^- (1^+, 2^+) control	0.94 ± 0.08	100
Davis 4^- (1^+, 2^+) elicitor	0.17 ± 0.02	18.08

ments of T_L-DNA as probes (Hirsch and Beggs 1984). The results obtained confirmed the transformation of both 'Red River' and 'Davis' cells with the described genes (Fig. 1).

The competence of both the transformed cells and the controls for active defence was assessed by testing a series of parameters that had been previously shown to be indicative of resistance (Storti et al. 1992), i.e. cell browning after elicitor treatment, peroxidase induction in dual culture, callose content and ion leakage in the presence of fusaric acid.

Elicitor treatment of 'Red River' callus transformed with the *A. tumefaciens* plasmid pGV2282, consequently showing a deletion of the *iaaM* gene (1^-) and carrying *iaaH* and *ipt* ($2^+ 4^+$), i.e. unbalanced in favour of cytokinin synthesis, showed a very clear browning that was indicative of a hypersensitive reaction (Fig. 2), while no effect was observed in cells transgenic for pGV2250, showing a deletion of the *ipt* gene (4^-) and carrying *iaaH* and *iaaM* (1^+, 2^+), or in control tissue. The induction of competence for active defense in cells derived from a susceptible genotype through the integration of the cytokinin-synthesizing gene was further confirmed by the inhibition of fungal growth in dual culture (Table 1 and

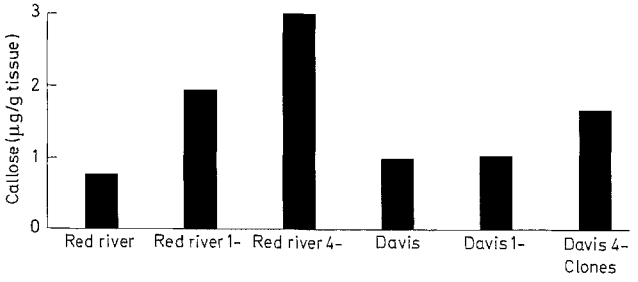

Fig. 5 Callose content (µg/g tissue) of calli of susceptible cv 'Red River' and resistant cv 'Davis' transformed or untransformed with the *iaaH* and *ipt* (1^-, 2^+, 4^+) and *iaaM* and *iaaH* (4^-, 1^+, 2^+) genes

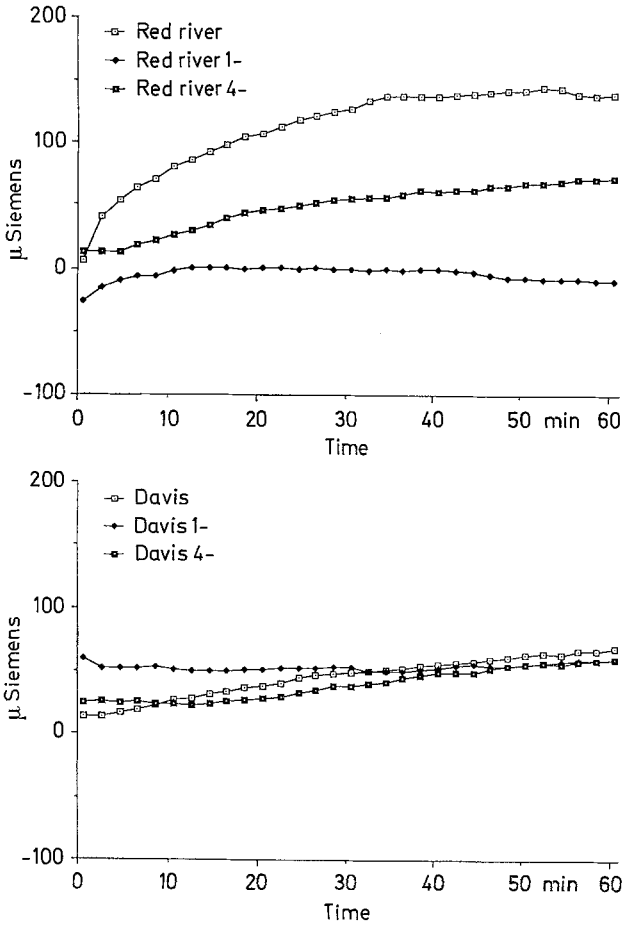

Fig. 6 Electrolyte leakage from tranformed and control callus of 'Red River' and 'Davis' in the presence of 3 M*M* fusaric acid

Fig. 3), a parameter for which a similar effect was observed in transgenic cells where the auxin level was increased. An additional confirmation of this effect came from the inhibition of conidia germination by the ethanolic extracts of 1^--transformed callus (Table 2) and by the induction of rishitin in the same genotype as detected by TLC analysis (data not shown), the extracts from 4^- transformants being ineffective with respect to these parameters. Moreover, soluble, ionically bound and convalently bound peroxidase activity (Fig. 4), whose induction is correlated to the hypersensitive reaction in our system (Buiatti et al. 1987; Storti et al. 1990, 1992), strikingly increased after 8 h of dual culture only in 'Red River' transgenic cells. This phenomenon was particularly evident when the phytohormone equilibrium was altered in favour of cytokinin synthesis; the value for control cells remaining unchanged or only slightly increasing with dual culture times. The analysis of callose content in transformed and control callus tissue demonstrated a dramatic increase in both *iaaM*/*iaaH* (4^-) and *iaaH*/*ipt* (1^-) transgenic cells with respect to the untransfored control (Fig. 5). When 1^- and 4^- were compared, 4^- was shown to have the higher callose content. Finally, ion leakage in 'Red River' cultures transgenic for *iaaH* and *iaaM* (4^-) challenged with 3 m*M* fusaric acid was lower than that found in untransformed cells, cytokinin-synthesizing clones being completely resistant to the toxic effect (Fig. 6).

With respect to the resistant 'Davis' cultivar, the endogenous changes in hormone levels were shown to have an effect on the parameters tested, although control cells from this cultivar confirmed the inborn competence for defense shown in a previous study (Storti et al. 1992). Browning after elicitor treatment did not differ between control and transgenic cells. Mycelial growth, which was inhibited by 'Davis' control callus, was unaffected when cells transgenic for *iaaH* and *ipt* were used but strikingly reduced when the equilibrium was altered in favour of auxin (Table 1). A similar result was obtained when conidia germination was analysed: in this case, the inhibitory action of extracts from control callus treated with elicitor was strikingly reduced by *ipt* transformation and only slightly enhanced by the increase in auxin synthetic capacity (Table 2). Peroxidase activity in dual culture remained unaltered by a change in the equilibrium towards cytokinins, being particularly increased at later hours when auxin synthesis was increased (Fig. 7). Callose content in 'Davis' callus transgenic for the *iaaM* and *iaaH* genes (4^-) was higher than in the control with no significant difference being observed in 1^- (Fig. 5). Finally, no significant effects were observed to be induced by transformation in 'Davis' as far as ion leakage was concerned (Fig. 6).

Discussion

Our data show that the integration of *Agrobacterium* hormone-related genes in susceptible cv 'Red River' can bring the activation of defense processes to stable competence particularly when the transformation results in a change of phytohormone equilibria towards a higher cytokinin concentration. 'Red River' cells transgenic for *iaaH* and *ipt* (1^-) acquire the ability to inhibit both mycelial growth in dual culture and germ-tube elongation of *Fusarium* conidia, have a higher callose content, exhibit peroxidase activation after pathogen challenge and are resistant to fusaric acid. On the other hand, 'Red River' cells transgenic for the auxin-synthesizing complex (4^-) only show an increase in callose content, the induction of peroxidase activity but to a lower extent than 1^-, and inhibition of fungal growth in dual culture.

On the contrary, in the resistant cultivar the inhibition of *Fusarium* growth in dual culture and of conidia germination is

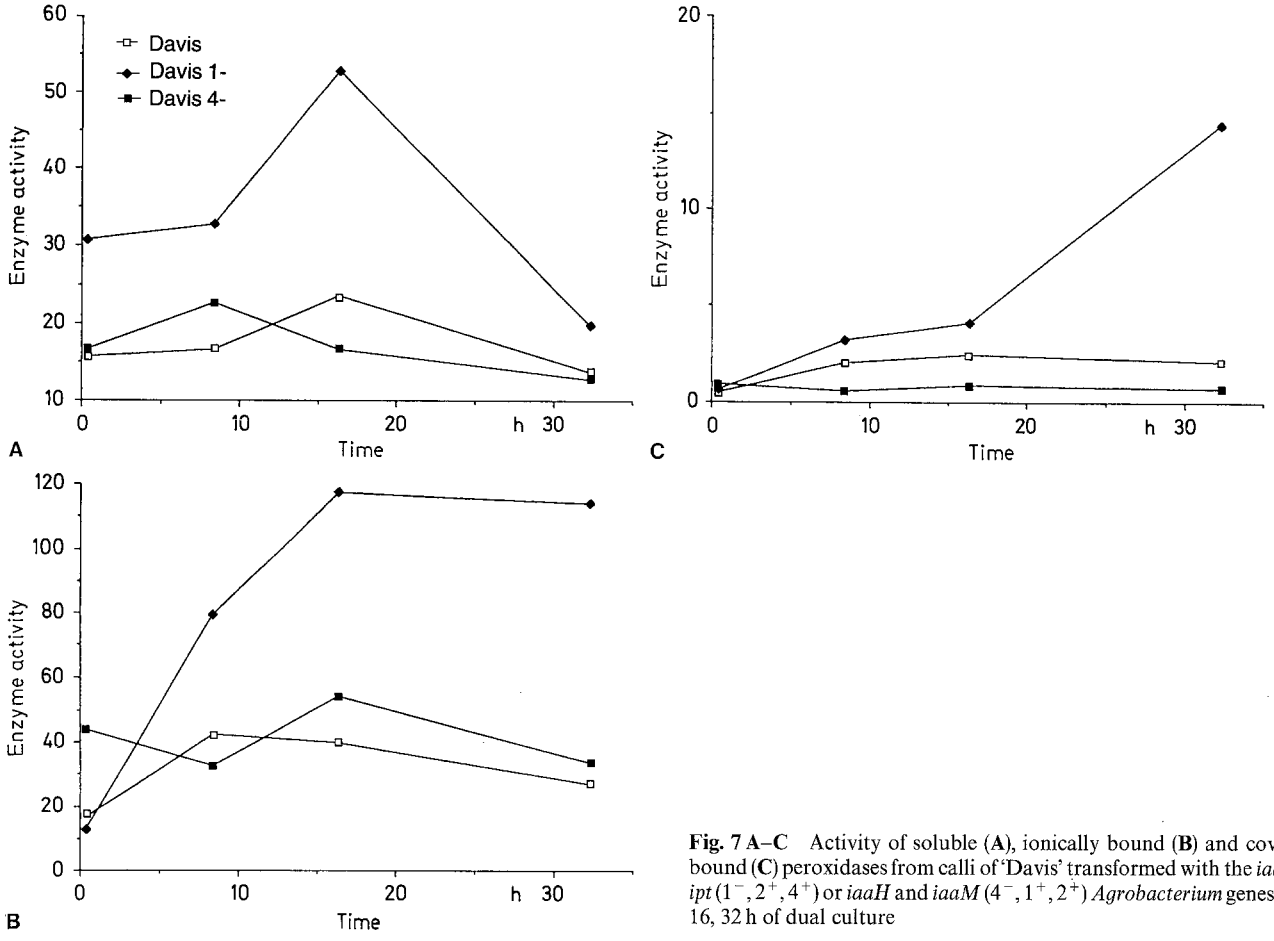

Fig. 7 A–C Activity of soluble (**A**), ionically bound (**B**) and covalently bound (**C**) peroxidases from calli of 'Davis' transformed with the *iaaH* and *ipt* ($1^-, 2^+, 4^+$) or *iaaH* and *iaaM* ($4^-, 1^+, 2^+$) *Agrobacterium* genes after 8, 16, 32 h of dual culture

higher when the hormone balance is modified in favour of auxins. No significant effect has been observed for ion leakage and peroxidase induction.

These results are consistent with data obtained from the literature concerning both differences in hormone contents between resistant and susceptible cultivars and the increase in specific phytohormone levels in the case of acquired resistance. High cytokinin contents have been correlated with resistance in host-parasite systems like wheat-*Puccinia* (Eidel'nant et al. 1983), wheat-*Erysiphe* (Vizarova and Muzikova 1981) and barley-*Erysiphe* (Vizarova 1985, 1987; Vizarova et al. 1988), with low auxin content being connected with susceptibility in the system *Pristiphora wesmaeli-Larix sibirica* (Fedorova and Gagulaeva 1976). It should be mentioned, however, that two cauliflower cultivars, resistant to *Xanthomonas campestris* showed high levels of both auxin and cytokinins (Dua et al. 1978). This result is consistent with our observation of a positive effect, although of lower intensity than in the case of cytokinins, of increases in auxin synthetic capacity in the susceptible cv 'Red River'. Increases in cytokinin contents were observed during systemic acquired resistance of tobacco (Balazs et al. 1977) and *Chenopodium amaranticolor* (Faccioli et al. 1984) to Tobacco Mosaic Virus, and of barley to *Bipolaris sorokiniana* (Sarhan et al. 1991).

On the other hand, data on the effect of exogenous treatments with auxins and cytokinins on infection by a series of pathogens are, on the whole, contradictory and certainly dependent on the system analysed. For instance, cytokinin treatment has been shown to have a positive effect in inducing resistance to Tobacco Mosaic Virus of tobacco (Kaplan et al. 1988) and to *Colletotrichum lagenarium* in cucumber (Mills et al. 1986), and to the synthesis of isoflavonoid phytoalexins in *Phaseolus* (Goossens and Vendrig 1982). However, a negative effect has been observed in the systems potato-*Verticillium* (Corsini et al. 1989), tobacco-*Phytophthora parasitica* (Kim and Chae 1985), rice-*Pyricularia oryzae* (Matsumoto et al. 1980) and apple-*Phytophthora cactarum* (Plich 1976). Increased endogenous or exogenous cytokinin levels have been shown to induce in tobacco shoots four mRNA species corresponding to plant defense-related genes (Memelink et al. 1987, 1990). Moreover, chitinase and chitinase mRNA accumulated in tobacco pith tissue incubated on medium supplemented with cytokinin or auxin, while both chitinase and β-1,3 glucanase were inhibited by a combination of both hormones (Shinshi et al. 1987; Mohnen et al. 1985). However, the existence of a correlation between hormone treatments and increased resistance to pathogens has not been investigated in these last cases. It should be noted that the effect of auxins, on the contrary, is generally positive when cytokinins decrease resistance, and vice versa. All of these results suggest that specific auxin/cytokinin balances may be required for optimal competence for defense. Within this frame, the apparent contradictions between observed results should be reconsidered in the view of the fact that endogenous contents generally not

measured in the experiments just mentioned certainly vary between the systems analysed. Therefore, the fact that the total auxin/cytokinin ratio is not known renders experiments not as comparable as in our case where only endogenous levels are modified in cultures grown on known media.

The hypothesis of a specific optimal equilibrium is further supported by our data on the effects of changing endogenous cytokinin ratios observed in transgenic cells of resistant cv 'Davis'. In this case the inhibition of both fungal growth in dual culture and conidia germination by callus extracts is higher in the case of equilibrium modifications towards high auxin concentrations, with no significant effect being observed for ion leakage and peroxidase induction. The low increase in peroxidase activity observed in cells transgenic for *iaaM* and *ipt* is delayed, and thus probably not connected with defense. This behaviour may be attributed to an overproduction of cytokinins in the control 'Davis' cells, which seems to be typical of resistant genotypes, an hypothesis that is being checked by direct determination.

Our results open the way to a better understanding of the physiological control of the activation of defense processes and thereby to the development of techniques for its modulation, possibly leading to a new way of constructing resistant genotypes. Within this frame studies on regenerated transgenic tomato plants aimed also at analysing the degree of "horizontality" of acquired resistance process are being carried out in our laboratory.

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