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Modification of competence for in vitro response to *Fusarium oxysporum* in tomato cells. I. Selection from a susceptible cultivar for high and low polysaccharide content

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Abstract. Plant cell walls play a major role in the outcome of host-parasite interactions. Wall fragments released from the plant, and/or the fungal pathogen, can act respectively as endogenous and exogenous elicitors of the defence response, and other wall components, such as callose, lignin, or hydroxyproline-rich glycoproteins, can inhibit pathogen penetration and/or spreading. We have previously demonstrated that calli from tomato cultivars resistant in vivo to *Fusarium oxysporum* f.sp. *lycopersici* show a high amount of polysaccharides in vitro. The aim of the present work was to assess the possible role of polysaccharide content and/or synthetic capacity in determining the competence of plant cells for active defence. For this purpose, tomato cell clones with increased and decreased polysaccharide (FL^+, FL^-) and callose (A^+, A^-) content have been selected by means of specific stains as visual markers and tested for the effect of these changes on the extent of response to *Fusarium*. The analysis of several parameters known to be indicative of active defence (cell browning after elicitor treatment, peroxidase and β-glucanase induction and inhibition of fungal growth in dual culture) clearly shows that FL^+ and A^+ clones have acquired an increased competence for the activation of defence response. The results are thoroughly discussed in terms of an evaluation of the relative importance of constitutive and/or inducible polysaccharide synthetic capacity for plant response to pathogens, and their possible regulation by plant physiological backgrounds.

Key words: *Lycopersicon esculentum* – *Fusarium oxysporum* f.sp. *lycopersici* – Polysaccharides – Active defence

Introduction

The relevance of plant cell walls in determining the fate of host-parasite interactions has frequently been stressed (Hahn et al. 1989; Smart 1991). A number of studies support the hypothesis that oligosaccharides released from plant cell walls by partial acid hydrolysis can elicit phytoalexin accumulation in different plant-pathogen systems (Hahn et al. 1981; Lyon and Albersheim 1982; Davis and Hahlbrock 1987). During pathogen attack, these elicitor-active fragments of the plant cell wall can be released by microbial pectic-degrading enzymes, such as fungal endopolygalacturonase (Lee and West 1981a, b; Robertsen 1987), pectate and pectin lyase (Collmer and Keen 1986; Linhardt et al. 1986). Elicitor-active oligosaccharide fragments can also be released from fungal walls by the plant pathogenesis-related (PR) proteins glucanases (Bailey 1980), chitinases and chitosanases (Barber et al. 1989; Kendra et al. 1989). Finally, other plant cell-wall components containing polysaccharides, such as callose, a mixture of heterogeneous β-1,3-glucans (Aist and Bushnell 1991), lignin (Hammerschmidt et al. 1984), and hydroxyproline-rich glycoproteins (Showalter et al. 1985; Corbin et al. 1987), are known to be synthesized in the early phases of plant response to pathogens and to inhibit the infection process.

All these observations, however, while showing correlations between polysaccharide content, and/or synthesis, and response to pathogens, do not allow an

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evaluation of the relative importance of polysaccharides in the network of processes which may eventually lead to defence.

The approach which is being followed by our laboratory is to define a "scale of importance" of the different processes by isolating mutants modified in single functions and testing the effects of these modifications on the response to the pathogen. This rationale closely resembles that introduced by biochemical geneticists involving mutants blocking single steps in metabolic pathways (Beadle and Tatum 1941).

Within this framework, tomato cell lines selected for increased elicitor recognition (Buiatti et al. 1987) were shown to respond better to *Fusarium oxysporum* and to display a pleiotropic phenotype, including a high hexopolysaccharide content, as determined by fluorescent staining with calcofluor or aniline blue (Storti et al. 1989). A higher polysaccharide content has also been found in calli from tomato cultivars (cvs) resistant to *Fusarium* in vivo when compared to susceptible ones (Storti et al. 1992). In line with our general approach, to better assess the role of polysaccharides in our system, we have therefore selected from susceptible cv Red River cell lines with an increased or decreased polysaccharide and callose content, using specific stains as visual markers. A series of parameters known to be indicative of active defence, such as cell browning after treatment with fungal elicitor, the induction of peroxidase and β-glucanase synthesis, and the inhibition of fungal growth in dual culture, were used to assess the changes in response to the pathogen in cell clones selected for increased (FL^+, A^+) and decreased (FL^-, A^-) polysaccharide and callose content, respectively.

Materials and methods

Plant and fungus cultures

The tomato cv Red River is a commercial cultivar susceptible to *F. oxysporum* f.sp. *lycopersici*. Establishment and growth of 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).

Selection experiments

For the selection of tomato cell lines with high (FL^+) and low (FL^-) polysaccharide content, cell suspensions of Red River were filtered through a 450 µm nylon mesh, and plated on Petri dishes (diameter 55 mm) at a concentration of 10 000 cells/dish in LS (Linsmaier and Skoog 1965) medium supplemented with 0.4 ppm 2,4-D and 1 ppm kinetin (A_1). The dishes were then incubated in a growth chamber at 24 ± 1 °C under continuous light. After 10 days, cell clones were transferred on the same medium containing 0.02% (w/v) Calcofluor white (Fluorescent brightener Sigma). After a 2 h incubation in the dark, clones were screened for fluorescence under 365 nm UV light and colonies with high and low polysaccharide content were isolated and plated on normal medium. The selection for high (A^+) and low (A^-) callose content was carried out in the same manner, except that the medium used consisted of 0.1 M Na_2PO_4 buffer pH 8.5 supplemented with 0.1% (w/v) aniline blue and dishes were incubated in the dark for 10 min prior to the screening under UV light at 365 nm. Colonies with high and low callose content were isolated and plated on normal solid medium. All the clones thus obtained were routinely transferred monthly.

Dual culture

Dual culture experiments were carried out by sowing *F. oxysporum* conidia on filter paper disks (3×10^5 conidia/disk). The disks were then plated on A_1 Petri dishes at the same distance from one explant of each of the two clones to be tested. Fungal growth was measured after 3 days and expressed as $D - R/D$, where R was the radius of the mycelium and D the distance between the center of the filter disk and the border of the callus.

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 of 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).

Peroxidase electrophoresis

Peroxidases isoenzymes were analysed as described by Storti et al. (1989). Peroxidase staining was performed with guaiacol and H_2O_2 according to Kay and Basile (1987).

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

F. oxysporum f.sp. *lycopersici* race 1 was surface-cultured in 150 ml of Czapek Dox Broth in 500 cc conical flasks at 24 ± 1 °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 fresh weight) 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. 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.

Isolation of acid-soluble proteins and 1,3 β-glucanase assay

The acid-soluble proteins were isolated according to De Tapia et al. (1986). Calluses of the different cell clones with or without *Fusarium* elicitor treatment were homogenized in a mortar in 1 ml/g tissue of cold phosphate-citrate buffer pH 2.8 (Na_2HPO_4 30 mM, Na citrate 80 mM, ascorbate 5 mM, β-mercaptoethanol 14 mM). The homogenate was filtered through a nylon cloth and centrifuged for 30 min at 20 000 g. The supernatants were desalted through G-25 Sephadex columns (Pharmacia PD-10).

β-glucanase activity was measured as the rate of reducing sugar production from laminarin (Sigma) according to Fisher et al. (1989) in calluses with or without *F. oxysporum* elicitor treatment as previously described. The assay mixture (12.5 mg laminarin in 2.5 ml of 50 mM Na acetate pH 5.0) was pre-

incubated for 10 min at 37 °C prior to the addition of the enzyme solution (maximum 20 µl), and then incubation was continued for 0.5, 10 and 15 min. Samples (0.5 ml) were taken from the assays and mixed with 0.5 ml of Somogyi solution (Somogyi 1952). The mixture was incubated for 15 min at 100 °C under water-saturated air. After cooling to room temperature, 0.5 ml of Nelson chromogenic reagent (Nelson 1944) was added and the absorbance at 660 nm determined. Glucose was used as a standard.

Callose extraction and determination

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

Callose determination was carried out according to Kohle et al. (1985). Samples with the addition of 1N NaOH to a final volume of 200 µl were mixed with 400 µl of 0.1% (w/v) aniline blue in water; 210 µl of 1N HCl were then added, and the final pH value was adjusted with 590 µl of 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 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 GmbH) in NaOH.

Results

To test the difference in polysaccharide content of selected cells, constitutive callose content was determined as described in Materials and methods in the cell clones selected for increased and decreased polysaccharide (FL^+, FL^-) and callose (A^+, A^-) content. As expected, and shown in Fig. 1, FL^+ and A^+ clones have a significantly higher callose content than FL^- and A^- clones, while the values obtained for Red River are intermediate.

The response to the pathogen was initially tested by treating the same calli with fungal elicitor, to visually analyze the hypersensitive response. As shown in Fig. 2 "plus" clones showed a very intense browning whereas "minus" ones were not responsive.

The activity of two classes of enzymes (peroxidases and β-glucanases) known to be synthesized in the early phase of plant response to pathogens in this and other systems (Kauffmann et al. 1987; Kombrik et al. 1988; Mohan and Kolattukudy 1990; Croft et al. 1990) was then analyzed as representative of active defence responses. Upon staining in vivo with guaiacol, an indicator of peroxidase activity in dual culture (Storti et al. 1989), FL^+ and A^+ clones showed a more intense red

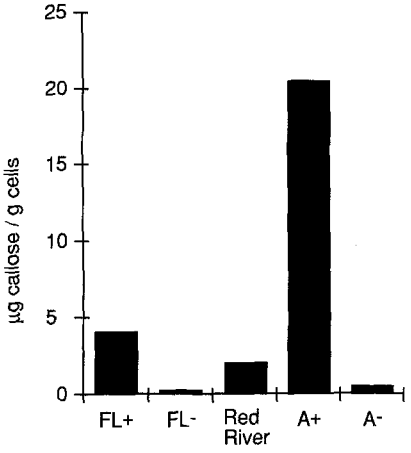

Fig. 1. Callose content (µg/g tissue) of cell clones selected for high (FL^+, A^+) and low (FL^-, A^-) callose or polysaccharide content and of the parental cv Red River

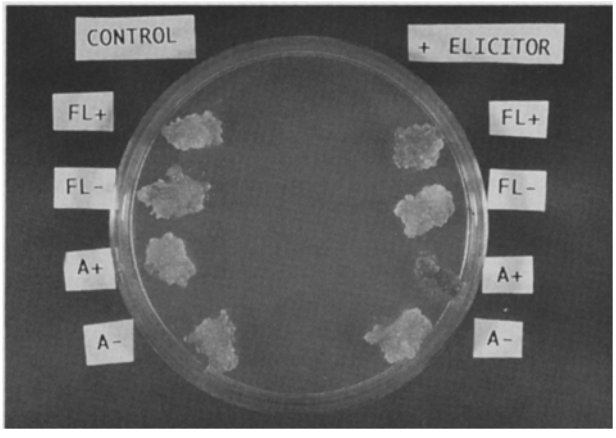

Fig. 2. Browning of calluses of selected clones after treatment with *F. oxysporum* cell wall components (+ elicitor). Controls were treated with distilled water

color than FL^- and A^- clones (data not shown). This observation was further supported by the direct analysis of ionically-, covalently-bound, and soluble peroxidases in selected and in parental cultures after 12 and 32 h of dual culture (Figs. 3 and 4 A, B, C). All three classes of enzymes were induced in the FL^+ clones, the soluble (Fig. 3A) and covalently-bound (Fig. 3C) fractions showing a peak after 12 h of dual culture, while the ionically-bound fractions (Fig. 3B) increased continuously until the end of the experiment. No induction was observed in the FL^- clones for the soluble and ionically-bound enzymes; a slight induction was observed for the covalently-bound fraction, but at a level significantly lower than in the FL^+ clones. The values for Red River were intermediate between the two

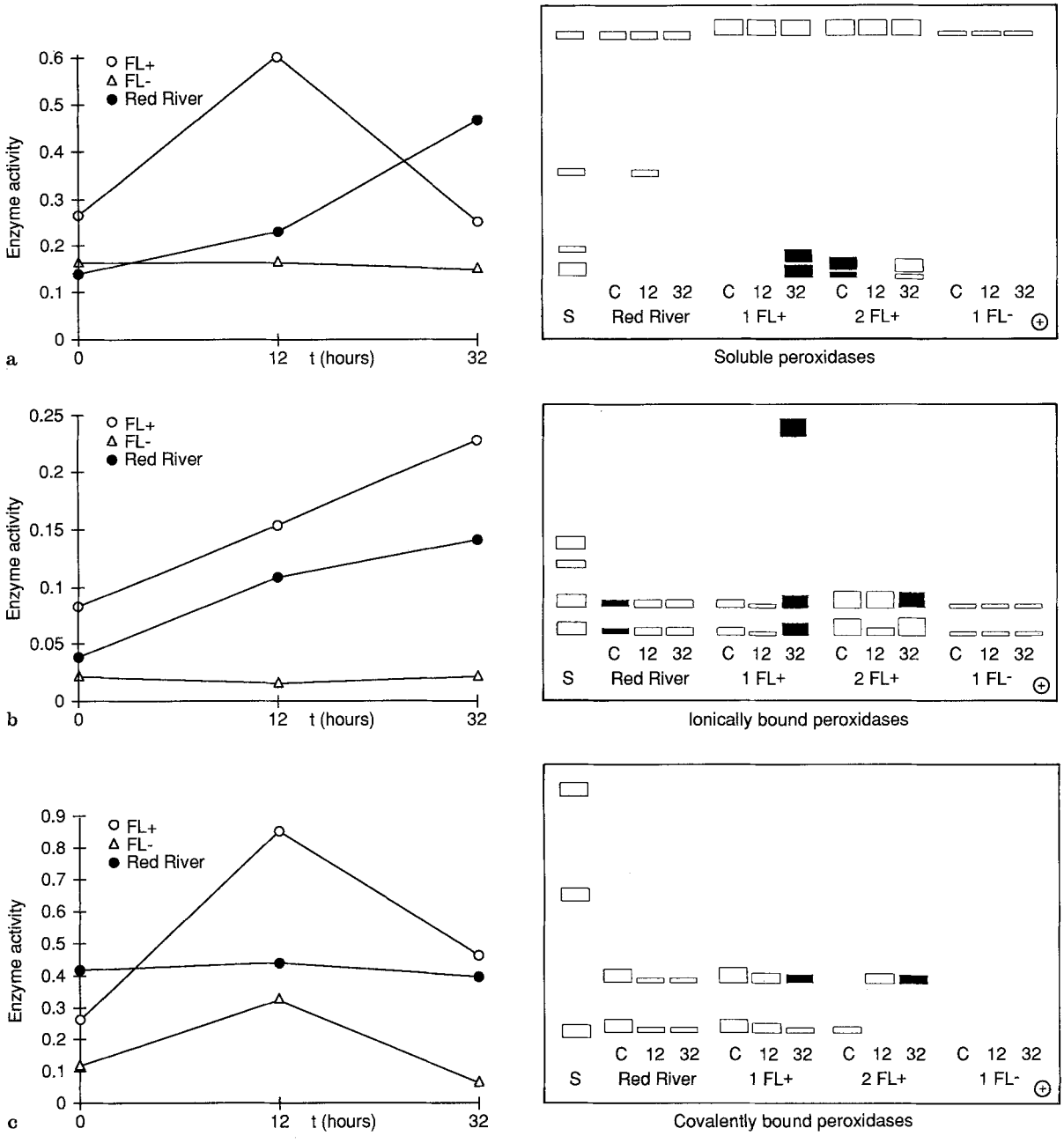

Fig. 3A–C. Soluble (**A**), ionically-bound (**B**) and covalently-bound (**C**) peroxidases from FL^+ and FL^- clones and from the parental cv Red River grown in dual culture with *F. oxysporum* f.sp. *lycopersici* for 12 and 32 h. The curves reported are representative of the behaviour of all FL^+ and FL^- clones

groups of clones. For A^+ and A^- clones, samples were taken after 8, 16 and 32 h of dual culture (Fig. 4A, B, C). In this case too the induction of the three classes of peroxidases was observed in the "plus" clones, reaching a maximum level after 8 h of dual culture for the soluble (Fig. 4A) and ionically-bound (Fig. 4B), and after 32 h for the covalently-bound (Fig. 4C), fraction. The same remarks made for the FL^- clones applied also to the A^- clones, thus confirming the lack of response for the "minus" group as far as peroxidase induction was concerned.

Moreover, electrophoretic analysis highlighted striking differences in isozyme pattern between clones and the appearance of new bands in the presence of the pathogen in FL^+ clones (Fig. 3A, B, C) and in A^+ clones (Fig. 4A, B, C).

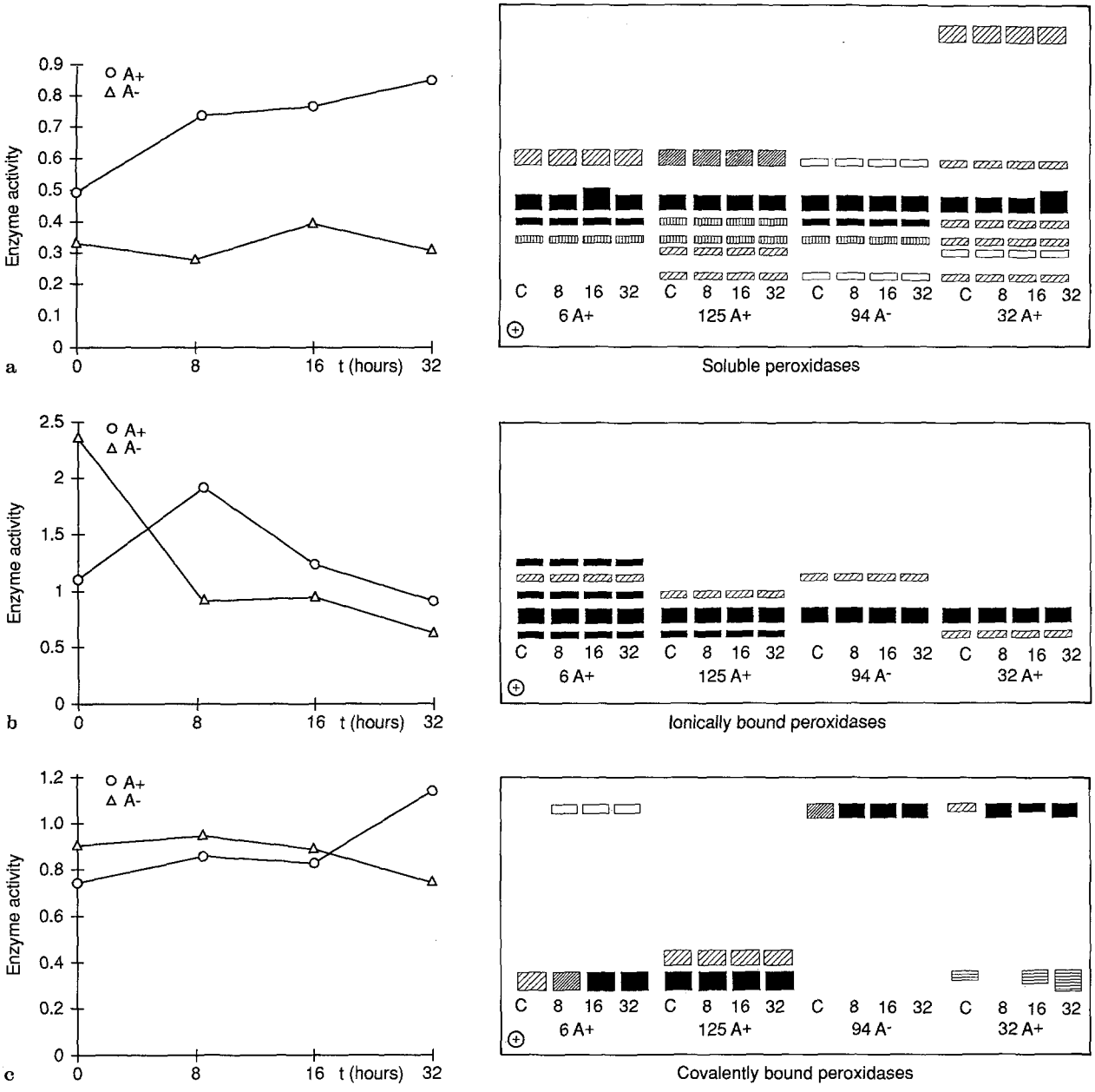

Fig. 4A–C. Soluble (**A**), ionically-bound (**B**) and covalently-bound (**C**) peroxidases from A^+ and A^- clones after 8, 16 and 32 h in dual culture. The curves reported are representative of the behaviour of all A^+ and A^- clones

Acid β-glucanases were also strikingly induced by *Fusarium* cell wall components (E1) both in FL^+ and in A^+ clones. In FL^- cells, treatment with the fungal elicitor seemed to decrease the enzyme activity, while in A^- cells β-glucanase activity was induced, though to a much lesser extent than in its A^+ counterpart (Fig. 5).

Finally, cells selected for increased polysaccharide content were tested for the ability to avoid fungal invasion in dual culture. Figure 6 shows that mycelial growth is inhibited only by 34 A^+ clones, a result which is representative of the behaviour of all the A^+ and A^- or FL^+ and FL^- clones tested. The inhibition of fungal growth was also quantified and expressed as the distance between the growing mycelium and the callus explant (D – R/D, where R was the radius of the mycelium and D the distance between the center of the filter disk and the border of callus). The data reported in Table 1 were the result of three independent series of experiments where A^+ was compared with A^-, FL^+ with FL^-, and Red River with all the other clones.

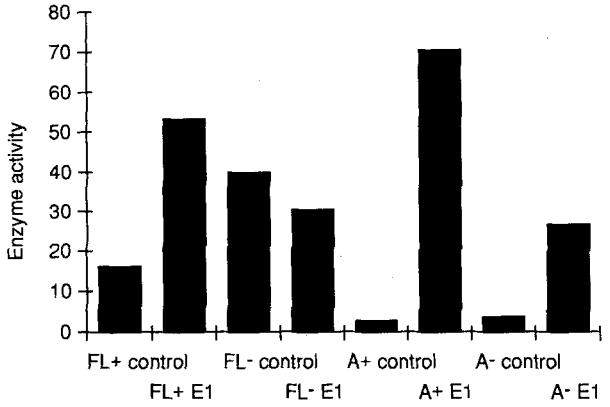

Fig. 5. 1,3-β-glucanase activity of cell clones selected for high (FL^+, A^+) and low (FL^-, A^-) callose or polysaccharide content and of the parental cv Red River, treated with fungal elicitor (E1) or with distilled water (control)

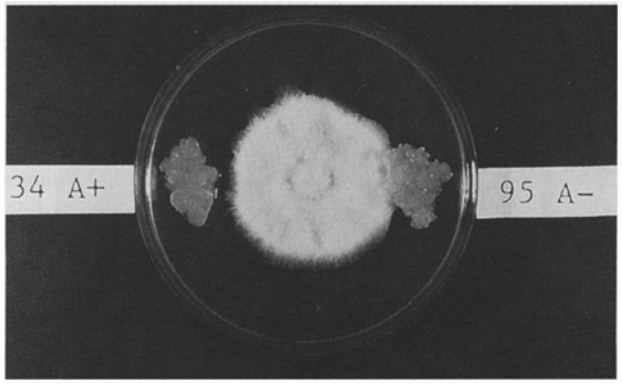

Fig. 6. An example of inhibition of fungal growth in dual culture by cells selected for high (34 A^+, on the left) or low (95 A^-, on the right) callose content

Table 1. Inhibition of *F. oxysporum* growth in dual culture experiments by tomato cell clones selected for high (FL^+, A^+) or low (FL^-, A^-) polysaccharide or callose content, respectively, and the parental cultivar Red River. Fungal growth inhibition was measured as D – R/D, where R represents the radius of mycelium and D the distance between the center of the filter disc and the border of callus

Selected Clones	D – R/D
A^+	0.393
A^-	0.325
FL^+	0.358
FL^-	0.270
Red River	0.270

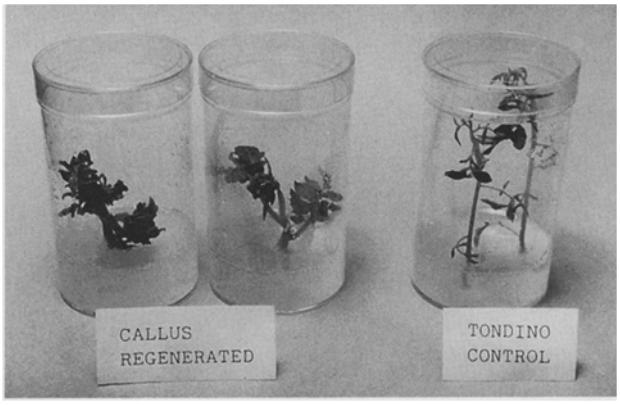

Fig. 7. Tomato plants of the cv Tondino regenerated from cell clones selected for a high polysaccharide content on calcofluor white (on the left) and normal Tondino plants (control)

Discussion

The results reported in this paper show that, when selected for increased polysaccharide or callose content, tomato cells from a cultivar susceptible to *F. oxysporum* acquire the ability to respond hypersensitively and to inhibit the pathogen in dual culture. This accords with the fact that the same "high polysaccharide content" phenotype has been demonstrated in cells from a commercial tomato cultivar resistant to *Fusarium* (Storti et al. 1992), and in cells specifically selected for increased response to the fungus from the same cultivar (Red River) used in the present work (Storti et al. 1989). These data are further supported by electron microscope observations on the same clones which show thicker cell walls in "plus" cell lines (Morassi Bonzi et al. 1993). Taken together the data suggest an important role of polysaccharide synthetic capacity (or constitutive amounts) in the control of tomato competence for the response to *Fusarium*, a role which is also supported by the known involvement of several classes of plant cell-wall components in the processes leading to active defence. It should be noted, moreover, that similar data have been obtained from other, different, host-parasite systems and that cells selected for increased response to *Fusarium*, and as previously mentioned showing a high polysaccharide content, also exhibited a higher hypersensitivity and increased phytoalexin synthesis when challenged with heat-released cell-wall components of another pathogen, *Phytophthora infestans*.

These data suggest a low specificity, i.e., a "horizontal" nature, in the increase of the plant response to pathogens in the analyzed cell lines. This may imply that the high polysaccharide contents observed in the selected cells may truly result from modifications of some basic physiological processes in selected cells. In fact, preliminary observations on plants regenerated

from FL^+ cell clones isolated from the regeneration-prone tomato cultivar Tondino seem to support this hypothesis. These plants (Fig. 7) show a strongly-modified, compact phenotype with intensely-green leaves, stems thicker than normal and aberrant root formation at the leaf base, modified fruit shape, and earlier flowering characters, which may all be indicative of modifications in phytohormone equilibria. A disequilibrium in the hormone balance towards auxin biosynthesis has been found to lead also to an increase in callose content (Storti et al. 1993; Pellegrini et al. unpublished results). Moreover, the alteration of auxin/cytokinin equilibria through transformation with the *A. tumefaciens* genes involved in phytohormone synthesis has been shown to induce competence for active defence in the tomato cultivar, Red River, used in the present work (Storti et al. 1993). Progenies from plants regenerated from FL^+ variants which seem to maintain the character of high polysaccharide content in leaf-derived callus, are at present being screened both in vitro for the characters analyzed in this paper and in vivo for resistance to the pathogen.

Whatever the results of further investigation and the eventual cause(s) of the increase in polysaccharides in selected cells, our data seem on the whole to point out the importance of gaining a deeper insight into the nature of the "physiological background conditions" which may be relevant to the choice between a compatible or incompatible outcome of host-parasite interactions. This view has been supported by a series of data by other authors including those related to the study of conditions leading to horizontal systemic acquired resistance (Madamanchi and Kuć 1991).

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