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Isolation of tomato cell lines with altered response to *Fusarium* cell wall components*

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Summary. To obtain Tomato cell lines with an altered capacity to respond to heat-released cell wall components (elicitor) of a tomato pathogen (*Fusarium oxysporum* f. sp. *lycopersici*), positive and negative selection experiments, using BUdR enrichment techniques, were carried out on suspension cultures of the susceptible, low phytoalexin producer cultivar Red River. Both high and low phytoalexin producing clones were isolated. Further tests demonstrated that not all phytoalexin-producing clones were more susceptible to the elicitor toxic effect, and that they were altered also in the speed of response to fungal cell wall components. Cells selected with *Fusarium* elicitor showed the same behaviour when challenged by *Phytophthora infestans* elicitor, thus suggesting in this case lack of specificity. The results are finally discussed with a view to using the technique both as a tool to study the genetics and physiology of host-parasite interactions and as a possible new method for the selection of pathogen resistant genotypes.

Key words: Elicitor – *Fusarium oxysporum* – in vitro selection – Pathogen resistance – Phytoalexin

Introduction

The use of plant tissue and cell cultures for the selection for resistance to pathogens has been proposed and discussed by several authors (Buiatti and Scala 1984; Ingram and MacDonald 1986). Some success has been achieved through in vitro selection for tolerance to fungal toxic compounds and/or in vivo screening of plants regenerated from tissue cultures (Buiatti and Scala 1984).

No other selection method has been tried however, up to now, in spite of the variety of processes which may be involved in plant defence mechanisms and which, if expressed in vitro, offer the basis for a more efficient selection of somaclonal genetic variability and/or for early screening purposes. Earlier work carried out in our and other laboratories (Buiatti et al. 1985; Buiatti and Scala 1984; Scala et al. 1985) has shown in several crop plants a correlation between in vivo resistance to pathogens and in vitro hypersensitivity reaction and synthesis of antimicrobial compounds (phytoalexins) following treatment with heat-released fungal cell wall components (elicitors). Although hypersensitivity and phytoalexin production are not necessarily correlated, selection for high and low response to elicitor treatments in terms of cell survival has been attempted in the present work with the aim of eventually obtaining high and low phytoalexin-producing cell lines. The rationale of the experiments was to isolate hypersensitivity-minus clones from cells surviving elicitor treatments and to obtain hypersensitivity-plus variants using on elicitor-treated cells the BUdR enrichment technique employed by other authors for the selection of auxotrophs (Shikkito et al. 1981; Shimamoto 1983).

The system tomato (*Lycopersicon aesculentum* L.) cv Red River-*Fusarium oxysporum* f. sp. *lycopersici* race 1 was chosen for these experiments as it had been shown (Scala et al. 1985) that tissue cultures of this particular genotype synthesized a low but significant amount of phytoalexins when challenged with fungal elicitors.

Materials and methods

Callus and suspension cultures

Seeds of *Lycopersicon aesculentum* cv Red River were surface-sterilized with 1% sodium hypochloride, sown on Linsmajer

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and Skoog (1965) medium (LS) and seedlings grown for 14 days at 24 ± 1 °C under continuous light (2,500 lux). Cotyledons were then aseptically excised and sown on LS medium supplemented with 0.4 ppm 2,4-D and 1 ppm kinetin. Cell suspensions were obtained through growth of the callus in the same liquid medium on a rotatory shaker.

Growth of Fusarium and Phytophthora and preparation of heat-released mycelial cell wall components

F. oxysporum f.sp. *lycopersici* race 1 was surface cultured in 150 ml Czapek Dox broth Difco in 500 cc conical flasks at 24 ± 1 °C for 21 days. *Phytophthora infestans* was cultured for 21 days in 100 ml pea juice broth in 500 cc conical flasks at 18 °C. Heat-released cell wall components (elicitors) were isolated according to a modified method by Garas et al. (1979) as described by Buiatti et al. (1985). Elicitor concentration was expressed as glucose equivalents measured with the phenol-sulphuric acid method (Hodge and Hofreiter 1962).

Selection experiments

For the selection of elicitor-resistant (hypersensitivity deficient) cell lines, cell suspensions were aseptically filtered through a 450 μm nylon mesh, plated on 55 mm ∅ Petri dishes at a concentration of 10,000 cells per plate in liquid LS medium (2.5 ml) supplemented with 0.4 ppm 2,4-D and 1 ppm kinetin and with various *Fusarium* elicitor concentrations (4 μg, 8 μg, 13 μg, 17 μg/10,000 cells) and grown for 6 days on a rotating shaker. After this period, cells were repeatedly washed with fresh medium without the elicitor and plated again on a solid medium.

For the isolation of putative elicitor-susceptible (hypersensitivity-plus) cells, 20 ml cell suspensions (20,000 cells/ml) in 100 ml conical flasks were grown on a rotatory shaker in the dark on the normal growth medium just described, supplemented with 11 μg/10,000 cells *Fusarium* elicitor and 29 μg/10,000 cells sterile 5-bromodeoxyuridine (BUdR). After 4 days, cell suspensions were repeatedly washed with fresh culture medium and cells were sown on solid medium in Petri dishes (10,000 cells per plate). Surviving colonies were scored in both cases and then routinely transferred and grown on normal solid medium.

Extraction and bioassay of phytoalexins

Antimicrobial compounds were extracted as described in a previous paper (Scala et al. 1983) from selected clones and Red River callus after treatment for different times with *Fusarium* or *Phytophthora* elicitors (100 μl = 0.85 mg/ml glucose equivalents). The presence of phytoalexins in ethanolic extracts was assayed by determining percentage inhibition of germ tube length of growing conidia of *F. oxysporum* f. sp. *lycopersici* race 1 on hanging drop glass slides. Incubation suspensions contained 170 μl Czapek Dox broth, 5 μl conidia suspension at 2×10^5 spores/ml and 10 μl ethanolic extracts. Slides were incubated in the dark for 12 to 18 h at 24 °C and then germ tube length was determined.

Results

Selection experiments

Figure 1 shows the "killing" effect of elicitor treatments in terms of number of colonies/10^5 plated cells found after treatment. To further test the acquired resistance, selected colonies resistant to the highest elicitor concentration (13 μg and 17 μg/10,000 cells) were exposed to a second elicitor treatment at higher concentrations (27 μg and 55 μg/10,000 cells). No difference in plating efficiency was found between treated and control cells in the case of selected clones, five of which were propagated for further experiments.

Also on surviving clones from BUdR experiments (putative hypersensitive-plus variants) loss of resistance to elicitor and/or resistance to BUdR were checked by

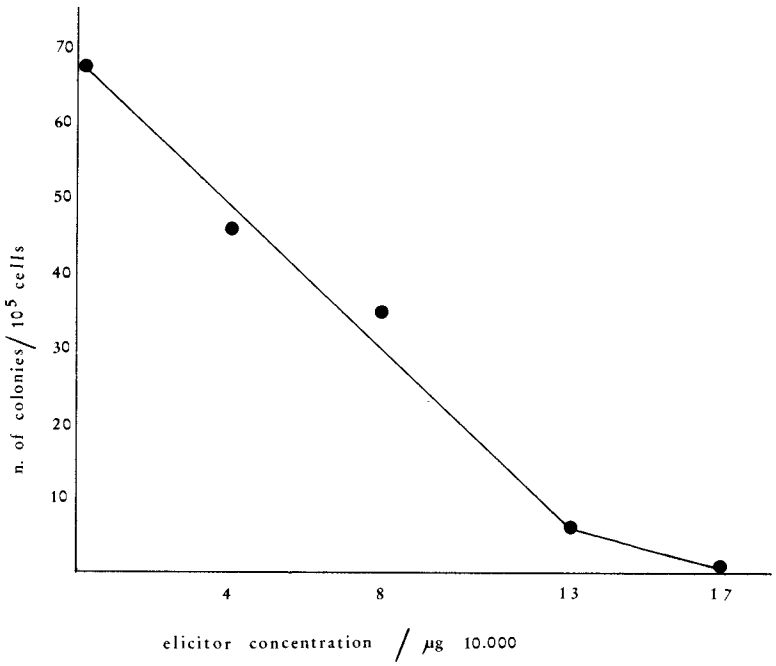

Fig. 1. Survival of plated tomato cells treated with different *Fusarium* elicitor concentrations

exposing them to the same concentrations of the two agents used in the selection experiments. None of the clones was found to be resistant to BUdR, while an apparently lower resistance to the elicitor was found in 5 out of 22 tested clones.

Phytoalexin production following elicitor treatment

As shown in Table 1, only one out of five elicitor-resistant clones tested showed significant phytoalexin synthesis after elicitor treatment as judged from the inhibition of *Fusarium* germinating conidia by callus ethanolic extracts. The inhibition was also strikingly lower than that induced by similar extracts from the non-selected parental callus.

Although, as previously mentioned, only a few clones selected with the BUdR technique were more susceptible than was the parental line to the elicitor, the five clones analysed for phytoalexin production all showed a higher reactivity to *Fusarium* elicitor than Red River. It should be noted that only two out of these five clones were found to have "hypersensitivity plus".

Table 1. Inhibition of *F. oxysporum* f. sp. *lycopersici* race 1 young conidia germ tube length after treatment with ethanolic extracts of putative high phytoalexin producing clones (F^+) and of putative elicitor resistant clones (F^-) challenged with *Fusarium* elicitor

Clone	Control	Treated	Inhibition (% of control)	Significance level
Red River	4.48±0.63	3.01±0.09	32.9	
1 F^+	1.83±0.09	0.27±0.05	85.5	***
5 F^+	8.20±0.26	3.67±0.30	55.2	***
12 F^+	10.06±0.29	3.48±0.26	65.4	***
21	1.74±0.06	0.68±0.03	63.8	***
14 F^+	6.65±0.29	3.42±0.29	48.6	*
6 F^-	5.84±0.30	5.56±0.27	4.8	**
7 F^-	5.90±0.29	6.43±0.30	−8.2	***
2 F^-	6.87±0.33	6.41±0.35	6.7	**
5 F^-	4.78±0.16	4.69±0.16	1.7	***
12 F^-	5.08±0.19	4.32±0.18	15.0	*

* $P<0.05$; ** $P<0.01$; *** $P<0.001$

Table 2. Percentage inhibition of *Fusarium* germinating conidia by ethanolic extracts of control and cells selected for high (F^+) and low (F^-) phytoalexin production treated for different times with *Fusarium* elicitor. NS: non significant

Strains	32 h	64 h	96 h	168 h
Red River	9.4% NS	9.6% NS	41.5%	43.3%
5 F^+	15.1%	27.5%	48.2%	62.2%
12 F^-	–	−13.3%	7.1% NS	3.3% NS

Partial characterization of selected lines

In an attempt to carry out a partial characterization of the obtained variants, two problems were tackled. Firstly, one "phytoalexin-plus", one "minus" and the parental cultivar were compared to ascertain whether changes in the timing of induction of phytoalexin synthesis were coupled with higher or lower production of antimicrobial compounds. As shown in Table 2, clone 5 F^+, a high phytoalexin producer, showed synthesis already after a 32 h elicitor treatment, while the same process was induced in the parental genotype only after 96 h, and the cell line 12 F^-, selected for resistance to the elicitor, never responded to the treatment.

The second question was then whether the changes observed in selected lines were specific or not to *Fusarium* elicitor. For this purpose calli from the parental cultivar, two high and two low phytoalexin producers, were treated with a *Phytophthora infestans* elicitor previously shown to interact with Red River cells (Scala et al. 1983) and phytoalexin production analysed after 60 h. As shown in Table 3, treatment with *Phytophthora infestans* cell wall components elicited a strikingly high phytoalexin production in only 60 h. One of the low phytoalexin producers (7 F^-) was completely resistant to *Phytophthora* elicitor while the other showed a response at significant levels.

Table 3. Inhibition of *Fusarium oxysporum* germinating conidia tube length after treatment with ethanolic extracts from Red River and high (F^+) and low (F^-) phytoalexin producing clones challenged with *Phytophthora* elicitor. NS: non significant

Strains	Red River	5 F^+	12 F^+	7 F^-	12 F^-
Treated	4.77±0.16	1.85±0.09	2.07±0.16	5.10±0.17	3.50±0.14
Control	5.28±0.17	3.71±0.15	4.20±0.20	4.73±0.15	4.06±0.16
Inhibition (% of control)	9.7	50.1	50.7	−7.8	13.8
Significance level		***	***	***	NS

*** $P<0.001$

Discussion

The data reported show for the first time that plant cells with an altered response to fungal heat-released cell wall components can be selected in vitro, leading to the isolation of phenotypically stable variant clones. Selection was particularly easy in our case for hypersensitivity deficient, low phytoalexin producing clones, i.e. cells unable to recognise fungal elicitors. Some of these clones, in fact, completely lack the capacity to be induced to produce phytoalexins even after relatively long elicitor treatments. Increase in hypersensitivity and high phytoalexin production on the other hand do not seem to be strictly correlated in negatively selected clones. Moreover, the amount of the increase in the synthesis of antimicrobial compounds and the speed of response to elicitor treatment varied between clones.

It can be said in general that different "high phytoalexin" clones seem to differ from each other in several respects. It should be noted, for instance, that preliminary experiments seem to show variation for tolerance to fungal toxic culture filtrates in terms both of in vitro growth inhibition and ion release. Finally, the selected changes in elicitor-plant cell interactions seem to be aspecific at least for *Fusarium* and *Phytophthora*. This suggestion, however, should be further tested with other biotic and abiotic elicitors in experiments with already known phytoalexin inducers (Scala et al. 1983, 1985).

Obviously, nothing can be said at the moment about the nature of the changes selected nor on their genetic or epigenetic nature. Experiments on both levels are being currently carried out in our laboratory. The results achieved may in any case offer a new, powerful tool for fine genetico-physiological analysis of the basis for phytoalexin production after plant-pathogen interaction. Moreover, if the genetic nature of the changes observed is confirmed in regenerated plants, the present work may open the way to new methodologies for in vitro selection of pathogen-resistant plants.

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