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A single dominant gene for *Fusarium* wilt resistance in protoplast-derived tomato plants

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Summary. Tomato plants resistant to the fungal pathogen, *Fusarium oxysporum* f. sp. *lycopersici,* race 2, were obtained using in vitro selection against fusaric acid, a non-specific toxin, as well as non-challenged cells. Protoplasts were isolated from cotyledonary tissue of tomato cv. 'UC-82', which is susceptible to *Fusarium* race 2. Protoplasts were challenged with the toxin, and the resistant calli were further subjected to the toxin. Plants regenerated from toxin-resistant calli were screened for resistance to the pathogen by using the *Fusarium* slurry inoculation technique. Seeds were collected from the surviving individuals, germinated and re-screened for resistance to the pathogen. Data obtained from this test showed a ratio of three resistant to one susceptible among R1 progenies. Further analysis of the R2 progenies confirmed that the *fusarium*-resistant plants were either homozygous or heterozygous dominant for the gene conferring the resistance. Similar results were recorded for tomato plants regenerated from cells that received no selection pressure. The nature of this single dominant gene-type of resistance is under investigation.

Key words: In vitro selection – Fusaric acid – *Lycopersicon esculentum* L. phytotoxin – Somaclonal variation

Introduction

In recent years, phytotoxins have been recognized as useful tools for the induction and selection of disease-resistant mutants in cell culture (Daub 1984; Wenzel 1985).

Phytotoxins are well characterized single molecules that are easy to use in selection schemes as opposed to the fungal or bacterial pathogen. Phytotoxins are classified into two major groups, those that are non-specific and those that are host-specific toxins. The first report on the use of non-specific toxin, methionine sulfoximine, in cell selection schemes was that of Carlson (1973) who selected tabtoxin-resistant mutants of tobacco. The selection of tissue cultures with resistance to a host-specific toxin (T-toxin) has been used to obtain maize plants resistant to Southern Corn Leaf Blight (Gengenbach et al. 1977). Since then, a number of investigators (Ling et al. 1985; for review, see Wenzel 1985) have used pathotoxins, in the form of crude and/or purified toxins, in selecting disease-resistant mutants. Equally important, disease-resistant mutants were also obtained without any selection pressure.

Fusaric acid, a non-specific toxin, is produced by many *Fusarium oxysporum* formae which cause wilt disease of many important crops. Of these, tomato is a very susceptible host to the pathogen *Fusarium oxysporum* f. sp. *lycopersici* which produces substantial quantities of fusaric acid in still cultures (Kern 1972) or in infected tomato (Kluepful 1957). The possible involvement of fusaric acid in disease development has been recently reviewed (Pegg 1981).

We now report the use of fusaric acid as a selecting agent in tomato cell cultures. *Fusarium*-resistant plants were obtained from fusaric acid-resistant calli as well as from non-selected calli. In both cases, the fusarium resistance was inherited as a single dominant gene.

Materials and methods

Selecting agent

Fusaric acid (Sigma Chemical Co. St. Louis) solutions were filter sterilized and added to autoclaved regeneration medium to obtain the desired concentrations.

Selection scheme

Protoplasts were isolated from 10–12 day old cotyledonary tissue (Shahin 1985) of a tomato cultivar, 'UC-82B' (Stevens et al. 1978), which is susceptible to *Fusarium oxysporum* f. sp. *lycopersici,* race 2. The protoplasts were cultured in liquid basal TM-2 medium (Shahin 1985) supplemented with 1.5 μM

zeatin riboside and 2 μM naphthalene acetic acid (NAA). Selection pressure was applied directly to the protoplasts upon plating. The fusaric acid (10–30 μM) was added to the protoplasts plating medium, and the surviving cell colonies were moved on TM-3 medium supplemented with 30 μM Fusaric acid. Thereafter, the resistant-calli were given another treatment with fusaric acid (30 μM) while undergoing shoot induction on TM-4G medium (same as TM-4 medium except sucrose was substituted with 2.0% glucose). Regenerated shoots were placed in TM-5 medium (Shahin 1985) in Magenta boxes for root induction.

Fungal culture and screening

The pathogen, *Fusarium oxysporum* f. sp. *lycopersici* Race 2 (obtained from Dr. Ben George, Heinz, USA) was grown on potato dextrose agar medium (Difco) in 100×15 mm plates incubated at 30 °C. After root development, the plantlets were removed from the agar, the root tips were clipped by a sharp blade, and the roots were dipped in a *Fusarium* slurry inoculum. Two-week old seedlings of 'Heinz 2152' (resistant to both races 1 and 2) and 'UC-82B' (susceptible to race 2), were always used as a positive and negative control, respectively. The inoculum was prepared by blending three plates of 14-day old culture in 100 ml water. The inoculated plantlets were then plated in moist sterile soil, covered with plastic cups, and placed in growth chamber for 1–2 days before being moved to the greenhouse. There, the plantlets were placed under 24 h stress lights and scored 3–4 weeks later.

Genetics studies

Resistant tomato plants (R0) were selfed, the seeds were germinated in soil and the seedlings (R1) were inoculated as above. Surviving individuals were potted in 6 inch pots and the R2 seeds were collected. Samples of this generation were screened for resistance to *Fusarium* as previously described, while other sample were crossed with the wild type (UC-82B). The resulting progenies were screened for resistance to the fungus.

Chromosome counts

Root tips from 5 day-old germinating seeds of R3 progeny were fixed directly in saturated aqueous alfabromonaphthalene for 3 h. The roots tips were softened in 5 N HCl for 15 min and then squashed in 2–3 drops of Toluidine Blue Stain as described previously (Marks 1973).

Results

Vigorous mini-calli were obtained on TM-3 medium supplemented with 30 μM fusaric acid (Fig. 1 A). In the two independent experiments (Table 1), a total of 409 mini calli were transferred to a toxin-containing shoot induction medium (TM-4G). After 4 weeks, 50 and 20 calli, from experiment 1 and 2, respectively, had survived the selection. These surviving calli were dense green with some of them bearing shoots (Fig. 1 B). When these surviving calli were subcultured on TM-4G medium plus fusaric acid for further shoot induction and shoot elongation (Shahin 1985), multiple shoots (>5) were abundantly produced. These shoots were further subcultured on fresh TM-4G medium (without fusaric acid) at least 3–4 times until well-developed shoots (>3 cm long shoot with two normal leaves and normal meristem) were available. These well developed shoots were rooted on TM-5 medium in Magenta boxes. The regenerated plants were pooled together from each experiment for fungal screening.

Each regenerated plant (R0) was tested for fusarium resistance using the *Fusarium* slurry technique described earlier. The plants were first scored for disease development 4–5 weeks after inoculation (Fig. 2). The resistance plants were allowed to grow for additional 3–4 weeks and then scored again to eliminate escapee. In some cases, many suspected resistant plants were dissected at the basal region of the stem to screen for the occurrence of vascular browning. The surviving plants were selfed (R1) and the resulting progenies were again screened for resistance to the *fusarium*-wilt fungus.

The segregation of resistance and susceptible among the progeny is presented in Table 2. These data are consistent with the ratios 3 resistant to 1 sensitive that are expected from self-fertilization of a heterozygous individual in the case in which resistance is conferred by a dominant allele of a single nuclear gene. Since the protoplasts were derived from diploid tomato plants (2n=24), the regenerated plants would be expected to be heterozygous for a dominant allele. These alleles have been assigned the general symbol *FmR* (for fusarium resistance) followed by the number of the

Table 1. Summary of the selection of *Fusarium*-resistant mutants from protoplast cultures of tomato cv. 'UC-82 B'

Plated protoplasts	Fusaric acid[a] (conc.)	Mini calli	Calli	*Fusarium*-resistant mutants[d]
4.0×10^6	20 μM	230[b]	50[c]	4
7.0×10^6	30 μM	179[b]	23[c]	3
5.0×10^6	None	400[e]	26	1
8.0×10^6	None	324[f]	100	5

[a] Fusaric acid was added to the protoplasts plating medium
[b] The number indicates those mini-calli that survived the selection pressure on TM-3 medium. These *Fusarium*-resistant mini-calli were moved on the shoot induction medium (TM-4G) for further selection against 30 μM fusaric acid
[c] The number represents those *Fusarium*-resistant calli that survived (sustained their growth and green color) on TM-4G medium supplemented with 30 μM fusaric acid
[d] The number represents those plants where the resistance to the pathogen was transmitted through meiosis
[e] 400 mini-calli were randomly selected from the TM-3 medium, and moved onto the shooting medium (TM-4G). Twenty-six green calli allowed to regenerate plants from which one disease-resistant mutant was obtained
[f] 324 mini-calli were randomly selected from the TM-3 medium and placed on TM-4G for shoot induction. 100 calli were allowed to regenerate plants from which 5 *Fusarium*-resistant mutants were obtained

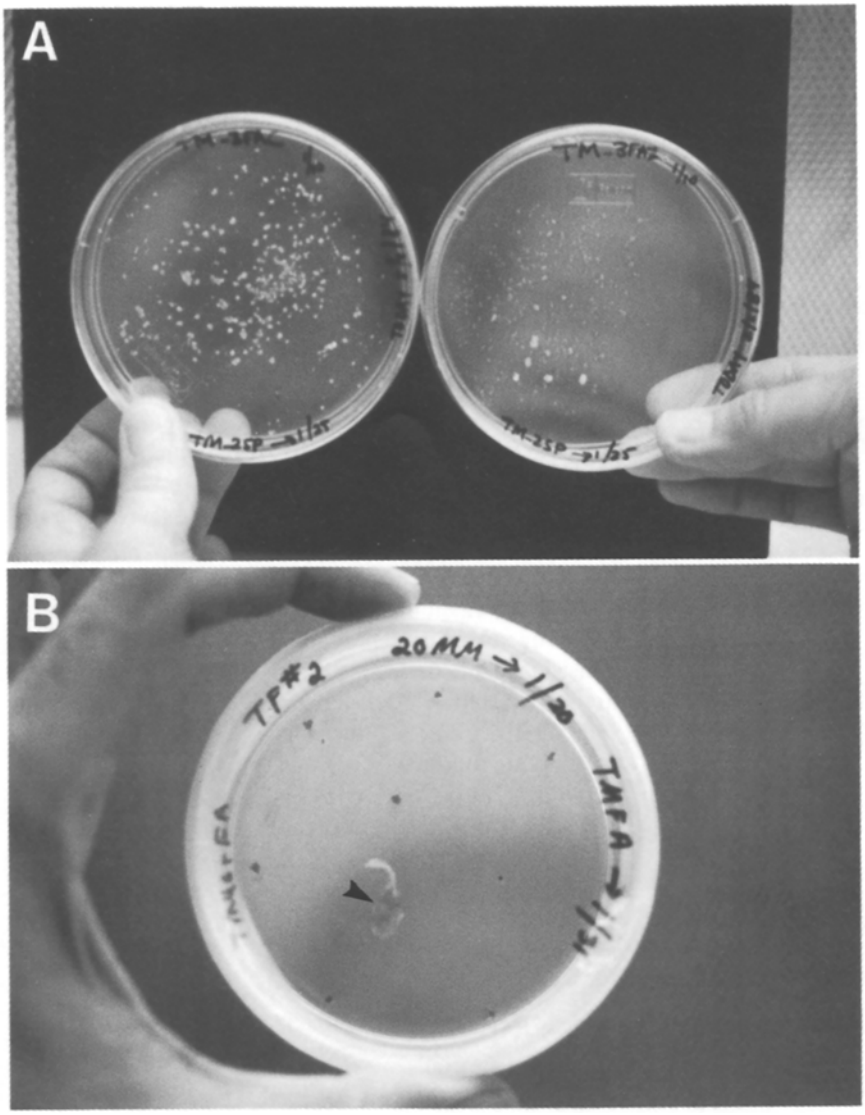

Fig. 1. **A** Fusaric acid-resistant minicalli (*right plate*) and non-treated minicalli (*left plate*). **B** Fusaric acid-resistant callus (*arrow*) undergoing shoot induction

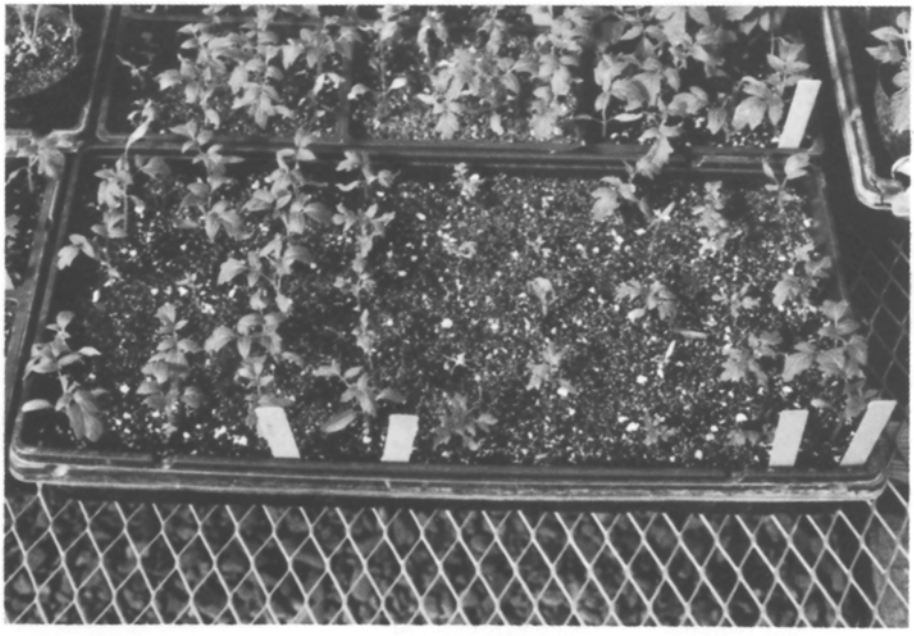

Fig. 2. Segregation for response to *Fusarium* wilt in R1-progeny obtained from self-fertilization of selected R0 individuals

Table 2. Segregation for the response to *Fusarium* wilt in R 1- progeny obtained from self-fertilization of selected Ro individuals (challenged with Fusaric acid) and non-challenged protoplasts of UC-82 B

Line[a]	Resistant		Susceptible		Ratio	x2	*P*
	Obs.	Exp.	Obs.	Exp.			
Fusaric acid							
FmR1/+[b]	30	31.5	12	10.5	3:1	0.29	0.50–0.80
FmR2/+[b]	20	21	8	7	3:1	0.19	0.50–0.80
FmR3/+[b]	18	18	6	6	3:1	0	
FmR4/+[b]	11	13.5	7	4.5	3:1	1.85	0.05–0.20
FmR5/+[c]	21	27	15	9	3:1	5.34	0.01–0.05
FmR6/+[c]	25	27	11	9	3:1	0.59	0.20–0.50
FmR7/+[c]	20	21	8	7	3:1	0.19	0.50–0.80
No treatment							
FmR8/+	29	31.5	13	10.5	3:1	0.79	0.20–0.50
FmR9/+	18	18	7	6	3:1	0.16	0.50–0.80
FmR10/+	22	21	6	7	3:1	0.19	0.50–0.80
FmR11/+	38	36	9	12	3:1	0.86	0.20–0.50
FmR12/+	21	21	7	7	3:1	0	
FmR13/+	20	21	8	7	3:1	0.29	0.50–0.80

[a] Since these disease-resistant lines were derived from diploid tomato protoplasts, they would be expected to be heterozygous for a dominant allele. For simplicity, these alleles have been assigned the general symbol *FmR* (for *Fusarium* resistance) followed by the number of the line

[b] Obtained from Experiment No. 1 (Table 1), where protoplasts were challenged with 20 μM fusaric acid

[c] Obtained from Experiment No. 2 (Table 1), where protoplasts were challenged with 30 μM fusaric acid

Table 3. Response to *Fusarium* wilt in R2 progeny of random R1 isolates obtained from self-fertilization of *FmR1/+*

R1 isolate	No. of R2 individuals		Presumed genotype of R1
	Resistant	Susceptible	
1	10	4	*FmR1/+*
2	9	5	*FmR1/+*
3	0	6	+/+
4	16	0	*FmR1/FmR1*
5	0	14	+/+
6	15	5	*FmR1/+*
7	0	9	+/+
8	4	0	*FmR1/FmR1*
9	16	0	*FmR1/FmR1*
10	12	4	*FmR1/+*
11	16	0	*FmR1/FmR1*
12	0	16	+/+
13	16	5	*FmR1/+*
14	14	4	*FmR1/+*
15	16	0	*FmR1/FmR1*
16	0	9	+/+
17	16	5	*FmR1/+*
18	21	7	*FmR1/+*
19	0	13	+/+
20	12	4	*FmR1/+*
21	15	6	*FmR1/+*
22	16	0	*FmR1/FmR1*
23	13	4	*FmR1/+*

line. This designation was tentatively adopted for simplicity even thought it is not known whether these mutations arose independently or whether they are on the same alleles of the *I2* gene (Stall and Walter 1965), a monogenic dominant resistance to race 2 found in a hybrid between *L. pimpinellifolium* and *L. esculentum.*

Resistant progeny were also obtained from plants regenerated from normal calli culture that were not treated with fusaric acid (Table 2). Again, the resistance is conferred by a single dominant gene.

R1 progeny produced by selfing the mutant*FmR1/+* were self-fertilized, and the seedlings were screened with *fusarium* slurry to determine the R2 segregation patterns. The results in Table 3 demonstrate that of 23 R1 plants, 6 were homozygous mutants (*FmR1/FmR1*), 6 were homozygous normal (+/+), and 11 were heterozygous (*FmR1/+*). The fit of the composition of the R1 to the theoretical pattern 1 : 2 : 1 confirms that the original regenerated plant was heterozygous for a dominant resistance allele.

Additional evidence for Mendelian inheritence is provided by the results obtained from selfed and backcrossed of random R2 isolates. The number of resistant and susceptible progeny resulting from these crosses are presented in Table 4. These data are con-

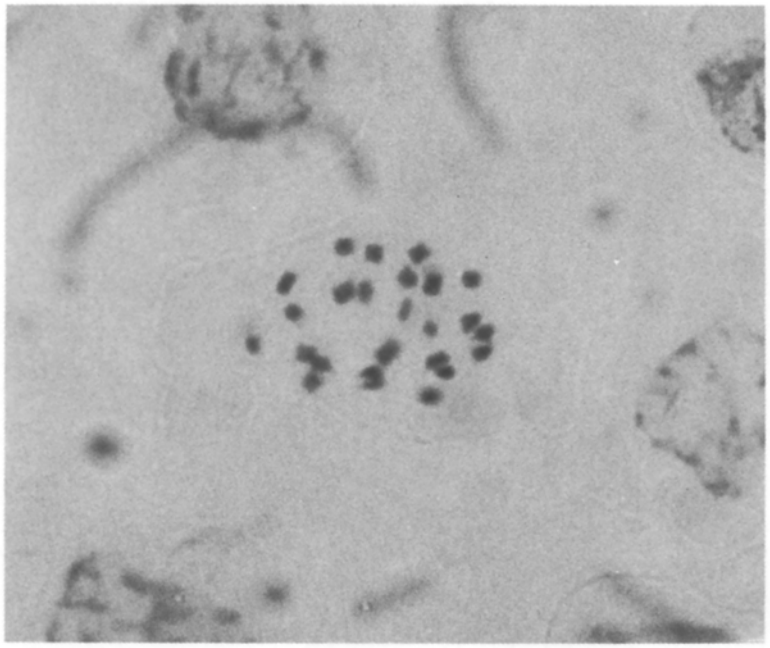

Fig. 3. Toluidine Blue squash preparation from a root of an R3 isolate (derived from the mutant FmR7/+) demonstrating the diploid chromosome number (2n=24)

Table 4. Segregation for response to *Fusarium* wilt reaction in crossed and self progenies of random R2 isolates

Cross mutant (R2 isolate)	Resistant		Susceptible		Expected ratio	Presumed R2 genotype
	Obs.	Exp.	Obs.	Exp.		
UC-82×FmR12/No. 10	10	10	10	10	1:1	*FmR12/+*
FmR12/No. 11×UC-82	0	0	6	6	0:1	+/+
FmR12/No. 19×UC-82	32	30	28	30	1:1	*FmR12/+*
UC-82×FmR12/No. 21	6	6	0	0	1:0	*FmR12/FmR12*
UC-82×FmR3/No. 2	7	9	4	3	1:1	*FmR3/+*
UC-82×FmR3/No. 11	8	9	1	0	1:0	*FmR3/FmR3*
UC-82×FmR3/No. 16	17	17	0	0	1:0	*FmR3/FmR3*
UC-82×FmR10/No. 4	13	13	0	0	1:0	*FmR4/FmR4*
FmR12/No. 12 (selfed)	10	9	4	3	3:1	*FmR12/+*
FmR3/No. 10 (selfed)	21	21	7	7	3:1	*FmR3/+*

Expected values have been calculated assuming *Fusarium*-resistance to be transmitted as a monogenic dominant character

sistent with the ratios 3 resistant to 1 susceptible and 1 resistant to 1 susceptible that are expected from self- and backcrosses, respectively, of a heterozygous individual where the resistance is conferred as single dominant. Additional analysis of the resulting progeny confirmed that the R2 isolates, FmR12 No. 11 and FmR12 No. 21, FMR3 No. 11 and FmR3 No. 16, respectively, are homozygous for susceptible and resistant.

Since high frequency of chromosome breakage and partial chromosome loss has been reported in plants regenerated from tissue culture (McCoy et al. 1983), it was desirable to determine the chromosome number in these mutants. To avoid the possibility of chimeral in the R0 progeny, chromosome counts were performed in *fusarium*-resistant plants of the R3 progeny. A normal diploid (2n=24) was observed in metaphase in root cells of all 13 mutants (Fig. 3).

Discussion

The results presented in this paper indicate that it was possible to isolate *fusarium*-resistant tomato lines from both fusaric acid-challenged and non-challenged protoplasts. In both cases, a single dominant type resistance

was recovered and transmitted through several sexual generations. This definitely establishes that these characters are due to mutations and not to stable epigenetic changes. Also, it confirms the usefulness of protoplasts as a source for either selecting or recovering disease-resistant mutants.

The recovery of disease-resistant mutants without selection pressure was not a surprise since there are reports of frequent recovery of disease-resistant regenerates from unselected cultures (Brettel et al. 1980; Larkin and Scowcroft 1983; Umbeck and Gengenbach 1983; Ling et al. 1985). It is noteworthy to mention that Evans and Sharp (1983) recovered 13 single gene mutations from 230 regenerated tomato plants obtained without any selection pressure. Whether any similar mutations (e.g. Jointless pedicle, green base, etc.) have ocurred in our *Fusarium*-resistant mutants remains to be seen.

The recovery of only dominant mutations is expected because the isolation of recessive mutations was essentially precluded by the use of diploid protoplasts. However, we can not rule out the possibility that a recessive mutation could have been isolated in the R2 progeny had it been that we discarded all susceptible plants in the R1 progeny. Since we recovered more than one mutation in each experiment, it is possible that these mutations did not originate independently (Table 1). It is likely that within the same experiment, these mutants might have been derived from the same callus since we harvested more than one shoot per callus. Genetic crosses can test the allelism of these mutants within the same experiment and among the 4 different experiments.

The evidence that we can obtain a single dominant gene for resistance (using fusaric acid) does not necessary confer on fusaric acid a role in disease development. Nonetheless, it could open the road to the use of other non-specific toxins for selecting disease-resistant mutants. In his review, Wenzel (1985) discussed several reports where resistant-mutants were recovered using non-host specific toxins. However, none reported the mode of inheritance of the recovered resistance or the nature of the defense mechanism(s) involved. Work is in progress to determine the mechanism(s) of the recovered resistance gene(s) (*FmR*) in comparison with that of the *I2* gene. More importantly, complementation tests are underway to determine if these mutations which arose from cell cultures are allelic to that of the *I2* gene.

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