Theor. Appl. Genet. 58, 55-58 (1980)

Reversion of Texas Male-sterile Cytoplasm Maize in Culture to Give Fertile, T-toxin Resistant Plants

R.I.S. Brettell and E. Thomas
Friedrich Miescher-Institut, Basel (Switzerland)

D.S. Ingram
Botany School, University of Cambridge, Cambridge (England)

Summary. Plants carrying Texas male-sterile (Tms) cytoplasm are normally sensitive to *Drechslera maydis* T-toxin. Tissue cultures were initiated from immature embryos of maize carrying Tms-cytoplasm, and plants were regenerated after selection for resistance to T-toxin. Fertile, T-toxin resistant plants were obtained from the unselected control cultures as well as from the selected material. In addition, one regenerant from an unselected culture was fertile and T-toxin sensitive. The progeny of the regenerants showed the phenotype of the female parent with respect to pollen-fertility, and T-toxin resistance. The data are consistent with the heritable changes observed being the result of the expression of an altered mitochondrial genome.

Key words: *Zea mays* L. – T-toxin – Texas male-sterility – Tissue culture – Mitochondria

Introduction

Various methods for the selection of toxin-resistant plant tissue and cell cultures have been described (Maliga 1978; Strauss et al. 1980). These offer a practical means of generating novel disease-resistant plants in cases where the disease-causing organism produces a toxin which itself is a determinant of pathogenicity (Brettell and Ingram 1979).

The technique of recurrent selection, using cultures grown on agar-stabilised media without mutagenic treatments, has been applied to tobacco (Maliga et al. 1973) and maize (Gengenbach and Green 1975; Brettell et al. 1979) for the isolation of toxin-resistant cultures. In maize, resistant lines were selected from Texas male-sterile cytoplasm cultures (Tms-cultures) which are normally susceptible to T-toxin produced by race T of the fungus *Drechslera maydis* (Nisikado) Subram. and Jain (=*Helminthosporium maydis* Nisikado). Plants regenerated from the resistant cultures were found to be resistant to T-toxin and to the disease Southern Corn Leaf Blight caused by *D. maydis* (Gengenbach et al. 1977). In this paper, we describe a further analysis of plants regenerated from selected Texas male-sterile maize tissue cultures and also show that plants resistant to T-toxin may be regenerated from unselected cultures.

Materials and Methods

Culture Initiation and Plant Regeneration

Complex shoot-forming cultures were initiated from immature embryos of maize as described in a previous paper (Brettell et al. 1979). Cultures carrying Texas male-sterile (Tms) cytoplasm were initiated from embryos resulting from the following crosses: (Wf9T/W22 × AI88N *rf rf*) × W22 *rf rf* and (Wf9T/W22 × AI88N *rf rf*) × AI88N *rf rf*. Cultures carrying normal (N) cytoplasm were initiated using embryos from self-pollinated plants of AI88N *rf rf*. All lines were maintained by serial subculture on the modified MS-medium (Murashige and Skoog 1962) recommended by Green and Phillips (1975), with biotin added at 4.0 $\mu g\ l^{-1}$ and 2,4-dichlorophenoxyacetic acid (2,4-D) added at 1.25 $mg\ l^{-1}$. Plants were regenerated by reducing the concentration of 2,4-D in the culture medium to 0.25 $mg\ l^{-1}$ for four weeks, and then transferring the expanding shoots to hormone-free medium in cylindrical plastic containers (50 × 50 × 60 mm) incubated at 26°C under light of 3000 lux (16 h day). Regenerant plantlets were picked out from the agar medium, placed in soil and allowed to develop in a phytotron (24°C, 12 h day (3500 lux): 20°C, 12 h night) for ten days before final transfer to glasshouse or field.

Selection of T-toxin Resistant Cultures

Details of the preparation of T-toxin and the selection procedure have been given previously (Brettell et al. 1979). T-toxin was extracted from cultures of *Drechslera maydis* race T and incorporated in the culture medium as described. Small sectors of viable tissue were selected from cultures carrying Tms-cytoplasm (Tms-

0040-5752/80/0058/0055/$ 1.00

cultures) that had been incubated for eight weeks on medium containing a toxic concentration of T-toxin extract (2 ml l^{-1}). Shoot-forming, T-toxin resistant cultures obtained from two such sectors were maintained on medium containing 4 ml l^{-1} T-toxin.

Analysis of Regenerant Plants and their Progeny

Plants were regenerated from cultures maintained for more than one year on agar medium. In addition to the Tms-cultures selected for T-toxin resistance, the experiments included Tms-cultures never exposed to T-toxin and cultures carrying normal cytoplasm (N-cultures). The toxin response of the regenerant plants was assessed by applying 30 µl undiluted toxin extract towards the base of a rapidly growing young leaf, a small area near the midrib of the leaf having been abraded to facilitate application. A positive (sensitive) reaction was characterised by the development of a long chlorotic streak, which was not observed when leaves of plants carrying N-cytoplasm were treated with toxin. The regenerant plants were also tested for pollen development, by removing anthers from the plant prior to dehiscence, and treating them with a solution of iodine (5 g l^{-1}) in potassium iodide (10 g l^{-1}). The percentage of pollen grains that were normally developed and gave a dark staining reaction was assessed microscopically.

Seed of the regenerant plants were sown in the field and glasshouse, and the resulting plants were likewise tested for their response to T-toxin and scored for the production of mature starch-filled pollen.

Results

A total of 126 plants were regenerated from cultures maintained for more than one year in vitro. These regenerants showed a tendency to tiller and were generally stunted, reaching a height of no more than 80 cm at maturity. In addition, many of the tassels were composed of a mixture of male and female flowers, a deviation from normal development that is sometimes observed in seed-derived plants grown in the glasshouse (Bonnett 1966).

Fifty-eight plants were regenerated from one Tms-culture line which had been selected for T-toxin resistance and maintained for at least three subcultures on medium containing T-toxin. All gave a resistant reaction when T-toxin was applied to a young expanding leaf (Table 1), and 56 had more than 50% starch-filled pollen. In two cases the apical inflorescence was composed mainly of pistillate spikelets and the few staminate florets contained shrivelled anthers which failed to produce pollen. These were distinguished from the truly sterile tassels characteristic of Tms cytoplasm, since the same abnormalities in development were also observed among the plants regenerated from N-cultures.

The most interesting results were obtained from control Tms-cultures never exposed to toxin, from which 31 fertile, toxin-resistant plants were regenerated, compared to 19 which were sterile and toxin-sensitive (Table 1). One plant regenerated from a Tms-culture never exposed to toxin, gave a sensitive toxin reaction, yet produced two tassels carrying well-developed fertile anthers, the only instance where there was an apparent break in the linkage between T-toxin sensitivity and male-sterility.

In contrast to the primary regenerants, the progeny of the plants regenerated from culture were uniform and had a normal morphology reaching a height of 2 m or more at maturity. In almost all cases they consisted of a single stem with an apical staminate tassel (no pistillate spikelets) and two or more lateral cobs. The plants all expressed the phenotype of the female parent with respect to

Table 1. T-toxin sensitivity and male-sterility in plants regenerated from embryo-derived maize cultures maintained on agar-stabilised medium for more than one year

Culture	Toxin reaction of regenerants	Pollen > 80% starch-filled	Pollen 50-80% starch-filled	No. mature pollen, tassel with majority of spikelets pistillate	No. mature pollen, tassel development normal
Texas male-sterile cytoplasm culture, selected for T-toxin resistance	sensitive	–	–	–	–
	resistant	45	11	2	–
Texas male-sterile cytoplasm culture, never exposed to T-toxin	sensitive	1	–	5	19
	resistant	22	9	4	–
Normal cytoplasm culture, never exposed to T-toxin	sensitive	–	–	–	–
	resistant	2	2	4	–
Total 126 plants					

Table 2. Analysis of the progeny of plants regenerated from embryo-derived maize cultures

	Female parent	Male parent	No. of crosses	No. of fertile T-toxin resistant plants	No. of sterile T-toxin-sensitive plants
A	Fertile T-toxin resistant plant from a Tms-culture selected for toxin-resistance	Fertile T-toxin resistant plant from a Tms-culture selected for toxin-resistance	8	58	0
B	Sterile T-toxin-sensitive plant from an unselected Tms-culture	Fertile T-toxin-resistant plant from a Tms-culture selected for toxin-resistance	3	0	36[a]
C	Fertile T-toxin resistant plant from an unselected Tms-culture	Fertile T-toxin resistant plant from an unselected Tms-culture	1	15	0
D	Fertile T-toxin resistant plant from an unselected N-culture	Fertile T-toxin resistant plant from a Tms-culture selected for toxin-resistance	1	24	0

[a] 3 plants showed partial fertility, but in each case less than 5% of the pollen produced was starch-filled

pollen-fertility and toxin-sensitivity (Table 2), and suggest that a dominant nuclear mutation is not the cause of fertility restoration in the primary regenerants. If a heterozygous dominant mutation were responsible, then a proportion of the progeny of self-pollinated, fertile toxin-resistant regenerants derived from Tms cytoplasm would be male-sterile; yet all were male-fertile (Table 2,A). On the other hand, a homozygous dominant mutation is excluded by the failure to transmit the fertility to male-sterile toxin-sensitive regenerants (Table 2,B).

Discussion

Gengenbach et al. (1977) described the regeneration of plants from cultures carrying Texas male-sterile cytoplasm (Tms-cultures) which had been selected for resistance to T-toxin by serial subculture on medium containing T-toxin. Fifty-two of 65 plants regenerated after five or more subcultures in the presence of toxin were fully male-fertile and toxin-resistant, whereas an unspecified number of plants regenerated from unselected control cultures were male-sterile and toxin-sensitive. Mitochondria isolated from toxin-resistant cultures were insensitive to the effects of the toxin, and in addition both male-fertility and toxin-resistance showed maternal inheritance, suggesting that the selection had occurred in an extranuclear (most likely mitochondrial) genome (Gengenbach et al. 1977).

In our experiments all plants regenerated from Tms-cultures exposed to T-toxin were resistant to the toxin, and the majority were also fully male-fertile. Thus far, the data are entirely consistent with those of Gengenbach et al. (1977). However, a significant number of T-toxin-resistant plants were also regenerated from control Tms-cultures which had been maintained for more than one year on agar medium without exposure to T-toxin. Again there was a close link between male-sterility and toxin-sensitivity. The failure of some of the toxin-resistant regenerants to produce fertile pollen may be ascribed to the developmental abnormalities often observed in the plants regenerated from cultures with normal cytoplasm. The fact that the first generation progeny of the regenerants were indistinguishable from plants that had been grown from seed without an intervening culture phase implies that these abnormalities may either be the result of physiological disturbances occurring in the tissue culture environment, or be due to chromosomal or gene mutations subsequently eliminated during sexual reproduction.

The analysis of the primary regenerants suggests that under the conditions of tissue culture there occurs in the Tms-cytoplasm material a stable genetic change which is responsible for the reversion to male-fertility and T-toxin resistance. This in itself is surprising in view of the lack of instability in the expression of Texas male-sterility under field conditions (Duvick 1965).

The analysis of the progeny of the regenerants excludes the involvement of a dominant nuclear mutation. The possibility of a recessive mutation is not consistent with the results of Gengenbach et al. (1977) when they regenerated male-fertile resistant plants from Tms-cultures and crossed them with pollen of the maintainer line

AI88N *rf rf*. In this case all progeny were male-fertile and toxin-resistant. Thus, we would support the hypothesis that the reversion of Tms-cytoplasm in culture to produce fertile T-toxin-resistant plants is the consequence of an alteration in the mitochondrial genome. This is reflected in the appearance of T-toxin-resistant mitochondria in selected cultures derived from Tms cytoplasm (Gengenbach and Green 1975; Brettell et al. 1979). The regeneration of one male-fertile, T-toxin-sensitive plant may reflect a true genetic separation of two closely linked characters (male-sterility and T-toxin-sensitivity), but alternatively might simply have been the result of reversion occurring during the development of the plant.

Kemble and Bedbrook (1980) have recently examined preparations of mitochondrial DNA from seed produced by two of our plants regenerated from Tms-cultures selected for T-toxin resistance. These showed the characteristic mitochondrial DNA banding pattern of Tms-cytoplasm. Thus a 2350 base pair DNA species found in N-cytoplasms is absent in the plants regenerated from selected Tms-cultures. This suggests that the reversion is not due to an increase in N-type mitochondrial genomes which might already be present in Tms-cytoplasm. To explain the heritable changes in the normally stable Tms-cytoplasm, we would propose another as yet undetected change in the genome of Tms-cytoplasm mitochondria. This might result from a mutation or loss of mitochondrial DNA. On the other hand, a recombinational event of the type first observed in yeast (Thomas and Wilkie 1968) may be involved. Evidence for mitochondrial recombination in higher plants has recently been described (Belliard et al. 1979).

In conclusion, the precise nature of the event responsible for reversion and how it may be influenced by the conditions of tissue culture remain obscure, and perhaps await a better understanding of the processes of DNA replication and recombination in the plant mitochondrial genome.

Acknowledgement

The authors are indebted to Brigitte Bolliger for technical assistance, to Drs. R.B. Flavell and R.J. Kemble and their colleagues at the Plant Breeding Institute, Cambridge, England, for valuable discussion, and to Dr. B. Gengenbach for supplying maize lines carrying Tms and normal cytoplasm. D.S. Ingram acknowledges receipt of an Agricultural Research Council research support grant. Work on *D. maydis* in the U.K. was carried out under M.A.F.F. Licence No. 11414/169 issued under the Destructive Pests and Diseases of Plants Order, 1965.

Literature

Belliard, G.; Vedel, F.; Pelletier, G. (1979): Mitochondrial recombination in cytoplasmic hybrids of *Nicotiana tabacum* by protoplast fusion. Nature **281**, 401-403

Bonnett, O.T. (1966): Inflorescences of maize, wheat, rye, barley and oats: their initiation and development. University of Illinois, College of Agriculture, Agricultural Experiment Station, Bulletin 721

Brettell, R.I.S.; Goddard, B.V.D.; Ingram, D.S. (1979): Selection of Tms-cytoplasm maize tissue cultures resistant to *Drechslera maydis* T-toxin. Maydica **24**, 203-213

Brettell, R.I.S.; Ingram, D.S. (1979): Tissue culture in the production of novel disease-resistant crop plants. Biol. Rev. **54**, 329-345

Duvick, D.N. (1965): Cytoplasmic pollen sterility in corn. Adv. Genet. **13**, 1-56

Gengenbach, B.G.; Green, C.E. (1975): Selection of T-cytoplasm maize callus cultures resistant to *Helminthosporium maydis* race T pathotoxin. Crop Sci. **15**, 645-649

Gengenbach, B.G.; Green, C.E.; Donovan, C.M. (1977): Inheritance of selected pathotoxin resistance in maize plants regenerated from cell cultures. Proc. Nat. Acad. Sci. **74**, 5113-5117

Green, C.E.; Phillips, R.L. (1975): Plant regeneration from tissue cultures of maize. Crop Sci. **15**, 417-421

Kemble, R.J.; Bedbrook, J.R. (1980): Low molecular weight circular and linear DNA in mitochondria from normal and male-sterile *Zea mays* cytoplasm. Nature **284**, 565-566.

Maliga, P. (1978): Resistant mutants and their use in genetic manipulation. In: Frontiers of plant tissue culture 1978 (ed.: Thorpe, T.A.), pp. 381-392. Calgary: The International Association for Plant Tissue Culture

Maliga, P.; Sz. Breznovits, A.; Márton, L. (1973): Streptomycin-resistant plants from callus culture of haploid tobacco. Nature New Biol. **244**, 29-30

Murashige, T.; Skoog, F. (1962): A revised medium for rapid growth and bioassays with tobacco tissue cultures. Physiol. Plant. **15**, 473-497

Strauss, A.; Gebhardt, C.; King, P.J. (1980, in press): Methods for selection of drug-resistant plant cell cultures. In: Tissue culture methods for plant pathologists (eds.: Ingram, D.S.; Helgeson, J.P.). Oxford: Blackwell

Thomas, D.Y.; Wilkie, D. (1968): Recombination of mitochondrial drug-resistance factors in *Saccharomyces cerevisiae*. Biochem. Biophys. Res. Commun. **30**, 368-372

Received February 15, 1980
Communicated by P. Maliga

Dr. R.I.S. Brettell
Friedrich Miescher-Institut
Postfach 273
CH-4002 Basel (Switzerland)

Dr. D.S. Ingram
Botany School
University of Cambridge
Downing Street
Cambridge, CB2 3EA (England)

Dr. E. Thomas
Rothamsted Experimental Station
Department of Biochemistry
Harpenden, Herts. AL5 2JQ (England)