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Induction of atrazine resistance and somatic embryogenesis in *Solanum melongena*

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Abstract The effects of atrazine on cotyledon cultures of *Solanum melongena* were investigated with a view to establishing a system for in vitro selection of resistant mutants. At herbicide levels producing little growth inhibition some chlorophyll loss occurred associated with the production of albino shoots. At 15 mg/l bleaching was more pronounced and was accompanied by the development of necrotic spots; however, efficient bleaching was associated with severe suppression of growth. Mutagenesis with EMS resulted in herbicide-resistant mutants based on the embryogenic ability of mutagenised explants placed on medium containing selective levels of sucrose (0.2%) and atrazine (15 mg/l). Different morphogenetic responses were observed when the levels of sucrose (0.2–5%) were altered. Somatic embryogenesis was observed at low sucrose concentrations (0.2–0.5%). Both embryogenesis and shoot regeneration occurred in 1% sucrose. Shoot regeneration was maximum in 2% sucrose and the regenerating ability decreased with a further increase in sucrose concentration (3–5%). However, lowering of sucrose concentration from 2% to 0.2% caused complete bleaching, permitting the selection of herbicide-resistant mutants.

Key words *Solanum melongena* · Mutagenesis · Atrazine resistance · Somatic embryogenesis · Shoot cultures

Introduction

Crop plants have severe sensitivity to herbicides inhibiting photosynthesis (Grassel et al. 1982). Triazine herbicides, such as atrazine and terbutryn, have been widely used for weed control because of their low cost and their effectiveness against a broad spectrum of weeds. However, some agronomically important crops are sensitive to triazine. The use of plant cell-cultures provides a new approach to the efficient production of resistant crops (Chaleff and Ray 1984; Maliga 1984).

A potential source of triazine resistance is provided by the chloroplasts of naturally occurring resistant weed biotypes which have been characterized in a number of species (Bandeen et al. 1982; Arntzen and Duesing 1984). It would be valuable to be able to isolate intact chloroplasts from a plant demonstrating chloroplast-encoded herbicide resistance or possibly increased photosynthetic efficiency (Cseplo et al. 1985; Menczel et al. 1986), then transforming these organelles to the recipient protoplast and regenerating a genetically modified plant.

A rapid and simple protocol for obtaining plastome-encoded antibiotic-resistant mutants of solanaceous plants was reported by Mc Cabe et al. (1990). Markers for studies on organelle inheritance and the interactions between nuclear and cytoplasmic genomes in higher plants have been developed by a number of workers (Cseplo and Maliga 1984; Fluhr et al. 1985). Other plastid mutants which have generated interest include those resistant to herbicides inhibiting photosynthesis (Cseplo et al. 1985; Menczel et al. 1986) and chlorophyll deficiency resulting from either large-scale deletions of plastid DNA (Day and Ellis 1985) or from point mutations (Hosticka and Hanson 1984; Svab and Maliga 1986). This approach is based on the use of an efficient plastome-targeted mutagen, Nitroso methyl urea (NMU) or ethyl methane sulphonate (EMS), and a highly regenerative leaf or cotyledon explant system in which the herbicide causes bleaching and suppresses adventitious shoot initiation. EMS induces mutations in plastid DNA. The plastid DNA mutations reported include changes that reduce plastid pigmentation and produce variegated plants. Atrazine at a concentration

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of 15 mg/l causes severe growth inhibition and only those mutants resistant to the herbicide are selected.

Previous reports of somatic embryogenesis showed that there are two stages in the development of somatic embryos: the induction of cells with embryogenic competence followed by the regeneration of plants (Kohlenbach 1978). Somatic embryos have been derived from zygotic embryos on MS medium supplemented with IAA in egg-plant tissue cultures (Yamada et al. 1967). The induction of a high frequency of somatic embryos by the unique action of NAA in leaf-derived callus of eggplant was reported by Gleddie et al. (1983). The appearance of somatic embryos, however, was restricted to media with NAA. This pronounced NAA specificity for embryogenesis is unique for eggplant. In the present investigation we describe the unique ability of low sucrose which reverts organogenesis to embryogenesis, and the selection of atrazine resistance using mutagenised organogenic explants, in *Solanum melongena*.

Materials and methods

Seeds of *S. melongena* cv Pusa Purple Round were soaked for 24 h in sterile distilled water at 25°C. The imbibed seeds were incubated in culture medium for 24 h in which 0.1% EMS had been dissolved, and were subsequently washed under tap water. Mutagenised seeds were surface-sterilized with 0.1% $HgCl_2$ for 3–5 min followed by three rinses with sterile distilled water and then inoculated on the surface of MS basal medium.

Cotyledons from 3-week-old normal and EMS-treated seedlings were implanted on shoot regeneration medium containing MS salts, 20 gm/l sucrose, 100 mg/l meso-inositol, 0.5 mg/l IAA, 3.0 mg/l BA supplemented with 15 mg/l atrazine (from Northern Minerals Limited Haryana). To visualize the photobleaching effect of atrazine, sucrose levels were gradually reduced from 2% to 0.2%. The pH of the medium was adjusted to 5.7 and solidified with 0.8% Difco bacto agar. A single cotyledon explant was placed in each culture tube and incubated at 25 ± 2°C with a 16-h photoperiod under fluorescent light ($50\ \mu EM^{-2}S^{-1}$) for 4–6 weeks. Resistance to the herbicide was confirmed by a leaf assay of differentiated plants on medium with 0.2% sucrose and 15 mg/l of atrazine.

Results

The effects of including atrazine at various levels in regeneration medium containing 2% sucrose concentration were investigated. A low atrazine concentration (5 mg/l) has little effect on growth and plant regeneration except for the occasional production of albino shoots. At a higher concentration (15 mg/l) bleaching was more pronounced and plants resembled albinos except for slight yellowing with several necrotic spots.

In an effort to maximize the expression of the chlorophyll photo-destructive effect of the herbicide, cotyledon cultures were subjected to a 15-mg/l atrazine concentration at a range of sucrose levels. Normal cotyledons when placed on medium containing 0.2% sucrose and 15 mg/l of atrazine were completely bleached. Mutagenized cotyledons cultured on medium containing selective levels of sucrose and atrazine showed direct somatic embryogenesis from green localised regions of the explants expressing resistance to herbicide. The percentage of explants expressing resistance and the number of embroys are given in Table 1.

When the concentration of sucrose was decreased from 5% to 2%, the number of shoots per explant was considerably increased. Embryogenesis was observed when the concentration of sucrose was further reduced gradually from 2% to 0.2%. Both organogenesis and embryogenesis occurred at a 1% sucrose concentration. Various stages of embryo development (i.e. globlular to heart shape) were observed; at 0.5% sucrose, heart-shaped embryos were predominant. The frequency of embroys and shoots obtained at various levels of sucrose are given in Table 2.

The somatic embroys obtained from these experiments developed into complete plantlets. The stability of resistance to the herbicide was confirmed by a leaf assay of differentiated plantlets on atrazine medium. Embryos appeared from the cut ends of the explant, thus confirming their resistance to the herbicide.

Reciprocal crosses between resistant and sensitive plants revealed a non-Mendelian inheritance pattern. A cross between atrazine-resistant and -sensitive plants produced all resistant phenotypes when the female parent was resistant. However, all the progeny were sensitive when the pollen of the resistant plant was used. The results obtained confirm that resistance is controlled by a maternally inherited mutation (Table 3).

Discussion

This investigation was initiated to provide a protocol for in vitro mutagenesis and for the selection of a valuable agronomic trait, atrazine resistance encoded on chloroplast DNA. In the event, the symptoms of herbicide injury were expressed in cotyledon cultures, and mutagenesis resulted in the efficient generation of triazine-resistant mutants using this approach. In vitro selection protocols for resistance to a range of herbicides affecting photosynthesis (Cseplo et al. 1985), in common with aminoglycoside antibiotics, have certain pecularities consequent upon the site of mutation and the primary symptom produced by these chemicals.

The general efficiency of the mutagens NMU and EMS for the production of chloroplast mutants has been reported by a number of workers (Fluhr et al. 1985; Mc Cabe et al. 1989; 1990; Dix et al. 1990; Janson et al. 1990; Rao et al. 1993). Triazine resistance is a plastid-encoded trait localised in the *PsbA* gene that codes for the 32 kDa thylakoid protein. The herbicide binds to wild-type protein thus displacing normally bound quinone. This alters the redox properties of electron transport and interferes with basic metabolic

Table 1 Induction of triazine resistance in *S. melongena*

Medium	Normal cotyledons		Cotyledons from EMS-treated seedlings	
	% Cultures responding	Mean no. embryos per explant	% Cultures responding	Mean no. embryos per explant
MS + 0.2% sucrose	100	5.55 ± 0.64	100	5.48 ± 0.45
MS + 0.2% sucrose with 15 mg/l atrazine	—	—	28	5.35 ± 1.08

Table 2 Effect of sucrose levels on morphogenic response of cotyledon cultures of *S. melongena*

Sucrose level	No. shoots per explant	No. embryos per explant	No. embryos + shoots per explant
2.0%	18.2 ± 1.27	–	–
1.5%	15.0 ± .040	–	–
1.0%	–	–	3.0 ± 1.08 + 6.35 ± 0.75
0.5%	–	7.62 ± 0.41	–
0.2%	–	5.55 ± 0.64	–

Table 3 Inheritance of atrazine resistance in *S. melongena*

Cross	% Germination	No. seedlings tested	
		Sensitive	Resistant
♀ AS × AR ♂	79	385	0
♀ AR × AS ♂	71	0	435

functions. Herbicide resistance in relation to the *PsbA* gene is used to illustrate the application of cpDNA modification to an agricultural character important to plant breeders. The critical importance of cpDNA to the photosynthetic process, and hence plant productivity, is clear (Rose 1991). CpDNA mutations represent valuable genetic resources for the dissection of the chloroplast genome, as well as for research on the manipulation and transformation of the chloroplast genome (Harring and DeBlock 1990). Cell cultures are sensitive to triazine and can be selected for resistance. Under low light, toxicity symptoms on plants treated with photosynthesis inhibitors can be prevented if the plants are supplied with respirable carbohydrates. The toxic effects of the herbicide were eliminated when non-mutagenised explants were cultured on a medium with 2% sucrose. When the sucrose concentration was reduced to 0.2%, the explants were completely bleached. When mutagenized explants were cultured on medium containing 0.2% sucrose and herbicide , embryos resistant to atrazine were obtained. Malone and Dix (1990) have studied the effects of various levels of simazine and sucrose with a view to establishing a protocol for in vitro selection for herbicide resistance and also studied mutagenesis in shoot cultures of strawberry. They were only successful in isolating solid albino mutants, and failed to develop conditions for resistance selection due to inefficient sorting out of the mutations in the cpDNA of the axillary buds. It appears that cytological events occurring during the release of dormancy of axillary buds in strawberry do not favour the sorting of resistant cpDNA molecules. In contrast, this sorting-out process readily occurs in the cells from which the adventitious shoots differentiate. Alternate culture systems, such as morphogenic explants, might prove more promising as reported in the present study.

In conclusion, we have developed culture conditions for inducing triazine resistance and somatic embryogenesis in eggplant. In order to visualize the photo-bleaching effect of atrazine, the sucrose concentration was reduced from 2% to 0.2%. Interestingly, at low concentrations of sucrose organogenesis reverted to embryogenesis. To our knowledge, this is the first report of triazine resistance and somatic embryogenesis in *S. melongena*. The system we describe for mutant selection may be beneficial for obtaining mutants of crop plants with improved agronomic traits and may provide an opportunity for more individual cells to undergo transformation, thus potentially increasing the number of individual transformants produced per explant in genetic transformation experiments.

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References

Arntzen CJ, Duesing JH (1983) Chloroplast-encoded herbicide resistance. In: Downey K, Voellmy RW, Ahmad F, Shultz J (eds) Advances in gene technology: molecular genetics of plants and animals. Miami winter Symposia, vol. 20. Academic Press, New York, pp 273–294

Bandeen JD, Stephenson GR, Cowett ER (1982) Discovery and distribution of herbicide-resistant weeds in North America. In: Le Baron HM, Grassel J, (eds) Herbicide resistance in plants. John Wiley and Sons, New York, pp 9–30

Chaleff RS, Ray TB (1984) Herbicide-resistant mutants from tobacco cell cultures. Science 223:1148–1151

Cseplo A, Maliga P (1984) Large-scale isolation of maternally inherited lincomycin-resistance mutations, in diploid *Nicotiana plumbaginifolia* protoplast cultures. Mol Gen Genet 196: 407–412

Cseplo A, Medgysey P, Hideg E, Demeter S, Marton L, Maliga P (1985) Triazine-resistant *Nicotiana* mutants from photomixotrophic cell cultures. Mol Gen Genet 200 : 508–510

Day A, Ellis THN (1985) Deleted forms of plastid DNA in albino plants from cereal anther culture. Curr Genet 9 : 671–678

Dix PJ, Mc Kinely CP, Mc Cabe PF (1990) Antibiotic-resistant mutants of *Solanum nigrum* In: Nijkamp HJJ, Vandes Plas LHW, Van Aartrij KJ (eds) Progress in plant cellular and molecular biology. Kluwer Academic Publishers, Dortecht Boston London, pp 169–174

Fluhr R, Aviv D, Galun E, Edleman M (1985) Efficient induction and selection of chloroplast-encoded antibiotic-resistant mutants in *Nicotiana*. Proc Natl Acad Sci USA 82 : 1485–1489

Gleddie S, Keller W, Setterfield G (1983) Somatic embryogenesis and plant regeneration from leaf explants and cell suspensions of *Solanum melongena* (egg plant) Can J Bot 61 : 656–666

Harring MA, De Block (1990) New roads towards chloroplast transformation in higher plants. Physiol Plant 79 : 218–220

Hosticka LP, Hanson MR (1984) Induction of plastid mutations in tomatoes by nitroso methyl urea. J Hered 75 : 242–246

Jansen CE, Snel EMA, Akerboom MJE, Nijkamp HJJ, Hile J (1990). Induction of streptomycin resistance in the wild tomato *Lycopersicon peruvianum*. Mol Gen Genet 220 : 261–268

Kohlenbach H (1978) Comparative somatic embryogenesis. In: Thorpe TA (ed) Frontiers of plant tissue culture, 1978, University of Calgary, Canada, pp 59–66

Maliga P (1978) Cell culture procedures for mutant selection and characterization in *Nicotiana plumbaginifolia*. In: Vasil IK (ed) Cell culture and somatic cell genetics of plants, vol. 1. Academic Press, New York, pp 552–562

Malone RP, Dix PJ (1990) Mutagenesis and triazine herbicide effects of strawberry shoot cultures. J Exp Bot 14 : 463–469

McCabe PF, Timmons AM, Dix PJ (1989) A simple procedure for the isolation of streptomycin-resistant plants in Solanaceae. Mol Gen Genet 216 : 132–137

McCabe PF, Cseplo A, Timmons AM, Dix PJ (1990) Selection of chloroplast mutants. In: Pollard JW, Walker JM (eds) Methods in molecular biology, vol. 6. Plant cell and tissue culture. The Humana Press, Totowa NJ, pp 467–675

Menczel L, Polsby LS, Steinback KE, Maliga P (1986) Fusion-mediated transfer of triazine-resistant chloroplasts: characterization of *Nicotiana tabacum* cybrid plants. Mol Gen Genet 205 : 201–205

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

Rao AV, Ashfaq Farooqui M, Jaya Sree T, Ramana RV, Sadanandam A (1993) EMS-induced streptomycin resistance in *Solanum melongena*. Theor Appl Genet 87 : 527–530

Rose RJ, Thomas MR, Fitter JF, (1990) The transfers of cytoplasmic and molecular genomes by somatic hybridisation. Aust J Plant Physiol 17 : 303–324

Svab Z, Maliga P (1986) *Nicotiana tabacum* mutants with chloroplast-encoded streptomycin resistance and pigment deficiency. Theor Appl Genet 72 : 637–643

Yamada TH, Nakagawa, Sinote Y (1967) Studies on the differentiation in cultured cells. 1. Embryogenesis in three strains of *Solanum* callus. Bot Mag 80 : 68–74