Theor Appl Genet (1984) 67:131–134

Somatic hybridization of an atrazine resistant biotype of *Solanum nigrum* with *Solanum tuberosum*

2. Segregation of plastomes

J. Gressel[1], N. Cohen[1] and H. Binding[2]

[1] Department of Plant Genetics, The Weizmann Institute of Science, Rehovot 76100, Israel
[2] Botanisches Institut, Christian-Albrechts Universität, Olshausenstrasse 40–60, D-2300 Kiel 1, Federal Republic of Germany

Received November 26, 1982; Accepted July 12, 1983
Communicated by G. Wenzel

Summary. Representative regenerated clonal plants from protoplast fusion of *Solanum tuberosum* L. and an atrazine resistant biotype of *S. nigrum* L. were studied to ascertain which plastomes each clone contained. DNA was isolated from fractionated chloroplasts, restricted with DNAases XHO-1, BGL-1, PVU-2 and BAM-H1, and the fragments separated by agarose gel electrophoresis for comparison. No difference could be found between resistant and susceptible biotypes of *S. nigrum* with all four enzymes. XHO-1, BGL-1, BAM-H1 differentiated between *S. nigrum* and *S. tuberosum*. All atrazine resistant regenerants, despite plant morphology, had the plastid DNA pattern of *S. nigrum* while all sensitive ones resembled *S. tuberosum*, even the subclone 38S having a *S. nigrum* morphology and chromosome number.

Key words: Plastome segregation – DNAase restriction – Protoplast fusion-triazine resistance – *Solanum*

Introduction

It was first suggested five years ago when maternally inherited triazine resistance first appeared in *Brassica campestris*, that this might be a useful and interesting trait for interspecies transfer by protoplast fusion (Gressel et al. 1978, 1982b). Triazine resistance is also maternally inherited in *Solanum nigrum* (Gasquez et al. 1981), a species that does not interbreed with *S. tuberosum* (potato). Because photosystem II fluorescence is affected in triazine resistant biotypes (Gasquez and Barralis 1979; Arntzen et al. 1982), resistance is presumed to be on the chloroplast genome. We recently described the variation in morphology, cytology and herbicide resistance within and between some of the 2,705 clonal regenerants received after bulk fusion experiments with protoplasts derived from *Solanum tuberosum* (potato) and a field selected triazine resistant biotype of *S. nigrum* (black-nightshade) (Binding et al. 1982).

The technique of DNAase restriction analysis of plastid DNA allows one to easily ascertain the origin of the plastome in regenerants. It emerged that all triazine resistant plants investigated bore *S. nigrum* DNA and triazine sensitive plants had *S. tuberosum* DNA. This was even true in regenerants of clone 38 that morphologically resembled *S. nigrum* but were triazine sensitive, indicating that susceptibility was due to acquisition of the potato plastome and not due to a back mutation in the *S. nigrum* plastome.

Materials and methods

The origins of the parental plants and the methods used for somatic hybridization, regeneration and clonal typing are all described in Binding et al. (1982). Plants were placed in the dark for three days to deplete starch from the chloroplasts. Five gram deveined and finely cut leaf samples were ground in a mortar and pestle in a minimal amount of a buffer containing 0.35 M sorbitol, 1 mM $MgCl_2$, 1 mM $MnCl_2$, 0.8 mM KH_2PO_4, 10 mM KCl, 50 mM HEPES, 2 mg · ml^{-1}, polyvinyl-pyrrolidone (PVP) M_r360,000, 100 µg ml^{-1} ethidium bromide, 5 mM mercaptoethanol, 2 mM sodium isoascorbate, pH7.6, and the buffer was slowly added to a volume of 20 ml. The slurry was filtered through cheese-cloth and Miracloth, centrifuged for 1 min at 2,500×g and the green upper part of the pellet resuspended and ultracentrifuged on a 20%, 45%, 60% w/v sucrose step gradient made up in the same buffer (but without mercaptoethanol, PVP, isoascorbate or sorbitol) for 1 h at 55,000×g (avg). The green parts were removed and NaCl added to give 0.1 mM, SDS to a volume of 0.5% and proteinase K (Boehringer) to 50 µg ml^{-1}. This was subsequently incu-

bated for 1 h at 37 °C. The nucleic acids were first deproteinized with phenol, then chloroform : isoamyl alcohol (24 : 1), then ether. The nucleic acids were precipitated by adding 0.1 vol 5 M NaCl and 2 vol ethanol to the aqueous phase. This was stored overnight at −20 °C. The nucleic acids were dissolved in 10 mM Tris-HCl, 1 mM EDTA (pH 8.0) and stored at 4 °C until use. These procedures are similar but not identical to those used by Frankel et al. (1979) and Fluhr and Edelman (1981) for Solanaceae. Some regenerant strains were not easily rooted and thus 3 g of leaves from the stem cultures were used. *S. nigrum* of both the normally susceptible and the selected resistant biotype (Gasquez and Barralis 1979; Gressel et al. 1982a) were grown from seed kindly provided by J. Gasquez. Greenhouse grown *S. tuberosum* cv. 'Desiree' and 'Mirka' (kindly provided by Hazera Seed Company) were used as standards instead of the parent potato clones. *Nicotiana sylvestris* DNA was similarly derived from greenhouse grown plants in order to have DNA from a less related member of the Solanaceae.

Prior to each run, aliquots of ca. 10 µg nucleic acids were digested with DNAase restriction enzymes at 37 °C in the manufacturer's (New England Biolabs) suggested buffer mix, for the duration stated in each figure legend. Digestion was stopped by adding gel application buffer. Agarose (Seakem ME-Marine Colloids) slab gels were prepared in 0.8 M Tris-acetate, 0.4 M sodium acetate, 20 mM EDTA, pH 7.8. All runs began at 90 V, until the DNA (with bromphenol blue and glycerol) had entered the gel and then at the voltage and for the duration stated in figure legends. Gels were stained with 1 mg · l^{-1} ethidium bromide, washed, and the fluorescent bands photographed.

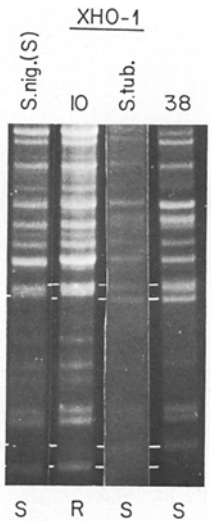

Fig. 1. An example of a restriction pattern with XHO-1. The 0.8% agarose gel was run for 14 h at 60 v. *S. nig.* (*S*) – susceptible *S. nigrum; S. tub.* – *S. tuberosum; S* – susceptible; *R*-resistant

Results and discussion

As described earlier (Binding et al. 1982) eleven primary clones from the fusion experiments with *S. tuberosum* and *S. nigrum* had sufficient qualities to suggest having chromosomal and/or extrakaryotic genetic traits of both *S. nigrum* and *S. tuberosum.*

Several resistant and sensitive segregants were found when the primary regenerants exhibited a mosaic nature under the influence of high levels of atrazine (Binding et al. 1982). These were subcloned separately from leaf pieces by protoplast isolation and regeneration.

The DNA was isolated from plastids of representative clonal types of resistant and sensitive *S. nigrum* of potato, and from *Nicotiana sylvestris.* The DNA was subjected to digestion by sequence-specific restriction enzymes (Figs. 1–3).

It is clear from the data, obtained by using all four enzymes, that there are no differences to be seen between the resistant and susceptible biotypes of *S. nigrum.* It can be concluded that the change to susceptibility is not due to a point mutation in a site of the plastid DNA where any of the restriction enzymes cut; this is expected as only a small proportion of the genome was cut. It also doesn't seem to be due to a major deletion in the plastid DNA as none of the bands of the resistant biotypes seem to have been displaced with respect to the wild-susceptible biotype.

It is clear from the data that *S. tuberosum* and *S. nigrum* are very closely related (Figs. 1–3) and that they are both highly dissimilar from *Nicotiana sylvestris* (Fig. 2). No differences could be discerned between *S. nigrum* and *S. tuberosum* with PVU-2 even though this enzyme cut at many places compared with BAM-H1 and BGL-1. XHO-1 also cut the plastid DNA at many sites and differences can be resolved at three band positions among the smaller fragments (Fig. 1). The differences between the two *Solanum* species were far more visible with BGL-1 and BAM-H1 which restricted the plastid DNA in fewer places. In each case there is only one major band displaced (Figs. 2 and 3). A similar close ressemblence was found in plastid segregants of fused potato and tomato (Schiller et al. 1982). As different enzymes were used in their study than in ours, we cannot say which of these three Solanaceae are more closely related.

These differences in electrophoretic patterns allowed us to quickly deduce whether plastomes in the protoplast regenerants originated from *S. nigrum* or *S. tuberosum.* All of the clones and sub-clones that were atrazine resistant had the *S. nigrum* restriction patterns; all that were susceptible had the *S. tuberosum* restriction patterns (Figs. 1–3, Table 1). This includes segregants of mosaics to resistance and susceptibility such as strain 31 (Fig. 2).

The correlation of plastid restriction patterns and atrazine response strongly indicated that it is controlled by the plastome. This conclusion could be further justified if co-segregation of homoparental plastids and

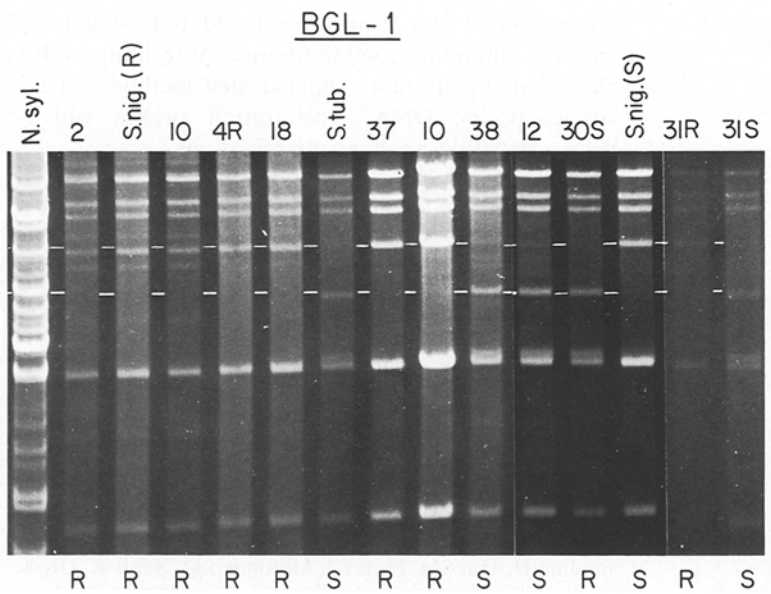

Fig. 2. An example of restriction patterns with BGL-1. The 0.8% agarose gel was run for 14 h at 60 V. The white markers drawn on the gel are at 14 and 9.2 kilo-base pairs as extrapolated from a lambda phage standard digested with Hind III. *S. nig.(R)* – resistant *S. nigrum; S. nig.(S)* – susceptible *S. nigrum; S. tub* – *S. tuberosum*

Table 1. Summary of morphological, cytological and DNA restriction patterns of parents and representative regenerant clones and subclones. Morphological and atrazine resistance data collated from Binding et al. 1982). An *S* or *R* near a clone number refers to a stable subclone which is atrazine susceptible or resistant, respectively

Clone no. and type	Atrazine sensitivity (S-susceptible) (R-resistant)	Restriction pattern (tb = *S. tuberosum;* ng = *S. nigrum*)[a]			
		XHO-1	BGL-1	BAM H-1	PVU-2
S. nigrum Type:	72 chromosomes; leaves rhomboid with denticulate margins and acutely branched; flowers without long calyx hair, corolla ¼ fused and about 9 mm diameter				
38	S	tb (2)	tb (3)	tb (2)	ng = tb (1)
22	R	ng (1)	ng (1)	ng (1)	ng = tb (1)
Morphological	variants – close to *S. nigrum:* > 90 chromosomes; leaves heart to rhomboid with variable to denticulate margins and acutely branched; flowers without calyx hair, corolla ½ to ¼ fused and about 9 mm diameter				
1	R	ng (1)	ng (1)		
2	R	ng (2)	ng (1)	ng (1)	ng = tb (1)
10	R	ng (3)	ng (4)	ng (1)	ng = tb (2)
18	R	ng (1)	ng (1)	ng (1)	
37	R	ng (3)	ng (2)	ng (1)	ng = tb
Hybrid types	> 96 chromosomes; leaves heart shaped to rhomboid with integer to denticulate margins and branch at *right* angles; flowers *with* long calyx hairs, corolla ¾ to zero fusion, and about 23 mm diameter. (Characters in italics are similar to potato)				
4R	R	ng (1)	ng (1)		
4S	S			tb (3)	
12	S	tb (3)	tb (3)	tb (3)	ng = tb (1)
28	R		ng (1)	ng (1)	
30S	S	tb (1)	tb (3)		ng = tb (1)
31R	R	ng (2)	ng (2)	ng (1)	ng = tb (1)
31S	S	tb (1)	tb (3)		
47R	R	ng (1)		ng (1)	

[a] The number in parenthesis refers to the number of separate gel fractionations

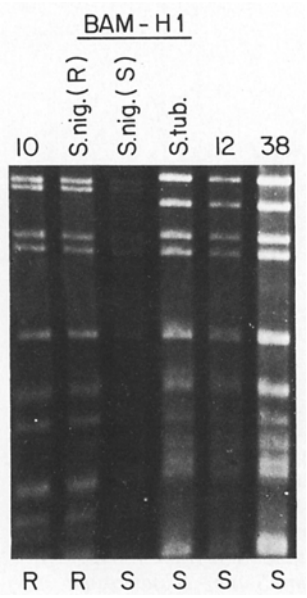

Fig. 3. An example of restriction patterns with BAM-H1. The 1.2% agarose gel was run for 9 h at 75 V. The white markers drawn on the gel are at 17.2 and 13.8 kilo-base pairs as extrapolated from a lambda phage standard digested with HindIII. *S. nig.(R)* – resistant *S. nigrum; S. nig(S)* – susceptible *S. nigrum; S. tub.* – *S. tuberosum*

mitochondria is excluded by restriction analysis of mitochondrial DNA.

The susceptible sub-clone No. 38S that clearly resembles *S. nigrum* in all morphological traits studied as well as in chromosome number possesses plastome DNA characteristic of *S. tuberosum* (Figs. 1–3, Table 1). Thus, it is not a genetic revertant to atrazine susceptibility but is a cybrid containing the nucleus of *S. nigrum* and the plastome of *S. tuberosum.* It developed either from a protoplast/cytoplast fusant or from a protoplast/protoplast fusant via the loss of the potato nucleus.

Segregation of the plastids was probably not totally finished in all fusants at the time of restriction analysis as one mosaic regenerant was obtained after re-isolation of protoplasts of hybrid clones (Binding, unpublished). The method of restriction analysis might not show up small amounts of heterology of plastid types. Schiller et al. (1982) claim that a cross contamination of less than 1–3% could not be detected with the ethidium bromide fluorescent techniques. It is clear that slightly more sophisticated methods such as species-specific DNA hybridization probes will be necessary to find smaller contaminations.

Acknowledgements. We are grateful to S. Bleichman and R. Fluhr who gave us initial instruction in DNA restriction analysis. This collaboration was supported by a travel grant to H. B. by the Deutsche Forschungsgemeinschaft.

References

Arntzen CJ, Pfister K, Steinback K (1982) The mechanisms of chloroplast triazine resistance: alterations in the site of herbicide action. In: Le Baron H, Gressel J (eds) Herbicide resistance in plants. J Wiley and Sons, New York, pp 185–214

Binding H, Jain SM, Finger J, Mordhorst G, Nehls R, Gressel J (1982) Somatic hybridization of a triazine resistant biotype of *Solanum nigrum* with *S. tuberosum.* 1. Clonal variations in morphology and atrazine sensitivity. Theor Appl Genet 63:273–277

Fluhr R, Edelman M (1981) Physical mapping of *Nicotiana tabacum* chloroplast DNA. Mol Gen Genet 181:484–490

Frankel R, Scowcroft WR, Whitfield PR (1979) Chloroplast DNA variation in isonuclear male-sterile lines of *Nicotiana.* Mol Gen Genet 169:129–135

Gasquez J, Barralis G (1979) Mise en evidence de la resistance aux triazines chez *Solanum nigrum* L. et *Polygonum lapathifolium* L. par observation de la fluorescence di feuilles isolees. CR Acad Sci, Ser D 288:1391–1393

Gasquez J, Darmency Y, Compoint JP (1981) Etude de la transmission de la resistance chloroplastique aux triazines chez *Solanum nigrum.* CR Acad Sci, Ser D 292:847–849

Gressel J, Zilkah S, Ezra G (1978) Herbicide action, resistance and Screening in cultures vs. plants. In: Thorpe TA (ed) Frontiers of plant tissue culture 1978. University of Calgary Press, Calgary Alta, pp 427–436

Gressel J, Ammon HY, Fogelfors H, Gasquez J, Kay QON, Kees H (1982a) Discovery and distribution of herbicide-resistant weeds outside North America. In: Le Baron H, Gressel J (eds) Herbicide resistance in plants. J Wiley and Sons, New York, pp 31–55

Gressel J, Ezra G, Jain SM (1982b) Genetic and chemical manipulation of crops to confer tolerance to chemicals. In: McLaren JS (ed) Chemical manipulation of crop growth and development. Butterworth, London, pp 79–81

Schiller B, Herrmann RG, Melchers G (1982) Restriction analysis of plastid DNA from tomato, potato and some of their somatic hybrids. Mol Gen Genet 186:453–459