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Somatic Hybridization of an Atrazine Resistant Biotype of *Solanum nigrum* with *Solanum tuberosum*

Part 1: Clonal Variation in Morphology and in Atrazine Sensitivity

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Summary. Plants were regenerated from protoplast fusion experiments with haploid *Solanum tuberosum* L. and an atrazine resistant biotype of the normally susceptible *S. nigrum* L. Sixty clones which were unlike the parents were selected by types of hairs and leaf pigmentation of young shoots and characterized by: chromosome numbers, response to atrazine, branching, hairs of the calyx, shapes and pigmentation of leaves, and morphology of flowers. Twenty five clones showed vegetatively stable differences from the parental clones; enough combinations of mixed characters suggested the clear origin from fusants of at least nine clones. Observed diversities within and between protoplast-derived clones are interpreted as expressions of variation during the development of the regenerants.

Key words: Somatic hybridization – Clonal variation – *Solanum* – Triazine resistance – Protoplast fusion

Introduction

Somatic hybridization is useful for investigations in intrageneric genetics, in developmental physiology and in plant breeding. In potato (*S. tuberosum*), the technology of protoplast regeneration has been established (Shepard and Totten 1977; Binding et al. 1978; Thomas 1981) so that this species is well suited for both basic and applied somatic hybridization programs, as a model plant and as an important crop (Melchers et al. 1978; Wenzel et al. in press). The experiments described herein were devised to obtain information on the fate of heterospecific genophores in fusion bodies of species belonging to different sections of the genus *Solanum*. *Solanum tuberosum* L. haploid (predominantly dihaploid) clones were used. The other parent was a biotype of *Solanum nigrum* (black nightshade) that was found to be resistant to the herbicide atrazine (Gasquez and Barralis 1979; Gressel et al. 1982 a). The trait is maternally inherited in *Solanum nigrum* (Gasquez et al. 1981) and in other weeds (Souza-Machado 1982). It causes a modification of chloroplast membrane properties (Arntzen et al. 1982) and is thus probably coded for on the plastid genome. In addition to the basic interest in transferring such a trait, the transfer of atrazine resistance into potato could be of great economic importance. An additional procedure to attain this goal would be by inactivating the nuclei of *S. nigrum* prior to fusion (Zelcer et al. 1978; Gressel et al. 1982 b). The two species are not known to interbreed in nature or in the laboratory.

Materials and Methods

The clones of *Solanum tuberosum* L. were established by the Max-Planck-Institut für Züchtungsforschung in Cologne. The clones HH 258, HH 345 and HH 439 were stable dihaploids. Mo 9 was characterized as a monohaploid; the material used, however, was a – predominantly – dihaploid derivative. The atrazine resistant biotype *Solanum nigrum* L. was provided by J. Gasquez, INRA, Dijon. Protoplasts were isolated from a single clone with 72 chromosomes per cell.

Axenic shoot cultures of both species were grown on agar medium B5 (Gamborg et al. 1968) without phytohormones (*S. tuberosum*) or with 2.5 μM 6-benzyladenine (*S. nigrum* and protoplast clones) in Petri dishes. Protoplasts were isolated from axenic shoot cultures; fusion was induced by incubation in 45% polyethylene -glycol M_r 1,500 (Kao and Michayluk 1974) and then in a calcium nitrate solution buffered at pH 10.5 (Keller and Melchers 1973; Binding 1974; Schieder 1974); the protoplasts were cultured mainly according to the protocol devised for potato (Binding et al. 1978), initially in liquid V-KM (Binding and Nehls 1977), later in soft agar (0.125%) as suspension cultures and, from the 18th day on, on solid agar V-KM. The regenerants were subcultured individually from the 30th day on and termed protoplast clones. It is not possible to decide if the protoplast clones of a certain experiment were really each derived from an individual protoplast or fusant. Monoclonal cell clusters may have disintegrated during the bulk culture period, but usually, the cells of a regenerant were in close contact. Agglutinated regenerants may have been subcultured as one individual.

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Shoot formation was obtained on agar medium B5 with 2.5 μM 6-benzyladenine and 10% liquid coconut endosperm (autoclaved); root formation was induced on MS medium (Murashige and Skoog 1962) with 10 μM β-indolylacetic acid prior to the transfer to soil. Protoplast isolation, fusion and regeneration are described in greater detail by Binding and Nehls (1982).

Protoplast clones that did not entirely resemble either parent were selected in early stages of shoot formation, especially by morphology of hairs (Fig. 1). Besides small multicellular glands which formed in all clones, long, pointed simple hairs are scattered over the shoots of potato. Leaves of *S. nigrum* are densely covered by hairs with unicellular glands. Simple hairs are much smaller in *S. nigrum* than in *S. tuberosum* and are mostly found just at the tips of the leaves. Unicellular glands are rarely formed in potato cultures. Criteria for selection were absence of unicellular glands or the presence of extremely long-stalked gland hairs. Chlorophyll deficient shoots were also cloned.

Protoplasts and regenerated clones were cultured at 25 °C under continuous cool white fluorescent illumination (1,500 lux) until transfer onto the agar medium. All other in vitro cultures were grown in a 14 h day length at 2,000–4,000 lux. Subclones were obtained by re-isolation of protoplasts and by inducing adventitious shoots on leaf disks.

Protoplast clones were screened for herbicide sensitivity by planting shoot tips having three small leaves on B5 agar medium with a reduced content of sucrose (1%), 2.5 μM 6-benzyladenine and 10^{-4} M atrazine (2-chloro-4-ethylamino 6-isopropyl-amino-s-triazine). Atrazine was added as a 10 mM ethanolic solution to the autoclaved medium before pouring into Petri-dishes. Each test plate also had shoots of *S. tuberosum* and *S. nigrum* (resistant) as controls. Sensitivity to atrazine was indicated by bleaching after 7 to 20 days. The triazine resistant *S. nigrum* shoot tips were unaffected by the concentration of atrazine used.

Plants were grown in the greenhouse for evaluation of morphological characters and seed formation. Seeds were harvested in most cases after bagging of flower buds. – Chromosomes were counted in meristems stained with aceto-carmine.

Results and Discussion

Protoplasts of *S. tuberosum* and *S. nigrum* (triazine resistant) were fused and cultured as outlined in 'Materials and Methods'. Shoot formation on regenerated calluses began as early as 10 d after transfer to B5 with liquid coconut endosperm. In some clones, shoots appeared much later (up to 17 months). A total of 2,705 clones regenerated shoots and were screened. They derived from six fusion experiments (Table 1). Most of the regenerants completely resembled the phenotype of *S. nigrum* and were discarded. No shoots of totally potato type plants were observed. Investigations to learn about the reasons for the lack of pure potato regenerants revealed that protoplasts of Mo 9 did not regenerate under the applied culture conditions and that PEG affected the protoplasts of all potato clones used in the described experiments.

Elongated gland hairs, high proportions of simple hairs, or chlorophyll deficiencies were found in 60 clones (numbered 1 to 60). Thirtyfive of these had stable differences from either of the parental clones and were saved.

The parental clones and the sufficiently investigated protoplast clones (a total of 24) were divided into four groups by chromosome numbers and morphological markers (Types 1 to 4; Table 2). The characters used for classification are illustrated in figures 2 to 6: a metaphase plate of protoplast clone 13 (Fig. 2), branched bases of young greenhouse plants (Fig. 3), typical leaves of all clones of interest – except three potato clones and protoplast clones not yet growing in the greenhouse (Fig. 4), calyxes of protoplast sub-clones of clone 4 illustrating segregation of hair peculiarities (Fig. 5), and flowers of all flowering clones (Fig. 6; cf. also Fig. 5).

All regenerant clones were assayed on atrazine agar. By this method, it was even possible to detect mixed sensitive/resistant mosaics (Fig. 7). This was confirmed by segregation of the traits in sub-clones of the mosaics. Sensitive clones, bleached by atrazine, recovered after transfer to atrazine-free media (Fig. 8). Atrazine sensitivity was found in ten protoplast clones: 4, 12, 13,

Table 1. Protoplast fusion experiments

Experiment No.	Parents: *S. nigrum* and	Calluses grown	Calluses with shoots	Numbers of protoplast clones of interest	
				Investigations advanced	Investigations restricted by retarded development
I	Mo 9	3849	1932	1, 2, 4, 12, 13, 21, 28, 30, 31, 37, 38, 45, 46, 47, 49, 54, 55	44, 48, 50, 51, 52, 53, 56 to 60
II	HH 258	167	96	18	
III	HH 345	78	56	10	
IV	HH 439	212	137	20, 25, 29	
V	Mo 9	814	421	22, 27	
VI	HH 258	260	63	–	

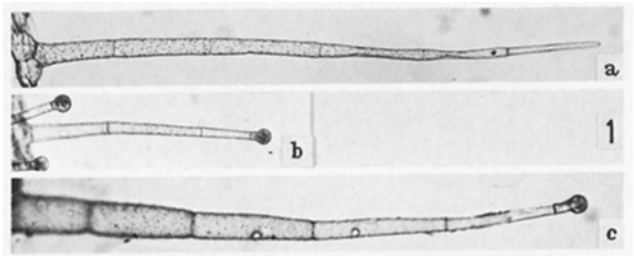


Fig. 1a–c. Leaf hair criteria for the selection of protoplast clones. **a** simple hair of *S. tuberosum;* **b** gland hair of *S. nigrum;* **c** long-stalked gland hair

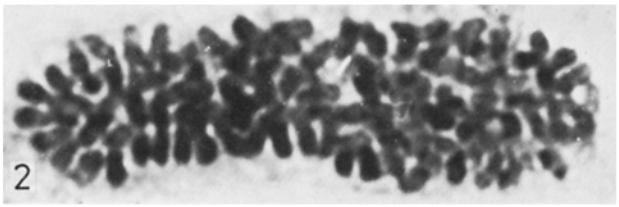
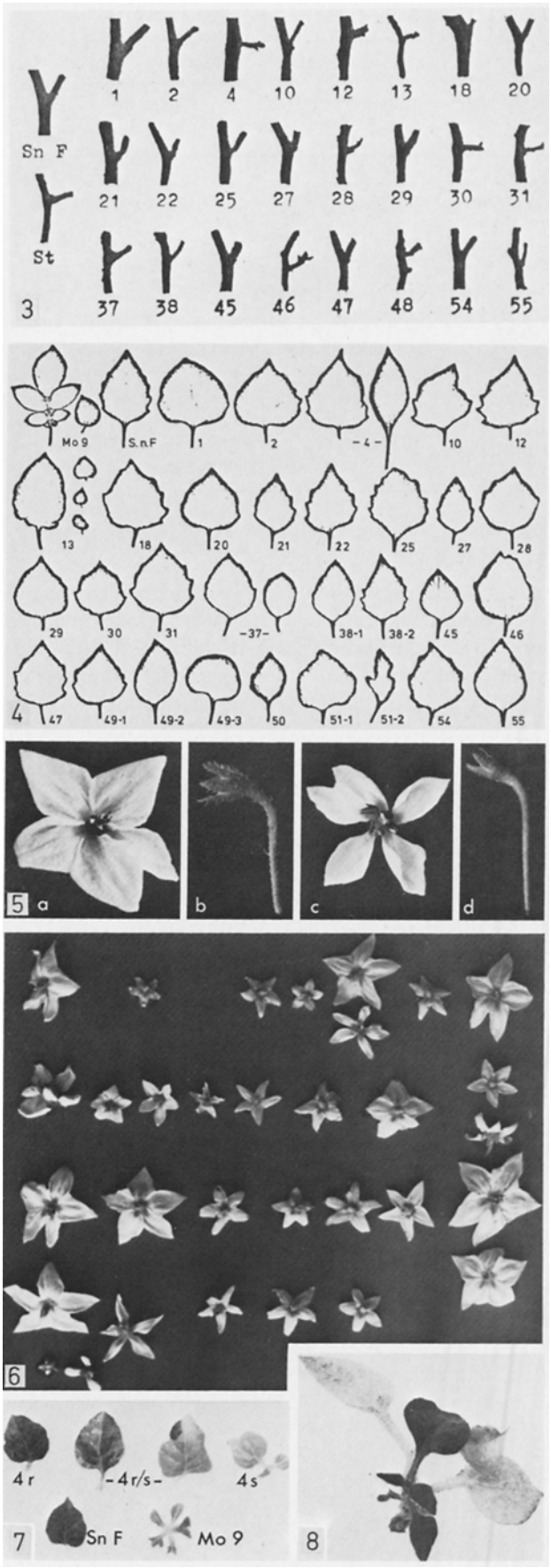


Fig. 2. Photograph of a metaphase plate of the protoplast clone 13 that contained 96 chromosomes. Not all chromosomes in the plain of focus

30, 31, 38, 44, 46, 47, and 55. DNase restriction analysis, presently in progress, has shown that plastid DNA of the sensitive clones 12, 31–1, and 38 have potato plastid DNA. All resistant clones tested have the same restriction pattern as triazine-resistant *S. nigrum* which is identical to that of the triazine sensitive *S. nigrum* wild type (J. Gressel, H. Binding, N. Cohen, unpubl.).

Morphological, pigmental or triazine resistant/sensitive mosaics were observed in 13 protoplast clones. The traits segregated to form sub-clones or seed progenies with vegetatively stable phenotypes (Table 3). None of them completely resembled either



Figs. 3–8. **3** Basal parts of young greenhouse plants for the demonstration of angles of branching (shadowgrams by a photocopier). **4** Leaf shapes of the protoplast clones (shadowgrams by a photocopier). Double numbers indicate sub-clones. Clone Mo 9 was chosen as reference for the potato clones because it was the potato fusion partner in experiment I of which all protoplast clones in Type 3 with strong evidence for hybrid nature derived. **5** Flowers of two sub-clones of protoplast clone 4, demonstrating shapes of corolla (a and c) as well as presence (b) and absence (d) of simple hairs on their calyxes. **6** Flowers of described clones. Top: Mo 9, *S. nigrum,* protoplast clones 1, 2, 4 (2×), 10, 12; second row: protoplast clones 13, 18, 20, 21, 22, 25, 28, 29 (2×); third row: Protoplast clones 30, 31, 37, 38, 45, 46, 47 (2×); fourth row: 49 (4×), 51, 54, 55. **7** A mosaic response to atrazine. The partial chlorotic bleaching of leaves of protoplast clone 4 after 10 d culture of shoots on agar medium with 10^{-4} M atrazine; 4r – a resistant sub-clone; 4r/s – mosaic leaves of the original clone; 4s – a sensitive sub-clone; Sn F – *Solanum nigrum* biotype F; Mo 9 – the clone of potato used in the fusion experiment. **8** The recovery of a sensitive sub-clone of clone 31 from atrazine inhibition conditions; 10 d on atrazine; 14 d on B5

Table 2. Classification of the investigated clones

	Clone number	Chromosome No.	Branching at base: angle	Leaf shape	Leaf margin	Long hair on calyx	Flower size	Corolla fused to a degree of about	Fertility (– means not or not yet)
Type 1 (*S. nigrum*)	21, 22, 27, 38, 55, *S. nigrum*	72	acute	rhomboid	denticulate	–	9±5 mm	1/4	+ (– in 55)
Type 2 (*S. tuberosum*)	parental potato clones Mo 9, HH 258 HH 345, HH 439	24	right	heart-shaped to rhomboid pinnae	pinnate, pinnae integer	+	25±5 mm	3/4	–
Type 3	4, 12, 13, 28, 30, 31, 46, 47, 49	ca. 96 or more	right	heart-shaped to rhomboid	integer to denticulate	+	23±8 mm	3/4 to zero	–
Type 4	1, 2, 10, 18, 20, 25, 29, 37, 45, 54	(ca. 90) mostly much more	acute (±)	heart-shaped to rhomboid	variable, mostly denticulate	–	9±6 mm	1/2 to 1/4	low (– in 29)

parent; hence, mosaic nature by just aggregation of parental cells is excluded. No mosaic markers appeared to be pleiotropic. It is suggested from the occurrence of mosaics that the variation occurred during the development of the protoplast clones. The genetic basis of the mosaic characters has not yet been clarified.

The protoplast clones of Type 3 expressed several biparental and intermediate characters in various combinations. The variation of morphological characters as well as the appearance of atrazine sensitivity were well beyond the clonal variation of protoplast-derived plants of the triazine resistant *S. nigrum* biotype as concluded from more than 1,000 protoplast clones. This also seems to hold true when considering the genotypic nature of the Type 3-clones in relation to those of *S. tuberosum;* clonal variation of morphological characters in protoplast regenerants of potato was limited to much smaller alterations as can be concluded from published data (Wenzel et al. 1979; Shepard et al. 1980; Thomas et al. 1982). These facts strongly support our conclusion that the protoplast clones 4, 12, 13, 28, 30, 31, 46, 47, and 49 are fusants. In Type 1, all protoplast clones differ from *S. nigrum* only by one peculiarity: clones 38 and 55 by expression of atrazine sensitivity; clones 21, 22, and 27 by chlorophyll deficiencies. The clones of Type 4 resemble to more or less degree polyploid protoplast-derived plants of *S. nigrum* and may be explained as variants of this species.

Table 3. Segregation of mosaic characters in sub-clones and seedlings[1]

Mosaic character (+)	Protoplast clone number													
	1	4	13	21	22	25	27	29	30	31	38	46	47	49
Atrazine: sensitive/resistant		+							+	+	+	+	+	
Branching Angle: acute/right														+
Leaf Shape: heart-shaped/ rhomboid/lanceolate		+	+			+								+
Leaf Margin: denticulate/ integer/pinnate		+	+								+			+
Hair on Calyx: long/short		+												+
Flower: larger/small; (+) medium/small								(+)						+
Rays of Corolla: broad/lanceolate		+											+	+
Pigmentation: green/chlorotic (c); pale-green (p); yellow-green (y); white (w)	y			y	w	w	y^1		c	w	p			
Pistil: as long as anthers/ a little (–), much + longer								+			(–)			

The appearance of at least eleven putative hybrids (nine of Type 3; 38; 55) and 14 clear variants among 60 primary clones demonstrates clearly that hair characters and degrees of pigmentation were valid for the selection. The lack of regeneration of *S. tuberosum* protoplasts under the applied conditions was helpful in this context.

The investigations are being continued to follow up the further developments of the various clones and to clarify the nature of the genetic traits which are responsible for the phenotypic characters. Analyses of patterns of plastid DNA restriction fragments, fraction I protein subunits and of isoenzymes in the protoplast clones, sub-clones and S_1 progenies are in progress.

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Literature

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

Binding, H. (1974): Regeneration von haploiden und diploiden Pflanzen aus Protoplasten von *Petunia hybrida* L. Z. Pflanzenphysiol. **74,** 327–356

Binding, H.; Nehls, R. (1977): Regeneration of isolated protoplasts to plants in *Solanum dulcamara* L. Z. Pflanzenphysiol. **85,** 279–280

Binding, H.; Nehls, R. (1982): Techniques of somatic cell hybridization by fusion of protoplasts. In: Techniques in somatic cell genetics (ed. Shay, W.J.), pp. 471–492. New York: Plenum Press

Binding, H.; Nehls, R.; Schieder, O.; Sopory, S.K.; Wenzel, G. (1978): Regeneration of mesophyll protoplasts isolated from dihaploid clones of *Solanum tuberosum* L. Physiol. Plant. **43,** 52–54

Gamborg, O.L.; Miller, R.A.; Ojima, K. (1968): Nutrient requirement of suspension cultures of soybean root cells. Exp. Cell Res. **50,** 151–158

Gasquez, J.; Barralis, G. (1979): Mise en évidence de la résistance aux triazines chez *Solanum nigrum* L. et *Polygonum lapathifolium* L. par observation de la fluorescence des feuilles isolées. C. R. Acad. Sci. (Paris) Sec. D **288,** 1391

Gasquez, J.; Darmency, Y.; Compoint, J.P. (1981): Etude de la transmission de la résistance chloroplastique aux triazines chez *Solanum nigrum*. C. R. Acad. Sci. (Paris) Sec. D **292,** 847

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

Gressel, J.; Ezra, G.; Hain, S.M. (1982b): Genetic and chemical manipulation of crops to confer tolerance to chemicals. In: Chemical manipulation of crop growth and development (ed. McLaren, J.D.), pp. 79–91. London: Butterworth

Kao, K.N.; Michayluk, M.R. (1974): A method for high-frequency intergeneric fusion of plant protoplasts. Planta **115,** 355–367

Keller, W.A.; Melchers, G. (1973): The effect of high pH and calcium on tobacco leaf protoplast fusion. Z. Naturforsch. **28c,** 737–741

Melchers, G.; Sacristan, M.D.; Holder, A.A. (1978): Somatic hybrid plants of potato and tomato regenerated from fused protoplasts. Carlsberg Res. Comm. **43,** 203–218

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

Schieder, O. (1974): Fusionen zwischen Protoplasten aus Mutanten von Sphaerocarpos donnellii. Aust. Bioch. Physiol. Pfl. **165,** 433–435

Shepard, J.P.; Totten, R.E. (1977): Mesophyll cell protoplasts of potato. Isolation, proliferation, and plant regeneration. Plant Physiol. **60,** 313–316

Shepard, J.F.; Bidney, D.; Shahin, E. (1980): Potato protoplasts in crop improvement. Science **208,** 17–24

Souza-Machado, V. (1982): Inheritance and breeding potential of triazine tolerance and resistance in plants. In: Herbicide resistance in plants (eds. Le Baron, H.; Gressel, J.), pp. 185–214. New York: Wiley

Thomas, E. (1981): Plant regeneration from shoot culture-derived protoplasts of tetraploid potato (*Solanum tuberosum* cv. 'Maris Bard'). Plant Sci. Lett. **23,** 84–88

Thomas, E.; Bright, S.W.J.; Franklin, J.; Lancester, V.A.; Miflin, B.J. (1982): Variation amongst protoplast-derived potato plants (*Solanum tuberosum* cv. 'Maris Bard'). Theor. Appl. Genet. **62,** 65–68

Wenzel, G.; Bapat, V.A.; Uhrig, H.: New strategy to tackle breeding problems of potato. In: Proc. intern. symposium on plant cell culture in crop improvement (ed. Sen, S.K.), New York: plenum Press (In press)

Wenzel, G.; Schieder, O.; Przwozny, T.; Sopory, S.K.; Melchers, G. (1979): Comparison of single cell culture-derived *Solanum tuberosum* L. plants and a model for their application in breeding programs. Theor. Appl. Genet. **55,** 49–55

Zelcer, A.; Aviv, D.; Galun, E. (1978): Interspecific transfer of cytoplasmic male sterility by fusion between protoplasts of normal *Nicotiana sylvestris* and x-ray irradiated protoplasts of male-sterile *N. tabacum*. Z. Pflanzenphysiol. **90,** 397–407

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