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Somatic Hybridization in *Petunia*

Part 2: Heteroplasmic State in Somatic Hybrids Followed by Cytoplasmic Segregation into Male Sterile and Male Fertile Lines*

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Summary. Two types of cytoplasmic hybrids were obtained by protoplast fusion. These contained either one or the other original parental nucleus and heteroplasmon, a mix of plasmons inducing cytoplasmic male sterility and fertility. In subsequent generations, following selfing, stable male sterile and male fertile lines segregated from single fertile cytoplasmic hybrid plants. These data demonstrated the existence of a heteroplasmic state in the somatic hybrids and the occurrence of cytoplasmic segregation of the heteroplasmon into homoplasmons following the first and the second meiotic cycles.

Key words: Petunia – Somatic hybridization – Heteroplasmic or homoplasmic state – Cytoplasmic hybrid (cybrid) – Cytoplasmic segregation – Cytoplasmic male sterility.

Introduction

One of the features of the protoplast fusion techniques as compared with conventional breeding is the possibility of achieving the heteroplasmic state in the extra-nuclear genetic elements in addition to, and independent of, the nuclei. This possibility allows one to directly study genome-plasmon interactions, as well as the expression and the transmission of the cytoplasmic genetic elements in subsequent generations. Thomas et al. (1979) reviewed recently the work on somatic hybridization in plants. In only a few cases was cytoplasmic inheritance the main objective of the study (Gleba 1978; Belliard et al. 1977, Zelcer et al. 1978; Izhar and Power 1979), and in even fewer cases was a follow-up study conducted to describe the relationships between the different plasmons and their fate in subsequent generations (Gleba 1979; Belliard et al. 1978; Izhar and Power 1979; Aviv et al. 1980).

Our studies deal with extra-nuclear genetics in crop species using cytoplasmic male sterility (cms) as the major marker. In a recent paper (Izhar and Power 1979) the transfer of cms through protoplast fusion from a cms *P. hybrida* line to a line of *P. axillaris* was reported. In the present paper we describe somatic hybrids, obtained by protoplast fusion, which contain a single parental nucleus and a heteroplasmon of cms and normal genetic elements. Following the first and second meiosis cytoplasmic segregation into stable cytoplasmic male sterile and normal lines occurred.

Materials and Methods

The details about the materials and the methodology used in these experiments were described previously by Izhar and Power (1979), and only the main points will be described here.

Two lines were used for protoplast isolation and fusion: *Petunia hybrida* (Hort.) Vilm. line 2426, a cms line from the collection at the Volcani Center, Bet Dagan, and *Petunia axillaris* (Lam) B.S.P. line 2785, a fertile line from Dr. K.C. Sink, Michigan State University, East Lansing, MI. The following lines were used for test-crosses in the genetic analysis: *P. hybrida* (Hort.) Vilm. line 2340, a normal fertile line which does not contain any male fertility restoration (mfr) alleles (Izhar 1978), and line 2439, a cm line isonuclear with line 2340.

The plants for the genetic analysis were grown at The Volcani Center in Bet Dagan from late 1976 to early 1979. The plants were grown in the greenhouse, usually under 16 h daylength. In the summer day temperatures were ca. 25°-30°C, and night temperatures were ca. 15°-20°C. In winter day temperatures were ca. 25°-30°C and night temperatures were maintained (by central heating) at a minimum of 15°C.

The term 'cybrid' is used in this paper for somatic hybrids with a single parental nucleus and a heteroplasmon which contain cytoplasmic genetic elements of both parental plasmons. The abbreviation 'cms' is also used in this paper for cytoplasmic male sterile;

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(S) plasmon refers to plasmon which induces cms as compared to normal (N) plasmon which induces male fertility; F_1 refers to somatic hybrid plants developed from protoplasts, F_2 refers to the progeny of the F_1 plants following selfing.

Results

The somatic hybrid plants described herein were obtained in the same fusion experiment carried out on February 6, 1976 and described by Izhar and Power (1979). Two types of reciprocal somatic hybrids were obtained and analysed genetically.

1 The 6.2-45 Cybrid Plant

This plant appeared among 80 plants developed from protoplasts (Izhar and Power 1979, Table 1). It was phenotypically identical to plants of the parental cms line 2426. However, unlike line 2426, this plant was partially male fertile and very weak.

Table 1. Segregation of male fertile vs male sterile plants in F_3. F_2 fertile plants were selfed and sterile plants were crossed to *P. parodii*

Line number	No. of male fertile plants	No. of male sterile plants
F_3 populations		
3302	3	0
3303	3	1
3304	2	1
3305	5	0
3306	2	0
3307	2	1
3308	3	0
3309	2	1
3310	2	0
3311	3	1
3312	1	0
3313	2	1
3314	4	1
3315	2	1
3316	4	0
3317	3	0
3318	4	0
3319	2	0
3320	6	0
3321	1	1
3322	4	1
3323	5	1
3324	4	0
3325	2	0
3326	7	1
3327	3	2
3329	4	1
Test crosses of F_2 plants × *P. parodii*		
3330	0	4
3331	0	7

A single fruit was obtained from this plant by selfing and a total of 34 F_2 plants were grown. Among the 34 plants most were partially or fully fertile and two plants were male steriles. The fertile plants were selfed and the male sterile plants were crossed with *P. parodii* as male parent. Twenty-seven F_3 populations and two populations of the cross with *P. parodii* were grown (Table 1). In 14 populations, segregation of male fertile plants and male sterile plants occurred. In 13 populations only fertile plants appeared. All the plants of all the F_3 populations listed in Table 1 were crossed with line 2340, a non-restorer fertile line. In all the 14 segregating F_3 populations the test-crosses of the fertile and male sterile segregants with line 2340 yielded only male sterile plants (Table 2). The test-cross of the fertile segregants (Table 2) to line 2439, a non-restorer cms line also yielded only male sterile plants. This suggests that no *mfr* alleles were present in the fertile segregants to account for the expression of male fertility. All the plants of all the 13 F_3 non-segregating populations were test-crossed with line 2340 as male parent and with cms line 2439 as female parent. The results of these crosses (Table 2) showed that the plants in these populations contained normal cytoplasm and no *mfr* alleles in the genome. The data in Table 3 represent an example of genetic analysis of single plants of five out of

Table 2. The scheme of test-crosses and selfing of the plants of the F_3 populations and the crosses with *P. parodii* listed in Table 1

Plants checked, and test-crosses	Results of test-crosses
All plants (fertile and sterile) of the 14 segregating F_3 populations crossed with line 2340 as male parent	All male sterile plants
All fertile plants of the 14 segregating F_3 populations crossed with line 2439 as female parent	All male sterile plants
All plants of 13 non-segregating fertile F_3 populations crossed with line 2340 as male parent. (Backcrosses of some plants of each population continued to BC_7)	All male fertile plants
All the above plants crossed with cms line 2439 as female parent	All male sterile plants
All fertile plants of the 13 non-segregating populations selfed. (Selfing of some plants of each population continued to F_7)	All F_4 populations were male fertile plants
All the male sterile plants from the crosses to *P. parodii* crossed with line 2340	All male sterile plants

Table 3. The genetic analysis of some F_3 fertile plants listed in Table 1. Each plant was crossed to cms line 2439, to line 2430 and was selfed to form F_4 population

cms tester line female parent		F_3 plants		Fertile tester line male parent	Fertile plants	Male sterile plants
		3305-2	×	2430	284	0
2439	×	3305-2			0	198
		3305-2	⊗		112	0
		3308-1	×	2430	279	0
2439	×	3308-1			0	110
		3308-1	⊗		173	0
		3316-1	×	2430	103	0
2439	×	3316-1			0	159
		3316-1	⊗		92	0
		3317-1	×	2430	108	0
2439	×	3317-1			0	89
		3317-1	⊗		145	0
		3324-1	×	2430	134	0
2439	×	3324-1			0	210
		3324-1	⊗		203	0

the 13 F_3 non-segregating populations. The number of plants used in the test-crosses was large enough, according to our previous experience in fertility restoration studies with petunia (Izhar 1978). Similar numbers of offspring were used in the analysis of the other F_3 plants listed in Table 1.

2 *The P. axillaris Cybrid Plants*

In the same fusion experiments described above (see also Izhar and Power 1979) male fertile plants identical to *P. axillaris* derived from protoplasts were grown. But unlike other plants in the same population which were apparently of parental type (*P. axillaris* line 2785) two plants (6.2-16; 6.2-31) showed segregation of male fertile and male sterile plants in the F_3 generation. The data of the genetic analysis of the segregating populations are presented in Table 4. Upon selfing, the fertile F_3 plants yielded only male fertile plants (F_5 is available now). The results of the test-crosses of the F_3 fertile segregants with line 2340 yielded only fertile plants and only male sterile plants were obtained in the test-crosses with the cms tester line 2439 (BC_5 is available at the present). In order to test the possible role of the *mfr* alleles present in line 2785 (Izhar 1978), test-crosses were made. The data obtained in these test-crosses showed that the amount of male fertility restoration alleles present was not enough to account for the fertility in the segregating generations.

Discussion

In a previous paper Izhar and Power (1979) described male sterile somatic hybrids combining the nuclear genome of *P. axillaris* line 2785 and the (S) plasmon of *P. hybrida* line 2426. In the present paper new types of somatic hybrids are described in which a mixture of the two parental plasmons occurred. The first type is a cybrid with the genome of the original line 2426 and a mixture of both the (S) and (N) plasmons. The heteroplasmic state in plant 6.2-45, which was a male fertile plants, was discovered when male fertile and male sterile segregants appeared in either the F_2 or the F_3 generation. The cytoplasmic segregation of male fertility and male sterility elements, which occurred in the first meiotic cycle (selfing of the 6.2-45 cybrid plant) is seen in F_2 generation. Two plants of the F_2 (Table 1) were homoplasmic with the (S) plasmon while 13 other plants were homoplasmic

Table 4. Two cybrid plants derived from protoplasts and their sebsequent generations. Line 2439 used as cms non-restorer tester and line 2430 as fertile non-restorer tester. The figures in the table are numbers of plants. (F) and (S) stand for fertile and sterile, respectively

Plants derived from protoplasts	6.2 – 16(F) ⊗			6.2 – 31(F) ⊗	
F_2 populations	63(F) Line 2830			49(F) Line 2843	
	2830–1 ⊗	2830–2 ⊗	2830–3 ⊗	2943–1 ⊗	2843–2 ⊗
F_3 populations	12(F) : 4(S)	8(F) : 1(S)	14(F) : 2(S)	14(F) : 2(S)	14(F) : 2(S)
F_3 plants (F) selfed[a]	all (F) –	all (F) –	all (F) –	all (F) –	all (F) –
All (F) F_3 plants × 2340[b]	all (F) –	all (F) –	all (F) –	all (F) –	all (F) –
All (S) F_3 plants × 2340[b]	– all (S)	– all (S)	– all (S)	– all (S)	– all (S)
2439 × F_3 plants (F)[c]	– all (S)	– all (S)	– all (S)	– all (S)	– all (S)

[a] For each F_3 plant selfed, at least 50 F_4 plants were observed
[b] For each F_3 plant crossed with 2430, at least 90 plants were observed
[c] For each F_3 plant crossed with 2439, at least 100 plants were observed

with the (N) plasmon. This was determined by the lack of segregation in either the F_3 or the test-crosses (Table 2). Heteroplasmic states existed in 14 other fertile F_2 plants, which upon selfing produced segregated F_3 populations (Table 1). Furthermore, heteroplasmic states still existed in the fertile segregants of the 14 segregating F_3 populations. This was indicated by the fact that although they were male fertile, all of them yielded male sterile plants in test crosses with line 2340, and with line 2439 (Table 2).

The second set of data showing heteroplasmic states followed by cytoplasmic segregation comes from two cybrid plants with heteroplasmon and the genome of *P. axillaris* line 2785. In this case, out of several single heteroplasmic F_2 plants (2830-1; -2; -3; 2843-1; 2), homoplasmic plants leading to stable cms and normal lines were obtained. The lack of cytoplasmic segregation in the F_2 generation in this case may be explained by the presence of *mfr* alleles in line 2785 (Izhar 1978). On the other hand it is evident from the data (Table 4) that the *mfr* alleles can not account for the segregation of stable normal and cms lines.

An important point in this work is to prove that the phenomena described are indeed due to a heteroplasmic state which is followed by cytoplasmic segregation. In other words, whether genetic elements of (S) and (N) plasmons are present in each cell of the cybrid plant and whether sorting out of these elements occurred during meiosis. That this is indeed the case can be demonstrated by the fact that cytoplasmic segregation also occurred in the progeny of the second meiotic cycle (in some of the F_3 populations) in plants each of which developed from a single zygotic cell (formed by selfing of the somatic hybrid plants). This therefore renders other explanations of the described phenomena such as mosaic or chimera in the somatic hybrid, unlikely.

At present it is difficult to draw conclusions about the phenotypic expression of the heteroplasmic state. In other words, does the heteroplasmic state always express fertility or are there dominance-recessive relations of the (N) over the (S) cytoplasmic elements? In this study the heteroplasmic state was only demonstrated in fertile cybrids which were selfed and showed cytoplasmic segregation. But, it is perhaps important to point out that no cytoplasmic segregation was achieved by crosses of any of the fertile cybrids with foreign pollen. Thus, it seems that selfing may be essential. The fact that it is impossible to self cms plants does not allow one to test whether the cms cybrids are heteroplasmic or homoplasmic in the same way the fertile cybrids are tested.

Nevertheless, recent data (Izhar unpublished) from other somatic hybridization studies in *Petunia* suggest that heteroplamic state in cybrids may express male sterility.

The achievement of somatic hybrid plants with heteroplasmons, followed by cytoplasmic segregation, suggests that both types of the cytoplasmic genetic elements have an equal chance to survive during cybrid development.

Furthermore, since cytoplasmic segregation of the heteroplasmon occurred equally in the background of the two different species, it seems that the (S) and (N) elements segregate independently of the genome.

The data presented here on *Petunia* differ in several respects from data accumulated in somatic hybridization experiments with *Nicotiana* in other laboratories. No heteroplasmic state (with respect to cms and fertility) was reported in *Nicotiana* (Gleba 1978; Belliard et al. 1977, 1978; Zelcer et al. 1978; Aviv et al. 1980). Cytoplasmic segregation of the heteroplastids population in somatic hybrids was observed following the first meiotic cycle (Gleba 1978; Belliard et al. 1978; Aviv et al. 1980) but in contrast to *Petunia* no cytoplasmic segregation of cms or normal plants were observed there. We may thus conclude that the interaction between the cms and the normal genetic elements in *Petunia* is different than that known so far in *Nicotiana*.

It has been shown in this paper that cms and normal genetic elements co-exist in heteroplasmic state in somatic hybrids in *Petunia*. The heteroplasmic state, although it undergoes cytoplasmic segregation to become homoplasmic, as expected (Birkey 1978), is still stable enough to survive more than two meiotic cycles. The relatively frequent sorting out into stable male fertile or sterile lines do not support the possibility of novel recombinations of extranuclear DNA in this case (Belliard et al. 1979). Biochemical characterization of the organelles' DNA in the cybrids and plants in the segregating generations is underway in an attempt to determine the site of the normal and the cms elements.

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