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Asymmetric hybridization in *Nicotiana* by "gamma fusion" and progeny analysis of self-fertile hybrids

I. Famelaer [1,*], I. Negrutiu [1], A. Mouras [2], H. Vaucheret [3] and M. Jacobs [1]

[1] Institute of Molecular Biology, Vrije Universiteit Brussel, B-1640 Sint-Genesius-Rode, Belgium
[2] Laboratoire de Biologie Cellulaire, Avenue des facultés, F-33405 Talence, France
[3] Laboratoire de Biologie Cellulaire, INRA-centre de Versailles, F-78026 Versailles Cedex, France

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Summary. Mesophyll protoplasts of the nitrate-reductase (NR)-deficient *Nicotiana plumbaginifolia* mutant, "*Nia26*," were fused with γ-irradiated mesophyll protoplasts of *Nicotiana sylvestris*, *V-42*. Hybrid selection was based on complementation of NR deficiency by transfer of the donor NR gene to *N. plumbaginifolia*. Regenerated hybrids had different numbers of donor chromosomes in a tetraploid background of *N. plumbaginifolia*. The transfer and expression of different isozymes from the donor were also observed. Six self-fertile regenerants were obtained from 21 independently isolated cell colonies. Progeny analyses revealed: (1) the linkage of NR and shikimate dehydrogenase (ShDh); (2) a stabilization of the transmission rate of NR; and (3) the obtainment of mono- and disomic addition lines in the first and second progeny of the original regenerants. Southern hybridization analyses demonstrated unequivocally the presence of the NR gene from the donor partner in progeny plants.

Key words: Asymmetric hybrids – γ-Radiation – Fertility – Addition lines – Linkage

Introduction

Parasexual hybridization experiments with a radiation-inactivated donor partner result in the formation of populations of asymmetric hybrids. This technique could thus be applied in breeding programs, since the manipulation of the introduced genetic variation could result in a quicker introgression of the desired trait(s).

Asymmetric nuclear hybrids were produced between distantly and more closely related species (Gupta et al. 1984; Gleba et al. 1988). All hybrids were male sterile, although they were characterized by the presence of only a few chromosomes from the donor partner (Dudits et al. 1980, 1987; Bates et al. 1987; Sidorov et al. 1987; Hinnisdaels et al. 1989). The sexual transmission of the selected characters in these studies could only be established by backcrossing. Therefore, it was not possible to stabilize the transmission of the chromosomes transferred in these hybrids. Self-fertile somatic hybrids may provide a one-step bridge for introgression of alien genes into the recipient partner.

The present study describes the isolation of self-fertile parasexual hybrids between a nitrate-reductase (NR)-deficient mutant of *N. plumbaginifolia* and a chlorophyll-deficient mutant of *N. sylvestris* as donor partner. A detailed progeny analysis demonstrated the transmission of the selected NR marker up to the third progeny, through the female and male meiotic cells.

Materials and methods

Protoplast isolation, fusion, selection, and regeneration

Shoot cultures of the NR-deficient mutant, *Nia26*, of *N. plumbaginifolia* (2n = 20, reversion frequency $4.04\ 10^{-7}$; Dirks et al. 1986) were cultivated as described by Negrutiu et al. (1983). Shoot cultures of *N. sylvestris*, *V-42* (2n = 24, chlorophyll-deficient mutant), were cultivated on R'SA medium (Negrutiu et al. 1983). Mesophyll protoplasts were isolated from both parents according to Negrutiu (1981). Donor protoplasts were irradiated in a Gamma cell 200 (Co^{60} source, dose rate 0.048 J kg^{-1} s^{-1}) with different doses, and fused with recipient cells according to Kao (1982). Culture and selection conditions of protoplasts and fusion products were carried out according to Dirks et al. (1986). Cell colonies were regenerated on RP0.25 or RP1 medium (Installé et al. 1985) with 0.25 mg or 1 mg/l zeatine. Regenerated plants were further cultivated on R'SA medium.

* To whom offprint requests should be addressed

Isozyme analyses

Polyacrylamide gel electrophoresis (PAGE) was carried out according to Jovin et al. (1964). Regenerated fusion products were analyzed for the presence of different donor isozymes according to Van Geyt and Smed (1984): phosphoglucomutase (PGM, EC 2.7.5.1), aspartate amino transferase (AAT, EC 2.6.1.1), malic enzyme (Me, EC 1.1.1.40), and isocitrate dehydrogenase (ICD, EC 1.1.1.42). Shikimate dehydrogenase (ShDh, EC 1.1.1.25) was analyzed according to Vallejos (1983).

Chromosome analysis

Root tips from in vitro-grown shoot cultures were incubated for 1 h at room temperature in a 0.1% α-bromonaphtalene, 0.05% $MgSO_4$ solution, and were fixed in Carnoy's solution (ethanol:acetic acid, 3:1). Fixed root tips were rinsed with water, digested at 37°C for 5–15 min in enzyme solution (0.1 *M* citric acid-sodium citrate buffer, pH 5.7, with 0.5% pectolyase, Sheishin Pharmaceutical Co. Ltd., Nibon Bashi Japan; 1% Onozuka R10-cellulase, Serva), and washed for 30 min in buffer solution. Chromosome analyses were further done according to Pijnacker and Ferwerda (1984).

Segregation analysis

Seeds were sterilized and sown on Mn medium as described by Dirks et al. (1986). Bleached NR-deficient seedlings were rescued on R'SA medium containing 10 m*M* ammonium succinate. The absence of NR activity was confirmed by enzymatic assay (Dirks et al. 1986) and by transferring the top apex on Mn medium. NR-deficient plants died after 4–5 weeks on this medium. Pollen fertility of regenerants was determined according to Alexander (1968).

Southern hybridization analysis

Genomic DNA from leaf material was isolated as described by Dellaporta et al. (1985) and digested with EcoRI. DNA fragments were separated by electrophoresis on 0.8% agarose gels and were transferred to high-bond N membranes (Amersham) by the Southern procedure. The blots were hybridized by standard procedures (Maniatis et al. 1982), with an 862-bp EcoRI/HindIII fragment of the plasmid pBMC102010 as probe. This plasmid was kindly provided by the laboratory of Dr. M. Caboche (INRA, Versailles, France) and contains a 1671-bp EcoRI insert of the NR gene from *N. sylvestris* (Vaucheret et al. 1989).

Results

Analysis of original regenerants (R_0)

Hybrid isolation and regeneration. A total of 26 nuclear hybrid colonies, complemented by the wild-type NR gene of the donor partner *N. sylvestris*, was isolated after selection, at a frequency of $2.2\ 10^{-5}$–$2.3\ 10^{-4}$ (Famelaer et al. 1986). Different plants were regenerated from each fusion product on RP0.25 and RP1 medium. The overall regeneration efficiency, as defined by Installé et al. (1985), was relatively better on RP0.25 medium, which is more suitable for regeneration of *N. plumbaginifolia* calli. Plants regenerated on this medium had a better phenotype and were retained for further analyses. Cell colonies isolated at the highest dose range (500–1,000 Gy), were subjected to different incubation periods on RP0.25 medium (14 or 51 days). Plants regenerated from these cell colonies that were subjected to a longer incubation period on this medium (51 days) generally had a more abnormal phenotype. A total of 28 regenerated plants from 21 isolated cell colonies was further analyzed.

Isozyme analyses. Regenerated hybrids were analyzed for the presence of several isozymes from the donor partner: ShDh, PGM, ICD, AAT, ME (Table 1). Different hybrids were characterized by the presence of one or more isozymes. Some hybrids, however, did not retain any of them. ME was not expressed in the hybrids that were analyzed. Isozymes were codominantly expressed in the somatic hybrid.

Chromosome analysis of regenerated hybrids. Chromosome analysis was facilitated by morphological differences of recipient and donor metaphase chromosomes: *N. plumbaginifolia* is characterized by telocentric and *N. sylvestris* by meta- or submetacentric chromosomes. The analysis of 21 hybrid plants from 15 independent cell colonies is presented in Table 1. According to these analyses, regenerants can be classified into two groups: a first group of 19 plants from 13 different cell colonies, with 43–56 chromosomes (37–42 recipient chromosomes), and a second group (2 plants from 2 independent cell colonies) with 61–67 chromosomes and a hexaploid set of recipient chromosomes. The average number of identifiable donor chromosomes in 15 independent regenerants is about 8.7. Donor chromosome fragments resulting from radiation-induced damage were observed in all plants. The total number and exact type of chromosomes are difficult to establish for several reasons: (1) small variations in chromosome numbers may exist within one regenerant; (2) radiation damage of donor chromosomes may result in recipient-like chromosomes; and (3) interspecific chromosome exchanges may result in reconstructed and deleted recipient chromosomes as well as in the loss of chromosomes. Chromosome translocations could not be observed.

Phenotype of regenerated hybrids. Regenerated hybrids phenotypically resembled the recipient partner with respect to leaf and flower morphology, inflorescence, and general appearance of the plant (Fig. 3). Plants regenerated from the same cell colony usually showed a different phenotype, e.g., slightly curved leaves in regenerant 5-2-1. This phenotype is even more pronounced in regenerants 5-2-2 and 5-2-4, which were regenerated following a longer incubation period on RP medium, i.e, 51 days versus 14 days for regenerant 5-2-1. Furthermore, these plants contained different chromosome numbers (Table 1).

Table 1. Chromosome and isozyme analyses of regenerated hybrids (R_0)

Dose Gy	Plant R_0 (a)	Chromosome no.	Recipient chromosomes	Donor chromosomes	Donor isozymes[b]
100	1-1	46	40	7–8	ShDh, PGM
200	2-1	48–49	39	9	ShDh, ICD
	2-2	47–48	40	8	ShDh
300	3-3	44	40	4	
	3-4	65–67	60	5–7	ND
	3-6	48–49	39–40	8–9	ICD
	3-8	53	40	13	ND
500	5-2-1	46–49	40	6–9	AAT
	5-2-2	40–41	36–37	4–5	AAT
	5-2-4	39–43	37–38	2–5	AAT
	5-3-1	46–48	40	6–8	AAT
	5-3-2	49–50	40	9–10	ND
	5-4-1	49	38–39	9–10	ShDh, ICD
	5-4-2	49	38–39	9–11	ND
	5-4-3	49–50	38–39	9	ND
	5-6-1	61–62	59	3–4	ND
1000	10-1-1	56	39	16–18	ShDh, ICD
	10-1-2	52–53	38–39	15–17	ND
	10-3	49–50	40–41	8– 9	
2000	20-1	43–44	36–39	5– 8	SHDh, AAT
	20-2	47	40	7	ICD
Somatic hybrid		44	20	24	ShDh, AAT, ICD, ME, PGM

[a] Code number of regenerants: the first number refers to the radiation dose; the second number to the isolated cell colony and to the regenerant, if only one plant has been retained. However, different regenerants, retained from the same cell colony, were all numbered separately (third number)
[b] ND – Not determined

Table 2. Transmission of NR in the first progeny (R_1)

R_0	Self		Transmission rate %	Backcross		% NR seedlings
	NR^+	NR^-		NR^+	NR^-	
1-1	1	–	–	103	34	75
2-2	27	30	47.3	43	29	59.7
2-1	15	–	–	12	10	54.5
3-3	41	82	39.8	140	90	61
3-6	128	183	41.1	67	36	65
5-2-1	5	4	–	10	7	58.8
5-3-1	–	–	–	–	4	–
5-4-3	–	–	–	5	4	–
10-3	8	4	–	18	12	60

[a] NR+ or –, presence or absence of NR. A few seeds obtained from regenerant 5-3-1 and 5-4-3 did not germinate

Segregation analysis: R_1 generation. Nine different plants from as many cell colonies could be selfed (6 plants) or backcrossed (nine plants), with the tetraploid revertant *RVNia26* (2n = 40) of *N. plumbaginifolia Nia26* (2n = 20) as male parent (Table 2). This revertant segregates in a 3:1 ratio for the NR gene (NR^+/NR^-; Dirks et al. 1986). All plants were only partially male- and female-fertile and were characterized, as compared to the revertant, by (1) a reduced pollen fertility (1%–90%); (2) a strongly reduced seed set; and (3) a reduced germination rate (0%–>90%). Two plants, however, 3-3 and 3-6, were almost normally self-fertile. Abnormal seedlings (1%–10%), which were not observed in the progeny of the revertant, could be observed in all first progenies. The transmission rate of the NR marker in the first progeny obtained by selfing is about 40%–47%, and in the progenies obtained by backcrossing, about 55%–75%. Self-fertility was restored after one backcross. The first progeny plants, obtained by selfing the original regenerants, were used for further analyses.

Progeny analysis: generation R_1 through R_3

Isozyme analyses; co-transmission of ShDh and NR. Analyses were concentrated on the presence of ShDh, PGM, and AAT in the most self-fertile R_1 progeny plants of regenerants 1-1, 2-2, and 5-2-1. PGM was absent in the first progeny plant of regenerant 1-1, and AAT was absent in second progeny plants of regenerant 5-2-1. ShDh was present in R_1 plants 2-2 $R_{1.1}$ and $R_{1.3}$, but absent in 2-2 $R_{1.2}$ and 2-2 $R_{1.4}$ (for an explanation of code numbers, see Table 4). Co-segregation analyses of ShDh and NR in the second and third generations are given in Table 3. Only a few R_2 plants from regenerant 1-1 and 2-2 were analyzed for the presence of ShDh, but all R_2 plants that were characterized by the presence of NR also retained the ShDh marker. NR-negative plants did not

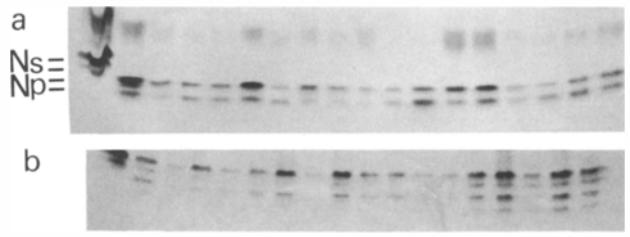

Fig. 1 a and b. Analysis of ShDh by PAGE in third-generation progeny plants of regenerants 2-2 and 1-1; Ns = *N. sylvestris;* Np = *N. plumbaginifolia;* third-generation progeny plants were randomly chosen for analysis. **a** NR-negative plants, **b** NR-positive plants; *arrows* indicate the specific ShDh bands for both parental plants

Table 3. Progeny analyses: co-transmission of NR and ShDh

R_0	R_x progeny analyzed [a]	No. of plants analyzed	Segregation pattern			
			NR^+ $ShDh^+$	NR^+ $SdDh^-$	NR^+ $ShDh^-$	NR^- $ShDh^+$
1-1	R_2 ($R_{1.1}$)	9	7	–	2	–
2-2	R_2 ($R_{1.1}$)	11	8	–	3	–
1-1	R_3 ($R_{2.1}$ $R_{1.1}$)	99	51	–	48	–
2-2	R_3 ($R_{2.1}$ $R_{1.1}$)	102	38	1	63	

[a] R_3 ($R_{2.1}R_{1.1}$), third generation-progeny of cell line 1-1 or 2-2, obtained by selfing plant number 1 of the first and second generation

Table 4. R_2 progeny

R_0	R_1 plants examined for fertility	R_1 plants analyzed for segregation [a]	Segregation values		Transmission rate %
			NR^+	NR^-	
1-1	1	$R_{1.1}$ $ShDh^+$	82	88	48
2-2	9	$R_{1.1}$ $ShDh^+$	15	25	35.3
		$R_{1.2}$ $ShDh^-$	138	24	85
		$R_{1.4}$ $ShDh^-$	89	31	74
3-3	11	$R_{1.1}$	207	282	42
		$R_{1.2}$	84	82	51
		$R_{1.4}$	171	342	33
		$R_{1.8}$	121	186	39
		$R_{1.10}$	784	1	99.9
		$R_{1.11}$	86	312	22
3-6	20	$R_{1.8}$	6	53	10
		$R_{1.18}$	82	188	31
		$R_{1.44}$	47	113	30
5-2-1	5	$R_{1.2}$ AAT^-	28	72	28
10-3	8		sterile		

[a] The most fertile R_1 plants (highest seed set in comparison with the revertant) were further analyzed for segregation of NR in the second generation. $ShDH^{+/-}$, presence or absence of ShDh. Code number of R_1 plants: e.g., 2-2 $R_{1.4}$, the fourth first-generation plant of regenerant 2-2

Table 5. R_3 progeny

R_0	R_1 progeny plant [a]	R_2 plants examined for fertility	R_2 plants analyzed for segregation	Transmission rate % [b]
1-1	$R_{1.1}$	7	1	21
2-2	$R_{1.1}$	5	1	18
	$R_{1.2}$	9	4	76–91
	$R_{1.4}$	2	2	66–97
3-3	$R_{1.1}$	19	9	18–48
	$R_{1.2}$	11	2	27
	$R_{1.4}$	15	4	18.6–33.7
	$R_{1.8}$	11	1	24
	$R_{1.10}$	16	14	94–100
	$R_{1.11}$	2	1	31.7

[a] Original R_1 plant, from which R_2 progeny has been obtained and analyzed for segregation in R_3

[b] Range of transmission rates in the third generation

retain this ShDh marker. All 51 NR-positive third-generation plants of regenerant 1-1 also retained the ShDh marker, and all 48 NR-negative plants lost it (Table 3). The same observation can be made in the third-generation progeny of regenerant 2-2. All 39 NR-positive plants except one retained the ShDh marker. The analysis of third-generation plants of regenerant 2-2 is given in Fig. 1. NR-negative plants were randomly chosen for analysis of ShDh.

Transmission of NR. Although many R_1 plants were self-fertile, only a few of them produced sufficient seed. Reduced fertility was observed in most progeny plants (17 out of 20, Table 4) of regenerant 3-6. All first-generation plants of regenerant 10-3 were actually sterile. An increased fertility in R_1 plants of regenerants 1-1, 2-2, and 5-2-1 coincided with a loss of donor isozymes (Table 4). On the other hand, a decreased transmission rate was observed along with increased fertility, e.g., progeny plants of regenerant 3-3 (Tables 2, 4, and 5). Abnormal seedlings could be observed in all R_2 families (1%–7%). First-generation plants were characterized either by (1) a higher transmission rate of NR to the second generation, e.g., 2-2 $R_{1.4}$ (74%), 2-2 $R_{1.2}$ (85%), 3-3 $R_{1.10}$ (99,9%); or by (2) a lower transmission rate in comparison with the original regenerants (Table 2 and 4).

The same observations can be made on the transmission rate of the NR marker to the third generation (Table 5): (1) second-generation progeny plants of regenerants 2-3 and 3-3 gave rise to more stable transmission rates in the third generation; (2) lower transmission rates were observed in R_2 plants from regenerants 1-1, 2-2, 3-3. Abnormal seedlings could again be observed in R_3 families (1%–10%).

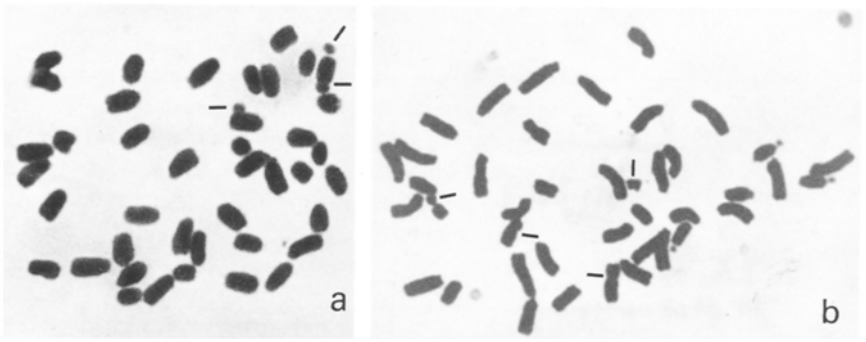

Fig. 2. **a** Chromosome plate of plant 3-3 $R_{1.11}$, which has 43 chromosomes; 1 submetacentric (*arrow*), and 2 fragmented donor chromosomes; **b** metaphase plate of plant 2-2 $R_{1.1}$. *Arrows* indicate *N. sylvestris* chromosomes

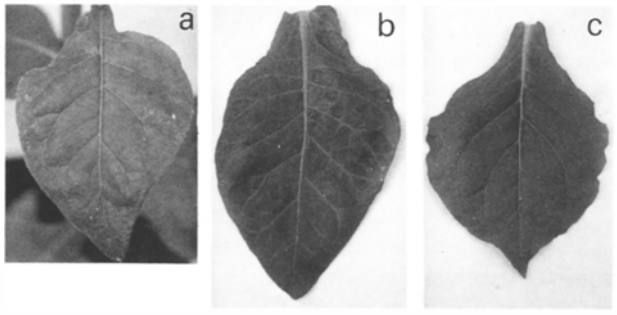

Fig. 3a–c. Leaves of asymmetric hybrids and revertant *RVNia26* of *N. plumbaginifolia Nia26;* **a** leaf of revertant; **b** leaf of plant 2-2 $R_{2.2}$; **c** 2-2 $R_{2.3}$

Table 6. Chromosome analyses of R_1 plants

R_0	R_1	NR/ ShDh	Chromosome no.	Recipient chromosomes	Donor chromosomes
1-1	$R_{1.1}$	+/+	43	38	5
2-2	$R_{1.1}$	+/+	44–45	40	5
	$R_{1.4}$	+/−	43	40	3
3-3	$R_{1.11}$	+/−	43	40	3
	$R_{1.4}$	+/−	41	40	1
5-2-1	$R_{1.2}$	+/−	43	39	4
10-3	$R_{1.1}$	+/−	48	40	8

Table 7. Chromosome analyses of R_2 plants

R_0	R_2	R_1	NR/ ShDh	Chromosome no.
2-2	$R_{2.1}$	$R_{1.1}$	+/+	43
	$R_{2.8}$	$R_{1.2}$	+/−	42
	$R_{2.1}$	$R_{1.4}$	+/−	43
3-3	$R_{2.3}$	$R_{1.4}$	+/−	41
	$R_{2.7}$	$R_{1.8}$	+/−	42
	$R_{2.4}$	$R_{1.10}$	+/−	41–42
	$R_{2.10}$		+/−	41–42
	$R_{2.7}$	$R_{1.11}$	+/−	42
5-2-1	$R_{2.13}$	$R_{1.2}$	+/−	42

Table 8. Chromosome analyses of R_3 plants

R_0	R_3	R_2	R_1	NR/ ShDh	Chromosome no.
2-2	$R_{3.2}$	$R_{2.1}$	$R_{1.1}$	+/−	42
3-3	$R_{3.7}$	$R_{2.3}$	$R_{1.4}$	−/−	40
	$R_{3.1}$	$R_{2.4}$	$R_{1.10}$	+/−	41–42
	$R_{3.2}$			+/−	40–41

Chromosome analysis. Analyses of R_1 plants demonstrated the retention of different numbers of donor chromosomes; e.g., R_1 plant 3-3 $R_{1.4}$ (monosomic addition line, 41 chromosomes, 1 submetacentric chromosome), 3-3 $R_{1.11}$ (43 chromosomes, 1 submetacentric chromosome, and 2 fragmented chromosomes (Fig. 2)). Progeny plants from colony 2-2 showed the same picture (Table 6).

The number of chromosomes in second- and third-generation plants seemed to be stabilized (41–43 chromosomes, Tables 7 and 8) in comparison with first-generation plants (41–45 chromosomes). Different progeny plants of regenerants 2-2 and 3-3 were characterized by the presence of a metacentric and/or submetacentric chromosome from the donor. Although small fragmented chromosomes showed a tendency to be lost, the presence of such a mini-chromosome could still be observed in some progeny plants of regenerants 2-2 and 3-3.

Phenotype of progeny plants. Although first-generation plants were characterized by a loss of donor chromosomes, they did not necessarily show enhanced vigor; for example, most R_1 progeny plants of regenerant 3-6 were characterized by a much lower seed set and vigor (Table 4). First- to third-generation progeny plants were phenotypically very similar to the recipient partner (Fig. 3). The distinct phenotype of curved leaves observed in regenerant 5-2-1 could also be observed in some of the progeny plants bearing the NR marker of the donor partner.

Southern analyses of progeny plants. Southern hybridization analyses demonstrated the presence of the NR gene

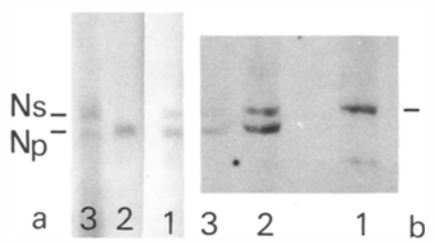

Fig. 4 a and b. Southern blots, showing the presence of the NR gene of *N. sylvestris* in: **a1** somatic hybrid; **a2** *N. plumbaginifolia;* **a3** plant 3-3 $R_{1.4}$; **b1** *N. sylvestris;* **b2** plant 2-2 $R_{2.1}$ $R_{1.4}$; **b3** plant 3-3 $R_{3.1}$ $R_{2.4}$ $R_{1.10}$. The hybridization pattern shows a single band in *N. plumbaginifolia* and *N. sylvestris;* the EcoR1/HindIII 862-bp fragment of the NR gene cloned in vector pBMC10.20.10 (H. Vaucheret et al. 1989) hybridizes with a larger EcoR1 fragment of about 4.3 kb in *N. sylvestris*

from *N. sylvestris* in several progeny plants. The analysis of a monosomic addition line (3-3 R1.4), plants 3-3 $R_{3.1}$ $R_{2.4}$ $R_{1.10}$, and 2-2 $R_{2.1}$ $R_{1.4}$, the somatic hybrid, the recipient, and donor partner is shown in Fig. 4.

Discussion

The results obtained in the present fusion combination are similar to those reported in a previous experiment with the same fusion partners but different mutants (Famelaer et al. 1989), e.g., (1) the regeneration of asymmetric hybrids possessing relatively high numbers of donor chromosomes in a tetraploid background of *N. plumbaginifolia*; (2) the inheritance and codominant expression of isozymes, not directly linked to the selectable marker; and (3) the isolation of perfect amphidiploid and fertile somatic hybrids in control experiments.

Several authors mentioned the retention of much lower donor chromosome numbers in other fusion combinations. e.g., between *N. tabacum* and *N. plumbaginifolia* (Bates et al. 1987), *Datura innoxia* and *Physalis minima* (Gupta et al. 1984), and *N. tabacum* and *Daucus carota* (Dudits et al. 1987). Our results are rather comparable to those obtained in interspecific fusion system, e.g., within *Solanum* species (Sidorov et al. 1987), *Lycopersicum esculentum* and *L. peruvianum* (Wijbrandi et al. 1987), and *N. plumbaginifolia* and *Atropa belladonna* (Gleba et al. 1988). Although parental chromosomes are structurally different, no translocation events could be observed in regenerants.

An important result of the present study is the obtainment of self-fertile hybrids. Several authors reported the isolation of asymmetric hybrids which, however, were self-sterile (Gupta et al. 1984; Bates et al. 1987; Dudits et al. 1987; Koornneef et al. 1987; Sidorov et al. 1987; Wijbrandi et al. 1987; Gleba et al. 1988). It is interesting to note that the male-sterile asymmetric hybrids isolated by Gleba et al. (1988), which possessed an additional number of *Atropa* chromosomes in a tetraploid background of *N. plumbaginifolia*, could be backcrossed to wild-type diploid *N. plumbaginifolia* plants. Self-fertile monosomic additions of *Atropa* to *N. plumbaginifolia* could be obtained in the progeny of the second backcross. These plants had an almost diploid number of *N. plumbaginifolia* chromosomes (Hinnisdaels et al. 1989). Reciprocal fusion experiments with *N. plumbaginifolia* and *N. tabacum* did not give any male-fertile hybrids (Koornneef et al. 1987; Bates et al. 1987). Moreover, in our previous experiment all original regenerants except two were male sterile (Famelaer et al. 1989). These different results coud be explained by the use of different plant species or mutants as fusion partners or by the use of suboptimal culture conditions.

In the present study, protoplasts and cell colonie were regenerated in a shorter time span (13–16 weeks) than in the previous study (19–20 weeks). The negative effects of a prolonged in vitro culturing on regeneration medium was observed in different regenerants from the same cell lines, e.g., cell colony 5-2, in which a loss of chromosomes coincided with a loss of fertility and a reinforcement of the curved leaf phenotype. This phenotype could result from the loss of a recipient chromosome and/or from the presence of a donor chromosome. These data suggest that the period of in vitro culture from protoplast to regenerant should, if possible, be short (see also Installé et al. 1985).

Progeny analysis of self-fertile hybrids, especially of regenerants 2-2 and 3-3, revealed a normalization of the transmission rate of the NR marker and of the donor chromosome numbers in the second and third generation. Some interesting observations were made in the progeny analyses of regenerant 2-2: (1) the co-segregation (linkage) of NR and ShDh in the second and third generation of two independently isolated cell colonies (Table 3); (2) the loss of the ShDh marker in first-generation progeny plants of regenerant 2-2; and (3) the stabilization of the transmission of NR and of the number of donor chromosomes in the second and third generation of those first-generation plants that lost the ShDh marker. These observations could only be explained by recombinational events and by transfer through the female and male gametes.

Similar data were obtained from the segregation and chromosome analyses of NR in descendents of regenerant 3-3. These data can be explained also by a transmission of the chromosome bearing the NR marker through the male gametes and the formation of mono- and disomic addition lines. The NR marker is most probably located on a submetacentric chromosome fragment present in regenerant 3-3 and its monosomic addition line(s). Recombinational events are not excluded, since distinct

chromosome fragments were observed in the original regenerant and in progeny plants stably segregating for NR. Although these high and stable transmission rates for NR could suggest a stable integration of the NR marker, the obtainment of disomic addition lines in a tetraploid backgroud of the recipient are rather in favor of stable addition lines.

Southern analysis of several progeny plants of regenerants 2-2 and 3-3, characterized by the presence of one or more *N. sylvestris* chromosome, confirmed the presence of the NR gene of *N. sylvestris*.

Although stable transmission rates were observed for the NR marker in some progeny plants of regenerants 2-2 and 3-3, some instability in the transmission rates could be observed as well. Riley (1960), for instance, who studied the meiotic chromosome pairing in disomic additions of rye to wheat, observed similar results: the alien chromosomes may fail to pair with each other, and bivalents were observed in 71%–94% of the meiotic cells, depending on the disomic addition line. Monosomic additions should, furthermore, be characterized by a much lower transmission frequency of the alien chromsome as a consequence of a competitive disadvantage of the pollen from the addition line (Riley 1960). This behavior, termed "addition decay" (Riley and Kimber 1966), can also be observed in the present study, e.g., regenerant 3-3, which is characterized by a higher transmission rate in comparison with addition line 3-3 R1.4 (Tables 2, 4, and 5).

The obtained results demonstrated the linkage of two genetic markers, NR and ShDh, and the isolation of self-fertile monosomic and disomic addition lines within one somatic plus one generative cycle. They also demonstrate that in vitro culture has no deleterious effects on fertility if optimal culture conditions are used. Although monosomic or disomic addition lines usually do not have an agronomic value per se, because of some limiting factors – e.g., chromosome instability, reduced fertility, addition of undesired traits – they have been used in many breeding programs as starting breeding material for the introgression of desirable characters (cf. review of Law 1983; Heijbroek et al. 1988). Recipient-donor fusion experiments with a radiation-inactivated donor partner (Zelcer et al. 1978) could thus effectively be combined with classical breeding technology.

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