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Co-segregation of nitrate-reductase activity and normal regeneration ability in selfed sibs of *Nicotiana plumbaginifolia* somatic hybrids, heterozygotes for nitrate-reductase deficiency

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Summary. The nitrate-reductase (NR) defective cell lines of *Nicotiana plumbaginifolia* isolated in our laboratory could not be regenerated into plants on the standard medium (Márton et al. 1982a). The normal regeneration potential, however, was restored in somatic hybrids obtained by fusing the NR^- (green) lines with a pigment deficient (P^-), but NR^+ line, A28. Somatic hybrid plants were fertile in two combinations (A28 + NA9 and A28 + NX9). As expected, segregation for NR^- and P^- was found after selfing the somatic F1 (SF1) obtained by protoplast fusion, and in the F2. The variable segregation ratios are explained by chromosome abnormalities. Co-segregation of the NR^- phenotype and the altered response to shoot induction on standard medium suggest the involvement of the nitrate-assimilatory pathway in determining shoot regeneration ability.

Key words: Nitrate reductase activity – Shoot induction – *N. plumbaginifolia* – Segregation

Introduction

Interest in nitrate metabolism and in generating auxotrophic mutations for plant cellular genetic studies has resulted in the isolation of nitrate-reductase deficient (NR^-) lines in a number of species.

Mutants isolated by screening seedlings were found to display residual NR activity (Oostindier-Braaksma and Feenstra 1973; Kleinhofs et al. 1980; Feenstra and Jacobsen 1980; Braaksma and Feenstra 1982). By tissue culture a number of cell lines were obtained which are fully defective in NR activity. Such mutants were obtained in *N. tabacum* (Müller and Grafe 1978), *Datura innoxia* (King and Khanna 1980), *Hyosciamus muticus* (Strauss et al. 1981), *N. plumbaginifolia* (Márton et al. 1982a; Negrutiu et al. 1983) and in *Petunia* (Steffen and Schieder, 1983).

In some of the NR^- cell lines isolated in *N. tabacum* (Müller 1983) and *N. plumbaginifolia* (Negrutiu et al. 1983) plants were regenerated and the inheritance of NR deficiency through meiosis has been studied. Plant regeneration, however, was not possible in some of the *N. tabacum* lines (Müller and Grafe 1978), in *Petunia* lines (Steffen and Schieder 1983), and in our *N. plumbaginifolia* cell lines which entirely lacked NR activity (Márton et al. 1982a).

Complemented somatic hybrids obtained by fusion regenerated normally in our lines (Márton et al. 1982b) and in *N. tabacum* (Glimelius et al. 1978). Restoration of shoot formation in complemented somatic hybrids suggested a causal relationship between the loss of NR activity and the loss of shoot regeneration ability. However, it could not be excluded that a mutation other than that responsible for the loss of NR activity was involved in the altered response to shoot induction due to unintentional selection in culture. As an example the work of Chaleff and Keil (1981) could be mentioned. They discovered that more than half of all tobacco cell lines isolated on the basis of resistance to the herbicide picloram were also resistant to hydroxyurea, traits which are genetically unlinked.

In this paper we provide evidence for the co-segregation of the NR^- phenotype and the altered response to shoot induction in the seed progeny of somatic hybrids.

Materials and methods

Plant material

Isolation and properties of the NR^- cell lines (Table 1) have been described earlier (Márton et al. 1982a, b). The chromosome numbers were not determined before protoplast fusion. In shoot induction tests a wild-type *Nicotiana plumbaginifolia* callus (DPN) was used which was initiated at the time of the

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isolation of the NR^- lines, and was maintained under identical conditions.

The pigment deficient line A28 was isolated in a haploid protoplast culture and was diploid at the time of protoplast fusion (Sidorov and Maliga 1982). Pigment deficiency in this line was shown to be recessive by fusion.

Callus culture and plant regeneration

The NR^- cell lines were maintained on the NH_4-S-RMOP-medium which is the Murashige and Skoog (MS) medium (1962) with the growth regulators naphthaleneacetic acid (0.1 mg/l) and benzyladenine (1 mg/l) (Sidorov et al. 1981), and was supplemented with 8.25 mM ammonium-succinate (Márton et al. 1982a).

Shoots from somatic hybrids were obtained on RMOP medium (same medium but ammonium succinate omitted). Shoot induction was also obtained on RMB medium, which is the same as the RMOP medium, except that it contains 1 mg/l BA but no NAA. The shoots were rooted on P medium (Maliga et al. 1982).

Determination of NR activity

NR activity was assayed in vivo as previously described (Márton et al. 1982a).

Protoplast isolation, fusion and culture conditions

Protoplasts were isolated and fused according to Menczel et al. (1981). Protoplasts were cultured in modified K3 medium containing 0.4 M glucose, 1 mg/l naphthaleneacetic acid and 1 mg/l benzyladenine. Details of protoplast culture have been described (Márton et al. 1982a, b).

Screening seedlings for NR^- and P^- phenotypes

The seeds were surface sterilized, treated with gibberelic acid (GA_3) (0.5 mg/ml; 1 h) and germinated on a medium containing the salts of the Murashige-Skoog medium; 8.25 mM ammonium-succinate, 3% sucrose, 0.7% agar, pH 5.6. On this medium the P^- plants were white, whereas wild-type and NR^- seedlings were green. NR activity and nitrate utilization were tested in calli induced from the seedlings on the RMNO medium, which is the MS medium with 3 mg/l indole-3-acetic acid, 0.1 mg/l dichlorophenoxyacetic acid and 0.04 mg/l kinetin (Márton and Maliga 1975), supplemented with 8.25 mM ammoniumsuccinate. The in vivo NR activity was measured in ten-day-old calli. Nitrate utilization by seedling calli was tested by their ability to proliferate on the RMNO medium without ammonium-succinate, a medium which does not support the growth of cells lacking NR activity (Müller and Grafe 1978; Márton et al. 1982a, b).

Determination of chromosome numbers

The chromosome numbers were determined in colchicine treated (0.5%; 3 h) root tips by the standard acetocarmine method.

Results

Somatic hybrids by protoplast fusion

Protoplasts obtained from calli of the NR^- cell lines (Table 1) were fused with diploid protoplasts from leaves of the recessive albino (P^-) mutant A28. Complemented green and NR^+ colonies were selected by culturing the fused products in ammonium-nitrate based K3 medium under light conditions. On this medium parental NR^- protoplasts did not divide whereas A28 protoplasts formed white calli. The frequency of somatic hybrid colonies (green colonies) was 3–10% in the fused population. No such green colonies were observed in mixed (non-fused) protoplast cultures or after the intra-line fusion of 10^6 protoplasts. Somatic hybrids in each combination contained levels of NR activity close to that of the wild-type (50–100%, Table 1). Each of these somatic hybrid clones regenerated shoots, as did the wild-type control tissue.

In regenerated somatic hybrid plants chromosome numbers were tetraploid (4x = 40), or varied around the tetraploid level (Table 1).

Marker segregation in the F2 and F3 generations

Regenerated somatic hybrids, with respect to the nuclear genetic information, but not necessarily with respect to the cytoplasm are equivalent to an F1 (with increased ploidy) and will be termed below as somatic F1 (SF1). The seed progeny of SF1 obtained by selfing will be termed F2 since in this case inheritance is not expected to differ from that of plants descended from generative hybrids.

Somatic hybrid plants from most fusion combinations were self sterile except for some of those derived from fusions with NX9 and NA9. Marker segregation was studied in the seed progeny (F2) of self-fertile NX9 + A28 and NA9 + A28 somatic hybrid plants (SF1).

Table 1. NR activity and chromosome numbers in the somatic hybrid (green) clones

Fusion combination	NR activity[a]	No. of plants[b]	Chromosome no.[c]	No. of "tetraploids"[d]
A28 + NA1	+	4 (3)	42–50	–
A28 + NA2	+	6 (5)	32–55	1
A28 + NA9	+	14 (6)	31–50	2
A28 + NA18	+	6 (3)	33–43	2
A28 + NA36	+	5 (1)	40–55	1
A28 + NX1	+	9 (5)	33–65	1
A28 + NX9	+	36 (13)	26–40	5
A28 + NX21	+	9 (2)	37–50	3
A28 + NX24	+	4 (4)	35–68	2

[a] *In vivo* NR activity was measured in four independent clones. Values varied between 50–100% of the wild-type level (260 nmoles NO_2^-/100 mg callus/1 h)

[b] In brackets: the number of clones from which the plants were regenerated

[c] Chromosomes were counted in 5 root tip cells per plant

[d] Plants with 40 chromosomes

Table 2. Segregation for NR^- and P^- phenotypes in F2

Fusion combination	Clone no.	Individual SF1 plant[a]	Chromosome no.	No. of F2 seedlings					
				Tested	NR^-	NR^+/NR^-	Tested	P^-	P^+/P^-
A28 + NX9	13	a	40	97	3	(31:1)	141	5	(27:1)
		d	36	58	0	–	183	1	(182:1)
		f	36	41	1	(40:1)	184	3	(60:1)
		g	26	115	4	(27:1)	168	8	(20:1)
		i	38	–	–	–	119	5	(22:1)
	5	a	37–38	59	0	–	110	3	(35:1)
	6	b	36	22	2	(10:1)	37	1	(36:1)
	8	a	36	64	0	–	133	2	(65:1)
	17	a	36–38	165	4	(40:1)	236	4	(58:1)
		b	34–35	45	0	–	107	3	(34:1)
	19	a	30	46	0	–	116	2	(57:1)
A28 + NA9	1	a	36–37	175	3	(57:1)	302	10	(29:1)
		e	38	214	0	–	917	17	(53:1)
		f	36	227	1	(226:1)	703	21	(32:1)
	10	b	36–38	27	0	–	88	4	(21:1)
		d	36–37	32	2	(15:1)	76	2	(37:1)
	9	a	36–38	19	0		110	5	(21:1)
		b	34–36	38	2	(18:1)	85	0	–
		c	32	85	3	(27:1)	98	1	(97:1)

[a] From each somatic hybrid clones 10–10 independent plants were isolated (letters) and potted into the greenhouse. The rest of the regenerated plants could not be grown to fertile plants

Table 3. Segregation for NR^- and P^- phenotypes in F_3

Fusion combination	Designation[a] of F_2 plants	No. of F_3 seedlings					
		Tested	NR^-	NR^+/NR^-	Tested	P^-	P^+/P^-
A28 + NX9	13/g–34 (38)	68	4	(16:1)	68	0	–
	13/g–26 (40)	134	0	–	3,032	0	–
A28 + NA9	10/b–12 (40)	69	4	(16:1)	62	2	(30:1)

[a] In brackets: the chromosome numbers of the F_2 plants

Since the chromosome numbers in regenerates from the same clone were different (Table 2), segregation was studied in seed lots from individual plants. Due to reduced fertility of the regenerates, in some cases only a small number of seedlings could be tested.

The frequency of the NR^- seedlings in the A28 + NA9 and A28 + NX9 combinations was 2.0% and 1.4%, respectively (excluding the progenies with NR^+ seedlings only, the frequencies were 2.9% and 2.0%). In the same population of F2 seedlings 2.5% P^- individuals were obtained.

Segregation was also studied in F_3 obtained by selfing F2 plants from segregating populations (Table 3). Two of the three plants turned to be heterozygotes, one of them for one (NR), one for both traits (P, NR).

Plant regeneration from calli derived from the NR^- seedlings

If factors other than the NR deficiency are involved in determining the loss of regeneration ability on normal shoot induction medium and the two markers are not linked, independent segregation of the two traits is expected after meiosis. In order to test the regeneration ability, undifferentiated calli derived from the leaves of 4 NA9 and 9 NX9 derived NR^- segregants were induced on NH_4-S-RMNO medium. These calli were subsequently subcultured three times on the shoot inducing NH_4-S-RMOP medium. No shoots were formed in any of the NR^- cultures. Shoot formation, however, was already abundant during the first subculture in the wild-type cultures under identical conditions (Fig. 1).

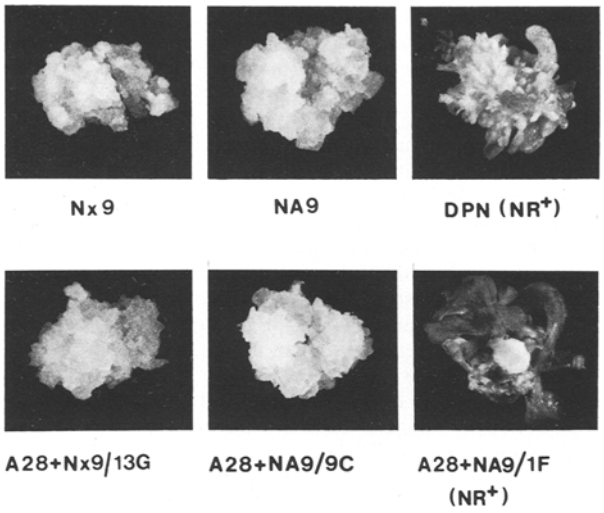

Fig. 1. Lack of shoot formation in NR^- cultures on NH_4-S-RMOP medium. NX9 and NA9 were the original NR^- cell lines; A28+NX9/13G and A28+NA9/9C were NR^- segregants in the F2. Wild-type NR^+ callus (DPN) and NR^+ segregant (F2) A28+NA9/1F are also shown

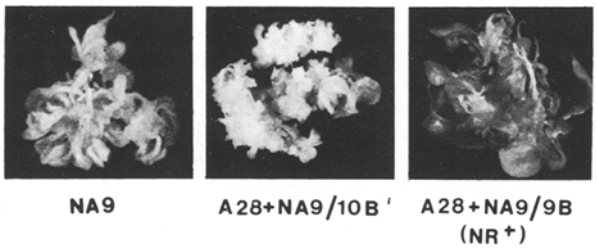

Fig. 2. Arginine (2 mM) induces shoot formation in the NR^- calli. NA9 was the original cell lines, A28+NA9/10B and A28+NA9/9B were NR^- and an NR^+ segregants in the F2. Calli are shown during the 3rd subculture on NH_4-S-RMB medium

Plant regeneration in the NR^- cultures was obtained (Fig. 2) if the ammonium succinate containing RMB medium was supplemented with arginine (2 mM). The number of shoots found in the NR^- clones was lower than in the wild-type culture. These shoots could be grown into plants after grafting. (Further data on regeneration of NR^- cell lines in Purnhauser et al. in preparation.)

Discussion

Segregation in the seed progeny of the NA9+A28 and NX9+A28 somatic hybrids confirmed that the NR^- phenotype in the NA9 and NX9 clones was the result of a recessive nuclear mutation. No information was obtained in 7 other somatic hybrid combinations due to the complete sterility of the regenerated plants. Sterility may be due to aneuploidy and chromosome abnormalities in the somatic hybrids (Table 2). Since most of the SF1 clones were aneuploids it seems probable that the NR^- cell lines from which the protoplasts were isolated for fusion contained cells with variable chromosome numbers. This would explain why all somatic hybrid plants of the NR^-+NR^- combinations in a previous experiment had aberrant morphologies, and were sterile (Márton et al. 1982 b). The addition of the genome of the morphologically normal, diploid line, A28 (instead of another aneuploid NR^- line) to the aneuploid NR^- genomes resulted in an improved morphology and fertility. The fact that several of the tetraploid somatic hybrids (Table 1) had fertility problems indicates that in the mutants, other changes than just alterations in chromosome numbers have occurred or those plants with 40 chromosomes were not real tetraploids.

The segregation ratios in autotetraploids can be predicted only if the meiotic behavior of chromosomes and the transmission of the gametes are known. Each chromosome is present in four copies which may become associated in a variety of ways (2 bivalents, 1 bivalent+2 monovalents, 1 monovalent+1 trivalent, four univalents or most frequently 1 quadrivalent). The frequencies of different associations, crossovers, and the distribution of chromosomes at anaphase II can result in different ratios after selfing an autotetraploid (duplex) plant. Assuming only quadrivalent associations of the homologous chromosomes and absolute linkage between the NR gene and the corresponding centromere, a ratio of 35 : 1 would be expected. In the case of maximal equational segregation the gene segregates independently from the centromeres due to crossovers and the segregation ratio would be ideally 19.3 : 1. In our case for both NR loci the actual segregation ratio was below this value (Table 2). This may be explained by the reduced transmission of the aneuploid gametes and the reduced germination ability of the NR^- seeds.

Concomitant changes in the NR activity and shoot regeneration ability in the SF1 and F2 plants indicate either a causal relationship between the two traits, or linkage. Isolation of 32 independent NR^- lines in our laboratory (M. Czako, personal communication) with similarly supressed regeneration ability seems to favour the first alternative.

Difficulties in plant regeneration in fully NR^- cell lines were also reported by Negrutiu et al. (1983), where less then 50% of the clones regenerated functional plants or by Steffen and Schieder (1983), where no regeneration could be obtained in *Petunia hybrida* cell lines. In another case difficulties in plant regeneration could be overcome by using high BA/NAA ratios and low ammonium-succinate concentrations in the culture medium (R. Grafe, personal communication).

Our finding that the addition of arginine to NH_4-S-RMB medium results in the partial restoration of shoot forming ability in the NR^- cultures also indicates that responsiveness to shoot induction can be restored by feeding an appropriate metabolite.

The existence of two types of NR^- lines, one with normal (apart from the leaky ones) and one with altered requirements for shoot induction however, cannot be excluded.

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