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Production and Characterization of Nitrate Reductase Deficient Mutants in *Petunia parviflora*

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Abstract - Nitrate reductase deficient (NR^-) mutant lines were selected indirectly by their resistance to 100mM chlorate in cell cultures of *P. parviflora*. A total of 585 chlorate resistant lines were confirmed by a second passage on a high concentration of chlorate. Frequency of spontaneous mutation was 9.7×10^{-7} in 3 month old suspension-cultured cells, and in non-selective media containing amino acids as sole nitrogen source. The frequency of mutation could be increased up to 11-fold by culture for 12 months. Out of 40 randomly selected calli, 22 were fully deficient in NR. The rest of the clones contained a decreased level of NR activity. Further characterization was carried out in 13 mutant lines which were fully deficient in NR and in 5 mutant lines containing residual (0-7.0%) NR activity, as compared to wild-type cells cultured on the same medium. The NR^- mutants were tentatively classified as defective in the NR apoenzyme (nia-type; 11 mutant lines including the 5 with residual NR activity) or in the molybdenum cofactor (cnx-type; 7 mutant lines) by the XDH activity. The cnx-type could be further classified into two groups. In one group (5 mutant lines) of these, the NR activity could be partially restored by nonphysiologically high (1.0mM) molybdate in the culture medium. Both types of NR^- mutants were unable to grow on minimal medium containing nitrate as sole nitrogen source, but grew well on amino acids. They also proved to be extremely sensitive to the standard medium (MSP_1) containing nitrate and ammonium. Shoot regeneration was obtained only in the NR^- mutants, which contained residual NR activity, but they so far have failed to grow into plants.

Key words - Nitrate reductase deficient (NR^-) mutant, *Petunia parviflora*, NR apoenzyme, Molybdenum cofactor

Introduction

The assimilation of nitrate in higher plants is an important metabolic process and therefore forms the subject of extensive physiological and biochemical investigations (Muller and Grafe, 1978). Nitrate reductase (NR) (NADPH: nitrate oxidoreductase, EC 1.6.6.3) catalyzes the initial controlling step in nitrate assimilation by the reduction of nitrate to nitrite using NADH as the electron donor. Nitrite is reduced to ammonium by nitrite reductase (NADPH: nitrite oxidoreductase, EC 1.6.6.4) and ammonium is finally assimilated into amino acids (Kleinhofs *et al*., 1985).

NR also reduces chlorate to chlorite which is toxic to plant cells (Marton *et al*., 1982). Nitrate reductase deficient (NR^-) cells, on the other hand, are resistant to chlorate, so it can be used for indirect selection of NR^- cells. NR^- mutants have been obtained in several plant species by screening for resistance of plants to chlorate (Oostindier-Braaksma and Feenstra, 1973; Feenstra and Jacobsen, 1980; Braaksma and Feenstra, 1982). However, except for certain barley mutants reported by Bright *et al*. (1982), all NR^- mutants isolated at plant level were shown to maintain residual NR activity. In contrast, fully NR^- mutant lines have been obtained from *in vitro* culture systems, such as cell suspension or protoplast cultures, of *P. hybrida* var. Mitchell (Steffen and Schieder, 1984), *N. tabacum* (Muller and Grafe, 1978; Evola, 1983) and *N. plumbaginifolia* (Marton *et al*., 1982; Negrutiu *et al*., 1985; Pelsy *et al*., 1988).

Such NR^- mutants were obtained either spontaneously (Marton *et al*., 1982 ; Pelsy *et al*., 1988) or after treatment with mutagens such as N-ethyl-N-nitrosourea (Muller and Grafe, 1978; Marton *et al*., 1982; Evola, 1983), X-rays (Steffen and Schieder, 1984), UV light (Negrutiu *et al*., 1985) and gamma rays (Grafe *et al*., 1986; Pelsy *et al*., 1988).

NR^- mutants were shown to be double recessive mutants for both nia- and cnx-types (Muller, 1981,1982). The nia-type is defective in the apoprotein of NADH-NR, therefore, unable to synthesize a functional apoenzyme but able to synthesize an active molybdenum cofactor. On the other hand, the cnx-type possesses the apoprotein but lacks NR activity due to an inability to synthesize the molybdenum-containing cofactor that is essential for the function of NR. This molybdenum cofactor deficiency also causes the loss of xanthine dehydrogenase (XDH : EC 1.2.1.37) activity (Mendel and Muller, 1979; Mendel *et al*., 1981,1982a,b; Buchanan and Wray, 1982). The cnx-type mutants

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could be further classified into two groups. In one of these, NR activity could be partially restored by adding high concentrations of molybdate in the culture medium (Mendel *et al.*, 1981; Marton *et al.*, 1982; Steffen and Schieder, 1984). On the contrary, the other cnx-type can not be repaired under the same molybdate concentrations (Marton *et al.*, 1982; Xuan *et al.*, 1983; Steffen and Schieder, 1984; Mendel *et al.*, 1986).

NR^- mutants are complete auxotrophs which have a strong requirement for nitrogen in a reduced form such as amino acids (Muller and Grafe, 1978) or ammonium succinate (Muller, 1983). Because of this property, NR deficiency has readily been utilized as a selective marker for the production of somatic or gametosomatic hybrids via complementation to nitrate reductase proficiency (Kohn *et al.*, 1985; Pirrie and Power, 1986; Brunold *et al.*, 1987; Tempelaar *et al.*, 1987; De Vries *et al.*, 1988). By complementation analysis of somatic hybridization or genetic crosses, both nia and cnx mutant types complement to give somatic hybrids with restored NR activity (Steffen and Schieder, 1984; Pelsy *et al.*, 1988).

P. parviflora has many useful characters, such as a prostrate, trailing vigorous growth habit but this species exists in no other form than the albino for somatic hybridization studies. The production of an NR^- mutant in *P. parviflora* was attempted with the aim of obtaining an alternative selectable marker since, using *P. parviflora* in the albino form gave hybrid callus but no plant regeneration.

Materials and Methods

1. Initiation and establishment of callus and cell suspension

P. parviflora seeds, vernalized at 4℃ (dark, 2 weeks) were surface

Table 1. Media composition used for this study

Components ($mg \cdot L^{-1}$) \ Media	MSO	MSP_1	UM	MSZ	MSD_3	AA	AAP_1	AAD_3	AA-chlorate	$MSNO_3$	$MSDNO_3$	NF_1	NF_2	NF_3
$CaCl_2 \cdot 2H_2O$	440	440	440	440	440	440	440	440	440	440	440	440	440	440
NH_4NO_3	1650	1650	1650	1650	1650	-	-	-	-	-	-	-	-	-
KNO_3	1900	1900	1900	1900	1900	-	-	-	-	3970	3970	-	-	-
KCl	-	-	-	-	-	2940	2940	2940	-	-	-	2940	2940	2940
$KClO_3$	-	-	-	-	-	-	-	-	12.255	-	-	-	-	-
KH_2PO_4	170	170	170	170	170	170	170	170	170	170	170	170	170	170
$MgSO_4 \cdot 7H_2O$	370	370	370	370	370	370	370	370	370	370	370	370	370	370
KI	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83	0.83
$CoCl_2 \cdot 6H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
H_3BO_3	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2	6.2
$Na_2MoO_4 \cdot 2H_2O$	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25	0.25
$MnSO_4 \cdot 4H_2O$	22.3	22.3	22.3	22.3	22.3	22.3	22.3	22.3	22.3	22.3	22.3	22.3	22.3	22.3
$CuSO_4 \cdot 5H_2O$	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025	0.025
$ZnSO_4 \cdot 7H_2O$	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6	8.6
$FeSO_4 \cdot 7H_2O$	27.85	27.85	27.85	27.85	27.85	27.85	27.85	27.85	27.85	27.85	27.85	27.85	27.85	27.85
Na_2EDTA	37.25	37.25	37.25	37.25	37.25	37.25	37.25	37.25	37.25	37.25	37.25	37.25	37.25	37.25
Glycine	2.0	2.0	2.0	2.0	2.0	-	-	-	-	-	-	-	-	-
Myo-inositol	100	100	100	100	100	100	100	100	100	100	100	100	100	100
Nicotinic acid	0.5	0.5	5.0	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Pyridoxine NCl	0.5	0.5	10.0	0.5	0.5	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Thiamine HCl	0.1	0.1	10.0	0.1	0.1	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
Casein hydrolysate	-	-	2000	-	-	-	-	-	-	-	-	-	-	-
L-glutamine	-	-	-	-	-	877	877	877	877	-	-	-	-	-
L-arginine	-	-	-	-	-	228	228	228	228	-	-	-	-	-
L-glycine	-	-	-	-	-	75	75	75	75	-	-	-	-	-
L-aspartic acid	-	-	-	-	-	266	266	266	266	-	-	-	-	-
6-BAP	-	0.5	-	-	1.0	-	0.5	1.0	0.5	0.5	1.0	-	1.0	0.5
Kinetin	-	-	0.25	-	-	0.2	-	-	-	-	-	-	-	-
Zeatin	-	-	-	1.0	-	-	-	-	-	-	-	1.0	-	-
IAA	-	-	-	-	2.0	-	-	2.0	-	-	2.0	-	2.0	-
NAA	-	2.0	-	-	-	-	2.0	-	2.0	0.1	-	-	-	2.0
2,4-D	-	-	2.0	-	-	1.0	-	-	-	-	-	-	-	-
GA_3	-	-	-	-	-	0.1	-	-	-	-	-	-	-	-
Sucrose	30,000	30,000	30,000	30,000	30,000	20,000	20,000	20,000	20,000	20,000	20,000	30,000	30,000	30,000

sterilized and subsequently germinated on agar-solidified MSO medium (Table 1). Following the removal of leaves from axenic shoots, obtained 3-4 weeks after germination, the remaining stems were cut into pieces (1.0-1.5cm length) and transferred (4-5/50mL medium, 6 oz jars) to agar-solidified UM or MSP_1 media (Table 1) for callus induction. Cultures were maintained at 23-25℃ under a continuous illumination of 500-1,000 lux (daylight fluorescent tubes).

Friable stem callus was transferred to AA liquid medium (5 g f.wt./80mL in 250mL flask; Table 1) and maintained on a rotary shaker (80 cycles/min) under the same conditions as used for cell suspension cultures of albino *P. parviflora*. Cell suspensions were subcultured every 7 days. Whilst callus cultures were maintained by subculturing (every 3-6 weeks) on agar-solidified AA medium as a renewable source for cell suspensions.

2. Selection of NR^- mutants

In order to assess the effects of cell suspension age on spontaneous NR^- mutation, cells harvested, at intervals of 3 months (3, 6, 9, 12 months after initiation), from cell suspensions of *P. parviflora* were used to select for NR^- mutants. Selection of NR^- mutants was performed indirectly by selecting for chlorate resistant mutants on AA-chlorate medium (Table 1) which contained 100mM $KClO_3$ as a selective agent and with amino acids as sole nitrogen source.

Cells were harvested from exponential growth phase suspensions (5-6 days post subculture). Spent AA medium was removed from cells by filtration through a 20μM nylon sieve. The cells were washed twice with 100mL of liquid AA-chlorate medium and resuspended in the same medium at a final density of 100mg (approx. 1.0×10^6 cells)/mL medium. This cell suspension (2mL) was spread on the surface of AA-chlorate medium (30mL) in 9cm petri dishes. For each assessment, 3 flasks were used and cells in each flask were placed in 5 petri dishes.

Controls were prepared using friable callus grown on agar-solidified MSP_1 medium 7 days after subculture. Callus was forced through a sieve (mesh size 1.0mM) to obtain a suspension of smaller cell aggregates. Cells were washed and inoculated (15 dishes) using the same methods as used for suspension-cultured cells. After 1 week, dishes were inverted and kept for further 3 weeks. Any green calluses that developed were transferred (10-15calli/30mL) to the same medium and results were assessed after 4 weeks.

3. Characterization of putative NR^- mutants

Surviving calluses (chlorate resistant) were transferred (10-15calli/30mL) to, and propagated on the surface of agar-solidified AAP_1 medium in 9cm petri dishes and used for the following analyses. Callus pieces (approx. 3mM diam.) of wild-type *P. parviflora* established on MSP_1 medium were cultured (10-15calli/30mL) on $MSNO_3$ medium (Table 1), containing nitrate as sole nitrogen source, for 2 months (1 subculture in between) in order to eliminate NR^- cells which might have been induced spontaneously and survived on MSP_1 medium. Surviving calluses (NR^+) were then cultured (10-15calli/30mL) on solidified AAP_1, $MSNO_3$ or MSP_1 media and used as a control, and NR^- mutant callus of *N. tabacum* (nia-130) cultured on AAP_1 medium (Table 1) was also used for the same purpose.

Assessment of growth of chlorate-resistant cell lines on $MSNO_3$ medium

The growth of 40 randomly selected chlorate-resistant calluses was tested on $MSNO_3$ medium in order to confirm their lack of ability to utilize nitrate. Small pieces (3mM diam.) of each callus line were cultured on $MSNO_3$ medium in 5 × 5 grids (5mL/well). Each line was replicated 5 times. Results were assessed after 4 weeks culturing with each week observation of cell growth.

Assessment of NR activity in calluses

NR has been known to catalyze the initial controlling step in nitrate assimilation, reducing nitrate to nitrite (Cove, 1976,1979; Kleinhofs *et al.*, 1985). Selected mutants unable to utilize nitrate as nitrogen source must have a reduced or no NR activity. For this reason, NR^- mutant calli were tested for their NR activity. In vivo NR activity was estimated by anaerobically incubating cells with nitrate and determining the production of nitrite using the modified method of Jaworski

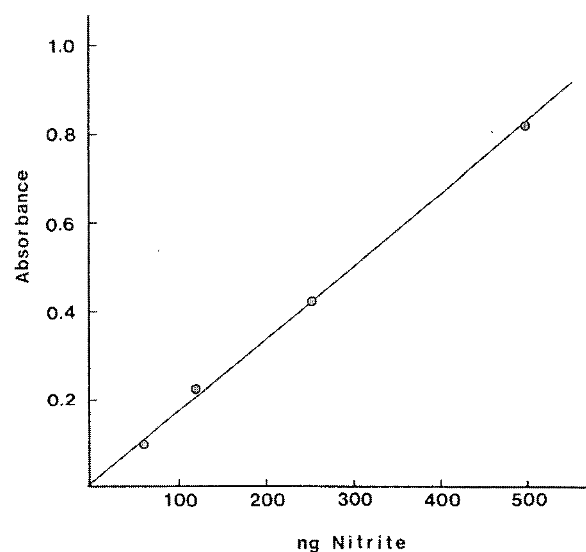

Fig. 1. Calibration curve for nitrate reductase activity assay (Jaworski, 1971). Absorption of light at 540nm was recorded.

(1971). Callus samples (100mg) of 18 selected lines were individually placed in 3mL of incubation solution containing 37.5mM KNO_3, 16.6mM Na-phosphate (pH = 7.5) and 2.5% (v/v) ethanol for reaction and incubated under vacuum for 2 h at 25℃ in the dark. Nitrite released into the solution was determined by adding 1mL of 1.0% (w/v) sulfanilamide in 3.0 N HCl and 1mL of 0.02% (w/v) aqueous N-1-naphthylethylenediamine dihydrochloride to 0.5mL of supernatant obtained. After 30 min, the absorbance of the clear supernatant was read at 540nm using a Unicam SP 600 spectrophotometer. Readings were calibrated against a standard curve of nitrite absorbance (Fig. 1). NR activity is expressed as nmoles NO_2^- formed per 100mg fresh weight of cells in 2 hours.

Assessment of xanthine dehydrogenase (XDH) activity

It has been proven that XDH and NR shared a common molybdenum-containing cofactor and genetic defects in the cofactor abolished both enzyme activities (Arst *et al*., 1970; MacDonald *et al*., 1974; Scazzocchio, 1974). NR- mutants could be therefore defective not only in the apoenzyme (nia-type) but also in the molybdenum cofactor (cnx-type). For this reason, NR^- mutants were tested for their XDH activity. Soluble protein extracts were prepared from callus samples 8 days after inoculation on AAP_1 medium, and the protein samples (100 μl) were loaded to each well of prepared gels. Following electrophoresis, gels were stained for XDH isoenzyme. All procedures were as described by Lee (1989).

Assessment of the response of the NR^- mutant lines to molybdate

It has been shown that NR activity could be restored in some of the cofactor-defective mutants by adding high concentrations of molybdate in the medium (Steffen and Schieder, 1984). Therefore, to possibly classify NR^- mutants further, 5 callus pieces (3mM diam.) taken from each of 18 NR^- selected lines were cultured on solidified MSP_1 medium (in 5 × 5 grids) which contained 1.0mM Na_2MoO_4. Results were assessed after 1 month, and NR activity of surviving callus was reassessed.

4. Shoot regeneration assessments

Regeneration assessments were carried out on individual NR^- mutant lines following their prolification (3-4 weeks) on solidified AAP_1 medium. Small pieces of such callus (4-5mM diam.) were placed on solidified AAD_3 medium (Table 1) and/or the following agar-solidified media: MSZ, MSD3, $MSNO_3$, $MSDNO_3$, NF_1 and NF_2 media (Table 1) each supplemented with 0, 5.0, 10.0, 15.0 or 20.0mM ammonium succinate. Experiments were performed with 3 replicates, and calli were cultured for two months with one subculture. The long-term culture of NR^- mutants, on AAD_3 medium, was also undertaken for up to 7 months with monthly subcultures. All cultures were maintained at 27℃ under continuous illumination (3,000 lux).

5. Cytological analysis

Due to the difficulty of inducing shoots, the cytological analysis of 6 NR^- lines was performed using actively growing cell suspensions in liquid AA medium. Actively growing callus and cell suspensions, of selected putative hybrids were incubated in an aqueous saturated solution of bromonaphthalene for 2 h at 24℃. After two washes in distilled water, cells were fixed in acetic alcohol (24 h), washed twice in distilled water, hydrolyzed in 1 M HCl (15 min), then finally washed (twice) in distilled water. The hydrolyzed cells were stained and examined. Twenty cells, scored for metaphase plates, were examined and cell chromosome numbers recorded.

6. Culture of NR^- mutants

Following NR^- mutant culture experiments were performed in order to determine the possibility of utilizing NR^- mutants, obtained in this study, for future somatic or gametosomatic hybridization as a selectable marker, as well as to determine the effectiveness of NR^- mutant selection system employed. Calluses of the 18 NR^- mutants (see Table 4) and wild-type *P. parviflora* (log-phase material) pre-grown (7-8 days) on AAP_1 solidified medium were used for these assessments.

Assessment of the response of the NR^- mutant lines to different nitrogen sources

In order to determine the cultural response of the NR^- mutants (nia-3,10,11 and cnx-6), compared to wild-type in *P. parviflora*, to different nitrogen sources, NF_3 medium (nitrogen-free medium ; Table 1), and MSP_1 (NO_3/NH_4), AAP_1 (NH_4) and $MSNO_3$ media (NO_3) were used. Five callus pieces (approx. 3mM diam.) were inoculated in a 9cm petri dish which contained 30mL of the agar-solidified media. The initial fresh weight of 5 callus portion was estimated by the weight differences of petri dish between pre- and post-inoculation. Each treatment was with 3 replicates. The fresh weight gain of callus was estimated every 7 days up to 49th day after inoculation by weighing 15 calli (5 calli × 3 replicates) per treatment.

Assessment of changes in NR activity during culture

In order to determine any possible correlation between culture duration and NR activity, calluses of NR^- mutants (nia-2,9,11 and cnx-1)

and wild-type *P. parviflora* were cultured on agar-solidified, non-selective AAP_1 medium. Callus pieces (3mM diam.) were inoculated (5calli/30mL) on solidified AAP_1 medium and cultured as in 5.2.1. Prior to inoculation, the initial NR activity of each cell line was assessed. NR activity was determined using samples 100mg samples of callus with 3 replicates. Assessments were carried out every 4 days, including day 2, up to 24th day after inoculation.

Testing of nurse culture effects

Cell suspensions [100mg f.wt. (approx. 1.0×10^6 cells)/mL] were prepared from calli of the NR^- mutants (nia-4,6 and cnx-2,4) and wild-type and used for following two kinds of experiments. Each experiment was carried out with 3 replicates.

Firstly, in order to determine whether reciprocal cross-feeding between different types (nia- and cnx-type) of NR^- mutants would stimulate both NR^- mutants to utilize nitrate by metabolic cooperation, mixture (1:1) of nia-type and cnx-type cell suspensions (4 combinations) were prepared and cultured (2mL/30mL of medium) on solidified $MSNO_3$ medium for 4 months without subculture.

Secondly, the effectiveness of chlorate as selective agent was studied with mixed cultures of wild-type and NR^- mutants. Cell suspensions of wild-type and NR^- mutants were mixed (100:1; 4 combinations) and cultured (2mL/30mL of medium) on solidified AAP_1 medium. After 1 month, random samples of 100 calli per each treatment were cultured (25calli/30mL) on solidified AA-chlorate medium for 1 month. Any surviving calli were again transferred (25calli/30mL) on $MSNO_3$ solidified medium and cultured for 1 more month. This experiment was also performed in order to determine whether reciprocal cross-feeding between NR^- mutants and wild-type would stimulate the passage of wild-type or NR^- mutants through the sequential selection procedure by metabolic cooperation.

Testing of NR^- mutant stability

NR^- mutant calluses (the 18 lines as described in Table 4) were tested every 3 months on $MSNO_3$ and AA-chlorate media in order to find the stability of NR^- mutants or the presence of nitrate-utilizing cells. Callus pieces (3mM diam.) of each NR^- line were transferred (5calli/30mL) to solidified $MSNO_3$ and AA-chlorate media with 2 replicates. One of two petri dishes per each NR^- line was cultured for 1 month but the other for 5 months without subculture.

Results

1. Selection of NR^- mutants

During the first selection, callus, presumably from wild-type cells, ceased division and became brown. On the other hand, a few green-coloured calli, considered to be NR^- mutants, were capable of growth on AA-chlorate medium and were readily visualized against the background of brown/dead callus.

Following 2 subsequent selections, a total of 585 chlorate resistant calli developed in the chlorate-containing selection plates with the best results (327 colonies) using 12 month old suspension-cultured

Table 2. Effect of culture duration on selection frequency of NR^- mutants

Culture duration (month)	Total no. of NR^- mutants selected	Frequency of selected NR^- mutants (per 10^7 cells tested)[z]
0	0	0
3	29	9.7
6	73	24.3
9	156	52.0
12	327	109.0

[z]For each treatment, 15 dishes each containing 200mg of cells were used (1 g callus ≒ 1.0×10^7 cells).

Table 3. Classification of randomly selected NR^- mutants by the level of NR activity

NR- mutants tested		No. of NR^- mutants			
Origin (selected from)	Total no. of NR mutants	NR activity[z]			
		0	0-10	10-20	20-30
3 month cultured cells	10	4	3	1	2
6 month cultured cells	10	6	2	1	1
9 month cultured cells	10	5	4	1	0
12 month cultured cells	10	7	2	0	1

[z]NR activity was determined in samples of 100mg callus, and expressed as nmoles NO_2^-/2 h, 100mg f. wt.

cells (Table 2). In contrast, no colonies were selected from the control dishes. These data clearly indicated that the frequency of spontaneously induced NR^- cells was enhanced with time in culture.

2. Characterization of NR^- mutants

Growth on $MSNO_3$ medium

Of forty chlorate resistant cell lines (Table 3) cultured on $MSNO_3$ medium, none survived. This confirmed that the selected cell lines were not capable of utilizing nitrate and were therefore NR^- mutants.

NR activity

Twenty-two cell lines were fully deficient, while in the other 18 lines residual NR activities (0-7.0%) were found (Table 3). By contrast, the cell lines used as wild-type was characterized by a relatively high constitutive level of NR activity. Wild-type used in this study exhibited considerable NR activity [corresponding to approx. 33.5% of the level induced (395.8) on $MSNO_3$ medium] even when grown on AAP_1 medium (Table 4). This high basal level of NR activity allowed to detect the NR deficiency of mutants and could be responsible for the high chlorate sensitivity of wild-type cells which favoured the selection of NR^- mutants. NR activity was also assessed from readily available another NR^- mutant, *N. tabacum* nia-130, and was about 6.3.

In addition, the intensity of pink colour, which appeared as a result of the reaction of NO_2^- released in incubation medium to the reagent solution for nitrite assay, was in direct proportion to NR activity at 540nm. Therefore, this could be also used to distinguish the NR^- mutants from wild-types.

Table 4. Growth properties and biochemical characteristics of NR^- mutant lines in *P. parviflora*

Cell lines		Chlorate resistance[z]	NR activities[y]	XDH activities[x]	Growth on[w]				Organogenesis[v]
					MSP_1 + 1.0mM Na_2MoO_4	AAP_1	$MSNO_3$	MSP_1	
Wild-type *P. parviflora*		R_0	132.4±19.7 395.8±47.0[a] 382.6±40.2[b]	+	+++	+++	+++	+++	+
N. tabacum (nia-130)		R_2	6.3±2.6	+	+	+++	0	+	+
NR^- mutant *P. parviflora*	nia- 1	R_2	0	+	0	+++	0	0	-
	nia- 2	R_2	0	+	0	+++	0	0	-
	nia- 3	R_2	0	+	0	+++	0	0	-
	nia- 4	R_2	0	+	0	+++	0	0	-
	nia- 5	R_2	0	+	0	+++	0	0	-
	nia- 6	R_2	0	+	0	+++	0	0	-
	nia- 7	R_1	2.3±1.4	+	0	+++	0	0	-
	nia- 8	R_1	3.7±0.8	+	+	+++	0	+	+
	nia- 9	R_1	5.6±0.9	+	+	+++	0	+	-
	nia-10	R_1	8.0±0.7	+	+	+++	+	+	+
	nia-11	R_1	9.2±1.1	+	+	+++	+	+	+
	cnx-1	R_2	0	-	0	+++	0	0	-
	cnx-2	R_2	0	-	0	+++	0	0	-
	cnx-3	R_2	0	-	++	+++	0	0	-
	cnx-4	R_2	0	-	++	+++	0	0	-
	cnx-5	R_2	0	-	+++	+++	0	0	-
	cnx-6	R_2	0	-	+++	+++	0	0	-
	cnx-7	R_2	0	-	+++	++	0	0	-

[z](R0) Not capable of growth on 100mM chlorate, (R1) Capable of growth on 100mM chlorate without necrosis, (R2) Fully resistant up to 100mM chlorate.
[y]NR activity of cell lines, all grown on AAP_1 medium, except: ⓐ $MSNO_3$, ⓑ MSP_1 medium.
[x]XDH activity: (+) Possession, (-) Absence.
[w](0) No growth, (+) Able to grow initially, but finally dead with necrosis, (++) Able to grow without necrosis, (+++) Active growth.
[v](+) Organogenesis on AAD3 medium (nia-10), or on MSDNO3 medium supplemented with 5.0-20.0mM ammonium succinate (nia-8,11), (-) No organogenesis.

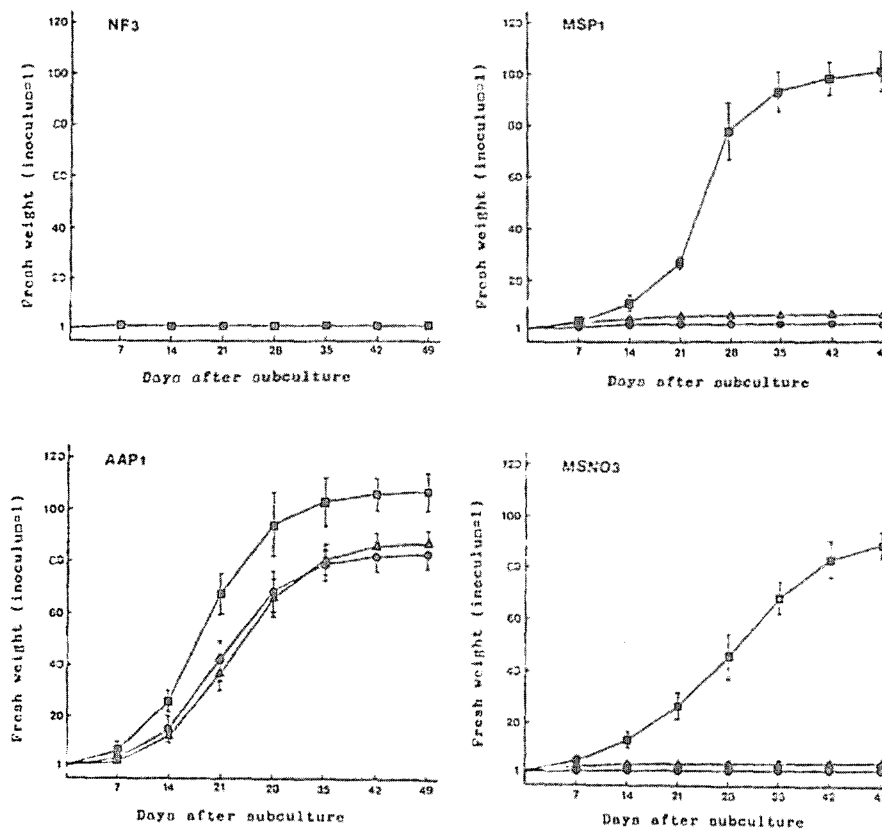

Fig. 2. Increase in fresh weight of cells during culture on four kinds of media containing different nitrogen sources.

Key: 1. Cell lines :

- ■ Wild-type.
- ▲ NR^- cell lines possessing residual NR activity (nia-10,11).
- • NR^- cell lines possessing no detactable NR activity (nia-3, cnx-6).

2. Each value = mean ± standard error.

n = 3 (wild-type) or 6 [two types (▲ ,•; see 1) of NR^- mutants each with 2 cell lines and 3 replicates]. Where n denotes number of replicates.

Classification of the *P. parviflora* NR^- mutants by XDH activity

The XDH activity was assessed from 18 randomly selected NR^- mutants. On this basis, 7 NR^- mutant lines were found to be deficient in both NR and XDH activities and were therefore designated as cnx-types. A lack of XDH activity reflected a defect in the molybdenum cofactor. Eleven lines which had XDH activity were likely to be defective in the apoenzyme and were therefore designated as nia-types (no active apoenzyme; Table 4).

Classification of NR^- mutants by their response to molybdate

All 18 NR^- mutant lines, tested by growth on MSP_1 medium (containing KNO_3 and NH_4NO_3) and supplemented with 1.0mM Na_2MoO_4 gave five cnx-types (cnx-3-7) which could grow on this medium and they had approximately 12-17% of the total NR activity as found in the wild-type, grown on nitrate medium (Table 4). The other cnx-types and nia-types tested did not grow under these conditions.

3. Shoot regeneration

Attempts to regenerate whole plants from the NR^- mutants failed. Small shoots or primordia-like structures were observed for these NR^- mutant lines (nia-8,10,11), which exhibited residual NR activities, on AAD_3 medium or $MSDNO_3$ medium, containing 5.0-20.0mM ammonium succinate, but were not grown to plants. However, no organogenesis was obtained in the fully NR^- cell lines.

4. Cytological analysis

The NR^- mutant lines (nia-1,5,8 and cnx-1,3,5) had a normal *P. parviflora* chromosome number of 2n=2x=18 with the exception of 2 cell lines (nia-8, cnx-3) which were polyploid (2n=4x=36).

5. Culture of NR$^-$ mutants

Response of the NR$^-$ mutant lines to different nitrogen sources

The results of the growth responses of each of the four NR$^-$ mutant lines (nia-3,10,11 and cnx-6) when compared with that of the wild-type cell line on various nitrogen-containing media are presented in Fig. 2. Wild-type cells were found to grow better on mixtures of amino acids (in AAP_1 medium) as sole nitrogen sources than in the presence of other reduced nitrogen compounds (in MSP_1 or $MSNO_3$ media). AAP_1 medium was preferably used as non-selective medium in this study because of the better growth of wild-type and NR$^-$ mutants, as compared to MSP_1 medium. When grown on AAP_1 medium, the NR$^-$ mutants formed a bright, friable callus and were thus morphologically similar to the wild-type line. These results indicated that the utilization of amino acids as nitrogen source was independent of whether or not the cells possessed active NR.

On the other hand, NR$^-$ mutant cells were unable to utilize nitrate. When grown on $MSNO_3$ medium containing nitrate as sole nitrogen source, they behaved like nitrogen-starved cells with approximately a 4-fold increase in cell fresh weight. Two kinds of NR$^-$ mutants, which contained no NR$^-$ activity and residual NR activity, showed similar responses on all media.

Effect of culture duration on NR activity

Results are presented in Fig. 3. The NR activity in wild-type was found to be relatively high and dependent on the age of culture. Maximum activity of NR in wild-type occurred in log-phase cells and was usually attained at about 8 days after transfer of the cells to new AAP_1 medium.

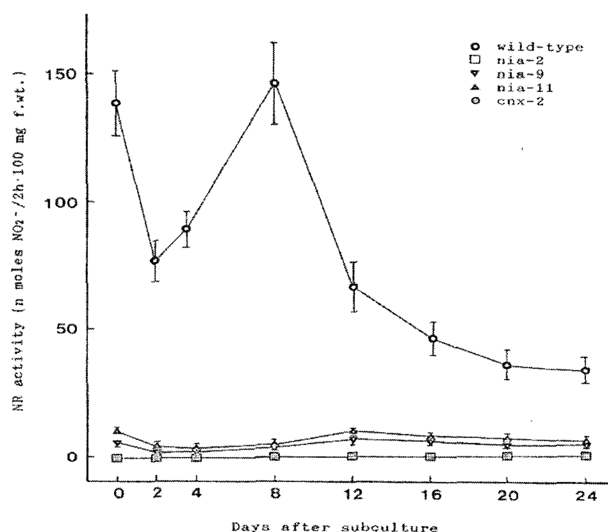

Fig. 3. Effect of culture duration on NR activity of wild-type and NR$^-$ mutant cells grown on AAP_1 medium.
Each value = mean ± standard error.

In two full NR$^-$ mutants (nia-2 and cnx-1), no in vivo NR activity was detected during growth on AAP_1 medium. Thus, the NR$^-$ mutant lines differed from the wild-type line in lacking a constitutive NR activity. This result implied that the abolition of NR activity was independent of the induction by nitrate and could not be the result of an impaired nitrate uptake.

The NR$^-$ mutant lines (nia-9,11), containing residual NR activity, regularly exhibited a low level of NR activity. NR activities of these cell lines were, however, slightly increased at about 12 days after culturing on new AAP_1 medium.

Effect of nurse culture

Following two kinds of experiments, no growing sectors were observed, showing that nitrate utilization due to metabolic cooperation, not only between two types (nia- and cnx-type) of NR$^-$ cells but also between wild-type and NR$^-$ mutants, did not occur. This result also showed that the sensitivity of wild-type cells to chlorate was not abolished by close contact with resistant cells (NR$^-$ mutants).

Stability of NR$^-$ mutants

All 18 NR$^-$ cell lines maintained on AAP_1 medium, in the absence of the selective agent, were repeatedly tested on AA-chlorate and $MSNO_3$ media and found to remain unchanged. As a more sensitive test for the presence of nitrate-utilizing cells, NR$^-$ cell were cultured for 5 months under the same conditions, but no growing callus sectors were observed from all the cell lines. These results showed that either no revertants occurred or only such revertants that were unable to successfully complete with the NR$^-$ cells and, therefore, had been eliminated from the cell population.

Discussion

Albino mutants have been most frequently utilized for the production of somatic hybrids especially in *Petunia* (Cocking *et al.*, 1977; Power *et al.*, 1979. 1980). However, the resulting hybrid calli have often shown a lack of regeneration capability, presumably based on the non-regenerable albino parents. It was therefore thought that the production of NR$^-$ mutants would give an alternative source for somatic or gametosomatic hybridization in the genus Petunia. In this study, NR$^-$ mutants for *P. parviflora* have been produced for the first time.

The fact that wild-type *P. parviflora* exhibits considerable NR ac-

tivity in the absence of nitrate implies that the abolition of NR activity found in the mutant lines can not be due simply to a defect in nitrate uptake or induction mechanisms. It must instead result from a defect in genes, such as the structural genes for NR or genes responsible for biosynthesis of the molybdenum-containing cofactor.

In the present study, the successful isolation of three NR⁻ groups was confirmed by biological analyses. In their genetic and physiological behaviour, all lines could be classified as nia, cnx A or cnx B mutants which have been described previously for *P. hybrida* (Steffen and Schieder, 1984) and *N. tabacum* (Muller and Grafe, 1978; Buchanan and Wray, 1982; Grafe and Muller, 1983).

The nia-type clones (nia-1-11) were classified as defectives in the apoenzyme since the presence of the functional molybdenum cofactor was shown by XDH activity. The cnx-type clones lacked XDH activity and were, therefore, thought to be impaired in the synthesis of the molybdenum-containing cofactor necessary for NR and XDH activity. Thus, the cnx mutation affected a gene which controlled a step in the synthesis of the molybdenum cofactor. In the cnx-type clones, NR activity could be restored by including nonphysiologically high levels of molybdate (1.0mM) in the culture medium. The genetic event leading to the cofactor defect in these cnx-type mutants (cnx-3-cnx-7) seems to be analogous to the cnx A class mutants of *N. plumbaginifolia* (Pelsy *et al*., 1988).

The NR deficiency was stably and continuously expressed in cell culture. Such fully NR⁻ mutants represent excellent biochemical markers, such as the resistance to chlorate and the inability to use nitrate as sole source of nitrogen. In addition, the selection and counter-selection conditions were stringent. All these parameters are essential with respect to the potential use of NR⁻ mutants as genetic markers for the production of future somatic or gametosomatic hybrids.

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Literature Cited

Arst, H.N., Jr., D.W. MacDonald and D.J. Cove. 1970. Molybdate metabolism in *Aspergillus nidulans*. I. Mutations affecting nitrate reductase and/or xanthine dehydrogenase. Mol. Gen. Genet. 108: 129-145.

Braaksma, F.J. and W.J. Feenstra. 1982. Isolation and characterization of nitrate reductase-deficient mutants of *Arabidopsis theliana*. Theor. Appl. Genet. 64: 83-90.

Bright, S.W.J., P.B. Norbury and J.L. Wray. 1982. A conditional-lethal cnx-type nitrate reductase-deficient barley mutant. In: Nitrate Assimilation. Abst. Intl. Symp. Nitrate Assimilation-Molecular and Genetic Aspects. Gatersleben, GDR. pp.34.

Brunold, C., S. Krüer-Lebus, M.W Saul, S. Wegmuller and I. Potrykus. 1987. Combination of kanamycin resistance and nitrate reductase deficiency as selectable markers in one nuclear genome provides a universal somatic hybridizer in plants. Mol. Gen. Genet. 208: 469-473.

Buchanan, R.J. and J.L. Wray. 1982. Isolation of molybdenum cofactor defective cell lines of *Nicotiana tabacum*. Mol. Gen. Genet. 188: 228-234.

Cocking, E.C., D. George, M.J. Price-Jones and J.B. Power. 1977. Selection procedures for the production of inter-species somatic hybrids of *Petunia hybrida* and *Petunia parodii*. II. Albino complementation selection. Plant Sci. Lett. 10: 7-12.

Cove, D.J. 1976. Chlorate toxicity in *Aspergillus nidulans*: studies of mutants altered in nitrate assimilation. Mol. Gen. Genet. 146: 147-159.

Cove, D.J. 1979. Genetic studies of nitrate assimilation in *Aspergillus nidulans*. Biol. Rev. 54: 291-327.

De Vries, S.E., W.J. Van Everdink, M.A. Ferwerda, E. Jacobsen, L.P. Pijnacker and W.J. Feenstra. 1988. Studies on gene mapping in the potato using somatic hybrids of *Solanum tuberosum* L. + *Nicotiana plumbaginifolia*. In: Progress in plant protoplast Reserch. (Eds. Puite, K.J., Dons, J.J.M., Huizing, H.J., Kool, A.J., Koornneef, M. and Krens, F.A.). Kluwer Academic Publishers, Dordrecht. pp. 215-218.

Evola, S.V. 1983. Chlorate-resistant variants of *Nicotiana tabacum* L. II. Parasexual genetic characterization. Mol. Gen. Genet. 189: 455-457.

Feenstra, W.J. and E.J. Jacobsen. 1980. Isolation of a nitrate reductase deficient mutant of *Pisum sativum* by means of selection for chlorate resistance. Theor. Appl. Genet. 58: 39-42.

Grafe, R., A. Marion-Poll and M. Cabiche. 1986. Improved *in vitro* selection of nitrate reductase-deficient mutants of *Nicotiana plumbarginifolia*. Theor. Appl. Genet. 73: 299-304.

Grafe, R. and A.J. Müller. 1983. Complementation analysis of nitrate reductase deficient mutants of *Nicotiana tabacum* by somatic hybridization. Theor. Appl. Genet. 66: 127-130.

Jaworski, E.G. 1971. Nitrate reductase assay in intact plant tissues. Biochem. Biophys. Res. Comm. 43: 1274-1279.

Kleinhofs, A., R.L. Warner and K.R. Narayanan. 1985. Current prog-

ress towards an understanding of the genetics and molecular biology of nitrate reductase in higher plants. Oxford Surv. Plant Mol. Cell Biol. 2: 91-121.

Khon, H., R. Schieder and O. Schieder. 1985 Somatic hybrids in tobacco mediated by electro-fusion. Plant Sci. 38: 121-128.

Lee, C.H. 1989. Somatic and Gametosomatic hybridization in the family *Solanaceae*. Ph.D. Thesis, Univ. of Nottigham, England.

MacDonald, D.W., D.J. Cove and A. Coddington. 1974. Cytochrome-C reductases from wild-type and mutant strains of *Aspergillus nidulans*. Mol. Gen. Genet. 128: 187-199.

Marton, L., T.M. Dung, R.R. Mendel and P. Maliga. 1982. Nitrate reductase deficient cell lines from haploid protoplast cultures of *Nicotiana plumbaginifolia*. Mol. Gen. Genet. 186: 301-304.

Mendel, R.R., Z.A. Alikulov, N.P. Lvov and A.J. Müller. 1981. Presence of the molybdenum cofactor in nitrate reductase-deficient mutant cell lines of *Nicotiana tabacum*. Mol. Gen. Genet. 181: 395-399.

Mendel, R.R., Z.A. Alikulov, N.P. Lvov and A.J. Müller. 1982a. Molybdenum cofactor in nitrate reductase-deficient tabacco mutants. II. Release of cofactor by heat treatment. Plant Sci. Lett. 25: 67-72.

Mendel, R.R., Z.A. Alikulov, N.P. Lvov and A.J. Müller. 1982b. Molybdenum cofactor in nitrate reductase-deficient tabacco mutants. III. Induction of cofactor synthesis by nitrate. Plant Sci. Lett. 27: 95-101.

Mendel, R.R., L. Marton and A.J. Müller. 1986. Comparative biochemical characterization of mutants of the nitrate reductase/molybdenum cofactor loci cnxA, cnxB and cnxC of *Nicotiana plumbaginifolia*. Plant Sci. 43: 125-129.

Mendel, R.R. and A.J. Müller. 1979. Nitrate reductase-deficient cell lines *Nicotiana tabacum*: Further biochemical characterization. Mol. Gen. Genet. 177: 145-153.

Müller, A.J. 1981. Nitrate reductase mutants and the cell culture approach to higher plants genetics. In: Problems in General Genetics, vol. II. Proc. 14th Intl. Cong. Genet. (Ed. Beliaev, D.K.). Mir, Moscow. pp. 229-237.

Müller, A.J. 1982. Genetic studies on nitrate reduction in *Nicotiana* and *Hordeum vulgare*. In: Nitrate Assimilation. Abs. Intl. Symp. Nitrate Assimilation-Molecular and Genetic Aspects. Gatersleben, GDR. pp. 9

Müller, A.J. 1983. Genetic analysis of nitrate reductase-deficient tobacco plants regenerated from mutant cells : Envidence for duplicate structureal genes. Mol. Gen. Genet. 192: 275-281.

Müller, A.J. and R. Grafe. 1978. Isolation and characterization of cell lines of *Nicotiana tabacum* lacking nitrate reductase. Mol. Gen. Genet. 161: 67-76.

Negrutiu, I., D. De Brouwer, R. Dirks and M. Jacobs. 1985. Amino acid auxotrophs from protoplast cultures of *Nicotiana plumbaginifolia*, Viviani. 1. BUdR enrichment selection, plant regeneration and general characterisation. Mol. Gen. Genet. 199: 330-337.

Oostindier-Braaksma, F.J. and W.J. Feenstra. 1973. Isolation and characterization of chlorate resistant mutants of *Arabidopsis thaliana*. Mutat. Res. 19: 175-185.

Pelsy, F., J. Gabard, I. Cherel, C. Meyer, A. Marion-poll, A.J. Müller and M. Caboche. 1988. Selection and characterization of nitrate reductase-deficient mutants of *Nicotiana plumbaginifolia*. Plant Cell Tiss. Org. Cult. 12: 231-233.

Pirrie, A. and J.B. Power. 1986. The production of fertile, triploid somatic hybird plants (*Nicotiana glutinosa* (n) + *N. tabacum* (2n)) via gametic : somatic protoplast fusion. Theor. Appl. Genet. 72: 48-52.

Power, J.B., S.F. Berry, J.V. Chapman, E.C. Cocking and K.C. Sink. 1979. Somatic hybrids between unilateral cross-incompatible *Petunia* species. Theor. Appl. Genet. 55: 97-99.

Power, J.B., S.F. Berry, J.V. Chapman, E.C. Cocking and K.C. Sink. 1980. Somatic hybridization of sexually incompatible petunias: *Petunia parodii, Petunia parviflora*. Theor. Appl. Genet. 57: 1-4.

Scazzocchio, C. 1974. The genetic determination of molybdoflavoenzymes in *Aspergillus nidulans*. J. Less-common Metals 36: 461-464.

Steffen, A. and O. Schieder. 1984. Biochemical and genetical characterization of nitrate reductase deficient mutants of *Petunia*. Plant Cell Rep. 3: 134-137.

Tempelaar, M.J., A. Duyst, S.Y. De Vlas, G. Krol, C. Symmonds and M.G.K. Jones. 1987. Modulation and direction of the electrofusion response in plant protoplasts. Plant Sci. 48: 99-105.

Xuan, L.T., R. Grafe and A.J. Müller. 1983. Complementation of nitrate reductase-deficient mutants in somatic hybrids between *Nicotiana* Species. In: Protoplasts 1983. (Eds. Potrykus, I., Harms, C.T., Hinnen, A., Hutter, R., King, P.J. and Shillito, R.D.). Poster Proc. 6th Intl. Protoplast Symp., Basel, Switzerland. pp. 21.

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