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# Complementation analysis of nitrate reductase deficient mutants of *Nicotiana tabacum* by somatic hybridization

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**Summary.** Mutant cell lines lacking nitrate reductase activity were analyzed genetically. Protoplasts from one apoprotein defective (nia) and four cofactor defective (cnx) mutants were fused in all possible pairwise combinations with the aid of polyethylene glycol. Complementing hybrids were detected by their ability to grow with nitrate as sole nitrogen source and confirmed by measuring their nitrate reductase activity. Strong complementation was observed in all types of nia + cnx hybrids, whereas the cnx mutants failed to complement each other. From the results it can be concluded that the mutants studied are recessive and that the four cnx mutants are alleles of the same pair of duplicate loci (cnxA1, cnxA2).

**Key words:** Complementation analysis – Somatic hybridization – Protoplast fusion – Nitrate reductase deficient mutants – *Nicotiana tabacum*

## Introduction

Mutants lacking nitrate reductase (NR) activity have been isolated from amphihaploid cell cultures of *Nicotiana tabacum* by direct selection for chlorate resistance (Müller and Grafe 1978). These mutants proved to be fully dependent on nitrogen in a reduced form and thus were the first non-leaky cell culture auxotrophs available in higher plants. Of the 40 independent $NR^-$ mutants isolated by us, 36 were defective in the NR apoprotein (nia type) and 4 were of the cnx type, which is characterized by a molybdenum cofactor deficiency leading to simultaneous loss of NR and xanthine dehydrogenase activities (Mendel and Müller 1979; Mendel et al. 1981, 1982; Müller and Mendel, in preparation).

Fifteen of the nia cell lines were regenerated to fertile plants and genetically analyzed through crosses. All of them proved to be recessive double mutants in the duplicate structural genes for the NR apoprotein (Müller, submitted). The 4 cnx cell lines failed to give fertile plants. Therefore, we attempted to analyze them genetically by somatic hybridization via protoplast fusion. This method has already been used for demonstrating genetic complementation between the nia-63 and the cnx-68 lines (Glimelius et al. 1978).

The present paper describes further complementation studies and gives evidence that the 4 cnx mutants are recessive and allelic to each other.

## Material and methods

### *Cell culture and media*

For cell culture and hybrid selection, modified MS media were used (Müller and Grafe 1978). These media contained 1 mg/l 2,4-D and 0.2 mg/l kinetin and the following nitrogen sources: amino acid mixture (AA medium), 20 mM $NH_4$-succinate ($NH_4$Su medium), 30 mM $NO_3^-$ ($NO_3$ medium), or 40 mM $NO_3^-$ + 20 mM $NH_4^+$ ($NO_3/NH_4$ medium).

The mutant cell lines studied (cnx-68, cnx-101, cnx-109, cnx-135, nia-115) were isolated from mutagenized cell cultures of the amphihaploid clone 21 of *Nicotiana tabacum* cv. 'Gatersleben' (Müller and Grafe 1978, and unpublished). They were maintained as callus cultures on AA or $NH_4$Su media.

For protoplast isolation, fast growing cell suspensions were used. These were grown in AA medium (line cnx-109) or $NH_4$Su medium (other lines) on a rotary shaker (240 rpm) in darkness at 27 °C and subcultured twice a week.

### *Protoplast isolation*

Suspension grown cells were harvested at the second day after subculture and washed once with a solution containing 0.3 M sorbitol and 0.05 M $CaCl_2$, pH 7.2 (= sorbitol/Ca solution). Cells were incubated in a solution containing 3% cellulase

(Meicelase P), 1% pectinase (Macerocyme) and 0.5 M mannitol (3 to 5 h, 27 °C, rotation 70 rpm). Protoplasts were separated from the undigested cell material by filtration, concentrated by centrifugation, and resuspended in the sorbitol/Ca solution.

*Protoplast fusion*

The fusion method described by Glimelius et al. (1978) was modified. Glass Petri dishes (50 mm) were used. Nine droplets of the protoplast suspension were placed into each dish. To each droplet, two droplets of a solution containing 0.15 M sorbitol, 0.03 M Ca $Cl_2$, 0.075 M KCl, 0.025 M Tris-HCl and 75% (w/v) PEG M.W. 1550, pH 7, were added. After 5 min the concentration of PEG was decreased by successively adding two times 2 ml of this solution, without PEG, and finally 2 ml of the sorbitol/Ca solution. After removal of the solution, protoplasts attached to the bottom of the glass dish were additionally washed once with the protoplast culture medium.

*Protoplast culture*

Protoplast were cultured as described (Glimelius et al. 1978). Regenerated cells started to divide after 1 to 3 days and formed microcolonies of up to 20 cells within 15 days. Between the 5th and the 15th day, fresh medium was added several times. After 2 to 3 weeks, the cultures were transferred to a shaker.

*Selection and characterization of hybrids*

Cultures of fusion-treated protoplasts were harvested after 3 to 4 weeks, washed once with liquid $NO_3$ medium and plated into $NO_3$ medium (about $10^3$ microcolonies per 50 mm dish). In addition, part of each culture was plated into AA medium to control viability. Plating media were solidified with 0.6% Difco-agar. Dishes were kept in darkness at 27 °C. Five weeks after plating, growing colonies were identified by their size (> 2 mm in diameter) and transferred to $NO_3/NH_4$ agar medium. NR activity of the $NO_3/NH_4$-grown callus was determined as described (Müller and Grafe 1978).

## Results

*Fusion and hybrid selection*

The 4 cnx lines were fused with each other and with the nia-115 line in all possible combinations. For each of these 10 pairwise combinations, several independent fusion treatments were performed (Table 1). The fusion-treated protoplast populations were cultured in a nonselective AA medim where they formed microcolonies. To select complementing hybrids, 3 to 4 week old colonies were plated into $NO_3$ agar medium. This medium supports growth of $NR^+$ cells, but prevents further division of $NR^-$ cells (Müller and Grafe 1978).

All nia + cnx combinations resulted in the formation of colonies capable of growing in $NO_3$ medium. These were counted 5 weeks after plating when the biggest ones were 8 mm in diameter. The frequency of nitrate-utilizing colonies (= no. of growing colonies per no. of plated colonies) was about 1% (maximum value: 78 growing colonies per 2900 plated colonies). Altogether 25 fusion-treated nia + cnx populations were plated and none of them failed to develop nitrate-utilizing colonies.

**Table 1.** Complementation for nitrate utilization and NR activity in somatic hybrids between $NR^-$ mutants

| Fusion combination | No. of fusion-treated p-populations[a] | | No. of colonies | | No. of isolated cell lines[a] | |
|---|---|---|---|---|---|---|
| | Tested | Growing in nitrate | Tested[b] ($\times 10^3$) | Growing in nitrate | Tested | $NR^+$ |
| nia-115 + cnx-68 | 7 | 7 | 27 | 263 | 34 | 34 |
| + cnx-101 | 2 | 2 | 6 | 38 | 4 | 4 |
| + cnx-109 | 6 | 6 | 32 | 295 | 19 | 19 |
| + cnx-135 | 10 | 10 | 24 | 253 | 18 | 18 |
| cnx-68 + cnx-101 | 10 | 0 | 28 | 0 | | |
| + cnx-109 | 10 | 0 | 37 | 0 | | |
| + cnx-135 | 8 | 0 | 26 | 0 | | |
| cnx-101 + cnx-109 | 7 | 0 | 27 | 0 | | |
| + cnx-135 | 5 | 0 | 19 | 0 | | |
| cnx-109 + cnx-135 | 2 | 0 | 6 | 0 | | |

[a] The number represents plated cell cultures derived from independently (i.e. in separate dishes) fusion-treated protoplast populations (only cultures showing full viability in nonselective control platings are included)

[b] Approximate number of microcolonies plated (in samples of about $10^3$/5 cm-dish)

[c] Number of cell lines tested for in vivo NR activity. NR activity was measured between days 6 and 8 in second subcultures of $NO_3/NH_4$ medium

**Table 2.** Complementation of $NR^-$ mutants

| | nia-115 | cnx-68 | cnx-101 | cnx-109 | cnx-135 |
|---|---|---|---|---|---|
| cnx-135 | + | – | – | – | – |
| cnx-109 | + | – | – | – | |
| cnx-101 | + | – | – | | |
| cnx-68 | + | – | | | |
| nia-115 | – | | | | |

However, no nitrate-utilizing colonies were formed when the same fusion and selection procedures were applied to the cnx+cnx combinations (Table 1). Forty-two fusion-treated cnx+cnx populations consisting of about $1.5 \times 10^5$ microcolonies were plated into $NO_3$ medium. (Populations that grew badly in the control dishes containing nonselective AA medium were not counted.)

Growing colonies were also not obtained when pure cultures of mutant protoplasts (about $10^4$ per line) were plated into $NO_3$ medium.

*Complementation for NR activity*

Altogether 75 nitrate-utilizing colonies were transferred from the selection plates to $NO_3/NH_4$ agar medium, where all of them continued to grow. After additional subculture and growth for 6 to 8 days, these cell lines were tested for in vivo NR activity. All of them exhibited NR activity (Table 1), which shows that the colony size (>2 mm in diameter) is a sufficient criterion for the identification of $NR^+$ lines. The level of NR activity in the presumably hybrid lines varied between 40 and 135% of the wild-type level, which was about 380 nmol $NO_2^-$/100 mg f.w./2h. Thus, genetic complementation in nia+cnx hybrids resulted in complete or nearly complete restoration of NR activity.

Attempts were made to use the in vivo assay directly (i.e. without previous selection for nitrate utilization) for detecting complementation in the mixed cell populations derived from fusion-treated protoplasts. Twenty-five day old cultures that contained about 2% hybrid colonies exhibited NR activities of up to 5 nmol $NO_2^-$/100 mg g.w./2h after induction by nitrate. However, this method failed to detect lower hybrid frequencies which were easily demonstrated by the growth test.

## Discussion

The results demonstrate that somatic hybridization and subsequent selection for nitrate-utilizing ($NR^+$) cells can be used for complementation analysis of a series of $NR^-$ cell lines of tobacco. In particular, they show that in hybrids between the nia-115 and any of the cnx lines studied, NR activity is restored due to genetic complementation, and that the 4 cnx lines do not complement each other (Table 2).

Evidence for complementation/noncomplementation was provided in the following way: Complementing combinations were identified by showing that the fusion treatment significantly increases the frequency of nitrate-utilizing colonies. This was usually achieved by a single fusion dish, because the fusion method employed gave hybrid frequencies of up to 3%, and neither in the untreated controls nor in the fusion-treated cnx+cnx populations could revertants be detected. A more difficult task was to provide unequivocal evidence for noncomplementation. The conclusion that the cnx mutants tested do not complement each other rests on the fact that other possible explanations for the observed lack of $NR^+$ colonies in fusion-treated cnx+cnx populations were excluded by suitable controls. To ensure that, for each of the cnx+cnx combinations tested, a sufficient number of hybrid cells were produced and that these would have developed to visible $NR^+$ colonies if they had been complementing, the following measures were taken: (1) the fusion experiments were repeated several times, (2) fusion-treated cnx+cnx populations that showed bad colony formation in the nonselective control platings were discarded, (3) the ability of the cnx protoplasts to form proliferating hybrids and the efficiency of the fusion and selection procedures were controlled by simultaneously fusing each cnx line with the nia-115 line.

The nia+cnx fusions had a second important function. They allowed one to decide whether the mutants are recessive or dominant. The finding that all nia+cnx combinations studied are complementing shows that the mutants involved are recessive. It can therefore be concluded that the failure of the 4 cnx mutants to complement each other is not due to dominance but due to allelism. Thus, the 4 cnx mutants tested are allelic to each other and nonallelic to nia-115.

The conclusion that the cnx mutants studied belong to one complementation group (cnxA) is consistent with the previously demonstrated similarities in their phenotype: all 4 cnx lines are partially repairable by high concentrations of molybdate, possess a molybdenum cofactor that is able to restore NR activity in extracts of the *Neurospora* nit-1 mutant, and appear to be defective in a function required for the insertion of molybdenum into the cofactor (Mendel et al. 1981). In *Aspergillus nidulans,* repair by molybdate is characteristic of the cnxE mutants (Cove 1979), which thus seem to be analogous to the cnxA mutants of *N. tabacum.*

Several arguments suggest that the cnx mutants studied here are double mutants in duplicate genes (cnxA1, cnxA2): (1) they occurred in amphihaploid cell cultures, (2) the nia mutants that were isolated from the

same cell cultures have been shown to carry two unlinked mutations which affect the duplicate structural genes for the NR apoprotein (Müller, submitted), (3) the cnx mutants occurred at a still lower frequency than the nia mutants: out of 40 $NR^-$ mutants isolated by us, only 4 were of the cnx type (Mendel and Müller 1979; Müller and Mendel, in preparation). Thus, in amphihaploid cells, two independent mutational events and possibly also some selective advantage of the single mutants are needed for a $NR^-$ mutant to occur. This may explain why the spectrum of $NR^-$ mutants in *N. tabacum* is very different from that in *Aspergillus nidulans* where the cnx mutants fall into 7 complementation groups and only about 10% of them belong to the cnxE group (Cove 1976, 1979). Recent results from *Nicotiana plumbaginifolia* (Marton et al. 1982) indicate that a similar broad spectrum of $NR^-$ mutants can be obtained when monohaploid plant cells are used.

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