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Genetic Analysis of Nitrate Reductase-Deficient Tobacco Plants Regenerated from Mutant Cells.

Evidence for Duplicate Structural Genes

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Summary. Fifteen nitrate reductase (NR)-deficient mutants that had been selected from amphihaploid cell cultures of *Nicotiana tabacum* and regenerated to fertile amphidiploid plants were genetically analyzed through crosses. All the 15 mutants proved to be allelic. Segregation among F_2 progeny from crosses between mutant and wild-type plants and among testcross progeny showed the regenerated plants to be homozygous double mutants. The NR deficiency is conferred by two unlinked recessive nuclear mutations, which thus define a pair of duplicate loci (*nia1 nia2*). These loci were identified as the structural genes for the apoprotein of NADH-NR. – Two mutants (Nia28, Nia30) were characterized further. Both Nia28 and Nia30 plants had less than 2% of wild-type NR activity, were resistant to chlorate and incapable of sustained growth on nitrate or in soil. However, they grew normally on media containing ammonium succinate (with or without nitrate). Growth test showed that Nia28 seedlings do not utilize nitrate, whereas Nia30 seedlings utilize nitrate at a very small rate. – The examination of single mutants (*nia1*-28/28 and *nia2*-28/28) revealed that either of the two loci is able to produce wild-type levels of NR activity. NR activity and chlorate sensitivity respond to nia^+ gene dosage only at the very early seedling stage. At later developmental stages, both the basal and the nitrate induced levels of NR activity were found to be independent of the number of nia^+ genes, indicating complete compensation of gene dosage effects by regulatory mechanism.

Introduction

Selection for chlorate resistance in amphihaploid cell cultures of *Nicotiana tabacum* yielded cell lines that lacked nitrate reductase (NR) and were auxotrophic for reduced nitrogen (Müller and Grafe 1978). Of the 44 NR^- cell lines so far examined, 36 were defective in the apoprotein of NADH-NR (Nia^- type) and 8 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; Buchanan and Wray 1982; Müller and Mendel 1983). Complementation analysis by somatic hybridization showed these mutants to fall into at least three groups representing different genes (*nia, cnxA, cnxB*) (Glimelius et al. 1978; Grafe und Müller 1983; Xuan et al. 1983). From 15 Nia^- cell lines, which after isolation from amphihaploid cell cultures had undergone spontaneous doubling of the chromosome complement, fertile plants and seed progeny were obtained (Müller 1978, 1983).

In this paper I report the genetic analysis of those regenerated Nia^- plants. Evidence is provided that in *N. tabacum* the apoprotein of NADH-NR is coded for by duplicate gene loci (*nia1, nia2*) and that all the 15 Nia^- lines studied are double mutants at these two loci. I also report a more detailed characterization of two mutants (Nia28, Nia30), showing that not only cultured cells but also whole plants of these mutants are incapable of sustained growth with nitrate as sole nitrogen source. A preliminary account of these studies has appeared (Müller and Mendel 1982). Further characterization of the 15 Nia^- mutants will be published elsewhere (Müller and Mendel 1983).

Mutants in the structural gene for the apoprotein of NADH-NR have also been described in barley (Kleinhofs et al. 1980; Warner and Kleinhofs 1981). However, these mutant plants are able to grow on nitrate and in soil. Of the NR-deficient whole plant mutants so far known, only the Cnx^- mutants isolated in pea (Feenstra and Jacobson 1980), *Arabidopsis thaliana* (Braaksma and Feenstra 1982) and barley (Bright et al. 1983) behave like conditional lethals.

Auxotrophs of a plant species that is well suited for cell culture work are an important tool for further genetic experiments. Results presented elsewhere (Pental et al. 1982; Müller 1983) show that protoplasts isolated from Nia30 plants can easily be regenerated to fertile mutant plants. Selection for nitrate utilization in such protoplast cultures has led to the isolation of revertants of Nia30 (Grafe and Müller 1982) and will be useful for studies on cell fusion and genetic transformation.

Materials and Methods

Nomenclature of Mutants and Crosses Nicotiana tabacum cv. "Gatersleben 1" was used as wild type. The 15 Nia^- lines (coded Nia20 through Nia40) have been selected from cultured cells of the amphihaploid plant clone H21 of cv. "Gatersleben 1" after nitrosomethylurea mutagenesis and regenerated to plants as described elsewhere (Müller 1983). (Note that the same lines were previously referred to as nia-120 through nia-140). Nia20r1, Nia20r2 etc. denote individual plants regenerated from the cell line Nia20. Regenerated plants may be considered to be a special type of M_1 plants. They represent the generation in which the mu-

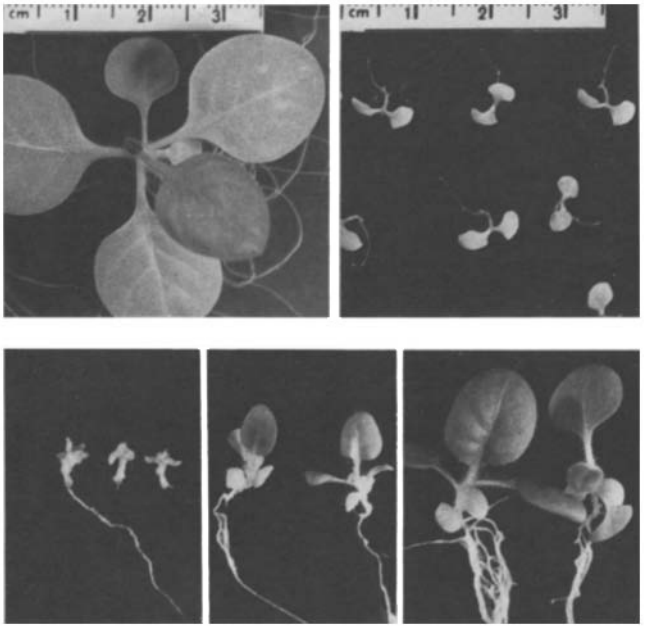

Fig. 1. Seedling tests for growth on nitrate and for chlorate resistance. *Top:* Wild-type (or F_1) and Nia28 seedlings after 37 days of growth on NO_3-O medium. The cotyledons of Nia28 are completely bleached. *Bottom:* Wild-type, F_1, and Nia28 seedlings germinated in 4 mM potassium chlorate and then grown in NH_4Su-O medium, at 22 days after germination. Note that cotyledons and hypocotyl of wild-type and F_1 are malformed and those of Nia28 are normal (1.5×)

tations to be studied arose. This generation is designated as R_1. Self-fertilization of the R_1 plants produces the R_2 generation etc.

Plant cultivation and Other Procedures. The N-free basal medium used for aseptic plant cultures contained the Murashige-Skoog salts (without KNO_3 and NH_4NO_3) plus 20 mg myo-inositol, 0.4 mg thiamine, and 20 g sucrose per litre. To indicate the nitrogen source added, the following abbreviations are used: NO_3 = 30 mM KNO_3, NO_3/NH_4 = 20 mM KNO_3 + 20 mM NH_4NO_3, NH_4Su = 20 mM NH_4Cl + 20 mM K-succinate (filter-sterilized!). Media were solidified with agar, unless otherwise stated. Aseptic plant cultures were kept under continuous fluorescent light (3,000 to 4,000 lux) at 26° C. Regenerated plants were maintained on NO_3/NH_4Su-O medium and propagated by cuttings. To obtain flowers, the aseptically grown Nia^- plants were either grafted onto wild-type stocks grown in soil or transferred to aerated hydroponics provided with nutrient solutions containing 1 mM $(NH_4)_2SO_4$ as sole nitrogen source. The solutions were supplemented with $CaCO_3$ or, in some cases, with K-succinate. Plants were grown to maturity in the greenhouse. In vivo NR activity was determined as described (Müller and Grafe 1978). One unit of in vivo NR activity is defined as the formation of 1 nmol nitrite per 2 h. Chlorate sensitivity was determined as follows: Sterilized seeds were germinated in petri dishes on filter paper moistened with 4 mM $KClO_3$. At 2 days after germination, the chlorate solution was removed and liquid NH_4Su-O medium was added. After another 20 days, the seedlings were classified according to their size and to the extent of malformations (class 0 = resistant = cotyledons and hypocotyl not malformed, class 4 = highest degree of sensitivity, see Fig. 1).

Results

Twenty-one plants regenerated from 15 Nia^- cell lines were studied for sexual transmission of the mutant characters. All these R_1 plants were chlorate resistant and deficient in NR activity, although not to the same extent. Plants regenerated from the Nia22, Nia29, Nia31 and Nia34 lines exhibited considerable residual activity. The R_1 plants were both selfed and reciprocally crossed with wild-type plants. Some of them did not set selfed seeds but gave F_1 seeds, and two R_1 plants (Nia25r17, Nia26r19) were male sterile, so that seeds were obtained from $R_1 \times WT$ crosses only. However, in each case, fully fertile plants were found among the F_1 progeny. These F_1 plants were selfed as well as backcrossed to R_1. Nia^- progeny were identified by their resistance to chlorate or by their reduced ability to grow on nitrate (Fig. 1).

Table 1. Segregation among progeny from crosses involving regenerated Nia28 and Nia30 plants (R_1 plants)

Cross	Pollen lethality[a]	No. of progeny	
		total[b]	% Nia^-
R_2:			
H21-3r7 selfed	0	290	0
Nia28r5 selfed	80	45	100
Nia28r6 selfed	75	68	100
Nia28r34 selfed	48	182	100
Nia30r2 selfed	0	288	100
Nia30r4 selfed	46	275	100
Nia30r19 selfed	0	542	100
Nia30r24 selfed	20	216	100
Nia30r26 selfed	0	440	100
F_1:			
WT × Nia28r5	80	30	0
WT × Nia30r2	0	29	0
Nia30r2 × WT	0	27	0
WT × Nia30r4	46	28	0
H21-3r7 × Nia30r26	0	30	0
F_2:			
WT/Nia28r5 selfed	0	589 (3)	7.1
WT/Nia30r2 selfed	0	755 (3)	5.8
Nia30r2/WT selfed	0	592 (2)	6.1
WT/Nia30r4 selfed	0	533 (3)	5.5
H21-3r7/Nia30r26 selfed	0	580 (3)	6.7
Testcrosses:			
WT/Nia30r4 × Nia30r24	20	820 (1)	24.4
Nia30r24 × WT/Nia30r4	0	344 (1)	23.3
Allelism test:			
Nia30r19 × Nia28r34	48	118	100
Nia30r19/Nia28r34 selfed	0	581 (2)	100

Progeny were tested for growth on nitrate

[a] Pollen lethality of male parent in %. (WT plants produced 1–6% nonstainable pollen. Accordingly, values below 6% are given as zero)

[b] in parentheses: no. of families. (Data were pooled after having been tested for homogeneity)

All F_1 plants were sensitive to chlorate and grew normally on nitrate. For all the 15 mutant lines studied, F_2 segregation ratios close to a 15:1 ratio and testcross segregation ratios close to a 3:1 ratio were obtained. Data from crosses involving Nia28 and Nia30 regenerants are summarized in Table 1 and Fig. 2. The results show that in each mutant line the NR deficiency is conferred by two unlinked recessive nuclear mutations and that the R_1 plants studied were homozygous double mutants. Thus, Nia28 plants for instance have the genotype *nia*1-28/28; *nia2*-28/28.

Allelism Test

The results of $F_1 \times$ Nia30 crosses given in Table 2 demonstrate that all the 15 Nia^- lines studied are double mutants at the same pair of duplicate loci (*nia1 nia2*). Allelism was confirmed by intercrossing the 15 lines in all possible combinations, using homozygous mutant plants. All hybrids thus obtained were chlorate resistant (data not shown). An example of these interline crosses (Nia30 × Nia28) is given in Table 1. The results of this cross show that the Nia30/Nia28 hybrids were noncomplementing also for growth on nitrate and that no Nia^+ recombinants occurred among their selfed progeny. Thus, the allelism of these two double mutants is indicated not only by noncomplementation but also by lack of recombination.

Identification of Nia28 and Nia30 Single Mutants

Using the seedling test for chlorate resistance, Nia^- progeny (even those with high levels of residual NR activity) could unmistakably be distinguished from all other types of F_2 progeny. When F_2 seedlings that had been identified as resistants were subsequently transferred to NO_3-O medium, they always exhibited bleaching of the leaves and (with the exception of Nia29 and Nia34 seedlings) stopped growing. In some cases, the classification was verified by progeny tests (Table 3). In addition to this qualitative assessment, the damage induced in germinating seeds by chlorate treatment was also assessed quantitatively. It was found that, with respect to the degree of chlorate sensitivity, the two mutant genes act as semidominants and interact additively (Fig. 2). Therefore, the degree of chlorate sensitivity could be used to select heterozygotes and single mutants.

F_2 seedlings exhibiting intermediate sensitivity to chlorate were grown to maturity and selfed (Table 3). The resulting F_3 progeny were scored for growth on nitrate and for chlorate resistance. Presumed homozygous single mutants, i.e. F_2 plants that produced non-segregating progeny of intermediate chlorate sensitivity, were intercrossed. By segregational analysis of the F_2 from these crosses, the two possible types of Nia28 single mutants were identified (Table 4). This provides the most conclusive evidence for duplicate loci and excludes the possibility that the dihybrid segregation patterns observed were simulated by the action of segregation distorters.

Data compiled in Table 3 show that single mutants at either locus as well as heterozygous double mutants were normal with respect to leaf NR activity, growth in soil, and growth on nitrate. In contrast, the chlorate sensitivity of germinating seeds increased with increasing number of nia^+ genes.

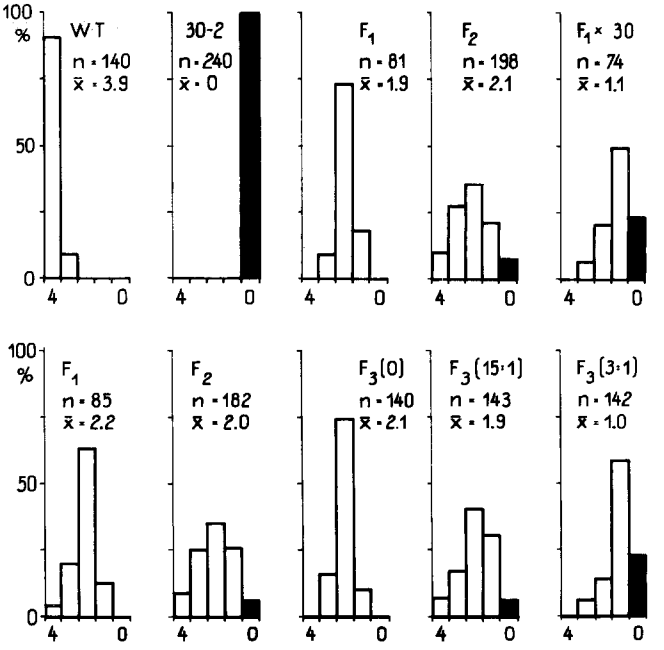

Fig. 2. Frequency distributions of chlorate sensitivity of germinating seeds (0 = resistant, 4 = highest degree of sensitivity). *Top:* Wild-type selfed, Nia30r2 selfed, as well as F_1, F_2 and testcross generation of WT × Nia30r2. *Bottom:* F_1, F_2, and selected F_3 families of WT × Nia28r5. For each population, the number of tested seedlings (n) and the mean degree of chlorate sensitivity ($\bar{x}$) are given

Table 2. Allelism test of 15 Nia^- mutants. (Segregation of chlorate resistance among progeny obtained after crossing F_1 plants from WT × R_1 crosses with a Nia30 plant)

Female parent	No. of progeny	
	total	% resistant
WT/Nia20r16	297	25.3
WT/Nia21r1	215	22.8
WT/Nia22r8	307	22.8
WT/Nia23r2	278	24.4
Nia25r17/WT	262	22.9
Nia26r19/WT	437	22.0
WT/Nia27r17	208	23.1
WT/Nia28r5	193	20.2
WT/Nia29r13	312	28.2
WT/Nia30r4	310	27.4
WT/Nia31r20	317	24.3
WT/Nia33r9	310	28.1
WT/Nia34r2	286	22.4
WT/Nia36r38	287	20.2
WT/Nia40r10	215	22.8

NR Activity

The in vivo NR activity of Nia28, Nia30, wild-type and F_1 plants was compared at various developmental stages. To avoid differences in growth rate between mutant and wild-type plants, nitrate-free media were used. Results show that Nia28 as well as Nia30 plants lack detectable NR activity at most developmental stages, both in roots and in shoots and even after addition of nitrate (Table 5). NR activity was also not detectable in Nia30 plantlets growing slowly with nitrate as the sole nitrogen source. Only in

Table 3. Genotype and phenotype of selected F_2 plants

Genotype[a] (nia1; nia2)	No. of plants	Chlorate sensitivity[b]	NR activity	Shoot height (cm)	No. of F_3 progeny tested for growth on nitrate		Mean chlorate sensitivity of F_3 progeny[b,d]
					total[c]	% Nia^-	
WT/Nia28r5 selfed:							
−/−; −/−	9	0	0–4	(grafts)	415 (9)	100	0
+/−; −/− or −/−; +/−	14	1	744	54	1,870(14)	23.8	1.1
+/−; +/−	2	1	710	48	312 (2)	6.7	2.2
+/+; −/−	5	1	736	51	1,318 (5)	0	2.0
−/−; +/+	1	1	696	62	238 (1)	0	2.1
WT/Nia30r4 selfed:							
−/−; −/−	9	0	0–5	(grafts)	420 (8)	100	0
+/−; −/− or −/− +/−	9	1	730	55	1,385 (9)	25.1	0.9
+/−; +/−	2	1	682	59	643 (2)	5.9	1.9
+/+; −/− or −/−; +/+	1	1	776	47	240 (1)	0	2.1
WT (control):							
+/+; +/+	5	4	752	51	240 (1)	0	3.9

All F_2 and WT plants were grown simultaneously in the greenhouse. Resistant (Nia^-) plants were grafted onto wild-type stocks. Shoot hight and in vivo NR activity (units/100 mg fr.wt.) were determined at 82 days after germination

[a] The genotype of each F_2 plant was inferred from the F_3 segregation. Homozygous single mutants were identified by intercrossing them, as shown in Table 4

[b] Effect of chlorate treatment of germinating seeds, as explained in Material and Methods

[c] The number of families is given in parentheses. The segregation ratio of each family did not differ significantly (1% level) from the expected value

[d] Frequency distributions are given in Fig. 2

Table 4. Identification of homozygous single mutants

Cross	No. of progeny	
	total	% Nia^-
f22 selfed	240	0
f23 selfed	238	0
f34 selfed	240	0
f23 × f22	98	0
f23 × f24	100	0
f34 × f22	100	0
f23/f22 selfed, plant 1	200	5.5
plant 2	200	9.0
plant 3	198	6.7
f23/f34 selfed, plant 1	186	6.5
plant 2	191	6.3
plant 3	189	4.2
f34/f22 selfed, plant 1	199	0
plant 2	195	0
plant 3	200	0

f22, f23 and f34 were F_2 plants obtained from the cross WT × Nia28r5 (see Tables 1 and 3). Progeny were tested for growth on nitrate. Segregation ratios do not differ significantly from the expected 15:1 ratio. Results show that f23 had the genotype nia1-28/28; nia2+/+ and that f22 and f34 had the genotype nia1+/+; nia2-28/28. (The locus designation is arbitrary)

young, still expanding leaves of older mutant plants grown in nitrate-free hydroponics (Table 5) or grafted on wild-type stocks (Table 3) could up to 2% of wild-type NR activity be detected. This activity was completely inhibited when KCN was present during the in vivo assay. It is therefore considered to be an enzymatic activity.

The NR activity of heterozygotes and single mutants generally was as high as that of comparable wild-type plants (Tables 5 and 3). However, when young seedlings kept in water (i.e. in the absence of an exogenous nitrogen source) were examined, both types of heterozygotes exhibited intermediate levels of NR activity.

Growth Response to Various Nitrogen Sources

The ability of the mutants to utilize nitrate was determined by comparing growth on nitrate with growth on N-free medium. Cultured cells of both Nia28 and Nia30 behaved like the Nia^- cell cultures studied previously (Müller and Grafe 1978), i.e. they did not show any detectable growth response to nitrate. Results given in Table 6 and similar results obtained from several other experiments with different seed samples indicate that in the case of Nia28 (but not of Nia30) the seedlings are also unable to utilize nitrate.

Although nitrate did not stimulate growth of Nia28 seedlings, it modified the nitrogen starvation symptoms in a characteristic manner. On NO_3-O medium, the initially green cotyledons bleached more rapidly and the roots formed were shorter as compared to seedlings on N-free medium (Fig. 1). When 30 day old Nia28 plants grown on NH_4Su-O medium were transferred to NO_3-O medium, all their leaves bleached simultaneously and became necrotic after about two weeks, whereas following transfer to N-free medium bleaching and necrosis started at the lower leaves and only slowly proceeded to the upper leaves. At 10 weeks after transfer to NO_3-O or N-free media, shoot axis, buds and roots were still viable and new lateral roots of normal morphology were being formed. Thus, the visible effects of nitrate were confined to the leaves; nitrate was not toxic to other Nia28 tissues.

Table 5. NR activity in wild-type, F_1, and mutant plants

Age (days)	Tested material	in vivo NR activity (units/100 mg fr.wt.)				
		WT	WT × Nia30	WT × Nia28	Nia30	Nia28
4	whole seedlings	212	119[a]	129[a]	0	0
24	shoots	31	29	27	0	0
	roots	38	35	31	0	0
	shoots (induced)	99	103	88	0	0
	roots (induced)	96	106	91	0	0
85	expanding leaves	411	393	423	0–6	0–6
	fully expanded leaves	390	386	404	0	0

Sterilized seeds were germinated in petri dishes on filter paper moistened with destilled water. Starting at 4 days after germination, plants were grown in liquid NH_4Su-O medium. Induction was performed by supplementing the medium with 50 mM KNO_3, 24 h prior to testing. Starting at the 32nd day, plants were grown in aerated hydroponics provided with nitrate-free nutrient solution. Mean values of 6 to 10 determinations are given

[a] Significantly different from the WT value (1% level)

Table 6. Growth response to various nitrogen sources of wild-type, Nia30 and Nia28 seedlings

Nitrogen source	Age (days)	Fresh weight (mg/plant)		
		WT	Nia30	Nia28
None	27	4	5	5
	50	5	5	5
20 mM KNO_3	27	112	17[a]	6[a,b]
	50	1,140	24[a]	6[a,b]
20 mM KNO_3 + 20 mM NH_4NO_3	27	94	22[a]	8[a,b]
	50	758	28[a]	9[a,b]
20 mM NH_4-succinate	27	86	78	81
20 mM NH_4-succinate + 20 mM KNO_3	27	97	106	104
20 mM NH_4Cl	27	8	9	8

The mutant plants were F_3 progeny from crosses WT × R_1. Each value is the mean of fresh weights for about 60 plants (in case of WT at day 50: for 12 plants) grown in four petri dishes on agar medium

[a] Mutant values that differ significantly (1% level) from the wild-type value

[b] Significant difference between Nia30 and Nia28

Nia28 shoots that had been grafted onto wild-type stocks accumulated nitrate and exhibited bleaching and necrosis of the intercostal regions of fully expanded leaves. No abnormal leaf necrosis was seen when Nia28 plants were grown to maturity in nitrate-free hydroponics.

Nia28 seedlings were incapable of sustained growth in soil, whether fertilized with ammonium sulphate or not. As shown in Table 6, they were also unable to grow on NO_3/NH_4-O medium, which supports normal growth of wild-type plants. Thus, Nia28 seedlings resembled the Nia^- cell cultures studied previously (Müller and Grafe 1978) in being sensitive to ammonium even in the presence of nitrate. However, Nia28 plants grew normally on NH_4Su-O or NO_3/NH_4Su-O media (Table 6). Growth on these media was slightly better than growth on glutamine (data not shown).

Different Alleles in Nia28 and Nia30

Both Nia28 and Nia30 plants were morphologically normal when grown on NH_4Su-O or NO_3/NH_4Su-O media. They were equally resistant to chlorate and gave the same results when tested for NR activity by the in vivo assay (Table 5). However, Nia30 plants differed from Nia28 plants in their response to nitrate (Table 6), which indicates that they are able to reduce nitrate, although at a very low rate. Nia30 seedlings growing on NO_3-O medium formed small, light green leaves and reached a fresh weight of about 50 mg within 6 months. Then their growth ceased. When older Nia30 plants were transferred from NH_4Su-O to NO_3-O medium, their leaves turned light green within a few days. However, neither the leaf necrosis typical of nitrogen-starved plants nor the nitrate-dependent leaf necrosis known from Nia28 plants occurred. After one year, the leaves were still light green or yellowish.

Using seedling growth on nitrate as a criterion, it was found that all progeny obtained from the five Nia30 regenerants had the Nia30 phenotype and all progeny obtained from the three Nia28 regenerants had the Nia28 phenotype (Table 1). This was also true for the Nia^- segregants occurring in the F_2 and F_3 from WT × R_1 crosses (Table 3), showing that the difference in nitrate utilization between Nia28 and Nia30 is due to different alleles and not to an additional mutation segregating independently of the *nia1nia2* genes. The hybrids obtained after crossing Nia28 with Nia30 were intermediate with respect to growth on nitrate; among their selfed progeny, Nia28-like and Nia30-like segregants occurred.

Discussion

N. tabacum is an amphidiploid species (2n = 48) which arose from the hybridization between *N. sylvestris* (2n = 24) and *N. tomentosiformis* (2n = 24) (Gray et al. 1974). Several of its characters are known to be controlled by duplicate genes, but functional diploidization has also been observed (Smith 1968). However, so far no data for structural genes of defined enzymes were available. The genetic analysis of 15 Nia^- mutants presented here shows that the apoprotein of NADH-NR is coded for by duplicate loci (*nia1 nia2*). Moreover, the characterization of single mutants revealed

that either of these loci is able to produce normal amounts of fully active NADH-NR. The two loci have been shown to be unlinked and are expected to be on homoeologous chromosomes. However, their chromosomal location as well as their relationship to the NR genes of the parental species of *N. tabacum* remain to be established.

The *nia1 nia2* duplicate loci have been identified as the structural genes for the haemoflavoprotein subunit of NADH-NR. The basis for this identification was the observation that some of the mutants shown here to be alleles of these loci (e.g. Nia28) have retained BVH-NR activity whilst lacking NADH-NR activity (Mendel and Müller 1979, Müller and Mendel 1983).

One objective of this study was to see whether the 200,000-dalton NADH-NR detectable in cell free extracts (Mendel and Müller 1979, 1980) is the only nitrate-reducing enzyme of *N. tabacum,* whether it completely accounts for the NR activity measured by the in vivo assay, and whether it is essential for growth with nitrate as the sole nitrogen source. Several higher plant species appear to have more than one NR. In barley, a second NR (NAD(P)H-NR) could be detected only after the dominant NADH-NR activity had been abolished by mutations in its structural gene (*nar1*). The presence of this *nar1*-independent NR enables the *nar1* mutants to grow substantially with nitrate as the sole nitrogen source (Kleinhofs et al. 1980; Warner and Kleinhofs 1981; Dailey et al. 1982).

The present results show that *N. tabacum* differs from barley in that NADH-NR is essential for growth on nitrate. Neither cultured cells nor seedlings of Nia28 exhibited a detectable growth response to nitrate (Table 6). The remote possibility that Nia28 has lost not only NADH-NR but also another, *nia1 nia2*-independent NR is excluded by the results of WT × Nia28 crosses, which give no indication of a third, independently segregating mutation that affects nitrate utilization. Seedlings (but not cultured cells) of another mutant, Nia30, were found to utilize nitrate, although at an extremely low rate, which is below the detection limit of the in vivo NR assay (Table 5) and which does not support sustained growth with nitrate as the sole nitrogen source. Since this limited capability for growth on nitrate is lacking in Nia28, it must be due to the presence in Nia30 of a leaky allele at the *nia1 nia2* loci, i.e. it indicates residual NADH-NR activity and not activity of a second NR.

The in vivo assay failed to detect NR activity in nearly all mutant tissues examined (Table 5). This shows that the in vivo NR activity found in wild-type plants is essentially due to the *nia1 nia2*-dependent NADH-NR. Only young, still expanding leaves of Nia28 and Nia30 plants often (but not always) retained up to 2% of the wild-type level of in vivo NR activity. The nature of this residual NR activity, which could not be detected in cell-free extracts, remains obscure. It might merely be a side effect of other oxidoreductases, such as peroxidases, which are known to be capable of nitrate reduction (Ivanova and Peive 1973).

Recent studies have shown that NADH-NR of higher plants is able to catalyze the reduction of Fe(III)-citrate (Campbell and Redinbaugh 1983). We found that mutants lacking NADH-NR activity can grow at a normal rate and develop normally green chloroplast when provided with a suitable source of reduced nitrogen. This suggests that NADH-NR is at least not essential for iron assimilation. Our mutants may be helpful in elucidating the physiological role of the iron-reducing activity of NR.

The results described here reveal new information about the mechanisms regulating the expression of the NR structural genes. Firstly, they demonstrate that not only cultured cells, as shown previously (Müller and Grafe 1978), but also whole plants of *N. tabacum* have an unusual high level of NR activity when grown in the absence of nitrate and under conditions that should ensure maximum N-metabolite repression. Secondly, they show an interesting pattern of gene dosage effects. Stepwise substitution of nia^+ genes for the null alleles provided a possibility to study the influence of gene dosage on NR activity. F_1 plants, single mutants, and even plants possessing only one wild-type allele at either of the *nia* loci were all normal with respect to growth on nitrate, growth in soil and NR level at most developmental stages (Tables 3 and 5). Only at the early seedling stages did the NR activity respond to the number of nia^+ genes, as shown by the in vivo NR assay and by the test for chlorate sensitivity. Thus, the initially constitutive expression of the nia^+ genes changes during seedling development to a strictly regulatory type of gene expression. Not only the basal but also the nitrate-induced level of NR activity were found to be independent of gene dosage and thus to be determined exclusively by regulatory mechanisms. One nia^+ gene (instead of four) seems to be sufficient to ensure maximum activity. Comparable findings for the NR structural genes of other organisms are still lacking. In barley, the nitrate-induced NR level of *nar1* +/− seedlings was found to be about one half of the wild-type level (Kleinhofs et al. 1980).

As to the origin of the double mutants it might be argued that the parental haploid plant actually was a *nia* single mutant and that the mutagenic treatment merely added the second mutation. To exclude this possibility, a diploid regenerant from the parental cell line H21-3 was crossed with a Nia30 plant (Table 1). The dihybrid segregation pattern found among the F_2 progeny of this cross shows that the parental cell line was $nia1^+$ $nia2^+$. It follows that both of these loci mutated after initiation of the parental cell culture and most probably after treatment of the cells with nitrosomethylurea, since mutants were revocered only after mutagen treatment (Müller 1983). The low frequency of recovered NR^- mutants (about 10^{-7}) is consistent with simultaneous induction of two independent mutations. The frequency of single mutations in NR-specific genes should have been much higher (about 10^{-3} to 10^{-4}), as judged by the result of recent studies in which monoploid cells of a diploid *Nicotiana* species (*N. plumbaginifolia*) were subjected to similar induction and selection conditions (Marton et al. 1982). Whether or not cells carrying a single *nia* mutation have some chlorate resistance, which could influence the occurrence of double mutants, is not clear.

The results of the phenotypic characterization of the Nia^- mutants are consistent with the idea that these double mutants arose independently of each other and therefore represent 15 different *nia1* alleles and 15 different *nia2* alleles. They are also consistent with the idea that within a given mutant line all cells (and plants) are identical with respect to the two *nia* loci. Above it has been shown that all the Nia30 regenerants (and their progeny) have the same phenotype, which is clearly distinguishable from the phenotype of Nia28 regenerants. Differences in phenotype have been found also among the other 13 Nia^- mutants (Müller and Mendel 1983). In addition, preliminary evidence is

available indicating that both *nia* loci contribute to these differences.

By showing the Nia^- cell lines to be due to recessive mutations that are transmitted in a normal Mendelian manner, the present results complement the results of other authors on the in vitro selection of dominant and cytoplasmic *N. tabacum* mutants (reviewed in Chaleff 1981). Besides reinforcing the general conclusion that selection in cell culture and subsequent plant regeneration can lead to fully fertile mutants plants, they allow the following specific conclusions: (1) Mutants that are lethal under normal conditions can efficiently be selected from cell cultures and regenerated to fertile plants. As shown previously (Müller 1978), complete loss of NR activity does not negatively influence plant regeneration, if a suitable source of reduced nitrogen, such as ammonium succinate, is provided. (2) The tendency of haploid cell and protoplast cultures to spontaneously double their chromosome complement can successfully be utilized for obtaining homozygous recessive mutants. (Monoploid cells of a diploid species should be still more suitable for this purpose.) (3) Cell culture selection enalbes the isolation of extremely infrequent genotypes, such as double mutants. Although the present results show that Nia^- mutants of tobacco can easily be recognized in the seedling stage by their chlorate resistance, it would have been almost impossible to obtain these double mutants by conventional methods. Their expected frequency among the M^2 progeny of highly mutagenized *N. tabacum* plants would be approximately $1/16 \times 10^{-7}$.

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References

Braaksma FJ, Feenstra WJ (1982) Nitrate reduction in the wildtype and a nitrate reductase-deficient mutant of Arabidopsis thaliana. Physiol Plant 54:351–360

Bright SWJ, Norbury PB, Franklin J, Kirk DW, Wray JL (1983) A conditional-lethal cnx-type nitrate reductase-deficient barley mutant. Mol Gen Genet 189:240–244

Buchanan RJ, Wray JL (1982) Isolation of molybdenum cofactor defective cell lines of Nicotiana tabacum. Mol Gen Genet 188:228–234

Campbell WH, Redinbaugh MG (1983) Ferric citrate reductase activity of nitrate reductase and its role in iron assimilation by plants. Proc 2nd Int Symp Iron in Plants. J Plant Nutrit (in press)

Chaleff RS (1981) Genetics of higher plants. Application of cell cultures. Cambridge University Press, Cambridge

Dailey FA, Warner RL, Somers DA, Kleinhofs A (1982) Characteristics of a nitrate reductase in a barley mutant deficient in NADH nitrate reductase. Plant Physiol 69:1200–1204

Feenstra WJ, Jacobsen E (1980) Isolation of a nitrate reductase deficient mutant of Pisum sativum by means of selection for chlorate resistance. Theor Appl Genet 58:39–42

Glimelius K, Eriksson T, Grafe R, Müller AJ (1978) Somatic hybridization of nitrate reductase-deficient mutants of Nicotiana tabacum by protoplast fusion. Physiol Plant 44:237–277

Grafe R, Müller AJ (1982) Revertants of the nitrate reductase structural gene mutant nia-130 of Nicotiana tabacum. Abstr Int Symp "Nitrate Assimilation – Molecular and genetic aspects". Gatersleben GDR, p 15

Grafe R, Müller AJ (1983) Complementation analysis of nitrate reductase-deficient mutants of Nicotiana tabacum by somatic hybridization. Theor Appl Genet (in press)

Gray JC, Jung SD, Wildman SG, Sheen SJ (1974) Origin of Nicotiana tabacum detected by polypeptide composition of fraction I protein. Nature (London) 252:266–267

Ivanova NN, Peive YV (1973) Nitrate reduction by higher plant peroxidase. FEBS Lett 31:229–232

Kleinhofs A, Kuo T, Warner RL (1980) Characterization of nitrate reductase-deficient barley mutants. Mol Gen Genet 177:421–425

Marton L, Dung TM, Mendel RR, Maliga P (1982) Nitrate reductase deficient cell lines from haploid protoplast cultures of Nicotiana plumbaginifolia. Mol Gen Genet 186:301–304

Mendel RR, Müller AJ (1979) Nitrate reductase-deficient cell lines of Nicotiana tabacum: Further biochemical characterization. Mol Gen Genet 177:145–153

Mendel RR, Müller AJ (1980) Comparative characterisation of nitrate reductase from wild-type and molybdenum cofactor-defective cell cultures of Nicotiana tabacum. Plant Sci Lett 18:277–288

Mendel RR, Alikulov ZA, Lvov NP, Müller AJ (1981) Presence of the molybdenum cofactor in nitrate reductase-deficicent mutant cell lines of Nicotiana tabacum. Mol Gen Genet 181:395–399

Mendel RR, Alikulov ZA, Müller AJ (1982) Molybdenum cofactor in nitrate reductase-deficient tobacco mutants. III. Induction of cofactor synthesis by nitrate. Plant Sci Lett 27:95–101

Müller AJ (1978) Nitrate reductase mutants and the cell culture approach to higher plant genetics. In: Problems in general genetics. Proc XIV Int Congr Genet, Moscow, vol II, 1. Mir Publishers, Moscow, p 229

Müller AJ (1983) Plant regeneration from selected tobacco cells deficient in nitrate reductase (submitted)

Müller AJ, Grafe R (1978) Isolation and characterization of cell lines of Nicotiana tabacum lacking nitrate reductase. Mol Gen Genet 161:67–76

Müller AJ, Mendel RR (1982) Nitrate reductase-deficient tobacco mutants and the regulation of nitrate assimilation. In: Plant Tissue culture 1982. Proc 5th Int Congr Plant Tissue and Cell Culture. Tokyo, p 233

Müller AJ, Mendel RR (1983) Expression of mutant alleles of the nitrate reductase structural genes in whole plants and cultured cells of Nicotiana tabacum. (Submitted)

Pental D, Cooper-Bland S, Harding K, Cocking EC, Müller AJ (1982) Cultural studies on nitrate reductase deficient Nicotiana tabacum mutant protoplasts. Z Pflanzenphys 105:213–227

Smith HH (1968) Recent cytogenetic studies in the genus Nicotiana. Adv Genet 14:1–54

Warner RL, Kleinhofs A (1981) Nitrate utilization by nitrate reductase-deficient barley mutants. Plant Physiol 67:740–743

Xuan LT, Grafe R, Muller AJ (1983) Complementation of nitrate reductase deficient mutants in somatic hybrids between Nicotiana species. Proc 6th Int Protoplast Symp, Basel (in press)

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