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Nitrate Reductase mRNA Regulation in *Nicotiana plumbaginifolia* Nitrate Reductase-Deficient Mutants

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Light and substrate regulation of nitrate reductase (NR) expression were compared in wild type and mutant lines of *Nicotiana plumbaginifolia*. Mutants affected in the NR structural gene (*nia*) or in the biosynthesis of the NR molybdenum cofactor (*cnx*) were examined. *nia* mutants expressing a defective apoenzyme, as well as *cnx* mutants, overexpressed NR mRNA, whereas *nia* mutants devoid of detectable NR protein had reduced or undetectable NR mRNA levels. Diurnal fluctuations of NR mRNA were specifically abolished in *nia* and *cnx* mutants, suggesting that the integrity of NR catalytic activity is required for the expression of diurnal oscillations. Unlike some fungal mutants, the *nia* and *cnx* mutants examined retained nitrate inducibility of NR expression. The possibility of autogenous control of NR expression in higher plants is discussed.

INTRODUCTION

Higher plants acquire the majority of their nitrogen from the environment by nitrate assimilation. Nitrate reductase (NR) (EC 1.6.6.1), catalyzing the first reaction of the nitrate reducing pathway, plays a critical role in nitrate assimilation. Studies in bacteria, fungi, and higher plants have shown that NR expression is highly regulated (Cove, 1979; Srivastava, 1980; Dunn-Coleman, Smarrelli, and Garrett, 1984).

NR-deficient mutants provide a useful tool for the study of the genetic control of nitrate assimilation. Mutants have been obtained in several plant species (Wray, 1986, 1988), and two main types are known: *nia* mutants, which are impaired in the structural gene of NR, and *cnx* mutants, which are impaired in molybdenum cofactor biosynthesis. In *Nicotiana plumbaginifolia*, a collection of 211 mutants has been isolated from mutagenized protoplasts by selection for chlorate resistance (Grafe, Marion-Poll, and Caboche, 1986; Gabard et al., 1987). Of these, 70 have been classified as *cnx* mutants and the others as *nia* mutants. Genetic analysis has provided evidence for seven complementation groups, one for the *nia* mutants (Gabard et al., 1987) and six for the *cnx* mutants (Gabard et al., 1988). In fungi, mutants impaired in nitrogen regulation of NR have been described, and three regulatory loci have been identified (Dunn-Coleman et al., 1984). NR-regulatory gene mutations have not yet been identified in higher plants, but *nia* and *cnx* mutations in plants may provide information for understanding NR regulation because regulatory perturbations of NR expression have been reported for *nia* and *cnx* mutations in fungi (Dunn-Coleman et al., 1984). In this work, two NR-regulatory mechanisms, namely light regulation and nitrate inducibility, were examined in different *N. plumbaginifolia* mutants.

Extensive studies of light regulation in higher plants have shown that NR activity rapidly increases when etiolated, nitrate-grown seedlings are exposed to white light (Duke and Duke, 1984). A diurnal rhythm in the accumulation of the NR mRNA has been reported for both tomato and tobacco (Galangau et al., 1988). NR mRNA accumulation increased rapidly during the dark period, reaching a maximum at the beginning of the day. At the end of the day, the level of NR mRNA was decreased by ≥100-fold in comparison with levels at sunrise in both species.

NR activity has been demonstrated to be nitrate-inducible in higher plants (Srivastava, 1980). A correlated increase of NR expression in response to nitrate induction at the protein level (Somers et al., 1983; Remmler and Campbell, 1986; Galangau et al., 1988) and at the mRNA level (Cheng et al., 1986; Crawford, Campbell, and Davis, 1986; Crawford et al., 1988; Galangau et al., 1988) was observed. In fungi, constitutive expression of NR in the absence of nitrate has been reported for several mutants affected in the apoenzyme or in molybdenum cofactor biosynthesis, supporting an autogenous regulation model (Cove, 1979; Tomsett and Garrett, 1981; Fu and Marzluf, 1988).

Here, we have analyzed the NR mRNA diurnal cycle in NR-deficient mutants of *N. plumbaginifolia*. Our results showed that the loss of this diurnal cycle correlates with

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the genetic impairment of the nitrate reduction process, and that the level of NR mRNA accumulation in the mutants depends on expression of immunologically detectable NR protein. We also demonstrated that *nia* and *cnx* mutants remain nitrate-inducible. We discuss the possibility of an autogenous regulatory mechanism in higher plants.

RESULTS

NR mRNA Contents in Leaves of NR-Deficient Mutants of *N. plumbaginifolia*

In addition to its physiological NADH-NR activity, NR displays partial activities that are classified either as dehydrogenases [transfer of electrons from NADH to artificial acceptors, e.g., NADH-cytochrome *c*(cyt *c*) reductase activity] or as terminal activities (transfer of electrons from artificial donors to nitrate). Immunologically detectable NR protein, measured with a monoclonal antibody in a two-site enzyme-linked immunosorbent assay (ELISA), is referred to in text as NR protein. Apoenzyme-deficient *N. plumbaginifolia* mutants had a wide range of NR protein contents (Gabard et al., 1987; I. Chérel, M. Gonneau, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted). Many of them did not have detectable NR protein or NR partial activities. However, all mutants expressing NR protein retained at least one of the partial activities (I. Chérel, M. Gonneau, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted). Molybdenum cofactor-deficient mutants all retained NADH-cyt *c* reductase activity (A. Marion-Poll, I. Chérel, M. Gonneau, M. T. Leydecker, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted). They were separated in two groups according to their NR protein content: the *cnxA* class, having higher amounts of NR protein, and the other *cnx* classes (*B*, *C*, *D*, *E*, and *F*), having lower amounts of NR protein. The apparent reduction of NR protein in these mutants is probably due to degradation processes or to poor recognition by antibodies, or both (A. Marion-Poll, I. Chérel, M. Gonneau, M. T. Leydecker, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted).

Thirty-seven *nia* and 18 *cnx* mutants were assayed for NR mRNA content. The *nia* mutants were representative of all the cases encountered during biochemical characterization (I. Chérel, M. Gonneau, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted). Of the 18 *cnx* mutants tested, nine were in the *cnxA* class and nine were in other classes. NR mRNA contents were assayed in leaf samples collected from grafted plants at the beginning of the day period. Mutants and the wild type were compared by RNA blot hybridization. Mutants are classified in Tables 1 and 2. Representative results from RNA blots are given in Table 3.

There are two classes of *nia* mutants. The first (Table 1) includes *nia* mutants that express detectable NR protein and at least one of the NR enzyme partial activities (I. Chérel, M. Gonneau, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted). Most of these (10) showed a significant increase in the NR mRNA level (2.5-fold to sixfold), as compared with the wild type (Figure 1A, Table 3). However, some mutants (D64, H22, and I5) showed an NR mRNA accumulation pattern similar to that of the wild type. These results may have been affected by sample variability, as the mutant morphology was dramatically affected by the NR impairment, resulting in chlorotic and crinkled leaves (Gabard et al., 1987; Saux et al., 1987). No significant differences in the NR mRNA levels were observed between those mutants retaining terminal activities and those retaining NADH-cyt *c* reductase activity. The second class (Table 1) comprises *nia* mutants devoid of NR protein and partial activities (I. Chérel, M. Gonneau, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted). Most of these (17) had NR mRNA levels that were reduced or undetectable (Figure 1B, Table 3). However, seven

Table 1. Classification of *nia* Mutants of *N. plumbaginifolia* Relative to Their NR mRNA Levels

	Mutants Expressing Protein		
mRNA Level	Term. Act.$^{+}$,[a] Cyt *c* Red.$^{-}$	Term. Act.$^{-}$, Cyt *c* Red.$^{+}$	Mutants not Expressing Protein (No Partial Activities)
Higher than WT^{0}	D51 D57 E56 F59 G3 H29 K5	E77 E122 F19	
Close to WT^{0}	D64	H22 I5	D55, I26 D80, I32 I2, 134 (I8)
Lower than WT^{0}			D99, F58 E12, (H8) E66, H14 E69, K26 E105, K32
Undetectable or traces			D7, E51 D65, F97 D71, K13 E23

NR mRNA levels in mutants were estimated relative to that of the wild type at the beginning of the day period (WT^{0}). They were tested both at the beginning of the day period and, except for mutants in parentheses, 8 hr to 11 hr later. Diurnal fluctuations of NR mRNA levels were abolished in all these mutants. Levels referred to are: higher than WT^{0}, *more than twofold*; close to WT^{0}, between 50% and twofold; lower than WT^{0}, less than 50%; and undetectable or traces, less than 10% of WT^{0}.

[a] Term. Act.$^{+/-}$, NR terminal activities present/absent; cyt *c* red.$^{+/-}$, NADH-cytochrome *c* reductase activity present/absent.

Table 2. Classification of *cnx* Mutants of *N. plumbaginifolia* Relative to Their NR mRNA Levels

mRNA Level	*cnxA*	*cnxB*	*cnxC*	*cnxD*	*cnxF*
Higher than WT[0]	B25	D126	F2	(D4)	D12
	D70			(D133)	E48
	D91			F91	
	D132			K4	
	E130				
	E134				
	F66				
	F88				
Close to WT[0]	F108			K15	

Levels are as in Table 1.

mutants had NR mRNA levels similar to that of the wild type.

Most of the *cnx* mutants (Table 2) had threefold to eightfold higher NR mRNA levels than did the wild type (Figure 2, Table 3). No significant differences were detected between the *cnxA* class and the other classes. *cnxA* mutants, therefore, appear to be the only ones to overexpress NR protein, correlating with NR mRNA overexpression (A. Marion-Poll, I. Chérel, M. Gonneau, M. T. Leydecker, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted). Some leaf samples, however, particularly from *cnxB*, *-C*, and *-D* mutants, had NR mRNA amounts comparable with that of the wild type. This may be attributable to a more severe effect of these mutations on phenotype.

Specific Disappearance of the Day/Night Rhythm of NR mRNA Accumulation in Both *nia* and *cnx* Mutants

NR mRNA Level in Wild-Type and Nia30 Tobacco Leaves during a Diurnal Cycle

Previous work with tobacco had demonstrated a sharp decline of NR mRNA level during the first part of the day, with NR mRNA being undetectable at the end of the light period. The NR mRNA pool then increased during the dark period, reaching a maximum level at the beginning of the next light period (Galangau et al., 1988). These diurnal NR mRNA fluctuations were investigated in Nia30, an NR apoenzyme-deficient mutant. Nia30 and wild-type tobacco plants were grown in the greenhouse under a 16-hr light/8-hr dark regime. Leaf samples were collected at the beginning of the day period, when NR mRNA levels are maximal, and 4 hr, 9 hr, 14 hr, and 19 hr later. RNA gel blot analysis of NR mRNA is shown in Figure 3. NR mRNA from the wild type displayed the diurnal cycle previously described. By contrast, no significant diurnal fluctuation occurred with NR mRNA from Nia30 leaves. In addition to the absence of diurnal oscillations, NR mRNA in this mutant accumulated to a higher level than in the wild type.

Comparative Levels of NR mRNA at the Beginning and the End of the Light Period in the Leaves of N. plumbaginifolia nia and cnx Mutants

As in tobacco and tomato, a sharp decline of the NR mRNA level in *N. plumbaginifolia* wild-type leaves was detected 8 hr to 11 hr after illumination. Therefore, we tested *N. plumbaginifolia* mutants only at these two times. Preliminary experiments did not reveal any difference in either NR mRNA level or rhythm between grafted and nongrafted wild types, justifying the use of nongrafted wild types.

Most mutants tested for NR mRNA content at the beginning of the day period (see Tables 1 and 2) were assayed for their NR mRNA diurnal cycle. Whereas the NR mRNA level at the end of the day period was <10% of the maximum value for the wild type, most of the mutants showed comparable contents of NR mRNA for the two timepoints of leaf sampling (Figures 1 and 2, Table 3).

Table 3. Relative NR mRNA Levels[a] at Two Timepoints of the Day/Night Cycle in NR-Deficient Mutants of *N. plumbaginifolia*

Complementation Groups	0 hr of Light	8-11 hr of Light
Wild type	1	<0.1
nia		
Negative for ELISA		
D55	0.7	0.9
D80	1.7	1.7
D99	0.3	0.5
E105	0.5	0.6
K13	<0.1	<0.1
Positive for terminal NR activities		
D51	3.7	3.7
D64	1.4	1.3
E56	6.2	5.4
K5	2.5	2.1
Positive for NADH-cyt *c* reductase activity		
E77	5.0	4.7
E122	4.7	4.7
F19	2.5	2.4
H22	1.3	0.9
I5	1.0	0.6
cnxA		
B25	8.0	4.5
D132	6.0	5.1
F108	1.9	2.6
cnxB D126	4.7	4.7
cnxC F2	4.2	3.6
cnxD K4	5.7	5.7
cnxF E48	3.8	3.8

[a] NR mRNA levels were calculated relative to the wild-type level at the beginning of the day period.

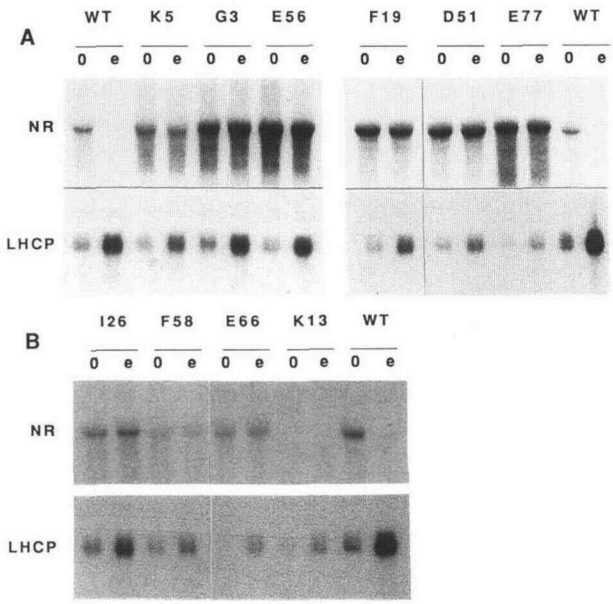

Figure 1. RNA Gel Blot Analysis of NR and LHCP mRNA Levels in Wild-Type (WT) and *nia* Mutant Leaves of *N. plumbaginifolia* at the Beginning (0) and the End (e) of the Light Period.

Grafted plants were grown in a controlled culture room under a light regime of 16 hr light/8 hr dark. Blots were hybridized with a 1.6-kb EcoRI fragment of tobacco NR cDNA (signals indicated by NR) and with a 0.83-kb PstI fragment of pea LHCP cDNA (signals indicated by LHCP).

(A) *nia* mutants expressing detectable NR protein and either NR terminal activities (K5, G3, E56, D51) or NADH-cyt *c* reductase activity (F19, E77).

(B) *nia* mutants devoid of detectable NR protein and NR partial activities.

To determine whether the loss of the diurnal cycle in mutants was specific to the NR mRNA, expression of the *cab* gene family, encoding the light-harvesting chlorophyll *a/b* protein (LHCP), was analyzed by using a pea LHCP probe (Coruzzi et al., 1983). No differences in the diurnal variations of LHCP mRNA levels were observed between wild-type and *nia* mutants (Figure 1). In all cases, the LHCP mRNA pool was higher at the end than at the beginning of the day period, in accordance with the results previously reported for tomato leaves (Piechulla, 1988) and tomato seedlings (Giuliano et al., 1988). Similar results were obtained with *cnx* mutants (data not shown). The lower LHCP mRNA levels observed in several mutants tentatively can be attributed to chlorosis affecting NR-deficient mutants.

Nitrate Inducibility of NR Expression in *N. plumbaginifolia* Mutants

Due to their NR impairment, mutants grown in vitro must be supplied with a reduced nitrogen source. In fungi, end products of the nitrate assimilation pathway, such as ammonium and glutamine, repress NR expression (Dunn-Coleman et al., 1984). In higher plants, nitrogen metabolite repression is not well documented, but glutamine has been shown to decrease NR expression in soybean (Curtis and Smarrelli, 1986) and squash (Langendorfer, Watters, and Smarrelli, 1988). To avoid nitrogen metabolite repression of NR expression, plants can be deprived of nitrogen prior to induction by nitrate. As nitrogen deprivation drastically affects NR expression (Galangau et al., 1988), we chose instead to use a nitrogen source displaying low repression. NR expression in the *N. plumbaginifolia* wild type and a *nia* mutant, therefore, was compared on different nitrogen sources. Glutamate appeared to have only a weak repressive effect on NR expression (data not shown) and, therefore, was supplied as nitrogen source for in vitro grown plants.

Representative mutants of the different classes characterized were tested for the nitrate inducibility of NR gene expression. NR protein and mRNA levels, as well as factors of induction (ratios of nitrate-induced versus uninduced levels of either NR protein or NR mRNA) are presented in Table 4. Due to the difficulty of propagating mutants in vitro, growth and morphology from one mutant to another varied. For this reason, one must be cautious in comparing NR protein and mRNA levels in the different mutants. However, for a same mutant, levels in the control treatment (20 mM KCl) and in the nitrate treatment (20 mM KNO_3) can be reliably compared. Total protein and RNA contents per gram of leaf tissue were found to be comparable in the two treatments (data not shown).

NR expression in the wild type and in mutants was not abolished in the absence of nitrate. In uninduced conditions, most of the mutants tested had low levels of NR mRNA. However, few mutants expressed higher amounts of NR mRNA, and the most pronounced situation was observed in mutants K4 and I9 (see Figure 5, Table 4). Except for *cnxA* mutants (discussed below), NR protein levels in the absence of nitrate were usually low (Figure 4, Table 3).

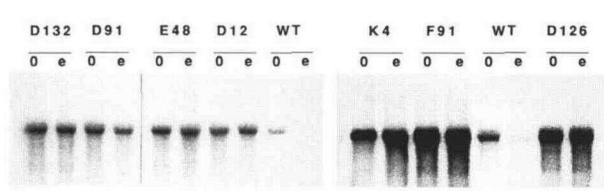

Figure 2. RNA Gel Blot Analysis of NR mRNA Levels in Leaves of *N. plumbaginifolia cnx* Mutants at the Beginning (0) and the End (e) of the Light Period.

Conditions of growth and analysis were the same as described in the legend of Figure 1. D132 and D91, *cnxA* mutants; E48 and D12, *cnxF* mutants; K4 and F91, *cnxD* mutants; D126, *cnxB* mutant.

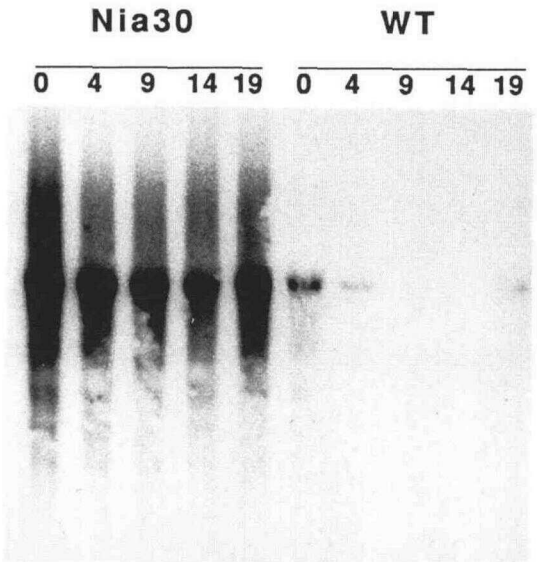

Figure 3. RNA Gel Blot Analysis of NR mRNA Levels in Nia30 and Wild-Type (WT) Tobacco Leaves during a Diurnal Cycle.

Plants were grown in the greenhouse under a light regime of 16 hr light/8 hr dark. Timepoints, numbered on a 24-hr time basis, are shown on top, 0 indicating the beginning of the light period. Blots were hybridized with a 1.6-kb EcoRI fragment of tobacco NR cDNA.

Both NR protein (Figure 4) and NR mRNA (Figure 5) expression remained nitrate-inducible in all the mutants tested (13 *nia* and 10 *cnx* mutants). In the *nia* mutants, factors of induction for NR protein (5.1-fold to 15-fold) were comparable with that of the wild type (8.1-fold) except for K25 (2.4-fold), whereas higher factors of induction were often observed for the NR mRNA (2.5-fold to 11.4-fold, compared with threefold for the wild type). It is worth noting that four out of the 13 *nia* mutants mentioned in Table 4 are mutants devoid of detectable NR protein. Even in these mutants, NR mRNA levels were typically increased by nitrate (Figure 5).

For five of the six *cnx* classes (*B*, *C*, *D*, *E*, and *F*), factors of induction comparable with those of the *nia* mutants were obtained (5.2-fold to 14.2-fold at the NR protein level and threefold to sixfold at the NR mRNA level). However, the remaining class (*cnxA*) displayed different features. Although the NR mRNA assay demonstrated a factor of induction (3.9-fold and 6.2-fold) similar to that of the other mutants, a reduced factor (1.9-fold to 2.6-fold) was observed in the NR protein assay. NR protein levels in the representative mutants were extremely high, as previously found in grafted plants (A. Marion-Poll, I. Chérel, M. Gonneau, M. T. Leydecker, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted), even in the absence of nitrate: uninduced NR protein levels in *cnxA* mutants were higher than nitrate-induced NR protein levels in the wild type (Figure 4, Table 4).

DISCUSSION

Presence of Defective NR Protein Correlates with NR mRNA Overexpression

nia mutants retaining nonfunctional NR protein or partial NR activities, as well as mutants of five of the six *cnx* complementation groups (*cnxA*, *-B*, *-C*, *-D*, and *-F*), had increased levels of NR mRNA. NR mRNA overexpression might be expected to correlate with NR protein overexpression. These different classes of mutants, except the *cnxA* group, expressed immunologically detectable NR protein at levels equal to or lower than that of the wild type (A. Marion-Poll, I. Chérel, M. Gonneau, M. T. Leydecker, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted), thus displaying a clear discrepancy between NR mRNA level and NR protein level. One explanation could be destabilization of NR protein in mutants. Results from HPLC analyses suggested that degraded forms of NR protein were present in *cnxB*, *-C*, *-D*, *-E*, and *-F* mutants (A. Marion-Poll, I. Chérel, M. Gonneau, M. T. Leydecker, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted). These degraded forms of NR protein may not be well recognized by antibodies, an inference supported by the observations that *cnx* mutants expressed NADH-cyt *c* reductase activity at levels comparable with or higher than the wild type (A. Marion-Poll, I. Chérel, M. Gonneau, M. T. Leydecker, C. Meyer, F. Pelsy, and M. Caboche, manuscript submitted). In *cnxA* mutants, however, NR protein accumulation correlates with NR mRNA accumulation. NR protein in these mutants is presumed to be more stable than in other *cnx* mutants (Mendel and Müller, 1985; Gabard et al., 1988).

Undetectable NR Protein Content Correlates with NR mRNA Underexpression

nia mutants devoid of detectable NR protein mostly appeared to have reduced, or even undetectable, NR mRNA contents. Some of these may be defective for NR mRNA synthesis. In addition to transcriptional defects, however, nonsense and frameshift mutations leading to premature translation termination might also destabilize NR mRNA, as is observed for β-globin mRNA in some human β-thalassemias (Orkin, 1987). The observation that some *nia* mutants lack detectable NR protein but retain wild-type NR mRNA levels may indicate that the synthesized NR apoenzyme is unstable or, alternatively, that structural modifications of the putative NR protein prevent recognition by monoclonal antibody. One mutant (E56) has been shown to fit this scenario (Meyer et al., 1987).

Table 4. Relative Nitrate-Induced and Uninduced NR mRNA[a] and Protein[b] Levels in NR-Deficient Mutants of *N. plumbaginifolia*

	NR mRNA Levels			NR Protein Levels		
Complementation Groups	$-NO_3$	$+NO_3$	$+NO_3/-NO_3$	$-NO_3$	$+NO_3$	$+NO_3/-NO_3$
Wild type	0.3	1.0	3.0	0.12	1.0	8.1
nia						
Negative for ELISA						
D55	0.4	1.6	4.0	ND[c]	ND	ND
D80	0.3	1.6	5.3	ND	ND	ND
D99	0.4	1.6	4.7	ND	ND	ND
E105	0.3	1.1	3.5	ND	ND	ND
Positive for terminal NR activities						
D51	NT[d]	NT	NT	0.70	3.6	5.1
E56	0.9	4.2	4.7	ND	ND	ND
K5	NT	NT	NT	0.14	1.50	10.7
K25[e]	0.7	5.4	7.5	0.09	0.22	2.4
Positive for NADH-cyt *c* reductase activity						
E77	0.8	7.8	9.8	0.02	0.23	11.5
E122	0.5	5.7	11.4	0.09	0.45	5.1
F19	1.2	9.1	7.6	0.13	1.35	10.4
H22	0.8	5.2	6.5	0.03	0.45	15.0
I9[e]	2.8	7.1	2.5	0.13	1.35	10.3
cnxA						
B25	0.8	5.0	6.2	8.5	19.6	2.3
D132	NT	NT	NT	6.8	13.0	1.9
F108	2.1	8.1	3.9	5.0	13.1	2.6
cnxB						
D126	1.5	9.0	6.0	0.16	1.76	11.0
D134[e]	NT	NT	NT	0.27	2.9	10.7
cnxC						
E137[e]	0.9	4.8	5.3	0.43	2.3	5.3
cnxD						
K4	5.7	17.6	3.0	0.12	1.14	9.5
cnxE						
D15[e]	1.5	4.5	3.1	0.42	2.2	5.2
cnxF						
D49[e]	NT	NT	NT	0.54	3.2	5.9
E96[e]	1.2	5.1	4.2	0.08	1.14	14.2

[a] NR mRNA levels were calculated relative to the nitrate-induced wild-type level at the beginning of the day period.
[b] NR protein levels were calculated relative to the nitrate-induced wild-type level by ELISA measurements with a monoclonal antibody. Note that they do not represent true quantitative values since mutant NR proteins may have modified affinities for antibodies.
[c] ND, nondetectable (<0.01).
[d] NT, nontested.
[e] These mutants are not classified in Tables 1 and 2, as they were not grafted.

Disappearance of the NR Diurnal Oscillation in NR-Deficient Mutants

NR mRNA diurnal oscillations in *N. plumbaginifolia* were examined at the two timepoints showing maximum amplitude variation in tobacco and tomato (Galangau et al., 1988). As expected, the wild type showed light regulation. By contrast, none of the *nia* or *cnx* mutants displayed significant diurnal oscillations in their NR mRNA content. In addition, for different timepoints of a complete cycle, the tobacco Nia30 mutant also had a nearly constant NR mRNA pool (Figure 3).

Recent work in our laboratory has shown a comparable disappearance of the NR diurnal fluctuations in tobacco plants treated with tungstate, an analog of molybdate that inhibits NR activity (Deng, Moureaux, and Caboche, 1989). Our results support the hypothesis that catalytic integrity of NR activity is required for the expression of the NR

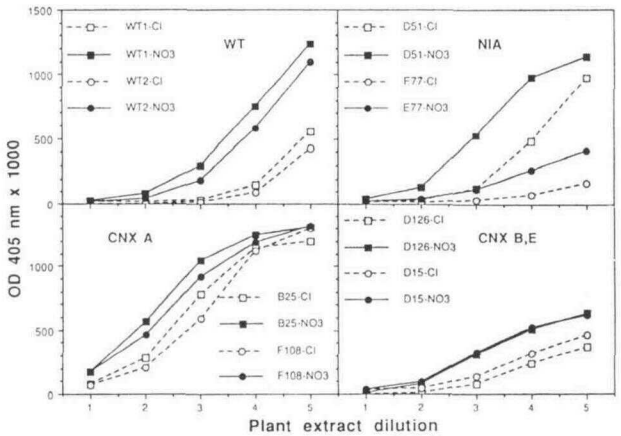

Figure 4. Nitrate Inducibility of NR Protein in NR-Deficient Mutants of *N. plumbaginifolia*.

Plantlets were grown aseptically on 20 mM glutamate and transferred onto fresh medium supplemented with either 20 mM KCl (uninduced control: -Cl) or 20 mM KNO_3 (induced sample: -NO3) 10 days before sampling. NR protein levels in leaf extracts were measured by a two-site ELISA, involving four successive incubation steps with (1) the monoclonal antibody, (2) the plant ammonium-sulfate precipitated crude extract, (3) a rabbit antiserum directed against maize NR, and (4) an anti-rabbit IgG-alkaline phosphatase conjugate. The optical density (OD) values have been measured after 10 min of alkaline phosphatase reaction with *p*-nitrophenylphosphate as substrate. The initial protein extract (0.2 mL of extract/g, fresh weight) has been used at the following dilutions: 1, 1/3125; 2, 1/625; 3, 1/125; 4, 1/25; 5, 1/5. WT = wild type, WT1 and WT2 being plantlets from two independent experiments. Representative mutants are presented: D51 and E77 for the *nia* mutants retaining NR terminal activities and NADH-cyt *c* reductase activity, respectively, B25 and F108 for the *cnxA* mutants, and D126 and D15 for the *cnx* mutants other than *cnxA*. Shapes of the curves displayed by these mutants are typical of their category.

mRNA diurnal fluctuations. Whether this effect in mutants is specific for NR mRNA was tested by studying another light-controlled protein, LHCP (Kloppstech, 1985; Piechulla and Gruissem, 1987; Giuliano et al., 1988; Nagy, Kay, and Chua, 1988; Otto et al., 1988; Paulsen and Bogorad, 1988; Piechulla, 1988). No change in the light control of LHCP mRNA can be observed in NR-deficient mutants.

Diurnal oscillations of a number of tomato genes (Giuliano et al., 1988) and of tobacco LHCP gene (Paulsen and Bogorad, 1988) have recently been demonstrated to be partially controlled at the transcriptional level. If this is true for NR mRNA, transcription of the NR gene may be responsible for the constant level of NR mRNA in mutants during a diurnal cycle. Transcriptional level control could also explain the apparent increase in NR mRNA in mutants expressing a defective NR protein.

Nitrate Inducibility of NR Expression Is Maintained in Mutants

To explain the findings that *Aspergillus nidulans* mutants have either inducible or constitutive synthesis of NADH-cyt *c* reductase activity, it has been suggested that the NR molecule somehow regulates its own synthesis (reviewed by Cove, 1979). A model has been proposed in which NR protein inactivates the product of *nirA*, a positive regulator of NR expression, in the absence of nitrate. Similar findings have been reported with *Neurospora crassa* mutants (Tomsett and Garrett, 1981), and autogenous control of NR has been shown to be exerted at the transcriptional level (Fu and Marzluf, 1988).

We examined 23 *N. plumbaginifolia* mutants for the nitrate inducibility of NR expression. Unlike many fungal mutants, all *nia* and *cnx* mutants tested were still nitrate-inducible and retained significant factors of induction by nitrate. Even in *nia* mutants in which NR protein was immunologically undetectable, NR mRNA expression remained typically nitrate-inducible.

NR expression in mutants, as well as in the wild type, was never found to be completely abolished in the absence of nitrate, but two mutants (one *nia* mutant, I9, and one *cnx* mutant, K4) expressed higher amounts of NR mRNA under uninduced conditions. This result may indicate that structural integrity of NR protein is required for its own regulation. It remains to be proven that the *nia* mutant is solely altered in the coding sequence for the *nia* gene, and, in the case of *cnx* mutants, that the molybdenum cofactor itself is not involved in regulation of NR expression. However, NR protein in these mutants was expressed at levels comparable with that of the wild type. In addition, none of

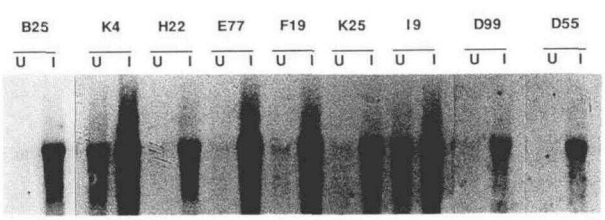

Figure 5. Nitrate Inducibility of NR mRNA in NR-Deficient Mutants of *N. plumbaginifolia*.

Conditions of in vitro culture and nitrate treatment were the same as described in Figure 4. Leaf tissue was collected at the beginning of the light period. RNA from control uninduced tissue (U) and from nitrate-treated tissue (I) were analyzed by RNA gel blot hybridization with a 1.6-kb EcoRI fragment of tobacco NR cDNA. B25 and K4, *cnxA* and *-D* mutants, respectively; H22, E77, F19, and I9, *nia* mutants expressing NADH-cyt *c* reductase activity; K25, *nia* mutant expressing NR terminal activities; D55 and D99, *nia* mutants devoid of detectable NR protein and of NR partial activities.

the *nia* mutants in which NR protein was immunologically undetectable expressed high uninduced levels of NR mRNA. Thus, from our results, we cannot exclude that NR expression in higher plants is autogenously regulated.

cnxA mutants were shown to overexpress both NR protein and NR mRNA. NR expression remained nitrate-inducible, but factors of induction were lower at the protein level, and their residual NR protein content in the absence of nitrate was consistently higher than the nitrate-induced NR protein level in wild type. However, their NR mRNA typically remained nitrate-inducible, with factors of induction comparable with that of the other mutants. To explain the discrepancy between the NR mRNA levels and the NR protein levels in the absence of nitrate, we suggest that *cnxA* mutants may have lost a putative post-transcriptional control of NR protein level. Such a control was first suggested by the discrepancy observed between NR protein levels and NR mRNA levels during nitrogen starvation of tobacco and tomato plants (Galangau et al., 1988). The possibility of translational control of NR expression is being examined in our laboratory.

METHODS

Plant Material

Plants of *Nicotiana tabacum* cv Xanthi, line XHFD8, and of *N. plumbaginifolia* cv Viviani, line pbH1D, were used as wild types. The Nia30 tobacco (*N. tabacum* cv Gatersleben) is an apoenzyme mutant, simultaneously mutated in the two homeologous genes encoding NR apoenzyme (Müller, 1983). *nia* and *cnx* mutants of *N. plumbaginifolia* cv Viviani were isolated from mutagenized haploid protoplasts (Grafe et al., 1986; Gabard et al., 1987) and aseptically propagated on solid B-N medium (Muller, Missonier, and Caboche, 1983) containing 10 mM diammonium succinate as nitrogen source and 10 mM KCl.

Growth Conditions for Day-Cycle Assays

Since growth of *cnx* and *nia* mutants on their own roots is very poor, mutant plants were grafted onto *N. tabacum* cv Wisconsin-38 plants and grown in the greenhouse with standard nutrient solution containing nitrate and ammonium (Coïc and Lesaint, 1971).

Wild-type and Nia30 tobacco lines were illuminated during a 16-hr period with natural light supplemented with incandescent lamps providing a total fluence rate of 200 $\mu mol \cdot m^{-2} \cdot sec^{-1}$ to 300 $\mu mol \cdot m^{-2} \cdot sec^{-1}$. Relative humidity was about 80%. Leaf samples were collected at the beginning of the light period, and 4 hr, 9 hr, 14 hr, and 19 hr later.

N. plumbaginifolia plants were transferred 7 days before sampling into a controlled culture room, under a light regime of 16 hr light at 25°C/8 hr dark at 17°C, with light supplied at a fluence rate of about 180 $\mu mol \cdot cm^{-2} \cdot sec^{-1}$ by a mixture of incandescent and fluorescent lamps (relative humidity about 80%). Leaves were collected at the beginning of the light period and 8 hr to 11 hr later.

Growth Conditions for Nitrate Inducibility Assays

Mutants were grown aseptically on solid medium B-N containing 20 mM potassium glutamate. Growth conditions of the controlled culture room were the same as in the day-cycle assay. Plantlets were then transferred onto fresh medium containing 20 mM potassium glutamate and either 20 mM KCl (uninduced control) or 20 mM KNO_3 (induced sample). Ten days later, rooting in the fresh medium was fairly good, and leaves were collected at the beginning of the day period for the NR mRNA assay, and 6 hr later for the NR protein assay.

RNA Isolation

Total RNA was extracted from leaves of grafted plants by a procedure described by Galangau et al. (1988). Since very small amounts of leaf tissue were available from the in vitro propagated plantlets, total RNA was prepared by a miniextraction procedure, adapted from Scherrer (1969). RNA fractionation by formaldehyde gel electrophoresis and transfer to Hybond C membranes (Amersham) were performed according to Thomas (1980). Comparisons of samples have been done on the basis of similar amounts of total RNA (typically 8 μg of total RNA per slot), and verified by ethidium bromide staining of the gels before transfer. When transferred onto Hybond N membranes (Amersham), RNAs were bound to the membranes by the UV-cross-linking procedure.

RNA Gel Blot Hybridization

NR mRNA was detected by hybridization with a 1.6-kb EcoRI fragment purified from a partial tobacco cDNA clone, previously described (Calza et al., 1987). RNA gel blot hybridization onto nitrocellulose membranes was performed as described by Galangau et al. (1988). In the case of RNAs purified from grafted plants, hybridizations were carried out under stringent conditions at 42°C in 50% formamide. Due to reduced levels of NR mRNA in the case of *N. plumbaginifolia* RNAs prepared from in vitro grown plantlets, hybridization stringency had to be lowered to 38% formamide at 42°C. A procedure adapted from Khandjian (1986) was used in the RNA gel blot hybridization experiments performed with nylon membranes.

LHCP mRNA was detected by hybridization with a 0.83-kb PstI fragment of a pea LHCP cDNA purified from a pAB96 clone (Coruzzi et al., 1983).

RNA gel blot hybridization signals were compared by the use of a combination of increasing times of autoradiogram exposure and densitometer scanning with Shimadzu equipment. In a control experiment, NR mRNA levels in leaf tissue of grafted wild type at the beginning of the day period were quantified relative to known dilutions of the NR cDNA fragment used as a probe, and were found in the range of 20 μg to 30 μg per gram of total RNA. NR mRNA abundance in leaf tissue of grafted plants and of plantlets grown in vitro were calculated relative to NR mRNA level at the beginning of the day period in, respectively, the grafted wild type and the nitrate-induced wild type grown in vitro. In the latter, NR

mRNA level was approximately 3 times lower than in the grafted wild type.

Extraction and Assay of NR Protein

The procedures described by Chérel et al. (1986) were used for extraction of proteins from *N. plumbaginifolia* leaves and for estimation of NR protein amounts by a two-site ELISA with a monoclonal antibody. ELISAs were performed twice on each mutant. For a same mutant, comparisons of NR protein levels in nitrate-induced and uninduced plants are quite reliable, but quantitative values relative to the wild type have to be taken as estimates since the affinity of antibodies for the NR protein may differ from one mutant to another, as shown by the different shapes of the curves. This is typically the case for all *cnxB*, *-C*, *-D*, *-E*, and *-F* mutants expressing an unstable NR protein, due to the absence of protection by the molybdenum cofactor. Relative values were obtained by interpolation in the linear part of the ELISA curves, at dilutions corresponding to 50% of optical density values at the plateau. In control experiments, the standard deviation of measurements was found to be lower than 23% of measurements, and the standard deviation of protein levels was lower than 12% of calculated values.

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