Post-Transcriptional Regulation of Nitrate Reductase by Light Is Abolished by an N-Terminal Deletion

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Higher plant nitrate reductases (NRs) carry an N-terminal domain whose sequence is not conserved in NRs from other organisms. A gene composed of a full-length tobacco NR cDNA with an internal deletion of 168 bp in the 5′ end fused to the cauliflower mosaic virus 35S promoter and appropriate termination signals was constructed and designated as ΔNR. An NR-deficient mutant of *Nicotiana plumbaginifolia* was transformed with this ΔNR gene. In transgenic plants expressing this construct, NR activity was restored and normal growth resulted. Apart from a higher thermosensitivity, no appreciable modification of the kinetic parameters of the enzyme was detectable. The post-transcriptional regulation of NR by light was abolished in ΔNR transformants. Consequently, deregulated production of glutamine and asparagine was detected in ΔNR transformants. The absence of in vitro ΔNR activity modulation by ATP suggests the impairment of ΔNR phosphorylation and thereby suppression of ΔNR post-translational regulation. These data imply that post-transcriptional control of NR expression is important for the flow of the nitrate assimilatory pathway.

INTRODUCTION

Nitrate reductase (NR; EC 1.6.6.1) is considered as a key enzyme in the nitrate assimilation pathway: it reduces nitrate to nitrite. In a second step, nitrite reductase (EC 1.7.7.1) catalyses the reduction of nitrite to ammonia, which is then used for the synthesis of amino and nucleic acids. Nitrate reduction is a process highly regulated by several environmental and internal factors, such as light, nitrogen source, circadian rhythm, and sugars (for review, see Hoff et al., 1994). Such environmental factors act mainly at the transcriptional level (Vincentz and Caboche, 1991). In addition, it has been demonstrated that rapid modification of the protein, in the presence of Ca^{2+} or Mg^{2+}, is responsible for the fine tuning of NR activity in response to changes in light or CO_2 status. These in vivo activity changes are assumed to be mainly the result of covalent modification of the protein caused by phosphorylation/ dephosphorylation of NR (for reviews, see Kaiser and Huber, 1994a; Lillo, 1994).

Plant NR has been shown to have a homodimeric structure. Each subunit contains three functional domains associated with one heme-Fe, one flavin adenine dinucleotide (FAD), and

one molybdenum cofactor (MoCo) component (for review, see Rouzé and Caboche, 1992). These groups are the redox centers that catalyze the transfer of electrons from the reductant (NADH or NADPH) to nitrate. The electrons travel successively through the FAD, heme, and MoCo domains before they finally reduce nitrate into nitrite.

The N-terminal region of the NR sequences varies considerably between higher plants and other organisms. In fungi, this region varies in size from seven to 121 amino acids (for *Ustilago maydis* and *Neurospora crassa*, respectively) and presents no significant common features, as shown in Figure 1. In higher plants, the length differences between N-terminal domains are less extreme. Their sizes range from 60 (soybean or bean leaf) to 99 (spinach) amino acids. Very few residues are identical, but the domains are largely hydrophilic and share a striking acidic stretch (Rouzé and Caboche, 1992; box in Figure 1). No homology with any other known sequence can be found. Therefore, to elucidate the potential role of the N-terminal domain in higher plants, we used transgenic plants to analyze the expression of a constitutive chimeric NR gene in which this domain has been deleted (ΔNR). Use of the cauliflower mosaic virus (CaMV) 35S promoter (Benfey et al., 1989) to drive this construct bypasses the normal transcriptional regulation of a wild-type NR allele, as demonstrated by Vincentz and Caboche (1991). The construct was introduced in the mutant E23 of *Nicotiana plumbaginifolia*, which is affected in the NR structural gene (*nia*) and impaired in the production of the full-

N-TERMINAL DOMAIN

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                    1                                              50
A.t.nia2    . . . . . . . . . . . . . . . MAASVDNRQYA.RLEPGLNGVVRSYK. . . . . . . . . .PPVPG.RSDSPKAHQNQTTN
A.t.nia1    . . . . . . . . . . . . . . . MATSVDNRHY. . . . .PTMNGVRHAFKPPLVPSPRSFDRHRHQNQTLDVILTETKIV
Bean leaf   . . . . . . . . . . . . . . . MAASVGNRQFA. . . .THMNGVVRS. .CGQ. . . . . . . . . . . . . . . . . . .DLKPS
Soybean     . . . . . . . . . . . . . . . MAASVDNRQYG. . . .THINAVVRA. .CGP. . . . . . . . . . . . . . . . . . .DFNTP
Bean root   . . . . . . . . . . . . . . . MAASVEHRPFT. . . .SHQHGVVRSFKSYP. . . . . . . . . . . . . . . . . . .DVPRP
N.t.nia1    . . . . . . . . . . . . . . . MAASVENRQFS.HIEAGLS. .RSFK. . . . . . . . . . . .PRSDSPVRGCNF.PS
N.t.nia2    . . . . . . . . . . . . . . . MAASVENRQFS.HLEAGLS. .RSFK. . . . . . . . . . . .PRSDSPVRGCNF.PS
Petunia     . . . . . . . . . . . . . . . MAASVENRQFS.HLEPGLSGVVRSFK. . . . . . . . . . . .PRSDSPVRGCNF.PL
Tomato      . . . . . . . . . . . . . . . MAASVENRQYT.HLEPGLSGVGRTFK. . . . . . . . .PRPDSPVRGCNFPPS
Squash      . . . . . . . . . . . . . . . MAASVDNRQYG.PLQPPLSGVVRSFK. . . . . . . . .NGPN.HRADSPVRGCNFPNS
Birch       . . . . . . . . . . . . . . . MAASVENRRFT.HHEPAVNGLVRTFK. . . . . . . . .PVPNSHRSDSPDLG. . . . . .
Spinach     . . . . . . . . . . . . . . . MAASVD.RQYH.P. .APMSGVVRT. . . . . . . . .PFSNHHRSDSPVRNGYTFSN
Rice        . . . . . . . . . . . . . . . MAASVQPRQ.FGHLEP.GSAPVRGAASSNGAKAYPPANGIPRRADSPVRGCGFPPL
Barley nar1 . . . . . . . . . . . . . . . MAASVEPRQPF.GRLD. . . . . .APATAPTARA.PGSNGIRRRADSPVRGCGFPSL
Barley nar7 . . . . . . . . . . . . . . . MAASVE. . . .Y.NRQVSAHPWPTNAQPKAAFDLFSSSGGGRRRSGA. . . . . . . . . .
Volvox      . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
A.niger     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .MATVTEVLTEPFTAQG
A.nidul     . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .MSTTVTQVRTGSIPK. .
Leptosph.   . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .MSVTTQQPAVVPLPSSP
Fusarium    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .METSTTTTLLQQERIPENS
Neurospora  MEAPALEQRQSLHDSSERQQRFTSLILPNGVGCSSREEPQGSGGLLVPHNDNDIDNDLASTRTASPTTTDFSSSSSDDNS
Ustilago    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .
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                              100                                          MoCo
                                                                          150
A.t.nia2    QTVFLKPAKVH. . . . . . . . . . . . . . . . . .DDDEDVSSEDENETHNSNAVYYKEMIRKSNAELEPSVLDPRDEYTAQSW
A.t.nia1    KETEVITTVV. . . . . . . . . . . . . . . . . . .DSYDDSSSDDEDESHNRNVPYYKELVKKSNSDLEPSILDPRDESTADSW
Bean leaf   . .LPL. . . . . . . . . . . . . . . . . . . . .DFDLDSSSSDDDENDDASYL. .KELVRKANAETEASVMDPRDEGTADQW
Soybean     . .LPS. . . . . . . . . . . . . . . . . . . . .DFDLDSSSDDEDQNDDASFL. .KELIQKANAETEASLLDPRDEGTADQW
Bean root   KKLPLPQPL. . . . . . . . . . . . . . . . . . . . . . . .SDST.NDNDEEAAVWKELVLKSSAGVEPSIFDPRDDGTSDQW
N.t.nia1    PNS.TNFQKKPNSTIFL. . . . . . . . . . . . . . .DYSSSEDDDDDDEKNEYLQMIKKG. .NSELEPSVHDSRDEGTADNW
N.t.nia2    PNS.TNFQKKPNSTIYL. . . . . . . . . . . . . . .DYSSSEDDDDDDEKNEYLQMIKKG. .NSELEPSVHDTRDEGTADNW
Petunia     NNELTNFQKKPNTTIYL. . . . . . . . . . . . . . .DCSSSEDDDDDDDKNEYLQMIRKG. .KLEVEPSVHDIRDEGTADNW
Tomato      SNHELPFQKKQNPPIYL. . . . . . . . . . . . . . .DYSSSEDEDDDDEKNEYVQMIKKG. .KTELEPSIHDTRDEGTADNW
Squash      NVDYNNNRPLKSSVKIQEAAA. . . . . . . . . . .EEMEDSCSEDENENE. . . .FRDLIVKG. .NRELEPSILDHRDEGTADNW
Birch       . . . . . . .RQIPSSPKKQVATG. . . . . . . . . . . . . .EDSSSEDENEND. . . .YKELIQKG. .NGELEPSILDPRDEATADNW
Spinach     PPSSNGVVKPGEKIKLVDNNSNSNNGSNNNNNRY.DSDSEEDDDENEMNVWNEMIKKG. .NSELEPSSVDSRDEGTADQW
Rice        VSPPPRKPP. . . . . . . . . . . . . . . . . . . . . .SDGSDDEEEEQEDWRELYGSHL. . .QLEVEPPVRDARNEGTADAW
Barley nar1 ISPPRKGPVA. . . . . . . . . . . . . . . . . . . . . . .EEEEDDDDEDDEGHEDWREAYGSHLQLEVEPSTRDPRDEGTADAW
Barley nar7 . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .DSDSDDEDSVPPDWRSLYSPR. . . . .LDVEPSVKDPRDEATSDAW
Volvox      . . . . . . . . . . . . . .MTIPELPLGGIVSQAVELGAPYE. . . . . . . . . .PPLTPDHPEWKVHVPAAAVDKKDQQDIPDNW
A.niger     . . . . . . . . . . . . . .VTLKSGPIKVHQEELPAVE. . . . . . . . . . . . . . .LSDIPLPPPSK.EPTEVLSIDKPIPDYH
A.nidul     . . . . . . . . . . . .TLKTSQIRVEEQEITELD. . . . . . . . . . . . . . . . . .TADIPLPPPSK.EPTEVLSLDKTIPDSH
Leptosph.   R. . . . . . . .LPIESHAGPRSTQLPPSPPETVNGDDPKSITST. . . . . . . . . .PAEPDFPLPPPAN.PKPQVLDIDKPIPDAH
Fusarium    EPISTHIHTHSLPPTPPGTAKPSRIGSFHDLQELSSVESP. . . . . . . . . . .RLDHIKPYPLPPKFSPKSVLKEDLKIPDNF
Neurospora  TTLETSVNYSHSSNTNTNTSCPPSP. . . . . . . . . . .ITSS. . . . . . . . .SLKPAYPLPPPST.RLTTILPTDLKIPDHL
Ustilago    . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . . .MSYPPSQPRKAVQPLTSDANIPDHW
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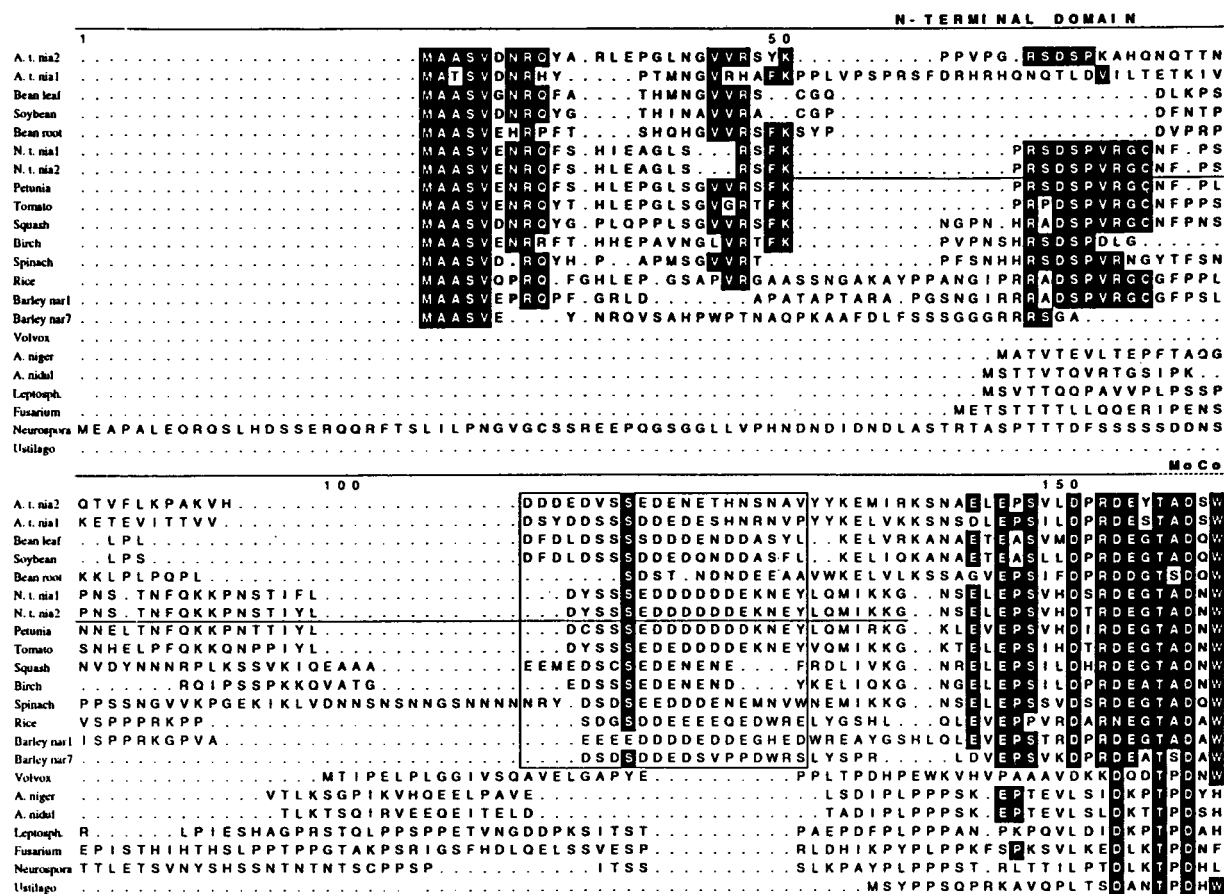
Figure 1. Sequences and Conserved Residues of N-Terminal Domains of Plant NRs and Homologous Redox Proteins.

The sequences were aligned, and gaps (dots) were introduced to maximize homology. The 56 amino acids deleted in the ΔNR construct are underlined for the tobacco *nia2* NR sequence. The position of the acidic domain conserved in all plant NRs is boxed. Highly conserved residues for a given position are indicated by white lettering on a black background. A.t., *Arabidopsis thaliana*; A. niger, *Aspergillus niger*; A. nidul, *A. nidulans*; Leptosph, *Leptosphaeria*; N.t., *N. tabacum*.

length NR transcript and protein (Pouteau et al., 1989). There-fore, in the transgenic E23 mutant transformed with the chimeric construct, the NR transcript and protein were derived only from the transgene. We describe here the production of E23 mutants with restored NR activity resulting from the in-troduction of the ΔNR transgene. Analysis of these plants showed that the ΔNR gene had lost post-transcriptional regu-lation by light.

RESULTS

Construction of Plant and Yeast Vectors Carrying the ΔNR Gene

A deletion was made in the cDNA from the NR structural gene *nia2* of tobacco (Vaucheret et al., 1989). Fifty-six amino acids

were removed from the N-terminal domain of NR (Figure 1). An acidic domain conserved only in plants is therefore lacking in the ΔNR protein. The deletion starts after the first 20 amino acids; therefore, the well-conserved first 10 amino acids (MAA-SVENRQF) of the NR sequence remain unchanged. Any effect on the translation initiation codon context should also be avoided (for review, see Gallie, 1993).

Because this 56–amino acid deletion might abolish NR cata-lytic activity, we decided to test the functionality of ΔNR in yeast. To produce ΔNR in yeast, the 2.6-kb cDNA, including the leader sequence and the ΔNR coding sequence, was introduced into the yeast galactose-inducible expression vector pKV482. These ΔNR-expressing yeast transformants exhibit NADH:cytochrome c reductase activity, partial NR activity, as well as NADH:NR activity if exogenous MoCo is provided (Truong et al., 1991). None of these data are shown. This confirmed the functional-ity of the electron transport chain in the ΔNR protein, and we proceeded to generate transgenic plants expressing ΔNR.

A 4.1-kb DNA fragment that included the leader sequence (138 bp), the ΔNR coding sequence, and 1.5 kb of the 3' noncoding sequence of the nia2 gene was fused to the 35S promoter of CaMV in the plant transformation vector pBinDH51 (Vincentz and Caboche, 1991), which carried a kanamycin resistance gene as a selectable marker, to create pBΔNR.

The nia E23 Mutant of N. plumbaginifolia Can Be Complemented for NR Activity by the Chimeric ΔNR Gene

The nia E23 mutant of N. plumbaginifolia does not produce any full-length NR mRNA due to the insertion of a Tnt1-like retrotransposon (Tnp2) in the first exon of the single-copy nia gene (Vaucheret et al., 1992). The E23 mutant cannot grow on nitrate but is able to use ammonium, the end product of the nitrate assimilation pathway. This mutant was transformed with the chimeric ΔNR gene via Agrobacterium-mediated gene transfer. A first screen was performed with kanamycin on a medium containing ammonium to minimize any selection pressure on the recovery of transgenic plants expressing the ΔNR gene. Selected kanamycin-resistant calli were regenerated and then tested for the ability to grow and develop into plantlets on a medium containing nitrate as the sole nitrogen source. Four primary transformants (R_0) were obtained, designated del2, del6, del7, and del8. Genetic analysis was performed on R_1 progeny grown on nitrate-containing medium because primary transformants are known often to give aberrant Mendelian segregation ratios for the transmission of inserted transgenes.

The selfed R_1 progeny were studied for the transmission of the kanamycin resistance marker and the ability to grow on nitrate. Both characteristics were stably transmitted to the progeny and behaved as dominant Mendelian markers. The two markers cosegregated in the progeny of transformed plants (data not shown).

R_1 plants deriving from primary transformants del2, del6, and del7 were all found to carry a single functional locus. Plants derived from del8 segregated two independent functional loci. This genetic analysis was confirmed by DNA gel blot analysis using neomycin phosphotransferase II (nptII) or NR cDNA as probes (data not shown). R_1 plants derived from transformants del2, del6, and del7 all displayed the same restriction pattern as the R_0 parents, whereas two different hybridizing bands were found to segregate among the progeny of plants del8. We concluded that a single functional locus is present in R_0 plants del2, del6, and del7, whereas del8 carries two functional loci.

Homozygous plants were selected from the progeny and used for further analysis. Transgenic plants grew vigorously in vitro and in the greenhouse. They were indistinguishable from the wild-type plants, except for transformant del2, which grew slower and periodically displayed chlorotic leaves. In the greenhouse, all transformants, with the exception of del2, were less fertile than wild-type plants (data not shown).

Analysis of ΔNR Polypeptides and RNA in Leaves of Transgenic Homozygous R_1 Progeny

R_1-complemented transgenic plants were grown to the rosette stage in a growth chamber with 8-hr days and 16-hr nights. Leaves from several plants were harvested, at the beginning and the end of the light period, for protein and RNA extraction. Analysis of the transgenic plants was facilitated by the absence in mutant E23 of any detectable NR mRNA when the 1.6-kb EcoRI nia cDNA fragment was used as probe (Vincentz and Caboche, 1991). As a control for mRNA loading, a probe for the gene encoding the nuclear β subunit of mitochondrial ATPase (Boutry and Chua, 1985) was used, and RNA levels were quantified using a PhosphorImager. The ΔNR mRNA detected in all transformants comigrated with the wild-type NR mRNA in that the deletion was too small to be visualized on RNA gel blots (Figure 2A).

In wild-type plants, the NR transcript accumulates toward the end of the dark period and decreases to almost undetectable levels at the end of the light period, as previously described (Galangau et al., 1988). Use of the CaMV 35S promoter
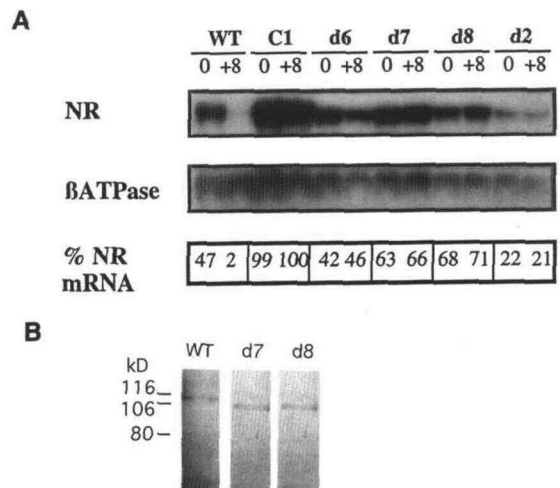

Figure 2. Analysis of NR mRNA and Protein of Leaves from Transgenic Homozygous R_1 Plants.

Leaves were harvested for RNA and protein extraction at different times—the beginning of the light period (0 hr) and the end of the day (+8 hr).

(A) RNA gel blot of total RNA (7 µg) using the 1.6-kb EcoRI NR cDNA probe. The blot was reprobed with a cDNA of a nuclear gene encoding the β subunit of mitochondrial ATPase. The percentage of NR mRNA after PhosphorImager data collection was standardized with respect to ATPase β subunit mRNA.

(B) Protein gel blot analysis of 5' AMP-Sepharose purified extracts using an anti-rabbit polyclonal antiserum raised against maize NR. Molecular mass numbers are given at left in kilodaltons.

WT, wild-type N. plumbaginifolia; C1, nia E23 mutant expressing the chimeric NR gene fused to the 35S CaMV promoter (Vincentz and Caboche, 1991); d (del), ΔNR transformants.

abolishes this regulation in C1 plants expressing the full-length NR chimeric transcript (Vincentz and Caboche, 1991). As shown in Figure 2A, this deregulation is also found in transgenic plants expressing ΔNR chimeric transcript under the control of the CaMV 35S promoter. The pool of NR mRNA varied by a factor of three between transformants del2 and del8, probably as a consequence of the different integration site of the chimeric gene in the genome (Sanders et al., 1987).

ΔNR proteins from the transformants were purified by chromatography on 5' AMP-Sepharose. Fractions containing NR activity were analyzed by SDS-PAGE and protein gel blotting using an anti–maize NR polyclonal antiserum. The ΔNR polypeptide in transformants del7 and del8 was 6 kD smaller than the 110-kD wild-type NR polypeptide (Figure 2B). This result agrees well with the predicted difference of 6.4 kD calculated for the sizes of the ΔNR and NR polypeptides.

Biochemical Properties of the ΔNR Protein

The estimated K_m value of the ΔNR protein for nitrate (300 μM) and the pH optimum (7.5) of the enzyme were not different from those of wild-type *N. plumbaginifolia* NR protein (data not shown). However, a difference in thermosensitivity was found between the NR and the ΔNR proteins when NR activity was assayed in vitro from ammonium sulfate–precipitated extracts. As shown in Figure 3A, the ΔNR protein lost its activity within 2 min of incubation at 30°C, whereas the wild-type NR control was still functional after 15 min at 30°C. The decrease in the assay temperature progressively reduced the difference, and at 15°C, both NR and ΔNR retained the same rate of activity for at least 40 min. This increased thermosensitivity of the ΔNR protein was not observed in the plant because the growth of ΔNR transgenic plants at 30°C for 3 hr had no effect on ΔNR protein activity (data not shown). To avoid the problem resulting from the thermosensitivity of the ΔNR protein, NR activity measurements during the following experiments were performed at 20°C.

The ΔNR transformants as well as the wild-type and C1 controls showed a good correlation between NR activity of total protein from leaf tissues, measured in crude extracts at 20°C, and NR protein levels (Figure 3B), measured by an ELISA test on an extract precipitated with 45% ammonium sulfate. The partial dehydrogenase activities of NR, such as NADH:cytochrome c reductase activity used to detect the functionality of NR FAD and heme domains (Chérel et al., 1990), were measured on the ammonium sulfate precipitate (Figure 4C) and also correlated well in both cases. However, this was not the case for terminal NADH:NR activity measured on the ammonium sulfate precipitates. In this case, NADH:NR activity was systematically decreased by a factor of three in the ΔNR extracts when compared with the wild-type and C1 controls (Figure 3B). We assumed that this decrease resulted from the loss of MoCo from some of the ΔNR enzyme during ammonium sulfate precipitation. Subsequent NR activity measurements
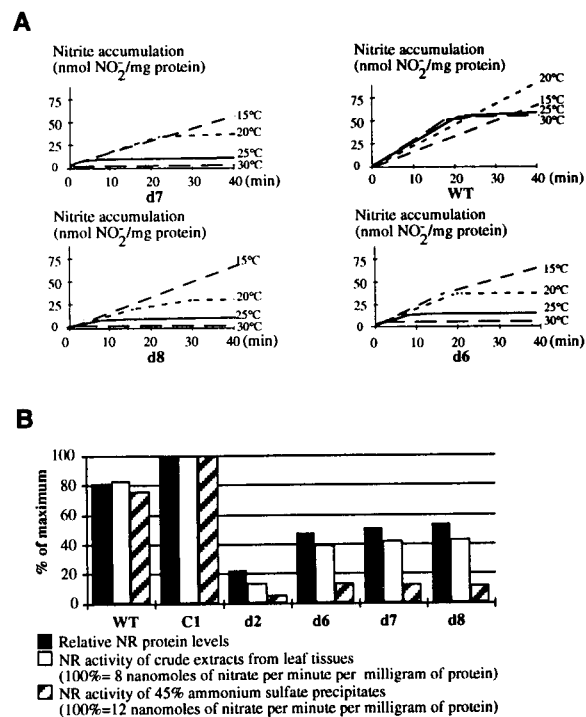
Figure 3. NR Activity.

(A) Thermosensitivity of NR activity observed during in vitro assay with ammonium sulfate precipitate extracts.

(B) NR activity and protein levels at the middle of the light period (+4 hr). NR activity was assayed during 10 min at 20°C. Abbreviations are as given in the legend to Figure 2.

were therefore performed on ammonium sulfate precipitate by assaying NADH:cytochrome c reductase activity.

The Level of ΔNR Protein in Transgenic Plants Is Not Affected after 70 Hr in the Dark

Previous experiments have demonstrated post-transcriptional regulation of the NR gene by light (Remmler and Campbell, 1986; Vincentz and Caboche, 1991). To study the effect of light on the expression of ΔNR, wild-type plants and plants transformed with chimeric genes (35S–NR and 35S–ΔNR) were kept in the dark for 70 hr and then transferred into white light. NR transcript, activity, and protein levels were compared.

In wild-type plants at the time points indicated in Figure 4A, NR mRNA decreased to undetectable levels after 70 hr of darkness and reaccumulated significantly within 4 hr of illumination, as shown in Figure 4B. In transgenic plants (C1, del2, del6, del7, and del8), in which transcription of the NR or ΔNR gene is driven by the CaMV 35S promoter, mRNA was still detectable under the same conditions. The lower level of NR transcript observed in dark-treated C1 plants is particular to this RNA

gel blot, as shown by the quantification of the mean of two independent experiments. Vincentz and Caboche (1991) have demonstrated with the same C1 line (and with other 35S–NR transformants) a higher accumulation of the transcript that is unaffected by such dark treatment. The absence of the NR transcript in dark-treated wild-type plants agrees with previous results (Deng et al., 1990; Vincentz and Caboche, 1991), indicating that light plays a role in transcriptional regulation of the NR gene. The control was performed by reprobing the RNA gel blot with a probe encoding the β subunit of the mitochondrial ATPase. The pool of this transcript declined in the dark (Figure 4B), although to a much lesser degree than did the pool of the wild-type NR transcript. As mentioned by others

(Vincentz and Caboche, 1991), this might reflect a general decline of transcriptional activity due to a lower metabolic state of the plant after 70 hr of darkness. In addition to its transcriptional stimulating effect, light regulates the translation and/or the stability of the NR protein (Vincentz and Caboche, 1991), which was observed with different 35S–NR transgenic plants. Despite the constant high-level production of NR mRNA, NR activity and protein declined to 10% of the control levels under light after a period of 60 hr of darkness (Vincentz and Caboche, 1991; Figure 4C).

In the various ΔNR transformants after 70 hr of darkness, ΔNR activity (NADH:cytochrome c reductase activity measured on the ammonium sulfate precipitate) and NR protein levels did not exhibit the same dramatic decrease observed in the wild-type and C1 controls (Figure 4C). This indicates that ΔNR transformants lost post-transcriptional control of NR activity by light and continued to accumulate ΔNR protein in the dark. The higher level of mRNA observed in transgenic plants after 4 hr of illumination is not correlated with higher ΔNR protein levels and suggests a limitation in the translation of ΔNR mRNA or an effect on protein stability.
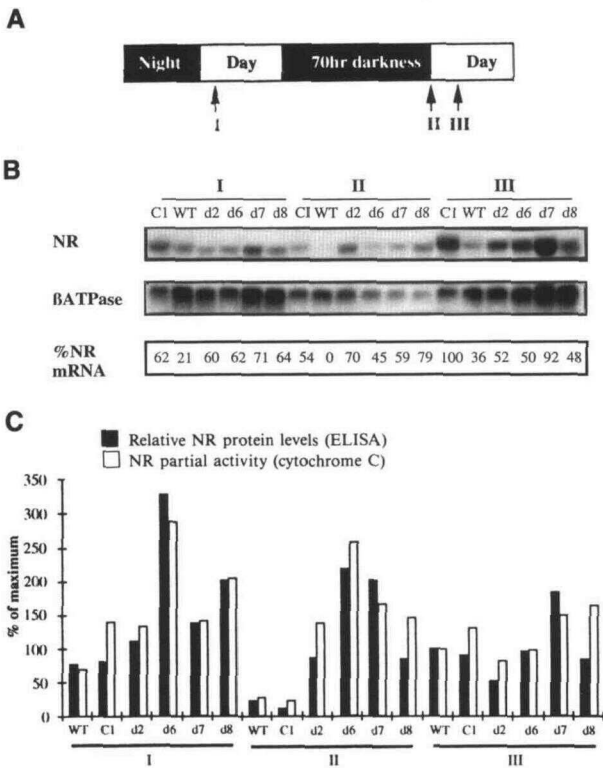
Figure 4. Effect of Light on the Expression of the Chimeric ΔNR Gene.

(A) Leaves were harvested at the beginning of a normal day/night cycle (I), after 70 hr of darkness (II), and following a dark period after 4 hr of white light illumination (III).
(B) Total RNAs (7 μg) were analyzed by RNA gel blotting as described in Figure 2. The same blot was used for hybridization with the two different probes (NR and ATPase β subunit). The percentage of NR mRNA after scanning data collection was standardized with respect to ATPase β subunit mRNA: the mean of two replicates is given here.
(C) Analyses of NR protein levels and partial (cytochrome c) and total NR activity were performed with ammonium sulfate–precipitated extracts as described in Methods.
Abbreviations are as given in the legend to Figure 2.

In Vivo Regulation of NR Activity by Light

A well-characterized post-transcriptional regulation of NR activity by light acts at the protein level. It has been shown that NR activity is modulated in vivo by light. This inactivated state of NR can be visualized only in a buffer with Mg^{2+}, and the addition of EDTA reverses this inactivation (Kaiser and Brendle-Behnisch, 1991). Such a buffer was used to study the effect of light on ΔNR activity (see Methods). Relative NR activity can also be stated as a ratio between NR activity measured with (fully activated) and without (inactivated) EDTA.

In vivo inactivation of NR activity was investigated by placing the plants in the dark for 30 min. When relative activity was measured for crude extracts, it was found that the controls (wild-type and C1) had an inactivation rate of 50 to 60%, respectively, as shown in Figure 5. This difference between light and dark relative NR activity was completely absent in ΔNR plant extracts.

Another significant finding was the high level of NR in an inactivated state (50%) in light-treated leaves of C1 and wild type. Because these plants were grown in a culture chamber with a light intensity of 230 μmol m^{-2} sec^{-1}, this result probably reflects a partially inactivated state of NR protein due to a limiting light intensity. The transgenic ΔNR plants displayed a different reaction because only 20 to 25% of ΔNR protein activity was in an inactivated state during the light period.

Although NR activity can vary significantly from experiment to experiment, the proportion of active NR could be accurately measured. This is demonstrated by the constant values of the relative NR activity between two experiments, as indicated by the small standard errors.
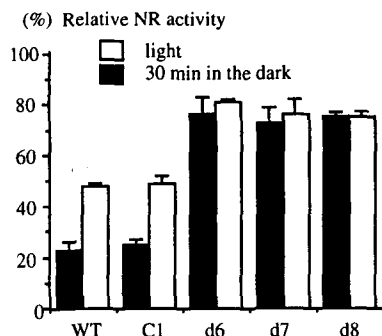
Figure 5. Effect of Light on NADH:NR Activity in Crude Leaf Extracts.

Extraction was performed in buffer A (50 mM Hepes-KOH, pH 7.6, 10 mM $MgCl_2$, 5 μM FAD, 1 μM leupeptin, 1 mM DTT), and the assays were performed in buffer B (50 mM Hepes-KOH, pH 7.6, 10 mM $MgCl_2$, 140 μM NADH, 5 mM nitrate). Relative NR activity is expressed as the ratio of NR measured with or without 15 mM EDTA. The means and the standard errors of three replicates are given. NR activity values for C1 and wild-type controls ranged from 1.1 to 5.2 nmol of nitrite per min per mg of protein during experiments, and similar variations were obtained with ΔNR activity data. This could be explained by the small size of the samples (1 g) and did not affect the relative NR activity, as demonstrated by the low standard deviation values. NR activity was assayed during 10 min at 20°C. Due to low NR activity, transformant del2 was not used for these experiments. Abbreviations are as given in the legend to Figure 2.

In Vitro Inactivation of the NADH:NR Activity by Preincubation with Mg-ATP

Kaiser and Spill (1991) have demonstrated that Mg-ATP causes a stable inhibition of NR in vitro that has all the characteristics of in vivo inactivation. In vitro inactivation of NR activity by Mg-ATP was investigated for each genotype. The transgenic ΔNR plants in this experiment exhibited a very different behavior when compared with C1 and wild-type controls. Indeed, preincubation with ATP inactivated only 16 to 24% of ΔNR activity, whereas controls lost between 60 and 65% of NR activity, as shown in Figure 6A.

This inactivation process of NR by preincubation with ATP has been described as strictly dependent on the presence of Mg^{2+} (Kaiser and Spill, 1991). As expected, the addition of 15 mM EDTA during the measurement of NR activity counteracted the majority of the inactivation (Figure 6B). Only slight discrepancies (7 to 15%) between samples preincubated with or without ATP were observed, and these differences were similar in controls and ΔNR extracts.

Nitrogen-Containing Metabolites Accumulate at Night in Transgenic Plants

The accumulation of nitrogen-containing metabolites in ΔNR transgenic plants was studied to evaluate the consequences of the absence of post-transcriptional regulation by light. The

transgenic ΔNR plants and the controls (wild type and C1) were grown to the rosette stage in controlled culture conditions with 8-hr days and 16-hr nights. Leaves from several plants were harvested at the beginning (0) and the end (plus 8 hr) of the light period for analysis.

As shown in Figure 7, important differences were observed at the beginning of the day. Analysis of the amino acid pools indicated an increased accumulation, by a factor of two to four, of asparagine and glutamine in transformants del6, del7, and del8, compared with wild-type or C1 controls that displayed very similar amino acid profiles. This accumulation was linked to a decrease in glutamic acid pools because glutamine and glutamic acid still represented on average 50% of the total amino acids for all plants. This suggests a limitation on the flow of this pathway by glutamate synthase or by the availability of α-ketoglutaric acids (carbon backbones) in the dark.
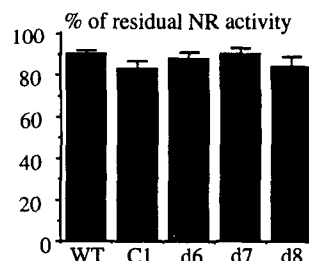
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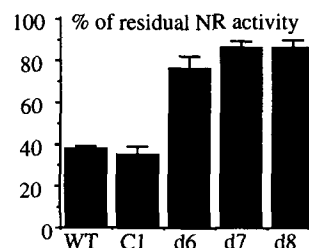
B

Figure 6. Effect of Mg-ATP Preincubation on in Vitro Modulation of the NADH:NR Activity in Desalted Crude Leaf Extract.

Leaf extracts were desalted with buffer A and assayed for NR activity with buffer B as described in the legend to Figure 5.

(A) Ratio of NR activity tested in buffer B measured after a 20-min preincubation in buffer A with or without 2 mM Mg-ATP.

(B) Ratio of NR activity measured in buffer B in the presence of 15 mM EDTA after a 20-min preincubation in buffer A, with or without 2 mM Mg-ATP.

NR activity present (nanomoles of nitrite per minute per milligram of protein) measured in buffer B in the presence of 15 mM EDTA and without preincubation with Mg-ATP was 3.7 for the wild type, 1.9 for C1, 5.6 for del8, and 5.9 for del6 and del7. NR activity was assayed during 10 min at 20°C. The means and the standard errors of three replicates are given. Abbreviations are as given in the legend to Figure 2.

A

A.A.	nanomoles per milligram of dry weight					
	WT	C1	del2	del6	del7	del8
0hr Asp	14.7 (2)	15.8 (2)	6.8 (1.1)	14 (0.6)	18.3 (0.9)	19.3 (2.3)
Asn	0.6 (0.1)	1 (0.5)	0.3 (0.1)	2.9 (0.2)	2.1 (0.6)	2 (0.5)
Glu	27.7 (1.1)	28.6 (2.1)	24.5 (3.3)	27.1 (1.7)	28.8 (0.5)	29.8 (2.7)
Gln	3.6 (0.5)	6.6 (2.2)	1.7 (0.2)	21.2 (1.2)	15.1 (2.6)	19.4 (3.8)
tAA	60.2 (1.1)	70.5 (7.9)	46.6 (3.5)	77.1 (3)	83.4 (3.2)	90.3 (6)
+8hr Asp	13.2 (1.8)	13.3 (1.7)	5.2 (0.5)	9.1 (1.6)	12.5 (2.3)	12.9 (1.7)
Asn	1.2 (0.1)	1.4 (0.2)	0.6 (0.1)	1.8 (0.7)	1.8 (0.2)	2.3 (0.3)
Glu	19.5 (2.2)	18.9 (2.8)	22.6 (1.6)	20.4 (0.6)	19.7 (1.3)	19 (1.5)
Gln	23.2 (2.4)	29.7 (9.4)	3.7 (0.9)	18.5 (5)	24.6 (2.8)	29.3 (2.7)
tAA	86.9 (6.1)	92.1 (10.5)	52.1 (1.1)	78.5 (7.6)	89.7 (5.7)	95 (1.8)
	% total amino acid					
0hr Asp	24.4	22.4	14.6	18.1	21.9	21.3
Asn	0.9	1.4	0.6	3.8	2.5	2.2
Glu	46	40.5	52	35.1	34.5	33
Gln	5.9	9.4	3.6	27.5	18.1	21.5
tAA	100	100	100	100	100	100
+8hr Asp	15.2	14.4	10	11.6	13.9	13.6
Asn	1.4	1.5	1.1	2.3	2	2.4
Glu	22.4	20.5	43	26	21.9	20
Gln	26.7	32.2	7.1	23.6	27.4	30.8
tAA	100	100	100	100	100	100

B

Ammonium content (nanomoles per milligram of dry weight)					
WT	C1	del2	del6	del7	del8
0 hr 7.8 (1.2)	9.2 (2.1)	5.9 (0.1)	16.4 (6.3)	11.7 (2)	10.9 (1.7)
+8 hr 25.1 (2.4)	25.9 (2.7)	13.3 (1.3)	31.7 (6.5)	21.4 (0.9)	21.3 (1.1)

Figure 7. Ammonium and Amino Acid Content of Controls and del2, del6, del7, and del8 Plants.

Analysis was performed on leaves of plants grown under short-day periods at the beginning of the light period (0 hr) and the end of the day (+8 hr).

(A) Variations in the levels of amino acids (A.A.) Asn, Asp, Glu, and Gln. Measurements are expressed as absolute values and as a percentage of the total amino acid content.

(B) Variations in the level of ammonium. Note the accumulation of Asn, Gln, and ammonium in ΔNR transformants harvested at the end of the night period.

Abbreviations are as given in the legend to Figure 2. Data represent the mean of three experiments; standard error is indicated within parentheses.
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Slightly higher levels of ammonium were also observed in these transformants.

These differences were most pronounced in the dark and decreased during the day. At the end of the day, relatively more glutamine was accumulated in wild-type and C1 controls (by a factor of three) compared with del6, del7, and del8 (by factors of one and 1.5). The total level of amino acids remained high in transgenic ΔNR plants, whereas controls exhibited a 30% fluctuation with a lower content at the beginning of the day. Transformant del2 did not behave like the controls or other ΔNR transgenic plants (Figures 7A and 7B). del2 plants had a very low level of glutamine (30 to 50% of the wild-type level), asparagine (60 to 70% of the wild-type level), and ammonium (50% of the wild-type level) over the entire day. This result is explained by the fact that in this transformant, the low level of expression of the transgene leads to a limitation of nitrate

reduction and subsequent amino acid synthesis, as previously observed for leaky NR-deficient mutants.

DISCUSSION

Complementation of NR Deficiency by the Chimeric ΔNR Gene

Transformation of the *nia* E23 mutant of *N. plumbaginifolia* with the ΔNR gene under the control of the CaMV 35S promoter restored NR activity. In the R_1 progeny of primary transformants, NR activity was found to be directly correlated to the ability to grow on nitrate. For example, transformant del2, which grew poorly in the greenhouse and occasionally displayed chlorotic leaves, exhibited the lowest level of NR mRNA and activity among transformants. This agrees with previous results (Vincentz and Caboche, 1991) obtained with transgenic E23 plants expressing the full-length *nia2* NR cDNA under the control of the CaMV 35S promoter. The 168 nucleotides that were removed and that represent 72% of the N-terminal domain of a *nia2*-derived NR cDNA are not required for the functionality of the NR protein. This is in accordance with the existence of NR without this N-terminal domain in fungi, such as *U. maydis* (Figure 1).

Role of the Deleted Region in the in Vitro Stability of the Protein

The ΔNR protein is less stable than the NR protein in vitro, as was demonstrated by its higher thermosensitivity and the significant loss of NADH:NR activity following ammonium sulfate precipitation from ΔNR transgenic plant protein extracts. The FAD and heme domains were not affected by this instability of the ΔNR protein because NADH:cytochrome *c* reductase activity was not changed (Figure 4C). The complementation by exogenous MoCo of ΔNR produced in yeast compared with NR protein was also very poor (1 to 2%). Taken together, these observations suggest that the MoCo domain is responsible for the instability of the ΔNR protein in ammonium sulfate precipitates, probably due to unstable MoCo binding.

This effect was not detected in vivo; similar to ΔNR transgenic plants, there was a good correlation between NADH:NR activity in crude leaf extracts and NR protein levels measured by ELISA on ammonium sulfate–precipitated extracts (Figure 3B). This might be due to the presence of plant factors that facilitate in vivo the integration of the MoCo into the NR protein. During precipitation with ammonium sulfate, such factors would be lost or nonfunctional, whereas in the plant they would help to maintain the ΔNR in an active form with a dynamic equilibrium between MoCo loss and integration. We are currently exploring the possibility of identifying such factors.

The ΔNR Gene Is Not Regulated Post-Transcriptionally by Light

Post-transcriptional regulation by light was abolished in ΔNR transgenic plants; the level of ΔNR protein was only slightly affected by 70 hr of darkness, whereas controls showed a sharp decrease in the level of NR protein. This might have been due to the absence of ΔNR degradation in the dark or to the absence of repression of its translation. Turnover experiments are planned to distinguish these two possibilities.

In vivo, N. plumbaginifolia NR activity in C1 and wild-type plants exhibited the same rapid activation/inactivation modulation during light/dark transitions as has been described for other plant species, such as maize (Remmler and Campbell, 1986), spinach (Kaiser and Brendle-Behnisch, 1991), barley (de Cires et al., 1993), and pea (Kaiser et al., 1993). These changes require Mg^{2+} to promote the inhibition of NR in the dark. It should be noted that relative NR activity (C1 and the wild type) is quite low in the light (50% of the maximum activity measured with 15 mM EDTA and 60% of the ΔNR activity). This suggests that the activation/inactivation process is finely modulated by external conditions and that the low-light intensity in the culture chamber (230 μmol m^{-2} sec^{-1}) is not sufficient to activate the NR protein pool fully. Our results clearly show the complete loss of this regulation for ΔNR transgenic plants, as demonstrated in Figure 5, in which ΔNR relative activity remained constant during light/dark transitions. The accumulation of nitrogen-containing metabolites during the night observed only in ΔNR transgenic plants is also consistent with the deregulation of the nitrogen assimilatory pathway. One hypothesis would be that ΔNR protein remains active during the night and that these plants continue to assimilate nitrate. Our current experiments focus on demonstrating that nitrogen-containing metabolites result from nitrate assimilation and not from protein degradation or blockage of alternative uses of reduced nitrogen. If this is the case, it suggests that NR, rather than other enzymes of the pathway, is a major control point.

ΔNR Activity Is Not Modulated in Vitro by ATP, Suggesting an Absence of Regulatory Phosphorylation

Changes in NR activity in vivo are assumed to be mainly the result of covalent modification of the protein caused by phosphorylation/dephosphorylation of NR. This conclusion is supported by the following results. The same modulation of NR activity occurring in vivo can be obtained in vitro by preincubating desalted spinach leaf extracts with ATP, which deactivates NR, or AMP, which has an activating effect (Kaiser and Spill, 1991). Spill and Kaiser (1994) have recently partially purified two proteins (100 and 67 kD) that are involved in this ATP-dependent inactivation of spinach leaf NR. The artificial lowering of the ATP levels in leaf or root tissues by anaerobiosis (dark), mannose, or the uncoupler carbonyl cyanide

m-chlorophenylhydrazon fully activates NR (Kaiser et al., 1993). The addition of okadaic acid, calyculin, or microcystin LR, which are type 1 and type 2A phosphatase inhibitors, prevents the reactivation of NR in the light (Huber et al., 1992; MacKintosh, 1992; Kaiser and Huber, 1994b).

In vitro, NR activity in desalted extracts from leaves of C1 or wild-type plants was converted from an active to an inactive form as a result of 20 min of preincubation with Mg-ATP (Figure 6A). These results suggest that N. plumbaginifolia NR, like the spinach NR enzyme, is regulated by an ATP-dependent mechanism.

The ΔNR protein inactivation by preincubation with ATP was much lower (~20%). Two hypotheses might be formulated to explain the remaining 20% inactivation. First, Huber et al. (1992) have demonstrated in vivo phosphorylation of spinach NR for serine residues in leaves. By peptide mapping, they have demonstrated the phosphorylation of four peptides. The modification of the phosphorylation status of three of them (two major and one minor) was correlated with NR activity changes. In the dark, they were phosphorylated and NR activity was inhibited for a period; in the light, they were less phosphorylated and the enzyme was more active. One NR phosphopeptide was phosphorylated independent of the external conditions. Such constitutive phosphorylation might still exist for ΔNR and lower its activity. A second hypothesis is that EDTA could chelate ions, exercising an inhibitory effect on NR activity. The rate of in vitro inactivation by ATP is similar to the in vivo inactivation rate found during the dark/light transition. Therefore, ATP appears to no longer play a main role in the modulation of ΔNR activity.

Role of the ΔNR Deletion in the Loss of Post-Transcriptional Regulation of NR

One hypothesis for the loss of post-transcriptional regulation in ΔNR transgenic plants is the presence in the deleted region of a serine residue(s) that is a target of a specific kinase. Indeed, two conserved serines are found in positions +68 and +119 of NR (Figure 1), but there is only a slight chance for them to be involved in regulatory phosphorylation. The serine residue at position +68 is not present in one of the two Arabidopsis genes. We have determined that the Arabidopsis nia mutant B29 (Wilkinson and Crawford, 1991), which expresses only this gene, still exhibits NR activity modulation during light/dark transitions (data not shown). The serine residue at position +119 is not present in the barley nar1 gene (Figure 1). This gene is responsible for the production of barley NR in leaves, which also exhibit modulation of NR activity in response to light or CO_2 changes (de Cires et al., 1993).

Two other explanations (not mutually exclusive) may also be proposed. (1) The presence of Mg^{2+} (or other divalent cations such as Ca^{2+}) is required to initiate NR inactivation. The acidic domain might be a region in which they bind NR, and its deletion would prevent inactivation of the ΔNR protein. (2) The deletion promotes tertiary structure changes that prevent

the recognition of the serine by the kinase. This last hypothesis is supported by the following data: biochemical and molecular experiments have localized the probable phosphorylation site to the MoCo domain (Huber et al., 1992; LaBrie and Crawford, 1994), and the deletion affects the binding of MoCo.

Hamano et al. (1984) have described a thiol protease that inactivates barley leaf NR. If such a protease recognized only the phosphorylated form of NR, a model might be proposed for degradation of inactivated NR. Such a hypothesis offers an explanation for the lack of degradation of ΔNR after a long dark period: ΔNR would not be specifically phosphorylated in the dark and could no longer be degraded by a specific protease. It would also explain why the NR protein is very stable in yeast extracts, in which such a selective degradation machinery is most likely not present.

METHODS

Plasmid Constructs

Construction of the Chimeric Nitrate Reductase Deleted Gene

Standard procedures were used for recombinant DNA manipulations (Maniatis et al., 1982). Oligonucleotides were prepared on an Applied Biosystems DNA synthesizer (Foster City, CA). The bacterial strain used was XL1-Blue (Bullock et al., 1987). Enzymes were used according to the supplier's recommendations.

An internal deletion in the complete nitrate reductase cDNA (carried by pCSL16; Vincentz and Caboche, 1991) corresponding to the nia2 nitrate reductase (NR) structural gene of Nicotiana tabacum (Vaucheret et al., 1989) was constructed in several steps. First, we amplified a 0.26-kb fragment by polymerase chain reaction (this fragment corresponded to the 5′ part of the NR cDNA), using pCSL16 as template with oligonucleotides D2 (5′-GGGGAATTCGACCGGGATAAACC-3′) and the universal M13 primer. D2 hybridizes with bases 46 to 60, following the ATG and introduces an EcoRI site at its 3′ end, which modifies the DNA sequence but not the amino acid codon. The amplified fragment contains 60 bp of the 5′ end of the NR cDNA, 138 bp of untranslated 5′ sequence (leader) with an SstI site at its 5′ end (Vincentz and Caboche, 1991), and 55 bp of the pBluescript M13+ phagemid vector (Stratagene). After EcoRI and SstI digestion, which released the 5′ portion of the NR cDNA and the leader, the fragment was cloned into the EcoRI-SstI sites of pBluescript M13+. A clone was verified by DNA sequencing and named pBD3.

A 4.5-kb NR DNA SalI-SstI fragment was gel purified from pCSL16. A partial EcoRI digest of this fragment released the 4.3-kb 3′ end of the NR cDNA (the EcoRI site is located 228 bp downstream of the ATG). This EcoRI-SalI fragment was ligated to the same sites of pBD3. This created pΔNR, which carries a 4.35-kb NR DNA fragment with an internal deletion of 168 bp at the 5′ end.

To construct a binary vector expressing ΔNR, the complete ΔNR sequence from pΔNR was gel purified as an SstI (blunt ended)-SalI fragment and cloned into the KpnI (blunt ended) and SalI sites of the plant transformation vector pBinDH51 (Vincentz and Caboche, 1991). This put the ΔNR cDNA under the control of the cauliflower mosaic virus 35S promoter and created the plasmid pBΔNR.

Construction of a Yeast Vector Expressing ΔNR

To produce ΔNR in yeast, we used the yeast galactose-inducible expression vector pKV482 (Delta Biotechnology, Nottingham, UK). We inserted the 2.65-kb blunt-ended SstI-PstI fragment of the ΔNR cDNA from pΔNR into the unique blunt-ended BglII site of pKV482. This created pYΔNR, in which the ΔNR coding sequence and the 138-nucleotides long 5′ untranslated region are under control of a modified phosphoglycerate kinase promoter. The normal upstream activating sequence was replaced by the gal4-dependent upstream activating sequence, leading to a galactose-inducible production of ΔNR. Transformation and expression of pYΔNR in yeast, protein extraction, and complementation of yeast extract with the molybdenum cofactor from xanthine oxidase were performed as described by Truong et al. (1991).

Plant Transformation, Regeneration, and Growth

The recombinant plasmid pBΔNR in Escherichia coli XL1-Blue was mobilized into Agrobacterium tumefaciens LBA4404 as described by Bevan (1984). The NR-deficient mutant E23 of N. plumbaginifolia was transformed as described previously (Vaucheret et al., 1990). Transformants were selected on 50 mg/L kanamycin and grown on B medium without nitrogen compounds (B-N), which was supplemented with 10 mM potassium nitrate as the sole nitrogen source, as previously described (Vincentz et Caboche, 1991). Plants able to utilize nitrate were then grown in the greenhouse and are referred to as primary transformants. Genetic analysis of the progeny obtained from selfing primary transformants (R_1 generation) was done on B-N medium supplemented either with 10 mM ammonium succinate and 50 mg/L kanamycin or with 10 mM potassium nitrate.

For physiological studies, plants were grown to the rosette stage in a controlled growth chamber under a short-day cycle. Culture conditions were 8 hr at 22°C with a light intensity of 230 μmol m^{-2} sec^{-1} (fluorescent lamps) and 16 hr of dark at 17°C.

Protein Extraction and Analysis

Extraction of total leaf protein, NR activity assays, and estimation of NR protein levels (ELISA) were performed as described by Chérel et al. (1986) and Galangau et al. (1988). NR activity is expressed as nanomoles of nitrite produced per minute per milligram of protein. Partial NR enzymatic activity (cytochrome c activity) was measured according to a previously described procedure (Chérel et al., 1990). For protein gel blot analysis, NR from 10 g of leaf tissue was purified on a 1-mL 5′ AMP-Sepharose (Pharmacia) microcolumn as described previously (Moureaux et al., 1989). Proteins were transferred to polyvinylidene difluoride 0.45-μm Immobilon-P membranes (Millipore, Bedford, MA) using a Millipore electrotransfer system. Immunodetection was performed with a polyclonal antibody raised against maize NR (Chérel et al., 1986) and a mouse anti–rabbit IgG linked to alkaline phosphatase. The K_m value for nitrate was calculated by measuring NADH:NR activity for 5 min with nitrate concentrations of 0, 0.5, 1, 2.5, 5, and 10 mM and using an enzyme kinetics program (D.G. Gilbert, Indiana University).

Amino Acid Analysis

A 3-g sample of leaf tissue was dried, and an aliquot of 100 mg was agitated for 1 hr in 5 mL of 96% ethanol at 5°C. After centrifugation

for 10 min at 27,000g, the ethanol fraction was saved. The process was repeated with 5 mL of 80 and 60% ethanol and finally with water. The four ethanol–water fractions were combined and stored at −80°C for analysis.

Amino acids and free ammonium were determined on the combined ethanol–water extracts. They were separated by ion exchange chromatography (Biotronik LC 5001 analyzer, Maintel, Germany; lithium citrate buffers; and ninhydrin postcolumn derivatization), identified using a mixture of amino acids (Benson standard P-ANB, Reno, NV), and quantified using the P.E. Nelson 2100 software (Perkin-Elmer). Three independent experiments were performed, and each measurement was made in duplicate.

In Vitro and in Vivo Modulation of NR Activity

To study the modulation of NR activity by darkness or Mg^{2+}, the specific conditions of extraction and activity assays were essentially as described by Kaiser and Huber (1994b). Approximately 1 g of leaf tissue was harvested 2 hr after the beginning of the day period (light sample) or 30 min after the subsequent dark period (dark sample) and ground in liquid nitrogen. Preliminary experiments determined that NR in wild-type N. plumbaginifolia is rapidly inactivated in darkened plants. The maximum state of inactivation was obtained after a 30-min dark period (E. Pigaglio, personal communication). The leaf powder was extracted in 4 mL of extraction buffer A (50 mM Hepes-KOH, pH 7.6, 10 mM $MgCl_2$, 5 µM flavin adenine dinucleotide, 1 µM leupeptin, 1 mM DTT). The entire procedure was performed at 0 to 4°C.

Enzyme activity was measured at room temperature in buffer B (50 mM Hepes-KOH, pH 7.6, 10 mM $MgCl_2$, 140 µM NADH, 5 mM nitrate). NR activity is expressed as nanomoles of nitrite produced per minute per milligram of protein.

To study in vitro modulation of NR by Mg^{2+}, leaf extracts (light sample) were immediately desalted on Pharmacia PD-10 columns equilibrated with extraction buffer A. Desalted leaf extracts were incubated at room temperature with effectors added as indicated in the legend to Figure 6. From preliminary experiments, we determined that a preincubation time of 20 min with 2 mM ATP gave the maximum rate of NR inactivation (E. Pigaglio, personal communication). NR activity assays were performed as described previously with buffer B. All experiments were performed at least in duplicate. Standard error was calculated using arc sine transformation as required for the treatment of percentage data (Sokal and Rohlf, 1981).

DNA and RNA Extraction and Gel Blot Analyses

Total DNA and RNA isolation and gel blot analyses were performed as described previously (Vincentz and Caboche, 1991). The probes used were the 1.6-kb internal EcoRI tobacco nia2 cDNA fragment for NR (Vaucheret et al., 1989) and the 1.6-kb EcoRI-SalI cDNA fragment for the nuclear-encoded β subunit of the mitochondrial ATPase from N. plumbaginifolia (Boutry and Chua, 1985). Quantification of the signals from the hybridized filter was performed with a PhosphorImager (Molecular Dynamics Inc., Sunnyvale, CA).

ACKNOWLEDGMENTS

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