

Constitutive expression of nitrate reductase allows normal growth and development of *Nicotiana plumbaginifolia* plants

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A nitrate reductase (NR) deficient mutant of *Nicotiana plumbaginifolia* totally impaired in the production of NR transcript and protein was restored for NR activity by transformation with a chimaeric NR gene. This gene was composed of a full-length tobacco NR cDNA fused to the CaMV 35S promoter and to termination signals from the tobacco NR gene. The transgenic plants we obtained were viable and fertile and expressed from one-fifth to three times the wild-type NR activity in their leaves. The analysis of chimeric NR gene expression in these plants showed, by comparison with wild-type plants, that the regulation of NR gene expression by light, nitrate and circadian rhythm takes place at the transcriptional level. However, unlike nitrate, light was required for the accumulation of NR protein in transgenic plants, suggesting that NR expression is also controlled at the translational and/or post-translational level.

Key words: 35S CaMV/gene regulation/nitrate reductase/nitrite reductase/transgenic *Nicotiana plumbaginifolia*

Introduction

Nitrate is the major nitrogen source for higher plants. Two successive enzymatic steps reduce nitrate to ammonium, generally in the leaves. First, nitrate is converted into nitrite in a two electron transfer reaction catalysed by nitrate reductase (NR, EC1.6.6.1), a cytoplasmic enzyme. Nitrite is then translocated to the chloroplast, where it is reduced by nitrite reductase (NiR, EC 1.7.7.1) to ammonium in a six electron transfer reaction (for reviews see Wray, 1988; Solomonson and Barber, 1990). Ammonium is subsequently incorporated into the amino acid pool mainly through glutamine and glutamate biosynthesis (Mifflin and Lea, 1977). The source of reductant for nitrate reduction is essentially generated from photosynthesis (House and Anderson, 1980).

Nitrate assimilation is an energetically expensive process which is finely regulated. In fungi, the synthesis of NR and NiR enzymes requires the simultaneous lifting of nitrogen repression (glutamine repression) and nitrate induction. These controls are mediated by two positively acting regulatory genes (for review see Schazzochio and Arst, 1989) the products of which probably act at the transcription level (Kudla *et al.*, 1990). In higher plants, NR activity and protein were shown to be regulated by several factors, among which the most important are light and the type of nitrogen source (Campbell, 1988). These controls appear to be achieved through the regulation of NR mRNA accumulation (for

review see Caboche and Rouzé, 1990). A high level of NR gene expression requires both nitrate (Galangau *et al.*, 1987; Crawford *et al.*, 1988) and light (Deng *et al.*, 1990). In etiolated squash cotyledons, NR expression is under phytochrome control (Rajasekhar *et al.*, 1988). In NR-deficient *Nicotiana plumbaginifolia* mutants, the NR mRNA is overexpressed (Pouteau *et al.*, 1989), suggesting that a nitrogen metabolite such as ammonium (Curtis and Smarelli, 1987) or glutamine (M.Deng, personal communication) down-regulates the gene in the wild-type. Finally, in tobacco and tomato the pool of NR transcript fluctuates over a 24 h dark–light cycle, with a maximum at the beginning of the day period and a minimum at the end of this period (Galangau *et al.*, 1988). These fluctuations appear to be controlled by an endogenous clock (Deng *et al.*, 1990). The NiR gene seems to be coregulated with the NR gene by light and nitrate, although the NiR mRNA cycles much less during a day–night period (Back *et al.*, 1988 and Faure, J.D., Vincentz, M. and Caboche, M. in preparation). The molecular basis of these controls (transcription and/or mRNA stability) are still largely unknown.

In order to evaluate the relative importance of transcriptional and post-transcriptional regulation on the expression of the NR gene, we have analysed the expression of a constitutive chimaeric NR gene in transgenic plants. This gene was designed to be transcribed into a native NR mRNA from a heterologous promoter in order to bypass the normal transcriptional regulation of a wild-type NR allele. To this end, a full-length tobacco NR cDNA was fused to the CaMV 35S promoter (Benfey *et al.*, 1989) and to poly(A) signals of the tobacco NR gene. This construct was introduced into the mutant E23 of *N.plumbaginifolia*, affected in the NR structural gene (*nia*), and which is impaired in the production of NR transcript and protein (Pouteau *et al.*, 1989). In transgenic plants, detectable NR transcript and protein must derive from the transgene, simplifying the study of its regulation. We describe here the production of E23 mutants restored for NR activity by the chimaeric NR gene. The analysis of these plants showed that most of the regulation of wild-type NR expression is under transcriptional control, but that light also affects the accumulation of the NR protein.

Results

Construction of a NR gene fused to the 35S CaMV promoter

The chimaeric NR gene construct used in this study is presented in Figure 1. A 2.8 kb cDNA covering the coding sequence plus the leader sequence (138 bp) from the NR structural gene *nia2* of tobacco (Vaucheret *et al.*, 1989a) was linked to 1.5 kb of 3' non-coding sequence of the same *nia2* gene, to generate a promoter-less NR gene (pCSL16). This gene was then fused to the 35S promoter of CaMV in the plant transformation vector pBinDH51 (see Materials and

methods), which carries a kanamycin resistance gene as a selectable marker, to create pBCSL16. This construct was electroporated into mesophyll protoplasts of the *nia* mutant NIA30 from tobacco and found to lead to the transient expression of NR activity (see Materials and methods), thus suggesting that the construct was functional.

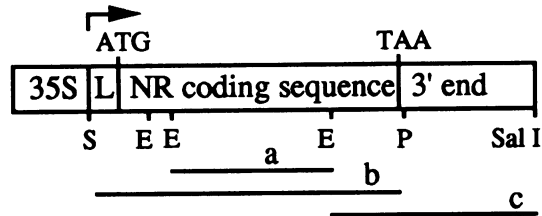


Fig. 1. Structure of the chimaeric NR gene. The CaMV 35S promoter (+528; +3) was derived from the expression cassette of pDH51 (Pietrzak *et al.*, 1986). The 3' end is from the *nia2* NR structural gene from tobacco. The arrow indicates the start of transcription. L = 5' untranslated leader sequence of the *nia2* tobacco gene. Fragments a, b, c were used as the probes for Southern analysis, Northern analysis and as the template for RNA antisense probe synthesis for 3' end mapping, respectively. Restriction sites are: E = *EcoRI*, S = *SstI*, P = *PstI*.

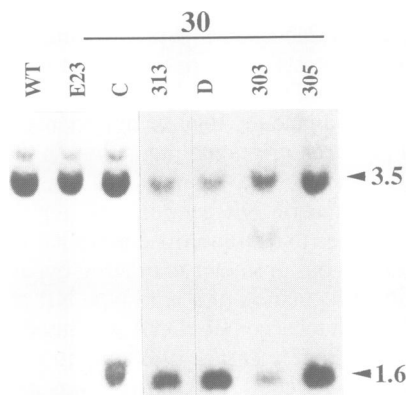


Fig. 2. Southern blot analysis of primary transformants. Total DNA (10 μ g) from five independent primary transformants was digested with *EcoRI* and hybridized to a probe which covers an internal 1.6 kb *EcoRI* fragment of the NR coding sequence (probe a in Figure 1). The 3.5 kb band represents the resident NR gene, the 1.6 kb fragment derives from the chimaeric NR gene. WT = wild-type *N.plumbaginifolia*; E23 = NR deficient mutant E23 of *N.plumbaginifolia*.

The E23 NR-deficient mutant of *Nicotiana plumbaginifolia* can be complemented for NR activity by the chimaeric NR gene

The NR-deficient mutant of *N.plumbaginifolia* E23 does not produce any full-length NR mRNA due to the insertion of a retrotransposon in the first exon of the *nia* gene (C.Meyer, personal communication). Therefore, it cannot grow on nitrate, but can still utilize ammonium, the end product of the nitrate assimilation pathway. This mutant was transformed with the chimaeric NR gene via *Agrobacterium*-mediated gene transfer. A first screen was performed on kanamycin, on a medium containing ammonium, so that no selective pressure was applied for the recovery of the chimaeric NR gene. Selected kanamycin resistant transformants were regenerated and then tested for the ability to grow and develop into plantlets on a medium containing nitrate as the sole nitrogen source. In one experiment, of 16 kanamycin resistant clones, 14 could grow on nitrate. Five representative primary transformants (30.C, 30.303, 30.305, 30.D, 30.313) were analysed in detail. The presence of a 1.6 kb DNA fragment internal to the cDNA (fragment a in Figure 1) was detected by Southern blot analysis of an *EcoRI* digestion of their genomic DNA (Figure 2), confirming the presence of the chimaeric gene.

The selfed progeny of the primary transformants (R1 progeny) was scored for the transmission of the kanamycin resistance marker and the ability to grow on nitrate. The results of the progeny tests are presented in Table I. Both characters were stably transmitted to the progeny and behaved as dominant Mendelian markers. The two markers, as expected, cosegregated in the progeny of transformed plants. Plants 30.C, 30.303 and 30.305 were found to carry a single active locus whereas plants 30.D and 30.313 carried at least two active loci. An example of segregation for growth on nitrate is shown in Figure 3A. It can be seen that the growth and the phenotype of the R1 progeny of clone 30.C are comparable to that of control wild-type plants, *in vitro* (Figure 3A) and in the greenhouse (Figure 3B) as opposed to the mutant E23 which does not survive on nitrate supplemented medium *in vitro* (Figure 3A). Efficient complementation for NR activity was therefore achieved by the introduced chimaeric NR gene.

Analysis of NR protein and RNA in leaves of transgenic progeny

R1 complemented transgenic plants were grown to the 'rosette' stage (Figure 3B) and leaves from several plants were harvested at the beginning of the light period for protein and RNA extraction. The analysis of transgenic plants was

Table I. Segregation analysis of Km resistance and growth on NO_3^- in the R1 progeny

	NH_4^+ - Km 100				NO_3^- - Km20				NO_3^-				Loci
	+	-	R	χ^2	+	-	R	χ^2	+	-	R	χ^2	
30.C	240	83	2.9	0.06	219	66	3.3	0.46	185	63	2.9	0.02	1
30.303	348	76	4.5	11 ^a	209	42	5.0	9.1 ^a	130	41	3.2	0.12	1
30.305	278	75	3.7	2.5	188	68	2.8	0.33	276	97	2.8	0.22	1
30.313	96	6	16.0	0.04	160	8	20.0	0.43	112	14	8.0	1.29	2
30.D	150	14	10.7	1.7	212	10	21.0	1.18	343	17	20.0	1.2	2

Seeds obtained by selfing the primary transformants (R1) were germinated on the different selective media: ammonium succinate (10 mM) and kanamycin (100 mg/l) (NH_4^+ - Km 100); nitrate (10 mM) and kanamycin (20 mg/l) (NO_3^- - Km 20); nitrate (10 mM) (NO_3^-). For clone 30.313, Km on NH_4^+ was 30 mg/l. (+) = growth, (-) = death. χ^2 values are given for theoretical segregation ratios (15:1; 3:1). Except for clone 30.303 (^a) P is >0.05. The number of loci refers to the NR chimaeric gene.

facilitated by the absence of any detectable NR mRNA in the mutant E23. Total RNAs were analysed by Northern blot using a 2.8 kb full-length NR cDNA probe (Figure 4A). The NR mRNA detected in all transformants comigrated with the wild-type NR mRNA. The pool of NR mRNA varied by a factor of 20 between transformants 30.313 and 30.D (Figure 4A) probably as a consequence of the different integration sites of the chimaeric gene in the genome (Sanders *et al.*, 1987). Figure 4B shows that there is a good correlation between NR activity and NR protein levels in the different transformants and that both can vary by a factor of 15 between clone 30.313 and clone 30.D. Four other transformants were analysed but none of them showed higher NR activity than that found in clone 30.D (data not shown). A correlation between growth on nitrate and the level of NR activity was also noticed: clone 30.313, which had the lowest activity, grew poorly and displayed chlorotic leaves (data not shown), whereas clone 30.C, with approximately the same activity as the wild-type, grew vigorously and was indistinguishable from the wild-type at the rosette stage of

development (Figure 3B). NR proteins from the transformants were purified by chromatography on 5'-AMP-Sepharese. Fractions containing NR activity were analysed by SDS-PAGE and immunoblotting using an anti-maize NR polyclonal serum. In the transformants 30.305 and 30.D, two polypeptides which comigrate with the wild-type NR polypeptides (Moureaux *et al.*, 1989) were detected by the polyclonal antiserum (Figure 4C). Authentic NR proteins were therefore synthesized in these transgenic plants. Similar results were obtained with the other transgenic plants (data not shown). The comparison of the data of Figures 4A and 4B also shows that, in the different transgenic plants, NR activity and protein levels are proportional to the steady-state levels of NR mRNA. We note that NR mRNA was detected in the roots of the transgenic plants, as was expected since the 35S promoter has been shown to be active in roots (Benfey *et al.*, 1989). However, no NR activity could be detected in protein extracts of roots of transgenic plants, presumably because of high protease activities in this organ (data not shown).

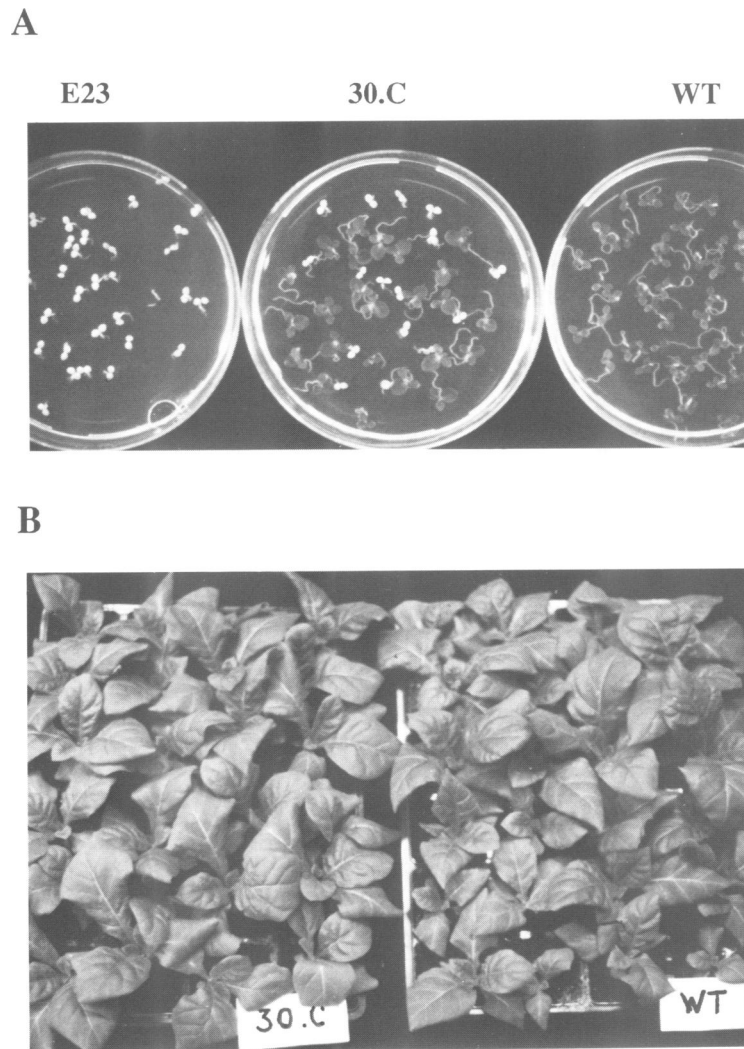


Fig. 3. Growth characteristics of the R1 progeny of clone 30.C. (A) *In vitro* on 10 mM nitrate as sole nitrogen source and (B) under greenhouse conditions as compared with *N.plumbaginifolia* wild-type (WT) and the mutant E23 (E23) control plants. NR deficient seedlings in the progeny of clone 30.C bleach and die on nitrate.

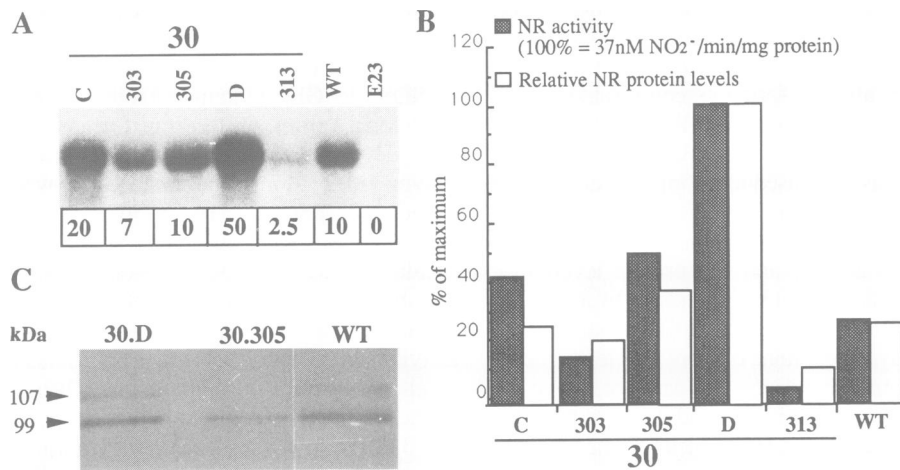


Fig. 4. Analysis of NR mRNA and protein of leaves from transgenic R1 plants. Leaves were harvested for RNA and protein extraction at the beginning of the light period. (A) Total RNA (6 μ g) was hybridized with a full-length 2.8 kb NR cDNA probe (probe b in Figure 1). NR mRNA levels were estimated by densitometric quantification and are given in arbitrary units at the bottom of the Northern blot. (B) NR activity and protein levels were measured as described in Materials and methods. (C) NR protein from transformants 30.305 and 30.D were purified on AMP–Sephrose, fractionated by SDS–PAGE and analysed by immunoblotting using a polyclonal maize NR antiserum. The arrows point to the two NR polypeptides.

Mapping of the 5' and 3' ends of the chimaeric NR transcript

The 5' end of the transcript was analysed by primer extension experiments using an oligonucleotide primer specific to the leader sequence of the *nia2* NR gene used in our construction. Figure 5A shows the results of such an experiment. The size of the cDNA product obtained with total tobacco RNA is close to 120 nucleotides which is in good agreement with the cDNA of 122 nucleotides previously described for the authentic *nia2* transcript in tobacco (Vaucheret *et al.*, 1989a). The extension products obtained with total RNA from the different transgenic plants are slightly longer because 5 nucleotides were added to the original leader sequence of the *nia2* NR gene during the construction of the chimaeric gene. The 3' end of the transcript was determined by RNase protection experiments. The DNA template used to generate the anti-sense RNA probe was fragment c (Figure 1). It covers 0.6 kb of the NR coding sequence and 1.5 kb of the *nia2* gene 3' non-coding sequence. Although protected fragments corresponding to two putative polyadenylation signals (Vaucheret *et al.*, 1989b) were detected, we focus here on one main fragment of 860 nucleotides common to *N.tabacum* and transgenic plants (Figure 3B), which points to a 3' end very close to the 3' end of a cDNA previously characterized (Vaucheret *et al.*, 1989a). These data strongly suggest that the 3' end processing of the NR chimaeric transcript and the wild-type NR transcript are similar, if not identical. Taken together these results show that an authentic NR mRNA is synthesized in the transgenic plants.

Since transgenic plants were expressing a functional NR transcript and protein, we further characterized the regulation of the chimaeric gene in order to get more insight into the regulation of a wild-type allele of the NR gene.

The chimaeric NR gene is constitutively expressed during a 24 h day–night cycle

In wild-type plants, as previously described (Galangau *et al.*, 1988), the NR transcript accumulates towards the end of the dark period and declines to almost undetectable levels at the

end of the light period (Figure 6A), whereas in the different transgenic plants the levels of NR mRNA are stable throughout this period (Figure 6A). Thus, the NR gene transcribed by the CaMV 35S promoter is clearly deregulated as compared to wild-type control plants. These results suggest that diurnal oscillations of NR mRNA are controlled at the transcriptional level. The NiR mRNA pool was also analysed, and appeared in wild-type and transgenic plants to fluctuate much less than the NR mRNA level in wild-type plants but in the same phase (Figure 6A). The low expression of NR in transformant 30.313 was correlated with a relative overproduction of the NiR mRNA when compared with the level of the wild-type (Figure 6A). As a control, the mRNA pool of the nuclear encoded gene for the β -subunit of mitochondrial ATPase (Boutry and Chua, 1985) was shown to be constant (Figure 6A). NR activity and protein levels in wild-type and transgenic plants (Figure 6C), appeared to reflect the mRNA pool (Figure 6A and C). In the wild-type, they decreased during the day by a factor of 2, while for the transgenic plants 30.305 and 30.D, they remained almost constant (Figure 6C).

Nitrate is not required for the expression of the chimaeric NR gene

We compared the pool of NR mRNA in leaves from wild-type and transgenic plants which had been grown *in vitro* on nitrate or ammonium supplemented medium. In wild-type plants grown on ammonium, NR and NiR mRNAs could not be detected, whereas on nitrate high levels of both mRNAs were synthesized (Figure 7A). In contrast, in the transgenic plants, independently of the type of nitrogen source, high levels of NR mRNA were accumulated (Figure 7A). This indicates that nitrate regulates the expression of the wild-type NR gene at the transcriptional level. As a control, the NiR mRNA was found to be detected only at high levels in plants grown on nitrate, and the pool of β -ATPase mRNA was unaffected by the type of nitrogen source (Figure 7A). Similar results were obtained with clones 30.303 and 30.C (data not shown). Interestingly, although the mutant E23 overexpressed the NiR mRNA (Figure 7A),

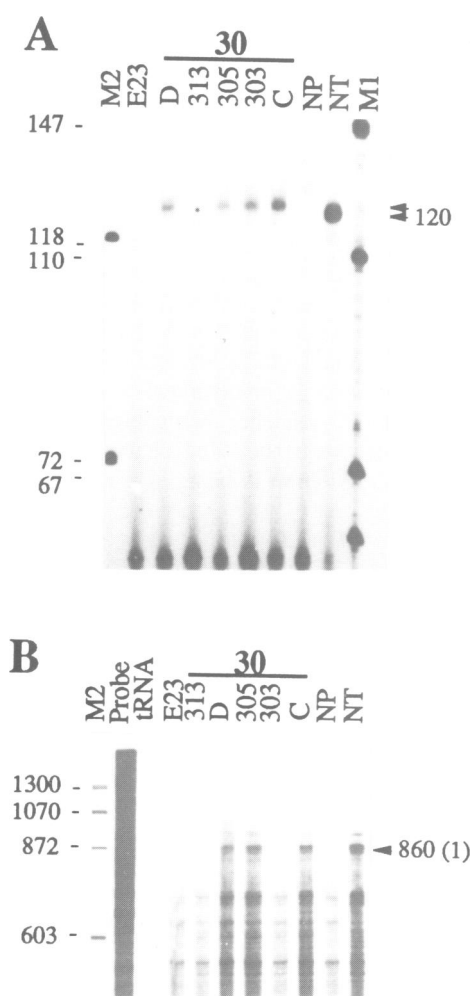


Fig. 5. 5' and 3' end mapping of the chimaeric NR gene transcript. (A) 5' end analysis by primer extension experiments. A 17-mer oligonucleotide specific to the leader sequence of the tobacco *nia2* gene was used as primer. Total RNA (20 μ g) was hybridized to an excess of 32 P-end labelled primer which was then elongated using reverse transcriptase. Control RNAs are from *N.tabacum* (NT) and *N.plumbaginifolia* (NP) and the NR deficient mutant E23 (E23). The arrow points to the extension products. Numbers are in nucleotides. The labelled size markers are: M1 = Bluescript SK⁺ vector digested by *Hpa*II; M2 = *Hae*III fragments of ϕ X. (B) 3' end analysis by RNase protection experiments. The antisense RNA probe was generated by T3 RNA polymerase from a DNA template overlapping the 3' end of the NR coding sequence and extending 1.5 kb downstream (fragment c in Figure 1). Total RNA (40 μ g or 80 μ g for clone 30.313) were hybridized to the probe prior to digestion with RNase A and T1. The arrow (1) points to the main specific protected fragment common to *N.tabacum* and transgenic plants. The rest is as in (A).

NiR mRNA levels in the complemented plants dropped to levels found in the wild-type (Figure 7A). NR activity and protein levels measured in the different plants (Figure 7B) reflected directly the mRNA pool (Figure 7A and B).

Light regulation of the NR chimaeric gene

In order to study light effects on the expression of the chimaeric gene, we analysed NR mRNA, activity and protein levels in leaves from plants kept in the dark for 60 h and then transferred into white light. In wild-type control plants, the NR mRNA amounts decreased to undetectable levels after 60 h of darkness, and reaccumulated significantly

within 4 h of illumination (Figure 8A). These observations agree with previous results (Deng *et al.*, 1990), and indicate that light plays a role in regulating the expression of the NR gene. In the transgenic plants 30.C and 30.305, the chimaeric NR gene transcript levels remained constant under the same conditions (Figure 8A). Similar results were obtained for clone 30.303 (data not shown). Thus, regulation of wild-type NR gene expression by light is transcriptional. As a control, the Northern blots were reprobated with a DNA probe coding for the β -subunit of mitochondrial ATPase. The pool of this transcript declined in the dark (Figure 6A) although much less than the pool of the NR transcript. As mentioned by others (Shih and Goodman, 1988), this might reflect a general decline of transcriptional activity due to a lower metabolic state of the plants after 60 h of darkness. Why this general effect is not seen for the NR chimaeric transcript is not clear. The pattern of NiR transcript fluctuation in both wild-type and transgenic plants was found to be comparable to that of NR mRNA in wild-type plants (Figure 8A). This shows that the NiR gene is correctly regulated by light in the transgenic plants. In leaves of wild-type plants, NR activity and protein declined to 10% of the control levels under light after 60 h of darkness (Figure 8C). This reduction reflects the rapid drop of NR mRNA. Upon transfer to light, NR activity and protein accumulated only slightly, while NR transcript was clearly induced (Figure 8A and C). This indicates that a delay is required for translation and assembly of an active NR protein. Although the NR transcript levels in leaves of transgenic plants 30.C and 30.305 were the same in light and dark (Figure 8A), a 3- to 4-fold drop of NR activity and protein in the dark was detected (Figure 8C). Besides, after dark treatment, light efficiently induced NR activity and protein accumulation (Figure 8C). These unexpected results reveal that light, apart from stimulating the transcription of the NR gene in the wild-type, also regulates NR mRNA translation and/or the stability of NR protein.

Discussion

Complementation of NR deficiency by the chimaeric NR gene

The NR-deficient *nia* mutant E23 of *N.plumbaginifolia* can be efficiently complemented for NR activity by transformation with a chimaeric NR gene consisting of a *nia2*-derived NR cDNA under the control of the CaMV 35S promoter and the poly(A) signals of the *nia2* gene. In the R1 progeny of primary transformants, NR activity was found to be directly correlated to the ability to grow on nitrate. For instance, clone 30.313 which grows poorly on nitrate and is chlorotic, expresses only one-fifth of the wild-type activity. On the other hand, clone 30.C, which expresses approximately the same activity as the wild-type, grows vigorously on nitrate. These results suggest that there is a minimum threshold of NR activity for efficient growth on nitrate. The transgenic plants analysed so far (nine independent clones) express at most three times the wild-type activity, which raises the possibility of a maximum threshold of NR activity compatible with growth on nitrate. The chimaeric NR construct can therefore be used as a new genetic marker for transient as well as stable transformation experiments. Work is in progress to use this construct as a negatively selectable marker on the basis of chlorate sensitivity.

Regulation of the expression of the NR gene as deduced from the comparison of wild-type and transgenic plants

As the mutant E23 is impaired in NR mRNA synthesis, the regulation of the expression of the NR chimaeric gene in the complemented mutant was directly accessible to analysis. The steady-state levels of NR mRNA in leaves of R1 transgenic plants expressing the chimaeric NR gene are constant throughout a day–night cycle, on different nitrogen sources (nitrate/ammonium) and under various light–dark treatments, which indicates that this gene is constitutively expressed. Under the same conditions, the expression of the *nia* gene in wild-type plants was shown to be highly regulated. The pool of a specific mRNA is determined by its half-life and the rate of transcription of the corresponding gene. Considering that the 35S promoter is constitutive (Lam and Chua, 1990; Elliot *et al.*, 1989) and that, in transgenic plants, the NR chimaeric gene is transcribed into a NR mRNA with genuine 5' and 3' ends (Figure 4), we should have detected any regulation of the half-life of the NR mRNA.

There is obviously no indication that this is the case, and therefore these results provide strong evidence that the regulation of the NR mRNA pool in the wild-type, in response to nitrate, light induction, and the endogenous clock are mainly taking place at the transcriptional level. Two other observations also support the hypothesis of transcriptional regulation. (i) A retrotransposon inserted in the first exon of the *nia* gene of *N.plumbaginifolia* leads to a truncated NR transcript with regulatory features similar to the NR transcript in wild-type plants (unpublished). (ii) Preliminary results in our laboratory show that a translational fusion

between the 5' portion of the *nia2* NR gene of tobacco and the bacterial NPTII gene respond to light induction and circadian rhythmicity in transgenic plants (A.Marion-Poll, personal communication).

In the different transformants analysed, the NR mRNA levels can be directly correlated to NR activity and protein levels under all conditions tested except light–dark treatments. In dark-adapted plants, NR protein levels decreased by a factor of 3–4 compared with light-maintained control plants, and reaccumulated quickly upon illumination, while no changes in the NR mRNA pool occurred (Figure 8). This suggests that light affects the translation of the NR mRNA and/or the half-life of the NR protein under these conditions. The possible involvement of the highly structured untranslated 5' sequence (Vaucheret *et al.*, 1989a) or the 3' untranslated sequence (Munroe and Jacobson, 1990) in translational regulation is being studied.

A highly regulated wild-type NR gene can be replaced by a deregulated NR gene without affecting vegetative development and seed production

The NR gene is highly regulated and is therefore believed to be a key enzyme which controls the flow of reduced nitrogen for optimal plant growth. Our results clearly show that the chimaeric NR gene driven by the 35S promoter is deregulated (constitutively expressed) compared with a wild-type allele of the NR gene and can functionally replace it. These data suggest that stringent regulation of NR expression is not essential and raises the question of why such deregulation is not detrimental to plant viability and development. Several explanations can be proposed to account for this result. (i) The availability of reducing equivalent and/or

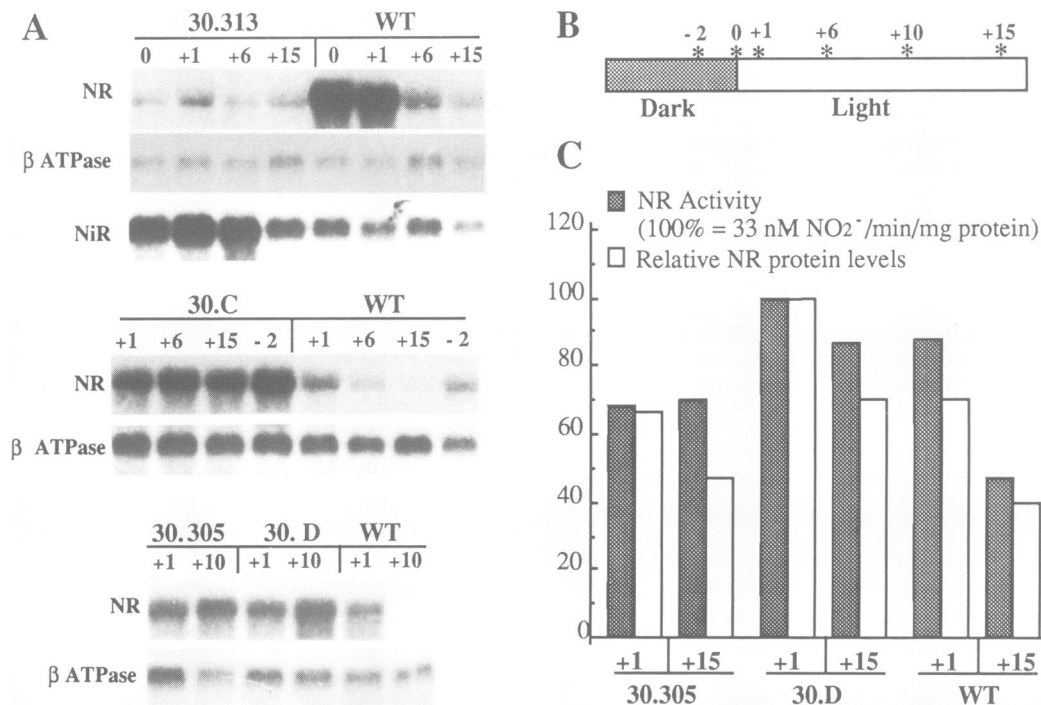


Fig. 6. Expression of the chimaeric NR gene during a 24 h day–night cycle. Leaves from different transgenic R1 plants were harvested at different time points of a 24 h light–dark cycle (16 h of light/8 h of darkness). (A) Northern blot of total RNA (6 μ g) using a full-length NR cDNA probe (Figure 1, probe b). After probe removal, the blots were re probed with a cDNA of a nuclear gene encoding the β subunit of mitochondrial ATPase. The nitrite reductase (NiR) probe was a partial NiR cDNA. The numbers at the top of each blot refer to the time-point of harvest during the cycle shown in (B). (C) NR activity and protein levels at the beginning and end of the light period were measured as described in Materials and methods. WT = wild-type *N.plumbaginifolia*.

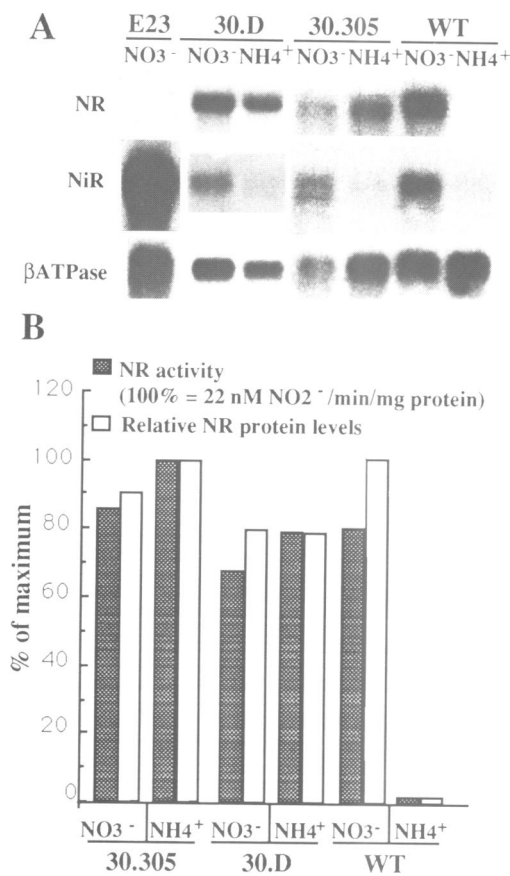


Fig. 7. Influence of the nitrogen source on the expression of the chimaeric NR gene. Transgenic R1 plants were grown *in vitro* on medium supplemented with nitrate (NO₃⁻) or ammonium (NH₄⁺) as sole nitrogen source. Leaves were harvested for RNA and protein extraction at the beginning of the light period. (A) Total RNA (6 µg) was analysed by Northern blotting as described in Figure 6. The same blot was used with the three different probes (NR; NiR; ATPase β subunit) with a washing step between each hybridization. (B) NR activity and protein levels were determined as described in Materials and methods. E23 is the NR-deficient mutant E23 of *N.plumbaginifolia* (grafted on wild-type tobacco stocks) and WT stands for wild-type *N.plumbaginifolia*.

substrate (nitrate) could be important regulatory elements of nitrate reduction and therefore the regulation of the *nia* gene would not be essential. (ii) The regulation of NR gene expression is essential, but redundant. Several steps of NR gene expression besides transcription contribute significantly to the regulation. The observation that post-transcriptional control prevents accumulation of NR protein in the dark supports this hypothesis.

A related possible explanation comes from the analysis of the expression of the NiR gene in transgenic plants. The expression of NR and NiR genes respond in similar ways to the nitrogen source and to light, suggesting that they are coregulated in the wild type (Faure *et al.*, in preparation). In the NR-deficient mutant E23, the NiR mRNA is overexpressed (Figure 7A). In contrast, in the transgenic plants expressing the NR chimaeric gene, the NiR mRNA level is down-regulated (Figures 6A, 7A, 8A). As described by others for the NiR gene (Faure *et al.*, in preparation) and the NR gene (Pouteau *et al.*, 1989), this is most likely a consequence of a negative feed-back regulation by an end product of nitrate assimilation such as ammonium or glutamine. Moreover, the NiR gene, as shown by mRNA pool fluctuations, is still correctly regulated by light and nitrate in transgenic plants, whereas the chimaeric NR gene is constitutively expressed. Both genes are therefore no longer coregulated. This means that in transgenic plants expressing a constitutive NR gene, the regulation of NiR gene may alone still control the nitrate reduction pathway.

To evaluate further the function of regulation of NR gene expression on plant physiology, experiments are under way to compare wild-type and transgenic plants under non-optimal growth conditions.

Materials and methods

Construction of the chimaeric NR gene

Standard procedures were used for recombinant DNA work (Maniatis *et al.*, 1982). Oligonucleotides were prepared on an Applied Biosystems DNA synthesizer. Bacterial strains were JM109 (Yanisch-Peron *et al.*, 1985) and XL1 Blue (Bullock *et al.*, 1987). Enzymes were used according to the supplier's recommendations.

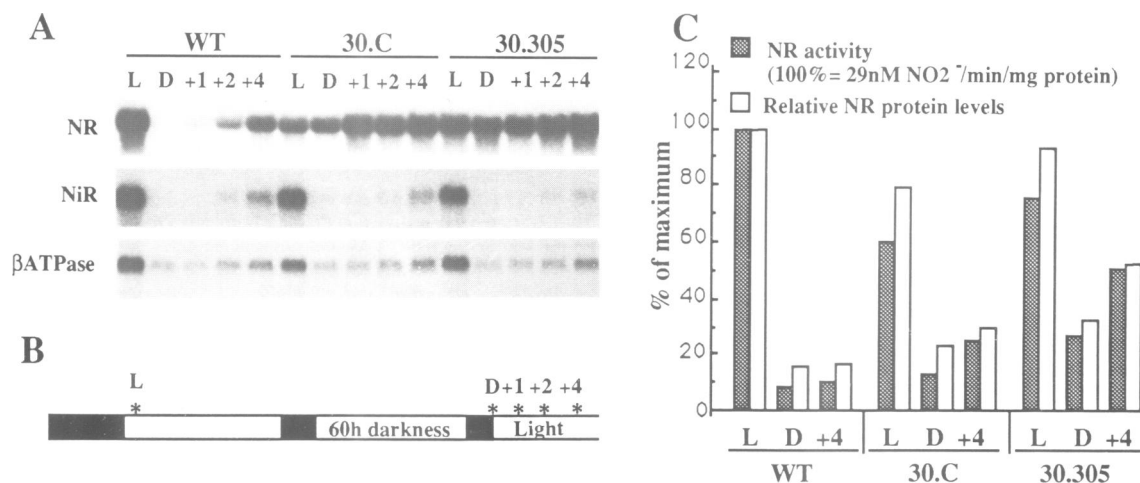


Fig. 8. Effect of light on the expression of the chimaeric NR gene. (B) Leaves from green R1 plants were harvested at the beginning of a normal day-night cycle (L), after 60 h of darkness (D) and following the dark period after 1, 2 and 4 h of white light illumination (+1, +2, +4). Total RNA (A) and NR protein (C) were extracted and analysed as described in Figure 7. The same blot was used for hybridization with the three probes. WT = wild-type *N.plumbaginifolia*.

A nitrate reductase (NR) complete cDNA corresponding to the *nia2* NR structural gene of *Nicotiana tabacum* (Vaucheret *et al.*, 1989a) was constructed in several steps. First, a 2.1 kb *EcoRI*–*SalI* fragment covering 0.6 kb of the NR coding sequence and 1.5 kb of 3' non-coding sequence from the *nia2* gene was ligated to a 1.4 kb *BamHI*–*EcoRI* fragment of a partial NR cDNA which derives from the part of the *nia2* gene carrying the three introns (Calza *et al.*, 1987) into the Bluescript M13 phagemid vector (Stratagene) in a trimolecular reaction to create pBES. A *HindIII*–*BamHI* fragment in pEMBL18 (pHB), containing 1 kb of the *nia2* promoter sequence, 138 bp of untranslated 5' sequence (leader) and 0.9 kb intron-less 5' coding sequence of the *nia2* gene, was used as the source for the 5' end of the cDNA as follows. A series of 5' deletions from an *XmnI* site (position –37) towards the initiation site of transcription (position +1; Vaucheret *et al.*, 1989b) were generated by *Bal31* exonuclease, followed by a repair step with T4 DNA polymerase and Klenow. A short double-stranded synthetic DNA fragment formed by two oligonucleotides of sequence 5'-CGTTCCTCC-3' (oligonucleotide A) and 5'-GGGAACGAGCT-3' (oligonucleotide B, which was phosphorylated) was then ligated in a 100-fold molar excess to the *Bal31* digested DNA. Oligonucleotide A corresponds to the seven first nucleotides of the leader sequence and the 3' sequence of oligonucleotide B is a *SacI* sticky end. After *BamHI* digestion (which excises the *nia2* gene sequence from pHB) gel purified fragments of ~1 kb were ligated into the *SstI* and *BamHI* sites of pBES to create a complete cDNA construct. A first screening of ampicillin resistant colonies in stringent conditions (Sartoris *et al.*, 1987) with oligonucleotide C of sequence 5'-CTGTTTGGGAACGAGCT-3' which is oligonucleotide B extended by 6 nucleotides into the leader sequence, was performed. Clones with the exact leader sequence plus an *SstI* site at the 5' end were then identified by DNA sequencing. One clone, pCSL 16 has been retained.

The complete 4.5 kb NR cDNA is bordered at its 5' and 3' ends by unique restriction sites which are respectively *SstI* and *SalI* sites. The cDNA was put under the control of the CaMV 35S promoter within the plant expression vector pRT103 (Töpfer *et al.*, 1987) to create pRTNR. To assess the functionality of the NR cDNA, pRTNR was electroporated by standard techniques (Guerche *et al.*, 1987) into mesophyll protoplasts of the NR-deficient mutant *Nia30* from tobacco. The complete NR sequence from pCSL16 was gel purified as an *SstI* (blunt-ended), *SalI* fragment and cloned into the *KpnI* (blunt-ended) and *SalI* sites of the expression cassette of the plant transformation vector pBinDH51. This puts the NR cDNA under the control of the CaMV 35S promoter and creates the plasmid pBCSL16. pBinDH51 has been constructed by inserting the expression cassette from pDH51 (Pietrzak *et al.*, 1986) as a 0.72 kb *EcoRI*–*KpnI* (blunt ended) by T4 polymerase and Klenow into the *SmaI* and *EcoRI* sites of pBin19 (Bevan, 1984).

Plant transformation and regeneration

The recombinant vector pBCSL 16 in *E. coli* XL1 Blue, was mobilized into *Agrobacterium tumefaciens* LBA 4404 as described (Bevan, 1984). The NR-deficient mutant E23 of *Nicotiana plumbaginifolia* was maintained aseptically *in vitro* on B-N medium supplemented with 10 mM ammonium succinate as the nitrogen source (Gabard *et al.*, 1987). Leaf discs of the mutant E23 were transformed essentially as described previously (Vaucheret *et al.*, 1990). Shoots emerging from calli grown on 10 mM ammonium as the sole nitrogen source and 100 mg/l kanamycin as selective agent were transferred onto B-N medium supplemented with 10 mM potassium nitrate as sole nitrogen source. Plantlets able to utilize nitrate were then grown in the greenhouse. These plants are referred to as primary transformants. Genetic analysis of the progeny obtained from selfing the primary transformants (R1 generation), was done as described previously (Vaucheret *et al.*, 1990) except that on nitrate containing medium only 20 mg/l of kanamycin was used for selection.

Plant growth conditions

R1 seeds obtained by selfing the primary transformants were germinated on B-N medium supplemented with 10 mM nitrate. For cycle studies and light induction experiments, plantlets able to utilize nitrate were placed in a controlled growth chamber under a 16 h light (25°C) 8 h dark (17°C) cycle with light intensity of 180 $\mu\text{mol}/\text{cm}^2/\text{s}$ (fluorescent lamps), and then to appropriate light or dark conditions. Alternatively plantlets were grown aseptically on B-N medium supplemented with 10 mM ammonium or 10 mM nitrate as nitrogen source for nitrate induction experiments. RNA and protein were extracted from leaves of plants grown to the 'rosette' stage. For the analysis of primary transformants with one active chimaeric NR locus, leaves from 5–10 R1 plants were harvested for each assay and for transformants carrying two active loci, leaves from 15–20 R1 plants were

harvested, cut into small pieces, mixed and frozen in liquid nitrogen. Five plantlets gave ~10 g of fresh leaf material.

DNA and RNA extraction, Northern and Southern blot analysis

Total DNA isolation and Southern analysis were as described previously (Calza *et al.*, 1987). Total RNA was extracted from 2–3 g of leaf tissue by grinding in liquid nitrogen and phenol extracted as described (Verwoerd *et al.*, 1989). RNAs were fractionated on 1.2% agarose gels containing formaldehyde (Maniatis *et al.*, 1982), transferred to Hybond-N membranes (Amersham) and cross-linked by UV irradiation for 5 min. Including ethidium bromide (20 $\mu\text{g}/\text{ml}$) in the denaturation mix allowed the visualization of RNA on the membrane after transfer. Hybridization with ^{32}P -labelled probes was performed in 50% formamide; 5 \times SSPE; 5 \times Denhardt's solution; 0.5% lauryl sarkosyl (v/v) and 200 $\mu\text{g}/\text{ml}$ denatured salmon sperm DNA for 16–24 h at 42°C. The filters were then washed once in 2 \times SSC; 0.5% sarkosyl at room temperature followed by one wash in the same buffer at 65°C for 30 min and one wash in 0.2 \times SSC; 0.5% sarkosyl at 65°C for 15 min. For rehybridization, the membranes were washed for 3 h according to Amersham's conditions. The relative amounts of mRNA were determined by densitometric scanning of the autoradiograms with a Shimadzu densitometer. The probes were labelled by random priming of gel-purified DNA fragments (Feinberg and Vogelstein, 1984). The probe for the nuclear-encoded β subunit of the mitochondrial ATPase from *N. plumbaginifolia* was a 1.6 kb *EcoRI*–*SalI* cDNA fragment (Boutry and Chua, 1985). The nitrite reductase probe was a 1.7 kb *EcoRI* tobacco cDNA fragment (Faure *et al.*, in preparation).

Primer extension analysis

The synthetic oligonucleotide P1 (Vaucheret *et al.*, 1989a) specific to the leader sequence of the *nia2* NR gene was end-labelled with T4 kinase (Biolabs) and 10 ng were hybridized with 20 μg of total RNA at 50°C for 1 h in 20 μl of 50 mM Tris pH 8.3; 0.3 M KCl. Primer extension was performed with 600 U of AMV reverse transcriptase (BRL) at 37°C for 1 h, in 60 μl 50 mM Tris pH 8.3; 8 mM MgCl_2 ; 0.1 M KCl; containing 0.5 mM of each dNTP; 50 U of RNase inhibitor (Boehringer). The extended primer was analysed on a 4% acrylamide, 8 M urea sequencing gel along with size markers. The cDNAs were visualized by autoradiography. The size markers were *HaeIII* fragments of ϕX174 RF labelled with T4 kinase and *HpaII* fragments from SK⁺ vector labelled with Klenow.

RNase protection assay

The DNA template for the RNA antisense was a 2.1 kb *EcoRI*–*SalI* fragment from pCSL16, which covers 0.6 kb of the coding sequence of the NR cDNA and 1.5 kb of the *nia2* NR gene 3' non-coding end (Figure 1). This fragment was cloned into the *EcoRI*–*SalI* sites of Bluescript M13 phagemid (Stratagene) to form pES3 which was checked by dideoxy sequencing. RNase A/T1 protection assays were performed essentially as described (Goodall *et al.*, 1990). The DNA template was generated by *EcoRI* digestion of pES3 and 1 μg was transcribed with 50 U of T3 RNA polymerase (BRL) in 10 μl of BRL's buffer at 37°C for 30 min. The reaction also contained 0.5 mM UTP, ATP and GTP, 50 μM CTP, 30 μCi [α - ^{32}P] CTP (3000 Ci/mM) and 50 U of RNase inhibitor (Boehringer). The DNA template was then digested in 100 μl of 10 mM Tris pH 7.5; 1 mM MgCl_2 with 30 U of RNase free DNase (Boehringer) for 15 min at 37°C. Approximately 10^5 c.p.m. of the antisense RNA probe were coprecipitated with 40 μg of total RNA. Hybridization was performed in 30 μl of 80% formamide; 40 mM PIPES pH 6.7; 0.4 M NaCl and 1 mM EDTA at 45°C overnight. RNase A/T1 digestion was carried out by addition of 300 μl of RNase buffer, which is 10 mM Tris pH 7.5; 0.2 M NaCl; 5 mM EDTA, containing 20 $\mu\text{g}/\text{ml}$ RNase A (Boehringer) and 0.8 $\mu\text{g}/\text{ml}$ RNase T1 (Boehringer) for 1 h at 26°C. The digestion products were analysed along with size markers on 4% acrylamide, 8 M urea sequencing gels.

Protein extraction and analysis

Extraction of total proteins of leaf tissues, NR activity assays and estimation of NR protein levels were done as described previously (Galangau *et al.*, 1988; Chérel *et al.*, 1986). NR activity is expressed in nM nitrite (NO_2^-) produced/min/mg total protein. NR protein amounts are expressed as a percentage of the maximum found in each experiment. This was done by comparing the dilution factor of each extract (standardized/mg of protein) in the linear part of the ELISA curve. For Western analysis, NR from 2 g of leaf tissues was purified on 200 μl of 5'-AMP-Sepharose (Sigma) microcolumns as described previously (Moureaux *et al.*, 1989). Proteins were transferred to PVDF 0.45 μm Immobilon-P membranes (Millipore) using a Millipore electro-transfer 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.

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