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Effects of constitutive expression of nitrate reductase in transgenic *Nicotiana plumbaginifolia* L. in response to varying nitrogen supply

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Abstract. Transformed *Nicotiana plumbaginifolia* plants with constitutive expression of nitrate reductase (NR) activity were grown at different levels of nitrogen nutrition. The gradients in foliar NO_3^- content and maximum extractable NR activity observed with leaf order on the shoot, from base to apex, were much decreased as a result of N-deficiency in both the transformed plants and wild-type controls grown under identical conditions. Constitutive expression of NR did not influence the foliar protein and chlorophyll contents under any circumstances. A reciprocal relationship between the observed maximal extractable NR activity of the leaves and their NO_3^- content was observed in plants grown in nitrogen-replete conditions at low irradiance (170 μmol photons $\cdot m^{-2} \cdot s^{-1}$). This relationship disappeared at higher irradiance (450 μmol photons $\cdot m^{-2} \cdot s^{-1}$) because the maximal extractable NR activity in the leaves of the wild-type plants in these conditions increased to a level that was similar to, or greater than that found in constitutive NR-expressors. Much more NO_3^- accumulated in the leaves of plants grown at 450 μmol photons $\cdot m^{-2} \cdot s^{-1}$ than in those grown at 170 μmol photons $\cdot m^{-2} \cdot s^{-1}$ in N-replete conditions. The foliar NO_3^- level and maximal NR activity decreased with the imposition of N-deficiency in all plant types such that after prolonged exposure to nitrogen depletion very little NO_3^- was found in the leaves and NR activity had decreased to almost zero. The activity of NR decreased under conditions of nitrogen deficiency. This regulation is multifactoral since there is no regulation of NR gene expression by NO_3^- in the constitutive NR-expressors. We conclude that the NR protein is specifically targetted for destruction under nitrogen deficiency. Consequently, constitutive expression of NR activity does not benefit the plant in terms of increased biomass production in conditions of limiting nitrogen.

Key words: *Nicotiana* – Nitrate nutrition – Nitrate reductase – Photosynthesis – Transgenic plant (tobacco)

Introduction

The factors limiting nitrogen (N) assimilation in plants are only poorly defined. It has long been supposed that nitrate reductase (NR, EC 1.6.6.1), the first enzyme of the nitrogen-assimilation pathway, is a major metabolic control point limiting the rate of N metabolism and in consequence plant growth. This is considered to be caused by several factors including the low level of NR protein in leaf cells and also by strict regulation of activity. Nitrate and light are effectors of NR-protein synthesis (Galangau et al. 1988). The quantity of NR protein in the cell varies diurnally, extractable foliar NR activity (measured in vitro) being maximum during the photoperiod and minimal at night (Deng et al. 1990). The activity of NR measured in vivo also appears to vary diurnally (Lillo and Henriksen 1984). In addition, NR activity has been shown to be modulated by covalent modulation involving phosphorylation/dephosphorylation modifications of the enzyme protein (Huber et al. 1992; Kaiser et al. 1993). The physiological significance of this type of regulation is however, unresolved. It has been known for many years that the availability of NO_3^- is also an important limitation of NO_3^- assimilation in situ (Shaner and Boyer 1976; Rufty et al. 1987; Gojon et al. 1991). The quantity of active NR enzyme might not, therefore, be the sole limiting step in N assimilation.

Genetic manipulation procedures have allowed modification of the expression of the NR gene in *Nicotiana plumbaginifolia* so that it is no longer regulated at the transcriptional level by NO_3^- or sugar. Transgenic *N. plumbaginifolia* plants which constitutively express NR were produced by Vincentz and Caboche (1991). These

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Abbreviations: Chl = chlorophyll; N = nitrogen; NR = NADH-nitrate reductase; WT = wild type

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plants were obtained by transformation of the NR-deficient mutant, E23, with the *nia-2* cDNA under the control of the 35S promoter from cauliflower mosaic virus (35S CaMV). In these plants, NR activity, measured in vitro, is 2–5 times that of the wild-type (WT; Foyer et al. 1994) and is no longer sensitive to regulation at the transcriptional level (Vincentz et al. 1993). These plants have similar leaf protein levels, total nitrogen, chlorophyll, starch and soluble sugars but they contain higher foliar glutamine and lower NO_3^- than the WT controls (Foyer et al. 1993; Quilleré et al. 1994). Photosynthesis, sucrose-phosphate synthetase and phosphoenolpyruvate carboxylase activities are not modified by the change in NR activity (Foyer et al. 1994). Constitutive NR expression allows normal growth and development in optimal growth conditions (Vincentz and Caboche 1991; Foyer et al. 1993; Quilleré et al. 1994). In the present study it was our aim to exploit these transgenic plants further. Since the level of NR protein present is no longer determined by the supply of the substrate, NO_3^-, N-deficiency should have no direct effect on NR-gene expression. We therefore compared foliar metabolism and the performance of the transgenic line (C) and the WT in conditions of varying NO_3^- nutrition and irradiance. This allowed us to explore the relative importance of the limitations imposed by substrate availability and modulation of NR activity.

Materials and methods

Plant material. Transgenic (line C) and WT *Nicotiana plumbaginifolia* L. (seeds kindly provided by Dr. Michel Caboche, Laboratoire du Métabolisme, Versailles, France) were grown in pots in a growth chamber with a 16-h photoperiod, 23° C day/18° C night and irradiance was 170 µmol photons·m^{-2}·s^{-1}. The pots were watered with a complete nutrient solution containing 10 mM nitrate and 2 mM ammonium (Coic and Lesaint 1975). When the plants were six weeks old, they were transferred to one of the following conditions of N nutrition; 12 mM ($NO_3^- + NH_4^+$), 0.2 mM NO_3^- and 0.05 mM NO_3^- in the absence of added ammonium. The plants were grown at two light levels: 170 and 450 µmol photons·m^{-2}·s^{-1}.

Leaf samples were taken prior to the imposition of these N and light treatments and then subsequently on the 9th (*to*+9) and 20th (*to*+20) days after the beginning of the treatments for the plants grown at 170 µmol photons·m^{-2}·s^{-1}, and on the 8th (*to*+8) and 14th (*to*+14) days for the plants grown at 450 µmol photons·m^{-2}·s^{-1}. Five plants were sampled for each time point except for Figs. 1–4 where single-plant data are given. The plants were weighed and the leaves were immediately frozen in liquid nitrogen and stored at −80° C until use.

Nitrate reductase assay. Leaf material was ground in a mortar with liquid N and 1 ml of extraction buffer (8 ml·g^{-1} FW containing 0.1 M potassium phosphate (pH 7.5), 1 mM EDTA, 1 µM FAD, 1 µM Na_2MoO_4, 2 mM β-mercaptoethanol, 1 µM leupeptin, and 0.1 g of polyvinylpyrollidone. The extract was centrifuged for 5 min at 12000·g and the soluble fraction assayed for NR activity according to Moureaux et al. (1989) in a reaction mixture containing 0.1 M potassium phosphate buffer (pH 7.5), 10 mM KNO_3 and 0.15 mM NADH, and 50 µl of the extract in a total volume of 500 µl.

Nitrate, protein and chlorophyll estimations. Nitrate, protein and chlorophyll were estimated using the same leaf extracts as for NR activity. Nitrate was determined by the method of Cataldo et al.

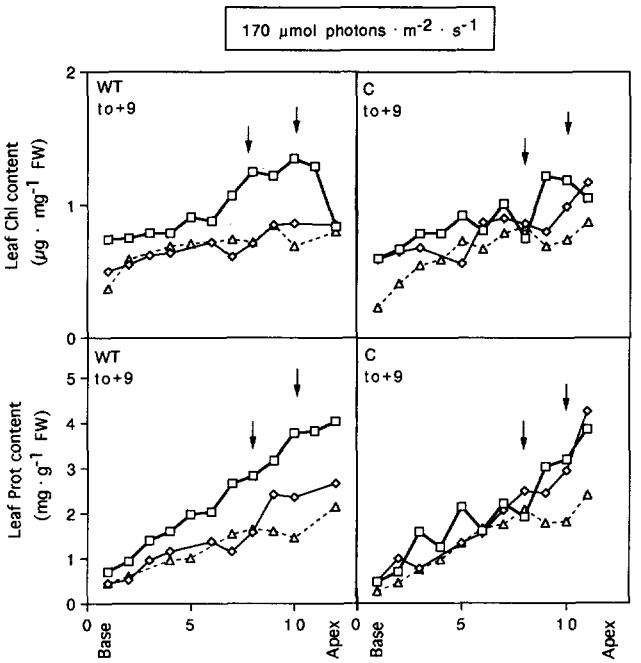

Fig. 1. The influence of nitrogen nutrition (12 mM NO_3^- □–□; 0.2 ◇–◇; 0.05 △–△) and leaf position on foliar chlorophyll and protein contents in low light-grown wild-type (*WT*) and transgenic (*C*) *N. plumbaginifolia* constitutively expressing NR. Plants were grown at 170 µmol photons·m^{-2}·s^{-1} with a 16-h photoperiod, 23° C day, 18° C night and 12 mM N for six weeks. Some of the plants were then transferred to either 0.2 or 0.05 mM N. Measurement were subsequently made 9 d after this transfer

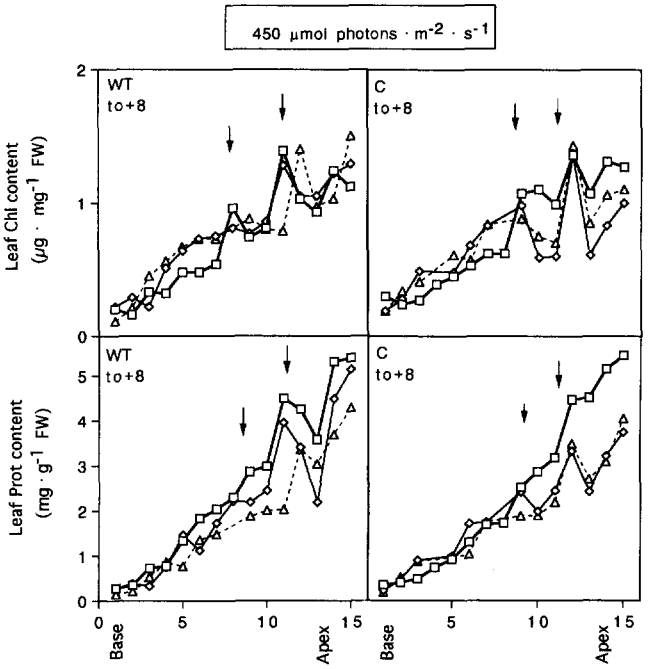

Fig. 2. The influence of nitrogen nutrition (12 mM NO_3^- □–□; 0.2 ◇–◇; 0.05 △–△) and leaf position on foliar chlorophyll (*Chl*) and protein (*Prot*) contents of moderately high-light-grown wild-type (*WT*) and transgenic (*C*) *N. plumbaginifolia* constitutively expressing NR. Plants were grown at 450 µmol photons·m^{-2}·s^{-1} with a 16-h photoperiod, 23° C day, 18° C night at 12 mM N for six weeks. Some of the plants were then transferred to either 0.2 or 0.05 mM N. Analyses were performed 8 d after transfer

Table 1. A comparison of the chlorophyll and protein contents of young low-light-grown leaves of wild-type (WT) *N. plumbaginifolia* plants and transformed plants constitutively expressing NR (C) grown at different levels of N nutrition

	WT			C		
	12 mM	0.2 mM	0.05 mM	12 mM	0.2 mM	0.05 mM
Chlorophyll ($\mu g \cdot mg^{-1}$ FW)	1.02 ± 0.19	0.84 ± 0.11	0.77 ± 0.10	1.04 ± 0.19	0.74 ± 0.26	0.8 ± 0.06
Protein ($mg \cdot g^{-1}$ FW)	11.28 ± 2.92	8.38 ± 0.87	6.64 ± 0.87	12.02 ± 5.07	8.037 ± 3.45	7.88 ± 0.99

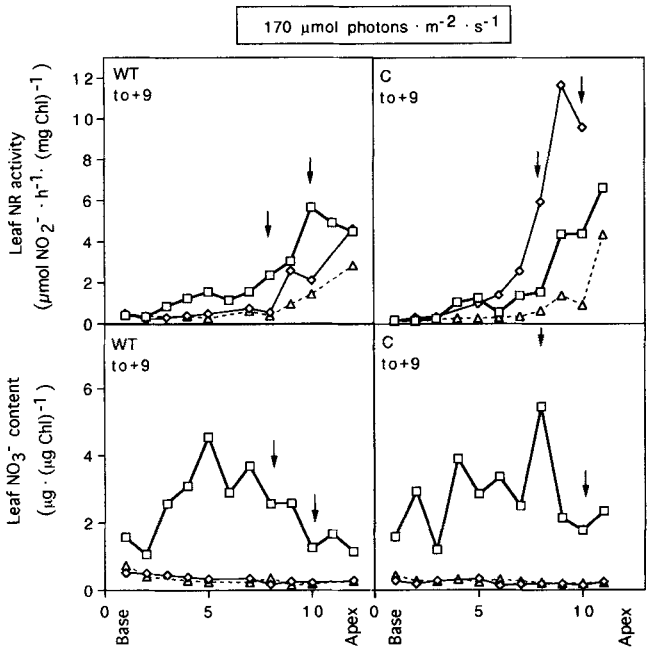

Fig. 3. The influence of nitrogen nutrition (12 mM NO_3^- □–□; 0.2 ◇–◇; 0.05 △–△) and leaf position on NR activity and foliar NO_3^- contents of low-light-grown wild-type (*WT*) and transgenic (*C*) *N. plumbaginifolia* constitutively expressing NR. All other conditions were as described in Fig. 1

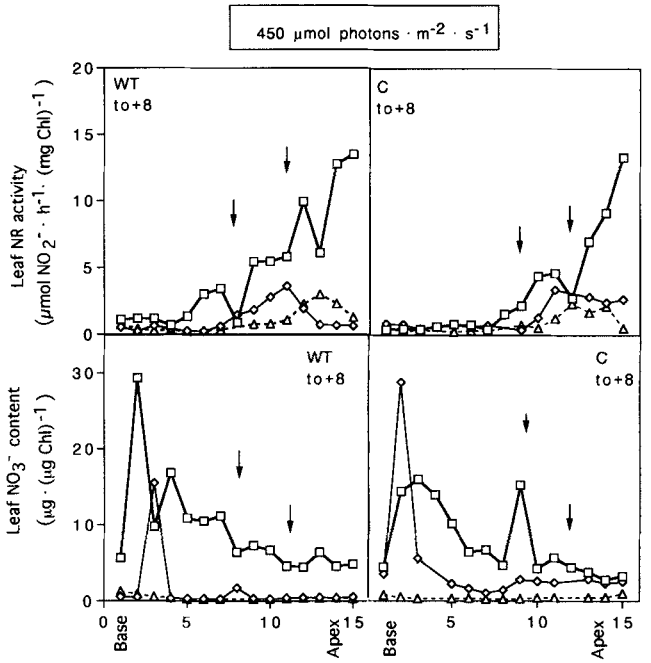

Fig. 4. Influence of nitrogen nutrition (12 mM NO_3^- □–□; 0.2 ◇–◇; 0.05 △–△) and leaf position on NR activity and foliar NO_3^- contents of moderately high-light-grown wild-type (*WT*) and transgenic (*C*) *N. plumbaginifolia* constitutively expressing NR. All other conditions were as described in Fig. 2

(1975), soluble protein via the method of Bradford (1976) and chlorophyll by the method of Arnon (1949).

Net CO_2 assimilation. Net CO_2 assimilation was measured on attached leaves using an infra-red gas analyzer (LCA2, Analytical Developmental Co. (ADC), Hoddesdon, UK) as described in Foyer et al. (1994).

Results

In order to determine the precise effects of N-deficiency on leaf metabolism in WT plants (showing regulated expression of NR activity) and in transformed plants (where expression of this enzyme was constitutive) all the leaves on the individual shoots were examined initially. Plants grown at 170 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (Fig. 1) or at 450 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (Fig. 2) were analysed for chlorophyll and foliar protein contents. Both increased from the base to the apex of the plant in the constitutive NR-expressors and the wild-type controls. The protein gradient with leaf position was much greater than that of chlorophyll but no differences were detected between the two irradiance levels. The N-nutrition greatly influenced the foliar chlorophyll and the protein contents. Plants grown at 0.2 or 0.05 mM NO_3^- for 8 or 9 d exhibited lower protein and chlorophyll contents than those grown at 12 mM NO_3^-. These differences were even more pronounced 20 d after the beginning of the treatment (data not shown). There were no significant differences in the chlorophyll or protein contents between the young leaves of the WT plants or constitutive NR-expressors in any of these conditions (Table 1).

In a similar manner to the studies on leaf order in relation to chlorophyll and protein content during N-deficiency, single whole shoots were analysed for foliar NR activity (Fig. 3) and NO_3^- contents, from plants grown at either 170 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (Fig. 3) or 450 µmol photons $\cdot m^{-2} \cdot s^{-1}$ (Fig. 4). Foliar NR activity increased from the base to the apex in both plant types. The activity

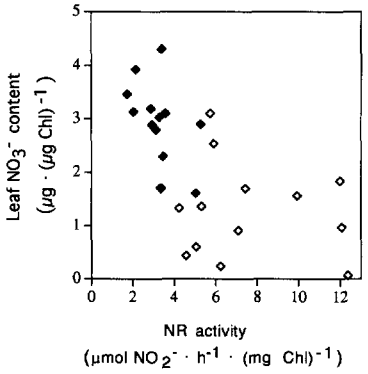

Fig. 5. The relationship between foliar NR activity and NO_3^- content for WT *N. plumbaginifolia* (◆—◆), and constitutive NR-expressors (◇—◇) grown at 170 μmol photons·m^{-2}·s^{-1} and 12 mM NO_3^-

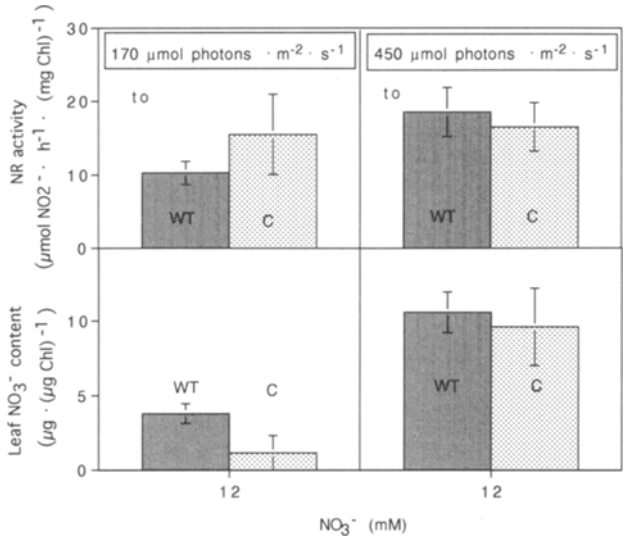

Fig. 6. The influence of the level of irradiance (170 and 450 μmol photons·m^{-2}·s^{-1}) on foliar NO_3^- content and NR activity in WT and transgenic (*C*) *N. plumbaginifolia* where the NR gene was constitutively expressed

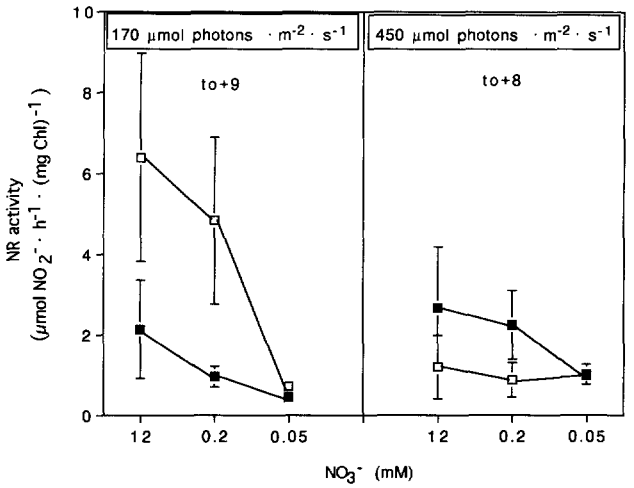

Fig. 7. The influence of N availability on foliar NR activity in WT (◆—◆) and transgenic (◇—◇) *N. plumbaginifolia* where the NR gene was constitutively expressed. Plants were grown at two levels of irradiance (170 and 450 μmol photons·m^{-2}·s^{-1}) with a 16-h photoperiod, 23° C day, 18° C night at 12 mM NO_3^- for six weeks. Some of the plants were then transferred to either 0.2 or 0.05 mM N. The activity of NR was measured 8 or 9 d after transfer

of the young leaves was, however, the most modified by N-availability. The gradient of foliar NR activity with the leaf position was much less pronounced in plants grown at 0.2 and 0.05 mM NO_3^- than at 12 mM NO_3^- at both irradiance levels. Moreover, the NR activities of young leaves were lower at 170 μmol photons·m^{-2}·s^{-1} in the WT controls grown at 12 mM NO_3^- than at 450 μmol photons·m^{-2}·s^{-1}. The maximum extractable NR activity of the young leaves from the transgenic line was not influenced by the irradiance level as might be predicted by the constitutive expression in this case. In both WT plants and constitutive NR-expressors, foliar NO_3^- contents were determined by N-availability and irradiance. In all plants, growth on either 0.2 and 0.05 mM NO_3^- caused the NO_3^- content of the leaves to drastically decrease at both irradiance levels. In plants grown at 12 mM NO_3^- the foliar NO_3^- contents were higher at 450 μmol photons·m^{-2}·s^{-1} than at 170 μmol photons ·m^{-2}·s^{-1}.

In the following experiments the leaves found in the section of the plants, delineated by the arrows in Figs. 1–4, are henceforward referred to as young leaves and characterised further. The relationship between foliar NO_3^- content and leaf NR activity is shown in Fig. 5. The leaves of the constitutive NR-expressors had higher extractable maximum NR activities and lower NO_3^- contents than the control plants when grown at 170 μmol photons·m^{-2}·s^{-1}. There is, thus, generally a reciprocal relationship between the two factors (Fig. 5). When plants were grown at 170 μmol photons·m^{-2}·s^{-1}, NR activity was higher in the constitutive NR-expressors than in the WT controls, but at 450 μmol photons·m^{-2}·s^{-1} the difference in NR activity between the transgenic and the WT controls disappeared (Fig. 6). The irradiance effect was also apparent in the foliar NO_3^- content which was lower in the constitutive NR-expressors than in the WT control at 170 μmol photons·m^{-2}·s^{-1} but not at 450 μmol photons·m^{-2}·s^{-1}.

Foliar NR activity decreased as a result of N-deficiency in the wild-type plants grown at 170 μmol photons·m^{-2}·s^{-1} and 450 μmol photons·m^{-2} ·s^{-1} (Fig. 7). The same trend was observed in the constitutive NR-expressors at low irradiance but at the higher light level the measured NR activity was very low in all cases, presumably because of the poorly understood phenomenon of gene suppression. This arises spontaneously in the transformed plants in high light. The frequency of this genetic malfunction is much decreased in low light.

The CO_2 assimilation rate was higher at 450 μmol photons·m^{-2} ·s^{-1} than at 170 μmol photons·m^{-2}·s^{-1} for both the transgenic plants and WT controls, but, was decreased to a similar level by N-deficiency in both the constitutive NR-expressors and WT plants (Fig. 8). No significant differences could be detected in shoot biomass accumulation between the constitutive NR-expressors

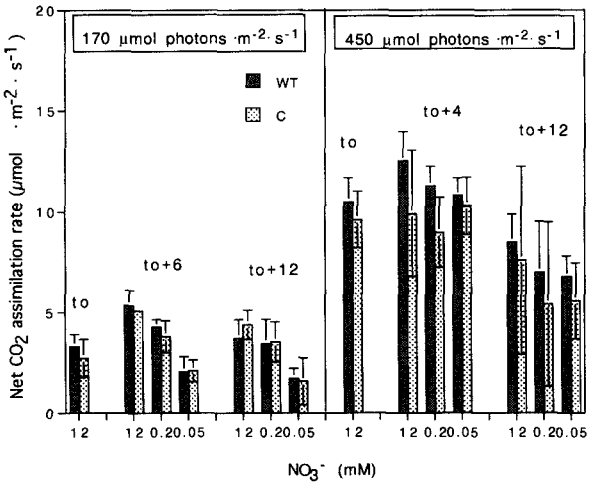

Fig. 8. The influence of N availability on net rate of CO_2 assimilation in leaves of WT and transgenic *N. plumbaginifolia* where the NR gene was constitutively expressed. Plants were grown at two levels of irradiance (170 and 450 µmol photons·m^{-2}·s^{-1}) with a 16-h photoperiod, 23° C day, 18° C night at 12 mM NO_3^- for six weeks. Some of the plants were then transferred to either 0.2 or 0.05 mM N. Assimilation of CO_2 was measured 6 or 12 d after transfer to different levels of N nutrition for plants grown at 170 µmol photons·m^{-2}·s^{-1} and 4 and 12 d after transfer for plants grown at 450 µmol photons·m^{-2}·s^{-1}

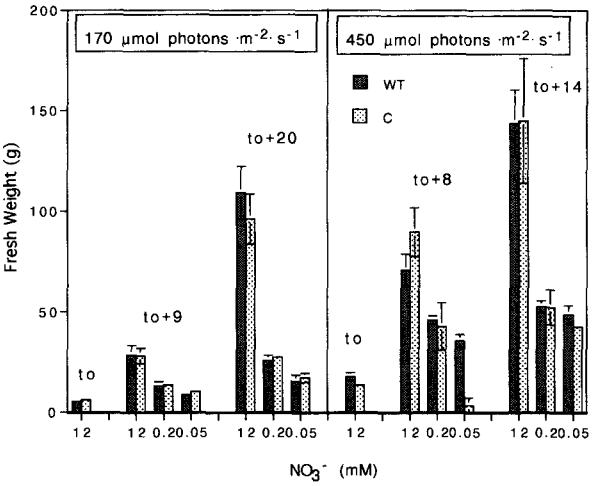

Fig. 9. The influence of N availability on biomass (fresh weight) production in WT and transgenic (*C*) *N. plumbaginifolia* where the NR gene was constitutively expressed. Plants were grown at two levels of irradiance (170 and 450 µmol photons·m^{-2}·s^{-1}) with a 16-h photoperiod, 23° C day, 18° C night at 12 mM for six weeks. Some of the plants were then transferred to either 0.2 or 0.05 mM N. Biomass was measured 9 and 20 d after transfer to different levels of N nutrition for plants grown at 170 µmol photons·m^{-2}·s^{-1} and the 8th and 14th days after transfer for plants grown at 450 µmol photons·m^{-2}·s^{-1}

and the WT controls. The N-deficiency and irradiance level both influenced growth (Fig. 9). Plants grown at 0.2 and 0.05 mM NO_3^-. accumulated much less biomass than plants grown at 12 mM NO_3^-. Similarly, plants grown at 170 µmol photons·m^{-2}·s^{-1} accumulated much less biomass than those grown at 450 µmol photons·m^{-2}·s^{-1}.

Discussion

The long-term consequences of N-deprivation are well characterised. They involve decreases in photosynthesis and chlorophyll content (Khamis et al. 1990) and alterations in leaf carbohydrate metabolism (Rufty et al. 1988). In our experiments, decreases in the chlorophyll and soluble protein contents in all the leaves of the WT plants were found 9 d after the beginning of N-deprivation. Similarly, the leaves of the constitutive NR-expressors were unable to maintain the levels of foliar chlorophyll and protein when subjected to N-deprivation. We conclude that constitutive NR expression does not increase the capacity of the plant to maintain chlorophyll and protein levels in the face of N-deprivation. No increases in apparent N-assimilation capacity result from the increased NR activity in the transformed plants in these conditions. The measured effect on the chlorophyll and protein levels in the WT leaves grown at moderate light levels was less than that on leaves from plants grown at very low light levels, presumably because of the higher NO_3^- reserves of the former than the latter.

The NO_3^- content of the leaves from the N-replete WT plants grown at moderate light levels (450 µmol photons·m^{-2}·s^{-1}) was much greater than that of leaves of plants grown at low light (170 µmol photons·m^{-2}·s^{-1}). In contrast, the constitutive NR-expressors grown at moderate high light had similar or lower NO_3^- levels to the WT controls grown at very low light.

The net CO_2 assimilation rate measured at 170 µmol photons·m^{-2}·s^{-1} was 2–3 times lower than at 450 µmol photons·m^{-2}·s^{-1}. Gastal and Saugier (1989) showed that an increase in irradiance leads to increased N uptake since the N uptake/C assimilation ratio remains constant. Increasing irradiance leads to enhanced NO_3^- assimilation rates because of (i) increased NO_3^- absorption (ii) induction of NR gene expression and (iii) direct modulation of NR activity. In WT plants, NR mRNA and NR-protein synthesis are stimulated by light, resulting in an increase in total extractable NR activity (Duke and Duke 1984). Furthermore, NR is activated in the light by protein phophorylation-dephosphorylation modulation. Total extractable NR activity was increased in the leaves of WT plants grown at 450 µmol photons·m^{-2}·s^{-1} compared to those grown at 170 µmol photons·m^{-2}·s^{-1}. This was largely due to stimulation at the level of NR gene transcription in this case. No such increase was possible or observed in constitutive NR expressors. In the latter, NR activity was similar or even lower in leaves of plants grown at 450 µmol photons·m^{-2}·s^{-1} than in those grown at 170 µmol photons·m^{-2}·s^{-1}. The measured differences in extratable NR activity between the transgenic line and the WT controls therefore decreased as the irradiance level was increased in our experimental conditions. Our results would indicate that the NR mRNA or NR protein in the constitutive NR-expressors may be less stable than in the WT. The total NR activity of the constitutive NR-expressors has been shown to be decreased in darkness compared to the light as it is in the WT (Vincentz and Caboche 1991; Foyer et al. 1994). Nitrate induces NR transcription in wild-type plants but is

also important in determining the stability of the transcripts (Srivastava 1980; Galangau et al. 1988). The gradient of NR activity observed from the base to the apex of the plant results in differences in the balance between net synthesis and degradation of the NR protein between the young and old leaves (Kenis et al. 1992). In our experiments there was a clear reciprocal relationship between the foliar NO_3^- content and the total extractable NR activity of the leaves. This gradient was decreased when plants were grown at low levels of NO_3^- in both the constitutive NR-expressors and the WT controls as the NO_3^- reserves were depleted. The net synthesis of NR protein in the young leaves might be reduced in conditions of low NO_3^- nutrition in the WT plants because of the absence of NO_3^--dependent stimulation of gene expression. Transcription could not be affected in the transgenic line in this way, however NR-protein synthesis might be diminished by the depletion of amino acids for synthesis. Degradation must also be increased due to the low NO_3^- content in both types of plant. Since the total protein level of the transgenic plants subjected to N-deficiency compared to N-replete conditions at low light is largely unchanged, we conclude that selective degradation of NR protein must occur in these conditions. Therefore, constitutively expressed NR, which does not require NO_3^- for its synthesis, might need NO_3^- for stability. This diminishs the benefit of a constitutive NR expression in conditions of low NO_3^- supply. Certain plant species (e.g. soybean, black alder and barley) contain a native constitutive NR protein in addition to the inducible form (Streit et al. 1987; Benamar et al. 1989; Kleinhofs and Warner 1990). The significance of the presence of such NR isoforms is as yet unknown. Our results indicate that the presence of a constitutive NR expression in *N. plumbaginifolia* did not confer any great advantage to plants subjected to N-deficiency. Uptake and availability of NO_3^- are major limiting steps to N assimilation in these circumstances. In consequence, biomass accumulation in the shoots was similar in all plant types regardless of the level NO_3^- nutrition.

In the constitutive NR-expressors the foliar glutamine content is about twice that of the WT (Foyer et al. 1994; Quilleré et al. 1994). We do not know how this amino acid is compartmentalised in the leaf cells but if we assume that the cytosolic glutamine content is increased this could have major consequences for NO_3^- uptake in the transgenic line since cycling of amino acids is considered to play a role in the regulation (inhibition) of NO_3^- uptake (Cooper and Clarkson 1989). An increased level of NR activity in the transgenic line might be counterbalanced by the negative effect of the increase in glutamine levels.

In addition, the post-translational modulation of NR activity by phosphorylation/dephosphorylation changes to the enzyme protein might also be important in decreasing NR activity (Huber et al. 1992; Kaiser et al. 1993). Hence, the differences in NR activity in vivo between the constitutive NR-expressors and the WT controls may be less than those estimated by measurements of total NR activity. Nevertheless, the measured differences in foliar glutamine and NO_3^- contents suggest that NR activity is greater in the constitutive expressors than in the WT.

This study with transgenic plants expressing constitutive NR confirms our earlier hypothesis that the overexpression of this single enzyme is insufficient to enhance N metabolism so as to influence biomass production (Foyer and Ferrario 1994). Other limiting steps clearly exist. When NR is deregulated other levels of control are imposed. This might occur, for example, at the level of NO_3^- uptake or transport and/or at the metabolic level of the co-regulation of N and C assimilation.

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