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Tungstate, a Molybdate Analog Inactivating Nitrate Reductase, Deregulates the Expression of the Nitrate Reductase Structural Gene

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ABSTRACT

Nitrate reductase (NR, EC 1.6.6.1) from higher plants is a homodimeric enzyme carrying a molybdenum cofactor at the catalytic site. Tungsten can be substituted for molybdenum in the cofactor structure, resulting in an inactive enzyme. When nitrate-fed *Nicotiana tabacum* plants were grown on a nutrient solution in which tungstate was substituted for molybdate, NR activity in the leaves decreased to a very low level within 24 hours while NR protein accumulated progressively to a level severalfold higher than the control after 6 days. NR mRNA level in molybdate-grown plants exhibited a considerable day-night fluctuation. However, when plants were treated with tungstate, NR mRNA level remained very high. NR activity and protein increased over a 24-hour period when nitrate was added back to N-starved molybdate-grown plants. NR mRNA level increased markedly during the first 2 hours and then decreased. In the presence of tungstate, however, the induction of NR activity by nitrate was totally abolished while high levels of NR protein and mRNA were both induced, and the high level of NR mRNA was maintained over a 10-hour period. These results suggest that the substitution of tungsten for molybdenum in NR complex leads to an overexpression of the NR structural gene. Possible mechanisms involved in this deregulation are discussed.

In higher plants, the regulation of NR[1] has been studied extensively under various physiological conditions. A number of factors such as light, mineral nutrition, growth regulators and environmental stress have been shown to affect its activity (21). The precise mechanisms by which these factors modulate NR expression are, at present, not well understood. Molecular tools such as NR monoclonal antibodies (8) and a NR cDNA probe (3) have been obtained in our laboratory to study the regulation of the NR gene expression in tobacco.

Higher plant NADH-NR (EC 1.6.6.1) is a nuclear gene-encoded homodimer, having a subunit mol wt of 105,000 to 115,000 and containing flavin adenine dinucleotide, heme-Fe, and molybdenum cofactor as prosthetic groups (4). Molybdenum is directly implicated in the electron transfer for nitrate reduction. Tungsten, a metal classified with Cr, Mo, and U in the Mendeleieff table, can compete with molybdenum for incorporation into the enzyme complex and results in enzyme inactivation (15, 17). In this work, we show that the substitution of tungstate for molybdate in the nutrient solution enhances strongly the expression of the NR structural gene.

[1] Abbreviations: NR, nitrate reductase; rbcS, ribulose bisphosphate carboxylase small subunit.

MATERIALS AND METHODS

Plant Material and Experimental Conditions

Tobacco seeds (*Nicotiana tabacum* cv Xanthi) were germinated and grown on a sand-peat mixture (1/1, v/v) in the greenhouse. A nitrate and ammonium-containing nutrient solution (10) was applied daily. After 1 month, the seedlings were transferred to a controlled culture room for experimentation under the following conditions: 85% RH, 16 h photoperiod (180 $\mu mol \cdot m^{-2} \cdot s^{-1}$, fluorescent lamps), 25°C/18°C day-night temperature cycle.

In the first series of experiments, plants were grown in aeroponic devices and supplied for 15 d with a "basal nutrient solution" (containing nitrate as sole nitrogen source): 3.9 mM KNO_3, 3 mM $Ca(NO_3)_2$, 1.1 mM KH_2PO_4, 0.75 mM $MgSO_4$, 0.2 mM NaCl, 0.05 mM $Mg(NO_3)_2$, and the micronutrients of the nutrient solution of Coïc and Lesaint (10). Tungstate (Na_2WO_4, 150 μM) was then substituted for molybdate (Na_2MoO_4, 0.28 μM) in the nutrient solution. Nutrient solutions were changed every day. Over a 12-d period after the addition of tungstate, plants were harvested at the beginning and the end of the light period.

In a second series of experiments, plants were grown in hydroponic cultures (four plants per container containing 8 L of nutrient solution constantly aerated and changed every day). Plants were fed with the basal nutrient solution for 15 d, and then fed with a nutrient solution lacking nitrogen compounds and molybdate (N-deficient nutrient solution). The macroelement composition of the N-deficient nutrient solution was as follows: 1 mM KH_2PO_4, 0.75 mM $CaSO_4$, 0.75 mM $CaCl_2$, 0.45 mM K_2SO_4, 0.4 mM $MgSO_4$, 0.3 mM K_2HPO_4, and 0.2 mM NaCl. After 5 d of N-starvation, molybdate (0.28 μM) or tungstate (150 μM) was added to the N-deficient nutrient solution. Eight days after the beginning of the N-starvation, at the end of the night period, starved plants supplied previously with molybdate were transferred to the basal nutrient solution; starved plants supplied previously with tungstate were either transferred to the basal nutrient solution containing tungstate (150 μM), or kept on N-deficient nutrient solution containing tungstate (150 μM). Control plants were maintained continuously in the basal nutrient solution without N-starvation. During the induction experi-

ment, plants were kept under continuous illumination and sampled at intervals.

Molecular and Biochemical Analysis of NR Expression

For sampling, four or five plants were harvested at random. The two youngest, fully expanded leaves were taken from each plant. After elimination of the main veins, leaves were cut into fragments (*ca.* 2 cm^2), mixed, divided into 3-g samples, and frozen immediately in liquid nitrogen. Samples were stored at −80°C until analysis.

Total RNA was extracted according to a procedure derived from Chirgwin *et al.* (9) as described previously (13). Northern blot analyses were performed according to Thomas (23). Identical amounts of 8 μg/sample) of total RNA prepared from different leaf samples were fractionated in formaldehyde agarose denaturing gels by electrophoresis and blotted on nitrocellulose filters. Tobacco NR mRNA levels were detected by hybridization with a ^{32}P-labeled partial cDNA clone of tobacco NR mRNA, pBMC 102010, previously described (3). In some cases, Northern blots were also hybridized with a cDNA probe coding for the ribulose bisphosphate carboxylase small subunit (rbcS) of *Nicotiana sylvestris* (19).

NR extraction was performed as previously reported by Galangau *et al.* (13). NADH-NR activity was measured according to Wray and Filner (24). NR protein amounts were estimated by a two-site ELISA measurement as described by Chérel *et al.* (8). The monoclonal antibody ZM96(9)25 directed against corn NR was used as the first coating reagent. After washes, the crude NR extract was added. A rabbit polyclonal antiserum directed against corn NR was used as the second antibody. An anti-rabbit immunoglobulin G alkaline phosphatase conjugate (Sigma) was then added and NR amount was titrated by performing the phosphatase assay. The specificity of the ELISA measurements was verified in all experiments with a corn NR monoclonal antibody described previously (7), 7(10)12, which gave no cross reaction with tobacco NR. For quantification, ELISA measurements were standardized with NR purified from the leaves of *N. tabacum* cv Xanthi. Protein contents were determined as described by Bradford (2). Nitrate concentrations were measured according to Cataldo *et al.* (5). All measurements were made two or three times.

RESULTS

Effect of Tungstate Treatment on NR Activity and Its Influence on Nitrate and Protein Contents

NR activity and soluble protein contents were measured in the leaves of control and tungstate-treated plants. In control plants, NR activity decreased by 30% over a 12-d growth period while soluble protein contents were nearly constant (Fig. 1, A and B).

Addition of tungstate (150 μM) to the nutrient solution induced a rapid decrease of NR activity in the leaves (Fig. 1A). After 24 h of treatment, NR activity had already decreased to 20% of the control and it continued to decline to a very low level (about 5% of the control). The decrease in NR activity was accompanied by a drop in the amount of

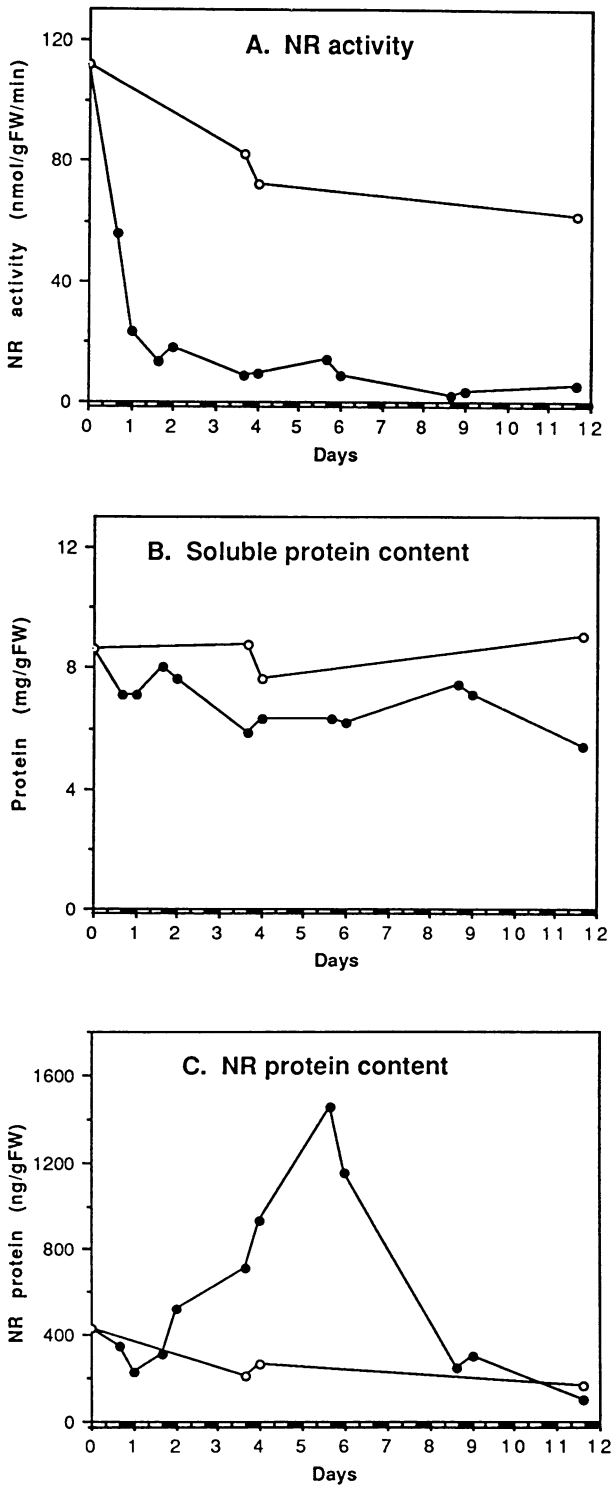

Figure 1. Effect of tungstate on NR activity (A), soluble protein (B), and NR protein content (C) in tobacco leaves. Plants were grown in aeroponic culture in a growth chamber as described in "Materials and Methods" and were supplied either permanently with 10 mM nitrate as sole nitrogen source (control, ○), or from the d 0, with the same nutrient solution containing 150 μM tungstate instead of 0.28 μM molybdate (●). At the days indicated, plants were harvested both at the beginning and the end of the light period. The light-dark regime (16–8 h) is represented by clear sections (light period) and dark sections (dark period) on the abscissa.

soluble protein in the leaves (Fig. 1B) and an increase in nitrate content (50% higher than the control after 12 d of treatment, data not shown). The growth of treated plants was also significantly decreased (data not shown).

Effect of Tungstate Treatment on NR Protein Level

Over a 12-d experiment, NR protein content in control plants exhibited some decrease (Fig. 1C), which paralleled somewhat to the loss of NR activity (Fig. 1A). When plants were grown in the presence of tungstate, the evolution of NR protein content was characterized by three distinct phases. During the first 3 d, it did not differ significantly from the control. It then increased strongly and reached a maximum (7-fold accumulation as compared to control plants) 6 d after the beginning of the tungstate treatment. During the following 6 d NR protein content decreased rapidly and became comparable to that in control plants.

Effect of Tungstate Treatment on NR mRNA Level

NR mRNA level in the leaves of control plants exhibited a characteristic diurnal pattern, being highest at the beginning and lowest at the end of the light period, as shown previously (13). When plants were grown with tungstate in the culture medium, the diurnal rhythm of NR mRNA level was markedly affected (Fig. 2). In treated plants, NR mRNA level at the beginning of the light period exhibited some increase as a function of the duration of treatment time. However, the NR mRNA level at the end of the light period was very strongly enhanced, reaching a maximum at the 9th d.

The variation of NR mRNA level during the light period was studied in more detail (Fig. 3) on the 9th d of tungstate treatment where the diurnal fluctuation of NR mRNA amount was most reduced (Fig. 2). In treated plants, NR mRNA level at the beginning of the light period was somewhat higher than in control plants, and this level remained high during the following light period, as opposed to control plants (molybdate-grown) where the amount of NR mRNA decreased rapidly during the light period and became almost undetectable after 8 h.

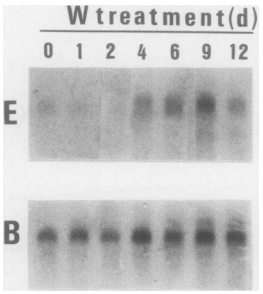

Figure 2. Effect of tungstate on NR mRNA level in tobacco leaves. Experimental conditions are given in the legend to Figure 1. Total RNA was extracted from the leaves sampled at the beginning (lane B) and the end (lane E) of the light period. These RNA preparations were used for Northern blotting experiments. Numbers indicated above the lanes refer to the days of tungstate treatment. Each lane corresponds to 8 μg total RNA fractionated by formaldehyde-agarose gel electrophoresis, and probed with a ^{32}P-labeled NR cDNA after transfer to a nitrocellulose membrane as described in "Materials and Methods."

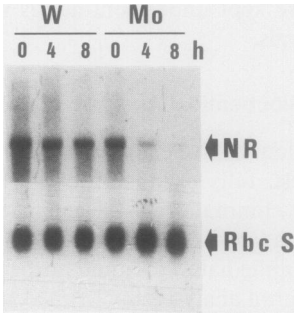

Figure 3. Variations in NR and rbcS mRNA levels in the leaves of tobacco plants grown with nitrate and molybdate (Mo) or nitrate and tungstate (W) during the light period of a day cycle. Experimental conditions are given in the legend to Figure 1. At the 9th day of tungstate treatment, plants were harvested at intervals during the light period. Northern blot analysis was performed on a sample of 8 μg of total RNA extracted from leaves. Fractionated RNAs were firstly probed with a NR cDNA fragment (*upper panel*) and then reprobed with a rbcS cDNA fragment (*lower panel*).

The mRNA level of rbcS from tobacco leaves did not vary significantly during the light period (Fig. 3) and tungstate treatment had no effect on the level of rbcS mRNA.

NR Induction by Nitrate in the Presence of Tungstate in N-Starved Plants

After 8 d of nitrogen deprivation, leaf nitrate content and NR activity were very low (Fig. 4, A and B). To study the influence of tungstate on the process of NR induction, N-starved plants were treated with tungstate before nitrate application to prevent the presence of interfering molybdate during the induction process. When N-starved plants were supplied with nitrate and molybdate, nitrate gradually accumulated in the leaves, and NR activity was induced, with an apparent lag of 2 h. After 24 h of continuous illumination, NR activity had increased to about 60% of that in control plants (constantly fed with nitrate and molybdate). In the presence of tungstate, nitrate accumulation was enhanced 2-fold as compared to N-starved plants supplied with nitrate and molybdate but NR activity was not induced.

NR protein levels increased to a similar extent in N-starved plants supplied with nitrate and molybdate, or in N-starved plants supplied with nitrate and tungstate (Fig. 4C).

As shown in Figure 5, NR mRNA levels in N-starved plants and in control plants sampled at the beginning of the light period were comparable. However, nitrate replenishment to N-starved plants caused a very important increase in NR mRNA level (Fig. 5). When N-starved plants were supplied with nitrate and molybdate, NR mRNA level reached a maximum after 2 h (corresponding to a 100-fold increase of the initial level). This maximum was followed by a marked decrease. After 12 h, the amount of NR mRNA was reduced to 10% of the maximum value. When N-starved plants were supplied with nitrate and tungstate, the amount of NR mRNA was also induced to a similar maximum level at 2 h. However,

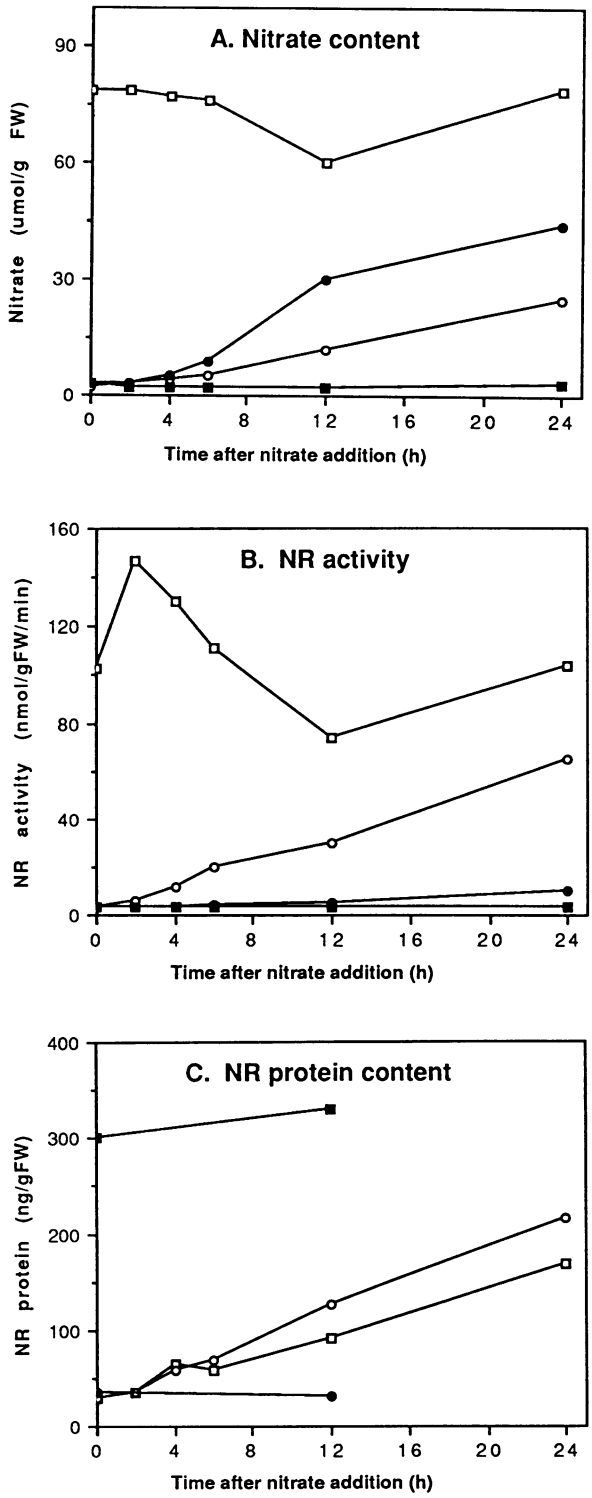

Figure 4. Changes in nitrate content (A), NR activity (B), and NR protein content (C) in the leaves of N-starved tobacco plants after nitrate replenishment. Plants were grown in hydroponic culture. Experimental conditions are described in "Materials and Methods." At time 0, N-starved plants were fed with 10 mM nitrate and 0.28 μM molybdate (○), or 10 mM nitrate and 150 μM tungstate (●), at the beginning of a continuous illumination following the normal dark period of a 16/8-h light-dark regime. Two types of controls were performed: plants permanently maintained on a nitrate and molybdate-containing nutrient solution (□) and N-starved plants, treated with tungstate alone (■). NR protein contents (C) in control plants were measured only at times 0 and 12 h.

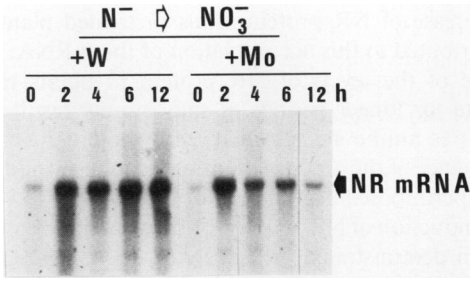

Figure 5. Northern blot analysis of NR mRNA level in the leaves of N-starved tobacco plants subsequently supplied with nitrate and molybdate (Mo) or nitrate and tungstate (W). Experimental conditions are described in the legend to Figure 4. Samples were collected at the time indicated (h) after nitrate application and RNA analysis was performed as described in the legend to Figure 2.

this high NR mRNA level remained constant during the subsequent 10 h (Fig. 5).

DISCUSSION

The effect of tungstate treatment on nitrate metabolism in higher plants has been studied in several laboratories. Tungstate was found to decrease the extractable NR activity (15). This effect of tungstate was attributed to the prevention of the formation of an active molybdenum cofactor indispensable for NR catalytic activity. This hypothesis was supported by the fact that NADH-Cyt *c* reductase activity of NR apoenzyme (partial activity independent of molybdenum cofactor) was not affected (24). Notton and Hewitt (17) provided further evidence for the synthesis of a tungsto-NR analog by radioactive ^{185}W labeling experiments. The inactivation of NR by tungstate was generally correlated with an accumulation of nitrate in plant tissues, implying that *in vivo* nitrate reduction really is affected (15). Measurements of NR protein in tungstate-treated plants by ELISA tests clearly confirm the synthesis of a catalytically inactive NR protein as reported previously (17, 24). Tungstate, therefore, inactivates NR without preventing the synthesis of the enzyme.

Over a 12-d experiment, an important, but transient, accumulation of NR protein was observed in tungstate-treated plants (Fig. 1C). In order to investigate whether the overproduction of NR protein reflects a modified availability of NR mRNA for the protein synthesis, Northern blotting experiments were carried out, using a previously characterized NR cDNA from tobacco as the probe (3). In control plants (molybdate-grown), it was found that the levels of NR mRNA fluctuate under a light-dark regime, as reported previously (13). When plants were treated by tungstate, the diurnal fluctuation of NR mRNA level was progressively attenuated. After 9 d of tungstate treatment, NR mRNA level at the end of the light period was much higher than that of control plants (Fig. 2). The substitution of tungsten for molybdenum in the NR protein, therefore, induces not only an accumulation of NR apoenzyme, but also that of the corresponding mRNA.

The increase of NR protein levels in treated plants can be thus attributed to this accumulation of the mRNA. As to the decrease of the levels of NR protein in plants treated by tungstate for longer periods, it may simply result from the lack of free amino acids available for protein synthesis, as a consequence of the absence of nitrate reduction, and/or result from superimposed degradation of NR protein.

The induction of NR activity, protein and mRNA by nitrate has been demonstrated previously (6, 13, 20, 22). Although the diurnal pattern of NR mRNA level was deregulated when tungstate was substituted for molybdate in the culture medium, NR mRNA and protein remained clearly inducible by nitrate. In addition, the level of NR mRNA induced by nitrate in N-starved molybdate-grown plants underwent an important decrease, appearing to be subjected to the diurnal regulation. However, this pattern of expression was not observed when tungstate was present in the nutrient solution. On the other hand, the enhancement of the accumulation of NR mRNA in N-starved plants subsequently supplied with nitrate and tungstate did not lead to any enhancement of the amount of NR protein (Fig. 4C), as compared to starved plants subsequently supplied with nitrate and molybdate. This is similar to the situation occurring after the longer periods of tungstate treatment as discussed above, and thus could be explained in the same way. However, this may also result from a specific inhibition of NR mRNA translation induced by N-starvation, as suggested by previous studies (13). Additionally, an overinduction of NADH-Cyt *c* reductase activity specifically linked to NR apoenzyme was observed in N-starved barley (24) and corn leaves (1) supplied subsequently with nitrate in the presence of tungstate, suggesting an overproduction of NR protein in these cases.

How could the substitution of tungstate for molybdate in the nutrient solution result in the overexpression of the NR structural gene at the mRNA level? Tungstate treatment did not affect the level of rbcS mRNA (Fig. 3), which indicates that the deregulation of NR expression is not due to a general disorder of the gene expression system. Whether this deregulated expression of NR is a direct consequence of modified transcription rate of the NR gene is not known. Nuclear runon experiments are currently being performed to study this possibility. It can also be envisaged that a modified rate of translation of NR mRNA leads to a modified stability of this mRNA. Cell-free translation studies could help to explore this possibility.

NR repression by nitrogen metabolites has been well demonstrated in fungi (12, 14). Exogenous supplies of amino acids reduced NR activity in tobacco cell culture (11) and in corn roots (18), and decreased both NR activity and NR protein level in squash cotyledons (16). Therefore, it is possible that the expression of higher plant NR is also regulated in response to the status of reduced nitrogen in the tissues. The blockage of nitrate reduction by tungstate may limit the nitrogen metabolite content to a low level, and thus result in overexpression of NR structural gene.

Alternatively, taking into account the inducer role of nitrate, it has been proposed that the higher nitrate content in tungstate-treated plants may result in NR overinduction (24). However, when N-starved plants were supplied with nitrate and molybdate, nitrate content increased continuously (Fig. 4A) while NR mRNA level decreased from 2 h to 12 h (Fig. 5). Therefore, this explanation appears unlikely.

Gene expression may be regulated at different levels including transcription, mRNA stability, translation and post translational modification. The present results suggest that tungsten substitution for molybdenum in the NR complex deregulates NR gene expression by increasing strongly both NR mRNA and NR protein levels. The generality of these observations in tungstate-treated plants is currently being further assessed in our laboratary by studying the consequence of mutations impairing NR activity on the expression of its own structural gene (results to be published). As discussed above, we assume that the regulation of NR expression involves a negative feed-back effect of one or several of the metabolites derived from the catalytic process of nitrate reduction, such as nitrite, ammonium or glutamine. Work is in progress to verify the validity of this hypothesis.

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