Plant Physiol. (1991) 97, 873–879
0032-0889/91/97/0873/07/$01.00/0

Received for publication January 15, 1991
Accepted June 30, 1991

Effect of Chlorate Treatment on Nitrate Reductase and Nitrite Reductase Gene Expression in *Arabidopsis thaliana*[1]

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ABSTRACT

The herbicide chlorate has been used extensively to isolate mutants that are defective in nitrate reduction. Chlorate is a substrate for the enzyme nitrate reductase (NR), which reduces chlorate to the toxic chlorite. Because NR is a substrate (NO_3^-)-inducible enzyme, we investigated the possibility that chlorate may also act as an inducer. Irrigation of ammonia-grown *Arabidopsis* plants with chlorate leads to an increase in NR mRNA in the leaves. No such increase was observed for nitrite reductase mRNA following chlorate treatment; thus, the effect seems to be specific to NR. The increase in NR mRNA did not depend on the presence of wild-type levels of NR activity or molybdenum-cofactor, as a molybdenum-cofactor mutant with low levels of NR activity displayed the same increase in NR mRNA following chlorate treatment. Even though NR mRNA levels were found to increase after chlorate treatment, no increase in NR protein was detected and the level of NR activity dropped. The lack of increase in NR protein was not due to inactivation of the cells' translational machinery, as pulse labeling experiments demonstrated that total protein synthesis was unaffected by the chlorate treatment during the time course of the experiment. Chlorate-treated plants still retain the capacity to make functional NR because NR activity could be restored by irrigating the chlorate-treated plants with nitrate. The low levels of NR protein and activity may be due to inactivation of NR by chlorite, leading to rapid degradation of the enzyme. Thus, chlorate treatment stimulates NR gene expression in *Arabidopsis* that is manifested only at the mRNA level and not at the protein or activity level.

Chlorate (ClO_3^-), the chlorine analog of nitrate, is used extensively as an herbicide. Over 4 million pounds of chlorate were used on California cotton fields in 1988 alone (34). Plants exposed to chlorate display various symptoms: root growth is severely inhibited and leaves yellow, wither, and die. The mechanism responsible for chlorate toxicity was first investigated in 1947 by Borje Åberg using young wheat plants (1). He found that chlorate was slow to act as a toxin, that not all cells were sensitive to chlorate, and that toxicity was enhanced by light. These results suggested to Åberg that some derivative of chlorate produced within the plant cell and not chlorate itself was the proximal toxin. Indeed, plants were found to reduce chlorate *in vivo,* and the reduction products chlorite (ClO_2^-) and hypochlorite (ClO^-) were shown to be rapidly acting toxins that poisoned all cell types tested. To elucidate how chlorate might be reduced in plant cells, Åberg noted that (a) chlorate is chemically similar to nitrate, (b) high quantities of nitrate depress the toxic effects of chlorate, and (c) the conditions and localization of chlorate toxicity correlate with the ability of plant cells to reduce nitrate. From these observations, Åberg formulated the hypothesis that chlorate toxicity is dependent on the reduction of chlorate to a toxic compound, probably chlorite, by the same mechanism that reduces nitrate to nitrite. Although there are some exceptions (10, 11), now most data strongly support Åberg's hypothesis and implicate NR[2] as the enzyme that reduces chlorate to chlorite. Chlorate is a substrate of purified NR and a competitive inhibitor of nitrate reduction (26, 32). Conversely, nitrate acts as a competitive inhibitor of chlorate reduction, and cyanate, which inhibits NR *in vitro,* reduces chlorate toxicity *in vivo* (23). Finally, many chlorate resistant mutants have been isolated and characterized, and most of these mutants have been found to be impaired in nitrate reduction (38).

The toxic effects of chlorate have been exploited not only as a means to eliminate plant life but also as a powerful tool to select for mutants with defects in nitrate reduction. Chlorate resistant mutants have been isolated from bacteria, fungi, and plants (21). These mutants are usually defective in NR due to lesions in either the NR structural gene or one of the six to seven genes that are required for the synthesis of a MoCo, an essential component of the NR holoenzyme (21). The first plant species that was used to isolate chlorate resistant mutants was *Arabidopsis thaliana* (4). Dozens of resistant mutants were isolated and found to comprise eight complementation groups (5). We have identified one of these loci, *chl3,* as the NR structural gene *NIA2* (37). Other loci have been shown to be required for MoCo synthesis (e.g. B25) (7). Such mutants have been invaluable for studying the structure, function, and regulation of NR (8, 12).

During these studies on the mechanism of chlorate resistance in *Arabidopsis,* an interesting observation was made: plants grown in the absence of nitrate are much more sensitive to chlorate than plants grown with high levels of nitrate (33). When plants are irrigated with 5 mM nitrate and treated with 2 mM chlorate, leaf chlorosis appears after 4 to 5 days, followed shortly thereafter by the death of the plant. When plants are grown on ammonia, in the absence of nitrate, they are much more sensitive to chlorate and display similar symptoms at one-hundredth the concentration of chlorate, 0.02 mM (our

[1] This work was supported by grants to N.M.C. from the Powell Foundation and the National Institutes of Health (1R29GM40672-01) and to S.T.L. from the San Diego Fellowship program.

[2] Abbreviations: NR, nitrate reductase; NiR, nitrite reductase; MoCo, molybdenum cofactor.

unpublished results). This result is surprising because chlorate toxicity depends on the reduction of chlorate to chlorite by NR, and plants grown in the absence of nitrate have low levels of NR activity. One explanation for this effect may reside in the specificity of NR: the K_m for chlorate is 50 to 100 times greater than for nitrate (26, 32). Thus, by acting as a competitive inhibitor of chlorate reduction, nitrate protects the plant. When plants are grown in the absence of nitrate, chlorate has no competition for NR binding and is efficiently reduced by the low levels of NR. As described above, this protective effect of nitrate against chlorate toxicity was one of the observations that originally led Åberg to propose that the nitrate reducing system in plant cells was involved in chlorate poisoning (1).

Another possible explanation for the heightened sensitivity of *Arabidopsis* plants grown without nitrate is that chlorate treatment stimulates the expression of the NR gene, thereby increasing the level of NR and the production of chlorite. It has long been known that nitrate is both the physiological substrate and inducer for NR (16, 35). Chlorate is also a substrate for NR; maybe it too acts as an inducer. To test this idea, we decided to investigate the possible effects that chlorate treatment might have on the expression of the NR gene. Previous experiments on the effects of chlorate have indicated that NR activity levels decrease after chlorate treatment (19), suggesting that chlorate does not induce NR gene expression. However, it has been shown that chlorite inactivates purified *Chlorella* NR *in vitro* (32). Thus, it is difficult to assess the effect of chlorate treatment on NR gene expression by measuring NR activity because the treatment may lead to inactivation of the enzyme. Therefore, we examined the effect of chlorate treatment on NR mRNA and protein levels through the use of recently obtained NR cDNA clones (14) and anti-NR antibodies (see "Materials and Methods"). Our results are described below.

MATERIALS AND METHODS

Plant Material and Growth

Arabidopsis thaliana (Columbia) seeds were obtained from Dr. George Redei (University of Missouri). The chlorate resistant mutant B25 and the parent (erecta) are both from the Landsberg ecotype and were a gift from Drs. Braaksma and Feenstra (University of Groningen) (7). Plants were grown in a 1:1 mixture of vermiculite and perlite with continuous light and subirrigated three times weekly with a nutrient solution containing: 5 mM potassium phosphate, pH 5.5, 2 mM $MgSO_4$, 1 mM $CaCl_2$, 0.1 mM Fe-EDTA, 50 μM H_3BO_3, 12 μM $MnSO_4$, 1 μM $ZnCl_2$, 1 μM $CuSO_4$, 0.2 μM Na_2MoO_4, and 2 mM $(NH_4)_2SO_4$. Chlorate treatments were performed using the same media and 50 mM $KClO_3$ (Fisher). Leaves and stems were harvested after 2 to 3 weeks of growth and stored at −80°C.

Protein Procedures

Leaf tissue was ground using Duall ground glass homogenizers in 0.1 M potassium phosphate, pH 7.5, 1 mM DTT, 1 mM EDTA, and the protease inhibitors leupeptin and pepstatin. The homogenate was centrifuged for 5 min at 10,000*g* and the supernatant was recovered and used as the protein extract for enzyme assays and immunoblots.

The NR enzyme assay was performed as described (29); one unit of activity is defined as the production of 1 μmol nitrite/min at 30°C.

Antibodies were prepared against the NR protein encoded by the *NIA2* gene of *Arabidopsis* by expressing the *NIA2* cDNA (from pAtc46) (14) in bacteria using the pET-3c vector (30) and injecting rabbits with the gel-purified NR protein isolated from induced bacteria.

NR protein was analyzed by immunoblotting. One hundred micrograms of protein from the protein extract was separated by 9% SDS-PAGE and then transferred electrophoretically to nitrocellulose in 25 mM Tris, pH 8.3, 192 mM glycine, and 20% methanol. The anti-NR antisera, biotinylated goat anti-rabbit antibody, and streptavidin-alkaline phosphatase were used for detecting the NR protein bound to the membrane as described in the manufacturer's protocol (Gibco BRL).

Nucleic Acid Procedures

RNA was isolated from leaf tissue as described (15) with the following changes. Tissue was homogenized in extraction buffer and phenol using a Brinkman polytron homogenizer. The homogenate was centrifuged and the aqueous layer collected. LiCl (8 M) was added to one-third volume and the milky solution left overnight at 4°C. This solution was centrifuged for 10 min at 10,000*g* and the supernatant discarded. The pellet was washed twice each with 2 M LiCl and 80% ethanol, air dried, and resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA. Poly(A)$^+$ RNA was purified from the total RNA sample using oligo(dT) cellulose chromatography, yielding 7 to 10 μg/g tissue (fresh weight) (36). RNA blot analysis was performed as described (13), except that poly (U) was omitted.

The *Arabidopsis* NiR cDNA clone was isolated from a λgt11 cDNA library following methods previously described (14) and using a spinach NiR cDNA clone as probe (pC113400) (3). The *Arabidopsis* NiR cDNA clone was used to isolate an *Arabidopsis* genomic clone and both clones were treated with DNaseI to construct nested deletion sets as described (2). Sequences were obtained using the dideoxynucleotide chain-termination method (31). DNA and predicted protein sequence analysis were performed using the MacVector programs from IBI.

RNA blots were hybridized with DNA probes labeled using random hexamers (17). The NR probe was a cDNA clone of the *Arabidopsis* NR gene *NIA2* (pAtc46) (14). The NiR probe was a cDNA clone of the *Arabidopsis* NiR gene (pALC-78). The tubulin probe was a genomic clone of the *Arabidopsis* β-tubulin gene (pATB-4) (24).

In Vivo Labeling

Fresh detached leaves were weighed and placed in 3 mL Murashige and Skoog media (Sigma) with 50 μCi [^{35}S]methionine and vacuum-infiltrated three times for 30 s. The leaves were incubated for 2 h at 30°C. After the incubation, the leaves were ground in 0.5 mL of 1 mM methionine, the extract centrifuged, and the supernatant added to 4.5 mL 5% TCA. This solution was filtered through glass fiber filters, which

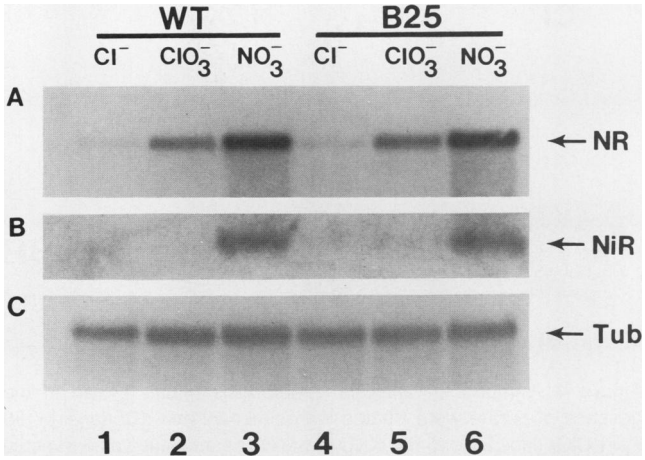

Figure 1. RNA blot analysis. *Arabidopsis* plants, wild-type (lanes 1–3) and B25 (lanes 4–6), grown in the absence of nitrate (with ammonia as the nitrogen source) were treated with either 50 mM KCl (lanes 1 and 4), 50 mM $KClO_3$ (lanes 2 and 5), or 5 mM KNO_3 (lanes 3 and 6). Leaf tissue was harvested after 16 h and poly(A)$^+$ RNA was isolated, electrophoresed, and transfered to a nylon membrane. The RNA blot was hybridized with ^{32}P-labeled DNA inserts from an NR *NIA2* cDNA clone (box A), a NiR cDNA clone (box B), and a β-tubulin genomic clone (box C) all from *Arabidopsis* as described in "Materials and Methods."

were then washed three times with 5% TCA and once with 95% ethanol. The filters were dried for 1 h at 65°C, placed in scintillation vials with Ecolume scintillation fluid (ICN), and radioactivity determined using a Beckman LS-930 scintillation counter. Background levels of [^{35}S]methionine incorporation were determined by incubating vacuum-infiltrated leaves at 0°C for 2 h before extraction.

RESULTS

We first determined the effect of chlorate treatment on leaf NR mRNA accumulation in *Arabidopsis*. Ammonia-grown plants, having low uninduced levels of NR activity, were subirrigated with nutrient media containing either 50 mM chloride (uninduced control), 50 mM chlorate, or 5 mM nitrate (induced control). Leaf tissue was harvested after 16 h, when no chlorosis or toxic effects had been observed. This time point was chosen to minimize metabolic effects due to toxicity and maximize the effect on NR gene expression. Poly(A)$^+$-RNA was prepared for RNA blot analysis as described in "Materials and Methods." The RNA blot was hybridized with radiolabeled NR cDNA (pAtc46), encoding the major leaf NR in *Arabidopsis* (*NIA2*) (9, 14, 37). As seen in Figure 1, NR mRNA levels increased after both nitrate (lane 3) and chlorate (lane 2) treatments compared with the chloride control (lane 1). Small increases of NR mRNA were also observed following treatment with lower levels of chlorate (5 mM) or at early time points (4–8 h) (our unpublished results). Thus, chlorate treatment results in an increased accumulation of NR mRNA.

The analysis of NR mRNA accumulation in response to chlorate treatment was also performed with a MoCo mutant of *Arabidopsis*. This chlorate resistant mutant, B25, is defective in nitrate and chlorate reduction due to a defective MoCo and has about 5 to 10% of wild-type NR activity (6). When B25 plants were grown on ammonia and then treated with nitrate, NR mRNA levels increased, as seen in Figure 1 (lane 6). When ammonia-grown plants were treated with chlorate, NR mRNA also accumulated (lane 5), the same response observed in wild-type plants. Thus, NR mRNA can be induced by nitrate and chlorate treatment even in a MoCo mutant.

NiR, the second enzyme in the nitrate assimilatory pathway, is also induced by nitrate (3). We wanted to test if NiR mRNA levels also responded to chlorate treatment. We isolated and partially sequenced *Arabidopsis* cDNA and genomic clones that are homologous to a spinach NiR cDNA clone as described in "Materials and Methods." Partial predicted protein sequences of the *Arabidopsis* DNA clones were compared with the predicted protein sequence of the spinach NiR cDNA. As shown in Figure 2, this comparison revealed that the two sequences are very similar, with 73% identity (93% if conserved amino acid changes are allowed), and that the *Arabidopsis* clones did indeed encode NiR. The NiR cDNA clone (pALC078) was then used to analyze NiR mRNA levels. The RNA blot in Figure 1 was hybridized with the radiolabeled NiR cDNA. As expected, the NiR mRNA levels increased after nitrate treatment (lanes 3 and 6), but chlorate treatment did not result in any accumulation of NiR mRNA (lanes 2 and 5). NR and NiR are thought to be coordinately regulated in response to nitrate (20, 28), but it appears that NiR mRNA does not respond to chlorate treatment as does NR mRNA.

We next investigated the effect of chlorate treatment on NR protein levels by immunoblotting. Anti-NR antibodies

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At  svlvAaaqttapAestaSVDAdRLEPRVElkDGFfiLKEkFReGINPqEK  84
sp  CQK-AVSPAAETAAVSPSVDAARLEPRVEERDGFWVLKEEFRSGINPAEK  82

At  VKIErePMKLFw-rwIeeLAkkSMEElDseKssKDDIDVRLKWLGLFHRR  133
sp  VKIEKDPMKLFIEDGISDLATLSMEEVDKSKHNKDDIDVRLKWLGLFHRR  132

At  KHqvYGkFMMRLKLPNwcdySaQTRYLASVIrKYGeDGCADVTTRQNWQI  183
sp  KHH-YGRFMMRLKLPNGVTTSEQTRYLASVIKKYGKDGCADVTTRQNWQI  181

At  RGVVLPelPEIlKGLaSVGLTSLQSGMDNVRNPVGNPiAGIDPeEIVDTR  233
sp  RGVVLPDVPEIIKGLESVGLTSLQSGMDNVRNPVGNPLAGIDPHEIVDTR  231

At  PyTNLlSQFiTANSqGNpdfTNLPRKWNvCVvGtHDLYEHPHINDLAYMP  283
sp  PFTNLISQFVTANSRGNLSITNLPRKWNPCVIGSHDLYEHPHINDLAYMP  281

At  AnKdGrFGFNLLVGGFFSpKRCEEAIPLDAWVpAdDVlPlCKAvLEAyRD  333
sp  ATKNGKFGFNLLVGGFFSIKRCEEAIPLDAWVSAEDVVPVCKAMLEAFRD  331

At  LGtRGNRQKtRMMWLIDEL                                  352
sp  LGFRGNRQKCRMMWLIDEL                                  350
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Figure 2. Protein sequence alignment. Partial predicted protein sequences from *Arabidopsis* cDNA and genomic NiR clones were aligned to the predicted protein sequence of the spinach NiR using the pam250 scoring matrix of MacVector. The *Arabidopsis* sequence is labeled At and the spinach sequence sp. Amino acid matches are signified with a vertical line. The alignment begins with the first amino acid of the mature spinach nitrite reductase (3).

Table I. *Anti-NR Antibodies Inhibit in Vitro NR Activity*

Arabidopsis plants were grown on a peat-soil mix for 3 weeks. A protein extract was made from the leaf tissue as described in "Materials and Methods." The indicated amount of serum (diluted to 1 μL final volume) was combined with 15 μL of the NR protein extract (specific activity 0.01 unit/mg) and incubated for 45 min at 0°C. Subsequently, Protein-A Sepharose was added, incubated for 20 min at 0°C, and cleared by centrifugation. NR enzymatic activity was determined in the supernatant as described in "Materials and Methods." The 100% NR activity level was 0.0003 unit.

Serum	NR Activity	
	Preimmune	Immune
μL	%	
1.0	100	26
0.5	100	32
0.2	98	35
0.01	94	90
0.0	100	100

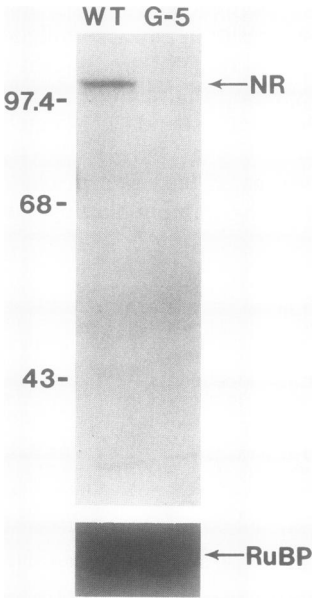

Figure 3. Immunoblot analysis. Extracts from wild-type and *chl3-5* NR deletion mutant plants (marked in figure as WT and G-5, respectively) grown in the presence of nitrate were separated by SDS-PAGE, transferred to nitrocellulose, probed with antibodies specific to NR, and developed as described in "Materials and Methods." The lower panel shows the same filter after staining with napthol blue black, revealing equal amounts of the large subunit of ribulose bisphosphate carboxylase in each lane as a loading control. The position of prestained mol wt markers (Bethesda Research Labs) is shown at the left.

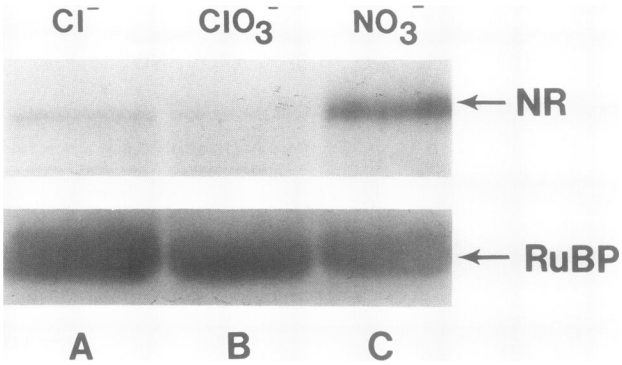

Figure 4. Immunoblot analysis. *Arabidopsis* plants grown in the absence of nitrate were treated with either 50 mM KCl (lane A), 50 mM $KClO_3$ (lane B), or 5 mM KNO_3 (lane C). Leaf tissue was harvested after 16 h and a crude protein extract was made, separated by SDS-PAGE, transferred to nitrocellulose, and probed with antibodies specific to NR as described in "Materials and Methods." The lower panel shows the same filter after staining with napthol blue black, revealing equal amounts of the large subunit of ribulose bisphosphate carboxylase.

were prepared as described in "Materials and Methods" and shown to be specific for NR: the immune sera inhibited NR activity *in vitro* (Table I) and bound to a 110 kD protein from extracts of wild-type plants but not from extracts of a mutant plant (*chl3-5*) in which the *NIA2* gene is deleted (Fig. 3) (37). As shown in Figure 4, ammonia-grown plants that were treated with nitrate (lane C) had a higher level of NR protein than uninduced (chloride treated) plants (lane A). The plants that were treated with chlorate (lane B) have much lower levels of NR protein compared with the plants treated with nitrate. Thus, even though NR mRNA levels increase following chlorate treatment, NR protein levels do not. Two explanations could account for these results: either chlorate treatment enhances the degradation of NR or interferes with the synthesis of the NR protein.

To complete our analysis of the effects of chlorate treatment on NR gene expression, we examined the levels of NR activity in protein extracts made from leaf tissue of chloride-, chlorate-, and nitrate-treated plants. As expected, nitrate-treated plants had over a fivefold increase in NR activity over the chloride-treated plants (Table II). Extracts from chlorate-treated plants, however, had virtually no NR activity, lower even than in extracts from chloride-treated plants (Table II). This reduced level of NR activity did not appear to be due to the action of an inhibitor of NR in the protein extracts. Extracts from chlorate-treated plants were mixed before assaying with extracts from nitrate-treated plants, and no inhibition of NR activity (present in the induced extract) was evident (Table II). For these experiments, we took the precaution to partially purify NR by 40% ammonium sulfate precipitation, which has been reported to remove an inhibitor of NR (7). Thus, chlorate treatment reduces the level of NR activity that can be assayed in leaf extracts from treated plants.

A possible explanation for the very low levels of NR protein in chlorate-treated plants that have moderate levels of NR mRNA is that the normal metabolism of the plant cell is

Table II. *Effect of Chlorate Treatment on NR Activity Levels*

Arabidopsis plants were grown on 2 mM $(NH_4)_2SO_4$ without nitrate as the source of nitrogen for 2 weeks and then treated with either 50 mM KCl (uninduced control) (line 1), 50 mM $KClO_3$ (line 2), or 5 mM KNO_3 (line 3). After 16 h, leaf tissue was harvested, extracts were made, NR was partially purified by 40% $(NH_4)_2SO_4$ precipitation, and used for the NR enzyme assay as described in "Materials and Methods." In the last line, equal volumes of extract from chlorate-treated plants and nitrate-treated plants were mixed before the assay. The specific activity recorded is per milligram of the nitrate-treated plant extract alone after subtracting the contribution from the chlorate-treated plant extract.

Extract	NR Specific Activity	Percent Activity
	unit/mg	
Chloride	0.005	17
Chlorate	0.0004	1
Nitrate	0.028	100
Nitrate + chlorate	0.028	100

disrupted so that cellular protein synthesis is impaired and no NR protein can be synthesized. We tested this possibility in two ways. First, we examined the rate of total protein synthesis in chlorate-treated plants by measuring the incorporation of methionine into total protein. As seen in Table III, after 16 h, leaves from plants treated with chlorate incorporate [^{35}S] methionine as well as leaves from untreated plants, indicating that total protein synthesis is not impaired by the 16 h chlorate treatment. We then tested the 16 h chlorate-treated plants to see if they still retained the capacity to synthesize NR. Plants treated with chlorate were subsequently irrigated with nitrate; 4 h later, *in vitro* NR activity was measured. As shown in Table IV, NR activity levels increased fivefold within 4 h of nitrate treatment (lines 1 and 2), thereby restoring NRA levels to 80% of the maximal level found in nitrate-treated control plants that had not been pretreated with chlorate (line 3). Thus, possible toxic effects of chlorate treatment on cellular metabolism do not appear to explain the absence of NR protein and activity in these plants.

DISCUSSION

We have found that chlorate treatment of *Arabidopsis* plants grown on ammonia results in an increase in leaf NR mRNA levels. However, no corresponding increase in NR protein occurs after chlorate treatment, and NR activity actually decreases to undetectable levels. These effects are observed 16 h after treatment, before any toxicity symptoms become apparent in the leaves. In addition to the effects seen in wild-type plants, chlorate treatment also elicits an increase in NR mRNA in a MoCo mutant (B25) that has low levels of NR activity. NiR mRNA levels do not respond to chlorate, remaining at undetectable levels after chlorate treatment. These observations describe some of the physiological effects that chlorate treatment has on the expression of genes in the nitrate assimilation pathway and provide clues on the mechanism of induction of NR.

It was surprising that chlorate treatment of *Arabidopsis* resulted in an increase of NR mRNA. Previous published work (19) as well as our own data showed that chlorate treatment of plants resulted in a reduction in NR activity, suggesting that chlorate represses NR expression. However, these results could be misleading because chlorite, the product of chlorate reduction, inactivates NR (32). Even if the NR gene was being induced by chlorate, the induction would be obscured by the inactivation of the enzyme by the reaction product chlorite. NR mRNA levels, on the other hand, should provide a more direct indicator of NR gene expression after chlorate treatment because NR mRNA levels respond quickly (within hours) to nitrate and can be induced even if NR is inactivated (25, 27). By measuring NR mRNA levels, we did observe an increase in response to chlorate treatment. That NiR and tubulin mRNA levels do not also increase indicates that the effect is at least partially specific. That the increase is observed in a MoCo mutant indicates that wild-type levels of active NR are not required for the effect.

Another surprise from our experiments was that NR protein levels did not increase in response to chlorate treatment even though NR mRNA levels did. One possible explanation for this finding is that cellular metabolism is being disrupted after chlorate treatment and that no protein (including NR) could be synthesized. Our results indicate that this is not the case. *In vivo* labeling experiments revealed that total protein synthesis appeared to be unimpaired in the chlorate-treated plants. In addition, it appears that the chlorate-treated plants are still capable of making active NR protein because subsequent treatment of these plants with nitrate restores NR activity to induced levels. A likely explanation for the absence

Table III. *Effect of Chlorate Treatment on Incorporation of [^{35}S] Methionine*

Arabidopsis plants were grown on 2 mM $(NH_4)_2SO_4$ (without nitrate) as the source of nitrogen for 2 weeks and then treated with either 50 mM KCl or 50 mM $KClO_3$ for h. Leaves were removed from the plants, then labeled with [^{35}S]methionine as described in "Materials and Methods." Each value below is the average of three measurements of acid precipitable material and the background incorporation (1.5×10^5 cpm) was subtracted from each value.

Treatment	cpm/g Fresh Weight
Chloride	$1.6 \pm 0.4 \times 10^6$
Chlorate	$1.3 \pm 0.1 \times 10^6$

Table IV. *Rescue of NR Activity in Chlorate-Treated Plants*

Arabidopsis plants were grown on 2 mM $(NH_4)_2SO_4$ without nitrate as the source of nitrogen for 2 weeks and then treated with either 50 mM $KClO_3$ (lines 1 and 2) or 5 mM KNO_3 (line 3). After 16 h, the $KClO_3$-treated plants were divided in two groups and treated with 25 mM KNO_3 (line 2) or 25 mM KCl (line 1). The nitrate-treated plants received no secondary treatment. After 4 additional h, leaf tissue was harvested and assayed for NR activity as described in "Materials and Methods."

Treatment	NR Specific Activity	Percent Activity
	unit/mg	
Chlorate + chloride	5.8×10^{-4}	13
Chlorate + nitrate	3.6×10^{-3}	80
Nitrate	4.5×10^{-3}	100

of NR protein in the chlorate-treated plants is that the NR protein is being rapidly degraded. After chlorate is taken up by the plant, it is reduced to chlorite, which inactivates NR. The inactivated NR may be much more unstable and rapidly degraded. The subsequent addition of nitrate to these plants inhibits the production of chlorite and protects the enzyme from inactivation. This hypothesis explains how one can get an increase in NR mRNA after chlorate treatment but no increase in NR protein and a drop in NR activity.

Because these experiments were done with whole plants, it is not possible to identify the active compound that is directly responsible for enhancing NR mRNA accumulation after chlorate treatment. Any compound that is taken up by the roots can be metabolized before it reaches the leaves where the effects were monitored. It is possible that a metabolite of chlorate may serve as an inducer. Our data argue against this possibility because the same chlorate response is observed in a MoCo mutant that is severely impaired in nitrate and chlorate reduction. If chlorate is the active compound, it would most likely be acting as an analog of nitrate. Nitrate binds to NR and induces the NR gene, presumably by binding to a receptor. Perhaps chlorate also induces the NR gene by binding to the same receptor. If this analogy is correct, it is possible that the binding to the receptor and induction of the NR gene would be weaker for chlorate than for nitrate because NR's affinity for chlorate is much lower than for nitrate. Consistent with this hypothesis was our finding that the chlorate response was weaker than the nitrate response and required higher levels of chlorate than nitrate. From our experiments, we cannot rule out the possibility that there is a small amount of nitrate present in our chlorate stocks that is acting as the inducer. However, the manufacturer's analysis of the chlorate indicates only 0.0008% nitrogen compounds. *Arabidopsis* is very sensitive to nitrate; we find that NR activity increases in leaves after irrigating the plants with only 0.2 mM nitrate, ½5 the level that we normally use (our unpublished results). Thus, it does not take much nitrate to induce NR. However, if nitrate were the active compound that was eliciting an increase in NR mRNA levels, we would have expected the chlorate treatment to increase NiR mRNA levels as well, but it did not.

These experiments confirm the importance of elucidating the regulatory mechanisms that control the expression of the nitrate assimilatory genes. In plants, there are very few clues about the identity of the regulatory genes and proteins in the nitrate assimilation pathway, including the nitrate receptor. One approach that has lead to the discovery of such genes in fungi was the analysis of mutants that could not use nitrate or that were resistant to chlorate. Two regulatory genes that induce the expression of the NR gene in *Aspergillus* and *Neurospora* have been identified and shown to be zinc finger DNA binding proteins (18, 22). Perhaps analysis of chlorate resistant mutants in plants will yield similar insights.

ACKNOWLEDGMENTS

We thank A. H. Rosenberg for the pET vectors, M. D. Marks for the *Arabidopsis* β-tubulin clone, and S. Rothstein for the spinach NiR clone.

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