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5′ Proximal Regions of *Arabidopsis* Nitrate Reductase Genes Direct Nitrate-Induced Transcription in Transgenic Tobacco[1]

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Nitrate reductase (NR) is the first enzyme in nitrate assimilation, a critical process for plant survival. The regulation of NR gene expression is complex, involving both internal and external factors. Of these, nitrate induction of NR gene expression has been studied most extensively and is well conserved among bacteria, fungi, and higher plants. We are interested in understanding the mechanism of nitrate induction of higher plant NR genes. Here we describe promoter analyses of the 5′ flanking regions of the *Arabidopsis* NR genes, *NR1* and *NR2*, with respect to nitrate induction of gene expression. To facilitate these analyses, a nitrate induction procedure using T_1 transgenic tobacco plants was established. Approximately 1.5-kb 5′ flanking regions of the two *Arabidopsis* NR genes (*NR1* and *NR2*) were fused to a reporter gene and its expression in transgenic plants was analyzed. Deletion analyses of these regions show that 238- and 188-bp 5′ flanking regions of the *NR1* and *NR2*, respectively, contain sequences responsive to nitrate induction.

Higher plants invest a substantial amount of energy to assimilate nitrogen, be it in the form of dinitrogen fixed by symbiotic, nitrogen-fixing bacteria or in the form of nitrate taken directly from the soil. Most plants lack the ability to host nitrogen-fixing bacteria and must assimilate nitrate, the predominant form of nitrogen in most soils. Prior to incorporation into amino acids, nitrate must be reduced first to nitrite by NR (NADH:nitrate oxidoreductase, EC 1.6.61) and then nitrite must be reduced further to ammonia by NiR (EC 1.7.7.1). The regulated expression of higher plant NR genes results from a complex interplay of external and internal factors. These factors include nitrate (substrate), light signals, photosynthates, and plant hormones (for reviews, see Caboche and Rouze, 1990; Solomonson and Barbar, 1990; Crawford et al., 1992). Because nitrate assimilation requires high energy input, the complex regulation of NR genes can be viewed most simply as an energy balance device between nitrate assimilation and carbon fixation. This view is well supported by experiments in which the expression of a reporter gene driven by a 5′ flanking region of NR gene is controlled by Suc availability and does not require light signals (Cheng et al., 1992; Vincentz et al., 1993).

Another aspect of the NR gene regulation, a more ancient trait, is nitrate induction. With rare exceptions (Streit et al., 1986), NR genes of bacteria, fungi, and higher plants all respond to nitrate induction (Kleihofs and Warner, 1990). This very characteristic of substrate inducibility of a plant enzyme has fascinated many plant physiologists ever since the discovery of the phenomenon (Tang and Wu, 1957). It is alluring to contemplate why this trait has been conserved during evolution while NR genes of higher plants have acquired additional effective means of regulation. In nature, nitrogen supply is often scarce. To obtain an adequate amount of nitrogen when available, it may be advantageous for the plants to elevate the otherwise low capacity for nitrate reduction to a much higher level. In fact, the response of higher plant NR genes to environmental nitrate is rapid; steady-state mRNA levels increase within 15 to 30 min of nitrate induction (Gowri and Campbell, 1989; Melzer et al., 1989; Cheng et al., 1991).

In addition to the two determining factors nitrate and carbohydrates, other factors also regulate NR gene expression. Light signals at red/far-red (Rajasekhar et al., 1988; Melzer et al., 1989) and at blue (Melzer et al., 1989) regions of the spectrum both increase the NR mRNA levels of etiolated seedlings. Cytokinin enhances, whereas ABA suppresses, the light induction of NR transcription in etiolated barley leaves (Lu et al., 1990, 1992). The biological significance of light signal and hormonal regulation is unclear. It is probable that these factors play important roles both during development of a plant and when it encounters environmental stresses. Finally, the expression of NR mRNA shows a diurnal pattern and is under the control of a circadian rhythm (Deng et al., 1990; Cheng et al., 1991). Although the nature of this oscillation is far from clear (Vincentz and Caboche, 1991; Pilgrim et al., 1993), it may fine tune the daily energy balance of the plant. Despite the extensive study of NR gene regulation, little is known about regulatory sequences involved in the complex regulation of higher plant NR gene expression.

To study the transcriptional regulation of a gene, promoter deletion analysis serves as the first and most effective step in defining biologically relevant sequences. In plants, stable transformation methods are used widely in such analyses and are considered to be a standard procedure. However, genes expressed at low levels and regulated in complex manners complicate such analysis. This has been the case

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Abbreviations: CAT chloramphenicol acetyltransferase; GUS, β-glucuronidase; MS, Murashige and Skoog salts; NiR, nitrite reductase; NR, nitrate reductase.

with the analysis of tobacco NR gene transcription. Only 20% of transgenic tobacco plants carrying the promoters of either tobacco *NIA-1* or *NIA-2* genes showed detectable levels of reporter gene expression. Among those transgenic plants in which the reporter gene is expressed, only 10% are regulated by nitrate (Vaucheret et al., 1992). Such low frequencies make the interpretation of results extremely difficult. In contrast, analysis of the transcriptional regulation of the NiR gene has fared well. It is generally agreed that NiR genes are expressed at a higher level than NR genes (Layzell, 1990). Deletion analysis of the spinach NiR promoter revealed that a 330-bp 5′ flanking region is sufficient in achieving high levels of expression in the presence of nitrate (Rastogi et al., 1993).

We have shown previously that the expression of two *Arabidopsis* NR genes is regulated in a coordinated fashion (Cheng et al., 1991). We demonstrated further that Suc can replace photosynthesis to increase NR gene transcription in *Arabidopsis*, thus separating the direct effect of light from that of the photosynthesis (Cheng et al., 1992). Here we demonstrate that the 5′ flanking sequences of the two *Arabidopsis* NR genes are necessary and sufficient for nitrate-induced transcription. Sequential deletions of each of the two 5′ flanking regions were fused to the reporter gene CAT. Analysis of the CAT gene expression in transgenic tobacco plants revealed that 238- and 188-bp 5′ flanking regions of *NR1* and *NR2*, respectively, are sufficient for the induction by nitrate.

MATERIALS AND METHODS

NR Promoter-CAT Gene Chimeric Constructs

The sequence flanking the translation start of the *NR1* gene constitutes a *Nco*I site. The 5′-ACATGG-3′ sequence flanking the translational start of *NR2* gene was mutagenized to 5′-CCATGG-3′ by the method of Kunkel (1985) to constitute a *Nco*I site. The *Nco*I site at the translation start sites of the *NR1* and *NR2* genes, with about 2 kb of 5′ flanking regions (designated NP1 and NP2), were fused to BlueCATKS (Schaffner and Sheen, 1991). A 5′ to 3′ nested deletion series was then generated by exonuclease III treatment (Henikoff, 1984). These deletion fragments were then subcloned into the binary vector pBI121-R as described previously (Cheng et al., 1992). A second reporter gene, GUS, driven by the cauliflower mosaic virus 35S promoter, is included in all constructs to serve as an internal control.

DNA Sequence Analysis

The 5′ flanking regions of the two NR genes were subcloned into pBC KS vector (Stratagene). Unidirectional deletion clones were generated by exonuclease III digestion (Henikoff et al., 1984). DNA sequences were determined by the dideoxy chain-termination method (Sanger et al., 1977). In all cases both DNA strands were sequenced.

Plant Transformation and Growth Conditions

Nicotiana tabacum SR1 was transformed with the deletion constructs via leaf-disc agrobacteria transformation procedures (Horsch et al., 1985). Regenerated shoots were selected on MS medium (Murashige and Skoog, 1962) containing 2% Suc and kanamycin at 100 μg/mL. Independent transformants were maintained in culture aseptically for 4 to 6 weeks under a 16/8 h light/dark cycle. The growth of agrobacteria was retarded by incorporating carbenicillin at 200 μg/mL in the medium.

Nitrate Induction Procedure

Explants from transformants chosen for nitrate induction experiments were transferred to ammonium medium (containing no nitrate) as previously described (Cheng et al., 1991). Each explant consisted of one or two shoot nodes. All the leaves were removed to minimize any residual CAT and nitrate (data not shown). The explants were grown under a 16/8 h light/dark cycle for 3 to 4 weeks. They were then shifted to constant light for 10 d to abolish possible circadian rhythm. One leaf was harvested from each plant. KNO_3 was added to the medium to a final concentration of 50 mM. After 24 h, another leaf comparable in size to that previously harvested was excised from each explant. The leaf material was frozen in liquid nitrogen immediately after harvesting and stored at −80°C.

Enzyme Assays and Protein Determination

From each leaf sample, 0.1 g was ground with 200 μL of ice-cold grinding buffer containing 20 mM Tris-HCl (pH 8.0) and 2 mM $MgCl_2$. The homogenate was centrifuged at 7000*g* for 10 min at 4°C to remove cell debris. The supernatant was used for CAT and GUS activity assays and for the determination of protein concentration. CAT assays were performed according to Seed and Sheen (1988). For each assay, 25 μL of leaf extract was incubated at 55°C for 5 min prior to the addition of 25 μL of assay mix containing 250 mM Tris-HCl (pH 8.0), 500 μM butyryl CoA, 200 μM chloramphenicol, and 0.1 μCi of [^{3}H]chloramphenicol (Du Pont-New England Nuclear). The reaction mixture was incubated at 37°C for 3 h and the reaction was terminated by extracting with 2 volumes of xylenes. The xylene layer was back-extracted twice with 1 volume of 10 mM Tris-HCl and 1 mM EDTA and counted in a liquid scintillation counter. The relative CAT activity is expressed in all tables and figures as cpm divided by 1000. Conversion to CAT enzyme activity units is described elsewhere (Seed and Sheen, 1988).

GUS activity of the leaf extract was determined using the fluorogenic assay according to Jefferson (1987). Leaf extracts were incubated in the presence of 1 mM 4-methyl umbelliferyl glucuronide at 37°C. Samples were taken and reactions were stopped at 0, 5, 15, 30, and 60 min. NR activity assays were performed as described by Warner and Kleinhofs (1981). Protein concentration was determined according to Bradford (1976).

RESULTS

5′ Flanking Sequences of the Two NR Genes and the End Points of Each Deletion

The sequences of NP1 and NP2 are shown in Figure 1, A and B, respectively. The end point of each deletion was

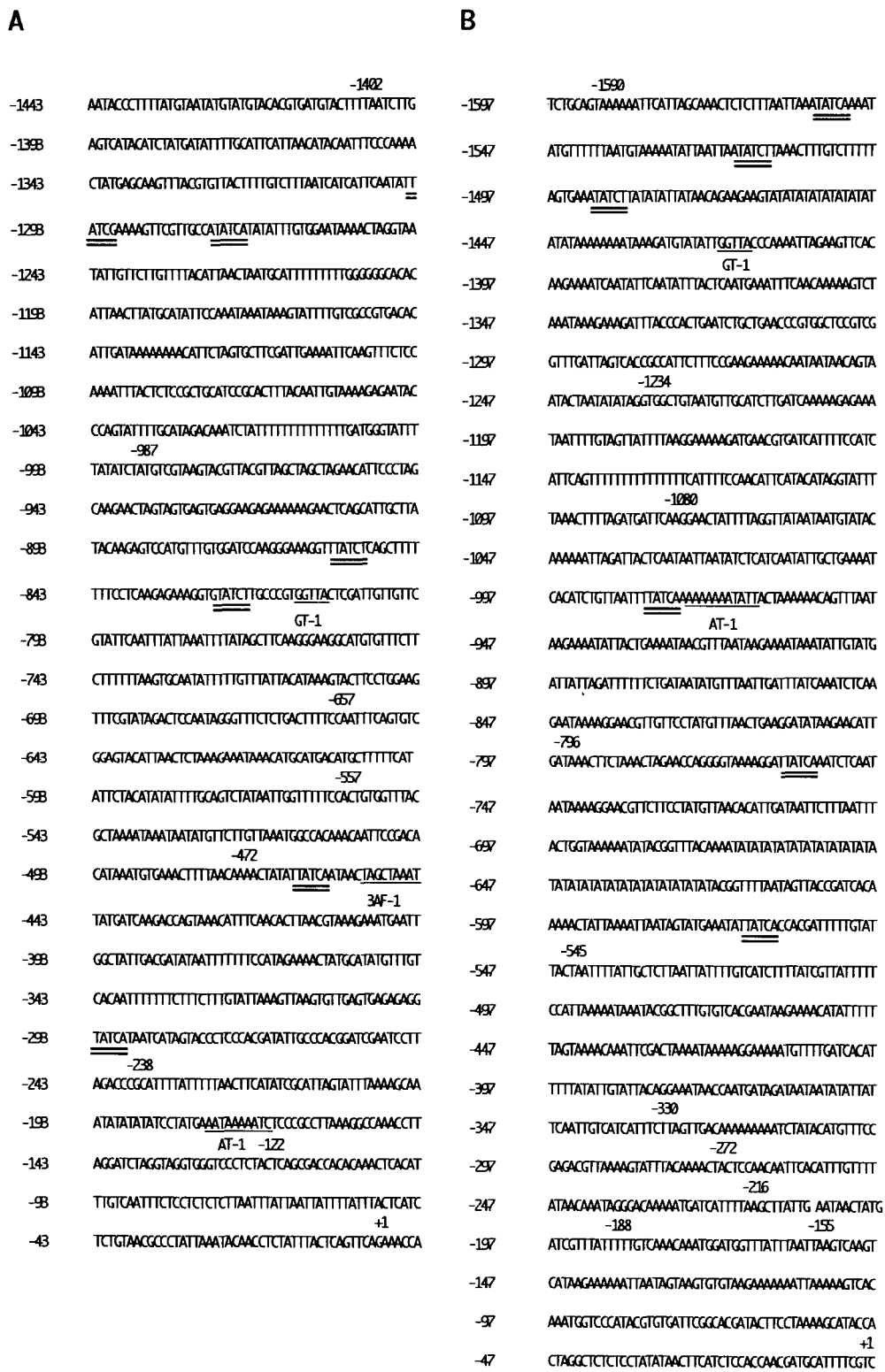

Figure 1. 5′ flanking sequences of the two NR genes and the end points of each deletion. A, Sequence is from *NR1*; B, sequence is from *NR2*. The end points of each deletion are shown above the lines. +1 is the transcriptional start site. Putative binding sites for protein factors are underlined and factor names are given, except the double-underlined sequences, which are for NIT-2. Note: Sequences from −1036 to +1 of *NR2* have been published by Wilkinson and Crawford (1991). The published sequences show large numbers of discrepancies when compared with ours. Therefore, we show the sequence here again with further upstream regions and also for easy comparison with *NR1*.

determined by sequence analysis. Underlined are sequence motifs shown in other plant genes to bind some of the known putative plant transcription factors, such as those that may be involved in tissue specificity and light regulation. Sequences highly similar to AT-1 (Datta and Cashmore, 1989) and GT-1 (Lam et al., 1990) are found in both promoter sequences; a 3AF-1 (Lam and Chua, 1990) site is found in NP1. In addition, long stretches of AT-rich tracts, which could be binding sites for high-mobility group chromosomal proteins (Jacobsen et al., 1990), occur frequently in both promoters (not marked due to their prevalence). The recognition sequence for NIT-2, a global regulatory factor of nitrogen metabolism in fungi (Fu and Marzluf, 1990a, 1990b), is present many times in both promoters. However, none of the above putative protein binding sites, including the NIT-2 binding sites, are located in regions of the 5′ flanking sequences shown to be necessary and sufficient for nitrate-induced expression (detailed below).

Transcription Activities of Each Deletion

In plant systems, significant variations in reporter gene expression are commonly observed among independent transformants. This variation does not correlate to copy number and has been attributed to "position effects." To obtain statistically valid quantitation, prior to nitrate induction experiments we analyzed reporter gene expression of large numbers (up to 30) of independent transformants for each deletion by measuring CAT activity in plants grown on MS medium (containing both ammonium and nitrate). To minimize variations due to plant age and the stage of the leaf development, 4- to 6-week-old plants and leaves of similar size were assayed for CAT activity. Background CAT activity in wild-type SR1 is about 10^3 cpm/mg protein. Approximately 25% of the transformants exhibit no CAT activity above background levels and were excluded from additional calculations. The results are shown in Tables I and II. To visualize the differences more easily, the means and the SE values of the CAT activities of each deletion are shown in bar graphs (Fig. 2). Although there are variations among the mean CAT activities of deletions from −1402 to −238 of NP1 and from −1590 to −330 and −272 to −155 of NP2, the differences are not significant. In contrast, CAT activities dropped significantly from −238 to −122 of NP1 and from −330 to −272 of NP2. This conclusion is based on *t* tests performed between each deletion within these regions (data not shown). Thus, we conclude that the 238-bp 5′ flanking region of NP1 and the 330-bp 5′ flanking region of NP2 are sufficient to express the full transcription activity of the two genes when grown in MS medium under the conditions stated in "Materials and Methods."

Comparison of the transcription activities of NP1 and NP2 shows that they are not significantly different. Similar levels

Table I. *CAT expression driven by NP1 deletions*
Relative CAT activity is in cpm × 10^{-3}/mg protein.

NP1 Length	No. of Independent Transformants	Relative CAT Activity	SE
−1402	10	17.2	4.29
−987	17	22.9	4.89
−657	8	17.7	5.21
−557	10	19.7	4.98
−472	19	16.0	3.70
−238	7	13.3	5.97
−122	9	1.84	0.23

Table II. *CAT expression driven by NP2 deletions*
Relative CAT activity is in cpm × 10^{-3}/mg protein.

NP2 Length	No. of Independent Transformants	Relative CAT Activity	SE
−1590	12	20.2	7.7
−1234	19	22.2	7.0
−1080	13	19.9	8.1
−796	18	23.0	6.2
−545	21	16.8	4.7
−330	14	19.1	6.6
−272	16	5.12	0.97
−216	22	3.01	0.59
−188	20	3.06	0.74
−155	17	1.77	0.74

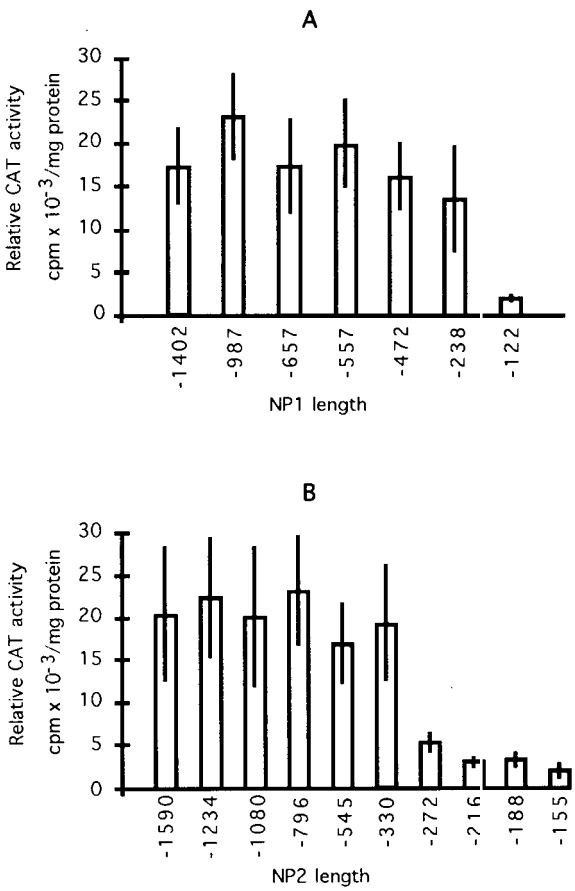

Figure 2. Reporter gene expression of each deletion construct on MS medium. Average relative CAT activities for each deletion construct are in open bars. SE values are shown as vertical lines in the bars.

of reporter gene expression correlate with our earlier observations that steady-state mRNA levels of NR1 and NR2 are similar (Cheng et al., 1991).

Responses of the 5′ Flanking Regions to Nitrate Induction

From the deletion analysis we learned that there is no significant difference among the deletions between −1401 to −238 of NP1 and between −1590 to −330 of NP2. Therefore, we concentrated the nitrate induction analysis on those deletions closer to the transcriptional start sites. Three to five independent transgenic plants were chosen from each deletion group to test nitrate inducibility. Each explant transferred from MS medium to ammonium medium consisted of only a stem and one or two lateral buds. This care, which was taken to minimize nitrate carryover in the explants, allowed us to obtain basal-level CAT activities (in the absence of nitrate) in these transgenic plants. The data are presented in Tables III and IV. Figure 3 compares the average CAT activities of ammonia-grown and nitrate-induced transgenic plants. All the plants carrying NP1 deletions from −987 to −238 exhibited averages of 6.3- to 7.8-fold induction by nitrate. In contrast, deletion −122 exhibited nearly no induction. These results suggest that NP1 sequences between −238 and −122 are necessary for nitrate induction. Similarly, deletions of NP2 from −796 to −330 exhibited 5.7- to 8.6-fold induction by nitrate. The CAT activity of deletion −272 and −188 continued to exhibit nitrate induction, although the -fold induction was only 2.7 and 1.8, respectively. These results suggest that NP2 sequences between −188 and −155 are responsive to nitrate induction.

Table III. *Nitrate induction of CAT expression driven by NP1 deletions*

Relative CAT activity is in cpm × 10^{-3}/mg protein. Numbers in parentheses are averages of the induction fold.

NP1 Length	Relative CAT Activity		Induction
	Ammonium	Nitrate	
			fold
−987	14.2	89.9	6.3
	5.53	32.4	5.9
	4.02	39.8	9.9
	23.8	76.5	3.2 (6.3)
−657	46.6	203	4.4
	10.6	168	15.9
	2.11	8.36	4.0
	2.53	8.76	3.5 (7.0)
−472	3.47	21.6	6.2
	4.60	65.9	14.3
	23.9	87.7	3.7 (8.0)
−238	1.94	14.1	7.3
	2.50	25.2	10.1
	1.72	10.3	6.0 (7.8)
−122	1.51	2.03	1.3
	1.28	1.66	1.3
	1.77	1.82	1.0
	1.65	2.24	1.4 (1.3)

Table IV. *Nitrate induction of CAT expression driven by NP2 deletions*

Relative CAT activity is in cpm × 10^{-3}/mg protein. Numbers in parentheses are averages of the induction fold.

NP2 Length	Relative CAT Activity		Induction
	Ammonium	Nitrate	
			fold
−796	36.5	172	4.7
	7.40	45.5	6.2
	2.93	22.9	7.8
	13.3	53.3	4.0 (5.7)
−545	3.50	31.4	9.0
	4.00	21.5	5.4
	40.0	254	6.3
	4.52	22.2	4.9 (6.4)
−330	5.25	66.0	12.6
	19.9	203	10.2
	3.25	9.57	2.9 (8.6)
−272	1.81	5.55	3.1
	0.98	3.20	3.3
	2.45	5.35	2.2
	2.00	4.16	2.1 (2.7)
−188	2.91	6.13	2.1
	1.06	1.72	1.6
	2.46	5.52	2.2
	1.27	1.73	1.4 (1.8)
−155	2.11	3.24	1.5
	2.39	1.93	0.8
	2.17	2.10	1.0
	2.09	1.88	0.9 (1.1)

Some plants exhibit higher basal CAT activities (Tables III and IV). To ensure that these were not caused by excess nitrate in the explants, we measured NR activity. The basal NR activities of all plants grown on ammonium were below 10 units/mg protein. When induced with nitrate, NR activities increased to over 150 units/mg protein. The induction of endogenous NR activity (15-fold) is consistent with that observed for reporter gene (CAT) expression (approximately 8-fold for each promoter). We cannot rule out the possibility that the high basal CAT activities in these plants were caused by excess nitrate contained in the explants due to the greater stability of CAT over NR. But consistent with the fact that these plants showed increases in CAT activity comparable to others upon nitrate induction, position effects are probably the cause of high basal CAT activities in some of the transgenic plants.

The increase in CAT activity upon 24 h of nitrate treatment was not due to the increase of total protein synthesis because the total protein content of leaf extracts before and after induction remained approximately 4 mg/mL. We also sampled some of the transgenic plants before and after the nitrate induction and assayed their GUS activity. Because GUS is under the control of the 35S promoter, its expression should not be affected by nitrate addition. Although GUS activity varied among independent transgenic plants (from 3 to 320 nmol mg^{-1} h^{-1}), no increase was observed between ammonium-grown and nitrate-induced plants (data not shown).

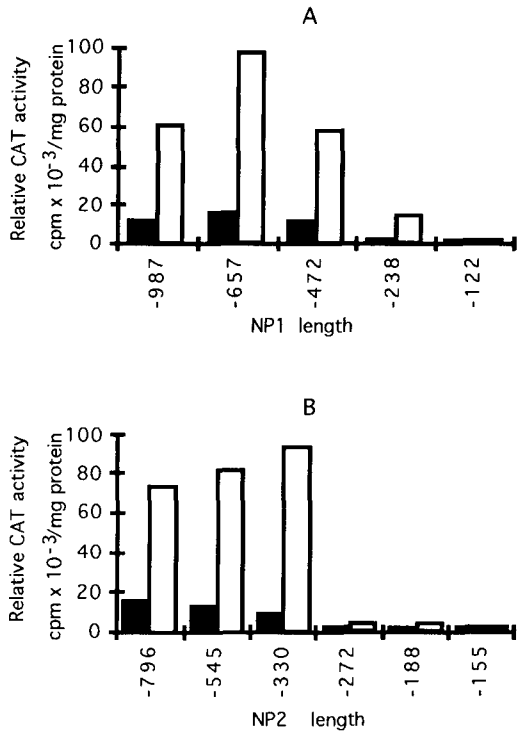

Figure 3. Nitrate induction of each deletion construct. The relative CAT activities of each deletion shown in Tables III and IV were averaged and represented in bars. The solid bars are before and the open bars are after nitrate induction.

DISCUSSION

Many external and internal factors affect the expression of NR genes in higher plants. Among these, photosynthetically active radiation (Cheng et al., 1992), Suc (Cheng et al., 1992; Vincentz et al., 1993), and cytokinin (Lu et al., 1990) affect NR gene expression, at least in part, at the transcriptional level. It has been demonstrated in numerous plant species that nitrate increases the levels of the steady-state NR mRNA (for reviews, see Caboche and Rouze, 1990; Solomonson and Barbar, 1990; Crawford et al., 1992). The increase of NR mRNA upon nitrate induction is, at least in part, due to increase of transcription. Callaci and Smarrelli (1991) performed run-on experiments and observed an increase in soybean NR gene transcription upon nitrate addition. Vincentz and Caboche (1991) showed that when tobacco NR cDNA containing the 5′ untranslated region is driven by the strong promoter of cauliflower mosaic virus 35S, the NR mRNA level is constitutively high. Based on this observation, the authors inferred that nitrate regulates the expression of NR genes at the transcriptional level.

Here we demonstrate that the 5′ flanking sequences of both the *Arabidopsis NR1* and *NR2* genes contain regions necessary and sufficient to confer nitrate induction on a reporter gene. When grown on MS medium, approximately 1.5 kb of the 5′ flanking regions driving expression of a CAT reporter gene express CAT activity at levels of more than 20-fold above wild-type (SR1) tobacco (Tables I and II). This high level of expression allowed us to compare the transcriptional activity of serial deletions when the transgenic plants were grown on MS medium. We analyzed up to 30 independent transgenic plants for each deletion construct. *t* tests based on the sample sizes and the variance values did not support the hypothesis that the transcription activities vary significantly between −1402 and −238 of NP1 and between −1590 to −330 of NP2. Therefore, it is not possible to determine if those putative transcription factor binding sites in these regions (Fig. 1, A and B) exert minor effects on the transcription activity of the NR genes. In contrast, the result showed clearly that 238 bp of NP1 and 330 bp of NP2 are important and sufficient to give full transcriptional activity under our experimental conditions.

Because the NR genes are extremely sensitive to nitrate induction and the CAT enzyme is very stable, we established an experimental protocol to minimize nitrate carryover and eliminate residual CAT activity. In our nitrate induction experiments, stems with one or two nodes were excised and all leaf material was removed prior to transferring to ammonium medium. CAT activity was then determined in newly grown leaves. This problem can also be overcome by germinating T_2 seeds directly on ammonium medium as described by Rastogi et al. (1993) in analysis of nitrate induction on NiR. Whether the differences between our results and those of Vaucheret et al. (1992) result from differences in the reporter gene, plant species, and/or the experimental protocol used for nitrate induction remains unclear.

The −238 of NP1 and −330 of NP2 are the shortest promoter deletions that still exhibit high transcriptional activity and are nitrate inducible. In the case of NP1, when deleted to −122 nitrate no longer has an effect. In the case of NP2, when deleted to −188 nitrate still gives a low but definite effect. To ensure that the small induction of −188 deletion is significant, we have analyzed 15 additional independent transformants and consistently have seen a small induction (2-fold with SE of 0.3). The CAT assay is linear over 2 to 3 orders of magnitude of enzyme concentration and is sensitive enough to detect small changes (Seed and Sheen, 1988). We have performed similar analysis with tobacco extracts and the assays are reproducible even at low activity, such as 150 cpm (approximately 1.5×10^{-6} units of CAT) with SE less than 0.05 when triplicate assays are conducted. Tobacco extracts from SR1 plants have a backgrounds less than 100 cpm.

The data presented in Tables III and IV and Figure 3 are consistent with at least two interpretations. In one, the nitrate-responsive element resides between −238 and −122 of NP1 and between −188 and −155 of NP2, and an additional nitrate-responsive element may also be located between −330 and −188 of NP2. If this is the case, then nitrate induction of NR1 and NR2 is, at least in part, transcriptional. Alternatively, it is also possible that the nitrate-responsive elements are located 3′ to −122 of NP1 and 3′ to −155 of NP2 and that one or more binding sites for other activators that are required for efficient transcription are located between −238 and −122 of NP1 and between −330 and −155 of NP2. In this case we cannot unambiguously ascribe transcriptional

mechanisms to nitrate induction. To distinguish these two possibilities and to further define the nitrate-responsible elements, we are in the process of making 3′ deletions fusing to a heterologous basal promoter. It is formally possible that the nitrate-responsive elements on NR1 and/or NR2 may reside within the 5′ untranslated regions of the corresponding mRNAs, altering mRNA stability or translational efficiency. We view this as unlikely, since the results of Vincetz and Caboche (1991), in which expression of a tobacco NR gene containing the entire 5′ untranslated region directed by the 35S promoter, do not show nitrate induction.

When sequences of 238- and 330-bp 5′ flanking regions of NP1 and NP2 were compared among themselves and with that of the 330-bp 5′ flanking region of the spinach NiR gene (Back et al., 1991), no homologous sequence motifs were apparent. Curiously, unlike NiR, which contains multiple NIT-2 binding sites in its nitrate-inducible region, no NIT-2 binding site was found within these regions of the two *Arabidopsis* NR genes.

Although further analyses are required to identify the nitrate-responsive elements, results presented here show that nitrate acts, at least in part, to induce the transcription of the *Arabidopsis* NR genes. In addition, we demonstrate that 238- and 188-bp 5′ flanking regions of the *NR1* and *NR2* genes, respectively, contain nitrate-responsive sequences.

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