The Regulatory Region Controlling the Nitrate-Responsive Expression of a Nitrate Reductase Gene, NIA1, in Arabidopsis

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Nitrate reductase (NR) is the enzyme that catalyzes the first step of nitrate assimilation. It is well known that the expression of NR genes is rapidly induced in various plants by nitrate. Previously, the activity of a tobacco NR gene promoter was reported to be high in tobacco plants grown on medium containing ammonium as the sole nitrogen source, but low in tobacco plants grown on nitrate-containing medium. This cast some doubt on the role of the NR gene promoter in the nitrate-inducible expression of this gene. Furthermore, in previous studies, transformation with genomic fragments containing NR loci restored the reduced NR activity in NR mutants to a limited extent, suggesting a complex regulation of NR gene expression. Here, we show that although the 1.9 kb promoter of an NR gene in Arabidopsis, NIA1, is not activated by nitrate, the expression of a GUS (β-glucuronidase) reporter gene inserted between the 5′- and 3′-flanking sequences of the NIA1 coding region is strongly induced by nitrate. When the 3′-flanking sequence was fused downstream of the GUS gene under the control of the 35S minimal promoter, its expression was also strongly induced by nitrate. Furthermore, dissection analysis of the 3′-flanking region revealed that the sequence downstream of the transcriptional terminator rather than the 3′-untranslated region plays a role in nitrate-inducible expression, indicating a requirement for the 3′-flanking sequence for the nitrate-inducible transcription of NIA1. We also show that the 2.7 kb promoter sequence of NIA2, another NR gene of Arabidopsis, cannot direct nitrate-inducible expression.

Keywords: Arabidopsis thaliana • Nitrate reductase • Nitrate response • Transcriptional regulation.

Abbreviations: GUS, β-glucuronidase; MS medium, Murashige and Skoog medium; NIR, nitrite reductase; NR, nitrate reductase; NRE, nitrate-responsive cis-elements; RT-PCR, reverse transcription-PCR; UTR, untranslated region.

Introduction

Nitrate is a major nitrogen source for land plants and acts as a signaling molecule that rapidly triggers changes in gene expression, metabolism and growth in plants (Crawford 1995, Stitt 1999, Crawford and Forde 2002, Gojon et al. 2009). The list of nitrate-inducible genes contains genes encoding enzymes involved in nitrate assimilation, including nitrate reductase (NR) and nitrite reductase (NIR), and nitrate transporter genes. The nitrate induction of NR and NIR gene expression is a primary response as it is not blocked by the protein synthesis inhibitor cycloheximide (Gowri et al. 1992, Price et al. 2004). Furthermore, the nitrate induction of NR and NIR gene expression has been observed even in mutants that are deficient in the NR activity necessary for the first step in nitrate assimilation, suggesting that nitrate but not nitrogen-containing metabolites functions as the signal (Pouteau et al. 1989, Scheible et al. 1997, Wang et al. 2004).

To better understand the mechanism underlying nitrate-responsive gene expression, it was necessary to elucidate the nitrate-responsive cis-elements (NREs) required for the nitrate-dependent activation of transcription. For this purpose, the NR and NIR gene promoters from several plant species and the promoter of a nitrate transporter gene, NRT2.1, in Arabidopsis thaliana were analyzed. A 150 bp region within the NRT2.1 promoter was found to be sufficient to mediate both the positive and negative effects on transcription that are observed in the presence of nitrate or nitrogen-containing metabolites, respectively (Girin et al. 2007). This suggested an intimate linkage between this sequence and the nitrogen status in Arabidopsis. However, the involvement of this sequence in the primary response to nitrate had not previously been addressed. On the other hand, analyses of NIR gene promoters from various higher plants have revealed that NRE(s) are located in the regions proximal to transcription start sites in these promoters (Neininger et al. 1994, Sander et al. 1995, Crawford 1995, Stitt 1999, Crawford and Forde 2002, Gojon et al. 2009).
Rastogi et al. 1997, Dorbe et al. 1998, Sivasankar et al. 1998, Warning and Hachtel 2000). Furthermore, we recently showed that a sequence in the proximal region of the Arabidopsis NIR gene (NIR1) promoter functions as an authentic NRE that is both necessary and sufficient for the nitrate response and is also conserved in several NIR gene promoters from higher plants (Konishi and Yanagisawa 2010).

In contrast to the NIR gene promoters, analyses of the NR gene promoters have yielded inconsistent results. The tobacco (Nicotiana tabacum) NR gene promoter is not activated but in fact was shown to be down-regulated in the presence of nitrate (Vaucheret et al. 1992, Vaucheret and Caboche 1995). Moreover, the NR gene promoter from birch (Betula pendula) could direct an increase in reporter activity only to a limited extent in the presence of nitrate (Hachtel and Strater 2000). However, reporter genes under the control of the promoters of two Arabidopsis NR genes (NIA1 and NIA2) are expressed in response to nitrate in transgenic tobacco plants (Lin et al. 1994). Furthermore, the elements necessary for nitrate induction, 5’-(A/T)3(A/C/G)TCA-3’, were identified in these promoters by linker scanning analysis, although the sufficiency of these elements has not yet been addressed (Hwang et al. 1997). A possible explanation for such a discrepancy might be the different locations of the NRES in distinct NR gene loci from different plant species.

The confusion in our understanding of the regulation of NR expression is further aggravated by the results of other experiments. Several reports have shown that the genomic fragments containing both the NR-coding region and a putative promoter region from tobacco, tomato and Arabidopsis could not fully complement the defects of NR-deficient mutants (Vaucheret et al. 1990, Wilkinson and Crawford 1991, Dorbe et al. 1992). In an experiment where two tobacco NR genes were introduced into an NR-deficient mutant of tobacco, only 15 out of the 266 transformants analyzed could utilize nitrate, and the highest NR activities observed were only 1–4% of the wild-type levels (Vaucheret et al. 1990). In experiments using a Nicotiana plum-baginifolia NR mutant, the introduction of genomic DNA fragments harboring the NR gene loci of tobacco and tomato resulted in the recovery of only 1–6% of NR activity in 70 transformants (Vaucheret et al. 1990) and a recovery of <17% of NR activity in 17 transformants (Dorbe et al. 1992), respectively. A similarly low restoration of NR activity was observed for an Arabidopsis mutant in which the maximum activity from 47 transformants was 25% of the wild-type NR levels (Wilkinson and Crawford 1991). These results indicate that the sequence required for full expression of the NR gene is located outside of the regions used in these complementation studies.

In our present study, we show that a genomic sequence downstream of the 3’ end of NIA1 is both necessary and sufficient for the expression of this gene in Arabidopsis in response to nitrate. The downstream sequence also facilitates the robust expression of a reporter gene in mature leaves in the presence of nitrate, further indicating the relevance of this downstream sequence to proper NIA1 expression. Furthermore, we also show that the 2.7 kb promoter sequence of NIA2 which was used in the previous complementation analysis of an Arabidopsis NR mutant (Wilkinson and Crawford 1991) cannot direct nitrate-responsive expression.

**Results**

**The 1.9 kb NIA1 promoter is not activated by nitrate**

Given that previous reports have shown that the NIA1 and NIA2 promoters can direct nitrate-responsive expression (Lin et al. 1994, Hwang et al. 1997), we generated transgenic NIA1pro-GUS-NOS Arabidopsis lines harboring the GUS (β-glucuronidase) gene under the control of a 1.9 kb NIA1 promoter sequence (Fig. 1A). We chose the NIA1 promoter as the induction of this gene by nitrate is more pronounced than that of NIA2 (Wang et al. 2000, Wang et al. 2003, Price et al. 2004, Scheible et al. 2004). Although the intergenic region between NIA1 and the upstream gene (At1g77765) is 5.7 kb, we used the proximal 1.9 kb fragment because a previous study had shown that the proximal 1.5 kb fragment had been able to respond to nitrate (Lin et al. 1994). We compared the GUS activity levels in the leaves of the NIA1pro-GUS-NOS lines grown on medium containing ammonium succinate, potassium nitrate, ammonium nitrate or glutamine as nitrogen source for 12 d. As a positive control in this experiment, we used a transgenic Arabidopsis line harboring the GUS gene under the control of the 5’- and 3’-flanking sequences of the Arabidopsis NIR1 gene (the NIR1-GUS line; Konishi and Yanagisawa 2010). GUS activity was found to be much higher in the leaves of NIR1-GUS plants grown on nitrate-containing medium compared with those cultivated on ammonium succinate medium (Table 1). This indicated that differences in GUS activity in ammonium-grown and nitrate-grown plants reflect the nitrate responsiveness of the NIR1 gene promoter. In leaves of the NIA1pro-GUS-NOS seedlings on nitrate-containing medium, we detected a severe reduction in GUS activity, which was <2% of the levels in these plants grown on ammonium succinate medium (Table 1). This finding indicated that the expression of the GUS reporter gene was not induced but rather was repressed by nitrate in the NIA1pro-GUS-NOS lines. The data we obtained for the NIA1 promoter are similar to those previously reported for the tobacco NR gene promoter (Vaucheret and Caboche 1995). This raises the question of the precise role that the NR gene promoters may play in nitrate-induced gene expression in Arabidopsis.

We next monitored the changes in total GUS activity in a cotyledon and in the first leaf during the development of NIA1pro-GUS-NOS plants grown on 1/2MS (half-strength Murashige and Skoog) medium (containing 10 mM KNO3 and 10 mM NH4NO3). Although a marked increase in the size of the cotyledons and first leaves in these lines was evident, the total GUS activity in these organs was mostly
constant and at a low level over a 12 d period (Fig. 1B, C). Furthermore, histochemical GUS staining of 9-day-old NIA1pro-GUS-NOS seedlings revealed that the NIA1 promoter activity was restricted to the hydathodes and was completely absent from leaf blades (Fig. 1E, G–I). These observations were therefore consistent with our finding of no increase in the total GUS activity during leaf development in NIA1pro-GUS-NOS plants.
To investigate the effects of nitrate treatment on the expression of the GUS gene in the NIA1pro-GUS-NOS lines, seedlings grown in nitrogen-free liquid medium were treated with 10 mM KNO₃ or KCl for 1 h. No increase in GUS mRNA in response to nitrate was detected in these seedlings, but the levels of endogenous NIA1 and NIA2 transcripts were clearly found to have increased after 1 h of nitrate treatment (Fig. 1J). In seedlings of the NIA1pro-GUS-NOS line grown on ammonium succinate medium, we again did not detect any elevation of the GUS mRNA levels following nitrate treatment (Supplementary Fig. S1), indicating that the NIA1 promoter is not activated by nitrate regardless of the pre-culture conditions. Collectively, therefore, our data indicate that the 1.9 kb NIA1 promoter is not activated by nitrate and is unlikely to be responsible for strong expression of the NIA1 gene on nitrate-containing medium.

### A sequence downstream of the NIA1 coding region is required for nitrate induction

Based on our initial findings, we hypothesized that the cis-element(s) required for the full expression of the NR genes might reside outside of the respective promoter regions. To test this possibility, we generated another construct, NIA1pro-GUS-NIA1d, in which the 4.5 kb sequence between the stop codon of NIA1 and its neighboring gene (At1g77750) was placed downstream of the GUS gene (Fig. 1A). We then produced transgenic Arabidopsis lines harboring this construct and detected comparable or higher GUS activity levels in the seedlings grown on medium containing nitrate, compared with those grown on medium containing ammonium succinate as the sole nitrogen source. This was a dramatic improvement over the situation in NIA1pro-GUS-NOS lines. Therefore, these data suggested that multiple cis-elements in the NIA1 promoter and the sequence downstream of the coding region of NIA1 are involved in the full expression of this gene. The cis-element(s) in the promoter sequence appear to be involved in driving NIA1 expression in hydathodes and its high expression in seedlings when grown on ammonium medium. In contrast, cis-element(s) within the sequence downstream of the coding region of NIA1 are suggested to mediate short-term nitrate responses and high gene expression in leaf blades of seedlings grown on nitrate-containing medium.

### The downstream NIA1 gene sequence is sufficient to confer nitrate-responsive expression

To investigate whether the 4.5 kb sequence downstream of the NIA1 stop codon could induce nitrate-responsive expression independently of the NIA1 promoter sequence, we generated a construct (min-GUS-NIA1d in Fig. 2A) in which the downstream sequence of NIA1 was fused to the 3' end of the GUS gene under the control of the 35S minimal promoter truncated at −72. We cultivated transgenic Arabidopsis plants harboring this construct on ammonium- or nitrate-containing medium, and then measured their GUS activity levels. The results revealed that the addition of the downstream sequence of NIA1 to the introduced DNA produced a strong stimulatory effect upon the reporter gene. GUS activity was found to be 4- to 87-fold higher when these seedlings were grown on nitrate-containing medium (Fig. 2A), indicating that the

### Table 1 GUS activity in the leaves of NIA1pro-GUS-NOS and NIA1pro-GUS-NIA1d lines grown on media containing different nitrogen sources

<table>
<thead>
<tr>
<th>Construct</th>
<th>Line</th>
<th>GUS activity (pmol min⁻¹ mg protein⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Ammonium</td>
</tr>
<tr>
<td>Nir1-GUS</td>
<td>b2</td>
<td>12.0± 5.6</td>
</tr>
<tr>
<td></td>
<td>#5</td>
<td>1,165.2± 182.7</td>
</tr>
<tr>
<td></td>
<td>#11</td>
<td>3,890.1± 408.7</td>
</tr>
<tr>
<td></td>
<td>#12</td>
<td>6,683.0± 4,856.9</td>
</tr>
<tr>
<td>NIA1pro-GUS-NIA1d</td>
<td>#53</td>
<td>2,631.1± 963.2</td>
</tr>
<tr>
<td></td>
<td>#141</td>
<td>2,378.6± 1,279.0</td>
</tr>
<tr>
<td></td>
<td>#142</td>
<td>1,972.6± 607.3</td>
</tr>
</tbody>
</table>

*Transgenic plants were grown for 12 d on medium containing either 2.5 mM ammonium succinate (Ammonium), 5 mM potassium nitrate (Nitrate), 5 mM ammonium nitrate or 5 mM glutamine as the sole nitrogen source. The first pair of true leaves was used in each case for the GUS assay. Values are the means ± SD (n = 3).

*Values that are significantly different from those of seedlings grown with ammonium alone (P < 0.05).

*Values relative to GUS activity in seedlings grown with ammonium alone.
4.5 kb sequence contained NRE(s). Furthermore, the min-GUS-NIA1d lines showed significant GUS activity on ammonium-containing medium whereas the min-GUS-NOS lines did not show any detectable GUS activity (Fig. 2A). This observation may indicate that the downstream sequence also harbors a nitrate-independent enhancer element. Alternatively, the NRE in the downstream sequence might respond to nitrate that is endogenously present at a low level. Our results did indicate, however, that the 4.5 kb downstream sequence is sufficient to mediate high NIA1 expression on nitrate-containing medium.

Analysis of the GUS transcript levels revealed that the min-GUS-NIA1d transgene also exhibits a short-term nitrate response (Fig. 2B). This result clearly demonstrated that the cis-regulatory element(s) responsible for the long- and short-term nitrate response of NIA1 is located downstream of the stop codon for this gene and could act independently of the NIA1 promoter.

**The downstream NIA1 gene sequence functions in the regulation of transcription**

As the 4.5 kb NIA1 downstream sequence contains both the 3' untranslated region (UTR) and the terminator sequence, the results we obtained using this fragment could not discriminate between transcriptional and post-transcriptional effects. Although many studies have reported on the nitrate inducibility of the NR gene transcripts in various plants (Cheng et al. 1986, Crawford et al. 1986, Calza et al. 1987, Gowri and Campbell 1989), few reports have demonstrated that this gene expression was indeed induced at the transcriptional level (Callaci and Smarrelli 1991). In fact, it has been shown that the transcript levels of another Arabidopsis NR gene

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**Table 1:**

<table>
<thead>
<tr>
<th>Construct</th>
<th>GUS activity (pmole MU min⁻¹ mg protein⁻¹)</th>
<th>Nitrate/Ammonium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Ammonium</td>
<td>Nitrate</td>
</tr>
<tr>
<td>NIA1pro-GUS-NIA1d</td>
<td>#53</td>
<td>737.5 ± 914.7</td>
</tr>
<tr>
<td>min-GUS-NIA1d</td>
<td>#3495</td>
<td>1.7 ± 1.8</td>
</tr>
<tr>
<td></td>
<td>#3498</td>
<td>4.5 ± 2.8</td>
</tr>
<tr>
<td></td>
<td>#3521</td>
<td>1.0 ± 0.3</td>
</tr>
<tr>
<td></td>
<td>#3523</td>
<td>2.9 ± 1.9</td>
</tr>
<tr>
<td>min-GUS-NOS</td>
<td>#2</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>#5</td>
<td>ND</td>
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<td></td>
<td>#7</td>
<td>ND</td>
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</table>

Fig. 2 The downstream NIA1 gene sequence is sufficient to mediate the nitrate response. (A) Schematic representation of reporter constructs and GUS activity levels in ammonium- or nitrate-grown seedlings. Black boxes indicate untranslated regions. Numbers indicate the nucleotide positions relative to the translation start codon. min, the 35S minimal promoter truncated at −72; NOS, nopaline synthase terminator sequence; ND, not detected. Transgenic plants were grown for 12 d on medium containing either 2.5 mM ammonium succinate (Ammonium) or 5 mM potassium nitrate (Nitrate) as the sole nitrogen source. The first pair of true leaves was used for the GUS assay. Values are the means ± SD (n = 3). (B) Quantitative RT–PCR analysis of the GUS transcripts and endogenous NIA1 and NIA2 transcripts in the min-GUS-NIA1d line. Treatment was carried out as in Fig. 1J. Two independent transgenic lines were used, and line numbers are indicated. Values are means of three independent biological replicates ± SD and are relative to those measured in untreated (0 h) seedlings. The gene expression levels were normalized to the endogenous UBQ10 transcript levels.
NIA2 are subjected to post-transcriptional control (Pilgrim et al. 1993). To investigate in our present analyses whether the NRE in the 4.5 kb downstream NIA1 sequence is involved in transcriptional regulation, we split this region into two portions using an internal EcoRI restriction site. One partial sequence contained the 3’-UTR and the 573 bp segment immediately downstream of the NIA1 3’ UTR, whereas the other 3.7 kb fragment contained the sequence downstream of the EcoRI site. By ligating these separate regions downstream of the GUS reporter gene, we generated the constructs NIA1pro-GUS-EcoRI and NIA1pro-GUS-NOS-EE (Fig. 3A). The transgenic lines harboring the NIA1pro-GUS-NOS-EE construct showed 6- to 14-fold higher GUS activity on medium containing nitrate as the nitrogen source (Fig. 3A). On the other hand, the GUS activity in the leaves of the nitrate-grown transgenic lines harboring the NIA1pro-GUS-EcoRI construct was <20% of that observed in the ammonium-grown plants (Fig. 3A).

In addition to the aforementioned results, we further found that the total GUS activity per cotyledon and first leaf of the NIA1pro-GUS-NOS-EE line increased during the development of true leaves, as observed for the NIA1pro-GUS-NIA1d line (Fig. 3B, D). However, the total GUS activity in the cotyledons and the first leaf of the NIA1pro-GUS-EcoRI line fluctuated only within a 4-fold range during an 18 d period (Fig. 3C), as also observed with the NIA1pro-GUS-NOS line (Fig. 1C). These results suggested that the cis-element(s) required for the robust expression of NIA1 in mature leaves on nitrate-containing medium is located in the region downstream of the EcoRI site (positions +4,134 to +7,890).

We additionally assessed the nitrate induction of GUS mRNA in transgenic seedlings. We found that the reporter gene did not respond to nitrate in the NIA1pro-GUS-NIA1d line (Fig. 3B, D), while its expression showed a marked increase in the NIA1pro-GUS-NOS line in response to nitrate (Fig. 3F). Taken together, these data suggest that the region from +4,134 to +7,890 contains the sequence responsible for the nitrate-induced transcription of NIA1.

Complementation of the nia1 nia2 double mutant with a 6.7 kb sequence for the NIA2 locus and the sequence downstream of NIA1

Arabidopsis harbors two NR genes, NIA1 and NIA2 (Wilkinson and Crawford 1993), but the major NR activity originates from NIA2 in this plant, as the single mutant for this gene retains only 10% of the wild-type NR activity (Wilkinson and Crawford 1991). Complementation of the nia2 mutant with a genomic DNA fragment for the NIA2 locus that contains the 2.5 kb promoter and the 0.7 kb sequence downstream of its coding region has been reported to result in a partial rescue of NR activity. Among 47 transgenic lines previously tested, the highest reported NR activity recovery was 25% of that of the wild type (Wilkinson and Crawford 1991). We thus hypothesized that an NRE deficiency in the NIA2 genomic fragment used in the previous complementation analysis might be associated with this poor recovery. Accordingly, we introduced a 6.7 kb fragment of the NIA2 locus (Fig. 4A) that contained the genomic DNA fragment used in the previous complementation experiment and the same 6.7 kb fragment fused to the downstream NIA1 gene sequence (+4,134 to +7,890; Fig. 4A) into a double mutant of NIA1 and NIA2. We used the previously established G’4-3 double mutant line, which retains only 0.5% of the wild-type NR activity (Wilkinson and Crawford 1993).

We initially analyzed whether the introduced NIA2 in the transgenic lines is expressed in response to nitrate treatment. Since G’4-3 contains a genomic deletion spanning the NIA2 locus (Wilkinson and Crawford 1991), the NIA2 transcript in these transgenic lines arises only from the introduced transgenes. Nitrate treatment did not affect the level of the NIA2 transcript in the transgenic lines harboring the 6.7 kb fragment alone (Fig. 4B, Supplementary Fig. S2) but markedly increased in the transgenic lines harboring the 6.7 kb fragment attached to the downstream sequence of NIA1 (Fig. 4C, Supplementary Fig. S2). Thus the NIA2 promoter used was also nitrate non-inducible, suggesting that the NRE(s) for NIA2 is located outside of the 6.7 kb region. Our data also indicated that the NRE(s) present in the downstream sequence of the transcriptional terminator of NIA1 can endow the NIA2 expression with nitrate responsiveness. Although G’4-3 is a double mutant of NIA1 and NIA2, the non-functional NIA1 transcripts were detected in this experiment. The expression of NIA1 was induced similarly by nitrate in all analyzed lines (Fig. 4B, C).

We then analyzed the NR activity of four independent lines for each construct. Under our growth conditions, G’4-3 has no detectable NR activity and the introduction of the 6.7 kb fragment alone resulted in a recovery of up to 21% of the wild-type NR activity (Fig. 4E). This is consistent with the results of the previous study in which 25% of the wild-type NR activity levels was the maximum activity obtained by analysis of 47 independent transgenic lines (Wilkinson and Crawford 1991). However, when the 6.7 kb fragment linked to the downstream NIA1 sequence was introduced, 85% of the wild-type NR activity was observed only in one line (Fig. 4E).

Discussion

Previously, the Arabidopsis NIA1 and NIA2 promoters were shown to be activated by nitrate (Lin et al. 1994, Hwang et al. 1997), but our current data are not consistent with this finding. Although we used 1 h nitrate treatments to monitor changes in the mRNA levels of the reporter genes, the reporter activities were always assessed after 24 h nitrate treatments in previous reports. We contend, however, that such reporter activity might reflect an overall increase in protein synthesis after prolonged exposure to nitrate, thus introducing an artifact into the results. Furthermore, the fact that the activity of the NR gene promoters is higher on medium containing ammonium as the sole nitrogen source compared with nitrate-containing...
Fig. 3 The downstream sequence of the NIA1 gene is involved in nitrate-inducible transcription. (A) Schematic representation of reporter constructs and GUS activity in ammonium- or nitrate-grown seedlings. Black boxes indicate untranslated regions. NOS, nopaline synthase terminator sequence. Numbers indicate the nucleotide positions relative to the translation start codon. Transgenic plants were grown for 12 d on medium containing either 2.5 mM ammonium succinate (Ammonium) or 5 mM potassium nitrate (Nitrate) as the sole nitrogen source. The first pair of true leaves was used for the GUS assay. Values are the means ± SD (n = 3). (B–D) Time course analysis of GUS activity in the cotyledons and first leaves of NIA1pro-GUS-NIA1d (B), NIA1pro-GUS-EcoRI (C) and NIA1pro-GUS-NOS-EE plants (D) grown on 1/2MS medium containing 1% sucrose. Values are the means ± SD (n = 3). Numbers preceded by ‘x’ indicate the values of the maximum GUS activity relative to those on the initial day of observation in this experiment (the second day for the cotyledon and the seventh day for the true leaf). (E and F) Quantitative RT–PCR analysis of GUS transcripts and endogenous NIA1 and NIA2 transcripts in the NIA1pro-GUS-EcoRI (E) and NIA1pro-GUS-NOS-EE (F) transgenic plants. Treatments were carried out as in Fig. 1J. Two independent transgenic lines were used for each construct, and line numbers are indicated. Values are the means of three independent biological replicates ± SD and are relative to those detected in untreated (0 h) seedlings. The gene expression levels were normalized to the endogenous UBQ10 transcript levels.
medium (Table 1; Vaucheret and Caboche 1995) indicates a complex regulatory system for these promoters in plants grown on different nitrogen sources. This characteristic of the NR gene promoters made it difficult to assess their nitrate responsiveness, which was in stark contrast to the NIR gene promoters that are dominantly controlled by nitrate (Neininger et al. 1994, Sander et al. 1995, Rastogi et al. 1997, Sivasankar et al. 1998, Dobke et al. 1998, Warning and Hachtel 2000, Konishi and Yanagisawa 2010). Moreover, the high activity of the NR gene promoters on ammonium medium is not an artifact caused by the use of reporter genes but reflects a specific aspect of NR gene regulation. This is evidenced by previous experiments in

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**Fig. 4** The effect of the NIA1 downstream sequence (+4,134 to +7,890) on the complementation of the nia1 nia2 double mutant, G’4-3, by the NIA2 gene. (A) Constructs used in complementation analysis. A 6.7 kb genomic DNA fragment containing the NIA2 gene (pNIA2, −2,745 to +3,965 relative to the translation start codon) or this fragment plus the downstream sequence of the NIA1 gene (pNIA2-NIA1d) was introduced into G’4-3 plants. (B–D) Quantitative RT–PCR analysis of NIA2 in the G’4-3 lines transformed with pNIA2 (B), G’4-3 with pNIA2-NIA1d (C) and in the wild type (D). Treatments were carried out as in Fig. 1J. Two independent transgenic lines were used for each construct, and line numbers are indicated. The wild-type line was also included to monitor the native expression levels of NIA2. Values are the means of three independent biological replicates ± SD and are relative to those detected in untreated (0 h) seedlings. The gene expression levels were normalized to the endogenous UBQ10 transcript levels. RT–PCR analysis of the endogenous NIA1 level was performed as a nitrate response control. (E) NR activity in G’4-3 transformed with the pNIA2 or pNIA2-NIA1d plasmid. Four independent transgenic lines were used for each construct, and line numbers are indicated. Values are the means ± SD (n = 3) and expressed relative to the value of the wild-type Columbia. The value for Columbia is 13.3 nmol NO₂ min⁻¹ g FW⁻¹. ND, not detected.
which the cotyledons of *Brassica napus* seedlings grown on medium containing ammonium as the sole nitrogen source had unusually high NR activity (Leleu and Vuylsteker 2004) and the NR protein levels detected in ammonium chloride-grown maize seedlings were comparable with those found in ammonium nitrate-grown seedlings (Oaks et al. 1988). The physiological significance of NR expression on ammonium medium in the absence of nitrate is unclear at present.

We showed that NRE is located in the 3’ downstream sequence of NIA1. This finding may provide, at least in part, an answer to the question raised more than a decade ago as to why the NR activities in NR mutants were restored only partially by transformation with genomic DNA fragments including the NR gene loci (Vaucheret et al. 1990, Wilkinson and Crawford 1991). Although we analyzed cis-elements for NIA1 gene expression in Arabidopsis here, these results are probably applicable to tobacco NR genes and also Arabidopsis NIA2. In the case of NIA2, we did show that the transcript levels from the introduced 6.7 kb fragment were not elevated by nitrate without the presence of the NIA1 downstream sequence (+4,134 to +7,890), suggesting that NRE(s) mediating nitrate-inducible NIA2 expression are present outside of this 6.7 kb region (Fig. 4B, C).

Our findings herein indicate that the sequence downstream of the NIA1 coding region enhances transcription in response to nitrate (Fig. 3). Typically, cis-elements acting as transcriptional enhancers can function not only within promoters, but also when located in coding sequences, introns or even sequences downstream of the coding regions. Indeed, in earlier studies of flies and mammals, it has often been reported that cis-elements are located downstream of a coding sequence (Trudel and Costantini 1987, Madisen and Groudine 1994, Fujioka et al. 1999, Sackerson et al. 1999). Moreover, in Arabidopsis it has also been demonstrated that the proper expression of a transcription factor involved in trichome formation, *GLABROUS1*, requires a sequence downstream of its stop codon (Larkin et al. 1993). Recent comprehensive analyses of transcription factor-binding sites in the Arabidopsis genome have further revealed the binding of transcription factors to sequences downstream of coding regions of their target genes (Zheng et al. 2009, Kaufmann et al. 2010).

We previously identified a pseudo-palindromic sequence, 5’-TGACcTTT-N10-AAGAg-3’ (completely conserved nucleotides and less conserved nucleotides are shown in uppercase and lowercase, respectively) as an NRE in the promoter of Arabidopsis NRI1. It is likely that nitrate induction of the genes encoding NR and NIR enzymes is mediated by the same transcription factor, because NR and NIR are enzymes in the same metabolic pathway. In fact, we found two sequences similar to the NRE of Arabidopsis NRI1 in the 3.7 kb downstream fragment of NIA1. One sequence is 5’-TGACcCTT-TGGAA-3’ at positions +5,103 to +5,084, and the other is 5’-TGACTcTT-N7-AAAGAg-3’ at positions +6,351 to +6,330. We are currently performing detailed analyses including deletion and mutational analyses, which will reveal whether these sequences are indeed NREs for NIA1 and would lead to deeper understanding of the molecular mechanisms underlying nitrate-responsive gene expression. We also tried to find candidates for NREs for tomato NR genes by comparing the downstream sequences of Arabidopsis NIA genes and tomato NR genes. However, we failed in identifying them because the 3’ downstream sequences of these genes are very divergent.

Although we show here that the sequence downstream of the stop codon of NIA1 contained NRE(s), cis-elements other than the NRE also probably contribute to the enhancement of nitrate-responsive expression. GUS activity appeared to be higher in the min-GUS-NIA1d lines compared with the min-GUS-NOS lines when grown on ammonium-containing medium, suggesting that separable nitrate-dependent and -independent cis-elements are present in the region downstream of NIA1. It is noteworthy in this regard that the complementation of the *N. plumbaginifolia* NR mutant using the NR gene from tomato resulted in a partial rescue of activity only when the introduced tomato NR gene showed enhanced expression on nitrate-containing medium (Dorbe et al. 1992). This phenomenon might reflect the fact that the introduced genomic fragment of the tomato NR gene contained an NRE but lacked some associated enhancer element(s). Furthermore, we also found that although the nitrate-responsive expression of NIA2 was restored by attachment of the 3’ downstream sequence of NIA1 to the 6.7 kb sequence for NIA2, the total NR activity obtained in our complementation test was lower than we expected (Fig. 4E). In fact, NR activity comparable with that in the wild type could be detected only in a transgenic line that possessed a higher basal expression level of the introduced NIA2 (Supplementary Fig. S2). Although, at this stage, it is difficult to explain this phenomenon fully, a possible explanation could be that the mechanism for nitrate-inducible expression in the short term might not be sufficient to support full NR activity under the growth condition where plants are continuously exposed to nitrate. The NRE and other cis-elements might cooperatively contribute to full expression of NR genes in various environments. Very recently, Wang et al. (2010) showed the presence of cis-elements in the NIA1 promoter that act cooperatively with the NRE. Further analyses would be necessary to reveal the complex regulatory mechanism for the expression of the NR genes.

In summary, we find that the transcription of NIA1 is regulated by NRE(s) located downstream of its transcribed region. Our results thus provide new insight into the mechanisms underlying nitrate-responsive gene expression in plants.

**Materials and Methods**

**Generation of reporter constructs**

Reporter constructs were generated by replacing the 35S promoter and the NOS terminator located upstream and
downstream of the GUS gene with appropriate DNA fragments (pCB302-3SS-Ω-GUS; Konishi and Yanagisawa 2007). The 1.9 kb fragment upstream of the translational start site and the 4.5 kb fragment downstream of the stop codon of NIA1 (At1g77760) were amplified by PCR using Arabidopsis genomic DNA, KOD plus DNA polymerase (Toyobo Co., Ltd.) and the primers 5′-GTGAAGCTTCGTAATTTTGCTGCGTGTGGT-3′ and 5′-TGGGGTCTAGTAAAACCTCGTGAACCGTCAAGCTCAG-3′ for the promoter sequence and 5′-TGGGCTAGTAAGCATAAGGAGAGGTTAAACCCAACATCGTCGAAGCTCAG-3′ for the downstream sequence. Some primers contained restriction sites (underlined) for cloning. To generate the plasmid pNIA1pro-GUS-NOS, the NIA1 promoter fragment was digested with HincII and Ncol, and inserted in place of the 3SS-Ω sequence in pCB302-3SS-Ω-GUS. To replace the NOS terminator in pNIA1pro-GUS-NOS with the sequence downstream of NIA1 and generate a reporter plasmid, pNIA1pro-GUS-NIA1d, we digested the PCR product for the downstream sequence with Xbal whose cleavage site was present in the stop codon of NIA1, created blunt ends with T4 DNA polymerase and then cut it with SpeI. On the other hand, pNIA1pro-GUS-NOS was digested with SacI and blunt ended with T4 DNA polymerase, and then digested with Xbal to remove the NOS terminator. Thus, the pNIA1pro-GUS-NIA1d construct was produced by ligation between the blunt-ended Xbal site of the downstream NIA1 fragment and the blunt-ended SacI site of pNIA1pro-GUS-NOS, and the SpeI site of the insert DNA and the Xbal site of pNIA1pro-GUS-NOS.

A reporter plasmid, pMin-GUS-NOS, was constructed by replacing the 3SS-Ω sequence of pCB302-3SS-Ω-GUS with the 35S minimal promoter truncated at −72, and then another reporter plasmid, pMin-GUS-NIA1d, was made by replacing the NIA1 promoter sequence of pNIA1pro-GUS-NIA1d with the 35S minimal promoter truncated at −72.

An additional reporter plasmid, pNIA1pro-GUS-EcoRI, was generated by the removal of a 3.7 kb EcoRI fragment from pNIA1pro-GUS-NIA1d, because EcoRI sites exist in the NIA1 downstream sequence. The insertion and deletion of the 3.7 kb EcoRI fragment at the position downstream of the 3′ end of the NOS terminator sequence in pNIA1pro-GUS-NOS was carried out using the floral transformation method described previously (Konishi and Yanagisawa 2007). In the T2 generation, the lines with a single T-DNA insertion site were selected based on the segregation of resistant and sensitive seedlings on glufosinate ammonium-containing medium. Next, the T3 lines homozygous for the T-DNA were selected and subjected to an NR assay.

Construction of binary vectors for complementation analysis

To generate the binary plasmid, pNIA2, for complementation analysis, a genomic fragment that ranged from a HincII site (−2,745, relative to the translational start site in NIA2) to an XbaI site (+3,965) was amplified by PCR, and used for replacement of a transcriptional unit (35S-Ω-GUS-NOS) in pCB302-3SS-Ω-GUS (Konishi and Yanagisawa 2007). Another binary vector, pNIA2-NIA1d, was made by inserting the 3.7 kb EcoRI fragment from pNIA1pro-GUS-NIA1d in the 3′ end of the NIA2 fragment.

Plant materials

The Arabidopsis ecotype Columbia was used as the wild-type strain. A double mutant of NIA1 and NIA2, G′4-3, was obtained from the Arabidopsis Biological Resource Center (Wilkinson and Crawford 1993). G′4-3 harbors a deletion of the entire NIA2 locus and a missense mutation within the NIA1 locus (Wilkinson and Crawford 1993).

Plant growth conditions

For time course analysis of leaf GUS activity, seeds were sown on 1/2MS plates (half-strength Murashige and Skoog salts, Gamborg’s vitamin, 0.5 g l−1 MES, pH 5.7, 0.8% agar) supplemented with 1% sucrose and cold treated for 3–4 d. Plates were then transferred to a growth chamber set at 23°C with continuous light (60 μE). On days 13 and 16, 1 ml of 10 mM KNO3 was added to each plate containing 40 ml of agar medium. For the analysis of the effects of various nitrogen sources, 2.5 mM ammonium succinate, 5 mM potassium nitrate, 5 mM ammonium nitrate or 5 mM glutamine was added to nitrogen-free 1/2MS medium [1/2MS salts from which ammonium nitrate and potassium nitrate were omitted, Gamborg’s vitamin and 0.5 g l−1 MES (pH 5.7)] supplemented with 1% sucrose and 0.8% agar. The first pairs of true leaves were sampled at 12 d after stratification.

For quantitative reverse transcription–PCR (RT–PCR) analysis, about 200–300 seedlings were grown in 20 ml of nitrogen-free 1/2MS liquid medium for 3.5 d under continuous light at 23°C, after which the medium was changed to fresh nitrogen-free 1/2MS liquid medium containing 10 mM KNO3 or 10 mM KCl. After 1 h of treatment, whole seedlings were collected. For the NR assay, T3 seedlings were grown on 1/2MS plates supplemented with 1% sucrose. On the 12th day, 1 ml of 10 mM KNO3 was added to each plate containing 40 ml of agar medium. Two days after the application of KNO3, cotyledons and the first to fourth leaves were collected and subjected to an NR assay.

Plant transformation

Arabidopsis transformations were carried out using the floral dipping method as described previously (Konishi and Yanagisawa 2007). In the T3 generation, the lines with a single T-DNA insertion site were selected based on the segregation of resistant and sensitive seedlings on glufosinate ammonium-containing medium. Next, the T3 lines homozygous for the T-DNA were selected and used in the analyses.

Histochemical GUS staining and quantitative measurements

Histochemical GUS staining was performed as described previously (Konishi and Yanagisawa 2007) and measurements of GUS activity using fluorescent substrate were carried out according to the method of Jefferson et al. (1987). Briefly, leaves were homogenized in 50 μl of extraction buffer (50 mM NaH2PO4-Na2HPO4, pH 7.4, 10 mM EDTA, 0.1% Triton X-100, 0.5 g l−1 MES, pH 5.7) supplemented with 1% sucrose and 0.8% agar. The first pairs of true leaves were sampled at 12 d after stratification.

For quantitative reverse transcription–PCR (RT–PCR) analysis, about 200–300 seedlings were grown in 20 ml of nitrogen-free 1/2MS liquid medium for 3.5 d under continuous light at 23°C, after which the medium was changed to fresh nitrogen-free 1/2MS liquid medium containing 10 mM KNO3 or 10 mM KCl. After 1 h of treatment, whole seedlings were collected. For the NR assay, T3 seedlings were grown on 1/2MS plates supplemented with 1% sucrose. On the 12th day, 1 ml of 10 mM KNO3 was added to each plate containing 40 ml of agar medium. Two days after the application of KNO3, cotyledons and the first to fourth leaves were collected and subjected to an NR assay.
0.1% sodium lauryl sarcosine, 10 mM β-mercaptoethanol. Enzyme reactions were then carried out in 50 μl of extraction buffer containing 1 mM 4-methylumbelliferyl-β-D-glucuronide at 37°C and were stopped by the addition of 450 μl of 0.2 M Na2CO3. Fluorescence of 4-methylumbelliferone was then measured using a DyNA Quant fluorometer (Hoefer Pharmacica Biotech Inc.) or Infinite M1000 microplate reader (TECAN Group Ltd.). Protein concentrations were quantified using Protein Assay reagent (Bio-Rad Laboratories).

RNA extraction and quantitative RT–PCR

RNA extractions and reverse transcription reactions were performed as described by Konishi and Yanagisawa (2010). PCRs were performed using a StepOne Plus™ Real Time PCR System (Applied Biosystems), using a KAPA SYBR Fast qPCR Kit (KAPA Biosystems). The primers used were as follows: 5'-GAAATTCGCAAAAGGAAAGTGG-3' and 5'-ACTGATCATAGGCCGC-3' for NIA1, 5'-ACGGACAGAAGGTACTGGA-3' and 5'-AACGTATCCAGCCTATTCC-3' for GUS, and 5'-AAGGGAAGAAGGTTG-3' and 5'-ACTGAATCATAGGCGGTG-3' for UBQ10. These tissues were then ground to a fine powder using a Multibead Shocker (Yasui Kikai, Osaka, Japan). Five volumes of 1366–1380.

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References


