

# Genomic Analysis of the Nitrate Response Using a Nitrate Reductase-Null Mutant of Arabidopsis<sup>1[w]</sup>

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A nitrate reductase (NR)-null mutant of Arabidopsis was constructed that had a deletion of the major NR gene *NIA2* and an insertion in the *NIA1* NR gene. This mutant had no detectable NR activity and could not use nitrate as the sole nitrogen source. Starch mobilization was not induced by nitrate in this mutant but was induced by ammonium, indicating that nitrate was not the signal for this process. Microarray analysis of gene expression revealed that 595 genes responded to nitrate (5 mM nitrate for 2 h) in both wild-type and mutant plants. This group of genes was overrepresented most significantly in the functional categories of energy, metabolism, and glycolysis and gluconeogenesis. Because the nitrate response of these genes was NR independent, nitrate and not a downstream metabolite served as the signal. The microarray analysis also revealed that shoots can be as responsive to nitrate as roots, yet there was substantial organ specificity to the nitrate response.

Nitrate is a potent signal that affects nitrogen and carbon metabolism as well as organ growth and development in plants (Crawford and Forde, 2002; Forde, 2002; Stitt et al., 2002; Foyer et al., 2003; for review, see Stitt, 1999). These effects are mediated at least in part by changes in gene expression that are elicited by nitrate (Stitt, 1999; Wang et al., 2000, 2001, 2003; Stitt et al., 2002). Within minutes, nitrate induces the expression of hundreds of genes encoding proteins that include nitrate transporters (NRTs) and the nitrate assimilatory enzymes nitrate reductase (NR) and nitrite reductase (NiR). Nitrate also induces genes encoding proteins that provide reductant (including ferredoxin [Fd], Fd reductase, and enzymes in the pentose phosphate and glycolytic pathways) and that redirect carbon metabolism from starch, fructan, and sugar synthesis to organic acid production (Scheible et al., 1997a, 2000, and references therein). Recent microarray analyses have demonstrated that the expression of many regulatory genes, including those

that encode transcription factors and kinases, are also affected (Wang et al., 2000, 2001, 2003). Some of the nitrate-regulated genes also respond to the loss of phosphate, potassium, or iron from the medium indicating that they respond more generally to nutrient status (Wang et al., 2001, 2002).

When considering mechanisms that might mediate nitrate responses, one must take into account that nitrate, in contrast to nonmetabolized ions such as potassium and calcium, is converted to other forms, namely nitrite and ammonium, and is ultimately incorporated into amino acids. Thus, downstream metabolites of nitrate may play an important role in nitrate responses or, in fact, may be the proximal signal. Several approaches have been employed to determine whether nitrate itself or other metabolites act as a signal. First, short-term treatments with low levels of nitrate that would be expected to have minimal impact on other metabolites have been used to study NR induction in roots (Tischner et al., 1993). Microarray experiments showed that over 1,000 genes, including those that encode NR and NiR, are induced or repressed within 20 min after treatment with 250  $\mu$ M nitrate in Arabidopsis roots (Wang et al., 2000, and references therein). The response in shoots was much lower. Second, the effects of other metabolites on nitrate responses have been studied. These studies have shown that nitrite and ammonium can depress gene expression (Scheible et al., 1997b; Dzuibany et al., 1998; Krapp et al., 1998; Zhuo et al., 1999). Ammonium can also induce expression of genes including those that encode phosphoenolpyruvate carboxylase (PEPC;

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Sugiharto and Sugiyama, 1992), Gln synthetase (GS; Hirose et al., 1997), a high-affinity sulfate transporter (Vidmar et al., 1999), the amino acid transporter AAP1 (Guo and Bush, 2003), and an Asn synthetase (ASN2; Wong et al., 2004). In a genomic analysis of the Arabidopsis root transcriptome, 270 genes were found to be differentially expressed in media containing nitrate versus ammonium nitrate (Fizames et al., 2004). In the case of organ growth, localized concentrations of ammonium or nitrate can elicit enhanced lateral root growth in barley (Drew, 1975) while only nitrate does this in Arabidopsis (Zhang et al., 1999; Forde, 2002; for review, see Bloom et al., 2002).

A third approach to study nitrate responses has used NR-deficient mutants from tobacco (*Nicotiana tabacum* [Muller, 1983] and *Nicotiana plumbaginifolia* [Negrutiu et al., 1983; Gabard et al., 1987]) and Arabidopsis (Wilkinson and Crawford, 1993). The *Nicotiana* mutants displayed a nitrate-induced shift from starch to organic acid production (Scheible et al., 1997a). Nitrate induction of PEPC (involved in organic acid synthesis) and long-term repression of a regulatory subunit of ADP-Glc pyrophosphorylase (AGPS, involved in starch synthesis) occur similarly in wild-type and NR-deficient tobacco (Scheible et al., 1997a). Other genes that support organic acid synthesis had higher levels of transcripts in mutants grown with 12 mM nitrate than wild type, consistent with the idea that nitrate is signaling these responses (Scheible et al., 1997a). In Arabidopsis, the NR double mutant G'4-3 (*nia1-1/nia2-5*) was used to show that nitrate serves as a signal for the induction but not the repression of the NRT gene *NRT2.1* (Filleur and Daniel-Vedele, 1999; Lejay et al., 1999). Another study used this mutant to show that nitrite can repress the *NIA1* NR gene and the *NRT1.1* transporter gene (Loque et al., 2003).

The use of NR-deficient mutants has been invaluable for examining nitrate signaling and nitrogen metabolism; however, a viable null mutant verified by DNA analysis has not yet been described. *Nicotiana* mutants with no detectable NR activity can be propagated when grafted onto wild-type plants, but the NR-deficient shoots produced leaves that yellowed and bleached (Saux et al., 1987). This did not appear to be due to nitrate poisoning (the mutants accumulated no more nitrate than wild type) or nitrogen starvation (the mutants have similar reduced nitrogen levels). The mutant shoots accumulated starch but had low levels of malate, Suc, and chlorophyll, and CO<sub>2</sub> fixation was reduced 5-fold. The Arabidopsis NR double mutant G'4-3 is not a true null as it shows detectable growth on nitrate and still retains some NR activity (1% of shoot wild type and 5%–10% of root wild type NR activity; Wilkinson, 1992; Lejay et al., 1999), presumably due to residual activity from the mutated *NIA1* gene. It would be useful to have a viable NR-null mutant with verified knock-out mutations in both NR genes in Arabidopsis to investigate the role of nitrate in signaling in roots and shoots. Just such a mutant is described below.

## RESULTS

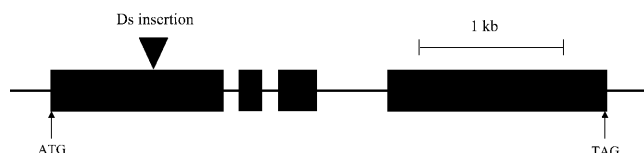
### Isolation and Propagation of an Arabidopsis NR-Null Mutant

To generate an NR-null mutant, two lines were crossed that had null mutations in either the *NIA1* or the *NIA2* NR gene. The first line (*nia2-5*, also named *chl3-5*) has a deletion in the *NIA2* gene (Wilkinson and Crawford, 1991). The second line (*nia1::Ds* or *nia1-2*) has a *Ds* insertion in the *NIA1* gene (Fig. 1; Parinov et al., 1999). The F<sub>1</sub> progeny were selfed, and F<sub>2</sub> progeny were planted on peat soil and then assayed for NR activity to identify homozygous double-mutant plants. No F<sub>2</sub> plants (72 were tested) had zero NR activity. These results suggested that the homozygous double mutant might not be viable on peat soil (organic, acidic soil with nitrate).

A second approach was adopted that employed a two-step screen. First, F<sub>2</sub> progeny that were homozygous for the *nia2* deletion (*chl3-5* mutation), and possibly heterozygous for the *nia1::Ds* mutation, were identified by NR assays. Second, progeny from these plants were grown on various media to find one that would support the growth of the double homozygous mutant, identified by PCR. This approach was successful. F<sub>2</sub> plants with only 5% to 10% of wild-type NR activity were selected and selfed. F<sub>3</sub> progeny from these low-NR plants were germinated on agarose plates. If ammonium was provided as the nitrogen source with an organic acid (succinate, Glu, or carbonate), all the progeny survived. Plants that were homozygous for both mutations were identified by PCR analysis of seedling DNA. For further propagation, plants could be transferred to and grown in 2-inch pots with vermiculite soil supplied with ammonium and a carbon source (succinate, Glu, or bicarbonate) as described in "Materials and Methods." If the double-mutant plants were transplanted to peat soil, they died.

### Characterization of the NR-Null Mutant

Several properties of the double mutant were examined. First, NR activity was measured. If the double mutant is truly NR-null, it should have no detectable NR activity. NR activity was determined in both shoots and roots in wild-type and mutant plants, and no activity was detected in the NR-null mutant in either



**Figure 1.** Structure of *nia1::Ds* mutant DNA. A schematic diagram of *NIA1* gene is shown with exons indicated as black boxes and with introns and untranslated regions as horizontal lines. The position of the *Ds* insertion at nucleotide 841 (Parinov et al., 1999) is indicated by the black triangle.

**Table 1.** NR activity in wild-type and NR mutants

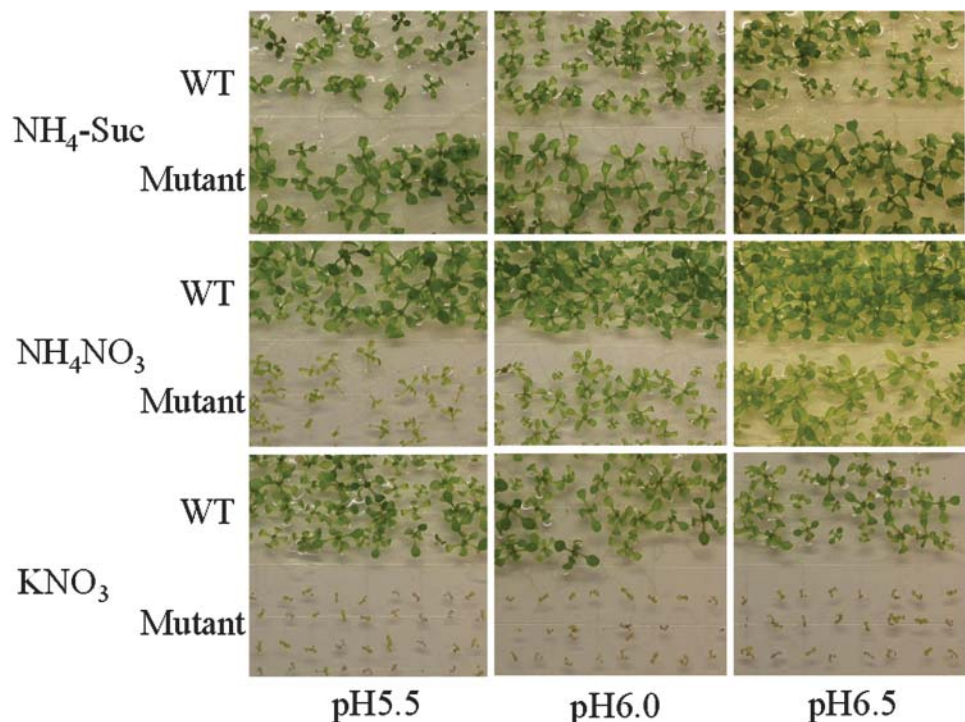
Arabidopsis were grown in pots with vermiculite soil irrigated with medium containing 2.5 mM (ammonium)<sub>2</sub>succinate. After 10 d, plants were irrigated with medium containing 5 mM NH<sub>4</sub>NO<sub>3</sub>. After 24 h leaves were collected and assayed for NR activity as described by Redinbaugh and Campbell (1983). NR activity is expressed as nmol nitrite/(mg protein min). ND, Not determined. For root measurements, plants were grown hydroponically with 2.4 mM (ammonium)<sub>2</sub>succinate for 10 d, then irrigated with 5 mM NH<sub>4</sub>NO<sub>3</sub> for 6 h before harvesting roots.

Genotype	NR Activity Shoot	NR Activity Root
Wild type	28.1 ± 0.3	4.7 ± 1.5
G5 ( <i>chl3-5</i> )	2.6 ± 0.7	ND
G'4-3( <i>chl3-5/nia1-1</i> )	0.14 ± 0.02	ND
NR-null mutant ( <i>chl3-5/nia1-2</i> )	0.00 ± 0.00	0.00 ± 0.00

shoots or roots (Table I). The double mutant also gave no detectable mRNA for *NIA1* and *NIA2* by real-time quantitative PCR analysis (as described in "Materials and Methods"). Based on these findings, we concluded that the double mutant is NR null.

The growth properties of the NR-null mutant were analyzed next. Plants were grown with different nitrogen sources at different pHs on agarose plates. Suc was included in the media, and plants were grown with 16 h light. After 13 d of growth, the plants were examined and shoot fresh weights were determined (Figs. 2 and 3). As a comparison, wild-type plants were tested and found to grow best with NH<sub>4</sub>NO<sub>3</sub> and to grow well with nitrate as the sole nitrogen source at all pHs tested. Growth on ammonium as the sole nitrogen source showed a significant pH preference (better at

**Figure 2.** Growth of wild-type and NR-null mutant plants. Seedlings were grown on agarose medium as described in "Materials and Methods," with 2.5 mM (ammonium)<sub>2</sub>succinate, 2.5 mM NH<sub>4</sub>NO<sub>3</sub>, or 5 mM KNO<sub>3</sub> as the sole nitrogen source at pH of 5.5, 6.0, or 6.5 as indicated. Pictures were taken after 13 d of growth at 25°C under 16 h light.



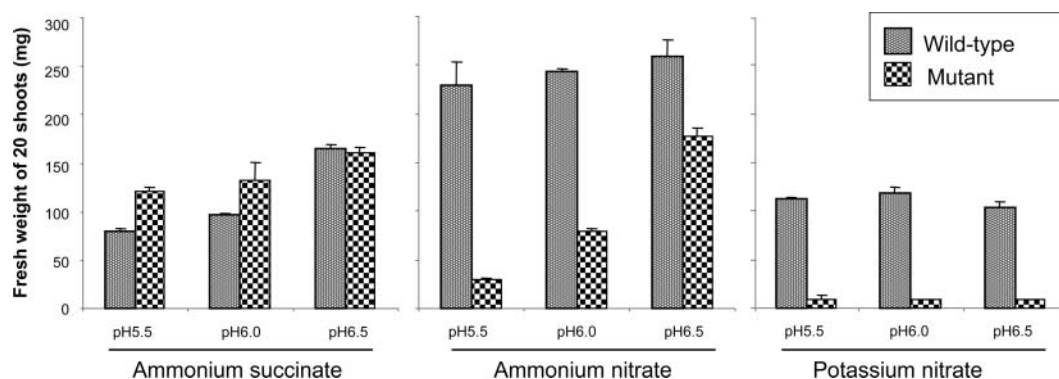
pH 6.5 than pH 5.5). The NR-null mutants behaved differently. As expected, they did not grow on nitrate as the sole nitrogen source. With NH<sub>4</sub>NO<sub>3</sub>, they showed a strong preference for more basic pH. With ammonium as the sole nitrogen source, the mutant grew better at more basic pHs. We interpret these data in the following way: Because the NR-null mutant cannot reduce nitrate, it cannot use nitrate and has to be provided another nitrogen source. Ammonium can satisfy the nitrogen requirement if the acidity produced from ammonium assimilation is compensated. This can be done by making the external medium pH 6.5 or by providing an organic acid such as succinate. This helps explain why the NR nulls do not grow on peat as this soil is too acidic (pH of peat soil was measured at 5.5–5.7) and is unlikely to contain high enough levels of neutralizing organic acids.

#### Analysis of Nitrate Regulation in the NR-Null Mutant

Having an NR-null mutant allowed us to examine the dependence of several nitrate responses on nitrate reduction. If the response occurs in both wild-type and mutant plants, we can conclude that nitrate itself can serve as a signal. If a process does not rely on nitrate but on a downstream metabolite as the signal, then the NR-null mutations should affect that response. Just such an effect was observed in the mobilization of starch by nitrate as described below.

#### Starch Mobilization

When plants are supplied abundant nitrogen, starch and sugars are mobilized into organic acids to support



**Figure 3.** Fresh weights of wild-type and NR-null mutant plants. Seedlings were grown as described in Figure 2 legend. After 13 d, fresh weights of shoots were determined from 20 seedlings. Average values of duplicate measurements are shown in the histogram with SE indicated.

amino acid biosynthesis. To investigate the role of NR in this process, starch mobilization (for review, see Smith et al., 2003) was examined in nitrogen-deprived plants that were resupplied with nitrogen. Plants starved for nitrogen accumulate starch in their leaves (Rufty et al., 1988; Schulze et al., 1994), which is depleted when nitrogen is resupplied. In our experiments, *Arabidopsis* plants were grown hydroponically in a nutrient media containing 3 mM nitrogen and 0.5% Suc in a diurnal cycle of 16 h light and 8 h dark for 10 d as described in "Materials and Methods." At day 10, plants were nitrogen-starved for 3 d and then treated with 250  $\mu$ M nitrate, ammonium, or Gln in the absence of Suc. Leaves were harvested 7 h later for starch determination. During the 3-d nitrogen starvation, starch levels increased almost 4-fold in leaves (Table II). Upon resupply of nitrogen, the accumulated starch was depleted (Table II). The nitrate treatment reduced starch levels by 19% after 7 h in the wild-type plants but showed no reduction of starch in the NR-null mutant. The same concentration of ammonium reduced starch levels by 26% in wild type and in the mutant. Gln treatment also resulted in a reduction in starch in both wild-type and mutant plants. These results indicate that the metabolism of nitrate or a downstream metabolite such as ammonium or Gln

supports or signals starch mobilization in *Arabidopsis* leaves during nitrogen resupply.

#### Microarray Analysis of Nitrate-Regulated Gene Expression

To broaden the analysis of NR's role in the nitrate response, a genomic analysis of mRNA levels in shoots and roots of both wild-type and NR-null plants was performed. Growth conditions were chosen that minimize the difference between wild-type and mutant plants; plants were grown hydroponically with (ammonium)<sub>2</sub>succinate as the sole nitrogen source at pH 6.5 with Suc for 10 d (compare fresh weights of wild-type and mutant plants shown in Fig. 3). By day 10 both wild-type and mutant plants had depleted all detectable ammonium from the medium (Table III; Fig. 4).

At day 10, plants were treated with 5 mM KNO<sub>3</sub> for 2 h. For the control, plants were treated with 5 mM KCl for 2 h. Roots and shoots were then harvested. Total RNA was isolated then reverse transcribed into cDNA. Affymetrix GeneChip ATH1 Genome Arrays (Santa Clara, CA) containing more than 22,500 probe sets were used as described in "Materials and Methods." Two biological replicates were performed for each sample. The complete lists of data showing averaged

**Table II.** Starch levels in shoots of wild-type and NR-null mutant

Plants were cultivated for 10 d in media with nitrogen (control) then transferred into N-free medium for additional 3 d (N-starved). At day 13, plants were treated with 250  $\mu$ M of the indicated nitrogen sources (nitrate, ammonium, and glutamine) for 7 h, then the starch content in leaves was determined as described in "Materials and Methods." Starch contents were expressed as  $\mu$ mol of Glc equivalents per milligram of fresh weight. SEs were calculated from five to seven replicate measurements. The percentage of starch content in relation to N-starved plants (day 13) is given in parentheses.

Treatment	Time Days	Starch Content $\pm$ STERR (Percentage of N-Starved)	
		Wild Type	NR-Null Mutant
Control	10	10.0 $\pm$ 1.1 (27%)	7.8 $\pm$ 0.8 (23%)
N-starved	13	36.4 $\pm$ 1.4 (100%)	33.9 $\pm$ 1.8 (100%)
Nitrate	13 + 7 h	29.6 $\pm$ 1.8 (81%)	34.8 $\pm$ 1.5 (103%)
Ammonium	13 + 7 h	26.9 $\pm$ 0.91 (74%)	25.0 $\pm$ 1.1 (74%)
Gln	13 + 7 h	30.1 $\pm$ 1.7 (82%)	22.6 $\pm$ 1.3 (67%)

**Table III.** Depletion of ammonium in cultures of wild-type and mutant plants

Seedlings were grown under hydroponic conditions with 2.5 mM (ammonium)<sub>2</sub>succinate as the sole nitrogen source. Ammonium concentrations (in  $\mu\text{M}$  and given as averages of triplicates with *SES*) were determined at the indicated days.

Culture Age	Wild Type	NR-Null Mutant
0 d	5,503 $\pm$ 185	5,503 $\pm$ 185
9 d	9.36 $\pm$ 1.27	5.95 $\pm$ 5.25
10 d	0.00 $\pm$ 0.00	0.00 $\pm$ 0.00

signals, signal ratios (between nitrate-treated and chloride-treated plants), and call values (A, absent signal; P, present signal; I, induced; D, depressed; NC, no change) are given in Supplemental Tables I (wild-type root), II (wild-type shoot), III (mutant root), and IV (mutant shoot), available at [www.plantphysiol.org](http://www.plantphysiol.org).

These data sets allowed us to determine the number and identity of genes that respond to nitrate in both wild-type and NR-null plants. The Affymetrix software MicroArray Suite 5.0 was used to identify genes that showed significant changes in mRNA levels (either induced [I] or depressed [D]) in both genotypes. Only genes that were expressed in both replicates (2 P calls in either the nitrate-treated or control experiments) and had call values I or D in two replicates were included. In roots, 288 genes were induced (I call) and 189 genes were depressed (D call) in both mutant and wild-type plants (Table IV). Using a 2-fold minimum cut off, 151 genes were induced and 35 were depressed in roots of both genotypes. In shoots, 209 genes were induced and 54 genes depressed in both wild-type and mutant plants (105 induced and 0 depressed 2-fold or more in shoots of both genotypes). In total, 595 genes responded to nitrate (either induced or depressed in at least one organ) in both the mutant and wild type. The list of 595 genes is provided in Supplemental Table V. These results show that there is an abundance of genes that respond to nitrate independent of nitrate reduction.

Our first analysis of these 595 genes determined which metabolic or cellular functions were most impacted by this group of genes. A bioinformatic analysis was performed with the Discover Biological Themes tool (described in L.V. Lejay, R.A. Gutierrez, and G. Coruzzi, unpublished data) to identify particular metabolic pathways or cellular functions that are over-represented in the group of 595 genes. The program used for this analysis and its documentation are available at the Web site of Dr. Gutierrez at New York University Department of Biology at [http://128.122.133.135/cgi-bin/rodrigo/go\\_browser.cgi](http://128.122.133.135/cgi-bin/rodrigo/go_browser.cgi). Genes were placed into functional categories using the scheme developed by the Munich Information Center for Protein Sequences (MIPS; <http://mips.gsf.de>). To determine statistical significance, the frequency of the nitrate-responsive genes in each category was compared with the frequency of genes from the entire

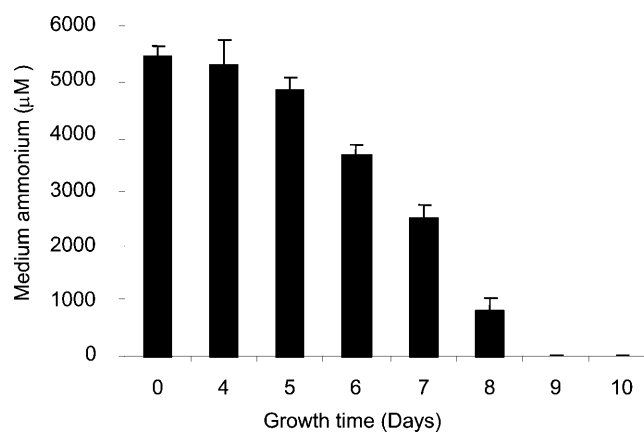
ATH1 list in each category to generate a *P*-value using the hypergeometric distribution.

The group of 595 nitrate-responsive genes is over-represented most significantly in the categories of energy, metabolism, glycolysis and gluconeogenesis, amino acid metabolism, nitrogen and sulfur utilization, and transport facilitation (Supplemental Fig. 1). The *P*-values for these categories are quite significant (from  $6 \times 10^{-6}$  to  $4 \times 10^{-8}$ ). The list of responsive genes in each of the major categories is provided along with their response call values (Supplemental Table VI).

To extend our analysis further, we examined response ratios and expression values for sets of genes involved specifically in nitrate uptake, nitrate/nitrite reduction, and ammonium assimilation. Overall, the nitrate response for these genes is similar for wild-type and mutant plants, but there are a few exceptions.

For the *NRT* genes, there are no striking differences in the response ratios or expression levels between wild-type and mutant plants (Supplemental Table VII; data from 20 min treatments with 250  $\mu\text{M}$  nitrate [column labeled WT-20 m] are included for comparison; Wang et al., 2003). *NRT1.1*, *NRT2.1*, and *NRT2.2* are strongly induced in roots, and only *NRT2.1* is strongly induced in shoots for both genotypes. In roots *NRT2.4* was moderately induced while *NRT2.5* was moderately repressed, and in shoots *NRT1.1* was moderately induced.

Nitrate-responsive genes involved in nitrate and nitrite reduction (Supplemental Table VIII) also show very similar response ratios in both genotypes. Just one gene (Fd reductase-At1g20020) showed a marked difference in the genotypes. This gene was moderately induced in mutant roots (3.8-fold) but no change in wild-type roots; however, these ratios are based on



**Figure 4.** Depletion of ammonium in hydroponic cultures of wild type. Seedlings were grown under aseptic hydroponic conditions as described in "Materials and Methods" with 2.5 mM (ammonium)<sub>2</sub>succinate as the sole nitrogen source. Samples of the media (50  $\mu\text{L}$ ) were taken at the indicated days, and ammonium concentrations were determined as described in "Materials and Methods." Values representing ammonium concentrations ( $\mu\text{M}$ ) in culture media were averages of triplicate assays with *SES* as indicated.

**Table IV.** Genes responding to nitrate in both wild-type and mutant plants

Criteria for Selection	Root No.	Shoot No.
Total number of genes on array	22,626	22,626
Significant expression in both replicates	14,253	13,750
Significant expression and increase in both replicates	288	209
Significant expression and decrease in both replicates	189	54
Significant expression and increase 2.0 or more	151	105
Significant expression and decrease of 2.0 or more	35	0

very low signals and may not be significant. Note that *NIA1* and *NIA2* signals are drastically reduced in the mutant and thus cannot be compared with wild-type data. Some signal is obtained from *NIA1*; we speculate that this arises from transcription upstream of the T-DNA insertion in this allele.

The ammonium assimilation genes (Supplemental Table IX) show a similar, low response to nitrate in both genotypes except for the GS genes in shoots, which typically show a very low response (less than 1.3-fold) in just one genotype. There are two exceptional genes: the Asn synthetase genes *ASN2*, which shows strong induction in shoots of both genotypes, and *ASN1*, which shows repression in wild-type roots and induction in mutant roots (note that *ASN1* signals are very low in the mutant).

### Analysis of Organ-Specific Responses

Because roots and shoots were analyzed separately in our experiments, it is possible to compare the nitrate response in these two organs. Previous experiments (Wang et al., 2003) comparing root and shoot responses using short time treatments (20 min) with low nitrate (250  $\mu$ M) revealed that shoots responded more weakly than roots (183 responsive genes in shoots compared with 1,171 responsive genes in roots). We did not know whether this was due to a delay or decrease in exposure of the shoots to nitrate (due in part to the transport of nitrate to the shoots) or to an intrinsic property of shoots that make them less responsive to nitrate. To address this issue, the present study used higher levels of nitrate (5 mM) for a longer time (2 h) to enhance exposure of the shoots to nitrate. Under these conditions, almost as many genes responded in shoots (897 genes) as roots (979 genes) in wild-type plants (Table V). These data show that given more nitrate for a longer time, shoots will respond similarly to roots in terms of the number of genes affected.

However, if one examines the identity of the responsive genes, a majority shows organ-specific responses (67% are induced in only one organ type and 82% are depressed in only one organ type; Fig. 5). Thus, the suites of responsive genes are quite different

in shoots and roots. Some of the organ-specific genes encode nitrate or ammonium assimilatory proteins (Supplemental Tables VII–IX). Two genes are especially interesting. They are *NRT2.2*, which is induced 74-fold in roots and is not expressed in shoots, and *ASN2*, which is induced 13-fold in shoots and less than 1.5-fold in roots. Bioinformatic analysis using the Discover Biological Themes tool revealed that genes induced specifically in shoots (Supplemental Table X) are overrepresented in the MIPS functional category of protein synthesis ( $P$ -value of  $4 \times 10^{-5}$ ; Supplemental Fig. 2). These genes encode primarily ribosomal proteins, initiation factors, and enzymes involved in rRNA synthesis and respond less than 2-fold to nitrate.

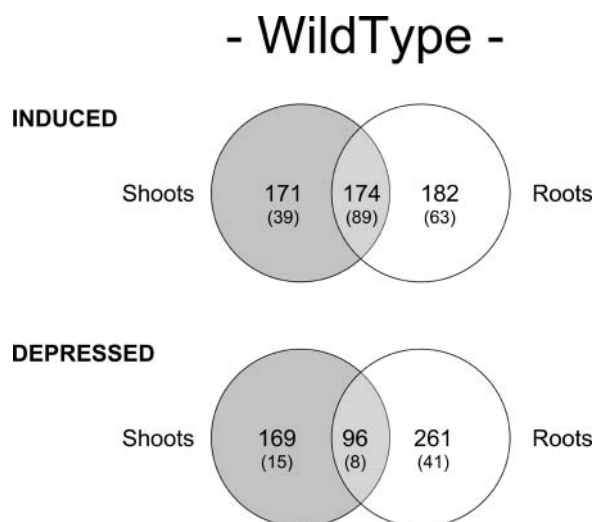
An important question that arises in the analysis of shoot-responsive genes (especially after a 2-h treatment of 5 mM nitrate) is, how many of these genes do not depend on nitrate reduction to be responsive to nitrate? To answer this question, genes were identified that are responsive specifically in shoots of wild-type plants and that respond in the same way in the NR mutant. There are a total of 65 genes that are induced specifically in shoots (i.e. not in roots) of both wild-type and mutant plants (Supplemental Table XI). This list includes metabolic and transporter genes, including those that encode a Ca-H exchanger and two sulfate transporters (Table VI shows a selection of genes with induction ratios more than 2 in wild-type plants). There are also regulatory genes (receptor-like kinases, transcription factors, and a putative His kinase) with an overabundance of two-component response regulator genes ( $P$ -value 0.007). The overabundance of genes involved in protein synthesis was not found in this group, suggesting that their response is NR-dependent. If one examines depressed genes, 29 genes are specifically depressed in shoots in both wild-type and mutant plants (Supplemental Table XII).

If the analysis of genes responding in both genotypes is broadened to include all shoot-responsive genes, including ones that also respond in roots, 209 genes are induced in shoots (Supplemental Table XIII), and 54 genes are depressed in shoots of both genotypes (Supplemental Table XIV). Bioinformatic analysis shows that the induced genes in the shoot

**Table V.** Genes responding to nitrate in roots and shoots in wild-type plants

Criteria for Selection	Root No.	Shoot No.
Total number of genes on array	22,626	22,626
Significant expression in both replicates	14,253	13,750
Significant expression and increase in both replicates	445	486
Significant expression and decrease in both replicates	534	411
Significant expression and increase 2.0 or more	201	174
Significant expression and decrease of 2.0 or more	93	35





**Figure 5.** Numbers of nitrate-responsive genes in shoots versus roots. Diagrams show the numbers of genes that were induced or depressed in roots only, shoots only, or in both roots and shoots in wild-type plants. Only data points that had call values of I (or D) in the two replicates and had a P call in both replicates in at least one of the treatment conditions were included. Numbers in parentheses correspond to genes that responded 2-fold or more.

(Supplemental Fig. 3) give similar functional categories to those found for responsive genes in both organs (energy, metabolism, glycolysis, and transport facilitation; Supplemental Fig. 1), except the shoot-responding genes included the functional categories of C-compound and carbohydrate metabolism and utilization, which were not found overrepresented in the more complete list. No categories were significantly overrepresented in the group of genes that were de-

pressed in shoots. Comparing genes induced in roots (Supplemental Fig. 4) and in shoots in both genotypes showed that very similar functional categories were overrepresented (energy, glycolysis and gluconeogenesis, metabolism, TCA cycle, amino acid biosynthesis, nitrogen and sulfur utilization, and C-compound and carbohydrate metabolism/utilization).

One additional analysis was performed on genes that are regulated by cytokinin. Cytokinin has been proposed to be a signal for nitrogen availability (for review, see Sakakibara, 2003). Nitrogen resupply to plants that have been deprived of nitrogen increases cytokinin synthesis. We examined the isopentenyl transferase (IPT, which catalyzes the first step in cytokinin biosynthesis) genes of Arabidopsis listed at The Arabidopsis Information Resource Web site ([www.arabidopsis.org](http://www.arabidopsis.org); Takei et al., 2001) in our microarray tables and found that only four gave detectable expression levels in both replicates (IPT2, IPT3, IPT7, and IPT9). Only one of these four genes showed a nitrate response in our conditions (IPT3; Table VII). This gene was strongly induced in roots and weakly induced in shoots in both wild-type and mutant plants. This is the same gene that was shown by promoter- $\beta$ -glucuronidase fusion constructs to be induced by nitrate in both roots and shoots (Miyawaki et al., 2004). The IPT3 promoter- $\beta$ -glucuronidase fusions showed expression in phloem tissue in both roots and shoots. Thus, any increase in cytokinin synthesis observed in response to nitrate in Arabidopsis may be due in part to the strong induction of IPT3.

We also examined a group of cytokinin-regulated genes identified by microarray analysis in Arabidopsis to determine the extent of overlap between cytokinin and nitrate-regulated genes (Rashotte et al., 2003). We

**Table VI.** Genes induced only in shoots of wild-type and mutant plants

Gene Description	Wild-Type Ratio	Mutant Ratio	Sequence ID
Putative cytochrome P450	8.1	8.1	At2g29090
Putative auxin-regulated protein	6.8	5.9	At2g21210
Ser/Thr kinase-like protein receptor	4.4	3.8	At4g11460
Glutaredoxin homolog	4.4	2.4	At4g15700
Putative transcription factor	4.3	2.6	At4g26150
Glutaredoxin-like protein	2.7	1.9	At5g18600
Nodulin-like protein	2.7	2.9	At2g16660
Putative His kinase	2.6	2.7	At2g17820
Response regulator 5	2.6	2.9	At1g19050
Multi resistance protein homolog	2.4	2.1	At3g60160
Putative hydroxymethyltransferase	2.3	2.0	At1g36370
Lipoxygenase AtLOX2	2.3	1.8	At3g45140
Gly-rich protein	2.3	1.5	At4g36020
CAX1-Ca exchanger	2.2	1.8	At3g51860
Putative auxin-inducible protein	2.2	1.6	At4g03400
Heat shock transcription factor HSF1	2.2	2.1	At3g24520
Receptor-like protein kinase	2.2	2.5	At5g60300
Sulfate transporter-SULTR2.2	2.1	2.0	At1g77990
TINY-like protein	2.1	1.5	At1g21910
Sulfate transporter-SULTR4.1	2.0	2.0	At5g13550
Trehalose-6-phosphate synthase	2.0	2.2	At1g60140

found that the vast majority of cytokinin up-regulated genes (104 total) and down-regulated genes (167 total) did not consistently respond to nitrate; however, there were a few genes that did. Table VII shows cytokinin up-regulated genes that were consistently induced at least 2-fold in one organ by nitrate (genes 2–7) and cytokinin down-regulated genes that were consistently depressed by nitrate at least 2-fold in at least one organ by nitrate (bottom two genes). Most of these genes are potential regulators reinforcing the proposal that nitrate and cytokinin signaling pathways have some overlap.

## DISCUSSION

The identification and characterization of an NR-null mutant with knockout mutations in both NR genes allow us to make several conclusions about nitrate reduction as well as nitrate signaling in Arabidopsis that have been unresolved. First, it is possible to isolate and propagate an Arabidopsis NR-null mutant that can complete its life cycle; thus, NR is not required for growth or reproduction as long as an alternative nitrogen source and a means to neutralize acidity are provided. Second, because the NR double mutant lacks any detectable NR activity and cannot grow on nitrate as the sole nitrogen source, *NIA1* or *NIA2* is necessary for nitrate reduction in Arabidopsis. Third, nitrate reduction is not required for many genes to respond to nitrate but is required for starch mobilization.

For many years it has been proposed that nitrate serves as a signal for metabolic and developmental processes. This proposal has been supported by analysis of gene expression, enzyme activities, metabolite levels, and root growth assays in both wild-type and NR-deficient plants. These NR-deficient lines usually contain some NR activity and thus still synthesize downstream products from nitrate. Our current findings add to this body of work by identifying genes on a genomic scale that respond to nitrate in both wild-type and NR-null plants. We were careful to limit our analysis in this study to those genes that respond in both genotypes (and not include genes that respond in only one genotype) because the NR-null mutant had

a different ecotype as one of its parents (the *nia1* mutant was in the Landsberg ecotype).

Our results demonstrate that the key nitrate assimilatory genes respond to nitrate independent of nitrate reduction. These genes encode the major nitrate transporters NRT1.1 and NRT2.1, NiR, two major Fd NADP reductases, and Fd3, one of the major Fds. (Note: Major is defined here as having the highest induced signals in the microarray dataset for that class of genes.) Thus, nitrate itself is a signal for the core nitrate assimilatory genes and key supporting genes.

For the ammonium assimilation genes, nitrate also acts as a direct signal but on a more selective group of genes. In roots, the major GS gene *GLN1-2*, the GS gene *GLN2*, the major GOGAT gene (*GLT1*), and one of the ASN genes *ASN2* responded to nitrate and did so in both genotypes. In shoots, all three GOGAT genes and only *ASN2*, which was the major ASN gene, were induced in both genotypes.

For the genes involved in carbon metabolism, many that produce energy for nitrate or nitrite reduction are induced by nitrate directly. These genes include those that encode the pentose-phosphate enzymes G6PDH, 6PGDH, transketolase, and transaldolase. They also include genes encoding glycolytic enzymes phosphoglycerate mutase (At1g78050, At1g09780, and At1g22170) and Glc-6-phosphate isomerase (At4g24620). They include genes involved in organic acid metabolism such as PEPC (At3g14940), pyruvate kinase (At2g36580), and isocitrate dehydrogenase (At4g35260 and At3g09805), as described for tobacco (Scheible et al., 1997b) but not for citrate synthase. Thus, the genes that generate energy by Glc or pentose oxidation and that provide organic acids for ammonium incorporation and pH homeostasis all respond to nitrate directly.

It is important to keep in mind when interpreting these results that because a gene responds to nitrate independent of nitrate reduction does not mean that this gene does not respond to other nitrogen signals as well. Some of the nitrate-responsive genes can also be induced by ammonium (including genes that encode PEPC, GS, a high-affinity sulfate transporter, AAP1, and *ASN2*; see introduction). The response for two of these genes, *ASN2* and *AAP1*, can be compared directly in Arabidopsis and is induced by ammonium

**Table VII.** Nitrate response ratios for cytokinin-regulated genes

Gene Descriptions	Wild-Type Shoot	Wild-Type Root	Mutant Shoot	Mutant Root	Sequence ID
Isopentenyl transferase-IPT3	2.3	23.0	1.8	6.6	At3g63110
Putative Ap2 domain protein	21.4	1.0	9.6	1.1	At4g23750
Response regulator 3	13.1	2.6	3.0	3.2	At2g41310
Putative AP2 domain transcription factor	12.9	0.6	6.5	0.9	At2g46310
Response regulator 5	2.6	1.2	2.9	1.7	At1g19050
Putative glucosyltransferase	2.1	1.4	1.7	1.8	At2g16890
Cytochrome P450	1.0	2.0	0.8	1.7	At1g67110
Ethylene response element binding factor 2	0.7	0.5	0.9	0.7	At5g47220
Scarecrow-like protein	0.8	0.5	0.9	0.5	At5g59450



and nitrate in both wild-type and mutant plants (Supplemental Tables X and XI; Guo and Bush, 2003; Wong et al., 2004). These findings indicate that these genes respond to multiple nitrogen signals (in this case nitrate and a downstream metabolite).

Many of the genes identified in this study that respond to nitrate in both genotypes were known to be responsive to short treatments with low nitrate (20 min with 250  $\mu\text{M}$ ), consistent with nitrate and not a metabolite being the signal. However, this was true for roots and not for shoots. The only genomic study of nitrate responses comparing roots and shoots published so far (Wang et al., 2003) found that only a fraction of the number of genes (15%) responded in shoots compared with roots after 20 min with 250  $\mu\text{M}$  nitrate. In this study, a treatment with 5 mM nitrate for 2 h produced a comparable response in both roots and shoots. These results showed two things. First, shoots can respond to nitrate in a quantitatively similar fashion to roots if given enough nitrate for long enough time. Second, treating roots with low nitrate for 20 min affects approximately the same number of genes as a treatment with high nitrate for 2 h. Roots are thus quicker to respond to nitrate.

Having both root and shoot data under conditions where the extent of the nitrate responses were roughly equivalent allowed us to compare the organ specificity of the nitrate response. The first analysis was performed with wild-type plants and thus does not distinguish between responses that are nitrate specific and those that are not. We found that, although there was extensive overlap, the majority of genes responded in only one organ type. This was a bit surprising to us given that nitrate metabolism is ubiquitous throughout the plant. The shoot-specific genes included transporters, regulators (but very few transcription factors), ribosomal proteins, and proteins involved in protein synthesis. None of the core nitrate or ammonium assimilation genes or related carbon metabolic genes was shoot specific except for the two Fd-GOGAT genes. *NRT2.5* is interesting as it was induced in shoots and depressed in roots. In interpreting these data, one should remember that, even though conditions were used that elicited a similar numerical response to nitrate in roots and shoots, some of the observed organ specificity could still be due to differences in the kinetics of nitrate exposure in these two organs.

Since the nitrate treatments in these experiments allowed ample time to produce downstream metabolites, the use of the NR-null mutant was necessary to establish which genes respond directly to nitrate. The shoot-specific genes that respond in both genotypes include those that encode transporters (includes two sulfate transporters) and regulators (includes several potential transcription factors and two-component response regulators).

In addition to a genomic analysis of the nitrate response, starch mobilization induced by nitrate resupply to N-starved plants was examined. This process was blocked in the mutant. Because the

downstream metabolites, ammonium and Gln, can induce starch mobilization in both wild-type and mutant plants, we conclude that nitrate reduction is necessary for this response. Thus, the mobilization of starch is not responding to nitrate directly but most likely to a change in intracellular ammonium or amino acid concentrations. This is consistent with the findings that ammonium depletes starch levels more readily than nitrate (Table II; Raab and Terry, 1995); however, it is not consistent with results reported for NR-deficient tobacco where starch mobilization was similar in wild-type and mutant lines (Scheible et al., 1997a). Perhaps the residual nitrate reduction in the NR-deficient tobacco accounts for the mobilization of starch in these experiments. It is unlikely that NR is involved as a regulatory protein in starch mobilization because ammonium can induce the response even in the mutant. It is also unlikely that the lack of response to nitrate in the mutant is due to a lack of nitrate uptake because uptake assays showed that the mutant was able to absorb nitrate albeit at a slower rate (123  $\mu\text{mol g}_{\text{fw}}^{-1} \text{h}^{-1}$  versus 174  $\mu\text{mol g}_{\text{fw}}^{-1} \text{h}^{-1}$  in wild type). A more complete description of uptake in the mutant is given in Unkles et al. (2004).

## MATERIALS AND METHODS

### Plant Material

Arabidopsis plants were of the Columbia ecotype unless otherwise indicated. The *nia1::Ds* (*nia1-2*) mutant was in the Landsberg *erecta* background and was obtained from the National University of Singapore (line no. SGT3811; Parinov et al., 1999). The *chl3-5* mutant (also known as G5; Wilkinson and Crawford, 1991) and the NR double-mutant G' 4-3 (*nia1-1/chl3-5*; Wilkinson and Crawford, 1993) have been described previously.

### Growth Conditions

For the microarray experiments, plants were grown under hydroponic conditions previously described (Wang et al., 2003). Briefly, plants were grown supported on liquid media containing 2.5 mM (ammonium)<sub>2</sub>succinate (equivalent to 5 mM NH<sub>4</sub>) as the nitrogen source and 0.5% (w/v) Suc for 10 d at 25°C with continuous light. A 1-mL solution of KNO<sub>3</sub> (treatment) or KCl (control) was added to the culture to final concentration of 5 mM; plants were grown for another 2 h then harvested for RNA extraction.

For growth on agarose plates, seeds were surface sterilized and then individually placed on 50 mL of agarose medium spaced at 5 mm apart in a 100-mm gridded square petri dish. After 24-h cold treatment at 4°C, the plates were incubated in a 25°C growth room under 16 h light. The medium in the plates is the same as described previously for liquid culture (Wang et al., 2003) with 2.5 mM (ammonium)<sub>2</sub>succinate, 2.5 mM NH<sub>4</sub>NO<sub>3</sub>, or 5 mM KNO<sub>3</sub> as the sole nitrogen source as indicated.

To propagate the NR-null mutant, seedlings are first grown on agarose plates with 2.5 mM (ammonium)<sub>2</sub>succinate as the sole nitrogen source for 10 d as described above. Seedlings are then transplanted onto 2-inch pots containing Vermiculite (medium coarse; Therm-O-Rock West, Chandler, AZ) and grown in 16 h light at 23°C. Before transplantation, the Vermiculite pots are rinsed twice from top with distilled water and autoclaved. The pots are then washed twice (about 60 mL/wash) from top with vermiculite pot medium composed of 10 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 6.5), 1 mM (ammonium)<sub>2</sub>succinate, 2 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.125 mM NaFeEDTA, 0.5% (w/v) Suc, 0.125 mM H<sub>3</sub>BO<sub>3</sub>, 0.03 mM MnSO<sub>4</sub>, 2.5  $\mu\text{M}$  ZnSO<sub>4</sub>, 2.5  $\mu\text{M}$  CuSO<sub>4</sub>, and 0.5  $\mu\text{M}$  Na<sub>2</sub>MoO<sub>4</sub>. After transplantation, pots are covered with transparent plastic for 5 d (without watering), the cover is removed, and the pots are watered twice weekly from top with vermiculite pot medium (about 100 mL/pot).

## PCR Screening of Plants

This protocol was developed to identify homozygous *nia1-2* mutant plants, which contain a *Ds* insertion in *NIA1*. A small seedling (2–3 d old) or a small piece of leaf (about 1 mm<sup>2</sup>) was homogenized into 30  $\mu$ L of PCR reaction mixture consisting of 10 mM Tris-HCl, pH 8.3, 50 mM KCl and 1.5 mM MgCl<sub>2</sub>, 1  $\mu$ M each of three PCR primers (5'-CGGTTCCGGTCATTGTAC, 5'-GTA-ATCGCCGCAAGGAACAG, and 5'-CGTGGGTGTGAGCATTTC), and 1 unit of Taq DNA polymerase. The mixture was diluted 5 $\times$  (5  $\mu$ L added to 20  $\mu$ L) successively for three times into the PCR reaction mixture. PCR reactions were performed using the three diluted reaction mixtures on an Eppendorf MasterCycler Gradient machine (Eppendorf Scientific, Westbury, NY) for 30 cycles (94°C, 45 s; 60°C, 2 min; and 72°C, 3 min). Wild-type *NIA1* gene produces a 0.5-kb PCR product and the mutant *NIA1* gene produces a 1-kb fragment.

## Ammonium Assay

The concentrations of ammonium in growth media were determined colorimetrically using the phenol-hypochlorite method described by Solorzano (1969).

## Nitrate Assay

Tissues were ground into frozen powder in liquid nitrogen. About 50 mg of the frozen powder was transferred into a previously weighed 2-mL microfuge tube. The tube was weighed again to obtain the exact amount of tissue powder in the tube. After adding 1 mL of water to the tube, it was boiled for 15 min with lid closed. The boiled samples were centrifuged in an Eppendorf microcentrifuge (Eppendorf Scientific) for 10 min at maximum speed. The supernatant was assayed for nitrate by the hydrazine-sulfate method as described by Kamphake et al. (1967).

## Starch Mobilization

Wild-type plants were cultivated hydroponically under sterile conditions with 16 h light/8 h dark for 10 d as described previously (Wang et al., 2003). The medium is composed of 10 mM KH<sub>2</sub>PO<sub>4</sub>/K<sub>2</sub>HPO<sub>4</sub> (pH 6.5), 2 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 0.125 mM NaFeEDTA, 0.5% (w/v) Suc, 0.125 mM H<sub>3</sub>BO<sub>3</sub>, 0.03 mM MnSO<sub>4</sub>, 2.5  $\mu$ M ZnSO<sub>4</sub>, 2.5  $\mu$ M CuSO<sub>4</sub>, 0.5  $\mu$ M Na<sub>2</sub>MoO<sub>4</sub>, and nitrogen source as indicated. For wild-type plants, the nitrogen source was 0.5 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub> and 1 mM KNO<sub>3</sub>; for the NR-null mutant the nitrogen source was 3 mM NH<sub>4</sub>HCO<sub>3</sub>. At day 10, plants were nitrogen starved for 3 d (transferred to the same medium with no added nitrogen). At the end of the N starvation, plants were transferred to Suc-free medium (same as growth medium except that Suc is omitted) with 0.25 mM KNO<sub>3</sub>, 0.125 mM (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, or 0.25 mM Gln as the sole nitrogen source. After 7 h of treatment, shoots were collected, weighed, and frozen in liquid N<sub>2</sub>. Starch content was determined as described (Stitt et al., 1989; Schulze et al., 1991).

## RNA Preparation

Total RNA was prepared from roots or shoots using an RNeasy Plant Mini kit (Qiagen, Valencia, CA) and quantified with a Genesis 6 spectrophotometer (Thermo Spectronic, Rochester, NY).

## Real-Time Quantitative PCR

Real-time PCR was performed using a LightCycler system from Roche Diagnostics (Indianapolis). Template cDNA samples were prepared using the SuperScript First-Strand Synthesis System kit (Invitrogen, San Diego) for reverse transcription with 3  $\mu$ g of total RNA in a reaction volume of 20  $\mu$ L. The cDNA synthesis reaction mixture was diluted 20 times before being used for PCR. Primers for the PCR reactions were designed to have a melting temperature of about 60°C to 65°C and to give a PCR product between 175 and 250 bp. The oligo primers used were as follows:

### NIA1

forward primer, 5'-ATCGTCAAAGAAACCGAAGTC

reverse primer, 5'-ACGGAGCATGGATGAGTT

### NIA2

forward primer, 5'-GGTTACGCATATTCGGGAG

reverse primer, 5'-CATGCACGAACAGCAATC

The QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA) was used for the PCR reactions. Each PCR reaction contained 2  $\mu$ L of cDNA and 0.5  $\mu$ M of each primer. The initial denaturing time was 20 min., followed by 45 PCR cycles consisting of 94°C, 25 s; 60°C, 25 s at a temperature drop rate of 10°C/s and 72°C, 25 s. A melting curve was run after the PCR cycles. Quantification was performed with LightCycler Relative Quantification software 1.0 (Roche Applied Science, Indianapolis).

## Target Preparation/Processing for GeneChip Analysis

Procedures for target preparation and processing for GeneChip Analysis were as previously described (Wang et al., 2003).

Upon request, all novel materials described in this publication will be made available in a timely manner for noncommercial research purposes, subject to the requisite permission from any third-party owners of all or parts of the material. Obtaining any permissions will be the responsibility of the requestor.

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