Microarray Analysis of the Nitrate Response in Arabidopsis Roots and Shoots Reveals over 1,000 Rapidly Responding Genes and New Linkages to Glucose, Trehalose-6-Phosphate, Iron, and Sulfate Metabolism[1[w]]

Rongchen Wang, Mamoru Okamoto, Xiujuan Xing, and Nigel M. Crawford*

Section of Cell and Developmental Biology, Division of Biological Sciences, University of California at San Diego, La Jolla, California 92093–0116

The genomic response to low levels of nitrate was studied in Arabidopsis using the Affymetrix ATH1 chip containing more than 22,500 probe sets. Arabidopsis plants were grown hydroponically in sterile liquid culture on ammonium as the sole source of nitrogen for 10 d, then treated with 250 μM nitrate for 20 min. The response to nitrate was much stronger in roots (1,176 genes showing increased or decreased mRNA levels) than in shoots (183 responding genes). In addition to known nitrate-responsive genes (e.g. those encoding nitrate transporters, nitrate reductase, nitrite reductase, ferredoxin reductase, and enzymes in the pentose phosphate pathway), genes encoding novel metabolic and potential regulatory proteins were found. These genes encode enzymes in glycolysis (glucose-6-phosphate isomerase and phosphoglycerate mutase), in trehalose-6-P metabolism (trehalose-6-P synthase and trehalose-6-P phosphatase), in iron transport/metabolism (nicotianamine synthase), and in sulfate uptake/reduction. In many cases, only a few select genes out of several in small gene families were induced by nitrate. These results show that the effect of nitrate on gene expression is substantial (affecting almost 10% of the genes with detectable mRNA levels) yet selective and affects many genes involved in carbon and nutrient metabolism.

One of the major nutrient responses in plants is the induction and repression of gene expression in response to nitrate (for review, see Redinbaugh and Campbell, 1991; Stitt, 1999; Wang et al., 2000). Nitrate serves as an important source of inorganic nitrogen for plants. It also signals rapid changes in metabolism that include the induction of the synthesis of nitrate assimilatory enzymes and shifting from starch biosynthesis to the production of organic acids to assimilate ammonium (for review, see Stitt, 1999; Foyer et al., 2003). Ammonium is synthesized from nitrate by the actions of nitrate reductase (NR) and nitrite reductase (NiR; for review, see Crawford, 1995; Crawford et al., 2000). Nitrite is fairly toxic to plants, and it does not accumulate to significant levels. The reduction of nitrite in shoots occurs in chloroplasts with reduced ferredoxin (Fd) serving as reductant. In roots, reduction occurs in plastids with reductant being produced via the pentose phosphate pathway. The uptake of nitrate involves nitrate transporters from two gene families, *NRT1* and *NRT2* (for review, see Crawford and Glass, 1998; Forde, 2000; Galvan and Fernandez, 2001; Glass et al., 2001; Williams and Miller, 2001). Four genes make up the *NRT1* family, and seven make up the *NRT2* family. The most studied genes are *NRT1.1* (*CHL1*), a nitrate-inducible gene encoding a dual-affinity nitrate transporter, *NRT2.1*, encoding the most significant component of the inducible, high-affinity, nitrate uptake system (iHATS), and *NRT1.2*, encoding a constitutive, low-affinity transporter.

Given the importance of nitrate as a source of nitrogen, it is not surprising that plants respond to nitrate in the environment. It was first shown in 1957 that NR activity is inducible by nitrate (Tang and Wu, 1957). Subsequently, NR and NiR genes were shown to be inducible as well as genes indirectly involved in nitrite reduction (uroporphyrin III methyltransferase [UPM1], Fd, NADH-ferredoxin reductase [FNR], 6-phosphogluconate dehydrogenase [6PGDH], and Glc-6-phosphate-1-dehydrogenase [G6PDH]; Stitt, 1999; Wang et al., 2000). In addition, specific isoforms of Gln synthetase (GS), Glu synthase (GOGAT), and phospho*enol*pyruvate carboxylase (PPC) are also known to be induced. This analysis has been extended to a genomic level; two microarray analyses have been reported using cDNA arrays containing 5,524 Arabidopsis genes/clones (Wang et al., 2000) and 1,280 tomato (*Lycopersicon esculentum*) genes (Wang et al., 2001). In Arabidopsis, 25 genes were found to be induced 2-fold or more by low nitrate for a short period of time (250 μM for 20 min), and 41 were induced 2-fold or more by high nitrate for a

[1] This work was supported by the National Institutes of Health (grant no. GM40672) and by the National Science Foundation (grant no. IBN–0115586).

[w] The online version of this article contains Web-only data. The supplemental material is available at http://www.plantphysiol.org.

* Corresponding author; email ncrawford@ucsd.edu; fax 858–534–1637.

Article, publication date, and citation information can be found at www.plantphysiol.org/cgi/doi/10.1104/pp.103.021253.

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

longer time (5–10 mM nitrate for 2 h; 20 mM for plants grown in submerged culture with high ammonium; Wang et al., 2000). In addition, eight genes showed reduced levels of mRNA (2-fold or greater) after the high-nitrate treatment. The most striking finding from the Arabidopsis experiments were that the most responsive genes were those devoted to nitrite reduction (e.g. NiR, UPM1, FNR, G6PDH, and 6PGDH); these genes responded to low levels of nitrate and were induced to the greatest extent. Many new nitrate-responsive genes were identified by this analysis, including two genes encoding enzymes of the nonoxidative pentose phosphate pathway, a calcium antiporter, an MYB transcription factor, two putative protein kinases, an Asn synthetase, and nonsymbiotic hemoglobin. Genes that were repressed by high nitrate for 2 h included the ammonium transporter AMT1;1 and the MADS-box transcription factor ANR1. The array analysis in tomato roots added 14 responsive genes (for plants deprived of nitrogen for 48 h then supplied with 4.8 mM nitrate for 1–96 h), including genes encoding water channels, phosphate and potassium transporters, potential regulatory proteins, stress response proteins, and ribosomal proteins (Wang et al., 2001).

Previous studies have shown that the nitrate response has several key properties. It is rapid (occurs within minutes), is independent of protein synthesis, and occurs at nitrate concentrations below 100 μM (for review, see Redinbaugh and Campbell, 1991; Stitt, 1999; Wang et al., 2000). Pharmacological studies have indicated that Ca^{2+} is involved, and protein kinases and phosphates also play a role (see ref. in Wang et al., 2000). We have been studying the nitrate response in Arabidopsis and have been creating a comprehensive list of genes that display rapid responses to nitrate at low concentrations, conditions that should reveal the primary response to nitrate. This project was accelerated with the release of the ATH1 Affymetrix chip (Affymetrix, Inc., Santa Clara, CA), which has 22,626 known or predicted genes from Arabidopsis (http://www.affymetrix.com/products/arrays/specific/arab.affx). This chip was used to analyze the rapid nitrate response in Arabidopsis roots and shoots. The results and analysis of these data are given below.

RESULTS AND DISCUSSION

Our previous microarray data of nitrate-induced genes was generated using whole plants submerged in liquid with 20 mM ammonium as the nitrogen source for 10 d (Wang et al., 2000). In the experiments described in this paper, plants were grown hydroponically with only the roots submerged in liquid. The nitrogen source was 5 mM ammonium [in the form of 2.5 mM $(NH_4)_2$succinate], which was completely consumed by 10 d. Carbon in the form of 0.5% (w/v) Suc was also included in the media. After 10 d, the media were supplemented with either 250 μM KNO_3 for induction or 250 μM KCl for the control for 20 min. Roots and shoots were harvested and total RNA was prepared. RNA was analyzed using the Affymetrix GeneChip Arabidopsis array ATH1 (contains 22,626 Arabidopsis probe sets) as described in "Materials and Methods." Two biological replicates (two independent sets of cultures) were performed for each organ. The complete array data for each sample are presented in the supplementary tables (see http://www.plantphysiol.org).

Comparison of Root and Shoot Responses

The data from the array give signal (RNA) levels and induction ratios, which were averaged for roots and for shoots using the biological replicates. An overall view of the expression data is presented as a scatter plot (Fig. 1). Signal intensities from nitrate-treated samples (y axis) were plotted over control samples (x axis) for roots (Fig. 1A) and shoots (Fig. 1B). Guide lines are provided in the plots showing when signal ratios are 1, 2, and 0.5 (-2). The data show that after 20 min of nitrate treatment, many genes are both induced and repressed greater than 2-fold in both roots and shoots. In addition, the scatter is much greater for roots than in shoots, indicating that the RNA response to nitrate is much larger in roots under these conditions. Care should be taken in interpreting the data points for signal intensities less than 100 (boxed off in the plots), which, as described below, is an approximate limit of reliable detection. If a signal is below 100, it is often not meaningful, and the resulting ratios can be artificially high.

To get an accurate count of the number of genes that are induced or repressed by the nitrate treatment, an assessment of the sensitivity of detection is needed. If signal values fall below a reliable detection limit, the values are not useful for determining signal ratios. To determine which genes show a reliable level of RNA, the Affymetrix Microarray Suite software (MAS version 5.0) was used to analyze the signals for each probe set. The results of this analysis are summarized in Table I. First, those genes that showed reliable signal levels in both biological replicates were identified. When a transcript was reliably detected, it was given a detection call of "P" (present), and when it was not detected, it was given a detection call of "A" (absent). For roots, 13,846 genes of a total of 22,626 had P call values in both root samples, and 13,221 genes had P call values for both shoot samples (Table I). Thus, a little more than one-half of the genes represented on the ATH1 chip gave reliable signals. Next, changes in RNA levels in response to nitrate (i.e. induction or repression) were assessed using Wilcoxon's signed rank tests as described (Affymetrix Statistical Algorithms Reference Guide). When the increase in signal level in response to nitrate is significant, a call of "I" (increase) is given. When a decrease in signal is significant, a call of "D" (decrease) is given. When this test was per-

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

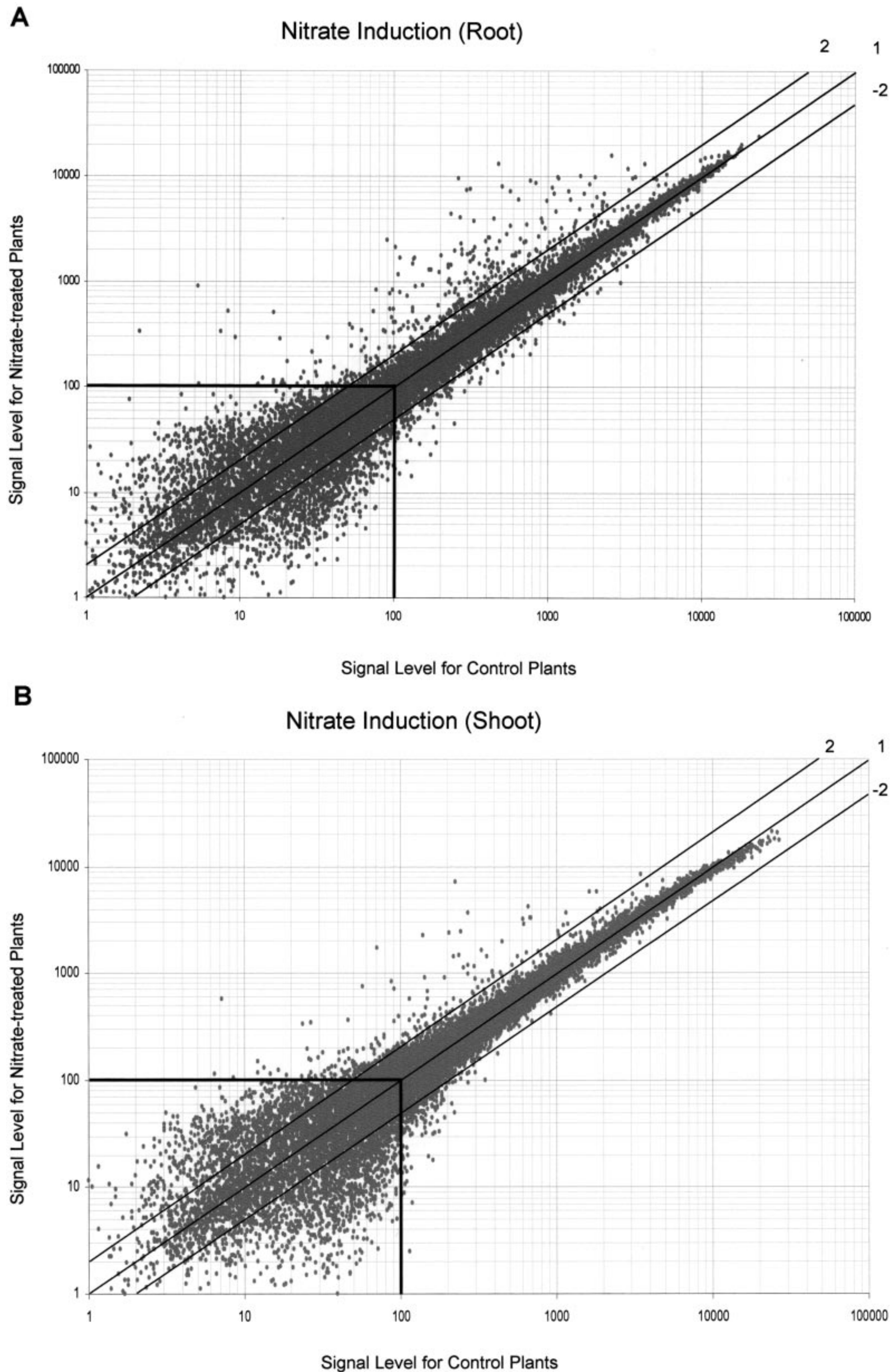

Figure 1. Scatter plots of ATH1 array data. Plotted are signal intensities for roots (A) and shoots (B). Values are averaged from two biological replicates for nitrate-treated samples and chloride-treated (control) samples. Guide lines are given showing signal values of 100 and showing signal ratios of 1 (no change), 2 (induced), and −2 (repressed).

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

Table I. *Genes responding to nitrate in roots versus shoots*

Criteria for Selection	Root No.	Shoot No.
Total no. of genes on array	22,626	22,626
Significant expression in both replicates	13,846	13,221
Significant expression and increase in both replicates	555	171
Significant expression and decrease in both replicates	621	12
Significant expression and increase 2.0 or more	251	76
Significant expression and decrease of 2.0 or more	78	2

formed, the following results were obtained. In roots, 555 genes had signals that significantly increased, and 621 had signals that decreased. In shoots, 171 genes showed increased signals, and 12 showed a decrease. If a threshold value of 2 was used for the signal ratio, the number of genes with increased signals was 251 in roots and 76 in shoots. The number of genes with decreased signals was less: 78 with decreased signals in roots and 2 with decreased signals in shoots. Thus, there is a 6-fold greater number of genes responding to nitrate in roots using the Wilcoxon's test (1,176 responsive genes in roots versus 183 in shoots) and a 4-fold greater response in roots using a cutoff value of 2 (Fig. 2 showing 329 in roots versus 78 in shoots). These results provide quantitative evidence on a genomic scale showing that roots have a more dramatic response to nitrate than shoots after 20 min.

Analysis of Nitrate Transporter Genes

Compared with the 25 nitrate-responsive genes identified previously for 250 μM nitrate at 20 min (Wang et al., 2000), the ATH1 data provide more responsive genes by more than an order of magnitude. To analyze the genes from the ATH1 array in more detail, data for specific groups of genes were extracted from the array tables and studied. First, data for the nitrate transporter gene families *NRT1* and *NRT2* gene families were examined (Fig. 3). Of 11 *NRT* genes, only four showed significant induction in roots (*NRT1.1*, *2.1*, *2.2*, and *2.4*; note that for *NRT2.6*, the signal calls were "absent," indicating that expression levels were too low to be reliable). Of these four genes, *NRT2.1* and *NRT2.2* show the most dramatic induction ratios. In fact, *NRT2.2* had the third highest induction ratio of any gene in roots; however, the signal from the chloride sample for this gene was not reliable ("absent") so that the value of the ratio is not reliable. If one considers only genes that had significant ("present") signals for both the induced and control samples, *NRT2.1* was the 10th most induced gene in the complete array behind those encoding G6PDH, NiR, phosphoglycerate mutase (PGM), and six unknown genes. The strong induction of *NRT2.1* is noteworthy because this gene is subject to repression by ammonium or high nitrogen. In our previous experiments where plants were grown on 20 mM ammonium, induction of *NRT2.1* was not observed at 250 μM nitrate for 20 min or 2 h (Wang et al., 2000). It took 5 to 10 mM nitrate for 2 h to induce *NRT2.1*. The fact that *NRT2.1* is induced so strongly in the ATH1 experiments described here indicates that the ammonium repression of *NRT2.1* is very weak in these plants, presumably due to the fact that the ammonium has been reduced to undetectable levels by the time the plants were treated with nitrate. In shoots, only two NRT genes showed response to nitrate (*NRT2.1* and *NRT2.5*), but the response by *NRT2.5* was very small, and the ratio for *NRT2.1* is not reliable because the control signal was "absent." Thus, shoot NRT genes show very little response to 250 μM nitrate after 20 min.

Another interesting finding shown in Figure 3 is the distribution of RNA signals for each family. Based on signal levels, *NRT1.1* and *NRT2.1* account for 78% of the total NRT signal in nitrate-treated roots, indicating that these two genes produce the vast majority of nitrate transporter mRNA in nitrate-treated roots of these 10-d-old seedlings. In control roots, *NRT1.1* and *NRT1.2* account for 64% of the NRT mRNA. For shoots, the data show that the mRNA from the NRT1 family of genes makes up over 90% of the total NRT mRNA in treated and control plants. These results indicate that, at least at the mRNA level, *NRT1.1* and *NRT2.1* dominate in roots that are freshly treated with nitrate, and NRT1 genes predominate in shoots of plants regardless of the nitrate treatment. It should be noted that for these experiments plants were grown with Suc in the media, which raises the level of *NRT1.1* mRNA in roots (Lejay et al., 1999).

It is interesting to compare the results from the ATH1 analysis with those reported previously for NRT2 genes in Arabidopsis using quantitative PCR (Orsel et al., 2002). In the study by Orsel et al. (2002), plants were grown on 1 mM KNO_3 with short days and without Suc, and then analyzed at 29 d and after flowering. Orsel et al. (2002) reported that NRT2.1 mRNA predominates in roots with *NRT2.4*, *2.5*, *2.6*, and *2.3* showing detectable levels in that order. Our results (obtained for 10-d-old seedlings grown with 24 h of light with Suc) show that NRT2.1 mRNA levels also dominate in nitrate-treated roots followed by *NRT2.4*, *2.5*, and *2.2*. In shoots, Orsel et al. (2002) reported that *NRT2.4* had the highest mRNA levels with detectable signals from *NRT2.5*, whereas our nitrate-treated plants showed that *NRT2.5*, *2.7*, and *2.1* gave equivalent signals, and *NRT2.4* gave no detectable signal. This comparison shows that *NRT2.1* encodes the majority of NRT2 mRNA in roots in the presence of nitrate, whereas shoots show dif-

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

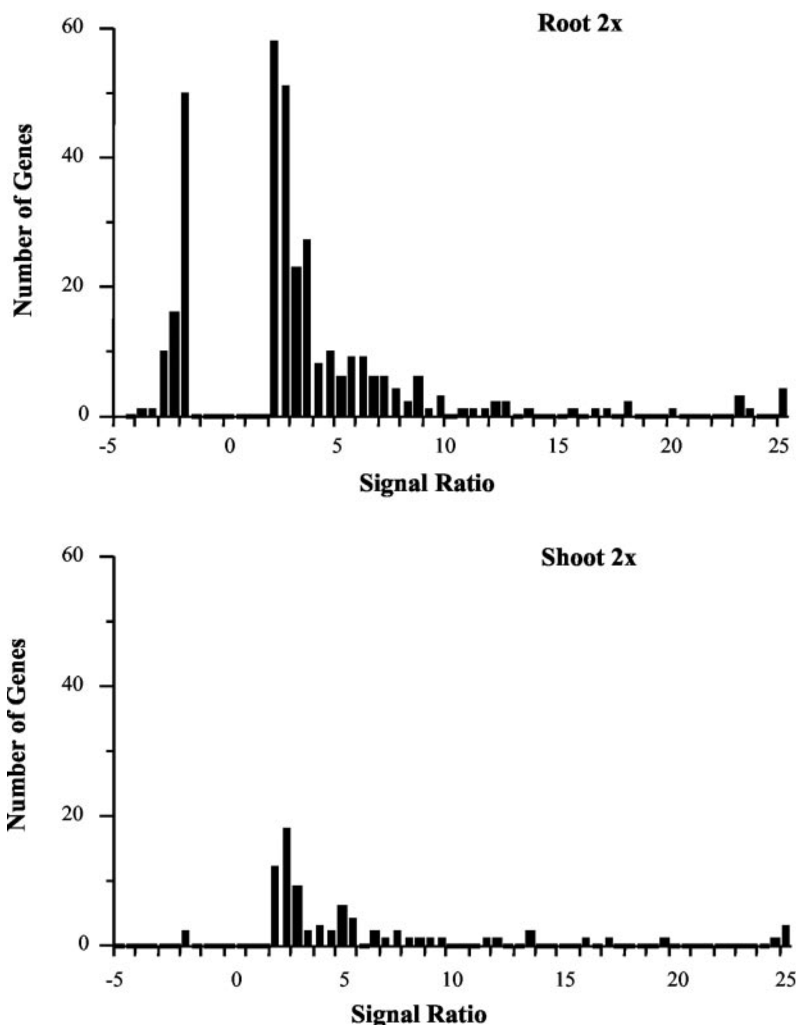

Figure 2. Numbers of induced/repressed genes in roots and shoots. Histogram pair shows the number of genes that were given a call of present (reliable signal intensity) for RNA samples from both biological replicates and had average ratios 2.0 and above and −2.0 and below. Note that the signal ratios were determined by the Affymetrix MAS software by comparing each probe pair on the induced array with the corresponding probe pair on the control array and, thus, are not simply the ratio of the average signal intensities shown in Figure 1. Bars above the 25 value correspond to values that were greater than 25 and were grouped together.

ROOT Ratio	SHOOT Ratio	Gene Description	ROOT Nit-Signal	ROOT Ctrl-Signal	SHOOT Nit-Signal	SHOOT Ctrl-Signal	Sequence ID	Probeset ID
5.5	NC	NRT1.1	9891	2060	8050	6858	At1g12110	264348_at
-1.3	NC	NRT1.2	1490	1928	1412	1481	At1g69850	260414_at
NC	NC	NRT1.3	252	447	973	874	At3g21670	258181_at
NC	NC	NRT1.4	127*	169*	2600	2510	At2g26690	267612_at
17.8	19.6	NRT2.1	7399	298	334	24*	At1g08090	260623_at
55.1	NC	NRT2.2	332	7*	9*	12*	At1g08100	260624_at
NC	NC	NRT2.3	31*	49*	52*	80*	At5g60780	247592_at
5.2	NC	NRT2.4	1790	404	19*	24*	At5g60770	247591_at
NC	1.2	NRT2.5	599	727	557	337	At1g12940	261198_at
NC	NC	NRT2.6	72*	28*	17*	55*	At3g45060	252604_at
NC	NC	NRT2.7	154*	162*	481	504	At5g14570	250151_at

Figure 3. NRT family of genes. Shows average signal ratios and signal intensities for the NRT nitrate transporter gene families. Note that the signal ratios were determined by the Affymetrix MAS software by comparing each probe pair on the induced array with the corresponding probe pair on the control array and, thus, are not simply the ratio of the average signal intensities. NC, No change as determined by the Affymetrix MAS software. A value is given for the ratio only if both RNA samples had a call of I or D. An asterisk by a signal value indicates an "Absent" call.

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

ROOT Ratio	SHOOT Ratio	Gene Description	ROOT Nit-Signal	ROOT Ctrl-Signal	SHOOT Nit-Signal	SHOOT Ctrl-Signal	Sequence ID	Probeset ID
12.6	3.2	Nitrate reductase (NIA1)	7213	575	5710	1626	At1g77760	259681_at
1.9	2.4	Nitrate reductase (NIA2)	9529	4951	8341	3473	At1g37130	261979_at
23.4	24.3	Nitrite reductase (NiR)	13272	483	7127	227	At2g15620	265475_at
15.5	13.5	Urophorphyrin III Methylase (UPM1)	8056	508	3622	270	At5g40850	249325_at
9.3	4.2	Ferredoxin NADP reductase (FNR)	6043	648	2855	616	At4g05390	255230_at
8.3	4.8	Ferredoxin NADP reductase (FNR)	9819	1168	1990	450	At1g30510	261806_at
NC	NC	Ferredoxin NADP reductase (FNR)	145	157	162	125	At4g32360	253479_at
NC	NC	Ferredoxin NADP reductase (FNR)	309	335	4276	4176	At5g66190	247131_at
NC	NC	Ferredoxin NADP reductase (FNR)	17*	42*	4611	4191	At1g20020	261218_at
NC	NC	Ferredoxin NADP reductase (FNR)	292	320	377	370	At5g66810	247062_at
3.0	2.8	Putative ferredoxin	12986	4293	5787	1823	At2g27510	265649_at
NC	NC	Ferredoxin	149	168	1517	1208	At4g14890	245347_at
NC	NC	Ferredoxin	119*	120	1236	1172	At1g32550	256468_at
NC	NC	Putative protein ferredoxin	106	114	172	191	At5g07950	250555_at
NC	NC	Ferredoxin precusor isolog	1256	1540	7656	6510	At1g10960	260481_at
NC	NC	Ferrodoxin precursor	1342	1261	11243	13017	At1g60950	259727_at
35.0	36.3	Glucose-6-phosphate 1-dehydrogenase	9617	261	2234	139	At1g24280	264859_at
10.9	5.1	Glucose-6-phosphate 1-dehydrogenase	8601	837	3271	679	At5g13110	245977_at
7.5	5.2	6-phosphogluconate dehydrogenase	9514	1189	4141	666	At5g41670	249266_at
5.9	2.6	6-phosphogluconate dehydrogenase	15533	2633	3662	1155	At1g64190	262323_at
3.7	NC	Transketolase	11917	3377	9943	8834	At3g60750	251396_at
NC	NC	Transketolase precursor	1267	1208	301	295	At2g45290	245089_at
2.6	1.4	Transaldolase	9589	3586	2741	1764	At5g13420	250234_at
NC	NC	Similar to transaldolase	1165	1138	1153	1194	At1g12230	260967_at

Figure 4. Nitrate/nitrite assimilatory genes. Shows average signal ratios and signal intensities for NR, NiR, and genes involved in nitrite reduction, including those in the pentose phosphate pathway. An asterisk by a signal value indicates an "Absent" call.

ferent patterns of NRT2 gene expression depending on the age and growth conditions.

Analysis of Nitrate Assimilation Genes

The next group of genes that was examined encodes nitrate assimilatory enzymes involved in reduction of nitrate to nitrite and nitrite to ammonium (Fig. 4). The two NR genes, *NIA1* and *NIA2*, were both induced in roots and shoots as expected. In roots, induction of *NIA1* was much higher (15-fold) than for *NIA2* (2-fold). This differential induction of *NIA1* and *NIA2* has been described previously (Cheng et al., 1991). *NiR* was also dramatically induced in both organs as previously described (Wang et al., 2000). In fact, *NiR* was the third most induced gene in shoots and the eighth highest in roots. *UPM1*, which encodes an enzyme that makes a cofactor for NiR, was also highly induced in both organs. In our previous work, two FNR and one Fd genes, involved in supplying reductant to NiR, were found to be induced (Wang et al., 2000). In the ATH1 array, the

ROOT Ratio	SHOOT Ratio	Gene Description	ROOT Nit-Signal	ROOT Ctrl-Signal	SHOOT Nit-Signal	SHOOT Ctrl-Signal	Sequence ID	Probeset ID
NC	NC	GS-GLN1-1	11441	12799	9932	12666	At5g37600	249581_at
NC	NC	GS-GLN1-2	15888	13029	9202	10947	At1g66200	256524_at
NC	NC	GS-GLN1-3	4715	5020	3396	3458	At3g17820	258160_at
NC	NC	GS-GLN1-4	2531	3064	974	1060	At5g16570	250100_at
NC	NC	GS-GLN1-5	79*	81*	105*	112*	At1g48470	261305_at
2.0	NC	GS-GLN2	3735	1907	10200	10154	At5g35630	249710_at
NC	NC	GS-GLN3	1246	1137	547	576	At3g53180	251973_at
NC	NC	GOGAT Fd-GLU1	85*	58*	2707	2295	At5g04140	245701_at
NC	NC	GOGAT Fd-GLU2	3079	2772	1117	1263	At2g41220	266365_at
2.6	1.6	GOGAT NADH-GLT1	9757	3755	2340	1346	At5g53460	248267_at
NC	1.4	AS-ASN1	87*	77*	867	675	At3g47340	252415_at
1.8	2.0	AS-ASN2	630	336	719	316	At5g65010	247218_at
NC	NC	AS-ASN3	858	803	403	387	At5g10240	250484_at

Figure 5. Ammonium assimilatory genes. Shows average signal ratios and signal intensities for GS, GOGAT, and Asn synthetase (AS). An asterisk by a signal value indicates an "Absent" call.

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

ROOT Ratio	SHOOT Ratio	Gene Description	ROOT Nit-Signal	ROOT Ctrl-Signal	SHOOT Nit-Signal	SHOOT Ctrl-Signal	Sequence ID	Probeset ID
20.1	32.3	phosphoglycerate mutase	7596	315	1713	71*	At1g78050	262180_at
3.7	NC	putative phosphoglycerate mutase	1600	411	278	214	At1g22170	255924_at
1.3	NC	putative phosphoglycerate mutase	4624	3513	2006	1688	At1g09780	264668_at
2.5	1.4	glucose-6-phosphate isomerase	5625	2295	1424	1032	At4g24620	254141_at
13.5	NC	trehalose-6-phosphate phosphatase	1667	110*	56*	23*	At1g78090	260059_at
3.7	NC	trehalose-6-phosphate phosphatase	953	235	150	71*	At5g65140	247228_at
2.7	NC	trehalose 6-phosphate synthase	461	170	272	204	At1g23870	263019_at
1.6	NC	trehalose-6-phosphate synthase	1156	702	633	485	At1g60140	264246_at
1.6	NC	trehalose-6-phosphate phosphatase	383	242	79*	24*	At5g10100	250467_at
1.6	NC	trehalose-6-phosphate phosphatase	480	280	447	371	At5g51460	248404_at
4.3	NC	phosphoenolpyruvate carboxylase kinase	1484	362	337	176	At3g04530	258570_at
2.2	2.1	phosphoenolpyruvate carboxylase (PPC)	7322	3392	422	197	At3g14940	257217_at
2.9	2.1	chloroplast NAD-dependent malate dehydrog.	6882	2338	3795	1856	At3g47520	252407_at
4.3	1.7	2-oxoglutarate/malate translocator precursor	8328	1881	6610	3820	At5g12860	250278_at
5.9	NC	nicotianamine synthase	1802	303	207	159	At5g04950	250832_at
2.7	NC	nicotianamine synthase	559	249	148*	178	At5g56080	248048_at
3.4	NC	putative high affinity sulfate transporter	1350	442	267	300	At1g78000	262133_at
3.1	NC	putative sulfate transporter	2335	709	1247	822	At1g80310	260302_at
3.5	1.8	5 -adenylylphosphosulfate reductase	1787	608	2399	1441	At1g62180	264745_at
2.6	1.3	putative molybdopterin synthase large subunit	1784	589	986	664	At2g43760	260572_at
2.3	1.6	molybdopterin synthase (CNX2)	888	368	908	581	At2g31950	263472_at
-3.2	NC	aquaporin-"NIP2,1"	254	840	163	223	At2g34390	267024_s_at

Figure 6. Other metabolic genes. Shows average signal ratios and signal intensities for additional and novel metabolic and transporter genes. An asterisk by a signal value indicates an "Absent" call.

same result was obtained. Two of six FNR genes and one of six Fd (or Fd-like) genes were induced by nitrate in both shoots and roots. The induction of the two FNR genes was substantial in roots. Another group of genes involved in producing reductant for nitrite reduction encodes enzymes in the pentose phosphate pathway, which generates NADPH. As described previously, genes encoding two enzymes in the oxidative arm of the pathway, G6PDH and 6PGDH, and genes encoding enzymes in the nonoxidative arm, transketolase and transaldolase, were induced (Wang et al., 2000). The ATH1 array gave the same result (Fig. 4). The induction was strongest in the root except for one of the G6PDH genes. This gene (At1g24280) was the second most induced gene in shoots and the fifth highest in roots.

Last, genes involved in ammonium assimilation were examined (Fig. 5). We found that one GS gene (*GLN2*), one GOGAT gene (NADH dependent, *GLT1*), and ASN2 are induced in roots after 20 min. In shoots, no GS gene, only one GOGAT gene (*GLT1*), and two ASN genes (*ASN1* and *ASN2*) were induced, although the response was low. These results are similar to what we reported previously with the glass slide arrays where one GS, one Asn synthetase (ASN2), and two NADH-GOGAT genes were found to be induced either at 20 min or 2 h (Wang et al., 2000).

Analysis of Glycolysis-Related Genes

The most interesting and novel finding in our data was the induction of genes that are involved or potentially involved in glycolysis. First, two genes encoding the glycolytic enzymes PGM and Glc-6-phosphate isomerase (G6PI) were induced. Glycolytic enzymes and their corresponding genes are not known to respond to nitrate. Both PGM and G6PI are involved in isomerization reactions that involve little change in free energy and, thus, are not rate-limiting steps in glycolysis. It is interesting, however, that G6PI serves both glycolysis and the pentose phosphate pathways. G6PI interconverts Glc-6-P and Fru-6-P, both intermediates in glycolysis. Fru-6-P is also a product of the pentose phosphate pathway, and Glc-6-P is the initial substrate. Glycolysis and pentose phosphate oxidation occur in the same compartments (cytosol and plastids) and are integrated (Dennis and Blakeley, 2000). It is possible that G6PI induction helps recycle carbon back to the pentose phosphate pathway and, thus, aids in the generation of NADPH by pentose phosphate oxidation. Another product of the pentose phosphate pathway is glyceraldehyde-3-P. This is also a glycolytic intermediate, which when oxidized to glycerate-3-P, becomes the substrate for PGM. Perhaps PGM induction is needed to help metabolize glyceraldehyde-3-P generated by the pentose phosphate pathway. The induction of G6PI is modest, but induction of one PGM gene is very strong, being in the top 20 induced genes in roots and the top three induced genes in shoots. There are six potential PGM genes identified in the ATH1 array, and three of these genes are induced by nitrate.

The next six genes involve the metabolism of trehalose-6-P. Trehalose is a disaccharide of Glc and

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

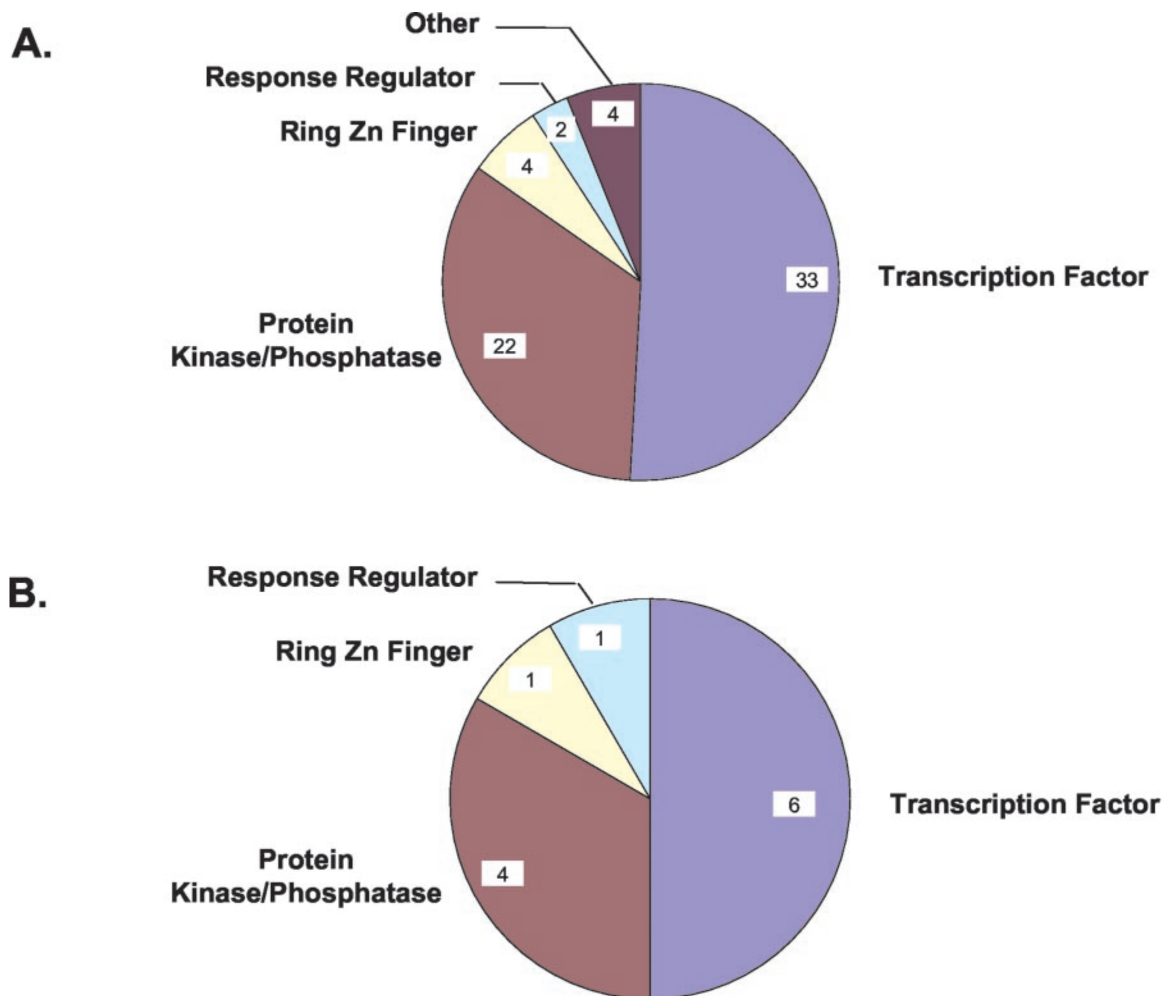

Figure 7. Potential regulatory genes. Pie charts showing the number of genes identified on the ATH1 array in one of several categories (transcription factors/DNA-binding proteins, protein kinase/phosphatases, response regulators, RING Zn finger proteins, and other) for (A) roots and (B) shoots showing 2.0-fold or greater induction or repression.

is synthesized in two steps catalyzed by trehalose-6-P synthase (TPS) and trehalose-6-P phosphatase (TPP). In yeast (*Saccharomyces cerevisiae*), trehalose-6-P regulates glycolysis. In plants, its role is not known, but trehalose-6-P is implicated in controlling photosynthetic capacity (Paul and Pellney, 2003), and mutations in one of 11 possible TPS genes in Arabidopsis leads to an embryo lethal phenotype (for review, see Eastmond et al., 2003). Genes encoding enzymes that are implicated in both steps of trehalose synthesis are induced in roots but not shoots (Fig. 6). The induction of one of the TPP genes is quite substantial. There are a total of 10 potential TPP genes and 11 potential TPS genes in the ATH1 array. This result suggests that nitrate may be altering trehalose-6-P levels, which may, in turn, affect glycolysis if it behaves in plants as it does in yeast. Given the strong connection between nitrate reduction and pentose oxidation, it is also possible that trehalose-6-P is a regulator of the pentose phosphate pathway.

Analysis of Other Metabolic and Regulatory Genes

As has been reported before, nitrate signals a shift from starch biosynthesis to organic acid production (for review, see Stitt, 1999; Foyer et al., 2003). Three enzymes involved in this process, PPC kinase, PPC, and NAD-dependent malate dehydrogenase (NMDH), all show gene induction in roots, and the latter two show induction in shoots (Fig. 6). There are four PPC genes on the ATH1 array, and only one shows induction. The NMDH induction is interesting because it has been found in the nitrate assimilation gene cluster in *Chlamydomonas reinhardtii* (Quesada et al., 2000). Together with the malate-oxalacetate translocator (also induced in roots and shoots, Fig. 6), NMDH is thought to export reducing power from the chloroplast to the cytosol to support nitrate reduction.

As observed previously (Wang et al., 2000), the nicotianamine synthase gene is induced by nitrate.

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

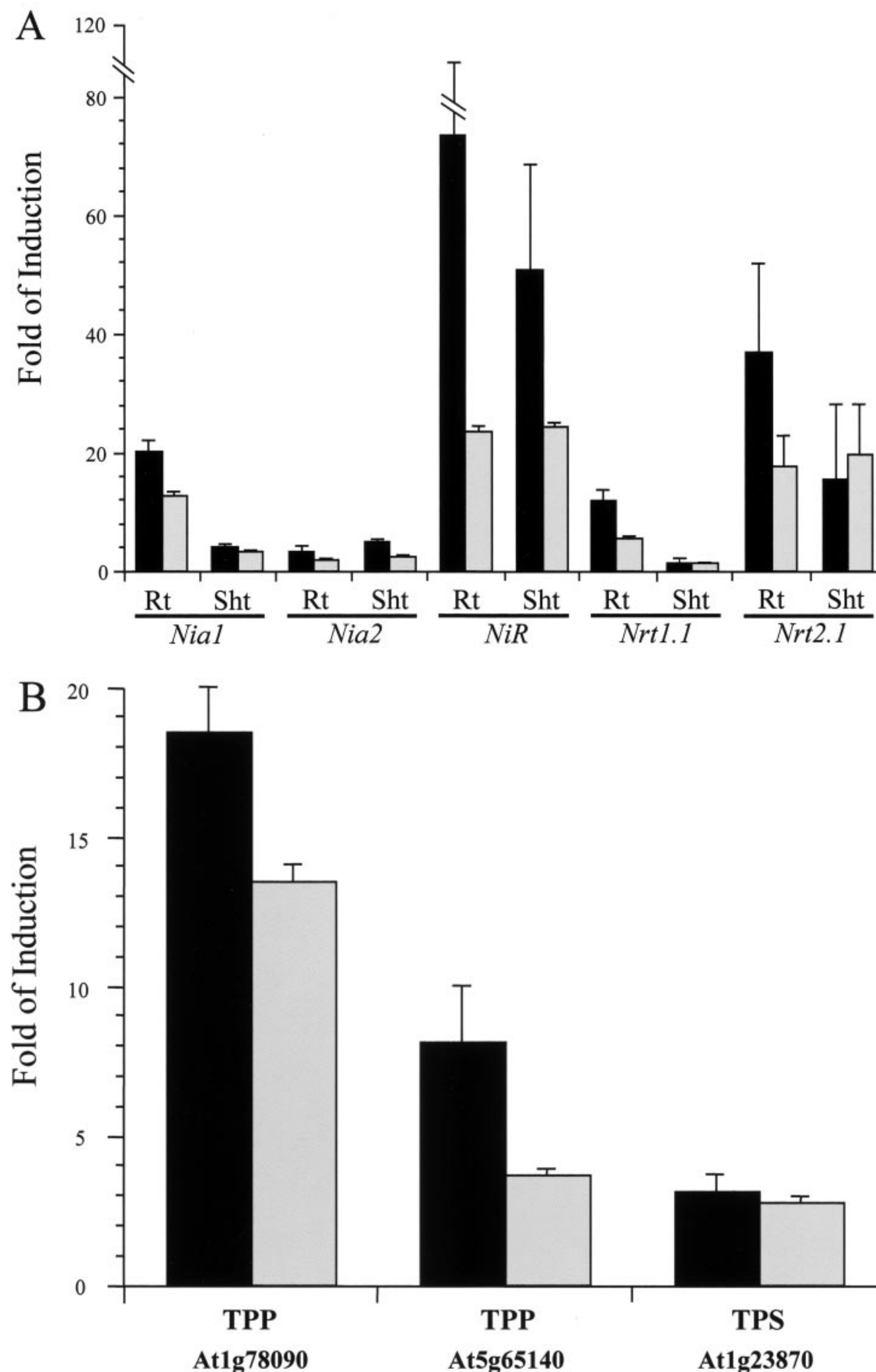

Figure 8. Comparison of real-time PCR and ATH1 array data. Histograms shows ATH1 signal ratios and quantitative real-time PCR ratios for select genes. The array data are averages of two biological replicates, and the error bar shows the spread between the two data points. The PCR data were obtained from two independent reactions for each biological replicate (total of four reactions) and averaged. Error bars = SE. Ubiquitin 10 was used as the standard to normalize each sample. A, Induced genes involved in nitrate uptake and nitrate or nitrite reduction. B, Induced genes involved in trehalose-6-P synthesis.

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

Induction of two nicotianamine synthase genes is observed with the ATH1 array in roots. Nicotianamine is involved in iron (Fe) acquisition, transport, and homeostasis in plants (van Wiren et al., 1999; Pich et al., 2001). Fe is required for many of the enzymes in nitrate assimilation including NR, NiR, and Fd. Perhaps nitrate is inducing the synthesis of nicotianamine to facilitate the transport of Fe to support synthesis of nitrate assimilatory enzymes.

Another set of genes induced by nitrate is implicated in sulfur metabolism. Two putative sulfate transporter genes and a 5-adenylylphosphosulfate reductase gene are induced in roots; the latter is also induced in shoots. 5-Adenylylphosphosulfate reductase is part of a novel pathway in plants for the assimilation of sulfate (Leustek et al., 2000). A linkage between sulfur and nitrogen metabolism has been known for many years, with a deprivation of one leading to a disruption of the metabolism of the other (Reuveny et al., 1980; Prosser et al., 2001). It has also been shown in barley (*Hordeum vulgare*) that high levels of nitrate or ammonium can induce a high-affinity sulfate transporter gene and sulfate uptake in nitrogen-starved plants, suggesting that a nitrogen metabolite may affect sulfate transporter gene expression (Vidmar et al., 1999). The data in Figure 6 suggest that nitrate itself can induce sulfate uptake and assimilation genes and, thereby, may increase sulfate assimilation.

Two genes involved in molybdopterin biosynthesis are induced in roots and shoots (Fig. 6). Molybdopterin serves as a cofactor for NR, and many of the genes involved in the synthesis of this molecule have been identified (Mendel and Hansch, 2001).

Lastly, none of the 35 aquaporin water channel genes on the ATH1 array are induced by nitrate in either roots or shoots. However, one is significantly repressed in roots (NIP2.1, Fig. 6). In fact, it is the third most repressed gene in the list of expressed genes in the ATH1 list. In tomato roots, several aquaporin genes were found to be induced by nitrate (Wang et al., 2001); however, this induction was observed 6 to 48 h after treatment so that these results are not comparable with the ATH1 data, which were taken 20 min after treatment.

In addition to the metabolic genes described above, many potential regulatory genes were found to respond to nitrate after 20 min. If the analysis is restricted to genes that show reliable signals and response ratios equal to or greater than 2-fold, 65 genes in roots and 12 genes in shoots were identified as possible regulatory genes. As an example, the two most highly induced genes in roots are a putative protein phosphatase (At4g32950, 8.9-fold increase) and a putative protein kinase (At1g49160, 8.6-fold increase). A putative Leu zipper transcription factor (At5g65210, similar to the tobacco [*Nicotiana tabacum*] transcription factor TGA1a; Katagiri et al., 1989) that is induced by nitrate in tomato roots (Wang et al., 2001) is also induced 4-fold in roots and 1.8-fold in shoots in the ATH1 data. A summary of the results showing how many genes in each class for roots and shoots is shown (Fig. 7).

Verification of the ATH1 Array Data

We used several criteria to verify the array data. First, two biological replicates were performed, and only genes that showed reliable signals and signal ratios in both replicates were considered. Second, we performed real-time quantitative PCR on a select group of genes and compared the results with those obtained from the array (Fig. 8). The induction ratios from the PCR data generally exceeded those from the array; however, if the overall difference is used to normalize the data, then the correlation between the PCR and array data are very good. Those genes that were the most highly induced (e.g. *NiR*, *NRT2.1*, and *TPP-At1g78090*) gave the highest induction ratios in both methods, whereas those that were the least induced (e.g. *NRT1.1* in shoots) gave the lowest ratios in both methods.

CONCLUSIONS

The use of the ATH1 array, which contains probes for most of the known and predicted Arabidopsis genes, has lead to the most thorough analysis of nitrate-induced genes to date. Using this method, over 1,000 genes are found to respond to low levels of nitrate after only 20 min. New connections between nitrate and glycolysis, trehalose-6-P synthesis, sulfate uptake, and reduction have been uncovered. An interesting feature of nitrate regulation observed in these experiments is that only select members of small gene families respond to nitrate in a significant way. Examples of this can be observed for the NRT1, NRT2, FNR, Fd, GS, PGM, and TPP genes. Other members of these families respond to nitrate but to a much lower extent. Thus, it appears that only a fraction of the potential target genes that encode nitrate-regulated enzymes have retained the nitrate response. Future efforts will be devoted to understanding the mechanisms that mediate nitrate responses for these genes.

MATERIALS AND METHODS

Plant Growth Media

The growth medium contained the following components (final concentration): 10 mm potassium phosphate (pH 6.5), 2.5 mm $(NH_4)_2$succinate, 2 mm $MgSO_4$, 1 mm $CaCl_2$, 0.1 mm $FeNa_2EDTA$, 0.5% (w/v) Suc, and micronutrients (50 μm H_3BO_3, 12 μm $MnSO_4$, 1 μm $ZnCl_2$, 1 μm $CuSO_4$, and 0.2 μm Na_2MoO_4). Component solutions were autoclaved ($FeNa_2EDTA$ was filter sterilized) separately and added to sterile deionized water before use.

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

Hydroponics Growth Conditions

Arabidopsis ecotype Columbia was grown at 25°C to 27°C under constant illumination with agitation at 120 rpm for 10 d. Plants were grown in a 50-mL glass beaker containing a segment (20 mm in height) of a 50-mL Falcon centrifuge tube with triangular openings at bottom to allow for liquid flow. On top of the tube was a disc of polypropylene mesh (250-micron pore size, 25 mm in diameter). Beakers initially contained 25 mL of sterile medium (described above) and approximately 50 surface-sterilized seeds evenly distributed on the mesh disc. On d 5, 5 mL of medium was removed from the beaker to allow shoots to stay above the medium liquid. The beaker top was covered with three layers of aluminum foil to keep the culture sterile.

Nitrate Treatment

On d 10, 50 μL of 100 mm KNO_3 (induced) or 100 mm KCl (control) was added to the beaker (final concentration of 250 μm) and incubated under growth conditions (as described above) for 20 min. At least three beakers were used for nitrate treatment or KCl treatment. At the end of the 20 min incubation, root and shoot were separated and frozen in liquid nitrogen for total RNA preparation.

RNA Preparation

Total RNA was prepared by using an RNeasy Plant Mini Kit (Qiagen, Chatsworth, CA) and quantified with an LKB Biochrom Ultraspec II spectrophotometer.

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 transcriptase-PCR with 3 μg of total RNA. The cDNA synthesis reaction mixture was diluted 25 times before being used for PCR. Primers for the PCR reactions were designed to have a melting temperature of about 63°C to 69°C and to give a PCR product between 175 and 250 bp. The genes tested for expression levels by quantitative PCR were as follows: *NRT1.1*, forward primer (5′-AAAGCTGCCACACACTGAAC) and reverse primer (5′-ATTGTGCGACTGATAATGTCGT); *NIA1*, forward primer (5′-GGTGC-TGGTGTTTCTGGTCACT) and reverse primer (5′-GGGTCTGGTCGGGT-GTTC); *NIA2*, forward primer (5′-CGAGACACACAACAGCAACGC) and reverse primer (5′-GCAACGGGACAGGGGTGA); *NiR*, forward primer (5′-CCGGTAGCCAGTTCTGCG) and reverse primer (5′-CCTATTCGTCC-CCCGACGT); *NRT2.1*, forward primer (5′-GGCTAACGTGGATGGGAGTG) and reverse primer (5′-CGGCGTAGCAGCAGAGC); *TPP-At1g78090*, forward primer (5′-CCATCTTGGGTTTGAAAACCATC) and reverse primer (5′-GGCTGCTTCAAGAAGTTTCA); *TPP-At5g65140*, forward primer (5′-TAACATGTTCGAACGAATCATC) and reverse primer (5′-TTAGCCATT-TTTTTCACTGTTCTT); and *TPS-At1g23870*, forward primer (5′-GAGTTT-CAGAGTTCTGTCACTGTC) and reverse primer (5′-TTCGGCATTAGG-GGTTTTGATGAT). The internal control gene was *ubiquitin 10*: forward primer, 5′-GTCCTCAGGCTCCGTGGTG; and reverse primer, 5′-TGCCATC-CTCCAACTGCTTTC.

The LightCycler-FastStart DNA Master SYBR Green I Kit (Roche) was used for the PCR reactions. Each PCR reaction contains 2 μL of cDNA and 0.5 μm of each of the primers. The initial denaturing time was 10 min., followed by 35 PCR cycles consisting of 94°C for 0 s, 63°C for 5 s, and 72°C for 10 s. A melting curve was run after the PCR cycles followed by a cooling step. Quantification was performed with LightCycler Relative Quantification Software 1.0.

Target Preparation/Processing for GeneChip Analysis

Isolated total RNA samples were processed as recommended by Affymetrix (Affymetrix GeneChip Expression Analysis Technical Manual, Affymetrix, Inc.). In brief, total RNA was initially isolated using an RNeasy Plant Mini Kit (Qiagen). Total RNAs were quantified with a portion of the recovered total RNA adjusted to a final concentration of 1 μg μL^{-1}. All starting total RNA samples were quality assessed before beginning target preparation/processing steps by running out a small amount of each sample (typically 25–250 ng $well^{-1}$) onto a RNA Lab-On-A-Chip (Caliper Technologies Corp., Mountain View, CA) that was evaluated on an Agilent Bioanalyzer 2100 (Agilent Technologies, Palo Alto, CA). Single-stranded, then double-stranded cDNA was synthesized from the poly(A^+) mRNA present in the isolated total RNA (10 μg of total RNA starting material each sample reaction) using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen Corp., Carlsbad, CA) and poly (T)-nucleotide primers that contained a sequence recognized by T_7 RNA polymerase. A portion of the resulting double-stranded cDNA was used as a template to generate biotin-tagged cRNA from an in vitro transcription reaction (IVT), using the Bio-Array High-Yield RNA Transcript Labeling Kit (T_7; Enzo Diagnostics, Inc., Farmingdale, NY). Fifteen micrograms of the resulting biotin-tagged cRNA was fragmented to strands of 35 to 200 bases in length following prescribed protocols (Affymetrix GeneChip Expression Analysis Technical Manual). Subsequently, 10 μg of this fragmented target cRNA was hybridized at 45°C with rotation for 16 h (Affymetrix GeneChip Hybridization Oven 320) to probe sets present on an Affymetrix ATH1 array. The GeneChip arrays were washed and then stained (streptavidin-phycoerythrin) on an Affymetrix Fluidics Station 400, followed by scanning on a Hewlett-Packard GeneArray scanner (Hewlett-Packard, Palo Alto, CA). The results were quantified and analyzed using MicroArray Suite 5.0 software (Affymetrix, Inc.).

Received January 29, 2003; returned for revision February 27, 2003; accepted March 19, 2003.

LITERATURE CITED

Cheng C-L, Acedo GN, Dewdney J, Goodman HM, Cankling MA (1991) Differential expression of the two *Arabidopsis* nitrate reductase genes. Plant Physiol **96:** 275–279

Crawford NM (1995) Nitrate: nutrient and signal for plant growth. Plant Cell **7:** 859–868

Crawford NM, Glass ADM (1998) Molecular and physiological aspects of nitrate uptake in plants. Trends Plant Sci **3:** 389–395

Crawford NM, Kahn ML, Leustek T, Long SR (2000) Nitrogen and sulfur. *In* B Buchanan, W Gruissem, RL Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp 786–849

Dennis DT, Blakeley SD (2000) Carbohydrate metabolism. *In* B Buchanan, W Gruissem, RL Jones, eds, Biochemistry and Molecular Biology of Plants. American Society of Plant Physiologists, Rockville, MD, pp 630–675

Eastmond PJ, Li Y, Graham IA (2003) Is trehalose-6-phosphate a regulator of sugar metabolism in plants? J Exp Bot **54:** 533–537

Forde BG (2000) Nitrate transporters in plants: structure, function and regulation. Biochim Biophys Acta **1465:** 219–235

Foyer CH, Parry M, Noctor G (2003) Markers and signals associated with nitrogen assimilation in higher plants. J Exp Bot **54:** 585–593

Galvan A, Fernandez E (2001) Eukaryotic nitrate and nitrite transporters. Cell Mol Life Sci **58:** 225–233

Glass ADM, Brito DT, Kaiser BN, Kronzucker HJ, Kumar A, Okamoto M, Rawat SR, Siddiqi MY, Silim SM, Vidmar JJ et al. (2001) Nitrogen transport in plants, with an emphasis on the regulation of fluxes to match plant demand. J Plant Nutr Soil Sci **164:** 199–207

Katagiri F, Lam E, Chua NH (1989) Two tobacco DNA-binding proteins with homology to the nuclear factor CREB. Nature **340:** 727–730

Lejay L, Tillard P, Leptit M, Olive FD, Filleur S, Daniel-Vedele F, Gojon A (1999) Molecular and functional regulation of two NO_3^- uptake systems by N- and C-status of Arabidopsis plants. Plant J **18:** 509–519

Leustek T, Martin MN, Bick JA, Davies JP (2000) Pathways and regulation of sulfur metabolism revealed through molecular and genetic studies. Annu Rev Plant Physiol Plant Mol Biol **51:** 141–165

Mendel RR, Hansch R (2001) Molybdoenzymes and molybdenum cofactor in plants. J Exp Bot **53:** 1689–1698

Orsel M, Krapp A, Daniel-Vedele F (2002) Analysis of the NRT2 nitrate transporter family in Arabidopsis: structure and gene expression. Plant Physiol **129:** 886–896

Paul MJ, Pellny TK (2003) Carbon metabolite feedback regulation of leaf photosynthesis and development. J Exp Bot **54:** 539–547

Pich A, Manteuffel R, Hillmer S, Scholz G, Schmidt W (2001) Fe homeosta-

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.

sis in plant cells: does nicotianamine play multiple roles in the regulation of cytoplasmic Fe concentration? Planta **213:** 967–976

Prosser IM, Purves JV, Saker LR, Clarkson DT (2001) Rapid disruption of nitrogen metabolism and nitrate transport in spinach plants deprived of sulphate. J Exp Bot **52:** 113–121

Quesada A, Gomez-Garcia I, Fernandez E (2000) Involvement of chloroplast and mitochondria redox valves in nitrate assimilation. Trends Plant Sci **5:** 463–464

Redinbaugh MG, Campbell WH (1991) Higher plant responses to environmental nitrate. Physiol Plant **82:** 640–650

Reuveny Z, Dougall DK, Trinity P (1980) Regulatory coupling of nitrate and sulfate assimilation pathways in cultured tobacco cells. Proc Natl Acad Sci USA **77:** 6670–6672

Stitt M (1999) Nitrate regulation of metabolism and growth. Curr Opin Plant Biol **2:** 178–186

Tang PS, Wu HY (1957) Adaptive formation of nitrate reductase in rice seedlings. Nature **179:** 1355–1356

van Wiren N, Klair S, Bansal S, Briat JF, Khodr H, Shioiri T, Leigh RA, Hider RC (1999) Nicotianamine chelates both Fe-III and Fe-II: implications for metal transport in plants. Plant Physiol **119:** 1107–1114

Vidmar JJ, Schjoerring JK, Touraine B, Glass ADM (1999) Regulation of the *hvst1* gene encoding a high-affinity sulfate transporter from *Hordeum vulgare*. Plant Mol Biol **40:** 883–892

Wang R, Guegler K, LaBrie ST, Crawford NM (2000) Genomic analysis of a nutrient response in Arabidopsis reveals diverse expression patterns and novel metabolic and potential regulatory genes that are induced by nitrate. Plant Cell **12:** 1491–1510

Wang Y-H, Garvin DF, Kochian LV (2001) Nitrate-induced genes in tomato roots. Array analysis reveals novel genes that may play a role in nitrogen nutrition. Plant Physiol **127:** 345–359

Williams LE, Miller AJ (2001) Transporters responsible for the uptake and partitioning of nitrogenous solutes. Annu Rev Plant Physiol Plant Mol Biol **52:** 659–688

Downloaded on December 5, 2020. - Published by https://plantphysiol.org
Copyright (c) 2020 American Society of Plant Biologists. All rights reserved.