Nitrogen transporter and assimilation genes exhibit developmental stage-selective expression in maize (Zea mays L.) associated with distinct cis-acting promoter motifs

Christophe Liseron-Monfils1, Yong-Mei Bi2, Gregory S Downs1, Wenqing Wu2, Tara Signorelli2, Guangwen Lu2, Xi Chen3, Eddie Bondo1, Tong Zhu3, Lewis N Lukens3, Joseph Colasanti3, Steven J Rothstein3, and Manish N Raizada1*

Department of Plant Agriculture; University of Guelph; Guelph, ON Canada; 2Department of Molecular and Cellular Biology; University of Guelph; Guelph, ON Canada; 3Syngenta Biotechnology Inc.; Research Triangle Park; Greensboro, NC USA

Keywords: Zea mays, maize, juvenile, adult, phase change, promoter, motif, nitrogen, transporter, assimilation, transcriptome, nitrite transporter, nitrate response element, NRE, G-box, E-box

Abbreviations: NRT, nitrate transporter; NiTR, nitrite transporter; AMT, ammonium transporter; NAR2, NRT2 complex accessory protein; NR, nitrate reductase; NiR, nitrite reductase; GS, glutamine synthetase; Gln, GS1-encoding gene; GOGAT, glutamate synthase

Nitrogen is considered the most limiting nutrient for maize (Zea mays L.), but there is limited understanding of the regulation of nitrogen-related genes during maize development. An Affymetrix 82K maize array was used to analyze the expression of ≤ 46 unique nitrogen uptake and assimilation probes in 50 maize tissues from seedling emergence to 31 d after pollination. Four nitrogen-related expression clusters were identified in roots and shoots corresponding to, or overlapping, juvenile, adult, and reproductive phases of development. Quantitative real time PCR data was consistent with the existence of these distinct expression clusters. Promoters corresponding to each cluster were screened for over-represented cis-acting elements. The 8-bp distal motif of the Arabidopsis 43-bp nitrogen response element (NRE) was over-represented in nitrogen-related maize gene promoters. This conserved motif, referred to here as NRE43-d8, was previously shown to be critical for nitrate-activated transcription of nitrate reductase (NIA1) and nitrite reductase (NIR1) by the NIN-LIKE PROTEIN 6 (NLP6) in Arabidopsis. Here, NRE43-d8 was over-represented in the promoters of maize nitrate and ammonium transporter genes, specifically those that showed peak expression during early-stage vegetative development. This result predicts an expansion of the NRE-NLP6 regulon and suggests that it may have a developmental component in maize. We also report leaf expression of putative orthologs of nitrite transporters (NiTR1), a transporter not previously reported in maize. We conclude by discussing how each of the four transcriptional modules may be responsible for the different nitrogen uptake and assimilation requirements of leaves and roots at different stages of maize development.

Introduction

Plants have evolved multiple strategies to cope with wide variation in the types and concentrations of soil nitrogen including nitrate and ammonium.1,3 For example, at low external nitrate, plants employ high affinity transporters, while low affinity transporters are used when external nitrate is high.1 Following uptake, soil-derived nitrogen is either transported directly to the shoot or first assimilated into amino acids for long-distance transport and/or to provide nitrogen donor substrates for nitrogenous compounds.4 To help adjust for changing nitrogen demand throughout plant development,7 nitrogen incorporated in organic compounds can be scavenged from senescing tissues and re-converted into amino acids for transport through the phloem to growing tissues such as seeds. This process requires tissue specific expression of nitrogen assimilation enzymes and transporters.4 To allow for such fine-tuned control, plants have evolved nitrogen-related gene families that perform similar functions but in different tissues and stages of development.4,6 Such developmental control of nitrogen-related genes may involve distinct sets of transcriptional enhancers, but these are poorly characterized. In maize (Zea mays L.), very limited information has been reported on the transcriptional regulation of nitrogen uptake and assimilation genes across different tissue and stages...
of development, which is therefore the objective of the current study.

Nitrogen transporter genes have been most thoroughly characterized in Arabidopsis thaliana (L.). The Arabidopsis genome encodes at least 67 nitrate transporters, including 53 NRT1 genes, 7 NRT2 genes, and 7 ArtClc (chloride channel) genes. In maize, at least 4 genes encoding low affinity nitrate transporters (ZmNrt1.1A, ZmNrt1.1B, ZmNrt1.2, ZmNRT1.5A) have been reported, along with 4 genes encoding high affinity nitrate transporters (ZmNrt2.1, ZmNrt2.2, ZmNrt2.3, ZmNrt2.5).2,8-11 NRT2 requires interaction with co-transporter NAR2 (NRT3) proteins to be functionally active.12-14 The maize genome encodes at least 2 NAR2-encoding genes [ZmNar2.1 (ZmNrt3.1), ZmNar2.2 (ZmNrt3.2)].2,4 In higher plants, a chloroplast-localized nitrite transporter (GsNitrI-L) was reported in cucumber, with a functional ortholog in Arabidopsis;15 an NiR ortholog has not been reported in maize. The genomes of higher plants have been reported to encode up to 14 ammonium transporter paralogs (AMT1 family), with the highest affinity transporters (AMT1:1; AMT1:3; AMT1:5) typically localized to root hairs and outer root cells; the lower affinity transporter(s) (AMT1:4) are located in the root endodermis.4 In Arabidopsis, at least 5 gene families have been reported to transport amino acids and peptides, some of which also transport inorganic nitrogen.4

Nitrogen assimilation is similarly complex. Higher plants directly assimilate ammonium into glutamine in plastids.5 By contrast, nitrate is first converted to nitrite in the cytoplasm by nitrate reductase (NR), followed by reduction to ammonia in plastids by nitrite reductase (NiR).4 Ammonium is fixed onto glutamate to form glutamine by glutamine synthetase (GS; Gln gene family), of which a plastidic isoform (GS2) and a cytosolic isoform (GS1) exist. A single gene in maize encodes GS2 (Gln2) whereas at least 5 genes encode GS1 (Ghi1–1 to Ghi1–5), which are differentially expressed during development.16,17 Glutamine can subsequently react with 2-oxoglutarate to form 2 molecules of glutamate via glutamine 2-oxoglutarate amino transferase (GOGAT), also called glutamate synthase.18 Glutamate can serve as an amino donor for other amino acids and nitrogen-requiring compounds, or act as an amine acceptor in the GS-GOGAT cycle to regenerate glutamine. Plants have 2 types of GOGAT enzymes, NADH-GOGAT and Fd-GOGAT, which use NADH and ferredoxin as electron donors, respectively.18 Different GOGAT paralogs show constitutive or tissue-specific activity in plants, including in maize.17,18 Ferredoxin-GOGAT is localized to leaf chloroplasts, while NADH-GOGAT is expressed in non-photosynthetic tissues including root plastids.4 Following nitrogen assimilation, glutamine, glutamate, and other amino acids, including asparagine and aspartate, are transported by vascular tissues to growing organs.5 Nitrate and ammonium can also be stored in vacuoles before or after their long-distance transport.4

Transcriptional regulation of plant nitrogen-related genes is poorly understood, with very limited information available in monocots in particular. In dicots, including Arabidopsis, a 43 bp pseudo-palindromic nitrogen response element (NRE) was recently identified in the promoter of a nitrite reductase (NiR) gene and shown to be necessary and sufficient for nitrate-activated gene transcription.19 Subsequent research showed that a similar 43 bp NRE is present in the promoters of NiR genes from rice, maize, and sorghum, and that 4 copies of the maize NRE are necessary and sufficient for nitrate induction of a GUS reporter construct in Arabidopsis.20 The authors reported that they did not observe the NRE in genes other than nitrate reductase (NIA1) and nitrite reductase (NIRI) in Arabidopsis,21 consistent with a parallel study.22 NRE-like sequences were also identified downstream of the transcriptional terminator of the Arabidopsis nitrate reductase NIA1 gene.23 A different research group, however, reported the existence of 3 non-NRE motifs that were necessary and sufficient for nitrate induction of the Arabidopsis NIA1 promoter, specifically: a myb motif (C-G/C-GTT-G/A), ALFIN1 (core C/A-CAC), and an E-box (CAAGTG).24

Plant development above ground has been divided into distinct developmental phases based on characteristics including leaf shape, surface wax, the presence of trichomes, and underlying genetic networks.25,26 In maize, the juvenile vegetative phase (V) is from seedling emergence (Ve) to the formation of leaves 4 to 6 (V2 stage), which is followed by the adult vegetative phase (here ≥ V5). Flowering initiates the reproductive phase (R), which includes seed formation, the latter divided into stages based on the number of days after pollination (DAP). Much less information has been reported on whether these phases have any importance in categorizing root development.

The objective of this study was to characterize the global expression pattern of nitrogen transport and assimilation genes during the different phases of maize development, and to identify associated cis-element motifs over-represented in the promoters corresponding to each expression cluster. We analyzed data from a developmental expression series which we recently reported for maize.27 Specifically, the expression of 46 putatively unique nitrogen uptake and assimilation probes were analyzed in 50 maize tissues from seedling emergence to 31 d after pollination using an 82K customized maize B73 inbred microarray, with a focus on root and leaf expression. The expression of a subset of these probes was subsequently validated using quantitative real time PCR (qRT-PCR). Promoters associated with distinct expression clusters were subsequently used to screen for over-represented cis-acting elements using a new de novo motif discovery program customized and validated for maize called Promzea (Promoters of Zea).28

**Results**

**Tissue and developmental stage selective expression**

As we recently described,27 RNA was collected from 50 tissues from seedling emergence to 31 d after pollination (DAP; Table S1) and hybridized onto a customized maize Affymetrix 82K Unigene array. From all the probes present on the array, and after substantial trimming (Table S2; see below for details), a total of 65 nitrogen uptake and assimilation array probes were identified (Table 1) and analyzed, of which 46 probes were predicted to be unique. The expression patterns for these nitrogen-related probes from all 50-tissue samples are summarized (Fig. S1). Expression of 64 of the 65 probes showed
significant differential expression in at least 1 of the tissue-stages of development, with adjusted p-values ranging from 4.61e-04 to 4.51e-36, well below the cut-off of 0.05 (data not shown). Only 1 probe, ZmN-25, was not differentially expressed; this probe corresponded to an oligopeptide and nitrate transporter (GRMZM2G127134) in expression Cluster 2.

In reproductive tissues, consistently high nitrogen-related expression was observed in anthers (Fig. S1). Interestingly, probes corresponding to nitrate transporters (NiTR1),19 a gene class not previously reported in maize, were expressed at different stages of maize development (Fig. 1; Table S1). In vegetative tissues, 4 nitrogen-related gene expression clusters were detected (Fig. 1; Table S3). Cluster 1 probes showed peak expression in juvenile stage roots (Ve-V2), with several probes also peaking in vegetative stage leaves (Ve-V5), suggestive of a whole plant juvenility cluster. Cluster 1 probes matched genes encoding a high affinity transporter NRT2.1, the low affinity transporter NRT1.1, and ammonium transporters (Table 1). Cluster 2 probes were leaf-selective and more highly expressed in vegetative stage leaves (juvenile and adult: Ve-V5) than post-flowering leaves (R1-R31) (Fig. 1; Table S3). Cluster 2 probes matched low affinity nitrate transporters including NRT1.5, nitrite transporter NiTR1, and nitrate reductases (NR1, NR2) (Table 1). Cluster 3 probes were root selective and were expressed throughout development (juvenile to post-flowering) (Fig. 1; Table S3). Cluster 3 probes matched genes encoding glutamine synthetases (cytosolic isofrom GSI) including Gln4/Gln1–4 and/or Gln5/Gln1–5 as well as several glutamate synthases (GOGAT, Table 1). Also included in Cluster 3 were ammonium transporters, the low affinity transporter NRT1.1, and the companion protein (NRAR2.1) of the high affinity nitrate transporter complex. Finally, Cluster 4 probes were leaf selective and showed the most consistent expression in older leaves at later stages of development (adult to post flowering: V5-R31), with 3 probes showing peak expression in reproductive stage roots (Fig. 1; Table S3). Several Cluster 4 probes matched genes encoding nitrate reductase (NR1) and nitrate transporters, as well as 1 NAR2 paralog and the nitrite transporter NiTR1 (Table 1).

Validation of microarray expression

Using corresponding gene sequences from the maize genome, expression of 8 nitrogen-related probes was verified by quantitative real time PCR (qRT-PCR) for 6 of the developmental tissue-stages (Fig. 2; Fig. S2; Tables S4 and S5). The results showed that 75% of the qRT-PCR data sets (6 out of 8) correlated with the microarray results, having Pearson coefficients of correlation (r) ranging from 0.8 to 0.97 (Fig. S2; Table S5). The Pearson coefficients of correlation were significant for 5 of these 6 genes (P value < 0.05) while the sixth showed borderline significance (P value = 0.056); 2 were not correlated (Nrt1.1 and Nrt1) (Fig. S2; Table S5). Specifically, the high affinity nitrate transporter Nrt2.1 showed peak expression in V1 seminal roots, and a minor peak in V5 leaves (Fig. 2A), consistent with belonging to juvenility Cluster 1 (Fig. 1). Though not statistically correlated with Cluster 1, expression of low affinity transporter Nrt1.1 also showed peak expression in V1 seminal roots (Fig. 2B). Expression of nitrate reductase Nr2 showed leaf-selective expression (note expanded y-axis values), peaking in V1 leaves (Fig. 2C), consistent with belonging to Cluster 2. Though predicted by microarray data to belong to Cluster 2, qRT-PCR expression of Nr1 appeared to be more consistent with juvenility Cluster 1, with expression peaks in both juvenile roots and leaves, and little expression in reproductive-staged leaves and roots (Fig. 2D). Nar2.1 showed peak expression in V1 seminal roots, and higher expression in the 3 root tissues compared with the 3 leaf tissues examined (Fig. 2E), consistent with belonging to Cluster 3. Cluster 3 was also exemplified by NADH glutamate synthase (NADH-GOGAT), which showed very high expression in V1 and V5 seminal roots, but low expression at all leaf stages (Fig. 2F). Nar2.2 showed peak expression in reproductive stage (R1) leaves but low expression in R1 roots (Fig. 2G), consistent with belonging to reproductive stage Cluster 4. A high affinity nitrate transporter (GRMZM2G455124) also showed peak expression in R1 stage leaves, but also a second peak in R1 nodal roots (Fig. 2H), consistent with a reproductive stage expression cluster.

More generally, the qRT-PCR expression data was consistent with maize having distinct nitrogen gene expression clusters during vegetative development; this became apparent when pairs of genes were compared (Fig. 2). For example, Nr2 and NADH glutamate synthase showed opposite expression, peaking in juvenile leaves vs. juvenile roots, respectively (Fig. 2C, D). Similarly, expression of a high affinity nitrate transporter (Maize GDB ID: GRMZM2G455124), which peaked in reproductive-stage vegetative tissues (Fig. 2H), was opposite to Nr1 which peaked in juvenile stage tissues (Fig. 2D). Finally, Nar2.1, which showed peak expression in V1 seminal roots and exhibited low expression in reproductive stage (R1) leaves (Fig. 2E), was strikingly opposite to Nar2.2 which showed peak expression in R1 leaves (Fig. 2G).

Over-represented cis-acting promoter motifs

Four over-represented motifs were present in the promoters of the nitrogen-related genes expressed in Cluster 1 (early root/leaf cluster), of which 3 were similar to previously identified cis-acting elements (Table 2). Interestingly, 1 Cluster 1 motif was very similar to the conserved 8 bp distal element of the 43 bp pseudopalindromic nitrogen response element [(t/c)GACcCTT] identified in dicot and monocot nitrite reductase (NiR) genes, including maize, and shown to be necessary and sufficient for nitrate-activated gene transcription. Within Cluster 1, this 8 bp motif (consensus CGACcCTT), which we refer to as NRE43-d8 (NRE 43 bp, distal 8 bp, see Discussion), was over-represented in the promoters of genes encoding nitrate transporters (NRT1, NRT2.1) and ammonium transporters (Table 3). The second Cluster 1 element was similar to the G-box 10 and E-Box elements (Table 2). The core of the G-box 10 element is identical to the E-box element (CANNTG) which was recently shown to be critical for nitrate induction of the NIA1 (nitrate reductase) promoter in Arabidopsis. The third Cluster 1 element, NRG1 (Negative Regulator of Glucose-repressed Genes 1) (Table 2), was previously identified in yeast upstream of genes involved in regulating cell sugar status. For example,
the NRG1-like motif was over-represented in the promoters of \textit{Nrl1.1} and \textit{Nrl2.1} (data not shown).

With respect to the additional expression clusters, there were two over-represented motifs in the promoters of Cluster 2 (early...
Table 1. Microarray probes used in this study

| Expression cluster | Probe number | GenBank ID | Maize GDB ID | Corresponding gene annotation |
|--------------------|--------------|------------|--------------|-------------------------------|
| 4                  | ZmN-65       | BAA35120.1 | GRMZM2G077054; GRMZM2G085078; GRMZM2G375064 | NADH-dependent glutamate synthase |
| 4                  | ZmN-64       | AAD38068.1 | GRMZM2G076723; GRMZM2G443637 | Nitrate reductase (NADH; nr1) |
| 4                  | ZmN-63*      | BAB89087.1 | GRMZM2G064091 | Nitrate transporter |
| 4                  | ZmN-62       | CA93316.2  | GRMZM5G827496 | Nitrite transporter (NIT1 gene) |
| 4                  | ZmN-61       | AAN06953.1 | GRMZM2G043193; GRMZM2G080045; GRMZM2G338809 | Ammonium transporter (OsAMT3.3) |
| 4                  | ZmN-60       | AAD38068.1 | GRMZM2G076723; GRMZM2G443637 | Nitrate reductase (NADH; nr1) |
| 4                  | ZmN-59       | CA93316.2  | GRMZM5G827496 | Nitrite transporter (NIT1 gene) |
| 4                  | ZmN-58       | BAC65231.1 | GRMZM2G080045 | Ammonium transporter |
| 4                  | ZmN-57*      | BAB89087.1 | GRMZM2G064091 | Nitrate transporter |
| 4                  | ZmN-56       | AAM12786.1 | GRMZM2G179294; GRMZM2G163494 | High affinity nitrate transporter (nar2.1; nar2.2) |
| 4                  | ZmN-55       | AAF78499.1 | GRMZM2G455124 | High affinity nitrate transporter |
| 4                  | ZmN-54*      | BAB89087.1 | GRMZM2G064091 | Nitrate transporter |
| 4                  | ZmN-53       | BAB19757.1 | GRMZM2G122251 | Nitrate transporter (Glycine max nrt1.2) |
| 4                  | ZmN-52^      | AAD38068.1 | GRMZM2G076723; GRMZM2G443637 | Nitrate reductase (NADH; nr1) |
| 4                  | ZmN-51       | CAD41034.1 | GRMZM2G176253 | Nitrate transporter |
| 4                  | ZmN-50       | AAGS2554.1 | GRMZM2G137421 | Nitrate transporter (AtnT1) |
| 4                  | ZmN-49^      | AAD38068.1 | GRMZM2G076723; GRMZM2G443637 | Nitrate reductase (NADH; nr1) |
| 4                  | ZmN-48^      | AAD38068.1 | GRMZM2G076723; GRMZM2G443637 | Nitrate reductase (NADH; nr1) |
| 4                  | ZmN-47       | AAO00921.1 | GRMZM2G137421 | Nitrate transporter |
| 3                  | ZmN-46*      | BAC01257.1 | GRMZM2G077054; GRMZM2G085078; GRMZM2G375064 | NADH-dependent glutamate synthase |
| 3                  | ZmN-45*      | BAC01257.1 | GRMZM2G077054; GRMZM2G085078; GRMZM2G375064 | NADH-dependent glutamate synthase |
| 3                  | ZmN-44       | BAA35120.1 | GRMZM2G077054; GRMZM2G085078; GRMZM2G375064 | NADH-dependent glutamate synthase |
| 3                  | ZmN-43^      | BAC01257.1 | GRMZM2G077054; GRMZM2G085078; GRMZM2G375064 | NADH-dependent glutamate synthase |
| 3                  | ZmN-42^      | BAA35120.1 | GRMZM2G077054; GRMZM2G085078; GRMZM2G375064 | NADH-dependent glutamate synthase |
| 3                  | ZmN-41#      | P38561     | GRMZM5G872068; GRMZM2G036464 | Glutamine synthase gln4; gln5 |
| 3                  | ZmN-40#      | P38561     | GRMZM5G872068; GRMZM2G036464 | Glutamine synthase gln4; gln5 |
| 3                  | ZmN-39       | AAL05614.1 | GRMZM2G028736 | Ammonium transporter |
| 3                  | ZmN-38@      | AAD39317.1 | GRMZM2G127134 | Oligopeptide and nitrate transporter |
| 3                  | ZmN-37**     | BAC65231.1 | GRMZM2G080045 | Ammonium transporter |
| 3                  | ZmN-36       | P27968     | GRMZM5G878558 | Nitrate reductase (nr2) |
| 3                  | ZmN-35¥      | AAD39317.1 | GRMZM2G127134 | Nitrate transporter |
| 3                  | ZmN-34       | AAL05612.1 | GRMZM2G118950; GRMZM2G473697; GRMZM2G701289 | Ammonium transporter (OsAMT1.1; Osamt5.2) |
| 3                  | ZmN-33†      | BAC56914.1 | GRMZM2G086496 | Nitrate transporter (nrt1.1) |
| 3                  | ZmN-32**     | BAC65231.1 | GRMZM2G080045 | Ammonium transporter |
| 3                  | ZmN-31†      | BAC56914.1 | GRMZM2G086496 | Nitrate transporter (nrt1.1) |
| 3                  | ZmN-30       | AAM12786.1 | GRMZM2G179294 | High affinity nitrate transporter (nar2.1) |
| 3                  | ZmN-29       | AAB82307.1 | GRMZM2G173967 | Amino acid transport protein |
| 2                  | ZmN-28       | MZEFELGU   | GRMZM2G036609 | Ferredoxin-dependent glutamate synthase |
| 2                  | ZmN-27*      | P27968     | GRMZM5G878558 | Nitrate reductase (nr2) |
leaf cluster): LTRE (low temperature responsive element) and YAP1. The core of the LTRE element, also called C/DRE, mediates ABA-independent responses to cold, amplified by light via phytochrome signaling. The YAP1 element was first identified as the binding site of the yeast YAP1 protein, a conserved protein across eukaryotes including Arabidopsis. YAP1 was shown to regulate the yeast cell cycle in response to oxidative stress, but also able to regulate cell elongation. Over-represented in Cluster 3 promoters (root cluster) was a motif similar to the binding site of the Forkhead (FHL1) transcription factor from yeast, a repressor of ribosome-encoding genes during glucose starvation. Here, the FHL1 motif was over-represented in genes encoding nitrate and ammonium transporters, nitrate reductase, and glutamine synthases (data not shown). Finally, a previously unidentified motif was found in the promoters of Cluster 4 genes (late leaf cluster) with the consensus NANGAG.

**Discussion**

Nitrogen-related genes have distinct patterns of expression during maize vegetative development. Recent transcriptome studies have highlighted global gene expression patterns during maize development, examining gene expression along a maturation gradient of a juvenile leaf; in leaves during the transition from juvenile to adult vegetative stages; and in different organs and tissues across developmental stages. The focus of this study was to examine the expression of nitrogen transporter and assimilation genes across maize development, particularly in roots and leaves. Four vegetative

| Expression cluster | Probe number | GenBank ID | Maize GDB ID | Corresponding gene annotation |
|--------------------|--------------|-----------|--------------|-------------------------------|
| 2 ZmN-26          | P27967       | GRMZM2G268636 | Nitratreductase(NADH)         |
| 2 ZmN-25          | AAD39317.1   | GRMZM2G127134 | Oligopeptide and nitrate transporter |
| 2 ZmN-24          | AAG52554.1   | GRMZM2G137421 | Nitrate transporter(Atnt1)    |
| 2 ZmN-23          | CA93316.2    | GRMZM2G827496 | Nitrite transporter (NTR1 gene) |
| 2 ZmN-22          | CAD4114.1    | GRMZM2G138731 | Nitrate transporter (nt1–5)   |
| 2 ZmN-21          | P27968       | GRMZM2G87558  | Nitrate reductase (n2)        |
| 2 ZmN-20          | AAG52554.1   | GRMZM2G137421 | Nitrate transporter (Atnt1)   |
| 2 ZmN-19          | No Description | NA      | Nitrate and chloride transporter |
| 2 ZmN-18          | P49102       | GRMZM2G68636  | Nitratreductase(NADH)         |
| 2 ZmN-17          | AAD38068.1   | GRMZM2G076723; GRMZM2G443637 | Nitrate reductase (NADH; nr1) |
| 2 ZmN-16          | BAB89087.1   | GRMZM2G064091 | Nitrate transporter           |
| 2 ZmN-15          | AAL31925.1   | GRMZM2G453320 | Nitrate and chloride transporter |
| 2 ZmN-14          | AAL31925.1   | GRMZM2G453320 | Nitrate and chloride transporter |
| 2 ZmN-13          | BAA35120.1   | GRMZM2G077054; GRMZM2G085078; GRMZM2G375064 | NADH-dependent glutamate synthase |
| 2 ZmN-12          | Q05085       | GRMZM2G086496 | Nitrate transporter (nt1.1)   |
| 1 ZmN-11          | AAL05612.1   | AC208641.3 FG005; AC208641.3 FG008; GRMZM2G028736; GRMZM2G175140; GRMZM2G335218 | Ammonium transporter (Osamt1.1) |
| 1 ZmN-10          | No Description | NA      | Symbiotic ammonium transporter |
| 1 ZmN-9           | BAC56913.1   | GRMZM2G086496 | Nitrate transporter (nt1.1)   |
| 1 ZmN-8           | AAC32828.1   | GRMZM2G082343 | Symbiotic ammonium transporter |
| 1 ZmN-7           | AAC32828.1   | AC211704.3 FG003; GRMZM2G082343 | Symbiotic ammonium transporter |
| 1 ZmN-6           | BAC65231.1   | GRMZM2G080045 | Ammonium transporter           |
| 1 ZmN-5           | AAL31925.1   | GRMZM2G453320 | Nitrate and chloride transporter |
| 1 ZmN-4†          | AAL31925.1   | GRMZM2G453320 | Nitrate and chloride transporter |
| 1 ZmN-3†          | BAC56914.1   | GRMZM2G086496 | Nitrate transporter (nt1.1)   |
| 1 ZmN-2†          | BAC56914.1   | GRMZM2G086496 | Nitrate transporter (nt1.1)   |
| 1 ZmN-1†          | AY129953     | GRMZM2G010280 | High affinity nitrate transporter (nt2.1) |

Note: Within each cluster, probe numbers that share a common symbol are predicted to correspond to the same gene based on the sequence homology of the corresponding genes and their similar expression patterns.
gene expression clusters were identified (Fig. 1; Table S3), specifically an early stage-selective cluster (Cluster 1, roots and leaves), an early leaf-selective cluster (Cluster 2, juvenile to adult leaves), a root-selective cluster (Cluster 3, juvenile to reproductive roots), and a late leaf-selective cluster (Cluster 4, adult to reproductive leaves), which also included some late-stage root expression. We observed that, within a gene family, distinct subsets of nitrogen-related paralogs were preferentially expressed in leaves compared with roots (Fig. 1; Fig. 2; Table S3). For example, high affinity co-transporter Nar2.1, showed peak expression in roots (Fig. 2E), while paralog Nar2.2 showed peak expression in leaves (Fig. 2G). Furthermore, distinct paralogs were expressed at different developmental phases corresponding to previously defined juvenile, adult and reproductive phases of shoot development in maize.\textsuperscript{25,26} For example, qRT-PCR data showed that a high affinity nitrate transporter (Nrt2.1) was expressed in juvenile-stage roots and leaves (Fig. 2A) compared with a different high affinity nitrate transporter (GRMZM2G455124) which was expressed in reproductive-stage roots and leaves (Fig. 2H). It is noteworthy that nitrogen-related gene expression in roots also corresponded to these aboveground developmental shifts (Fig. 1; Table S3). The latter result may not be surprising, however, given that earlier studies have demonstrated that nitrogen stress responses are highly coordinated between the shoot and root systems of maize.\textsuperscript{2,38,39}

As a note added in proof, our root expression results are consistent with a recently published study by Garnett et al.\textsuperscript{11} which analyzed patterns of expression for nitrate uptake transporter paralogs in roots during maize development. Garnett et al. showed that low affinity (Nrt1 family) and high affinity (Nrt2, Nar2/Nrt3 family) nitrate transporters change in expression during maize development: peak abundance of nitrate transporter transcripts generally occurs in juvenile and reproductive stage roots. Furthermore, Garnett et al. showed that within each transporter gene family, different paralogs exhibit different expression patterns: for example ZmNRT1.1A peaks early in development in roots, whereas ZmNRT1.2 peaks in roots during reproductive stages.\textsuperscript{11} Direct comparisons are difficult to make between our study and Garnett et al., however, because the latter study employed inbred Gaspe Flint which has a much shorter life cycle and dramatically smaller shoot compared with the Syngenta inbred used here; the two studies also employed different nitrogen and growth conditions (hydroponics vs. coarse clay).

The developmental complexity of nitrogen-related gene expression observed in our study is also consistent with data emerging from other species. For example, in Arabidopsis, Nrt1 gene family paralogs are differentially expressed in roots, stems, leaves and reproductive tissues.\textsuperscript{6,40} Also in Arabidopsis, nitrogen-related genes including nitrite reductase (NIR1), nitrate reductase (NIA1), and a high affinity nitrate transporter (Nrt2.4), showed differential expression in young roots vs. young leaves during nitrogen starvation.\textsuperscript{41}

High expression in anthers

In terms of reproductive development, a particularly interesting finding from this study was the high level of gene expression of many nitrogen-related probes in anthers (Fig. S1). In fact, > 14/65 probes showed peak expression in anthers (VT stage), including probes that showed root or shoot-selective expression during vegetative development. Given that the normalization
method used, named RMA, standardizes the gene expression ranges between arrays (e.g., relative gene expression from anther RNA has the same range than leaf RNA after normalization), this result does not appear to be an artifact. It is also noteworthy that probes corresponding to nitrate transporters were expressed in late stage tassels (Fig. S1).

Nitrite transporter expression

As noted in the above section, transcripts of putative maize nitrite transporter (NiTR) orthologs were detected in the 2 leaf-selective expression clusters (Clusters 2 and 4; Fig. 1) with additional strong expression in the husk leaves surrounding the cob (Fig. S1; Table S3) but were excluded from Clusters 1 and 3 which included root expressed genes. This data is consistent with nitrite transporters being localized to chloroplasts. Here we note NiTRs in a separate section because this gene class has not previously been reported in maize. A gene encoding NiTR (Nar1) was initially reported in Chlamydomonas chloroplasts. In higher plants, a NiTR gene was first reported in cucumber (CsNitr1-L), along with a functional ortholog tested in Arabidopsis (At1g68570) where a knockout mutation showed a 5-fold increase in nitrite accumulation in leaves. The cucumber NiTR protein was localized to the inner envelope membrane of chloroplasts, where it was hypothesized to load nitrite from the cytoplasm into the stroma of the chloroplast during nitrate assimilation. Functional data will be needed to authenticate the genes corresponding to the putative maize NiTR(s) probes reported in this study.

Study limitations including discrepancies between microarray data and qRT-PCR data

In this study, the reader is cautioned not to interpret each probe as representing a unique locus. The microarray was designed using EST sequences prior to the major sequence release of the maize genome. As a result, more than 1 nitrogen-related probe could match the same gene; this scenario was possible for a total of 49 probe sets (75%; 49/65). These related probe sets could have originated from different portions of the same gene including alternative splice products (e.g., probes ZmN-2 and ZmN-3 which have the same array expression pattern, Table S3). Alternatively, the related probe sets may have originated from closely related paralogs due to the extensive number of duplications in the allotetraploid maize genome (e.g., nitrate transporter probes ZmN-31 and ZmN-33 have a different array expression pattern than ZmN-2 and ZmN-3) (Fig. 1; Table S3). The latter hypothesis could explain the low correlation observed for 2 microarray probes when compared with qRT-PCR data, specifically the probes corresponding to Nrt1.1 in Cluster 2 (probe ZmN-2) and Nr1 in Cluster 3 (Fig. S2). The microarray vs. qRT-PCR data might in fact represent duplicated paralogs (Table S4). Recent gene duplication phenomena (e.g., tandem duplications) were suggested by the observation that 18 nitrogen-related probes (27%; 18/65) matched more than 1 maize locus (Table 1). Based on sequence identities and expression patterns, we estimate that the maximum number of unique

| Cluster | Motif Logo | Consensus | Preferential position | Similarity | E-value | Alignment |
|---------|------------|----------|----------------------|------------|--------|-----------|
| 1       | ![Motif Logo](image1) | CGACCCNTT | -50 +1 | NRE core | 2.26E-09 | CGACCCNTT
|         |            |          |                     |            |        | -GACCCTT   |
| 2       | ![Motif Logo](image2) | CCAGGTGC | -300 -250 | G-BOX 10 reverse | 8.78E-11 | -GCACNTGG
|         |            |          |                     |            |        | -GGCCAGTGGGC |
| 3       | ![Motif Logo](image3) | CACGCCRCAG | -250 -200 | / | / | / |
| 4       | ![Motif Logo](image4) | NNANGCSGCW | -50 +1 | NRG1 reverse | 6.23E-07 | AANGGGTCG
|         |            |          |                     |            |        | -AGGTCC |
| 2       | ![Motif Logo](image5) | AGTCCGG | -500 -450/-350 -300 | LTRE | 6.88E-07 | AGTCCGG
|         |            |          |                     |            |        | -GTCCG |
| 3       | ![Motif Logo](image6) | TAGYCRGC | -250 -200 | YAP1 | 6.73E-07 | —TAGYCRGC
|         |            |          |                     |            |        | -NTTAGTMAGCN |
| 4       | ![Motif Logo](image7) | TRTCGGTACG | -350 -300 | FHL1 | 1.19E-06 | TRTCGGTACG
|         |            |          |                     |            |        | -AYCGTACAT |

Gene expression clusters correspond to Figure 1. The most common position(s) of the motif in the -500 bp promoter region is indicated (Preferential position). Matches to previously identified motifs in promoter databases are identified (Similarity) along with the similarity score (E-value) and alignment to the match (Alignment). Only the most over-represented motifs are shown. Abbreviations: n = any nucleotide, Y = C/T, R = A/G, S = G/C, W = A/T.
Table 3. NRE43-d8 motifs detected in nitrogen-related promoters from gene expression Cluster 1

| Maize annotation | Description | NRE43-d8 motif | Downstream nucleotides | Percentage identity |
|------------------|-------------|----------------|------------------------|---------------------|
| GRMZM2G010280    | High affinity nitrate transporter (nrt2.1) | -319 TGATCCCTT | -313 GGCTGATCCC AGGGGATGAG GCAAGCCCA | 93.24% |
| GRMZM2G453320    | Nitrate and chloride transporter | -424 CGACCTTT | -418 ATGATTTGCG GTCTCTCTTTT TGAAGAAAGGAA | 96.65% |
| GRMZM2G080045    | Ammonium transporter | -292 CGACCTTT | -286 TGCCGTGCG CGTCTCGAGT CGTCCCTACC | 100.00% |
| GRMZM2G082343    | Symbiotic ammonium transporter | -493 CCACCGTTT | -487 AGATCAGTCA CCAGGCTCA TGTCCACCATG | 91.12% |
| GRMZM2G028736    | Ammonium transporter (Osatm1.3) | -208 CGAACCCTT | -202 TATGTAATT TTGACTAG TCTCTTCTTTT | 94.29% |
| GRMZM2G175140    | Ammonium transporter (Osatm1.1) | -146 CGATCTTTT | -140 CTACAGTGGCA AGATAATAAT GGAGATCTC | 95.61% |
| GRMZM2G335218    | Ammonium transporter (Osatm2.2/Osam1.3/Osam5.1) | -160 CGATCTTTT | -154 CTCTCTCTCT TAGAGGCCAC TACCGGCGGC | 98.95% |

| Maize transporter consensus | | CGACCTTT |
| Dicot- nitrite reductase consensus (Konishi and Yanagisawa, 2010) | | tGACCCTTT |

probes analyzed in this study was 46 (out of 65) but we have presented data for all probes analyzed.

Other study limitations
On a cautionary note, the qRT-PCR data presented here (Fig. 2, Fig. S2) suggests that nitrogen-related gene expression in maize vegetative tissues may be more complex or nuanced than the 4 clusters identified by microarray analysis. For example, whereas Nar2.2 and a high affinity nitrate transporter (GRMZM2G455124) were grouped together into Cluster 4, the qRT-PCR expression data suggests overlapping but distinct patterns of expression: Nar2.2 peaked only in reproductive stage leaves (Fig. 2G) while the transporter peaked in both reproductive stage leaves and roots (Fig. 2H). Another significant limitation of our study was that it excluded a significant number of nitrogen-related genes (see Methods for explanation) including genes encoding: nitrite reductase (which is induced in both maize leaves and roots); mitochondrial glutamate dehydrogenase (GDH); and 4 of the 6 known maize nitrate synthases (GS) (GS2; GS1: Gln1–1; Gln1–2, Gln1–3). Therefore, the expression clusters described here should not be viewed as comprehensive. Finally, as the tissue sampling did not focus on senescing leaves, it likely caused probes related to scavenging and export of nitrogen to growing tissues (e.g., GS1), to be under-represented in this study.

Physiological relevance of each vegetative expression cluster
In the context of the above study limitations, an intriguing observation from this study is that there appears to be a higher-order physiological pattern to the expression clusters (Fig. 1). Specifically, each of the 4 vegetative expression clusters appears to be selectively over-represented for genes responsible for specific steps of the nitrogen uptake and assimilation pathway:

Cluster 1: In this cluster, which showed peak expression in juvenile stage roots and leaves, 7/8 putatively unique probes corresponded to nitrate/nitrate-chloride transporters (4/8) and ammonium transporters (3/8), rather than to nitrogen assimilation genes (Fig. 1; Table S3). qRT-PCR data confirmed that expression of both a low affinity transporter (Nrt1.1) and a high affinity transporter (Nrt2.1) peaked in juvenile roots (Fig. 2). It was logical that both nitrate and ammonium transporters showed high expression, as the plants were fertilized with both sources of inorganic nitrogen throughout their life cycle. Given that we identified a distinct set of over-represented motifs in the promoters corresponding to Cluster 1 genes (Tables 2 and 3; see below), together these results suggest that maize may have a transcriptional module devoted to coordinating early stage, whole plant, inorganic nitrogen uptake and transport, possibly including transport of nitrogen into the vacuole (e.g., nitrate-chloride transporter), and loading/unloading of nitrate and ammonium to/from xylem.60 The existence of an early root/shoot inorganic nitrogen expression module is consistent with a recent study61 which demonstrated that there is a peak in nitrate uptake and transporter expression in young maize roots when normalized to root dry weight, which results in an early stage peak in leaf nitrate concentration. Relative to root growth, the early stage peak was shown to correspond to a period of rapid shoot growth and hence increased nitrogen demand.61

Cluster 2: This cluster was leaf selective, with peak expression generally at earlier developmental stages (Fig. 1). A closer examination showed that 9/11 putatively unique probes in Cluster 2 corresponded to proteins with nitrate transport activity (5/11, including 1 unique nitrate-chloride transporter) as well as nitrate reductase activity (4/11) (Fig. 1; Table S3). qPCR
data confirmed that nitrate reductase gene Nr2 was leaf selective, with peak expression in V1 stage leaves (Fig. 2C). One interpretation of this data is that maize has an early stage leaf-selective module that is minimally responsible for unloading nitrate from xylem into leaf cell cytoplasm, followed by storage of nitrate in leaf vacuoles or conversion of nitrate to nitrite in the cytoplasm. Our data would suggest that these two steps are transcriptionally coordinated, perhaps facilitated by the motifs that were over-represented in the promoters associated with this module (Table 2). Maize leaf NR activity is associated primarily with mesophyll cells, with dramatic upregulation during greening.60

The expression of additional probes in Cluster 2, corresponding to 1 nitrate transporter and 1 ferredoxin-dependent GS (note: nitrite reductase was excluded from this study), suggests that other components of the nitrate assimilation pathway may also be part of this regulatory module. Given the existence of Cluster 4, which was selective for leaf reproductive stages, an attractive hypothesis is that Cluster 2 is coordinated with Cluster 1 to satisfy the high shoot/root ratio during early development.11 As noted above, Cluster 1 was over-represented for probes associated with early stage root and leaf nitrate (and ammonium) uptake and transport, but not assimilation (Fig. 1), and hence the 2 clusters may be complementary.

None of the Cluster 2 probes was associated with ammonium transport, even though 9 putatively unique probes corresponding to ammonium transporters were included in the study, and the plants were fed ammonium (Fig. 1). This result is consistent with previous observations demonstrating that de novo ammonia assimilation occurs primarily in the root.40,47,48

A final interesting observation for Cluster 2 is that the single ferredoxin-GOGAT affiliated probe in this study was expressed in this cluster (Fig. 1). This result is consistent with ferredoxin-GOGAT activity being dominant in leaf chloroplasts compared with NADH-GOGAT, which is expressed primarily in non-photosynthetic tissues.17,49

Cluster 3: Cluster 3 was the root selective cluster that was expressed both early and late in development (Fig. 1). Cluster 3 included 12 putatively unique probes, of which 3 probes corresponded to proteins with nitrate transporter activity and 1 with nitrate reductase (NR) activity. qRT-PCR results confirmed the root-selective expression of the high affinity co-transporter gene Nar2.1 in Cluster 3 (Fig. 2E; Table S5). In maize, though a considerable fraction of grain nitrogen originates from scavenged nitrogen from senescing leaves, 30–70% comes from post-silking uptake of soil nitrogen, especially nitrate.45,50 In fact, a recent study by Garnett et al.50 showed that there is a reproductive (anthesis) phase peak in root nitrate uptake associated with increased expression of nitrate transporters. Enzymatic nitrate reductase activity has been measured in maize roots, with peak levels occurring in root tips.51

The remaining 8 probes in Cluster 3 corresponded to genes encoding the ammonia assimilation pathway (which was missing in root Cluster 1), including: 1) 3 putatively unique probes corresponding to proteins with ammonium transport activity (these might enable soil uptake of ammonium, root vacuolar storage of ammonium, or cytoplasmic to plastid transport to facilitate the first step of ammonium assimilation); 2) 1 or 2 probe(s) corresponding to the next step in ammonium assimilation, specifically incorporation of ammonium with glutamate to form glutamine, catalyzed by glutamine synthetase (GS); 3) 3 putatively unique probes corresponding to glutamate synthase (NADH-GOGAT); and 4) 1 amino acid transporter, potentially to transport glutamate, glutamine, or other amino acids. There were no probes expressed in Cluster 3 that corresponded to proteins with nitrite transport activity, while probes for nitrite reductase were not included in the study as already noted.

Given that our study probes did not represent the complete set of nitrogen-related genes in the maize genome, one interpretation of Cluster 3 is that maize has a transcriptionally coordinated set of genes that act in early-to-late stage maize roots and which encode the complete nitrate and ammonia uptake and assimilation pathway. As already noted, the existence of an ammonia assimilation pathway in roots is consistent with previous studies demonstrating that the majority of de novo ammonia assimilation occurs in roots.48,51,53 With respect to nitrate, assimilation of nitrate is reported to be more prevalent in leaves than in roots.48 However, whereas feeding studies using maize roots showed a faster rate of amino acid accumulation when the nitrogen source was 15NH4+ rather than 15NO3-, the difference was thought to be caused by a lag period of induction of nitrate uptake rather than a slower steady-state rate of nitrate assimilation.52

As noted above, ferredoxin-GOGAT is reported to be primarily expressed in leaf chloroplasts, while NADH-GOGAT is expressed in non-photosynthetic tissues including root plastids.13,60 Consistent with these observations, we observed the expression of 3 putatively unique probes corresponding to NADH-GOGAT genes in Cluster 3, but none for ferredoxin-GOGAT (Fig. 1). qRT-PCR results confirmed that an NADH-GOGAT gene belonging to Cluster 3 was expressed in a root-selective manner (Fig. 2F; Table S5).

Cluster 4: This late developmental stage, leaf selective cluster, which also included probes that were expressed in late stage roots (Fig. 1), was biased for probes corresponding to the nitrate transport and assimilation pathway (12/15 putatively unique probes). Specifically, Cluster 4 included: 1) 7 putatively unique probes corresponding to nitrate transporter activity, perhaps enabling nitrate unloading from xylem to leaf cytoplasm, or transport of nitrate into vacuoles for storage; 2) 3 putatively unique probes corresponding to nitrate reductase; and 3) 2 probes corresponding to nitrite transporters. qRT-PCR confirmed that a high affinity co-transporter (Nar2.2) was upregulated in reproductive stage leaves (Fig. 2; Table S5). Combined with the observation that Cluster 4 was over-represented for specific candidate promoter motifs (Table 2), these data suggest that maize has a transcriptional module that acts to facilitate reproductive stage nitrate transport and assimilation in leaves, perhaps to supply grain demand for nitrogen.45,48,50

With the exception of one NADH-dependent GS probe, it is curious that probes corresponding to downstream nitrate assimilation genes were not apparently expressed in Cluster 4;
possible reasons include their exclusion from the experiment (see Methods) and/or that the downstream genes belong to a different regulatory module. In support of the latter hypothesis, nitrate reductase and nitrite reductase activities have been shown to be restricted to mesophyll cells in maize leaves, whereas glutamine synthetase (GS) and glutamate synthase (GOGAT) activities occur in both mesophyll and bundle sheath cells.\textsuperscript{24} Furthermore, whereas the activities of nitrate reductase and nitrite reductase are upregulated during greening, greening causes minor effects on GS and GOGAT activities.\textsuperscript{46} One interpretation of these results, when combined, is that the early (cytoplasmic) vs. late (chloroplast) steps in leaf nitrate assimilation belong to separate transcriptional modules, a hypothesis that will require further investigation.

As already noted in the Study Limitations section above, a limitation to our interpretation of Cluster 4 is that we under-sampled senescing leaves. In senescing leaves, ammonium is produced by protein degradation, and is subsequently assimilated into the amide group of glutamine by GS, for subsequent transport to growing tissues including grain.\textsuperscript{44} Two ammonium transporters were expressed in Cluster 4, which could potentially be involved in the export of ammonium from the vacuole into the cytosol following proteinolysis, as a substrate for GS.\textsuperscript{4} Another possible study limitation is that we sampled the second leaf above the ear to collect reproductive stage leaf data (Table S1). During maize grain filling, cytosolic GSI isoforms have been shown to be expressed in the leaf below the ear, which is dedicated to ear feeding.\textsuperscript{45} The single NADH-dependent glutamate synthase probe (ZmN-65; Table 1) observed in Cluster 4 leaves was highly expressed during the reproductive stages. The corresponding gene could play an important role in nitrogen remobilization from leaf to grain.

Promoter motifs underlying stage-selective gene expression

A prediction of this study is that each vegetative nitrogen gene expression cluster is associated with distinct motifs that are over-represented in the associated set of promoters (Table 2). Of particular interest was the 8 bp motif (consensus CGACCCCTT) that was over-represented in the promoters of Cluster 1 genes and which matched a sequence within the 43 bp nitrogen response element (NRE) (Table 3); the NRE was previously identified in the promoter of the Arabidopsis nitrite reductase (NiR) gene, and shown to be both necessary and sufficient for nitrate-induced gene expression.\textsuperscript{15} Within the 43 bp NRE, an alignment of monocot-dicot NiR promoters suggested that an 8 bp distal element (consensus (c/t)GaCeCTT) and a 5 bp proximal element [AAG(a/g)], separated by a 10 bp spacer, were somewhat conserved in the -100 bp to -240 bp promoter region,\textsuperscript{20} with the distal 8 bp element being dominant.\textsuperscript{19} Our predicted 8 bp Cluster 1 promoter motif matched the dominant distal element of the NRE and was similarly located in the -200 to -250 bp regions of Cluster 1 promoters (Tables 2 and 3). However, we failed to find the 5 bp proximal element in our study, as the Promzea software only searches for motifs ≥ 6 bp. Recently, a yeast 1 hybrid screen using the 43 bp Arabidopsis NRE as bait identified the NIN-LIKE PROTEIN 6 (NLP6) transcription factor (and perhaps related NLP proteins) as being responsible for binding and nitrate-inducible activation of NRE in vitro and in planta.\textsuperscript{21} Linker-scanner mutagenesis showed that the 8 bp distal element was especially responsible for nitrate activation by NLP6, in Arabidopsis protoplasts.\textsuperscript{23} NRE-like sequences including the 8 bp element were also previously identified in the 3′ region of the Arabidopsis nitrate reductase NIA1 gene\textsuperscript{25} where it was shown to help mediate nitrate activation of this gene by NLP6.\textsuperscript{21} In Arabidopsis, the NRE was only reported in nitrite reductase (NIR1) and nitrate reductase (NIA1) genes,\textsuperscript{21,22} but in our study it was found to be over-represented in the promoters of genes encoding nitrate transporters (NRT1, NRT2.1) and ammonium transporters (Table 3), a novel observation that suggests NLP6-like proteins may activate these promoters in maize. Here we refer to this conserved distal 8 bp motif as the NRE43-d8 motif. As NRE43-d8 was only over-represented in Cluster 1 promoters, one possibility is that it is an important cis-acting element of nitrogen-related genes during early-stage vegetative development in maize.

Prior to the discovery of the NRE 43 bp motif noted above,\textsuperscript{15} an even more distal motif, also called the NRE [core A(c/g)TCA], was shown to be conserved in the promoters of nitrate reductase (NR) and NiR genes in both dicots and monocots including maize, but not tested for sufficiency of nitrate induction.\textsuperscript{56} In maize, several of the distal NREs [A(g/c)TCA] were located in the −500 to −1000 bp promoter region,\textsuperscript{39} more distal than the region analyzed in this study, perhaps explaining why we did not recover this motif in our search.

Our analysis did however reveal another putative nitrogen-related motif of interest, the G-box 10 element, which was over-represented in Cluster 1 gene promoters (Table 2). The core of the G-box 10 element is identical to the E-box motif (CANNTG) which is necessary for nitrate induction of the NIA1 (nitrate reductase) promoter in Arabidopsis.\textsuperscript{24} The E-box motif and other non-NRE motifs that were identified in this study (Table 2) may be the yet to be identified binding sites for NLP7, a regulatory factor recently shown to bind 851 genes in Arabidopsis within 10 min of nitrate addition including genes encoding nitrate reductase (NIA1), GS2, and transporters NRT1.1, NRT2.1, and NAR2.\textsuperscript{22} Interestingly, nitrate triggers rapid nuclear retention of NLP7 leading to chromatin binding.\textsuperscript{22} NLP6 and NLP7 are related, but they have been reported to have different chromatin binding sites.\textsuperscript{22}

Significance for crop improvement

It is estimated that only 30–80% of nitrogen fertilizer is taken up by maize roots, with the remainder leached or volatilized.\textsuperscript{3,57-59} However, improving the nitrogen use efficiency of maize will require a better understanding of nitrogen uptake and assimilation in different tissues and stages of development. For example, in newer maize hybrids, an increase in nitrogen uptake efficiency has been attributed specifically to increased post-flowering nitrogen uptake.\textsuperscript{60} Unfortunately, the underlying transcriptional regulation of nitrogen-related genes during maize development has been poorly understood, with the first comprehensive study of nitrate transporters only recently reported.\textsuperscript{21} Here we identified distinct developmental modules of nitrogen-related gene expression in maize. We have discussed
the possibility that each module may be fine-tuned for the different nitrogen uptake and assimilation needs of each tissue and stage of development. Clustering of nitrogen-related genes by their developmental tissue and stage of expression appears to have assisted in the identification of putative cis-acting promoter motifs; this strategy might represent a path forward in other plants. These advances may assist in targeting long-term efforts for improving nitrogen use efficiency in maize.

Materials and Methods

Plant growth and tissue harvest

Syngenta inbred SRG200 seeds were grown in a greenhouse during the summer of 2007 at the University of Guelph, using the following conditions: 16 h light (–600 μmol m⁻² s⁻¹) at 28°C, 8 h dark at 23°C, and 50% relative humidity. Plants were grown semi-hydroponically in pots containing Turface® clay, and irrigated with a nutrient solution containing: 0.4 g/L 28-14-14 fertilizer [28% total N (1.6% nitrate, 0.4% ammonium, 26% urea), 14% P₂O₅, 14% K₂O], 0.4 g/L 15-15-30 fertilizer [15% total N (nitrate 8.8%, ammonium 2.95%, urea 3.25%), 15% P₂O₅, 30% K₂O], 0.2 g/L NH₄NO₃, 0.4 g/L of MgSO₄•7H₂O, and 0.03 g/L of micronutrient mix (S, Co, Cu, Fe, Mn, Mo and Zn). Three biological replicates per tissue/stage were harvested, always at –11 AM.

Microarray analysis

Details of the microarray and hybridization procedure have been described previously.²⁷ Briefly, RNA was isolated from 50 tissues/stages (Table S1) and hybridized onto maize B73 Affymetrix 82K Unigene arrays. The Syngenta GeneChip microarray was custom-designed in 2005. The starting array consisted of approximately 87,000 probe sets representing 82,000 Zea mays unigenes (as defined by Pubmed) or expressed sequence tag (EST) clusters, which were trimmed for further analysis (Table S2), resulting in only a subset of nitrogen-related genes to be reported in this study (see details below). Each probe set consisted of an average of 16 perfect-match probes, of 25 mer oligonucleotides. For each tissue-stage, 3 hybridizations were conducted using RNA isolated from 3 biological replicates representing 3 pools of plants. Total RNA was isolated and used for cDNA, cRNA synthesis, and labeling using standard Affymetrix protocols. Labeled cRNAs were then fragmented and applied to the maize custom GeneChip microarray for molecular hybridization.⁶¹ The array images with hybridization signals were acquired and quantified by GeneChip Operation System (GCOS) software (Affymetrix). The quality of the hybridizations was assayed using Expressionist (GeneData). Experiments were repeated for any arrays that initially failed to pass the quality assays. Array expression was normalized using the RMA method from Bioconductor.⁶² The significance of differential expression for the 82 K probes was measured by ANOVA using R. The P values of relative microarray expression were adjusted using the Bonferroni method.⁶³

Annotation and clustering of nitrogen-related genes

Clustering was conducted using K-means clustering of the relative expression data after RMA normalization. The number K of clusters was estimated by testing the uniformity of the cluster for K from 2 to 50 (for comparison of 50 tissues) or 17 (for comparison of leaf and root stages only). Array probes corresponding to nitrogen-related genes were retrieved using 3 methods. First, as the probes were designed from the maize Unigene set, the corresponding original GenBank sequences were used to retrieve matches, using nucleotide BLAST searches against the B73 maize genome (MaizeSequence.org, release 4a.53). Probe sets with no expression (relative expression < 100) in any of the 150 microarray experiments were removed from the annotation (26,989 probe sets). If 75% of the probes in the probe set (12/16) matched the same gene model, the probe set was identified as a match for that gene. If only 12%—< 75% of the probes in the probe set (11/16—11/16) matched the same gene model, the probes were considered as a partial match to that gene. A probe was required to have 85% sequence identity with the gene model to be considered as a valid match of that gene. A total of 33,664 probe sets that matched to unique gene models were mapped using these steps (Table S2). Exonerate alignment was used to annotate an additional 9,919 probes. Nucleotide BLAST was not successful in identifying EST matches because of the bias created by gene model issues related to gene-calling software (GeneBuilder or FGENESH).

The remaining 12,089 probe sets on the array showed expression, but did not map to the maize genome. After the elimination of the probe sets with either no expression, cross-hybridizing, or redundant probe sets, there were 22,787 high-quality annotated probes. Subsequently, the array probe sequences were re-screened for matches with EST sequences from NCBI using BLAST. Finally, nitrogen uptake and assimilation keywords were used to search gene annotations and protein domains in the B73 maize genome from MaizeSequence.org, and the search results were each matched to a GenBank protein with the greatest homology with the microarray probe set. After this pipeline, probes corresponding to nitrogen-related genes such as nitrite reductase were found to be missing which is a limit of the current study.

Quantitative real-time reverse transcription PCR (qRT-PCR)

The genes and PCR primers used for qRT-PCR validation are listed (Table S4). An aliquot of the RNA used for microarray hybridization was quality control tested using the Agilent 2100 Bioanalyzer. Total RNA was used to create cDNA. Subsequently, qRT-PCR was performed using Perfecta SYBR® Green FastMix ROX™ (Quanta BioSciences, Inc, Gaithersburg, MD USA) and a StepOnePlus™ Real-Time PCR System (Applied Biosystem-Life Technologies Corp, Carlsbad, CA USA). Amplification conditions were 95°C for 3 min, followed by 40 cycles of: denaturation, 95°C for 15 s; annealing (55°C for NAR2.1 and 60°C for the other primers) for 30 s; extension at 72°C for 1 min. The results were normalized using the Livak method to report relative expression.⁶⁶

Promoter motif discovery

Microarray probes were matched to known genes from the filtered B73 maize gene set from MaizeSequence.org as previously described.²⁸ Over-represented de novo motifs that were
present in the promoters (~500 to +1 region) of the genes within each expression cluster were then searched using a Perl-based motif discovery program developed by us for the maize genome, called Promzea. Briefly, Promzea filters and combines results from 3 motif discovery tools (Weeder, MEME, and BioProspector) and performs additional validation tests. Retrieved motifs are subsequently matched to previously defined motifs using Athamap and PLACE using STAMP software. Promzea predicts motifs ≥ 6 bp in length.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References

1. Crawford NM, Glass ADM. Molecular and physiological aspects of nitrate uptake in plants. Trends Plant Sci 1998; 3:389-95; http://dx.doi.org/10.1016/S1360-1385(98)01311-9
2. Gaudin ACM, McClymont SA, Raizada MN. The nitrogen adaptation strategy of the wild teosinte of maize. Zea mays subsp parviglumis. Crop Sci 2011; 51:2780-95; http://dx.doi.org/10.2135/cropsci2010.12.0866
3. Kant S, Bi YM, Rohsthein SJ. Understanding plant response to nitrogen limitation for the improvement of crop nitrogen use efficiency. J Exp Bot 2011; 62:1496-509; PMID:20926552; http://dx.doi.org/10.1093/jxb/erq297
4. Mascula-Daubresse C, Daniel-Vedelee F, Dechorgnat J, Chardon F, Gauthichon L, Suzuki A. Nitrogen uptake, assimilation and remobilization in plants: challenges for sustainable and productive agriculture. Ann Bot 2010; 105:1141-57; PMID:20299346; http://dx.doi.org/10.1093/aob/mcp028
5. Li P, Ponnala L, Gandorita N, Wang L, St Y, Tausta SL, Kehron TH, Pwartz N, Patel R, Myers CR, et al. The developmental dynamics of the maize leaf transcriptome. Nat Genet 2010; 42:1060-7; PMID:21037656; http://dx.doi.org/10.1038/ng.703
6. Dechorgnat J, Nguyen CT, Armengaud P, Jossier M, Diatloff E, Filleur S, Daniel-Vedele F. From the soil to the seeds: the long journey of nitrate in plants. J Exp Bot 2011; 62:1349-59; PMID:21913579; http://dx.doi.org/10.1093/jxb/erq094
7. Forde BG. Nitrate transporters in plants: structure, function and regulation. Biochim Biophys Acta - Biomembr 2000; 1465:219-35.
8. Quaggiotti S, Ruperti B, Borsa P, Destro T, Malagoli T, Pasternak S, Liang C, Zhang J, Fulton L, Graves PD, Fauteux F, Stroomvik M, Raizada MN. Promzea: a pipeline for discovery of co-regulatory motifs in maize and other plant species and its application to the anthocyanin and phlobaphene biosynthetic pathways and the Maize Development Atlas. BMC Plant Biol 2013; 13:42; PMID:23497159; http://dx.doi.org/10.1186/1471-2229-13-42
9. Zhang Y, Guan P, Chen M, Xing X, Zhang Y, Crawford NM. Multiple regulatory elements in the Arabidopsis NIA1 promoter act synergistically to form a nitrate enhancer. Plant Physiol 2010; 154:423-32; PMID:20668861; http://dx.doi.org/10.1104/pp.110.162586
10. Schnable PS, Ware D, Fulton RS, Stein JC, Wei F, Putterill S, Liang C, Zhang J, Fulton L, Graves PD, Fauteux F, Stroomvik M, Raizada MN. Promzea provides a web interface for Arabidopsis motif discovery that enhances interpretation of transcription factor binding site information in high-throughput genome-wide datasets. BMC Bioinformatics 2011; 12:16120; http://dx.doi.org/10.1186/1471-2105-12-16120

Supplemental Material

Supplemental material may be found here:
http://www.landesbioscience.com/journals/psb/article/26056/
35. Hermann-Le Denmat S, Werner M, Sentenac A, Thuriaux P. Suppression of yeast RNA polymerase III mutations by FHA1, a gene coding for a fork head protein involved in RNA processing, Mol Cell Biol 1994; 14:2905-13; PMID:8164651

36. Strable J, Borsuk L, Neerleton D, Schnable PS, Irish EE. Microarray analysis of vegetative phase change in maize, Plant J 2006; 56:1045-57; PMID:17864925; http://dx.doi.org/10.1111/j.1365-313X.2006.03661.x

37. Sekhon RS, Lin H, Childs KI, Hansey CN, Buell CR, de Leon N, Keppeler SM. Genome-wide atlas of transcription during maize development. Plant J 2011; 66:553-63; PMID:21229659; http://dx.doi.org/10.1111/j.1365-313X.2011.04527.x

38. Gaulin AC, McCloud SA, Holmes BM, Lyons E, Raizada MN. Novel temporal, fine-scale and growth variation phenotypes in roots of adult-stage maize (Zea mays L.) in response to low nitrogen stress. Plant Cell Environ 2011; 34:2122-37; PMID:21848860; http://dx.doi.org/10.1111/j.1365-313X.2011.02049.x

39. Peng Y, Niu J, Zhang F, Li C. Shoot growth correlations among traits. Crop Sci 2007; 47:1787-96; PMID:17652288; http://dx.doi.org/10.2135/cropsci2007.02.0096

40. Harel E, Lea PJ, Miffin BJ. Localization of enzymes of nitrogen assimilation in maize leaves and their activities during greening, Planta 1977; 134:195-200; http://dx.doi.org/10.1007/BF00384971

41. Kiyosiyama S, Nakashiki H, Uchida H, Tsuji A, Nishiyama S, Furutashibashi M, Takeda H, Ishikawa N, Watanabe S, To T, et al. Real time visualization of 15N-translocation in rice under different environmental conditions using position emitting tracer imaging system. Plant Physiol 2001; 127:1573-53; PMID:11299555; http://dx.doi.org/10.1104/pp.115.4.7143

42. Stritt M, Krapp A. The interaction between elevated carbon dioxide and nitrogen nutrition: the physiological and molecular background. Plant Cell Environ 1999; 22:583-621; http://dx.doi.org/10.1046/j.1365-3040.1999.00338.x

43. Peng Y, Niu J, Zhang F, Li C. Shoot growth correlations among traits. Crop Sci 2007; 47:1787-96; PMID:17652288; http://dx.doi.org/10.2135/cropsci2007.02.0096

44. Wagnér F, Radelof U. Performance of different small sample RNA amplification techniques for hybridization on Affymetrix GeneChips. J Biotechnol 2007; 129:628-34; PMID:17408796; http://dx.doi.org/10.1016/j.jbiotec.2007.02.015

45. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, Illius B, Gautier L, Ge Y, Gentry J, et al. Bioconductor: open software development for computational biology and bioinformatics. Genome Biol 2004; 5:R80; PMID:15167198; http://dx.doi.org/10.1186/gb-2004-5-10-r80

46. Hochberg Y. A sharper Bonferonni procedure for multiple tests of significance. Biometrika 1988; 75:800-2; http://dx.doi.org/10.1093/biomet/75.4.800

47. Berger RL, Hsu JC. Bioequivalence trials, intersection-union tests and equivalence confidence sets, Stat Sci 1996; 11:283-319; http://dx.doi.org/10.1214/ss/1032280304

48. Slater GS, Birney E. Automated generation of heuristics for biological sequence comparison. BMC Bioinformatics 2005; 6:31; PMID:15715233; http://dx.doi.org/10.1186/1471-2105-6-31

49. Schmitzgen TD, Livak KJ. Analyzing real-time PCR data by the comparative C(T) method. Nat Protoc 2008; 3:1011-8; PMID:18556601; http://dx.doi.org/10.1038/nprot.2008.73

50. Pavela G, Zambelli F, Peso G. WeederH: an algorithm for finding conserved regulatory motifs and regions in homologous sequences. BMC Bioinformatics 2007; 8:46; PMID:17286685; http://dx.doi.org/10.1186/1471-2105-8-46

51. Bailey TL, Elkan C. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology. Menlo Park, California: AAAI Press, 1994:28-36.

52. Liu X, Brutlag D, Liu J. BioProspector: discovering conserved DNA motifs in upstream regulatory regions of co-expressed genes. In: Altman RB, Dunker AK, Hunter L, Klein TE, eds. Pacific Symposium on Intelligent Systems for Molecular Biology. Menlo Park, California: AAAI Press, 2000:127-38.

53. Buelow L, Engelmann S, Schindler M, Heli R. AthaMap, integrating transcriptional and post-transcriptional data. Nucleic Acids Res 2009; 37(Database issue):D998-3; PMID:18842622; http://dx.doi.org/10.1093/nar/gkn709

54. Higo K, Ugawa Y, Iwamoto M, Korenaga T. Plant secondary structure prediction, Plant Signal Behav 2007; 2:006; PMID:18025040; http://dx.doi.org/10.1155/2007/52722