Gene Expression Biomarkers Provide Sensitive Indicators of in Planta Nitrogen Status in Maize[W][OA]

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Over the last several decades, increased agricultural production has been driven by improved agronomic practices and a dramatic increase in the use of nitrogen-containing fertilizers to maximize the yield potential of crops. To reduce input costs and to minimize the potential environmental impacts of nitrogen fertilizer that has been used to optimize yield, an increased understanding of the molecular responses to nitrogen under field conditions is critical for our ability to further improve agricultural sustainability. Using maize (*Zea mays*) as a model, we have characterized the transcriptional response of plants grown under limiting and sufficient nitrogen conditions and during the recovery of nitrogen-starved plants. We show that a large percentage (approximately 7%) of the maize transcriptome is nitrogen responsive, similar to previous observations in other plant species. Furthermore, we have used statistical approaches to identify a small set of genes whose expression profiles can quantitatively assess the response of plants to varying nitrogen conditions. Using a composite gene expression scoring system, this single set of biomarker genes can accurately assess nitrogen responses independently of genotype, developmental stage, tissue type, or environment, including in plants grown under controlled environments or in the field. Importantly, the biomarker composite expression response is much more rapid and quantitative than phenotypic observations. Consequently, we have successfully used these biomarkers to monitor nitrogen status in real-time assays of field-grown maize plants under typical production conditions. Our results suggest that biomarkers have the potential to be used as agronomic tools to monitor and optimize nitrogen fertilizer usage to help achieve maximal crop yields.

Agricultural food production worldwide has doubled over the last several decades to provide sufficient food supply for both animal and human consumption. The increased agricultural production has been made possible by the introduction of high-yielding crops and improved agronomic practices as well as a dramatic increase in the use of nitrogen-containing fertilizers. Annual worldwide consumption of nitrogen fertilizers is between 85 and 90 million metric tons and represents one of the major input costs in plant production (for review, see Good et al., 2004). Furthermore, worldwide nitrogen use is estimated to continue to rise by an additional 23.1 million tons by 2012 compared with 2008 (FAO, 2008). A consequence of the increased use of nitrogen fertilizers is increased input costs to growers and a heightened awareness of the potential impacts on the ecosystem (for review, see Hirel et al., 2007). The fundamental challenges for the future, therefore, will be continued sustainable agricultural productivity at a reasonable cost, with preservation of our natural resources and ecosystems.

In most commercial cereal crops such as maize (*Zea mays*), productivity is linked to fertilizer use. In typical field practices, a single application of nitrogen fertilizer is applied in early winter or at seed sowing, usually in high amounts to help optimize yield. Fertilizer capture by crop plants is relatively inefficient, with only 30% to 65% of applied nitrogen being utilized. Thus, more than 35% of the soil nitrogen is lost through a combination of processes, including leaching, denitrification and ammonia volatilization, and fixation in organic matter (Raun and Johnson, 1999; Tilman et al., 2002; Kant et al., 2011). Faced with rising energy costs and price increases for nitrogenous fertilizers, grain producers are under growing pressure to maximize fertilizer nitrogen use efficiency. Strategies to increase fertilizer uptake efficiency include improving plant varieties by classical breeding and biotechnological approaches, precision application to more accurately account for site-specific variations in residual soil nitrogen, and synchronizing application with the period of optimal plant nitrogen uptake (Raun and Johnson, 1999; Edgerton, 2009). Thus, an increased understanding of plant responses to nitrogen will be important in the refinement of strategies to improve agricultural productivity and sustainability.

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Plants respond to limiting nitrogen through a complex of physiological, morphological, and developmental responses. Genome-wide microarray analysis in Arabidopsis (*Arabidopsis thaliana*) under limiting nitrogen conditions shows extensive changes in primary and secondary metabolism, protein synthesis, and cellular growth processes and numerous changes to regulatory genes and other cellular pathways (Wang et al., 2003; Scheible et al., 2004; Bi et al., 2007; Peng et al., 2007; for review, see Kant et al., 2011). Less is known about the gene expression changes in response to nitrogen in cereals such as rice (*Oryza sativa*; Lian et al., 2006; Beatty et al., 2009), and there are no published reports in other crops such as maize.

The inability to quickly and accurately assess plant responses to varying nitrogen conditions in crops has hindered progress in the genetic improvement of yield potential through nitrogen utilization. To better understand and characterize responses to nitrogen in maize, we have utilized multiple whole-genome microarray experiments to identify gene expression biomarkers that are able to assess plant responses under limiting and sufficient nitrogen conditions. Using logistic regression statistical approaches, we have identified a common set of genes whose expression profiles quantitatively assess the extent of plant stress levels under varying nitrogen conditions independent of genotype, tissue type, developmental stage, and environment, including in plants grown under controlled conditions and in the field. Consequently, we have successfully used these biomarkers to monitor nitrogen stress and its correlated yield responses in real-time assays of field-grown maize plants under typical production conditions. To our knowledge, these results represent the first identification of sensitive molecular biomarkers for a complex abiotic trait and will serve as an important tool for dissecting the factors affecting nitrogen fertilizer utilization under standard agronomic conditions.

RESULTS

Identification of Nitrogen Response Biomarker Genes

Four independent microarray experiments were conducted as described (see "Materials and Methods"; Supplemental Table S1), totaling 216 independent corn samples. Leaf samples at the V6 development stage were collected from plants grown under sufficient (16 mM NH_4NO_3) or limiting (2 mM NH_4NO_3) nitrogen conditions or from plants that were recovering from limiting nitrogen conditions (see below). To minimize environmental factors, experiments were conducted at different times of the year and samples were collected at different times of the day. Likewise, to limit the genotype dependence of nitrogen-responsive genes, plants were derived from two different maize hybrids, representing three different inbred parents.

Multiple microarray data-filtering and -processing steps were performed to identify the core set of consistently nitrogen-responsive genes, as shown in Table I. Normalized microarray hybridization intensity values from randomly selected microarray samples from low and sufficient nitrogen treatments (30 samples each) were chosen as the training set for biomarker identification. For data filtering, probe sets that were expressed below $\log_2$ intensity = 9.0 were eliminated, while probe sets whose intensity value differed by at least ±1.5-fold (at *t* test $P \leq 0.01$) between the different treatments were further utilized. The 3,707 probe sets remaining after filtering (Table I; Supplemental Table S2) were subjected to a logistic regression approach, involving iterative selection over 30 cycles, as described in "Materials and Methods." Using this approach, a panel of 112 probe sets (representing 84 genes; see below) was selected for subsequent validation.

To determine if the expression values from the 112 probe sets identified would accurately differentiate plants treated with different nitrogen concentrations, the untested remaining 156 microarray samples from limiting and sufficient nitrogen-treated plants were evaluated. Figure 1 shows the calculated composite expression value (see "Materials and Methods") for each of the untested microarray samples (validation samples) and the original training samples. As shown, the expression of 112 probe sets clearly distinguishes all plant samples grown on limiting nitrogen from the sufficient nitrogen-treated samples in both the 60-sample training data set (Fig. 1A) and the remaining validation samples (Fig. 1C), with no exceptions. These data suggest that the 112 probe sets represent molecular biomarkers useful in determining the plant's response to nitrogen conditions.

Table I. *Statistical analysis and selection of nitrogen-responsive biomarker genes*

Data from microarray samples selected as the biomarker training set were subjected to multiple statistical analysis steps for data filtering. The number of starting (input) and remaining (output) probe sets at each filtering step are shown. TaqMan assays were developed for eight biomarker genes after validation by TaqMan PCR for a subset of the biomarker genes.

Phase	Data-Processing Step	Input Probe Set No.	Output Probe Set No.
Data filtering	$\log_2$ intensity (75th percentile) ≥ 9.0	83,247	24,466
	t test, $P \leq 0.01$	24,466	10,559
	Fold difference ≥ 1.5	10,559	3,707
Biomarker identification	Logistic regression (30 iterative selection cycles)	3,707	112
TaqMan validation	Gene selection for assay development	112	8

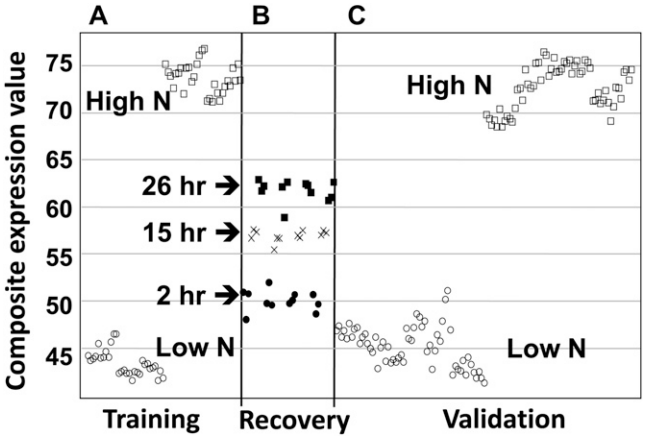

Figure 1. Composite expression values of the 112 nitrogen-responsive biomarkers for microarray training and validation testing samples. A, Training samples. B, Samples from recovery from limiting nitrogen treatment (2-, 15-, and 26-h recovery time). C, Validation samples from limiting or sufficient nitrogen treatment. The *y* axis shows the composite value for expression of the 112 probe sets in each sample. Note that each sample consists of three replications, plotted individually.

Nitrogen-Responsive Biomarkers Detect Rapid Changes in in Planta Nitrogen Status

Accurate differentiation of all tested microarray samples indicates that the biomarkers represent genes whose expression patterns respond to long-term growth under different nitrogen treatments. To determine if the biomarker genes would also respond to short-term differences in nitrogen concentrations during plant growth, we evaluated the composite expression values of plants that had been grown under limiting nitrogen conditions for 28 d and then were subsequently transferred to sufficient nitrogen (20 mM NH_4NO_3) conditions to recover from stress for 2, 15, or 26 h (Fig. 1B).

As shown in Figure 1B, the composite expression values for plants transferred from limiting- to sufficient nitrogen conditions for only 2 h were clearly distinguishable from plants that were treated with limiting nitrogen for the identical length of time but not transferred to sufficient nitrogen conditions. It should be noted that nitrogen-starved plants showed stunted growth and chlorosis and that no change in these phenotypes was observed in plants that were allowed to recover for only 2 h on sufficient nitrogen. This result indicates that the biomarkers are more sensitive indicators of in planta nitrogen status than phenotypic responses. Continued recovery of plants on sufficient nitrogen for 15 and 26 h is accompanied by an additional shift in the biomarker composite expression values and in the direction of the composite expression values of plants grown for a long term (until V6 stage) on sufficient nitrogen. These results further suggest that the biomarker genes can accurately quantify in planta nitrogen status over a range of treatment times and conditions.

Biomarker Genes Respond to in Planta Nitrogen Levels in Multiple Tissue Types

To more conclusively show that the biomarker genes respond to in planta nitrogen status, we directly assayed nitrogen levels (as percentage dry weight of tissue) in both leaves and roots, as shown in Figure 2. For these experiments, soil-grown plants were irrigated with different concentrations of nitrogen fertilizer (six different concentrations from 0.2 to 16 mM NH_4NO_3) until V6 stage, at which time leaf and root tissues were harvested for analysis. Tissue was ground and nitrogen content as a percentage of dry weight was determined (see "Materials and Methods"), as shown in Figure 2A. As expected, the nitrogen content increased with increasing concentrations of nitrogen fertilizer application. Similar increases in nitrogen content were observed in both leaf and root tissues (Fig. 2).

We also monitored plant morphological responses to differing nitrogen concentrations. Low concentrations of applied nitrogen fertilizer (0.2–4 mM NH_4NO_3) result in stunted growth, anthocyanin accumulation in

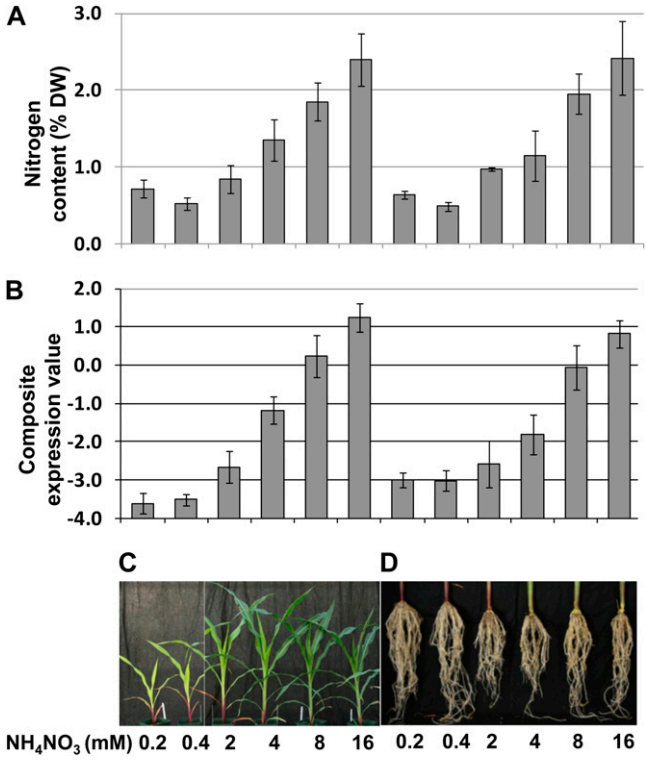

Figure 2. In planta responses to varying applied nitrogen conditions across multiple tissue types. Plants were grown in soil in a growth chamber to V6 development stage. The youngest mature leaf and total root tissues were collected for analysis. A, Nitrogen content as percentage of dry weight (DW). Note the linear increases in in planta nitrogen content in both leaf and root tissues. B, Biomarker composite expression value from TaqMan PCR assays of eight biomarker genes. Note that the composite scores are in log scale, as described in "Materials and Methods." C and D, Photographs of representative aerial tissues (C) and roots (D) from plants grown on varying nitrogen levels. Data are derived from three replications. Error bars show ±1 SE.

stalks, and leaf bleaching (Fig. 2C). In roots, biomass increased at low (0.2–0.4 mM NH_4NO_3) and sufficient (8–16 mM NH_4NO_3) nitrogen concentrations, with decreased root biomass at intermediate (2–4 mM NH_4NO_3) nitrogen treatments. These results are consistent with previously observed responses to limiting or sufficient nitrogen conditions (Scheible et al., 1997).

Biomarker responses were assayed at each nitrogen treatment level in both leaf and root to compare responses with intracellular nitrogen content and plant phenotypes. To simplify this and all subsequent screenings, we utilized a subset of the 112 biomarker genes to create a panel of high-throughput quantitative PCR gene expression assays (see "Materials and Methods"). Eight of the original biomarker genes (Supplemental Table S2) were chosen based on relatively large fold change of expression across limiting and sufficient nitrogen conditions and their amenability to quantitative expression assay development (data not shown). Figure 2B shows the biomarker composite expression response in both leaf and root over the multiple nitrogen concentrations. The composite expression values for these eight genes increase with increasing nitrogen concentration and, furthermore, clearly differentiate plants grown on each increasing nitrogen concentration. Interestingly, the biomarker panel, which was developed exclusively from leaf tissue samples, was also able to quantitatively distinguish the range of nitrogen treatments even in the root tissue samples.

The Biomarkers Are Specific to Nitrogen Response

For optimal utility, biomarker genes should respond uniquely to the intended stress or perturbation. To show that nitrogen status biomarkers developed here are not responsive to other stresses, we tested the effect of drought and nutrient depletion in leaf samples of plants grown to the V3 stage under aeroponic growth conditions. For drought stress, plants were grown for 4 weeks with sufficient nitrogen and daily hydration, followed by the removal of liquid medium for 4 d until leaf wilting was observed. As a control, an equal number of plants were grown for the same time with continued misting of roots with nutrient solution. As can be seen in Figure 3A, no difference was observed in the biomarker composite expression values between the two treatments, indicating that a nonrelated abiotic stress has no effect on our identified nitrogen-responsive biomarkers.

For nutrient depletion treatment, seedlings were germinated and then grown aeroponically for 5 d under full nutrient conditions. Seedlings were then either maintained under full nutrient conditions or the medium was depleted of nitrogen or potassium for continued growth over 30 h. As shown previously, nitrogen depletion of seedlings resulted in a marked change of the composite expression value of nitrogen biomarker genes relative to full nutrient-grown control plants. In contrast, depletion of potassium from the nutrient medium had no apparent effect on the biomarker genes (Fig. 3B), confirming the specificity of those biomarkers.

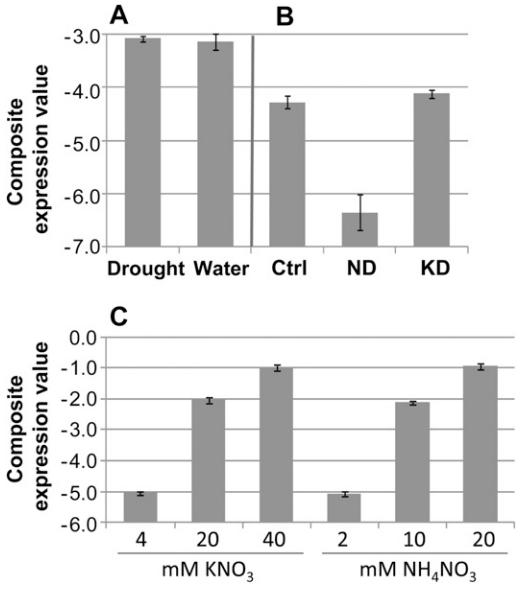

Figure 3. Biomarkers are specific for nitrogen and are independent of nitrogen source. Biomarker composite expression values are from TaqMan assays of eight biomarker genes. A, Plants were grown aeroponically to V3 leaf stage and then were either maintained (Water) or deprived of water for 4 d until leaf wilting was observed (Drought). B, Aeroponically grown seedlings were grown for 5 d under full nutrient conditions and then either maintained (control [Ctrl]) or deprived of nitrogen (ND) or potassium (KD) for 30 h. Note the similar biomarker composite scores for full nutrient control plants and potassium-deprived plants, whereas nitrogen-deprived plants show a significantly lower biomarker composite score. C, Plants were grown to V6 development stage in the greenhouse with continuous supplementation of either KNO_3 or NH_4NO_3 at three different total nitrogen levels, as indicated on the *x* axis. Note that the biomarker expression composite scores increase with increasing nitrogen levels in the irrigation solution and show similar responses to total nitrogen with either nitrogen source. Data are derived from three (A and B) or six (C) replications. Error bars show ±1 SE.

Agronomic practices can utilize different sources of exogenously applied nitrogen for crop fertilization treatments. To determine if the biomarkers could detect different sources of nitrogen, we compared the response of plants grown in the presence of three different NH_4NO_3 (2, 10, and 20 mM) or KNO_3 (4, 20, and 40 mM) concentrations. As shown in Figure 3C, the biomarker panel responded similarly to nitrogen depletion when either nitrogen source was used. Interestingly, the magnitude of the composite scores in each treatment was similar across the tested nitrogen concentrations, suggesting the presence of similar levels of available nitrogen despite the different sources used.

Biomarkers Differentiate in Planta Nitrogen Status across Multiple Field-Grown Hybrids

For nitrogen use efficiency, genetic variability and interactions of genotype responsiveness to fertilization level are readily observed (Gallais and Hirel, 2004). To

ascertain the genotype dependence of the original 112 nitrogen-responsive biomarkers, we examined the expression of these genes in 27 different maize hybrids grown in the field under both high (225 pounds per acre total nitrogen using 28% urea ammonia nitrate solution) and low (0 pounds per acre) levels of exogenously applied nitrogen fertilizer. For fertilizer-treated plants, hybrid seed was sown with a single application of fertilizer applied prior to seedling emergence. The plants were grown to V12 stage in triplicate plots in a single field site in Illinois in 2007, at which time ear leaf tissue was harvested and flash frozen in liquid nitrogen.

Figure 4 shows the composite expression values for all of the 27 hybrids grown under the two different nitrogen conditions. Despite the variability expected under natural field conditions and the relatively low replication in this experiment, the biomarker composite expression values correctly differentiate the nitrogen treatment levels in the majority of hybrid lines, with a clear positive trend in all hybrids except three (F06 + M07, F07 + M03, and F07 + M04). Although some variability was observed in absolute composite expression values between the different hybrids at each nitrogen condition, these results indicate that the biomarkers are quantitative across a wide range of germplasm and will be useful to ascertain nitrogen stress status in most hybrid lines under normal field production conditions.

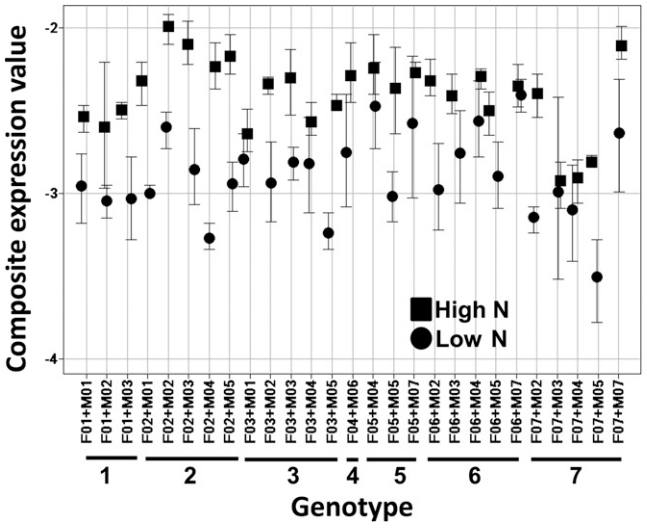

Figure 4. Biomarkers distinguish nitrogen status independent of genotype. Biomarker composite expression values are from TaqMan PCR assays of eight biomarker genes. The expression composite scores are shown for leaf tissues from 27 hybrids grown to V12 development stage in the field under both high (225 pounds per acre; squares) and low (0 pounds per acre; circles) nitrogen conditions. The 27 hybrid lines derive from a diallele crossing scheme where each tester group includes a female (F) tester line crossed to between one and five male (M) lines. Note that, although variability is observed among the expression composite scores across hybrids, in all cases the sufficient nitrogen and limiting nitrogen responses are correctly separated. Data are derived from three replications. Error bars show ±1 SE.

Real-Time Monitoring of Nitrogen-Responsive Biomarkers

Nitrogen availability under field conditions can vary from site to site as a result of residual applied fertilizer and an abundance of environmental factors, such as soil and rainfall conditions.

To test if biomarker composite expression scores can be used to monitor and quantify nitrogen stress in real time in a field site-specific fashion, we screened plants grown under varying nitrogen conditions in 16 field sites across three states (data not shown) in the U.S. Corn Belt during the summer of 2008. As an example, Figure 5 shows biomarker composite expression values along with the grain yield data (in bushels per acre) from hybrid plants of the same genotype grown at two locations (Monmouth and Alexis, IL) under five different applied nitrogen fertilizer regimes (0, 30, 60, 120, and 250 pounds per acre total nitrogen using 28% urea ammonia nitrate solution). In this case, ear leaf samples were collected for nitrogen biomarker response assays at the R2 development stage and plants in the same rows were subsequently grown to maturity for grain yield measurements. Measurement of yield response tends to be one of the most reliable measures of overall plant response to nitrogen fertilization.

The biomarker response in Monmouth, Illinois (Fig. 5A), increased with each increasing nitrogen treatment, while the grain yield of the same plots was also responsive to the added nitrogen (Fig. 5B). On the other hand, at the Alexis, Illinois, field site, the biomarkers did not show a response to added nitrogen (Fig. 5A). The lack of response to nitrogen in this field was confirmed by measuring grain yield, which was also unresponsive to added nitrogen (Fig. 5B). These results demonstrate that the expression patterns of only eight genes in the biomarker panel are sufficient to predict corn response in the field over a range of nitrogen levels.

Functional Characterization of the Nitrogen-Responsive Genes

The complexity of the molecular response to changing nitrogen conditions has been extensively studied by microarray analysis in Arabidopsis. Those previous studies have shown that up to 10% of the Arabidopsis transcriptome can be regulated by nitrogen (Wang et al., 2003; Peng et al., 2007). Our meta-analysis across multiple microarray experiments in maize identified a set of at least 3,707 nitrogen-responsive genes, representing approximately 7% of the unique genes on our microarray. Thus, similar to the Arabidopsis model, a large percentage of the maize transcriptome is responsive to nitrogen. To begin to understand the role of the 3,707 nitrogen-responsive genes, we performed a homology search of the maize microarray probe set sequences against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to map them to spe-

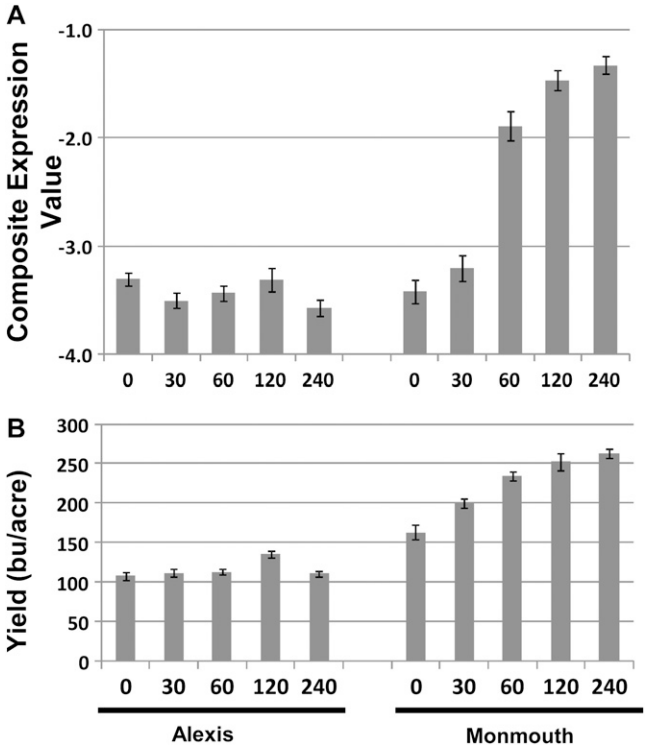

Figure 5. Real-time monitoring of biomarker expression and yield response to varying nitrogen conditions across field sites. A, The biomarker composite expression values from TaqMan PCR assay of eight genes were determined from ear leaf tissues collected at R2 stage. Tissues were harvested from three consecutive plants in the middle of two-row plots. B, Yield (in bushels per acre) was subsequently determined from the same plots as in A upon maturity. Five different levels of applied nitrogen (in pounds per acre using 28% urea ammonia nitrate solution; values across the *x* axis) at Alexis (left) and Monmouth (right), Illinois, in 2008 were evaluated. Note the linear response of both biomarker expression values and yield at Monmouth and the lack of responses at Alexis. Data are derived from 12 replications. Error bars show ±1 SE.

cific biological pathways. Enrichment analysis using a hypergeometric test identified 62 overrepresented KEGG pathways, including nitrogen metabolism, carbohydrate, amino acid, lipid, secondary metabolites, transport, signal transduction, and metabolism of cofactors (Table II). Interestingly, although hormone biosynthesis pathways were not enriched based on the hypergeometric test, genes in this category are highly abundant, representing approximately 5% of the nitrogen-responsive genes (Supplemental Table S2). Recent work in Arabidopsis indicates significant cross talk between nitrogen and hormone-responsive pathways, suggesting a role of hormones in early nitrogen status sensing and signaling (Vidal and Gutiérrez, 2008; Nero et al., 2009; Krouk et al., 2010).

Although the transcriptome response to nitrogen is large and complex, 112 biomarker genes were able to quantitatively indicate the nitrogen status in maize plants. These nitrogen biomarkers were mapped to 84 unique genes representing 20 functional gene categories. The categories containing the most genes included cell wall metabolism, lipid metabolism, electron transport, stress response, regulation of transcription, and many genes with unknown functions (Fig. 6; Supplemental Table S2). Some of the biomarker genes have previously been implicated in nitrogen stress, for example, a gene encoding ADP-Glc pyrophosphorylase small subunit I, which is a key gene in starch biosynthesis. Accumulation of starch is a commonly observed response to low levels of nitrogen in Arabidopsis (Foyer et al., 2003). Another biomarker is annotated as Tonoplast Intrinsic Protein1 (TIP1), whose transcript levels were found to indicate nitrogen stress in maize plants. Similarly, mRNA expression levels of several TIPs were highly affected by nitrogen treatment in Arabidopsis (Scheible et al., 2004). Biomarker genes annotated as chitinase, thaumatin-like protein, universal stress protein, and salt stress root protein RS1 related, which are implicated in the abiotic stress response in plants, are also observed. Not surprisingly, numerous genes involved in transcriptional regulation and signaling were also among the biomarker genes, including three Myb transcription factors. Similarly, Arabidopsis Myb transcription factor genes showed marked changes in transcript abundance in response to nitrogen treatment (Scheible et al., 2004). Myb transcription factors are implicated in the different developmental and physiological responses relevant to nitrogen utilization, including root development (Shin et al., 2007), circadian regulation of carbon-nitrogen synergy (Gutiérrez et al., 2008), and regulation of phenylpropanoid metabolism (Zhou et al., 2009).

DISCUSSION

Nitrogen use efficiency is considered one of the most significant rate-limiting steps for increasing yield in row crops. Additionally, nitrogen fertilizer application represents one of the most costly inputs, from both its financial impact to the grower and potentially to the environment. As such, extensive efforts have been undertaken to improve grain yield by the identification and breeding of nitrogen use efficiency quantitative trait loci into maize germplasm (for review, see Hirel et al., 2007). Likewise, efforts to manage nitrogen application rates and availability in the field are also important (for review, see Edgerton, 2009). The results presented here indicate that gene expression biomarkers can quantitatively measure the response of plants to differing nitrogen levels and may provide a new tool to more carefully manage nitrogen application rates and to mitigate limiting nitrogen conditions in real time in production fields.

Previous analyses have used a limited set of metabolic, biochemical, and molecular markers to characterize nitrogen metabolism in developing ears of maize (Hirel et al., 2007; Cañas et al., 2009, 2011). These studies have identified markers that are strongly

Table II. *Enriched KEGG pathways in 3,707 nitrogen-responsive genes*

Total count refers to the number of probe sets on the microarray with the indicated KEGG category annotation. Target count indicates the number of statistically significant probe sets with the indicated KEGG annotation. Expected target count refers to the number of probe sets expected at random for the indicated KEGG category. Geo_p refers to the *P* value for significance in a hypergeometric test for enrichment analysis.

Functional Category Description	KEGG Identifier	Total Count	Target Count	Expected Target Count	Geo_p
Nitrogen metabolism					
Nitrogen metabolism [PATH:ko00910]	KEGG:1.2.7	149	19	7.5	1.90E-04
Carbohydrate metabolism					
Aminosugars metabolism [PATH:ko00530]	KEGG:1.1.9	74	12	3.7	3.20E-04
Ascorbate and aldarate metabolism [PATH:ko00053]	KEGG:1.1.7.3	24	7	1.2	1.30E-04
Butanoate metabolism [PATH:ko00650]	KEGG:1.1.14.62	24	7	1.2	1.30E-04
Fru and Man metabolism [PATH:ko00051]	KEGG:1.1.5	203	24	10.3	1.00E-04
Gal metabolism [PATH:ko00052	KEGG:1.1.6.16	4	3	0.2	4.90E-04
Glycolysis/gluconeogenesis [PATH:ko00010]	KEGG:1.1.1.32	24	7	1.2	1.30E-04
Inositol phosphate metabolism [PATH:ko00562]	KEGG:1.1.17.23	877	68	44.3	3.60E-04
Propanoate metabolism [PATH:ko00640]	KEGG:1.1.13.57	24	7	1.2	1.30E-04
Pyruvate metabolism [PATH:ko00620]	KEGG:1.1.11.28	24	7	1.2	1.30E-04
Starch and Suc metabolism [PATH:ko00500]	KEGG:1.1.8	529	60	26.7	5.91E-09
Carbon fixation [PATH:ko00710]	KEGG:1.2.4.27	23	6	1.2	7.90E-04
Amino acid metabolism					
Ala and Asp metabolism [PATH:ko00252]	KEGG:1.5.2	163	25	8.2	7.51E-07
Gly, Ser, and Thr metabolism [PATH:ko00260]	KEGG:1.5.3	184	29	9.3	5.56E-08
His metabolism [PATH:ko00340]	KEGG:1.5.11.24	24	7	1.2	1.30E-04
Lys degradation [PATH:ko00310]	KEGG:1.5.9.34	24	7	1.2	1.30E-04
Phe, Tyr, and Trp biosynthesis [PATH:ko00400]	KEGG:1.5.15	122	17	6.2	1.40E-04
Trp metabolism [PATH:ko00380]	KEGG:1.5.14.27	24	7	1.2	1.30E-04
Tyr metabolism	KEGG:1.5.12	163	23	8.2	8.57E-06
Urea cycle and metabolism of amino groups [PATH:ko00220]	KEGG:1.5.16.38	24	7	1.2	1.30E-04
Val, Leu, and Ile biosynthesis [PATH:ko00290]	KEGG:1.5.7	87	15	4.4	2.90E-05
Lipid metabolism					
Arachidonic acid metabolism [PATH:ko00590]	KEGG:1.3.13.28	9	5	0.5	3.49E-05
Bile acid biosynthesis [PATH:ko00120]	KEGG:1.3.6.12	24	7	1.2	1.30E-04
Biosynthesis of steroids [PATH:ko00100]	KEGG:1.3.5	89	14	4.5	1.40E-04
Fatty acid metabolism [PATH:ko00071]	KEGG:1.3.3.34	24	7	1.2	1.30E-04
Glycerophospholipid metabolism [PATH:ko00564]	KEGG:1.3.10	147	18	7.4	4.70E-04
Linoleate 13-lipoxygenase [EC:1.13.11.12]	KEGG:1.3.14.3	22	7	1.1	7.28E-05
Sphingolipid metabolism [PATH:ko00600]	KEGG:1.3.12.31	4	3	0.2	4.90E-04
Secondary metabolites					
Alkaloid biosynthesis II [PATH:ko00960]	KEGG:1.10.9	90	14	4.5	1.60E-04
Diterpenoid biosynthesis [PATH:ko00904]	KEGG:1.10.4	51	9	2.6	9.40E-04
Flavonoid biosynthesis [PATH:ko00941]	KEGG:1.10.7.6	29	7	1.5	4.80E-04
Limonene and pinene degradation [PATH:ko00903]	KEGG:1.10.3.5	24	7	1.2	1.30E-04
Stilbene, coumarin, and lignin biosynthesis [PATH:ko00940]	KEGG:1.10.6	497	53	25.1	3.20E-07
Transport					
ATP-binding cassette transporters	KEGG:3.1.1.136	23	6	1.2	7.90E-04
Other ion-coupled transporters	KEGG:3.1.2	399	45	20.2	5.18E-07
DNA segregation ATPase FtsK/SpoIIIE, S-DNA-T family	KEGG:3.1.5.4	132	19	6.7	3.80E-05
Signal transduction					
Jak-STAT signaling pathway [PATH:ko04630]	KEGG:3.2.8.21	411	44	20.8	2.77E-06
Glutathione *S*-transferase	KEGG:3.2.2.158	108	16	5.5	1.00E-04
Two-component system	KEGG:3.2.1	175	22	8.8	8.01E-05
Protein folding and associated processing	KEGG:2.3.1.117	59	14	3	1.07E-06
E4.1.99.3, phrB; deoxyribodipyrimidine photolyase [EC:4.1.99.3]	KEGG:2.4.3.110	9	4	0.5	6.60E-04
Transcription	KEGG:2.1	202	22	10.2	6.20E-04
Metabolism of cofactors					
Nicotinate and nicotinamide metabolism [PATH:ko00760] \ E2.7.1.-; [EC:2.7.1.-]	KEGG:1.9.4.20	877	68	44.3	3.60E-04

(*Table continues on following page.*)

Table II. (*Continued from previous page.*)

Functional Category Description	KEGG Identifier	Total Count	Target Count	Expected Target Count	Geo_p
Porphyrin and chlorophyll metabolism [PATH:ko00860]	KEGG:1.9.10.64	3	3	0.2	1.20E-04
Riboflavin metabolism [PATH:ko00740]	KEGG:1.9.2.10	23	10	1.2	6.68E-08
β-Ala metabolism [PATH:ko00410]	KEGG:1.6.1.5	24	7	1.2	1.30E-04
Cyanoamino acid metabolism [PATH:ko00460]	KEGG:1.6.5	127	20	6.4	6.14E-06
Glutathione metabolism [PATH:ko00480]	KEGG:1.6.9.16	108	16	5.5	1.00E-04
Purine metabolism [PATH:ko00230]	KEGG:1.4.1.54	23	6	1.2	7.90E-04
Others					
1- and 2-methylnaphthalene degradation [PATH:ko00624]	KEGG:1.11.20	72	12	3.6	2.40E-04
1,2-Dichloroethane degradation [PATH:ko00631] \ E1.2.1.3; aldehyde dehydrogenase (NAD^+) [EC:1.2.1.3]	KEGG:1.11.8.3	24	7	1.2	1.30E-04
Benzoate degradation via CoA ligation [PATH:ko00632] \ E2.7.1.-; [EC:2.7.1.-]	KEGG:1.11.16.12	877	68	44.3	3.60E-04
Ethylbenzene degradation [PATH:ko00642]	KEGG:1.11.13	29	7	1.5	4.80E-04
Fluorene degradation [PATH:ko00628]	KEGG:1.11.14	252	26	12.7	4.80E-04
γ-Hexachlorocyclohexane degradation [PATH:ko00361] \ E3.1.3.2; acid phosphatase [EC:3.1.3.2]	KEGG:1.11.4.9	23	10	1.2	6.68E-08
Metabolism of xenobiotics by cytochrome P450 [PATH:ko00980] \ E2.5.1.18, gst; glutathione *S*-transferase [EC:2.5.1.18]	KEGG:1.11.21.10	108	16	5.5	1.00E-04
Tetrachloroethene degradation [PATH:ko00625] \ E3.3.2.10, EPHX2; soluble epoxide hydrolase [EC:3.3.2.10]	KEGG:1.11.9.5	9	5	0.5	3.49E-05
Cell division \ FTSK, spoIIIE; DNA segregation ATPase FtsK/SpoIIIE, S-DNA-T family	KEGG:4.2.1.6	132	19	6.7	3.80E-05
Insulin signaling pathway [PATH:ko04910] \ CBL; Cas-Br-M (murine) ecotropic retroviral transforming sequence [EC:6.3.2.-]	KEGG:4.4.1.24	411	44	20.8	2.77E-06
Insulin signaling pathway [PATH:ko04910] \ E2.7.1.40, pyk; pyruvate kinase [EC:2.7.1.40]	KEGG:4.4.1.34	23	6	1.2	7.90E-04
Antigen processing and presentation [PATH:ko04612] \ CALR; calreticulin	KEGG:4.5.5.12	9	4	0.5	6.60E-04
Transforming sequence [EC:6.3.2.-]	KEGG:4.5.6.59	411	44	20.8	2.77E-06

dependent upon the genetic background examined (Cañas et al., 2011). A large amount of phenotypic diversity is observed across maize lines, attributed mostly to significant differences in genome structural and gene presence/absence variation (Springer et al., 2009). It was critical, therefore, to evaluate the utility of the nitrogen response biomarkers across a range of maize lines. Although the biomarkers were originally identified using only two hybrids derived from three inbred parents, our data indicate that they can accurately detect differences in nitrogen response across the majority of the 27 hybrids tested (Fig. 4). This is remarkable in that these 27 hybrids are derived from 14 inbred parents, representing different heterotic groups that differ in genetic background from the original lines used to identify the biomarkers (data not shown). The utility of the biomarkers across numerous germplasm suggests that the biomarker genes may be candidates for transgenic approaches or may be used in association studies and expression quantitative trait locus mapping to identify potential loci useful in plant breeding approaches to improve nitrogen response. However, it should be noted that nitrogen-responsive gene expression does not necessarily indicate that a biomarker gene may help to confer nitrogen use efficiency.

Although the biomarkers distinguished low and high nitrogen concentrations across the majority of genotypes tested, it was not surprising to see quantitative differences in the biomarker composite scores in the different hybrids (Fig. 4), because of the genetic differences expected across the parental lines and due to within-site field variation. This result suggests that the biomarker response of an unknown hybrid would first need to be confirmed in the field with test strips that vary in the amount of nitrogen applied prior to broader use across field sites. Some of the variation in biomarker response observed may also be due to the low replication level (only three replications at one field location) in this study. In most applications, biomarkers would be used across numerous field locations and replications, which we expect would

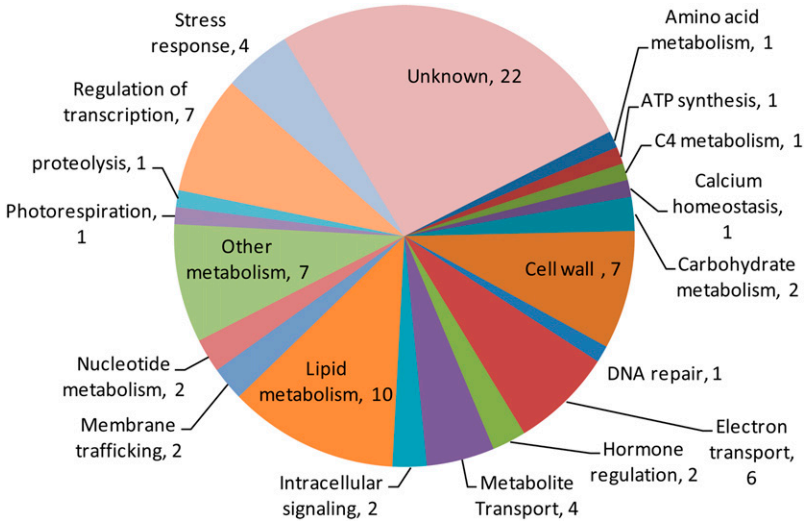

Figure 6. Functional categorization of 84 biomarker genes represented by the 112 probe sets. The biomarker probe set sequences were mapped to the UniRef protein database and then subsequently to KEGG function categories, as described in the text.

significantly reduce the measured variation. Despite the potential for some quantitative differences among hybrid genotypes, the linear responses of the biomarkers to nitrogen application rates in field-grown plants (Fig. 5) may have potential as an early indicator in cases where maximal yield response may not be achieved. However, more work is needed to establish a link of the biomarker response to the yield response.

The biomarker gene set was identified by statistical analysis of leaf gene expression data from plants grown to V6 stage, although the biomarker response was equally quantitative in roots and in leaf tissues from all development stages tested, including V3 (Fig. 3), V12 (Fig. 4), and R2 (Fig. 5), as well as multiple additional developmental stages (data not shown). In addition to the quantitative response across all tissues and developmental stages tested, the biomarkers respond extremely rapidly to changes in nitrogen conditions. Upon nitrogen supplementation to nitrogen-starved plants for only 2 h, the biomarker composite response was clearly distinguished from nitrogen-starved plants (Fig. 1). Prior to nitrogen supplementation, leaf wilting and chlorosis of the nitrogen-starved plants was observed (data not shown), which does not change visibly until much later (by at least 24 h; data not shown). Therefore, the biomarker response is much earlier and more sensitive than phenotypic observations. The change in biomarker composite scores continued throughout the 26-h experiment until nearly at the level of plants grown continuously under sufficient nitrogen conditions (Fig. 1). This expression response is not simply due to temporal factors, since control plants that were not supplemented with nitrogen were sampled at the same time with no change in biomarker response (data not shown). Therefore, the biomarker gene set includes both early nitrogen-responsive genes and genes whose expression continues to respond during continuous nitrogen exposure. These results also indicate that biomarkers more accurately reflect the physiological status of plants than do phenotypic observations.

Interestingly, only a small number of biomarker genes were sufficient to quantitatively assess nitrogen responses across the variety of conditions tested here. While a single gene alone was often able to assess nitrogen status in an individual experiment (data not shown), only the composite score approach using multiple genes was able to quantitatively assess nitrogen status across all experiments. This suggests that a biomarker panel and composite score may be considered an optimal approach when assaying complex traits such as nitrogen utilization across a variety of environments. The statistical approaches utilized to identify the 112-biomarker gene set from microarray expression data were purposefully designed to find a relatively large set of genes, and more stringent statistical criteria may have identified fewer candidate biomarkers. However, to enhance their utility in higher throughput applications, detection of the nitrogen responses via PCR assays was required. In doing so, we were able to narrow the gene list to the most consistent and highly responsive genes for use in subsequent biomarker applications.

The gene expression response to varying nitrogen conditions in Arabidopsis has been extensively studied using the commercial Affymetrix oligonucleotide microarrays (Wang et al., 2003, 2004; Scheible et al., 2004; Bi et al., 2007; Huang et al., 2010). Surprisingly, to our knowledge, there are only limited reports of gene expression analysis under limiting nitrogen conditions in the monocot crop rice (Lian et al., 2006; Beatty et al., 2009). We used a custom-designed Affymetrix oligonucleotide microarray, representing approximately 58,000 unique maize genes based on a combination of public and proprietary database sequences. Using this microarray, we identified 3,707 nitrogen-responsive genes based on our statistical meta-analysis across multiple experiments. Functional annotations could be assigned

to about half of the nitrogen-responsive genes, and analysis of the KEGG pathways confirmed the enrichment in multiple functional categories (Table II) previously observed in those other species, suggesting that maize responds to nitrogen conditions using similar biochemical pathways to those observed in the dicot Arabidopsis and the monocot rice. The list of 112 biomarker genes contains functional gene categories that generally mirror the larger list of nitrogen-responsive genes.

Gene expression biomarkers are used extensively in the human health and pharmaceutical industry for applications such as cancer diagnosis and for predicting patient responses to pharmacological treatments (Baker, 2005; Fielden et al., 2007). Gene expression biomarkers have also been explored in other areas, such as indicators of toxin exposure (Forrest et al., 2005) and longevity therapeutics (Spindler, 2006). In plants, DNA markers are routinely used in breeding applications to follow association and for the introgression of favorable gene alleles that impart desired agronomic traits (for review, see Collard and Mackill, 2008). However, the use of gene expression biomarkers for monitoring agronomic traits has been limited. To our knowledge, this is the first example of transcript biomarkers in plants that have been validated to be stress specific, highly quantitative, and generally applicable across a broad germplasm pool and environmental conditions. The demand for this type of biomarker panel for additional agronomic traits is widespread, and the results reported here provide a blueprint for their continued development.

MATERIALS AND METHODS

Plant Growth and Treatment Conditions

Field-Grown Plants

The maize (*Zea mays*) hybrids described in Figure 4 were derived from a design II mating scheme that used 13 inbred lines as female and 12 inbred lines as male. The inbred parental lines were in the 105- to 115-d maturity range with a span of about 200 growing degree units from planting to midsilk among lines. Three repetitions of the hybrids were grown in Champaign, Illinois, in 2007 in a split-split plot design with maturity (early, mid, and late) as the main plot, hybrid as the subplot, and nitrogen treatment (0 and 225 pounds per acre) as subsubplot in the field design.

Greenhouse-Grown Plants

Plants were grown using Hummert's Metro Mix 200 (approximately 120 g per pot) without starter fertilizer and supplemented with 2 or 20 mM NH_4NO_3 in full-strength Hoagland nutrient solution. Greenhouse conditions were a minimum of 70°F at night and 80°F to 85°F during the day, with average relative humidity maintained between 60% and 80%. The light cycle (14-h photoperiod) and temperature of the greenhouse were controlled by the MicroGrow ProCom system (MicroGrow Greenhouse Systems). Each pot was manually dispensed 100 mL of nutrient solution three times per week on alternate days starting at 10 d after planting. The plants were harvested after 28 d (V6 stage) for the low nitrogen treatment and after 21 d for the sufficient nitrogen treatment to ensure that plants were harvested at the same V6 developmental stage.

Growth Chamber-Grown Plants

Seeds were sown in 8-inch pots containing 50% Metro Mix 200 and 50% fritted clay (by volume) in a growth chamber with light intensity of 900 μE m^{-2} s^{-1}, 16-h photoperiod, 30°C/22°C day/night temperature, and relative humidity of 70%. Before V3 stage, the plants were watered once daily with 500 mL of water. After V3 stage, the plants were watered with 500 mL of Hoagland nutrient solution including NH_4NO_3 at specified concentrations (0.2, 0.4, 2, 4, 8, and 16 mM NH_4NO_3) on Monday, Wednesday, and Friday at 4:00 PM. At V6 and V8 stages, leaf and root tissues were collected and frozen immediately in liquid nitrogen.

Aeroponically Grown Plants

Seeds were sterilized in 50% bleach and then pregerminated overnight at room temperature in 0.1 mM calcium chloride with aeration. Seeds were then germinated vertically at 30°C for 30 h. Germinated seeds were grown under aeroponic conditions at 26°C/18°C day/night temperature, 17-h days, 45% relative humidity, and 800 μE m^{-2} s^{-1} light. Plants were started (day 0) on full nutrient solution (0.5 mM phosphoric acid, 2.25 mM calcium chloride, 0.75 mM magnesium sulfate, 4.5 mM ammonium nitrate, 2.4 mM potassium chloride, 1.0 mM sodium chloride, and micronutrients, pH 5.7) delivered aeroponically by an ultrasonic fog generator from Shira Frapa Canada. On day 5, plants were completely deprived of either nitrogen or potassium by removal of these ingredients from the aeroponic medium. Plants were then harvested 6 h, 30 h, and 7 d after nutrient depletion. Three technical replicates of 16 plants each were harvested from two ultrasonic fog generators (eight plants each) for each time point and nutrient condition; four plants were pooled to create two biological replicates. Leaf tissue was flash frozen in liquid nitrogen.

Microarray Experiments

Maize lines were grown in four independent greenhouse experiments as described above. Mature leaf tissue samples were flash frozen in liquid nitrogen. Approximately 100 mg of frozen ground plant tissue was treated with a cetyl-trimethyl-ammonium bromide procedure to isolate nucleic acids, which were subsequently treated with DNase for 1 h at 37°C. Total RNA was then purified using the RNeasy kit from Qiagen according to the manufacturer's instructions. RNA yield was analyzed using a NanoDrop-1000 spectrophotometer, and RNA integrity was visualized using the Agilent 2100 Bioanalyzer. RNA amplification for labeling was performed according to the manufacturer's recommendations using the TargetAmp one-round BiotinaRNA amplification kit from Epicentre. Twelve micrograms of labeled RNA was then fragmented according to the standard protocols for gene expression analysis provided by Affymetrix. Fragmented copy RNA samples were hybridized to Affymetrix microarrays according to the manufacturer's standard protocol.

The microarray data for plants grown under limiting nitrogen and sufficient nitrogen conditions, and plants grown under limiting nitrogen and then subsequently transferred to sufficient nitrogen conditions to recover from stress, have been deposited in the National Center for Biotechnology Information's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through accession number GSE32361 (http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE32361).

Biomarker Identification

Microarray hybridization intensity data were first normalized using Partek software. Intensity values were subsequently transformed into $\log_2$ scale. Randomly selected samples from low and sufficient nitrogen treatments in experiments I and II (30 samples each) were chosen as the training set for biomarker identification (Supplemental Table S2). Hybridization intensity data were used to eliminate probe sets with low expression intensity in both treatment groups, using as cutoff a $\log_2$ of 9 at the 75th percentile. A *t* test was then used to select probe sets with at least ±1.5-fold mean difference between the treatment groups at $P \leq 0.01$. The selected probe sets were randomly split into groups, each with no more than 60 probe sets. Logistic regression analysis was performed using the score selection option in SAS software to identify the top 10% of probe set candidates within each subgroup that best separate samples from the different nitrogen-treated groups. The top probe sets from all of the subgroups were then used as the selected variables for the first

randomization cycle. The regression cycle was repeated 30 times. The most frequent selected probe sets (present in at least 50% of the 30 cycles) were then tested by principal component analysis. Each element of the eigenvalue corresponding to the first principal component was used as the weight for each probe set's expression value, and a composite score for each sample was calculated as a weighted average of expression intensity in $\log_2$ scale over the selected genes (composite score = $\sum V_i * \log\_int_i$, where V_i is the eigenvalue of probe set i and $\log\_int_i$ is the probe set's intensity in $\log_2$ scale). The composite score calculation was simplified for validation analysis with smaller data sets (less than 20 genes) as follows: for a set of i nitrogen-induced and j nitrogen-suppressed genes, the composite score = $(\sum \log\_int_i - \sum \log\_int_j)/(i+j)$.

Quantitative Reverse Transcription-PCR

RNA was extracted using the EZNA RNA kit (Epicentre). RNA samples were then treated with Turbo RNase-Free DNase (Ambion) and the concentration was adjusted to 5 ng μL^{-1}. Primers and probe (Supplemental Table S2) for quantitative reverse transcription (RT)-PCR were selected using Primer Express version 2.0 software (Applied Biosystems). RT-PCR was performed using a 60°C annealing temperature and 40 PCR cycles using the TaqMan One-Step RT-PCR Master Mix Reagents Kit in an ABI7900HT TaqMan machine (Applied Biosystems) following the manufacturer's recommendations. Final concentrations of 300 nM primers and 200 nM probe were used for each assay, except for the 18S rRNA control, which used 100 nM primer and probe. Comparative gene expression (2^{-ddCt}) was used for data analysis, as described in the ABI Prism 7700 Sequencing System User Bulletin 2.

Nitrogen Content Analysis

Flash-frozen plant samples were milled to a fine powder using a ball mill at −80°C. A subsample of the frozen powder was lyophilized for carbon and nitrogen analysis. Twenty-milligram samples of the lyophilized powder were weighed and sealed into tin capsules for combustion analysis using a Thermo Scientific Flash 2000 instrument. Atropine obtained from CE Elantech was used to make a secondary standard of soy leaf powder, which was used in the determination of the percentage nitrogen and percentage carbon results.

Sequence data from this article can be found in the GenBank/EMBL data libraries under accession number GPL14616.

Supplemental Data

The following materials are available in the online version of this article.

Supplemental Table S1. Microarray experiments used to identify nitrogen-responsive probe sets.

Supplemental Table S2. Annotation of the 3,707 nitrogen-responsive transcripts.

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