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Full Length Research Paper

Genomics analysis of genes expressed reveals differential responses to low chronic nitrogen stress in maize

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A growth system was developed where N was the only growth-limiting factor. Whole-genome transcription profiling of leaf tissues were analyzed using the Affymetrix GeneChip. 129 clones showed significant change and 83 clones were classed accurately. Most induced clones were largely involved in various metabolism processes including physiological process, organelle regulation of biological process, nutrient reservoir activity, transcription regulator activity and multicellular organismal process. Putative high affinity nitrate transporter (nrt2.1) showed significant up-regulation under the severe low chronic nitrogen stress condition. Analysis of genes expression revealed several previously unidentified genes, including beta-D-glucosidase precursor gene (glu2), and Cyc3 cyclin3 gene. It suggests that under the said condition, nrt2.1 plays the most important role in N absorption and most of the other genes induced expression to enable the maize to have normal growth. A better understanding of the complex regulatory network for plant N responses among these genes will help and lead to improve N use efficiency.

Key words: Genomics analysis, genes expressed, low chronic nitrogen stress, maize.

INTRODUCTION

Nitrogen (N) is a constituent of numerous important compounds including amino acids, proteins (enzymes), nucleic acids, chlorophyll and several plant hormones. It is the most important inorganic nutrient for plant growth (Frink et al., 1999), such as root branching, leaf growth and flowering time (Zhang and Forde, 1998). The production of high-yielding crops is associated with the application of a large quantity of fertilizers at a substantial cost (Shrawat et al., 2004), and as such, N pollution is becoming a threat to global ecosystems because majority of N fertilizers are lost to the atmosphere or leached into groundwater, lakes and rivers (UNEP, 1999). Thus, developing crops that are less dependent on the heavy application of N fertilizers is essential for the sustainability of agriculture. Efforts have been directed to understanding the molecular basis of plant responses to N and to identify N-responsive genes in order to manipulate their expression and enable plants to use N more efficiently (Crawford and Forde, 2002).

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Abbreviations: ATH1, *Arabidopsis thaliana* homeobox gene 1; **NR,** nitrate reductase; **NiR,** nitrite reductase; **GS,** glutamine synthetase; **RT-PCR,** reverse transcription-polymerase chain reaction; **qRT-PCR,** quantitative RT-PCR; **FDR,** false discovery rate; **ELIP,** light-induced proteins; **glu2,** β-D-glucosidase precursor gene.

Nitrate is the major source of N in agricultural soils (Crawford and Glass, 1998). It serves as nutrient and signal (Stitt, 1999). It is taken up by the nitrate transporter gene family members, reduced to nitrite by nitrate reductase (NR), and to ammonium by nitrite reductase (NiR). Ammonium is then incorporated into amino acids, catalyzed primarily by glutamine synthetase (GS) and glutamate synthase (GOGAT) (Campbell, 1999). Nitrate can also induce the expression of genes including NRT1, NRT2, NR and NiR (Campbell et al., 1986). In addition to these metabolic genes, expression of some regulatory genes, sugar metabolism genes and other metabolic pathways genes is also affected by N levels.

Many studies of plant N-responses based on microarray gene expression were reported last years. Wang et al. (2000) used the Arabidopsis GEM1 microarrays to study the response of seedlings grown on ammonium to the addition of different nitrate levels, and identified 25 and 49 N-responsive genes to low or high nitrate induction, respectively. Subsequently, they (Okamoto et al. 2003) used the Arabidopsis whole-genome Affymetrix ATH1 microarray to study the addition of low level nitrate, and discovered more N-responsive genes. Scheible et al. (2004) used the ATH1 microarray combined with real-time RT-PCR to identify genes affected by N-deprivation or N-induction after 30 min or 3 h from N-starved seedlings. Price et al. (2004) used the ATH1 microarray to identify the individual contributions of nitrogen, sugar and nitrogen plus sugar on global gene expression. Yong-Mei et al. (2007) identified that the regulatory elements involved in N response were also important first steps towards understanding N regulatory networks when Arabidopsis are grown under chronic N stress.

These studies have provided valuable insights into N response. However, no studies have yet investigated the responses at the transcriptional level when *Zea mays* are grown under chronic N stress. Earlier reports involved investigations of transient changes in gene expression when nitrate was added to nitrate-starved seedlings. How maize could respond to a severe chronic N stress is not clear.

So, a growth system was developed where N was the only growth-limiting factor, the transcriptional changes of genes that were most affected by N limitation could be investigated. The objective of this study was to characterize the expression patterns of the genes representing a large segment of the maize genome in response to low chronic N stress, with the hope to advance the understanding of the physiological and biochemical processes underlying the response to low chronic N stress.

MATERIALS AND METHODS

Maize inbred line of Huangzaosi used in this experiment was the key maize inbred lines representing 'Tangsipingto' heterotic group. It has been used extensively in breeding programs (Pan et al., 2002). According to previous phenotypic analysis of yield and other agronomic traits, Huangzaosi is tolerant to low nitrate level and does not show great alteration in physiological properties under low nitrate conditions (Agrama et al., 1999; Cao et al., 2000).

Maize growth media

The growth medium contained the following components (final concentration): 40 μM KH_2PO_4 (pH 6.5), 130 μM K_2SO_4, 110 μM $MgSO_4.7H_2O$, 170 μM KCl, 0.16 μM EDTA-Fe, 0.17 μM H_3BO_3,0.02 μM $MnSO_4.H_2O$, 0.02 μM $ZnSO_4.7H_20$, 8.33E-05 μM $CuSO_4.5H_2O$, 0.167 μM $H_2MoO_4.4H_2O$. Component solutions were added separately to deionized water before use. Quartz sand was sterilized with formalin and subjected to open-air drying before use. First, diffirent nitrate content experiment was made to determine the severe low N-limiting treatment level.

Maize growth conditions

Seeds of Huangzaosi were rapidly germinated at 30℃ in an illumination incubator with constant deionized water for 7days. Then young seedlings were transplanted to a flower pot filled with sterilized quartz sand in a controlled environment, where temperature was held at 25 to 30℃ and a fresh 40-ml aliquot of liquid growth media was added to each flowerpot daily.

Nitrate treatment

For the severe low N-limiting treatment, each flowerpot received 1 ml of 0.33 mM nitrate (KNO_3) and 0.33 mM calcium nitrate ($Ca(NO_3)_2$), respectively, everyday and each control flowerpot received 4 ml of 0.33 mM nitrate (KNO3) and 0.33 mM calcium nitrate ($Ca(NO_3)_2$), respectively everyday, three replicates. Two weeks later, the young leaves were excised at the middle of the day and frozen in liquid nitrogen for total RNA preparation and the other samples were selected to detected agronomy indexes and chlorophyll content.

Isolation of total RNA, agronomy indexes and chlorophyll content detected

Total RNA was initially extracted using a TRIzol kit (TaKaRa) and then quantified with a portion of the recovered total RNA adjust to a final concentration of 1 μg μl^{-1}. Agronomy indexes were detected using traditional physical methods. Chlorophyll content was detected using SPAD-502 radiomete.

Microarray analysis

In this experiment, genes signals intensities were determined using the Maize Genome Array Affymetrix Chip which contains 17,734 probe sets to monitor approximately 14,850 maize transcripts representing 13,339 known or predicted genes from maize. Isolated total RNA samples were treated as described in the Affymetrix instruction manual (Affymetrix Gene Chip Expression Analysis Technical Manual).

In brief, an aliquot of 3 μg of total RNA was used to synthesize double stranded cDNA using the Super Script Double-Stranded cDNA Synthesis Kit (Invitrogen) and poly (T)-nucleotide primers that contained a sequence recognized by T7 RNA polymerase. A portion of the resulting double-stranded cDNA was used as a template to generate biotin-tagged cRNA from an *in vitro* transcription reaction (IVT), using the Bio-Array High-Yield RNA Transcript Labeling Kit. 15 μg of the resulting biotin-tagged cRNA was fragmented to strands of 35 to 200 bases in length according to the protocols from

Affymetrix. 10 µg of this fragmented target cRNA was hybridized to an Affymetrix maize array containing 17,734 transcripts.

Hybridization was performed at 45°C with rotation for 16 h. The maize genome arrays were washed and then stained on an Affymetrix Fluidics Station 400, followed by scanning on a Gene Array scanner. The hybridization data were analyzed using Gene Chip Operating software GCOS 1.4. A logistic scaling procedure and comparison analysis were performed on different arrays with significance analysis of microarray software (SAM; academic version 3.08) and Excel 2003. Between the severe low N-limiting and the N-sufficient samples, different numbers of significant genes could be obtained depending on the SAM median false discovery rate (FDR) and minimum fold change of 0% and a 2-fold, respectively.

Quantitative real-time PCR

Real-time PCR was performed using an iQ™5 Optical System (Bio-Rad). Template cDNA samples were prepared using the PrimeScript First Strand Synthesis System Kit (TaKaRa, Dalian, China) for reverse transcriptase-PCR with 500 ng of total RNA. Primers for the PCR reactions were designed to have a melting temperature of about 56 to 62°C and to produce a PCR product of between 100 and 150 bp. Several genes selected randomly were determined using quantitative real-time PCR. Primers were listed in Table 1. The house keeping gene was actin. Each PCR reaction contained 2 µl of cDNA and 0.5 µl of the primers. SYBR® PrimeScript™RT-PCR Kit from TaKaRa was used for the PCR reactions. The initial denaturing time was 2 min, followed by 40 PCR cycles consisting of 94°C for 10 s, 56 - 62°C for 20 s and 72°C for 10 s. A melting curve was run after the PCR cycles followed by a cooling step. Relative quantification was performed with Bio-Rad iQ5 Relative Quantification Software 2.0.

Gene ontology analysis

All the significant clones were analyzed through the Molecular Annotation System of *CapitalBio Corporation*, (http://bioinfo.capitalbio.com/mas/) which provides broad functional classifications for genes and gene products representing their corresponding biological processes, molecular function, cellular localization and so on. The P-value was set to be 0.05.

RESULTS AND DISCUSSION

Developing defined nitrogen growth conditions for genomics analysis

To apply chronic N stress, it is important to develop a defined nitrogen growth condition for maize inbred lines. It is difficult to maintain a constant N level in a soil system due to the different size and affinity of soil particles for nutrients. A "pure" hydroponic system could control the N level well but the root system is not supported by a substrate and it is bathed directly in the nutrient solution with poor aeration. To avoid those shortcomings, flower pots filled with sterilized quartz sand were used and nutrient solution was given in each flower pot every day in a controlled environment. It provided a buffering reservoir of nutrient solution in the root zone, while maintaining an adequate volume of air.

Maize inbred lines, two weeks of age, were evaluated for shoot dry-weight, leaves area, root dry-weight and chloro- phyll content under different N conditions, ranging from 0 to 21 mM.. Under these N conditions, 12 mM nitrate was found to give maximal growth (Figures 1A and B). For subsequent experiments, 12 mM nitrate was used to produce the N-sufficient condition and 3 mM nitrate to produce the severe low N-limiting condition under which plant growth measured by shoot dry-weight, leaves area, root dry-weight and chlorophyll content were reduced to approximately 78.2, 52.1, 16.1 and 14.8%, respectively, of that at 12 mM nitrate.

Under these conditions, the main agronomy characters were increasing during the N-concentration of 0 – 12 mM and decreasing when N-concentration was beyond 12 mM. The consequence was the obvious difference in growth under these N conditions. Which proved that increase of shoot dry-weight, leaves area; root dry-weight and chlorophyll content were all due to N supplying (data not shown).

Identification of differentially expressed genes by genomics analysis

In leaves of 2-week-old maize inbred lines grown under the N-sufficient condition (12 mM N) and the severe N-limiting condition (3 mM N) respectively were harvested to compare the gene expression levels under different, but stable N conditions. All samples were taken at the middle of the day to minimize diurnal changes in C and N metabolism based on the reports of Y.M.Bi et al. (2007), because the expression levels of nitrate assimilation genes are different at different points in time in one day (Scheible et al., 2000). RNA was extracted and hybridized to a custom designed Maize Genome Array Affymetrix Chip. This Array contained 17,734 probe sets to monitor approxi- mately 14,850 maize transcripts representing 13,339 known or predicted genes. On average, each gene contains 15 perfect match probes.

Significance analysis of microarray software (SAM; academic version 3.08) was used to identify genes differentially expressed between the severe low N- limiting and sufficient samples. Different numbers of significant genes could be obtained depending on the SAM median false discovery rate (FDR) and minimum fold change. The final number of significant genes of the following analyses was based on a median FDR of 0% and a minimum of a 2-fold expression difference (Table 2).

To confirm the results of the microarray analysis, 12 significant expression genes was tested by quantitative RT-PCR and found to be consistent with the microarray data. Clones of CD443909, U44087, BM072990 and so on of 11 clones showed down-regulation expression in both methods of GeneChip and qRT–PCR, while clone of BM331860 showed induced expression in both methods when maize were grown under the severe

Table 1. Sequence of primers used for qRT-PCR.

GB accession no.		Primer sequence (5'-to-3')
BM331860	F	GGTAACACAAATAACACGAGTCAAG
	R	GCTCTGGAGTGAAGAATAATGTGAA
U44087.1	F	CACGACACCACCTATCTTCTTCACC
	R	AAGATGAAGTCAGAGGGGAACCAGT
BM337131	F	TAGAATACACGAAACGGATTGCAAC
	R	AGGACAGACAAAGGCTACAGATACC
CD443909	F	GGTGCGTGCCCTGTTGA
	R	ACACACGCACTCCATTACCA
AW927712	F	CCTCGCCTTCCTTGACG
	R	AGCAGCCGGTTTTATCG
BM072990	F	AGGGTAAGATGAAATAGACCACGAG
	R	TGTTTTTCGAGACAATACAGTGCAG
CK370542	F	GGACCCTGGAGGAAGCACA
	R	AGCATGGACGCCCTGAACT
CO526660	F	TCGCAGTCGCAGCCAAAT
	R	ATGCCCTACCTCAACGAATACA
CO519149	F	CAGCAACAACGGGCAGAG
	R	TTTGAGGCGAACGAGTGG
BM334482	F	AGGCAAGAGCATAAACGAGA
	R	CATCAACAAAGTGGAGGGAC
BM379264	F	CAGACAGACCCAGATAGATGAGA
	R	ATGCTGTTGACGAGGACTGAT
BM380179	F	ATGAATGCACAACCCACAGCT
	R	AGTGCCCGTAATCACATCAGC
Actin	F	5'-GTGACCTTACCGACAACC
	R	5'-CCAATACCAGGGAACATAG

GB accession no. represents the gene bank accession number. 'F' means the forward primer, 'R' means reverse primer.

on a genomic scale to show that results of maize GeneChip are reliable (Figure 2).

Functional classification of significant genes under the severe chronic low N condition

Although maize growth measured by shoots dry-weight, leaves area, roots dry-weight and chlorophyll content were reduced to approximately 78.2, 52.1, 16.1 and 14.8%, respectively, under the severe chronic low N condition, the expression levels of most clones in the genome remained similar. 129 clones identified with expression levels were significantly different, of which 114 were induced and 15 were suppressed when compared to the N-sufficient condition. The result is similar to Y.M. Bi et al. (2007)'s study on Arabidopsis under different degrees of N deficiency, and they reported that much more genes were pronounced to be induced during the low long-term deficiency.

In order to further analyze the maize genes expression in the severe chronic low N condition, the Molecular Anno- tation System of *CapitalBio Corporation* was used, which provides broad functional classifications for genes and gene products representing their corresponding biological processes, molecular function, and cellular localization (Figure 3).

Gene ontology (Go) results showed that 15 genes with known Go were induced with up-regulation expression and 3 genes with down-regulation in severe chronic low N condition. The significant level was 0.05. The fold change values were normalized by log2 base, so genes were up-regulated when the fold value was beyond 1; in contrast, genes were down-regulated when the fold value was below 1.

Nitrate transporter and assimilation genes

With regard to the N transporter and assimilation genes, different trends were observed in the past reports.

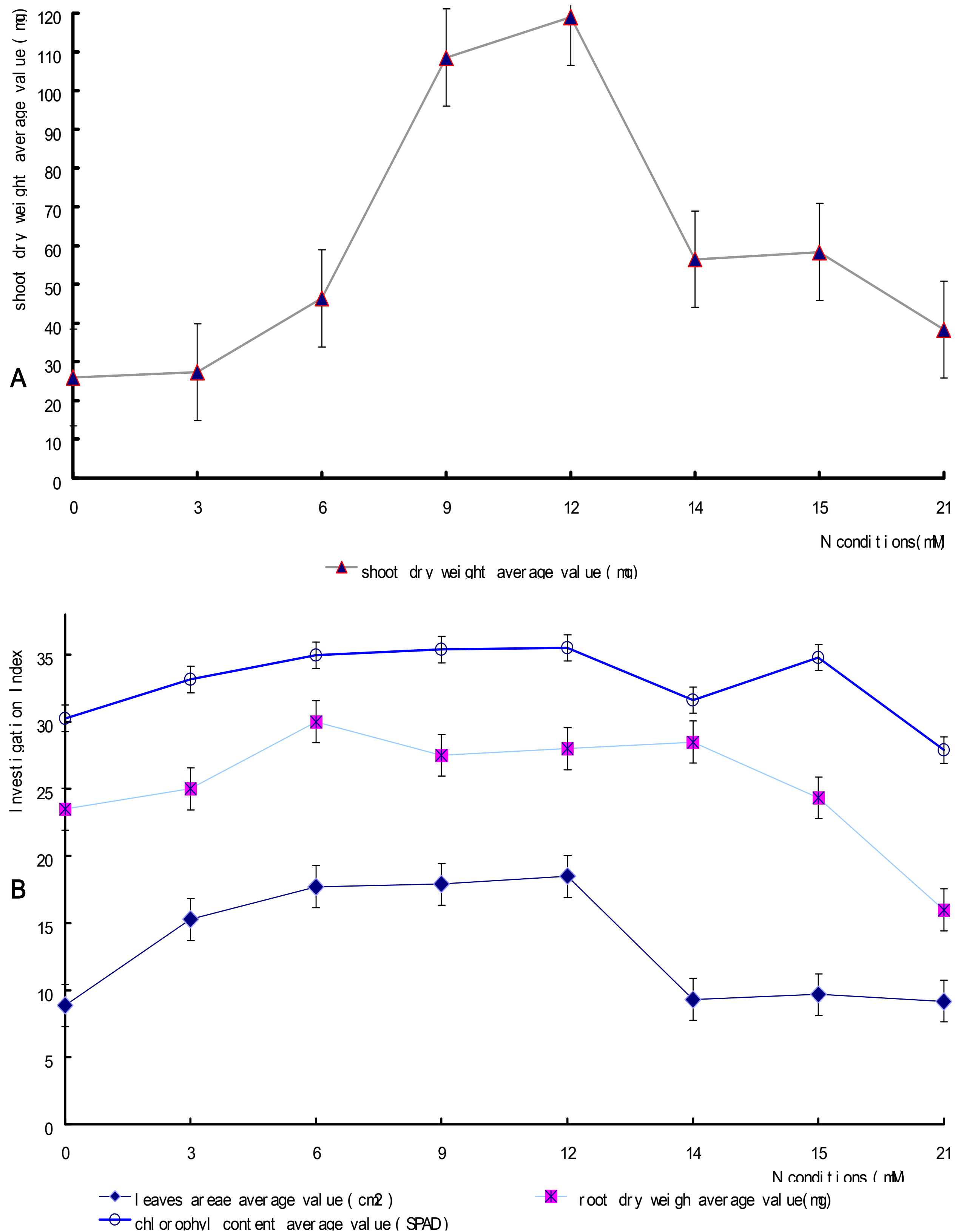

Figure 1. Two-week-old maize inbred lines under different N conditions which was the only limiting factor. A: average of shoot dry-weight (mg/plant) of 3 two-week-old maize inbred lines under different N-concentrations. B: Investigation indexes which included leaves area, root dry-weight and chlorophyll content under different N conditions. The N-concentrations include 0, 3, 6, 9, 12, 14, 1 and 21 mM of 3 biological replicates, respectively.

Table 2. Significant gene numbers at different cutoff levels.

Parameter	FDR 0%	FDR 0% minimum 2 fold
Severe low N versus sufficient N	833	129 (114[a], 15[b])

'a' is the number of genes up-regulated; 'b' is number of genes down-regulated.

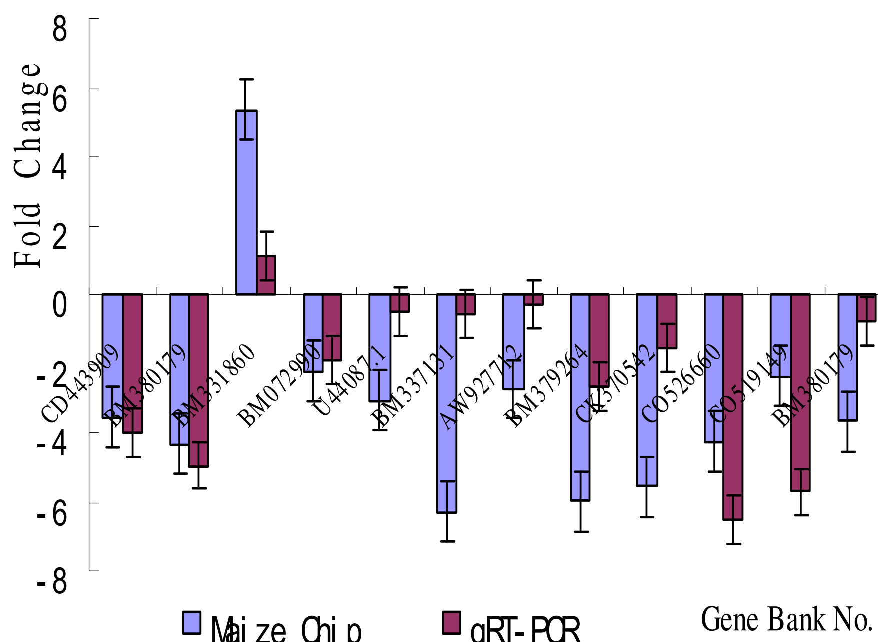

Figure 2. Comparative results of qRT–PCR and Maize array. Column diagrams represent the average fold change of genes expression under the severe chronic low N-limiting compared to the N-sufficient condition with different detection methods. The average values are normalized by log 2 base. Different color means different detection methods of gene chip and quantitative RT-PCR, respectively.

Yong-Mei et al. (2007) reported that the low affinity nitrate transporters gene family including NRT1 and NRT1.1 baselines expressions was higher, while NRT1.3 and NRT1.4 baselines expression were lower under N stress. Matsumura et al. (1997) proved that *Zea mays* putative high affinity nitrate transporter (nrt2.1) gene showed great expression under N-deficient condition.

In the severe chronic N-limiting condition of this paper when maize dry-weight was significantly reduced, none of the genes with low affinity nitrate NRT1 nor NRT1.1 showed significant expression. Only the high affinity nitrate transporter (AY129953.1: nrt2.1) gene showed increased expression, the fold change was 7.7 (Table 3). Many primary N metabolism genes were not detected. Under the severe chronic N-limiting condition, the expression of nrt2.1 can improve the N absorption in order to keep the maize normal growth.

22kD alpha zein5 gene

The storage protein genes of zeins are by far the most highly expressed genes in the endosperm, and are divided into α-zeins, γ-zeins and δ-zeins. Of these, α-zeins were the first storage protein genes to be described (Wienand et al., 1981; Pedersen et al., 1982) and are encoded by large multigene families, γ-zeins and δ-zeins are encoded by small gene families, relatively. Young-Min et al. (2001) study showed that only a few α-zeins genes are transcribed at high or detectable levels and the whole genes of γ- and δ-zeins are highly expressed.

The severe chronic low N stress led to marked changes in the expression of genes involved in protein storage in maize leaves, including large multigene families of zeins, but only one of these encoding 22 kD alpha zein5 genes showed significant up-regulation expression, which belongs to nutrient reservoir activity ontology; the fold change was 8.1 (Table 3). Other zeins of γ- and δ-zeins, however, showed no detectable expression levels. Early studies showed that α-zeins were encoded by a large multigene family, although estimates of its size and complexity varied significantly (Wilson and Larkins 1984; Wilson 1985). It revealed that in the severe chronic low N condition, the α-zeins protein will work through the expression of genes

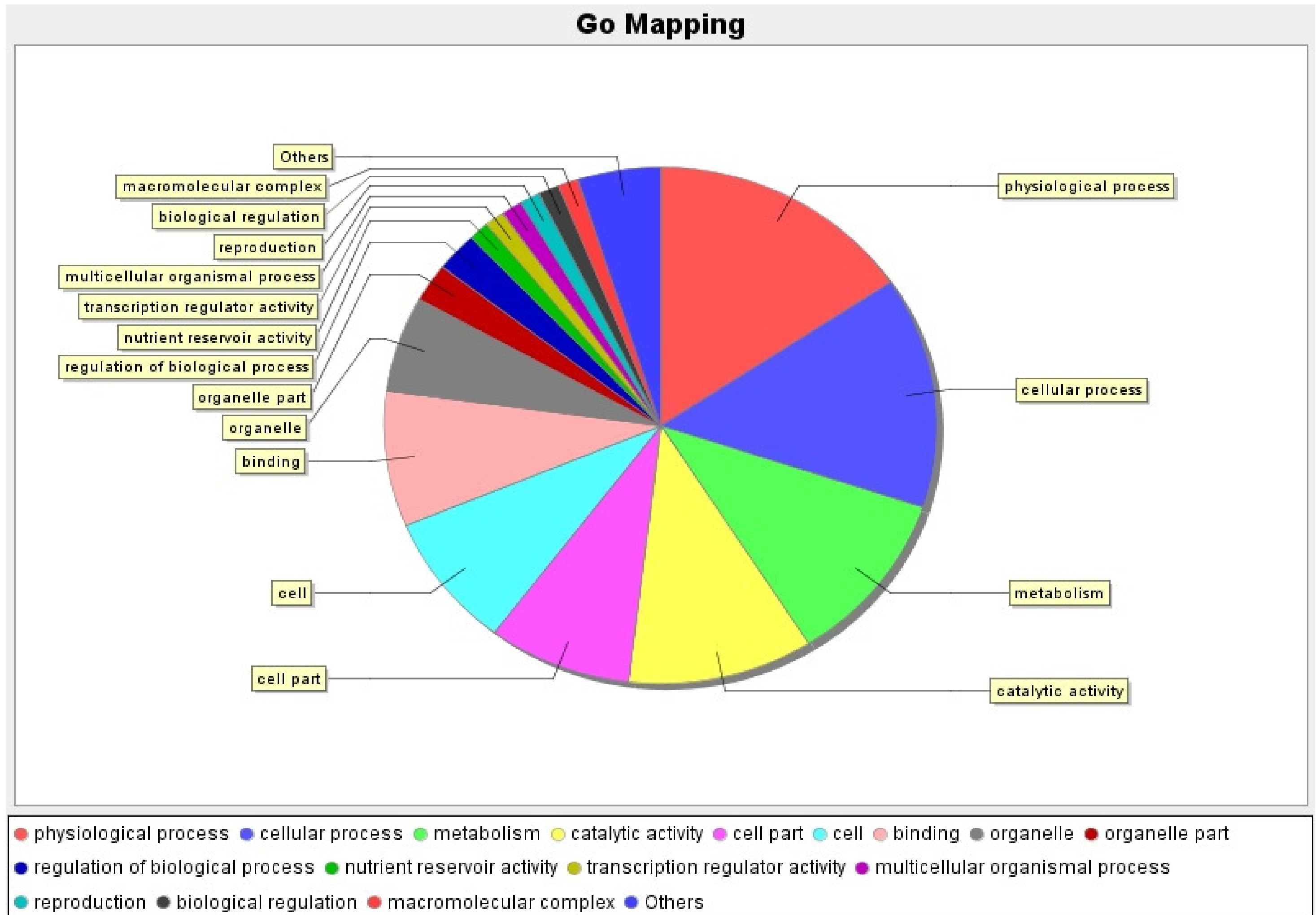

Figure 3. Broad functional classifications for genes and gene products representing their corresponding biological processes, molecular function and cellular localization.

just as 22 kD alpha zein5. It may be relative to maize development in the chronic low N condition.

R2R3MYB-domain protein gene of LOC541727

The regulation of gene expression is a fundamental process in all living organisms. Transcription factors are classified in structural families according to the presence of specific DNA-recognition motifs (Pabo and Sauer, 1992). The proteins containing the Myb-homologous DNA-binding domain are one of the transcription factors. Myb-homologous DNA-binding domains are widely found in transcription factors of eukaryotes. In plants, R2R3 Myb-domain proteins are involved in the control of form and metabolism. The Arabi- dopsis genome has more than 100 R2R3 Myb genes, but few have been found in monocottyledons, animals and fungi. In maize, more than 80 different R2R3 Myb genes were found (Pablo et al., 1999).

In this paper, only one gene of LOC541926 (AF099381) showed significant up-regulation under the severe chronic low N condition. The fold change was 6.2 (Table 3). It revealed that under different N supplying conditions, R2R3 Myb genes have different expression patterns. And in the severe chronic low N condition, LOC541926 gene's up-expression will regulate the other genes' expression to enable the maize growth.

Beta-D-glucosidase precursor gene (glu2)

In higher plants, the major functions of β-glucosidases are phytohormone activation (Brzobohaty et al., 1993) and cell wall catabolism (Leah et al., 1995). Most genes identified as putative β-glucosidase genes in *Arabidopsis thaliana* do not have a known function. Maize β-glucosidase is encoded by a locus called Glul that maps to chromosome

Table 3. Fold changes under different N conditions were presented: Low limiting N versus sufficient N.

GB	Fold	Gene	Gene ontology
BQ538226	13.3	ezma:24212	GO:0016538 cyclin-dependent protein kinase regulator activity; GO:0000074 regulation of progression through cell cycle.
CF045441	14	ezma:26671	GO:0003700 transcription factor activity; GO:0007275 development.
AF371277	8.1	LOC541926 (22kD alpha zein5).	GO:0045735 nutrient reservoir activity.
AW231741	11.1	ezma:17774	GO:0009269 response to desiccation; GO:0009737 response to abscisic acid stimulus; GO:0042538 hyperosmotic salinity response; GO:0006561 proline biosynthesis.
BM416729	7.6	ezma:18727	GO:0006333 chromatin assembly or disassembly; GO:0003677 DNA binding;GO:0001740 Barr body.
BM338441	7.2	ezma:7786	GO:0000398 nuclear mRNA splicing, via spliceosome; GO:0030532 small nuclear ribonucleoprotein complex.
AF099381	6.2	LOC541727 (ClonemMYBHX22 R2R3MYB-domain protein).	GO:0045449 regulation of transcription; GO:0005634 nucleus;GO:0003677 DNA binding.
CO528824	5.3	ezma:11088	GO:0015995 chlorophyll biosynthesis; GO:0016491 oxidoreductase activity; GO:0016630 protochlorophyllide reductase activity.
AY129953	7.7	nrt2.1	Putative high affinity nitrate transporter (nrt2.1).
U10076	6.6	LOC542305 (cyc3 cyclin3)	GO:0051301 cell division; GO:0000074 regulation of progression through cell cycle; GO:0005634 nucleus;GO:0007049 cell cycle.
AW927833	5.4	ezma:7106	GO:0005524 ATP binding;GO:0005622 intracellular; GO:0000103 sulfate assimilation; GO:0016779 nucleotidyltransferase activity; GO:0005739 mitochondrion; GO:0004781 sulfate adenylyltransferase (ATP) activity; GO:0009507 chloroplast;GO:0001501 skeletal development; GO:0016740 transferase activity.
CF636772	9.6	ezma:26682	GO:0001666 response to hypoxia;GO:0019825 oxygen binding.
U44087	9.6	glu2 (Beta-D-glucosidase precursor).	GO:0004553 hydrolase activity, hydrolyzing O-glycosyl compounds; GO:0005975 carbohydrate metabolism.
BM381172	5.5	ezma:4873	GO:0008270 zinc ion binding;GO:0016787 hydrolase activity.
BG319900	8.4	ezma:22901	GO:0015071 protein phosphatase type 2C activity.
CO526426	0.14	ezma:27463	GO:0016595 glutamate binding.
CB350631	0.07	LOC541918	Early light-inducible protein ELIP mRNA, complete cds; nuclear gene for chloroplast product.
BM382516	0.13	ezma:2447	GO:0016789 carboxylic ester hydrolase activity; GO:0016298 lipase activity; reproduction; GO:0008415 acyltransferase activity.

10 (Pryor, 1978) and shows extensive multiple allelism (polymorphism). Severe chronic low N condition led to marked changes in the expression of genes identified as putative β-glucosidase genes of β-D-glucosidase precursor gene (glu2) and not Glu1. The function of Glu2 has not been unidentified yet. It showed great induced of 9.6 when maize is grown under the severe chronic low N condition. Its expression may increase the beta-D-glucosidase activity and improve maize growth under N stress according the β-glucosidases' function of stress

metabolism.

Cyc3 cyclin3 gene (LOC542305)

The presence of maturation in advance is an indicator of stress, and N deficiency may lead to increased cyclins synthesis in those that are able to induce maturation of Xenopus oocytes and act as mitotic cyclins in this system (Renaudin et al., 1994). Maize obviously has maturation in advance under the severe N-limiting condition. Four genes involved in cyclins are able to induce maturation of Xenopus oocytes, but only Cyc3 cyclin3 involved in the regulation of progression through cell cycle was up-regulated just over 2-fold under severe chronic low N stress, the fold change was 6.6. Ezma:24212 which participates in the regulation of progression through cell cycle and has cyclin-dependent protein kinase regulator activity, was up-regulated under severe N stress, the fold change was 13.3. The expression of the ezma: 17774 was increased about 11.1-fold under severe N stress, and the ezma: 17774 gene has been shown to be able to response to abscisic acid stimulus, over-expression of the ezma: 17774 gene resulted in an enhanced accumulation of proline. It is clear that the accumulation of cyclins is closely correlated to the N stress, and Cyc3 cyclin3, Ezma: 24212 and ezma: 17774 gene is involved in the regulation of this process. However, how specific this regulation is for N stress response and the quantitive relationship among these genes is unclear.

Diaz reported that leaf yellowing is one of the typical responses plants have when N deficiency occurs (Diaz et al., 2006), while chlorophyll levels were significantly reduced (14.8% reduction) under the severe chronic low N condition. Many genes involved in chlorophyll metabolism including protochlorophyllide reductase, protochlorophyllide reductase and hydroxymethylbilane synthase genes were not changed in transcriptive levels. This is different from the results of Yong-mei (2007).

Here we showed that after the severe chronic low N treatment, the early light-induced proteins (ELIP) gene was down-regulated expression and the fold change was 0.07 (Table 3). The early light-induced proteins (Elips) in higher plants are related to light-harvesting chlorophyll (LHC) a/b-binding proteins (cab). The Elip family members in higher plants and green algae are nuclear-encoded proteins that accumulate in thylakoid membranes in response to various abiotic stresses (Montane et al., 1999). The primary function of the cab proteins is the absorption of light through chlorophyll excitation and transfer of absorbed energy to photochemical reaction centers (Heddad et al., 2006). The gene of ezma: 11088 involved in chlorophyll biosynthesis showed to have up-regulation when maize are grown in the severe chronic low N condition, the fold change was 5.3. Association of the significantly reduced chlorophyll levels with the down-regulated expression of the ELIP gene will decrease photoprotective anthocyanins levels. We proposed that the severe chronic low N treatment will reduce the maize photosynthesis and promote leaf yellowing process. But, the complex relationships between ELIP and ezma: 11088 are still unknown and need further research in future.

Conclusion

We used a maize whole genome array for a global evaluation of gene expression under the severe chronic low N condition. The differentially expressed genes identified provide additional insights into the coordination of the complex N responses of maize.

From the results, we propose that there is a special N response mechanism on the crop of maize, and most genes which are directly involved in N absorption and use are not found to change significantly. In contrast, a number of genes participate in physiological process, cellular process, metabolism, catalytic activity, cell part, binding, organelle part, organelle regulation of biological process, nutrient reservoir activity, transcription regulator activity, multicellular organismal process, biological regulation, macromolecular complex and others, they respectively play crucial roles in maize normal growth. Nrt2.1 plays an important role in maize N absorption under the severe chronic low N condition. A better understanding of the complex regulatory network for maize N responses will ultimately lead to strategies to improve efficiency of N use in maize.

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