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Comparative analysis of nitrogen metabolism-related genes in two inbred maize lines with different low-nitrogen tolerance

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Abstract

Maize is an important crop and its nitrogen use efficiency (NUE) has been an issue for plant scientists and breeders for many years. To meet the demand of genetic diversity in cultivating local elite maize cultivars, researchers need to isolate germplasms with a high NUE. In this study, 30 maize inbred lines were screened under hydroponic conditions, and three inbred lines that tolerate low nitrogen concentration were identified. A comparative analysis of genes involved in N uptake, transport, and assimilation between two lines with different tolerances revealed that the low-nitrogen-tolerant inbred line MBST exhibited higher expressions of nitrate and ammonium transporters, especially *ZmNRT3.1B*, than less tolerant B73. This suggests that the MBST line had a more efficient high-affinity transporter system. We also showed that low-nitrogen conditions down-regulated the expressions of master genes, *ZmNLPs*, which were negatively correlated with the expressions of the nitrate transporters *ZmNRTs.* This indicates the existence of regulators that antagonize the function of ZmNLPs. Some genes related to N assimilation and carbon metabolism were also more expressed in MBST. This research shows that the low-nitrogen-tolerant line, MBST, transported nitrate and ammonium more effectively than the B73 line. The NUE was also higher in MBST than in B73.

Additional key words: gene expression, high-affinity transporter, nitrogen use efficiency, *Zea mays.*

Introduction

Inorganic nitrogen fertilizers are often applied to the soil for crop yield improvement. However, research data has shown that only about half of the amount applied to the soil is absorbed by plants, whereas the remaining half is washed into rivers and lakes, resulting in water pollution and leading to great pressure on the environment. Hence, cultivating crop cultivars with high nitrogen use efficiency (NUE) can decrease the need for fertilizer usage and benefit both farmers and the environment (Guan 2017, Li *et al.* 2017).

Maize is the most important crop in world. The mechanisms of the uptake and utilization of nitrate and ammonium in maize have been widely studied. Due to the correlation between N fertilizer applied to the soil and the crop yield, scientists in China have divided high NUE maize cultivars into two major types (Shen and Wang 2016): those with high N absorption efficiency and high yield under conditions of low N input and those whose yield increases only with an increase in N fertilizer application. Undoubtedly, the latter type will result in an increase in the release of nitrogen to the environment, causing severe environmental pollution. Therefore, it

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Abbreviations: AMT - ammonium transporter; CK - control; CPK - Ca^{2+}-sensor protein kinases; CR - crown root; FENR - ferredoxin-NADP reductase; GARP domain - a single Myb-related DNA-binding domain, which was named using the initials of the GARP domain-containing proteins GOLDEN2, ARR-B, and Psr1; Gln - glutamine; Glu - glutamic acid; GPDH - glucose-6-phosphate dehydrogenase; GPI - glucose-6-phosphate isomerase; GPT - glucose-6-phosphate translocator; GS - glutamine synthetase; HATS - high-affinity transport system; HRS - hypersensitive to low Pi-elicited primary root shortening; LATS - low-afinity transport system; LBD - lateral organ boundary domain; LN - low nitrate; NIGT - nitrate inducible GARP-type transcriptional repressor; NIR - nitrite reductase; NLP - nodule inception-like protein; NPF - nitrate transporter/peptide transporter family; NR - nitrate reductase, NRE - nitrate-responsive *cis*-element; NRT - nitrate transporter; NUE - nitrogen utilization efficiency; PR - primary root; SNP - single nucleotide polymorphisms; SR - seminal root; TF - transcription factor.

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is very important to select cultivars that have high NUE under low nitrogen conditions. Some inbred lines with high NUE have been developed, including ZD958, Ye478, and Gaspe Flint (Wang *et al.* 2005, Garnett *et al.* 2013, Han *et al.* 2015). However, to meet the demand of genetic diversity in local elite maize cultivars, researchers need to isolate more germplasms with high NUE.

In recent decades, some of the genes involved in NO_3^- and NH_4^+ transport and assimilation have been identified. For example, there are 53 members of the nitrate transporter/peptide transporter family (NRTs/NPFs) in *Arabidopsis*, 93 in rice, and 97 in maize (Plett *et al.* 2010, Léran *et al.* 2014, Guan 2017, Li *et al.* 2017). So far, only a few of these have been well elucidated *in planta*. According to the affinity of NRTs/NPFs for nitrate, they have been divided into two subclasses, namely high-affinity transport systems (HATS) and low-affinity transport systems (LATS). Generally, members of the gene family of nitrate transporter 2 (NRT2s) are classified as HATS, functioning at low environmental nitrate concentrations, whereas NRT1 family members are classified as LATS, functioning at high nitrate concentrations (Garnett *et al.* 2013, Li *et al.* 2017, Guan 2017). Interestingly, some NRT1s, such as AtNRT1.1 in *Arabidopsis*, serve as dual-affinity transporters, involved in both HATS and LATS (Liu and Tsay 2003). An ortholog of AtNRT1.1 in maize, ZmNRT1.1B, is a pH-dependent monophasic high-affinity nitrate transporter (Wen *et al.* 2017). Many NRT2 family members require nitrate assimilation-related protein (NAR, a respective gene which belongs to the *NRT3* gene family), as a partner to transport nitrate (Laugier *et al*. 2012, Li *et al*. 2017, Kiba *et al*. 2018). When NO_3^- is transported in plants, it is further reduced to nitrite (NO_2^-) by nitrate reductase (NR) and then to NH_4^+ by nitrite reductase (NIR). Plants acquire environmental NH_4^+ through ammonium transporters (AMTs). Inorganic ammonium is converted into its organic form by two enzymes, glutamine synthetase (GS) and glutamate synthase (GOGAT) through the GS-GOGAT cycle (Li *et al.* 2017). Proteins, nucleic acid, and other N-containing compounds usually obtain elemental N through glutamic acid (Glu) and glutamine (Gln; Plett *et al.* 2010, Guan 2017, Li *et al.* 2017). In addition to NO_3^- and NH_4^+ transporters, urea transporters are also present in plants. For example, *ZmDUR3* is a high-affinity urea transporter that can functionally complement yeast *dur3* and *Arabidopsis Atdur3* mutants, which also respond to nitrate depletion, but not to urea, suggesting that it is regulated by the nitrate signaling pathway (Zanin *et al*. 2014, Liu *et al.* 2015).

In recent years, some regulators controlling the transcription of *NRTs* and *AMTs* have been functionally characterized in *Arabidopsis*. For example, it has been shown, that after long-term nitrogen starvation resupplying nitrate to the medium evokes high Ca^{2+} content in the cytoplasm, which triggers the activity of Ca^{2+}-sensor protein kinases (CPKs) to phosphorylate AtNLP. Phosphorylated AtNLP then enters the nucleus to induce the expression of downstream genes, such as *AtNRT*, *AtNR*, and *AtNIR* (Castaings *et al*. 2009, Konishi and Yanagisawa 2013, Yan *et al*. 2016, Liu *et al*. 2017). Very recently, *ZmNLPs* were identified in maize and their biological roles were verified in *Arabidopsis Atnlp7-4* mutants (Cao *et al*. 2017, Wang *et al*. 2018), but the target genes regulated by *ZmNLPs* are not yet known. Other transcriptional regulators are lateral organ boundary domain (LBD) and nitrate-inducible GARP-type transcriptional repressor 1 (AtNIGT1). The GARP proteins have roles as repressors to down-regulate N-responsive genes, such as *AtNRT2.1* and *AtNRT2.4*, under high nitrate concentrations (Riechann *et al*. 2000, Kiba *et al*. 2018, Maeda *et al*. 2018). Plant hormones also cooperate with N signals to harmonize plant growth (Medici and Krouk 2014, Guan 2017). Regrettably, there has been little research on these master genes in maize. Further research is required to determine whether different classes of plants (monocot *vs*. dicots) share common nitrogen signalling mechanisms.

Transcriptome data from maize has shown that perturbing nitrogen input influences the expression of many genes. Not only the genes of N transport and assimilation mentioned above, but also genes involved in carbon metabolism and stress defense, as well as long non-coding RNAs (Lv *et al.* 2016, Nazir *et al.* 2016). Although many genes have exhibited up- or down-regulated expression patterns, the results usually depend on the genotype of the maize being studied. To identify conserved genes that confer resistance to nitrogen stress, gene expression studies need to be performed more broadly, in different genotypes and especially in those with low nitrogen tolerance.

In the current study, we firstly screened low-nitrogen (LN)-tolerant inbred lines grown in hydroponic culture. We then utilized quantitative PCR to detect changes in the transcription of *ZmNRT/NPF*s, *ZmATMs*, *ZmNLP*, N assimilation-related genes, carbon metabolism-related genes, and some other genes in two cultivars (B73 and MBST) with different LN tolerance. Our aims were to determine: *1*) how these genes responded to LN, *2*) the relationship between *ZmNLPs and ZmNRT/NPF*s, and *3*) the association between gene expression and the tolerance of maize to LN. This research can not only provide LN-tolerant germplasm resources, but also increase our theoretical knowledge of LN tolerance in maize.

Materials and methods

Plants and treatments: Seeds of 30 inbred maize (*Zea mays* L.) lines (Table 1 Suppl.) were surface sterilized by 3 % (v/v) H_2O_2 for 10 min, washed five times with sterilized distilled water before sowing on moist filter paper. The seeds were incubated in darkness at 22 - 24 °C for 3 d. Then seedlings were transferred into planting baskets (55 mm diameter, 2 plants per each baskets) to grow for 7 - 8 days in climate chamber with a 14-h photoperiod, day/night temperatures of 22/20 °C, and an irradiance of about 300 μmol m^{-2} s^{-1}. Before treatment, the remaining tissue of seeds were removed to make sure that the nutrients were absorbed only through roots. Then, similar seedlings were separated into two groups for different treatments. Two different N concentrations were used: normal N supply (CK) containing 1 mM NH_4NO_3

+ 5 mM KNO_3 (6 mM nitrate + 1 mM ammonium) and low nitrogen supply (LN) containing 0.5 mM NH_4NO_3 + 0.5 mM NH_4Cl (0.5 mM nitrate + 1 mM ammonium). Other nutrient elements were supplied according to the Hogland medium (Hoagland and Snyder 1933). The experiment was repeated 3 times, at least 20 plants per inbred lines were treated each time, liquid medium was changed every 2 d. Ten days after treatment, plants were harvested for phenotype observation such as fresh mass, dry mass (drying at 80 °C to constant mass), root and shoot length. Roots of inbred lines B73 (LN sensitive) and MBST (LN tolerance) were also harvested at 0, 2, 4, 6, 8, and 12 h after LN treatment and stored at -80 °C until use.

Bioinformatic analysis of genes related to N transport, N metabolism, C metabolism and some others: According to previous reports (Plett *et al.* 2010, 2016, Garnett *et al.* 2013, Gu *et al.* 2013, Léran *et al.* 2014, Zanin *et al.* 2014), database in *MaideGDB* website (https://www.maizegdb.org/), phytozome website (https://phytozome.jgi.doe.gov/pz/portal.html) and PLAZA https://bioinformatics.psb.ugent.be/plaza/versions/plaza_v4_monocots/, CDS sequences of *ZmNRTs*, *ZmNPFs*, *ZmAMTs*, *ZmNLPs*, *ZmNR*, *ZmNIR* and carbon metabolism-related genes were searched. A 43 bp nitrate-responsive *cis*-elements (NREs) of *ZmNIR* (G*RMZM2G079381*) and *AtNIR* were used as references to identify potential NRE elements in the promoter (-3000 ± 1 upstream the translation initiate site of ATG) of *ZmNRT1s*, *ZmNRT2s* and *ZmNRT3s* (Liseron-Monfils *et al.* 2013, Konishi *et al.* 2014, Von Wittgenstein *et al.* 2014). Conserve sequences were found at http://meme-suite.org/tools/meme and http://plantpan2.itps.ncku.edu.tw/ (Grant *et al.* 2011, Bailey *et al.* 2015, Chow *et al.* 2016). Some other genes which showed response to LN in other maize cultivars reported by Nazir *et al.* (2016) were choose to detect the expression in B73 and MBST plants.

Isolation of DNA and RNA, cDNA synthesis and plasmid extraction: Genomic DNA of B73 and MBST was extracted using a plant DNA extraction kit (*Tiangen*, Beijing, China) according to the manufacture's manual. The total RNA of each sample was isolated using a *MiniBEST* universal RNA extraction kit (*TaKaRa*, Dalian, China) and digested with RNase-free rDNase I (*TaKaRa*) according to the manufacturer's protocol. The DNA and RNA concentration and quality were evaluated using *NanoDrop One* (*Thermo Fisher*, Shanghai, China) and 1 % (m/v) agarose gel. The cDNA was synthetized using *PrimeScript*™ RT reagent with gDNA eraser (*TaKaRa*, Dalian, China). Plasmid was isolated using *Plasmid Mini* preparation kit (*Beyotime*, Shanghai, China).

Quantitative reverse transcription PCR: After cDNA synthesis, quantitative PCR was performed in an *ABI 7500* real-time PCR system with *7500* software *v2.0.4*. A PCR mix (0.02 cm^3) was done by the 2× *TB Green Premix Ex Taq* II (*TaKaRa*, Dalian, China) user manual. Thermo-amplification was performed in a 96-well plate (*Applied Biosystems*, Carlsbad, USA) following our previous procedure (Yu *et al.* 2015). Using *actin* as an internal control, expression of a target gene was compared to an internal gene (Chen *et al.* 2018). Results were obtained from three biological repeats (mixed together) and three technical repeats. Gene specific primers are listed in Table 2 Suppl.

Cloning promoter of *ZmNRT3.1B* in B73 and MBST: According to sequences of B73, specific primers (Table 2 Suppl.) were designed to amplify -3338 to +1 regions of *ZmNRT3.1B (pZmNRT3.1B*) in B73 and MBST. Amplicons of PCR were cloned into the pGEM-T easy vector (*Promega*, Madison, WI, USA), positive clones were sequenced by *General Biosystems* (Chuzhou, China). The dNTP and *PrimerSTAR GXL* DNA polymerase were bought from *TaKaRa*, Dalian, China.

Measurement of total nitrogen, protein and sugar: Total content of nitrogen, proteins and sugars were determined. About 0.5 g dry material was digested with 18.4 M H_2SO_4, and the total nitrogen was measured by an automatic *Kjeldahl* apparatus (*UDK 159*, *VELP Scientifica*, Usmate, Italy). Total soluble protein was extracted according to the method described by Rocha and de Meis (1998). The concentration of protein was determined by using *Protein Assay* (*Bio-Rad*, Shanghai, China) at 595 nm (a *PerkinElmer*, *Enspire 2300 Multilabel* reader). One gramme of fresh maize tissue was homogenized in 10 cm^3 of distilled water, and total sugar was measured at 620 nm by anthrone colorimetry (Koehler 1952). The experiments were repeated at least three times.

Statistical analysis of biomasses of roots and shoots, gene expressions and correlation among *ZmNTRs* and *ZmNLPs* were carried out using *SSPS17*. Significant differences between samples were tested using the Student's *t*-test. A correlation heatmaps of *ZmNLPs* and *ZmNRTs* were drawn by the *R* language.

Results and discussion

We screened 30 inbred lines (Table 1 Suppl.) grown in hydroponic culture and found that B73, 2FACC, and many other inbred lines exhibited growth retardation, chlorosis of the base leaf tip, and light green coloration of the entire plant under LN conditions (Fig. 1 and Fig. 1 Suppl.). Further, length of shoot was decreased in B73 and fresh and dry masses in B73 and in 2FACC (Table 1). While root length was decreased MBST and N-PH-P, no other obvious phenotypic variation were found in MBST, 6M502, and N-PH-P under LN compared with plants growing under CK (Fig. 1 and Fig. 1 Suppl.). Hence, B73, 2FACC, and some other inbred lines are LN-sensitive lines, while, *e.g.*, MBST, 6M502, and N-PH-P are LN-tolerant lines. Previous studies also have shown that LN-tolerant cultivars maintain their biomass under LN conditions (Gaudin *et al.* 2011, Garnett *et al.* 2015). In the present study, the primary roots (PR) of MBST plants grown under LN conditions were shorter than in plants grown under CK

Table 1. Traits of five inbred lines under normal (CK) and low nitrogen (LN) conditions. Means ± SEs, $n \geq 60$, * - a significant difference between the same material under CK and LN conditions ($P < 0.05$, *t*-test). B73 and 2FACC are LN-sensitive inbred lines, MBST, 6M502, and N-PH-P are LN tolerant lines.

Inbred lines	Shoot length [cm]		Root length [cm]		Fresh mass [g plant^{-1}]		Dry mass [g plant^{-1}]	
	CK	LN	CK	LN	CK	LN	CK	LN
B73	41.50 ± 0.5	32.65 ± 4.65*	16.50 ± 0.60	17.95 ± 0.35	3.91 ± 0.01	2.64 ± 0.55*	0.31 ± 0.02	0.21 ± 0.06*
2FACC	40.88 ± 2.38	36.90 ± 1.5	27.80 ± 1.85	24.90 ± 3.55	4.87 ± 0.56	3.42 ± 0.29*	0.31 ± 0.03	0.24 ± 0.01*
MBST	37.34 ± 0.38	38.78 ± 2.44	28.99 ± 2.62	23.03 ± 1.93*	4.40 ± 0.21	4.05 ± 0.58	0.25 ± 0.01	0.25 ± 0.03
6M502	38.73 ± 2.51	35.13 ± 2.82	18.43 ± 2.16	18.53 ± 0.89	4.08 ± 0.81	3.53 ± 0.23	0.23 ± 0.05	0.24 ± 0.02
N-PH-P	34.42 ± 2.94	34.40 ± 2.48	24.68 ± 3.36	19.80 ± 2.72*	3.15 ± 0.03	2.84 ± 0.61	0.21 ± 0.01	0.21 ± 0.04

(Table 1, Fig. 1 Suppl.), the number and length of other types of roots (seminal roots and crown roots) increased. Under LN conditions, the primary roots of B73 were slightly longer than those grown under CK, but the density of laterals was unchanged and average number of SRs and CRs decreased by 10 - 15 %. Lynch (2013) proposed, that at different developmental stages root systems can adapt differently to the resources available in the soil. Early in seedling development, it is benefit to have a network of shallow roots to capture topsoil nutrients (Lynch 2013, Postma *et al.* 2014). When comparing the root systems of B73 and MBST plants, the network of shallow roots, such as CRs and SRs, was more extensive in of MBST than in B73 (Fig. 1 Suppl.). However, the deeper primary roots of B73 may beneficial for acquiring nutrients in deep soils. Based on these findings, we chose MBST and B73 to perform a comparative analysis of the transcriptions of N-related genes.

In the maize genome, 97 NRT/PTF members are divided into 8 clades according to their sequence similarity (Plett *et al.* 2010, 2016, Léran *et al.* 2014). In the present study, *ZmNRT* genes were named following the nomenclature used by Plett *et al.* (2010). *ZmNTR1s* (10 members), *ZmNTR2s* (4 members), and *ZmNTR3s* (2 members) are homologs of *AtNRT1s* (LATS), *AtNRT2s* (HATS), and *AtNRT3s* (AtNRT3 functions as a chaperon of AtNRT2; Plett *et al.* 2010, 2016, Léran *et al.* 2014). Other genes were named following the nomenclature used by Léran *et al.* (2014).

Of the ten *ZmNTR1s* investigated, the expression of two genes (*ZmNTR1.1C* and *ZmNTR1.4B*) was not detected in the roots of both lines, while the others were detected in at least one line (Fig. 2). Of the expressed *ZmNRT1s*, *ZmNRT1.1A* and *1.2* were highly expressed in both lines, while *ZmNRT1.1B* and *1.1D* were moderately expressed. These four members accounted for more than 90 % of the total *ZmNTR1* expression. Under LN conditions, the expressions of all 7 *ZmNTR1s* increased in the roots of B73

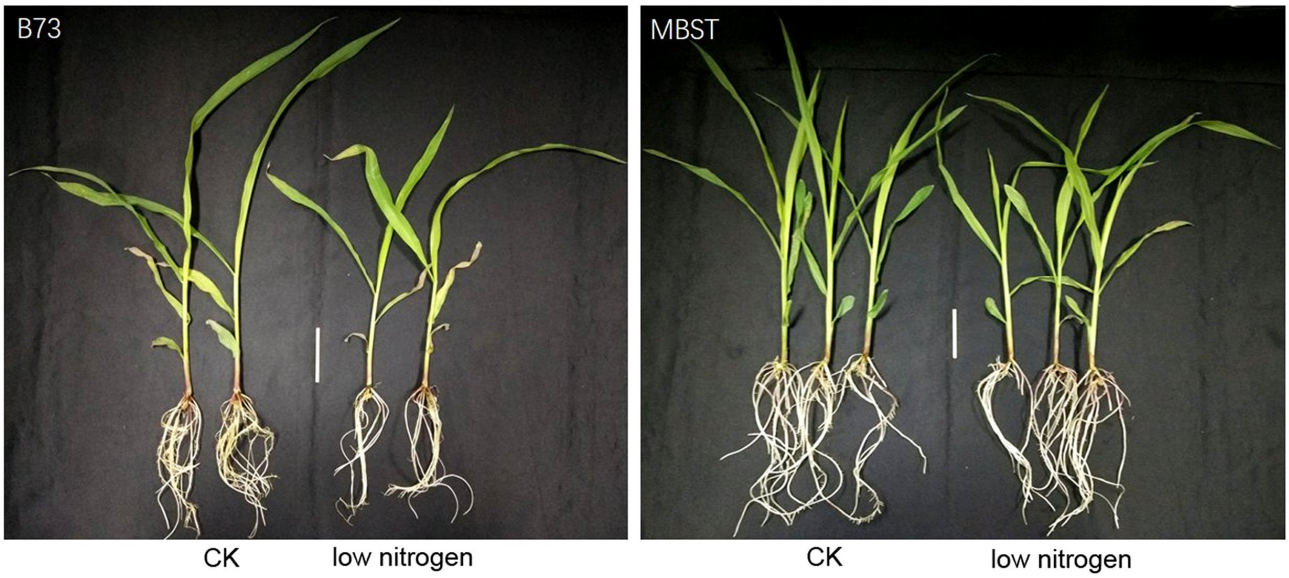

Fig. 1. The phenotype of B73 (sensitive) and MBST (tolerant) inbred lines under normal (CK) and low nitrogen (LN) conditions. CK- 6 mM nitrate + 1 mM ammonium; LN - 0.5 mM nitrate + 1 mM ammonium. Plants were harvested after 10 d of LN treatment applied to 9-d-old seedlings; the *bars* are 5 cm. Phenotypes of 6M502, N-PH-P, and 2FACC are shown in Fig. 1 Suppl. Data are not shown for other inbred lines.

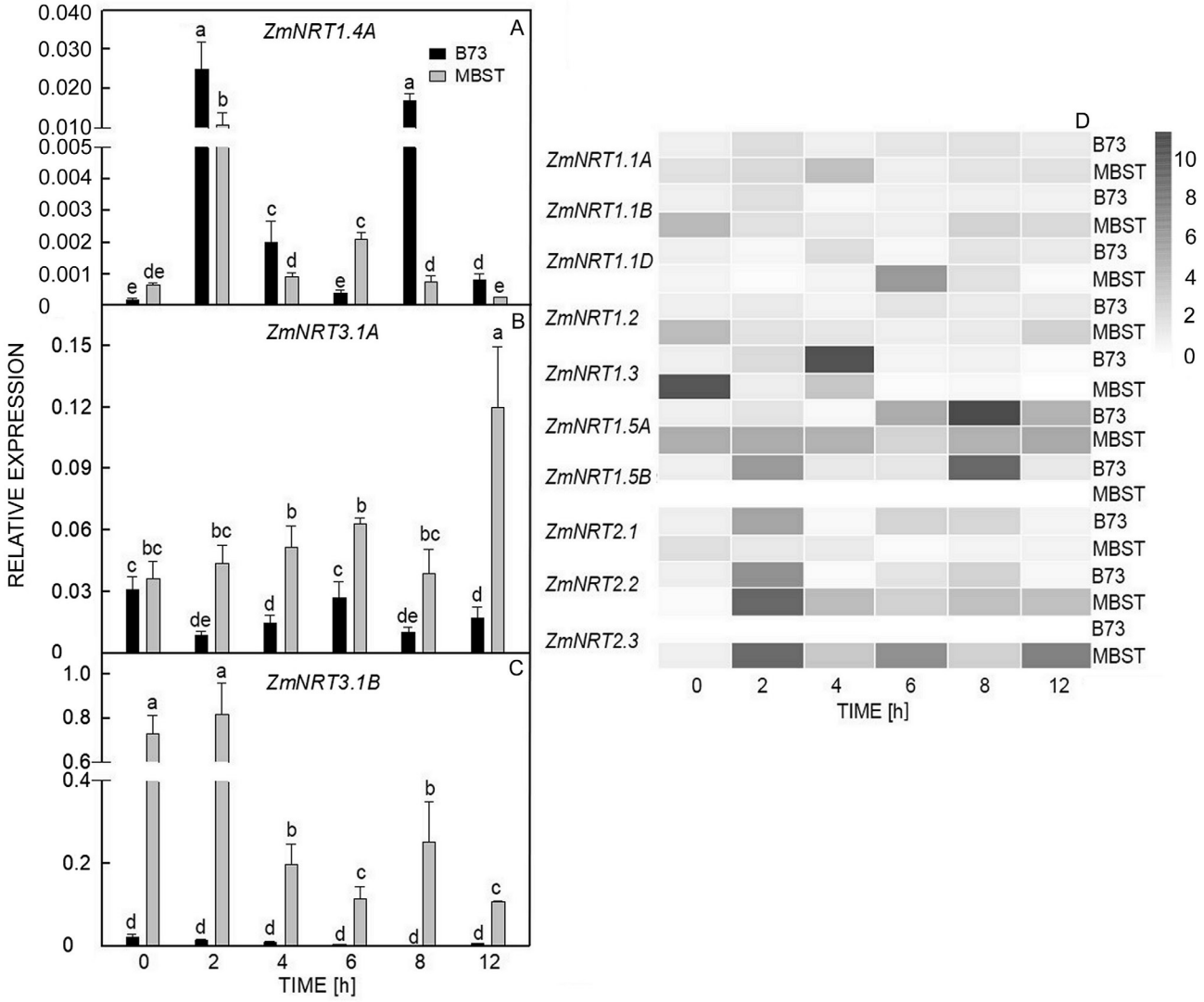

Fig. 2. Nitrate-dependent expression patterns of *ZmNRTs.* Maize plants were pre-cultured under 6 mM nitrate and 1 mM ammonium for 9 d before transfer to a 0.5 mM $NO_3^-$and 1 mM NH_4^+. Gene expressions were detected after low nitrogen treatments for 0, 2, 4, 6, 8, or 12 h. *A* - *ZmNRT1.4A* expressions in B73 and MBST at different time points; *B* - *ZmNRT3.1A* expressions; *C* - *ZmNRT3.1B* expressions. Gene expressions were normalized to *actin* expression (*GRMZM2G126190*). Means ± SDs, $n = 3$. Different letters indicate significant differences at $P < 0.05$ (the Student's *t*-test). *D* - The relative expression heatmap of *ZmNRTs.*

plants to a certain extent over a short period of time and then decreased. After 12 h of LN treatment, there were no significant differences compared with plants grown under CK (Fig. 2*D*). Although, the expressions of *ZmNRT1.1A*, *NRT1.1B*, and *NRT1.2* in MBST plants decreased under LN conditions, the overall levels were still higher than those in B73 plants (Fig. 2*D*). Similar differences in expression patterns of *ZmNRT1.4A* were detected between both lines (Fig. 2*A*). Our results were consistent with previous reports that the expression of most *ZmNRT1s* were unchanged or declined under long-term LN or nitrogen-deficient conditions (Wen *et al.* 2017). This may be because NRT1s are LATS, which are not the main transport proteins under LN conditions and therefore, it is expected that their expression will be reduced.

ZmNRT2.1 and *2.2* were the main *ZmNRT2* (HATS) members expressed in the two lines. Their expression increased rapidly under LN conditions. *ZmNRT2.2* was persistently induced in the MBST line, but its expression in B73 plants decreased after long-term treatment (Fig. 2*D*). Previous studies have shown that *ZmNRT2.1* expression is induced by low nitrate input in a different maize lines, but it is not correlated with LN tolerance (Santi *et al*. 2003, Sorgonà *et al*. 2011). However, the induced expression of *ZmNRT2.2* is positively correlated to genotypes with LN tolerance (Garnett *et al.* 2015). Our results were consistent with the above results, suggesting that *ZmNRT2.2* is indeed the main transporter under LN conditions.

It is interesting to note that *ZmNRT3.1A* and *ZmNRT3.1B* were moderately expressed genes (about one order of magnitude lower than the internal control

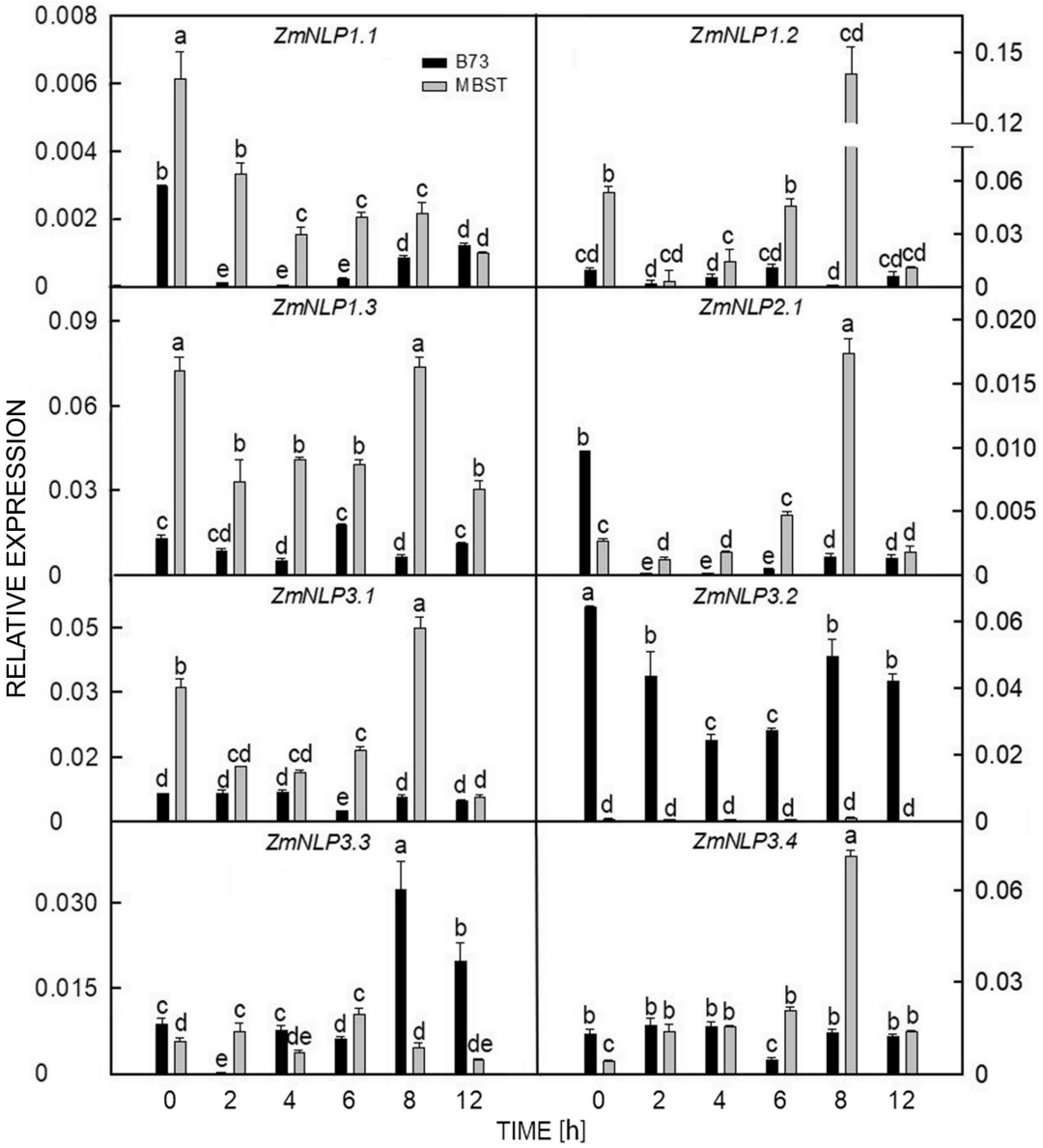

Fig. 3. Expression patterns of *ZmNLPs* under low nitrogen treatment for 0, 2, 4, 6, 8, or 12 h. Expressions of *ZmNLPs* are shown as *black* columns for B73 and as *gray* columns for MBST. All expressions were normalized to *actin* (*GRMZM2G126190)* expression. Seedlings were pre-cultured under 6 mM nitrate and 1 mM ammonium for 9 d before transfer to a 0.5 mM NO_3^- and 1 mM NH_4^+. Means ± SDs, $n = 3$. Different letters indicate significant differences at $P < 0.05$ (the Student's *t*-test).

actin) in B73 (Fig. 2*B*,*C*). These results are consistent with RNA-Seq data in the *Expression Atlas* (https://www.ebi.ac.uk/gxa/genes/) and the report by Garnett *et al*. (2015). However, *NRT3.1B* expression was significantly higher in the MBST line than in the B73 line (Fig. 2*C*). To determine the reason for this difference, we cloned the promoter (from -3338 to +1) of *ZmNRT3.1B* in MBST, and investigated differences in the promoter between the two lines. Five single nucleotide polymorphisms (SNPs) and two small insertions/deletions were found between -3000 and -2000 (Fig. 2 Suppl.). These may be the cause of the transcriptional differences between the two lines, since *cis*-elements in the promoter recruit proteins that regulate gene expression. Further experiments are required to determine which polymorphism in the promoter leads to the variation in expression. NRT3s serve as chaperones for NRT2s and in *Atnrt3* (*nar2.1*) mutant or knock-down lines of *Arabidopsis* and rice, *NRT2*s expression is also down-regulated (Okamoto *et al.* 2006, Yong *et al.* 2010, Yan *et al.* 2011). This may be why higher *ZmNRT3.1s* expression led to increased *ZmNRT2.2* and *2.3* expressions in MBST. Further experiments are needed to identify the mechanism whereby ZmNRT3.1s promote the expression of *ZmNRT2.2.* Considering that all detected NRT2·NRT3s serve as HATS, specifically in nitrate influx in other plants, we speculate that higher expressions of *ZmNRT2.2* and *ZmNRT3.1B* may increase the efficiency of NO_3^- uptake in the MBST line.

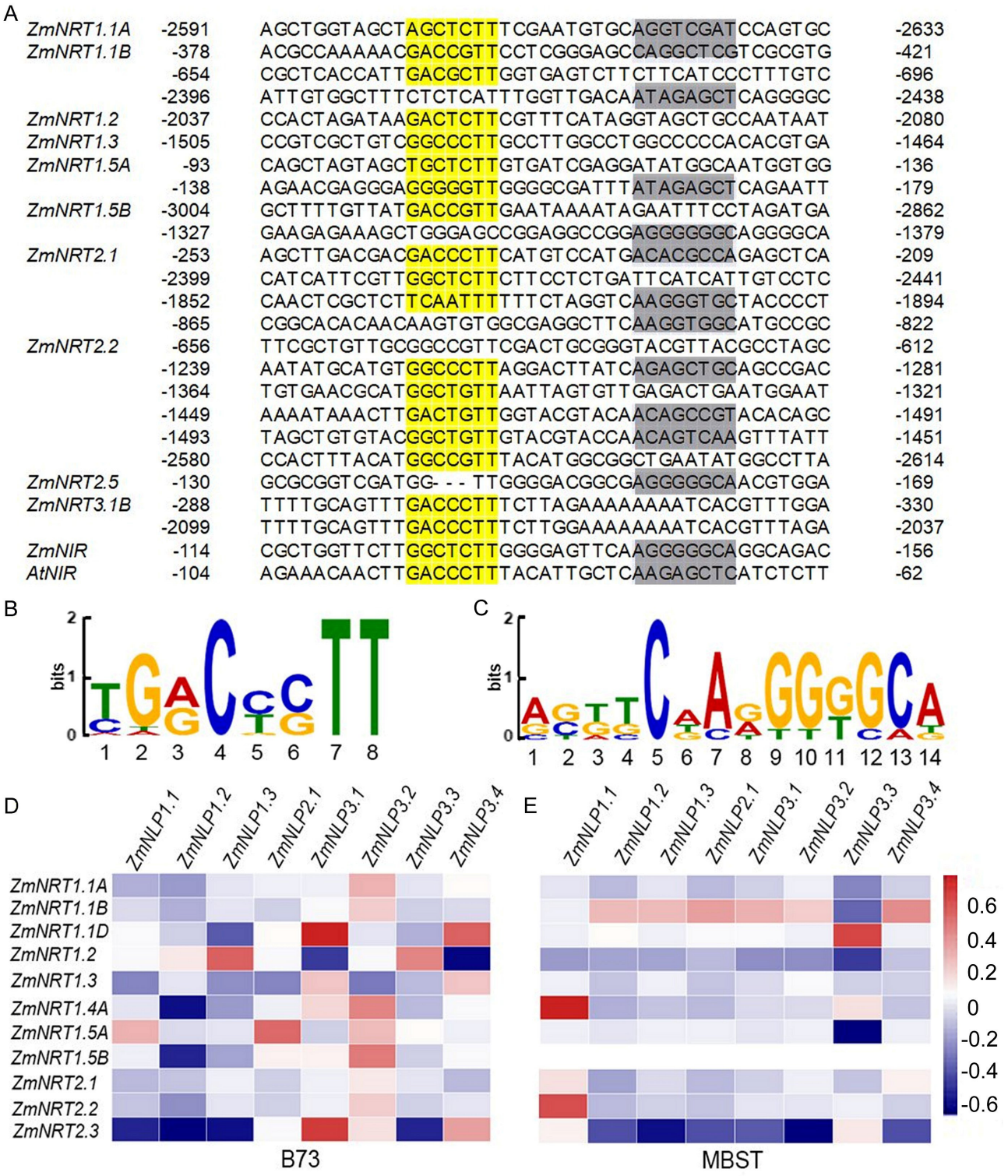

Fig. 4. The *ZmNLP* negatively correlates with *ZmNRTs*. *A* - nitrate-responsive *cis*-elements (NREs) in *ZmNRT* promoters. *Yellow* regions are NRE-A motifs and *gray* regions are NRE-B motifs. Numbers are upstream sites of ATG (+1). *B* and *C* - Logos of NRE-A and -B motifs (http://meme-suite.org/tools/meme). The 43-bp nodule inception-like protein binding sites in *Arabidopsis* (*AtNIR)* and maize (*ZmNIR*) were used as a reference (Liseron-Monfils *et al.* 2013). *D* and *E* - The heatmap of the relationship between *ZmNLPs* and *ZmNRT* mRNAs. Relative expressions of *ZmNLPs* and *ZmNTRs* were analyzed after 2, 4, 6, 8, or 12 h of low nitrogen treatment in inbred maize lines B73 and MBST. All expressions were normalized to *actin* (*GRMZM2G126190)* expression. Means ± SDs, $n = 3$. Positive correlations are shown in *red* and negative in *blue*.

In addition to the HATS and LATS members mentioned above, there are other *ZmNPF* members in maize that may carry out transmembrane transport of oligopeptides, auxins, and sugars (Plett *et al.* 2010, Guan 2017, Li *et al.* 2017). Previous studies have shown that ZmNPF8.8 (ZmPTR1) is an oligopeptide transporter, expressed in the scutellum of germinating seeds and in the mature zone of roots (Tnani *et al.* 2013). The functions of other *ZmNPFs* have not yet been identified. In the present study, the expression of 44 members of the *ZmNPF* family were detected (gene names as Léran *et al.* 2014). Of these, 14 were expressed in roots, but the remaining 30 were not expressed under our experimental conditions (Table 2 Suppl.). Among these 14 *ZmNPF* family members, *ZmNPF7.2, 8.8,* and *8.9* were highly expressed in the roots of B73 plants, whereas *ZmNPF5.4*, *7.2*, *7.4*, *8.8*, and *8.9* were the main members expressed in the roots of MBST plants (Fig. 3 Suppl.). The expression of *ZmNPF7.2* and *8.8* was down-regulated in the two inbred lines, suggesting that they responded to LN signals. From the expression patterns of the *ZmNPFs,* we can conclude that only a few of them may be involved in acquiring oligopeptides, auxins, and sugars in maize roots.

Studies in the model plant, *Arabidopsis*, have shown that AtNLPs are transcription activators when nitrate is resupplied after long term N deprivation (Castaings *et al*. 2009, Konishi and Yanagisawa 2013, 2014, Yan *et al*. 2016, Liu *et al*. 2017). Wang *et al.* (2018) also investigated the transcription profiles of *ZmNLPs* under nitrate re-supply after prolonged N starvation. They showed that *ZmNLP3.2* is the most abundantly expressed *ZmNLP* family member in different organs of maize, and that most of the *ZmNLPs* members are not regulated by the nitrate resupply. However, the variation in expression of *ZmNLPs* when maize is shifted from normal N to LN growth conditions is not known. Moreover, the relationship between *ZmNLPs* and *ZmNRTs* under these conditions is also unknown.

Under our experimental conditions, we found that the predominantly expressed gene members differed between the two inbred lines. In the B73 line, *ZmNLP3.2* was the most abundantly expressed *ZmNLP*. Its expression was approximately 7 % of the actin expression. However, in the MBST line, *ZmNLP1.2* and *1.3* were the two most abundantly expressed *ZmNLP* gene, with expressions similar to the expression of *ZmNLP3.2* in B73. *ZmNLPs* were found to have different LN response patterns in the two lines. In the B73 line, with the exception of *ZmNLP3.3*, ZmLNP expression decreased slightly at first and was then gradually restored during 12 h of treatment. However, in the MBST line, *ZmNLP* levels demonstrated a down-up-down pattern during 12 h of LN treatment. The accumulation of *ZmNLP* mRNA was less in both inbred lines under LN than in control plants. We conclude that sustained, long-term, LN conditions inhibit the expression of *ZmNLPs.*

AtNLPs induce downstream gene expression by binding to nitrate-responsive *cis*-elements (NREs). To uncover the relationship between *ZmNLPs* and *ZmNRTs*, we investigated NREs in the promoters (from -3000 to +1, where the A of the translation start site, ATG, is +1) of *ZmNRTs* (Fig. 4). Several putative NREs were identified in the promoter region of *ZmNRT2.1* and *ZmNRT2.2*. The left half motif of the NRE (named NRE-A in this study and highlighted in yellow in Fig. 4*A*,*B*) was more conservative than the right half (named NRE-B and shaded gray in Fig. 4*A*,*C*). Several NREs were identified in the promoters of *ZmNRT2.1* and *ZmNRT2.2*, but no NREs were found in the promoters of *ZmNRT.1.4A* and *ZmNRT2.3* (Fig. 4*A*).

Next, we investigated whether there is any correlation between the number of NRE motifs in the promoter and the expression of *ZmNRTs*. A correlation analysis of the expression of *ZmNLPs* and *ZmNRTs* showed a complicated relationship in the two inbred lines (Fig. 4*D*,*E*). In the B73 line, *ZmNLPs* showed a negative relationship with *ZmNRT2*s (Fig. 4*D*), while in the MBST line, *ZmNLP1.1* and *ZmNRT2.1, 2.2* were positively correlated, but the other *ZmNLPs* were negatively correlated with *ZmNRT2s* (Fig. 4*E*). Thus, it appears that *ZmNRT* expression was not correlated with the number of NREs, which suggests that only a few of the NREs are functional *in vivo*.

The negative relationship found between *ZmNRTs* and *ZmNLPs* in the present study challenges previous studies that have shown that *AtNLPs* are positive regulators of *AtNRT2s*. Our results indicated that there may be other factors overcoming the influence of NLPs. The recent work of two groups in *Arabidopsis* help us explain our results (Yan *et al*. 2016, Maeda *et al*. 2018). The GAPA-type transcription factors, AtNIGT1/HRS1s, are brakes for *AtNRT2.1* and *2.4* under high nitrate conditions. Importantly, the transcriptions of *AtNIGT1/HRS1s* are positively correlated with external nitrate concentrations and are also induced by *AtNLPs*. Hence, under limited nitrate availability, the content of AtNIGT1/HRS1s is low, whereas the downregulation of NLPs leads to a decrease in AtNIGT1/HRS1s. Therefore, it may be that that repressor effect of AtNIGT1/HRS1s outweighs the effect of NLPs under LN conditions. The results of the current research may also be explained by a model involving the antagonistic functions of NLPs and NIGT1/HRS1s. In other words, under LN conditions, the decreased content of ZmNIGT1/HRS1s may lead to the enhanced expression of *ZmNRTs*.

AMTs are coded by a small gene family in plants, with 6, 12, and 8 members in *Arabidopsis*, rice, and maize genomes, respectively (Plett *et al.* 2010, Kiba and Krapp 2016). Based on data in *MaizeGDB* database, *ZmAMT2A* has alternative splicing forms, which lead to asymmetric amplification and therefore, we only detected the remaining 7 members. Altogether, 6 of the 7 genes detected were expressed in the roots of the two inbred lines. In B73, most of the detected transcripts were *ZmAMT1C* and *2C,* whereas in MBST*, ZmAMT1C, 2C*, and *2D* were predominantly expressed. With the exception of *ZmAMT1A,* the transcriptions of the *ZmAMTs* were higher in MBST than in B73 (Fig. 5). The results also showed that *ZmAMT1B* and *1C* mRNA was up-regulated in MBST after 12 h of treatment, while mRNA of the others was down-regulated. However, in B73, *ZmAMT1A* and *ZmAMT2D* expressions increased, while expressions of the other *ZmAMTs* were unchanged or decreased

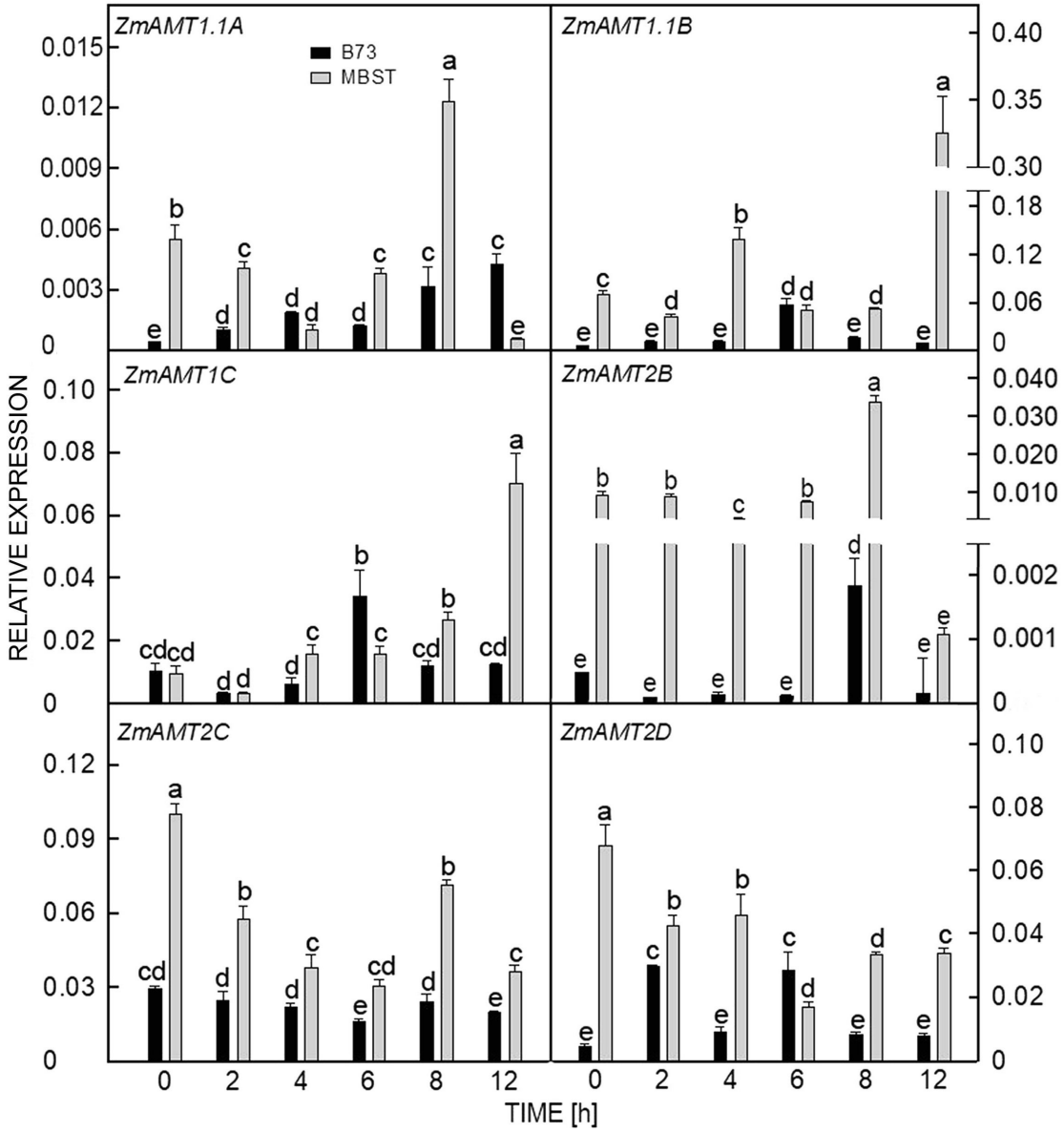

Fig. 5. Expressions of *ZmAMT*s under low nitrogen treatment for 0, 2, 4, 6, 8, or 12 h. Expressions of *ZmAMTs* are shown by *black* columns for B73 and *gray* columns for MBST. All expressions were normalized to actin (*GRMZM2G126190*) expression. Means ± SDs, $n = 3$. Different letters indicate significant differences at $P < 0.05$ (the Student's *t*-test).

(Fig. 5). Generally, our results showed that the expressions of *ZmAMTs* were relatively higher in the LN-tolerant line MBST, than in B73.

In the present study, only the nitrate content was changed in nutrient solution, but this nitrate could affect the abundance of *ZmAMTs*. The higher content of *ZmAMT* mRNA may lead to increased accumulation of ZmAMT protein, thus enhancing NH_4^+ transport activity. ZmAMT1.1a and ZmAMT1.3 are the major high-affinity ammonium transporters and their expression is persistently induced by ammonium (Gu *et al.* 2013). The results of this study suggested a higher efficiency of NH_4^+ capture in MBST than in B73, to partly compensate for limited nitrate availability and facilitate improved growth.

In addition to NO_3^- and NH_4^+ transporter genes, we also measured the relative abundance of nine N assimilation-related genes (Fig. 6). The results showed that four of these genes were expressed at moderate to high levels (Fig. 6*B*), while the others were expressed at low levels (Fig. 6*A*); seven of these nine genes were more highly expressed in MBST than in B73; and the content of *ZmNIR* and *ZmGS* (*GRMZM2G098290*) mRNA increased significantly ($P < 0.001$, *t*-test) in MBST, but decreased in B73. As mentioned above, NO_3^- is converted to NO_2^- by NR and then to NH_4^+ by NIR. NH_4^+ then serves as the substrate for GS and GOGAT enzymes, which catalyze Glu and Gln biosynthesis (Li *et al*. 2017). In MBST, the expression of these genes were higher than or at least similar to their

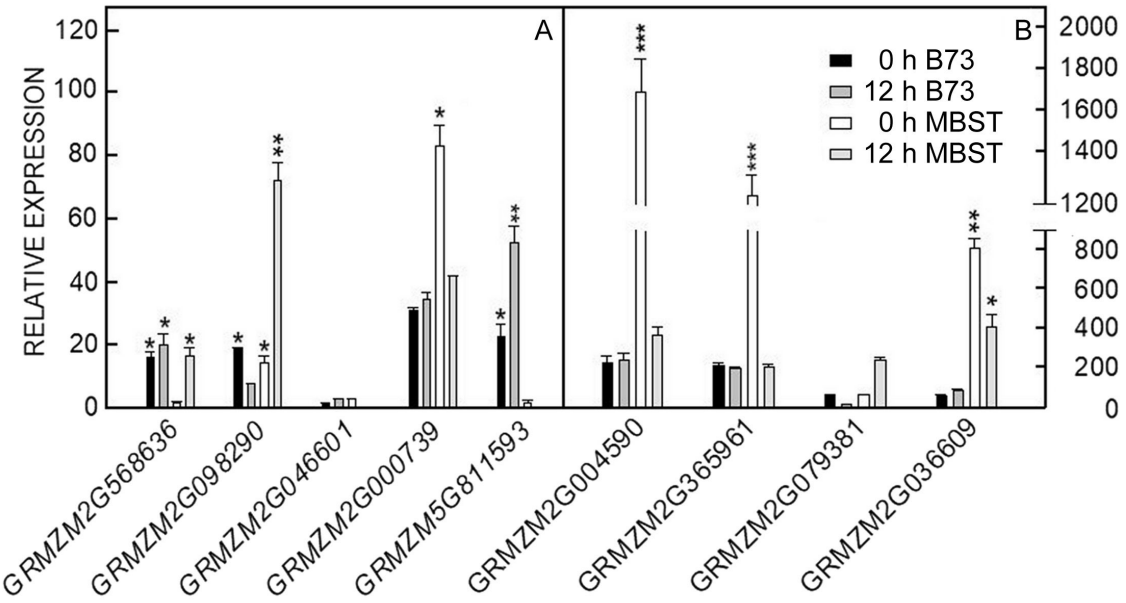

Fig. 6. Changes in expressions of N metabolism-related genes after 12 h of low nitrogen treatment. *A* - Genes with a low expression; *B* - genes with a high expression. *GRMZM2G568636 - nitrate reductase*, *GRMZM2G098290 - glutamine synthetase*,*GRMZM2G046601 - glutamine synthetase*, *GRMZM2G000739 - urophorphyrin methylase 1*, *GRMZM5G811593 - peptide transporter*, *GRMZM2G004590 shikimate kinase 1*); *GRMZM2G365961 - arogenate dehydrogenase isoform 2*, *GRMZM2G079381 - nitrite reductase*, and *GRMZM2G036609 - ferredoxin-dependent glutamate synthase*. All gene expressions were normalized to *actin* (*GRMZM2G126190*) expression. Means ± SDs, $n = 3$. Significant differences at * - $P < 0.05$, ** - $P < 0.01$, and *** - $P < 0.001$ (the Student's *t*-test).

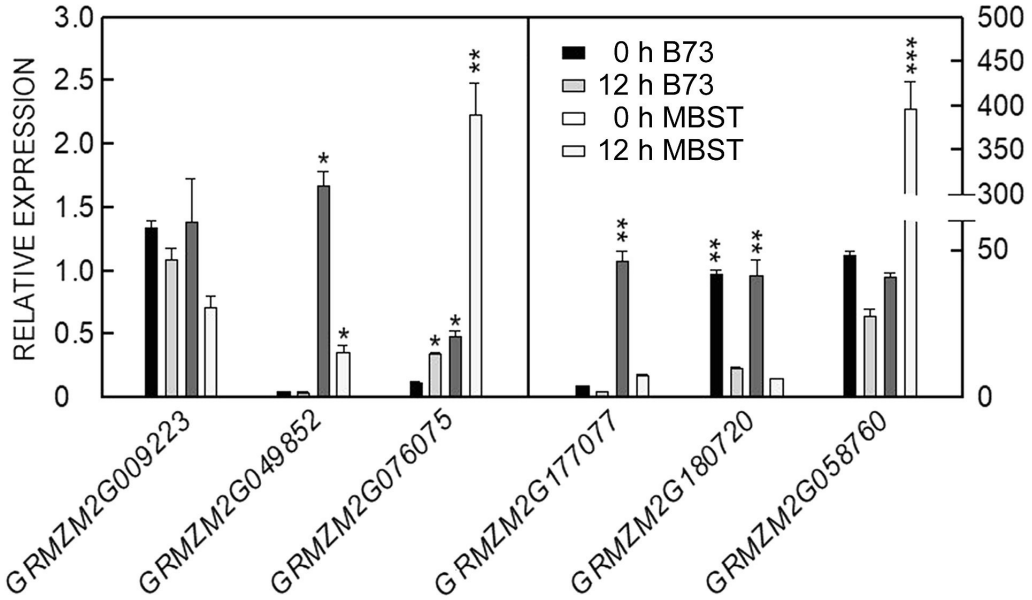

Fig. 7. Changes in the expressions of C metabolism-related genes and some other genes after 12 h of low nitrogen treatment. *A* - Genes with a low expreion; *B* - genes with a high expression. *GRMZM2G009223 - glucose-6-phosphate translocator 2*, *GRMZM2G049852 - protein detoxification 49*, *GRMZM2G076075 - glucose-6-phosphate isomerase 1*, *GRMZM2G177077 - glucose-6-phosphate-1-dehydrogenase*, *GRMZM2G180720 - glucose-6-phosphate translocator 2*, and *GRMZM2G058760 - ferredoxin-NADP reductase root isozyme 1*. All gene expressions were normalized to actin (*GRMZM2G126190*). Means ± SDs, $n = 3$. Significant differences at * - $P < 0.05$, ** - $P < 0.01$, and *** - $P < 0.001$ (the Student's *t*-test).

expression in B73 after 12 h of LN treatment, suggesting that MBST has a higher capacity than B73 to assimilate NO_3^- under the conditions of our experiment.

Two of the four detected carbon metabolism-related genes were highly expressed (Fig. 7*B*). The mRNA content of *ZmGPI* (*GRMZM2G076075*) and *ZmGPDH* (*GRMZM2G177077*) were significantly different between the two lines ($P < 0.05$). In both lines, *ZmGPTs* (*GRMZM2G009223*, *GRMZM2G180720*) had the same expression profile, being down-regulated by 12 h of

LN treatment. Our results showed that the expression of all four genes tested were influenced by N concentration in nutrient solution.

The expression of other gene, such as *GRMZM2G058760* (*ZmFENR*) was significantly up-regulated after LN treatment ($P < 0.001$, Fig. 7*B*) in the MBST line. FENR plays a key role in regulating the relative amounts of cyclic and non-cyclic electron flow to meet the demands of the plant for ATP and reducing power (NADPH, Küchler *et al.* 2002). A higher ratio of NADPH/$NADP^+$ means that the cell has ample energy for growth, which is especially important in rapid growth stages, such as in the maize seedling. The expression of *GRMZM2G049852* increased dramatically in the MBST line after LN treatment. As a defense-related gene, it may confer tolerance of MBST to LN (Nazir *et al.* 2016).

Although, limited genes related to carbon or energy metabolism were detected in this study, it is well known that there is an interaction between nitrogen and carbon metabolism (Nunes-Nesi *et al.* 2010, Lynch 2013). Nazir *et al*. (2016) reported that most proteins fluctuate with nitrogen supply, which also affected carbon metabolism. In the present study, the content of total nitrogen and soluble proteins in B73 was lower than in MBST (Fig. 4 Supppl.), while content of soluble sugars was higher in B73 (Fig. 4 Suppl.). Saccharides are resources for protein, nucleic acid, and lipid biosynthesis and have complicated effects on plant development. Larger amounts of soluble sugars accumulated in B73 may reflect reduction of their use for biosynthesis of others compounds.

Recently, Li *et al*. (2018) reported that in high NUE rice cultivars or transgenic lines, the expressions of genes such as *NRT1.1B, NRT2.3A, AMT1.1*, *NR*, *NIR*, and *GS* are very high. Our results also showed that genes homologous to these in maize had rather high expressions in the LN-resistant line MBST (Fig. 2, 5, and 6). From our results and those of previous reports (Guan 2017, Li *et al.* 2017, 2018), we conclude that plants may share common regulatory mechanisms for nitrogen utilization. The functions of some master genes, such as *NLP* and *NIGT1/HRS1*, have not been fully elucidated in maize (Li *et al*. 2018, Kiba *et al.* 2018, Wang *et al.* 2018) and therefore, further research is needed to determine how these genes regulate nitrogen utilization and growth in maize.

Conclusions

In this study, we identified three LN-tolerant, inbred maize lines at the seedling stage. Based on the transcriptions of genes involved in NO_3^- and NH_4^+ transport and assimilation and carbon metabolism, we conclude that the LN-tolerant line MBST have an increased capacity to transport external NO_3^- and NH_4^+ into cells and to assimilate N. Under LN conditions, the negative correlation between *ZmNLPs* and *ZmNRTs* suggests there are other regulators antagonizing the ZmNLPs. Further research is necessary to uncover the relationship between ZmNRT2s and ZmNRT3s and to identify the mechanisms by which they regulate N signals under LN conditions.

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