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Full Length Article

Identification of MicroRNAs Associated with Nitrogen Use Efficiency and Fertility in Rice

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Abstract

A study was conducted to identify target genes through differential expression of microRNAs (miRNAs) under low nitrogen (low-N) level (0.01 mM NH_4NO_3) for tolerant and sensitive rice samples, which may provide valuable information for breeding and improving production under nitrogen stress condition. MiRNA microarray data GSE38213 were downloaded from Gene Expression Ominibus database, including 2 low-N tolerant rice samples and 2 low-N sensitive rice samples. After the data were preprocessed and normalized, limma package in R was applied to identify the differentially expressed miRNAs. Then the target genes of the most significant up-regulated miRNAs were collected by using Plant miRNA Database. In addition, the high-reliable signal nucleotide polymorphisms (SNPs) of target genes were screened out by Rice Genome Browser in TIFR (The Institute for Genomic Research). Functional analysis was performed to the genes with SNPs. As the results, total 22 differentially expressed miRNAs were identified and the most significant ones were osa-miR530-3p, and osa-miR319a. Additionally, high-reliable SNPs were only discovered in 4 target genes of osa-miR530-3p. Functional analysis revealed that the 4 target genes of osa-miR530-3p were associated with plant growth, development and respiration. In conclusion, differentially expressed osa-miR530-3p and its target genes that had high-reliable SNPs were identified in present study. Besides, miR167 and miR319 were found to be associated with fertility. These miRNAs identified in the present study are potential targets for developing high yield low-N tolerant rice.

Keywords: Differentially expressed microRNAs; Low-N tolerant rice; Microarray data; Functional analysis.

Introduction

Nitrogen (N) is one of the major elements for crops. It has been reported that the nitrogen supply has a great impact on the rice yield during vegetative stage under field conditions (Senaratne *et al.*, 1995). The food demand is growing with rising global population in recent years. Correspondingly, the worldwide amount of nitrogen fertilizer used on field has increased compared with the last decades (Peccia *et al.*, 2013). People have noticed that the low efficiency in absorption and utilization of N will bring severe environmental problems, including leaching of nitrate and emission of nitrous oxide and ammonia (Koutroubas *et al.*, 2003).

Since rice is a main crop in China, its nitrogen-use efficiency becomes the focus of attention (Zhu and Chen, 2002). In Past, people were focused to develop new varieties of rice to improve the nitrogen fertilizer use efficiency and reduce the amount of nitrogen fertilizer (Tirol-Padre *et al.*, 1996). The molecular plant breeding integrating biotechnology, genomic research, molecular marker applications and conventional plant breeding practices hold great promise for future crop improvement (Moose and Mumn, 2008). It was noticed that increased nitrogen use efficiency in rice occurred by the tissue-specific expression of alanine aminotransferase (Shrawat *et al.*, 2008). An early nodulin gene OsENOD93-1 has been involved in improving the nitrogen-use efficiency in transgenic rice, OsENOD93-1 can increase shoot dry biomass and seed yield of transgenic rice plants (Bi *et al.*, 2009). However, these studies were limited and require further investigation on various aspects of the crop nitrogen use efficiency. In recent years, some researchers paid attention to microRNA (miRNA) to reveal the underlying mechanism of crop growth.

By fully or partially complementary binding with the 3'-UTR of target mRNA, miRNA can cause mRNA degradation or translational inhibition and regulate the expression of target genes (Jones-Rhoades, 2004; Lai, 2002). Previous studies have reported that miRNAs take part in a series of important biological processes, such as cell proliferation, apoptosis and differentiation (Bartel and Bartel, 2003). In addition, miRNAs have been applied to improve plant nutritional value and metabolic engineering (Tang *et al.*, 2007). MiRNA plays an important role in the plant life process. Therefore, it will be worthwhile to

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explore molecular basis of nitrogen use efficiency in rice germplasm. The obtained information will provide valuable information for genetic modifications of crops for nitrogen-use efficiency.

In the present study, miRNA expression profiles of low-N tolerant rice and low-N sensitive rice were compared to screen differentially expressed miRNAs. Then the target genes of the most significant up-regulated miRNAs were identified and the target genes with high-reliable SNPs were further investigated for functional analysis. Our findings might provide direction in future molecular plant breeding.

Materials and Methods

MiRNA Microarray Data

MiRNA microarray data GSE38213 (Nischal *et al.*, 2012) were downloaded from Gene Expression Omnibus database and 4 rice germplasm lines were available, including 2 low-N tolerant and 2 low-N sensitive germplasm lines. The germplasm lines were grown in the nutrient medium and the nitrogen in the form of NH_4NO_3 was maintained as 0.01 mM (low-N condition). The pH of the nutrient solutions was adjusted to 6.0 and the solutions were changed every three days. Twenty-day-old plants were sampled for expression studies of miRNA. Data was collected based on GPL1461322 [miRNA-2_0] Affymetrix Multi-species miRNA-2_0 Array platform. Chip annotation information was also downloaded with raw data.

Data Preprocessing and Differential miRNA Expression Analysis

Affymetrix proble-level data in CEL files were converted into gene expression profiling and were normalized using the affy package (Troyanskaya *et al.*, 2001, Fujita *et al.*, 2006) in the R statistical language (Kembel *et al.*, 2010) Then, limma package (Smyth, 2005) in R was used to identify the differentially expressed miRNA between the 2 low-N tolerant rice samples and 2 low-N sensitive samples. P values < 0.05 and |logFC (fold change)| > 1 were set as the cut-offs to screen out differentially expressed miRNA and the most significant up and down-regulated miRNA were selected out for further analysis.

Retrieval of Target Genes

Target genes of the most significantly up- and down-regulated miRNAs were retrieved in Plant miRNA Database (PMRD) (Zhang *et al.*, 2010). The PMRD contains sequence information, secondary structure, target genes, expression profiles and a genome browser.

SNPs of Target Genes

Single nucleotide polymorphisms (SNPs) are widespread in the plant genome. The SNP-based molecular markers are applied in gene mapping, map-based cloning and molecular marker-assisted breeding (Talukdar and Zheng, 2007). In the present study, high confident SNPs were identified in TIFR (the institute for genomic research) database with Rice Genome Browser (Chan *et al.*, 2007) for each target gene.

Table 1: Total 15 and 7 differentially expressed miRNAs in low-N tolerant samples and low-N sensitive rice samples

ID	P value	logFC
osa-miR530-3p	0.04236	-3.99733
osa-miR821b	0.00716	-2.69782
osa-miR821a	0.0082	-2.38092
osa-miR821c	0.01252	-1.8745
osa-miR169a	0.02966	-1.75648
osa-miR1318	0.01891	-1.68621
osa-miR156k	0.03594	-1.62925
osa-miR167b	0.03791	-1.61574
osa-miR1432	0.02523	-1.59814
osa-miR160f	0.04897	-1.58309
osa-miR167h	0.03051	-1.54386
osa-miR167j	0.03325	-1.48952
osa-miR167d	0.03887	-1.37778
osa-miR167i	0.04921	-1.31223
osa-miR167e	0.04821	-1.29496
osa-miR395h	0.04167	1.441964
osa-miR1850.1	0.04319	1.46727
osa-miR1423b	0.03881	1.528063
osa-miR815c	0.04764	1.559218
osa-miR529b	0.02776	1.563049
osa-miR815b	0.02716	1.61189
osa-miR319a	0.01887	1.686422

Functional Analysis for Genes with SNPs

Functional analysis was conducted to genes with SNPs to illustrate the alterations in response to low-N stress and the regulatory mechanisms of N utilization.

Results

Differentially Expressed MiRNAs

Raw data were pre-processed and normalized before differential expression analysis. A total of 22 miRNAs were identified as differentially expressed miRNAs, including 15 up-regulated miRNAs in low-N sensitive samples and 7 up-regulated miRNAs low-N tolerant samples. In addition, osa-miR530-3p and osa-miR319a which were the most significantly up-regulated in low-N sensitive and tolerant samples respectively were identified (Table 1).

Target Genes of Differentially Expressed miRNAs

The target genes of osa-miR530-3p and osa-miR319a were collected from PMRD. Finally, total 13 and 5 target genes were obtained for osa-miR530-3p and osa-miR319a, respectively (Table 2).

Table 2: Target genes of osa-miR530-3p and osa-MIR319a

osa-miR530-3p		
Target	Alignment	
LOC_Os02g48310.1	miRNA:CAACGUAGACGGAGACGUGGA	target: GUUGCAUCUGAAUCUGCAUUU
LOC_Os02g52390.1	miRNA: CAACGUAGACGGAGACGUGGA	target: GUUGCAGCUGCUGCUGCGCCU
LOC_Os04g02280.1	miRNA:CAACGUAGACGGAGACGUGGA	target: GUUGCAUCUGCUACUGCAUCC
LOC_Os05g09650.1	miRNA:CAACGUAGACGGAGACGUGGA	target: GUUGCAUUUGGUUCUGCAUUU
LOC_Os08g02180.1	miRNA:AACGUAGACGGAGACGUGGA	target: UUGCUUCCGCUUCUGCAUCU
LOC_Os08g34140.1	miRNA:CAACGUAGACGGAGACGUGGA	target: GUUGCAUCUGUUUGUGCAUUU
LOC_Os10g01984.1	miRNA:CAACGUAGACGGAGACGUGGA	target: GAUGCAUCUGCUCCUGCACUU
LOC_Os10g08970.1	miRNA:CAACGUAGACGGAGACGUGGA	target: GUUGCAUCUGAAUCUGCAUUU
LOC_Os10g34860.1	miRNA:CAACGUAGACGGAGACGUGGA	target: GUUGCAUCUGAAUCUGCAUUU
LOC_Os11g16460.1	miRNA:AACGUAGACGGAGACGUGGA	target: CUGCAUCUGCAUCUGCAUCU
LOC_Os11g47660.1	miRNA: CAACGUAGACGGAGACGUGGA	target: GUUGCUUUUGCCUCUGCAUCA
LOC_Os12g06210.1	miRNA: CAACGUAGACGGAGACGUGGA	target: GCUGCAGCUGCCUCUGCGCCU
LOC_Os12g32200.1	miRNA: AACGUAGACGGAGACGUGGA	target: CUGCAUCUGCCUAUGUACUU
osa-MIR319a		
Target	Alignment	
OC_Os03g57190.1	miRNA: CCCUCGUGGGAAGUCAGGUU	target: AGGGGGACCCUUCAGUCCAA
LOC_Os06g41500.1	miRNA: CCUCGU-GGGAAGUCAGGUU	target: GGAGCAAUCCUUCGGUCCAA
LOC_Os07g05720.1	miRNA: CCCUCGUGGGAAGUCAGGUU	target: AGGGGGACCCUUCAGUCCAA
LOC_Os08g16660.1	miRNA: CCCUCGUGGGAAGUCAGGUU	target: AGGAGUACCUUUCAGUUCAG
LOC_Os12g42190.1	miRNA: CCCUCGUGGGAAGUCAGGUU	target: CGGGGCACACUUCAGUCCAA

SNPs of Target Genes

SNPs of these target genes were searched in TIGR database with Rice Genome Browser. High-reliable SNPs were only acquired for the 4 target genes of osa-miR530-3p, including LOC_Os04g02280.1, LOC_Os05g09650.1, LOC_Os08g34140.1 and LOC_Os11g16460.1 (Fig. 1).

Functional Analysis for Genes with SNPs

These 4 genes were discovered encoded F-box domain containing protein, coenzyme Q biosynthesis protein, soluble N-ethylmaleimide-sensitive factor activating protein receptor (SNARE) and oxido-reductase, respectively. Gene functional analysis revealed that these 4 genes were involved in growth, development and respiration, which might contribute to the sensitivity of rice to low-N condition.

Discussion

In the present study, a number of differentially expressed miRNAs between low-N tolerant rice and low-N sensitive rice were screened out and then the target genes were analyzed, which may explain the diversity in low-N tolerance.

Previous study has found the involvement of miR169 in stress response (Sunkar, 2010). It was further reported that miR169 is involved in nitrogen-starvation responses in Arabidopsis (Zhao *et al.*, 2011). GmNFYA3 which is a target gene of miR169, has been reported to be a positive regulator of plant tolerance to drought stress (Abbas *et al.*, 2013). MiR156k is responsive to salt and drought in rice and regulates flowering time and shoot development (Kruszka *et al.*, 2012). One of the previous research has reported that miR-156-targeted SPL genes and CYP 78A5/KULH on plastochron length and organ size in *Arabidopsis thaliana* (Wang *et al.*, 2008). Xie *et al.* (2012) report that increase of miR156 regulates temporal expression changes of numerous genes during leaf development in rice. Ru *et al.* (2006) find enhanced expression of microRNA167 inhibits reproductive development through degradation of auxin response factor 8 mRNA and repressing auxin response factor 6 translation. ARF6 and ARF8 have been reported in regulating gynoecium and stamen development in immature flowers and their transcripts are cleaved by miR167 (Zhang *et al.*, 2008). These miRNAs were up-regulated in low-N sensitive rice compared with low-N tolerant rice according to our analysis.

MiR529 is involved in the development of rice grains (Zhu *et al.*, 2008) and reproductive system (Schwab, 2012, Jeong *et al.*, 2011). In addition, the miR529 targets have been discovered more constraint by strong purifying selection and evolved conservatively with a slow rate; meanwhile, its target genes most belong to SBP (squamosa promoter binding protein-like) family (Ling and Zhang, 2012). MiR395 mediates signal interactions between auxin and nutrition or stress in rice roots (Meng *et al.*, 2010). MiR395 targets three out of four isoforms of ATP sulfurylase, the first enzyme of sulfate assimilation, as well as a low-affinity sulfate transporter, SULTR2,1, is strongly induced by sulfate deficiency (Kawashima *et al.*, 2011). Schommer *et al.* (2008) report that miR319 regulates jasmonate biosynthesis and thus leaf growth and senescence. The previous study has confirmed that miR319 regulates transcription factors of the TCP family; meanwhile, the balance between miR319 and its targets controls leaf morphogenesis and several other plant

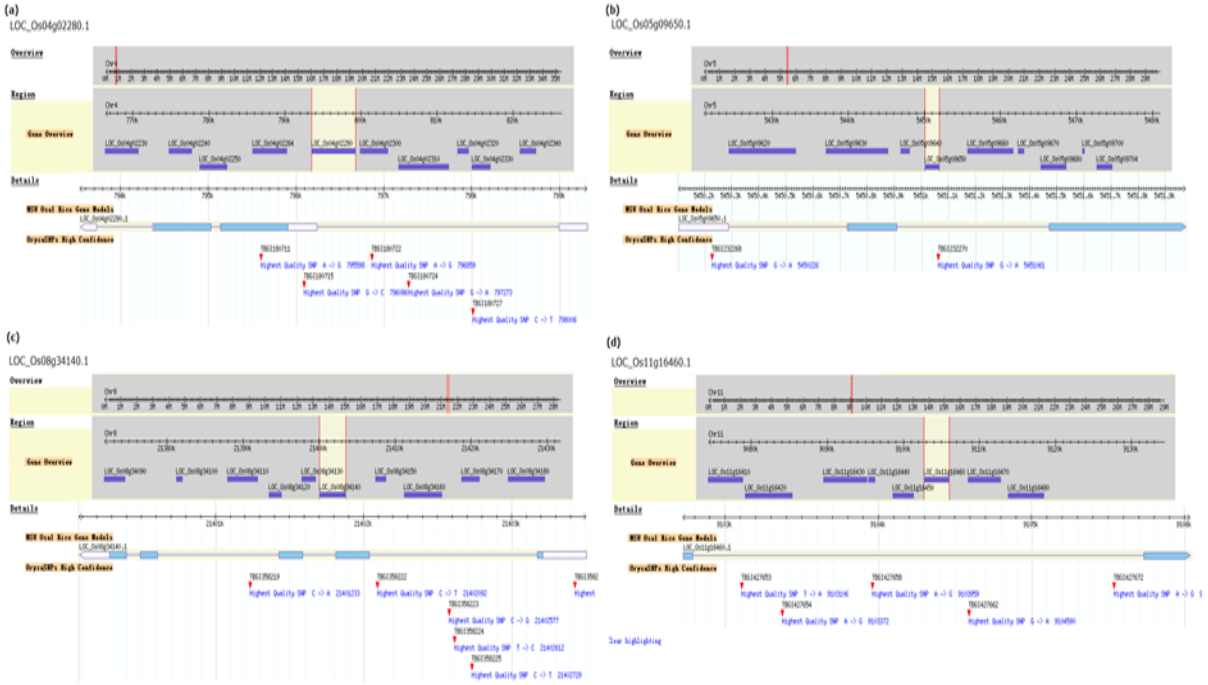

Fig. 1: The chromosomal locations and polymorphism sites of 4 target SNPs of osa-miR530-3p (a) LOC_Os04g02280.1 (F-box domain containing protein); (b) LOC_Os05g09650.1 (ubiquinone biosynthesis protein COQ4); (c) LOC_Os08g34140.1 (vesicle transport v-SNARE protein); (d) LOC_Os11g16460.1 (oxidoreductase)

developmental processes (Schommer *et al.*, 2012). These miRNAs were up-regulated in low-N tolerant rice.

Moreover, the up-regulation of miR167 and down-regulation of miR319 have also been observed in mutant type with reduced fertility compared with wild type in one previous study (Ao *et al.*, 2012). This may explain the poor yield of low-N sensitive rice. These miRNAs have the potential to modulate the productivity of rice as well as nitrogen utilization efficiency.

Our results revealed that osa-miR530-3p was the most up-regulated miRNA in low-N sensitive rice samples and osa-miR319a was the most up-regulated miRNA in low-N tolerant rice samples. Their target genes were further analyzed and 4 target genes of osa-miR530-3p, LOC_Os04g02280.1, LOC_Os05g09650.1, LOC_Os08g34140.1 and LOC_Os11g16460.1 had high-reliable SNPs. This finding indicates that these genes may play important roles in nitrogen tolerance.

LOC_Os04g02280.1 encodes an F-box domain containing protein. F-box proteins are components of SCF ubiquitin-ligase complexes in which they bind substrates for ubiquitin-mediated proteolysis (Kipreos and Pagano, 2000) and determine the specificity in substrate recognition (Kong *et al.*, 2004). F-box proteins are involved in plant growth, development, (Dharmasiri *et al.*, 2005a, b; Kepinski and leyser, 2005), senescence (Woo *et al.*, 2001). Recent studies have shown that the F-box proteins also play important roles in plant defense responses (Kim and Delany, 2002).

LOC_Os05g09650.1 encodes a coenzyme Q biosynthesis protein. Coenzyme Q acts as a linker in the electron transport chain. It transfers electron via quinone/phenol structural transformation. Respiration is the center of the plant metabolism (Miyagawa *et al.*, 2000) and the electron transfer is the most important part in this process. Therefore, it can be inferred that the coenzyme Q biosynthesis protein may take an important role in respiration as well as metabolism.

The protein product of LOC_Os08g34140.1 is a SNARE. SNAREs are widely studied proteins in membrane trafficking, docking, and fusion. Previous studies have found that the SNAREs are involved in a variety of fundamental processes, such as plant cell plate formation (Zheng *et al.*, 2002), cytokinesis (Heese *et al.*, 2001), shoot gravitropism, pathogen defense (Collins *et al.*, 2003).

The protein product of LOC_Os11g16460.1 is an oxidoreductase. It catalyzes the oxidation or reduction of substrates, which is a fundamental step in metabolism. The oxidoreductase is important to the plant growth and development. The reactive oxygen species (ROS) has a very reactive chemical nature, and it not only takes part in plant defense (Bolwell, 1999), but also causes serious injury to cells. Oxidoreductase has the ability to precisely control the generation and removal of ROS. Besides, it may be implicated in signal transmission. Therefore, these four proteins encoded by target genes of osa-miR530-3p play important roles in plant life

process and may regulate nitrogen tolerance of rice in our study.

Conclusion

With the development of molecular biology and biotechnology, understanding the interactions between plants and all kinds of adversity from the molecular level becomes a reality, which opens up new avenues for the improvement of crop. In the present study, differentially expressed miRNAs were identified which might contribute to the difference in low-N tolerance in rice. And target genes were also discussed. These finding are beneficial for understanding the underlying regulatory mechanisms and guiding future molecular plant breeding.

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