INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
19–0030/2019/22–4–721–726
DOI: 10.17957/IJAB/15.1121
http://www.fspublishers.org

Full Length Article

Transcriptome Analysis Identifies Nitrogen Utilization Genes in Chinese Fir

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Abstract

To screen for the genes related to nitrogen utilization efficiency (NUE), plants of the Chinese fir (*Cunninghamia lanceolata* (Lamb.) Hook) clone X6 were subjected to low nitrogen (LN) stress and control (CK; optimal nitrogen) treatments. The transcriptomes of their roots were obtained using RNA sequencing to determine the differences in gene expression between both conditions. A total of 4,300 significant differentially expressed genes (SDEGs) were detected by comparing the transcriptomes of the plants in the LN and CK groups. All SDEGs of the plants in the two groups were annotated using the Gene Ontology and Kyoto Encyclopedia of Genes and Genomes databases. The pathway for nitrogen metabolism was screened, and eight unigenes were selected as putative genes related to NUE in Chinese fir. Seven enzymes, namely nitrate transporter (NRT), ammonium transporter (AMT), nitrate reductase (NADH, NR1), glutamine synthetase (GS), ferredoxin-nitrite reductase (NIR1), glutamate synthase (NADPH/NADH, NADH-GOGAT), and glutamate dehydrogenase 2 ($NAD(P)^+$, GDH2) were encoded. Our result showed that *NRT*, *AMT*, *NR1* and *GS* could be used to improve nutrient acquisition and utilization in Chinese fir. © 2019 Friends Science Publishers

Keywords: Chinese fir; Low nitrogen stress; Root transcriptomes; Genes; Nitrogen utilization efficiency

Introduction

Nitrogen is an important nutrient, necessary for plant growth and development. Its deficiency has been a persistent problem in most forest ecosystems and has become an important limiting factor of their productivity (Vitousek and Howarth, 1991).

Chinese fir (*Cunninghamia lanceolata* [Lamb.] Hook) is an excellent fast-growing timber species found in southern China, where it has been widely cultivated for over 3,000 years. It is of great value for the forestry industry, accounting for 20–30% of the total commercial timber currently produced in China (Ma *et al.*, 2016). In recent years, the continuous planting of this tree has led to a decline in soil fertility, which has seriously affected wood yield. Nitrogen application is an important means of supplementing the soil with this nutrient. However, under the influence of the monsoon climate in south China, the nitrogen available in soil is easily lost through leaching, resulting in very low nitrogen utilization efficiency (NUE) (Qafoku *et al.*, 2004). The irrational use of nitrogen fertilizer is not only inefficient but also causes serious pollution of water, soil, and air (Peoples *et al.*, 1995). Therefore, appropriate fertilizer applications for improving NUE and reducing nitrogen emissions have been proposed globally. One of the best approaches to this problem is to identify and use genes involved in NUE in breeding (Sutton *et al.*, 2011; Mcallister *et al.*, 2012).

Plants absorb nitrogen mainly as ammonia (NH_4^+) and nitrate (NO_3^-) through the activity of enzymes coded by the ammonium transporter gene *AMT* and the nitrate transporter gene *NRT*, respectively (Näsholm *et al.*, 2009). After the uptake of nitrogen in its active forms, it is incorporated into amino acids *via* pathways catalyzed by the enzymes coded by the glutamine (Gln) synthetase (*GOGAT*) and glutamic acid (Glu) synthetase (*GS*) genes (Orsel *et al.*, 2002). Glutamate (the anion of Glu) is a precursor of nitrogenous compounds in organisms and it is converted by aminotransferases into different amino acids, nucleic acids, ureides, and polyamines, which are then assimilated by plant (Forde and Lea, 2007; Good *et al.*, 2007; Shrawat *et al.*, 2008). Studies on transgenic plants have shown that the over-expression of key genes involved in nitrogen metabolism can increase the protein content of the leaves, and such plants display morphological growth advantages (Zhou *et al.*, 2009). However, current information on NUE in Chinese fir is limited, and little is known about the genes involved in nitrogen metabolism in this plant.

There are many factors affecting NUE, mainly

To cite this paper: Wu, B., Y. Pan, G. Ma, J. Li, Z. Tong and Y. Lu, 2019. Transcriptome analysis identifies nitrogen utilization genes in Chinese fir. *Intl. J. Agric. Biol.*, 22: 721–726

environmental and genotypic. Therefore, there are two ways to improve NUE: by improving nitrogen status in the root environment, and by improving the absorption and utilization ability, which depend on plant genotype. The NUE at low nitrogen supply is higher than at high nitrogen supply owing to different factors limiting nitrogen metabolism in plants at low and high nitrogen supply levels. At high nitrogen supply level, the change in NUE is mainly attributed to the changes in plant's demand for nitrogen, which results in differences in nitrogen uptake, whereas at nitrogen deficiency, the change in NUE is mainly determined by remobility and assimilation efficiency of nitrogen by the plant (Coque and Gallais, 2006; Chardon *et al.*, 2010; Masclaux-Daubresse and Chardon, 2011). Therefore, the significant differentially expressed genes (SDEGs) screened under low nitrogen (LN) stress could represent NUE to some extent.

RNA sequencing (RNA-Seq) is a sensitive technology widely used in biological studies to discover key gene candidates. Li *et al.* (2017) identified some nitrogen utilization genes by RNA-Seq that are important for NUE in the tea plant. In the Chinese fir, RNA-Seq analysis has revealed several candidate genes involved in cellulose and lignin biosynthesis and in xylem development (Huang *et al.*, 2012; Zhang *et al.*, 2016). Studies have shown that nitrogen is a limiting factor for the growth of the Chinese fir (Zheng *et al.*, 2016). Therefore, the characteristics of nitrogen metabolism in this tree under LN conditions are crucial for determining NUE candidate gene (s). In the present study, it was aimed to identify NUE genes in Chinese fir, especially those responsible for nitrogen uptake from soil by roots and nitrogen assimilation into amino acids, using RNA-Seq technology.

Materials and Methods

Experimental Materials

A highly adaptive Chinese fir clone, X6, was used in the present study. In October 2013, stem cuttings of X6 were collected from a garden in the Kaihua Woodland, Zhejiang Province (118° 42′ E, 29° 13′ N), and propagated in a peat–perlite medium at the Germplasm Center of the Zhejiang Agriculture and Forestry University in China (119° 72′ E, 30° 23′ N). In the spring 2014, disease-free seedlings with completely developed root systems, similar in structure, and displaying uniform growth were selected as the experimental materials.

Experimental Strategies

In hydroponics, nutrient solutions can be accurately regulated. However, the long-term cultivation of Chinese fir in water and under anoxic conditions is still poorly understood. Therefore, an air mist cultivation device was designed for this species, which named an aeroponic system. In brief, it consists of a 100-L aeroponic barrel (40 cm deep) in which the plants were grown on the lid, while the roots developed in the barrel. The nutrient solution was pumped from a 25-L tank using a submersible utility pump, regulated by a time-delay relay, and it was misted over the roots at regular intervals of 10 min (1 min spray every 9 min) by sprinklers placed at the barrel bottom. The aeroponic barrel was placed onto a 0.5 m high platform to allow the nutrient solution to drain back by gravity and flow into the nutrient tank. Based on our previous study (Li *et al.*, 2018), half-strength Hoagland nutrient solution is suitable for the aeroponic culture of the Chinese fir seedlings. In the present study, seedlings were segregated into two groups and exposed to different treatments: LN stress (only nitrogen present in distilled water at less than 0.01 m*M*, was provided), and control (CK) conditions (5.03 m*M* nitrogen, which is the concentration present in half-strength Hoagland solution). Potassium chloride (KCl) and calcium chloride ($CaCl_2$) were used to maintain K^+ and Ca^{2+} concentrations, respectively, as these changes with nitrogen addition (Table 1). The components of the nutrient solutions were dissolved in distilled water.

The selected individual seedlings were planted in the aeroponic system, 10 cm × 10 cm apart, and cultured in water for 1 week to allow their recovery. After this period, 1/4-strength Hoagland nutrient solution was applied for cultivating strong plantlets. After 3 weeks, different nutrient solutions were used for the LN and CK treatments, which started in June, each containing five plants and run in triplicate. The nutrient solutions provided to each plant were changed every 3 weeks for 4 months. Seedlings were collected at the end of the experiment in October.

Experimental Methods

RNA Extraction and Transcriptome Sequencing

Root tips (about 1 cm) of the plants in the LN and CK groups were excised and total RNAs were extracted using cetyl trimethylammonium bromide + TRIzol reagent (Invitrogen, USA). The RNAs extracted from the three replicates were equivalently mixed to pool for transcriptome sequencing in an Illumina (USA) HiSeq 2000 platform at LC Sciences (USA).

To obtain high-quality clean data for *de novo* assembly, the raw reads were filtered by removing the adaptor sequences, low quality reads with more than 5% undetermined nucleotides, and reads in which more than 20% of bases had a Q-value < 30. The clean reads were assembled into contigs using Trinity (http://trinityrnaseq.sourceforge.net; Grabherr *et al.*, 2011). After Trinity *de novo* assembly and correction, the contigs without any gaps were linked into transcripts, and the longest transcripts were considered unigenes. The transcript abundance of unigenes was represented in reads per

Table 1: The composition of nutrient solutions used for low-nitrogen (LN) and control (CK) treatments of the Chinese fir. Half-strength Hoagland nutrient solution was used as basic culture solution

Salts	CK (mg/L)	LN (mg/L)
KNO_3	203	0
$Ca(NO_3)_2•4H_2O$	472.5	0
NH_4NO_3	40	0
$MgSO_4•7H_2O$	493	493
KCl	186	372
KH_2PO_4	136	136
$CaCl_2$	222	444
H_3BO_3	0.0031	0.0031
$CuSO_4•5H_2O$	0.000125	0.000125
$ZnSO_4•7H_2O$	0.043	0.043
$MnSO_4$	0.115	0.115
$CoCl_2•6H_2O$	0.000125	0.000125
KI	0.00415	0.00415
$Na_2MoO_4•2H_2O$	0.00125	0.00125
Fe_2EDTA	18.65	18.65
$Fe_2SO_4•7H_2O$	13.9	13.9

kilobase of exon model per million mapped reads (RPKM).

Functional Annotation

For functional annotation of the unigenes, the assembled sequences were compared against the National Center for Biotechnology Information (NCBI) non-redundant protein sequences, Swiss-Prot, Gene Ontology, EuKaryotic Orthologous Groups, and Kyoto Encyclopedia of Genes and Genomes (KEGG) databases, and against the Protein families (Pfam) database by HMMER (http://www.hmmer.org), with an E-value of 1E-10.

Identification of SDEGs

Transcriptome sequences of the plants were compared between the LN and CK groups. To infer the gene expression levels, the abundance of transcripts was normalized by using RPKM values and calculating the fold change in gene expression levels in the $\log_2$ scale, $\log_2$(FC). A p-value of RPKM < 0.05 and an absolute value of $\log_2(FC) \geqslant 1$ were considered the thresholds for SDEGs (Benjamini and Yekutieli, 2001).

Analysis of KEGG Pathways and Genes Related to NUE

The KEGG analysis (http://www.kegg.jp/kegg/pathway.html) was used to screen the main biochemical, metabolic, and signaling pathways in which the SDEGs were highly enriched. The pathway for nitrogen metabolism was identified and the genes likely related to NUE were screened.

Quantitative Analysis of SDEGs

To verify the reliability of the SDEGs identified by RNA-Seq analysis, the expression levels of two genes, namely ammonium transporter (*AMT3.1*, comp16516_c0) and nitrate transporter (*NRT2.1*, comp25897_c0), were determined by quantitative real-time polymerase chain reaction (RT-qPCR) using SYBR®PremixExTaq™ (Tli RNAseH Plus) (TAKARA Bio Inc., Japan). RNA samples used for RT-qPCR were aliquoted from those used for RNA-Seq. The *actin* gene of the Chinese fir was used as an internal control. The specific primer pairs used for the RT-qPCR of the three genes were: Actin-F: 5′-CAGCAACTGGGATGATATGG-3′ and Actin-R: 5′-ATTTCGCTTTCAGCAGTGGT-3′; AMT3-F: 5′-TAACACACACCCAAATCGCCTA-3′ and AMT3-R: 5′-ACATTCCTTCCAAATCTCAGCC-3′; and NRT2-F: 5′-AATGATTGAGTGGTGCCC-3′ and NRT2-R: 5′-TGCTTCCAAGATGTGAGG-3′.

Results

Transcriptome Sequencing (mRNA-seq) and Functional Annotation

The RNA samples obtained from the roots of clone X6 plantlets under LN stress and CK conditions produced 125.4 million reads. After removing the reads containing the adaptors and low-quality reads, the resulting 18.1 million clean reads were assembled into 32,814 unigenes by Trinity, 15,191 of which were annotated by the five published databases. The RNASeq reads and the assembly are publicly available at NCBI under the master accession number SRP077092.

Screening of SDEGs and Analysis of KEGG Pathways

The comparison of the transcriptome data of LN- and CK-treated groups resulted in 4,300 SDEGs; of those, 980 SDEGs were annotated by KEGG annotation and classification and related to 222 metabolic pathways. The SDEGs related to the nitrogen metabolism pathway in the Chinese fir were analyzed. In total, 12 unigenes displayed significant changes due to nitrogen deficiency. Of those, eight unigenes were more than 500 bp in length and were screened as putative genes related to NUE (Table 2).

Genes Related to NUE in Chinese Fir

The functions of putative NUE genes were analyzed. They encoded seven enzymes: nitrate transporter (NRT), ammonium transporter (AMT), nitrate reductase (NADH, NR1), glutamine synthetase (GS), ferredoxin-nitrite reductase (NIR1), glutamate synthase (NADPH/NADH, NADH-GOGAT), and glutamate dehydrogenase 2 ($NAD(P)^+$, GDH2). Among them, *NRT*, *AMT*, *NR1*, and *GS* were significantly up-regulated, whereas *NIR1*, *NADH-GOGAT*, and *GDH2* were significantly down-regulated. The up-regulated genes were divided into two categories. One comprised genes whose expression levels were promoted by nitrogen starvation and it included *NRT2.1* (comp25897_c0) and *AMT3.1* (comp16516_c0), with

Table 2: Unigenes of the Chinese fir involved in nitrogen metabolism

gene_ID	Name and annotation	Length (bp)	LN_rpkm	CK_rpkm	log_2(FC)
comp25897_c0	NRT2.1	2114	69.93	26.55	1.40
comp16516_c0	AMT3.1	1814	53.26	0.14	8.57
comp17440_c0	AMT3.1	1604	69.91	0.00	Inf
comp3927_c0	NR1 (NADH)	581	5.18	0.00	Inf
comp17457_c0	GS	751	6.74	0.00	Inf
comp14490_c0	NIR1	2758	36.25	285.91	-2.98
\comp21774_c0	NADH-GOGAT	7479	23.31	62.58	-1.42
comp18314_c0	GDH2 (NAD(P)+)	1650	0.99	13.89	-3.81

Inf indicates the expression level of 0 in control treatment. An absolute value for log_2 of the fold change, $log_2(FC) \geq 1$ and $P < 0.05$ were considered the thresholds for significant differentially expressed genes. See the (SRA) database (accession number: GSE117392) for information on gene sequences

log_2(FC) values of 1.4 and 8.57, respectively; the other group comprised genes whose expression was induced by nitrogen starvation and it included *AMT3.1* (comp17440_c0), *NR1* (comp3927_c0), and *GS* (comp17457_c0), with RPKM values in LN of 69.91, 5.18, and 6.74, respectively. The three down-regulated genes *NIR1*, *NADH-GOGAT*, and *GDH2* displayed log_2 (FC) values of -2.98, -1.42, and -3.81, respectively.

RT-qPCR Analysis of SDEGs

The results of the RT-qPCR showed that *AMT3* (*AMT3.1*, comp16516_c0) and *NRT2* (*NRT2.1*, comp25897_c0) were up-regulated under nitrogen stress, and their expression levels were higher by about 8.5- and 1.5-fold, respectively, with respect to the expression levels observed in CK plants (Fig. 1). The transcriptome sequencing data also revealed increased log_2 (FC) values for *AMT3* and *NRT2*, which were 8.57 and 1.40, respectively, indicating that RNA-Seq and RT-qPCR data were consistent.

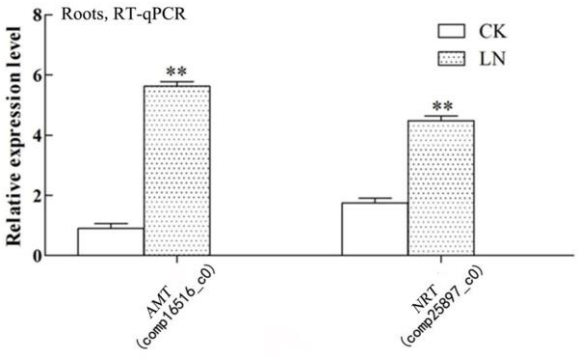

Fig. 1: The relative expression levels of genes in roots of the Chinese fir confirmed by quantitative real-time PCR (RT-qPCR). *AMT* and *NRT* represent the genes of the ammonium transporter and nitrate transporter, respectively. LK, low-nitrogen treatment; CK, control treatment. The symbol (**) represents extremely significant by Duncan's multiple range test at $P < 0.01$

Discussion

Nitrogen metabolism is an important physiological process in plants. Studies on NUE genes in other plants indicated that the expression of genes related to nitrogen metabolism forms the molecular basis for NUE (Gazzarrini *et al.*, 1999). Previous research on the Chinese fir focused mostly on finding nitrogen-efficient genotypes, and no genes related to NUE have been reported for this species.

The present study was based on the transcriptional differences between LN- and CK-treated plant groups to reveal the genes related to nitrogen stress. Nitrogen metabolism is an essential, complex process in plants comprising different pathways that may differ among tissues. In the present study, Chinese fir roots were used as study material, and therefore, the obtained genes were those expressed and functioning in the roots. The up-regulation of *AMT3.1* (comp16516_c0) was much higher under nitrogen deficiency than under control conditions, implying that *AMT3.1* is very important for nitrogen uptake by Chinese fir roots. In contrast, in rice, *OsAMT3.1* was expressed only in the aboveground plant, suggesting that it might be involved in NH_4^+ recycling during photorespiration in shoot leaves, while playing no crucial role in the absorption of NH_4^+ by roots (Zhao and Shi, 2007). The different roles of *AMT3.1* in rice and Chinese fir indicate that gene roles and expression profiles might differ among species.

NUE is a relative measurement of the plant capacity to acquire and utilize nitrogen for biomass production. Genotype determines nutrient uptake and utilization efficiency in a number of plant species, and key genes encode for improved nutrient acquisition and utilization. Because the clone X6 used in this study is a fast-growing clone with broad adaptability that can grow well in both nitrogen-rich and -barren soils, the genes obtained here were expected to play important roles in NUE of the Chinese fir. The pathway for nitrogen metabolism in X6 was screened, and eight SDEGs were selected by comparing gene expressions between LN- and CK-treatment groups.

Studies on *Arabidopsis thaliana* showed that nitrogen starvation can activate *NRT2.1* expression (Filleur and Daniel-Vedele, 1999), which can affect root growth and thereby directly affect the NUE (Garnett *et al.*, 2010). Many studies have shown that *GS* might be a key gene determining the NUE of crops (Fu *et al.*, 2013). These

results suggest that the four genes with up-regulated expression under LN stress found in the present study play important roles in the NUE of Chinese fir. NR activity is one of the major limiting factors of NUE when nitrogen is available as NO_3^- (Stitt, 1999; Tischner, 2001), and the increased expression of NR genes in pumpkin-grafted watermelon plants enhanced nitrate reduction, thus improving nitrate uptake and NUE (Nawaz *et al.,* 2017*).* However, there is not enough evidence on the effects of the three down-regulated genes *NADH-GOGAT*, *GDH2*, and *NIR1* on NUE. According to Nawaz *et al.* (2017), the expressions of NIR genes were increased, although not significantly, and promoted N assimilation of pumpkin rootstock-grafted watermelon plants. It should be noted, however, that the present results are based on studies of transgenes with constitutive expression. Because the genes involved in nitrogen metabolism often have different functions in different plant tissues or in different internal and external environments, the roles of these genes in NUE should not be evaluated based on the expression levels of individuals' transgenes alone.

Numerous studies have shown that the preference for NO_3^- or NH_4^+ varies among plant species. The capacity of plants to utilize both NO_3^- and NH_4^+ might provide great flexibility in their response to the changes in nitrogen supply from the environment (BassiriRad *et al.,* 2014). In forest soils, NH_4^+ is usually the predominant form of inorganic nitrogen. The mechanisms for NO_3^- and NH_4^+ uptake have been investigated in conifers such as Scots pine and European larch, and a clear preference for the uptake of NH_4^+ instead of NO_3^- has been reported (Malagoli *et al.,* 2000). The nitrogen acquisition strategies of the Chinese fir were examined, and NH_4^+ was the preferred form of nitrogen acquisition by Chinese fir grown in plantations (Li *et al.,* 2016). To date, two families of transporter genes, *NRT1* and *NRT2*, have been identified to be involved in NO_3^- uptake. The *NRT1* members studied thus far have low affinity for NO_3^- (except for NRT1.1, which has double affinity), while the *NRT2* members are involved in high-affinity NO_3^- transporters. A series of molecular and physiological studies have shown that high-affinity transporters play a central role in the efficient uptake of nitrogen under conditions of low nitrogen availability (Nacry *et al.,* 2013; Kiba and Krapp, 2016). In the present study, however, only one *NRT2.1* (comp25897_c0) was significantly up-regulated under LN stress, with the $\log_2$ (FC) value of 1.4. Two of the putative *AMT3.1* genes were significantly up-regulated under LN stress in the present study, and the results showed that *AMT*s were more sensitive to LN stress compared with *NRT*s in Chinese fir, suggesting that a variety of mechanisms might have been developed for the uptake of NH_4^+ during the evolution of Chinese fir, resulting in a more efficient AMT system than NRT system in this species.

Eight genes identified were involved in nitrogen metabolism in the Chinese fir. It has been reported that NO_3^- and NH_4^+ can function as nutrients or signals, depending on how long the plant is exposed to molecular nitrogen. and the expression profiles of the genes involved in NO_3^- signaling and metabolism were altered within 30 min in *Arabidopsis thaliana*, rice, and tea plant, but when these species were exposed to nitrogen for a long period, NO_3^- served as a mineral nutrient (Glass *et al.,* 2002; Nunes-Nesi *et al.,* 2010; Fallovo *et al.,* 2011; Liu *et al.,* 2017). Therefore, the duration of LN stress is a crucial factor. However, there has been no report on the effect of the LN stress duration in Chinese fir seedlings. In a previous study, it was observed that only a few Chinese fir seedlings exposed to LN stress started to display nitrogen deficiency symptoms after 4 months of exposure (unpublished). Therefore, in the present study, the LN stress treatment was performed for 4 months. It should be noted that the duration of the LN stress is the basis for the subsequent screening of SDEGs. If the stress period is too short, some genes might not be expressed. However, long-term stress exposure might lead to the omission of some key early response genes. Thus, the SDEGs screened in the present study might only represent some of the genes involved in the response of Chinese fir to LN stress. Based on the current knowledge, transcriptome dataset contained most of the genes required to encode the enzymes related to the response of Chinese fir to LN stress.

Conclusion

In the present study, eight unigenes were identified as putative genes related to NUE in the Chinese fir, which encoded seven enzymes: NRT, AMT, NR1, GS, NIR1, NADH-GOGAT, and GDH2. The results of present study showed that *NRT*, *AMT*, *NR1* and *GS* could be used to improve nutrient acquisition and utilization in Chinese fir.

Acknowledgement

This research was supported by the Opening Project of State Key Laboratory of Tree Genetics and Breeding of China (K2018205). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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[Received 08 Jan 2019; Accepted 22 Apr 2019; Published (online) 20 Aug 2019]