Identification of gene fragments related to nitrogen deficiency in *Eichhornia crassipes* (Pontederiaceae)

Minghui Fu[1*], Lihua Jiang[1], Yuanmei Li[1], Guohua Yan[1], Lijun Zheng[1] & Jinping Peng[2]
1. Department of Bioengineering, Guangdong University of Technology, Guangzhou, PR China; mhfugd@126.com
2. Department of Chemistry Engineering, Guangdong University of Technology, Guangzhou, PR China; 14998382@qq.com
* Correspondence

Abstract: *Eichhornia crassipes* is an aquatic plant native to the Amazon River Basin. It has become a serious weed in freshwater habitats in rivers, lakes and reservoirs both in tropical and warm temperate areas worldwide. Some research has stated that it can be used for water phytoremediation, due to its strong assimilation of nitrogen and phosphorus, and the accumulation of heavy metals, and its growth and spread may play an important role in environmental ecology. In order to explore the molecular mechanism of *E. crassipes* to responses to nitrogen deficiency, we constructed forward and reversed subtracted cDNA libraries for *E. crassipes* roots under nitrogen deficient condition using a suppressive subtractive hybridization (SSH) method. The forward subtraction included 2 100 clones, and the reversed included 2 650 clones. One thousand clones were randomly selected from each library for sequencing. About 737 (527 unigenes) clones from the forward library and 757 (483 unigenes) clones from the reversed library were informative. Sequence BlastX analysis showed that there were more transporters and adenosylhomocysteinase-like proteins in *E. crassipes* cultured in nitrogen deficient medium; while, those cultured in nitrogen replete medium had more proteins such as UBR4-like e3 ubiquitin-protein ligase and fasciclin-like arabinogalactan protein 8-like, as well as more cytoskeletal proteins, including actin and tubulin. Cluster of Orthologous Group (COG) analysis also demonstrated that in the forward library, the most ESTs were involved in coenzyme transportation and metabolism. In the reversed library, cytoskeletal ESTs were the most abundant. Gene Ontology (GO) analysis categories demonstrated that unigenes involved in binding, cellular process and electron carrier were the most differentially expressed unigenes between the forward and reversed libraries. All these results suggest that *E. crassipes* can respond to different nitrogen status by efficiently regulating and controlling some transporter gene expressions, certain metabolism processes, specific signal transduction pathways and cytoskeletal construction. Rev. Biol. Trop. 62 (4): 1637-1648. Epub 2014 December 01.

Key words: *Eichhornia crassipes*, SSH, deficient nitrogen, BlastX analysis, COG analysis, GO analysis.

Eichhornia crassipes (Mart.) Solms. is an invasive aquatic plant that originated in South America. It is one of the most productive plants in the world, and due to its rapid reproduction, strong assimilation of nitrogen and phosphorus (Reddy, Agami, & Tucker, 1989, 1990) and accumulation of heavy metals (Deng, Ye, & Wong, 2004; Odjegba & Fasidi, 2007; Caldelas, Iglesia-Turino, Araus, Bort, & Febrero, 2009), it can be used in phytoremediation of eutrophic lakes and rivers (Wang et al., 2013), industrial waste-water (Casabianca, Laugier,

& Posada, 1995), breeding waste-water (Lu, Fu, & Yin, 2008; Chen, Chen, Wan, Weng, & Huang, 2010) and landfill leachate (El-Gendy, Biswas, & Bewtra, 2006). However, the mechanisms for its high efficiencies of absorbance and utilization of nitrogen and phosphorus remain unknown.

There have been several reports on how *E. crassipes* responds to different environmental conditions. Xie, Wen, Yu, and Li (2004) found *E. crassipes* growth was stimulated by increased nutrient concentration, and the

plant could duly regulate biomass allocation to optimize resource acquisition in eutrophic environments. Ripley, Muller, Behenna, Whittington-Jones, and Hill (2006) also found that *E. crassipes* growth at low nitrogen and phosphorus levels could have the effect of decreasing phytosynthesis efficiency. Li, Gong, and Chang (2008) studied the effects of nitrogen form on *E. crassipes* growth and physiological responses by investigating the relative growth rate, the number of generated ramets, nitrate concentration, nitrate reductase activity, ammonium concentration, and glutamine synthetase activity. Reddy et al. (1989, 1990) evaluated the influence of phosphorus and nitrogen supply rates on growth and nutrient storage by *E. crassipes*. Notably, the existing studies were about morphological and physiological responses.

As far as we know, there are no reports describing the molecular response of *E. crassipes* to different nutrient conditions. In this study, we constructed forward and reversed subtraction cDNA libraries for *E. crassipes* under nitrogen deficient condition and identified gene fragments whose expressions were enhanced or lowered under this condition. Through the analysis of the functions of these genes, we could identify a potential molecular mechanism for *E. crassipes* adaption to different nutrient conditions and understand its invasion efficiency. These results also provide a theoretical basis for controlling *E. crassipes* growth from the nutrient metabolism perspective and could facilitate further development and utilization of *E. crassipes* as a polluted water remediation plant.

MATERIALS AND METHODS

Culture: Four or five 3-5cm high whole plants of *E. crassipes* with two or three leaves were collected from the lake in the West of Guangdong University of Technology. The plant roots were washed with water, sterilized with sodium hypochlorite, and rinsed with distilled water. Finally, the plants were cultivated in a tank (40cm*25cm*10cm) containing a preculture solution slightly modified according to the main components in Hoagland (0.25mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.25mM KNO_3, 0.1mM $MgSO_4 \cdot 7H_2O$, and 0.05mM KH_2PO_4) for 20 days at 28°C, under a 12/12-hour light (2000lx)/dark cycle. The solution was replaced every two days. After preculture, the plants were firstly cultured in maintenance culture (distilled water) for two weeks, and then cultured in experiment culture (1.8mM NH_4Cl solution or continued in distilled water) for 10 days.

Total RNA extraction and mRNA isolation: Total RNA of *E. crassipes* roots was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's instructions. The mRNA was purified with an Oligotex mRNA Kit (Qiagen); this mRNA was precipitated with 75% alcohol, air dried and solved in Diethylpyrocarbonate DEPC treated water. Total RNA integrity was examined by electrophoresis on a 1% agarose/EtBr gel.

SSH library construction: A forward subtraction library, where the driver cDNA is subtracted from test cDNA, consists of genes that are up-regulated in the test, while a reversed subtraction library, where the test cDNA is subtracted from driver cDNA, consists of down-regulated genes (Diatchenko et al., 1996). In our experiment, *E. crassipes* cultured in distilled water was regarded as the test condition, and that in ammonium solution was the driver. The forward and reversed subtraction libraries were constructed with a PCR-Select cDNA Subtraction kit (Clontech) according to the manufacturer's instructions. The subtracted cDNA obtained for differentially expressed genes was cloned into the pMD18-T vector for transformation into *Escherichia coli* DH5 cells. The recombinant white clones from LB agar were randomly picked and cultured in LB medium at 37°C overnight. To evaluate subtraction efficiency, the *E. crassipes* actin gene (GenBank accession No. KC505366) was compared with subtracted and unsubtracted cDNA by quantitative real-time

PCR analysis (qPCR). The qPCRs were carried out using actin-specific primers (Table 1). All qPCR reactions were performed in triplicate.

DNA sequencing and sequence data analysis: Positive white clones from each subtractive cDNA library were selected for sequencing. The sequences of clones were preprocessed to eliminate sequences of vector, adaptor, chimeric clone, pollution, repeating, and short Expressed Sequence Tag (EST) less than 100bp. Next, these informative sequences were clustered and assembled into unigenes. A unigene is a set of transcripts that appear to stem from the same transcription locus. There are two methods for clustering, supervised clustering and unsupervised clustering. Supervised clustering is that ESTs are classified with respect to known reference sequences, while unsupervised clustering is that ESTs are classified without any prior knowledge. In our experiment, we used a combination of supervised and unsupervised methods to cluster ESTs. The program Phrap (http://www.phrap.org/phredphrap/phrap.html) was used for ESTs assembly. The parameters of minmatch, minscore and repeat_stringency were set to 40, 40 and 0.96 respectively. Using NCBI BlastX and BlastP (Altschul, Gish, Miller, Myers, & Lipman, 1990) program (http://blast.ncbi.nlm.nih.gov/Blast.cgi), all these informative ESTs were searched against the NCBI nonredundant protein sequence (NR) database and COGs database (Tatusov, Koonin, & Lipman, 1997; Tatusov et al., 2003). The parameter e-value for searching NR database was set to 10^{-5} and for searching COGs database was set to 10^{-10}. After similarity search (e-value threshold is set to 10^{-5}), we used GO (http://amigo.geneontology.org/cgi-bin/amigo/blast.cgi) to classify all functions of informative ESTs and plotted GO annotations with WEGO (Ye et al., 2006) (http://wego.genomics.org.cn/cgi-bin/wego/index.pl).

Analysis of differential ESTs by qRT-PCR: For qPCR analysis, *E. crassipes* actin gene (GenBank accession No. KC505366)

TABLE 1
Primer sequences for PCR

Primer name	Primer sequences
actin-F1	5-GAGTTTGATCCTGGCTCAG-3
actin-R1	5-AGAAAGGAGGTGATCCAGCC-3
actin-F2	5-TCAATGTGCCTGCCATGTAT-3
Actin-R2	5-CCGTTCTGCAGTGGTAGTGA-3
18srDNA-F	5-GCATTTGCCAAGGATGTTTT-3
18srDNA-R	5-GCTATGTCCGGACCTGGTAA-3
FuM-G1-F	GTCTATAGGGCAGCGTGGTC
FuM-G1-R	CATCTTGGCCTTCTTCTTCG
FuM-G2-F	ATGCTCTCCACCTCATCCAG
FuM-G2-R	AAGTTGAACTCCCAAACGTAGC
FuM-G3-F	GGTATGTGCCGCTTTCCTAA
FuM-G3-R	GTATACTAGGGCGCGTCGAG
FuM-G4-F	AAACGGCTACCACATCCAAG
FuM-G4-R	CCTCCAATGGATCCTCGTTA
FuM-G5-F	GGTTGAGAACCAAGCCAAGA
FuM-G5-R	AGAGTTCGAGGACGAGGACA
FuM-G6-F	ATGTTCTCGCAGCGATCTTT
FuM-G6-R	GATTCCTTGTTGGTGGTGCT
FuM-G7-F	CTGGATTCCCTGATGGTGAC
FuM-G7-R	TTCCTTTGTATCGCGAGCTT
FuM-G8-F	TCTCAAGTGCCGAGGAAAGT
FuM-G8-R	AACAAGGATGAGGCTGATGG
FuM-G9-F	CACCATTGACAACGTGAAGG
FuM-G9-R	TGAAGGGTGGATTCCTTCTG
FuM-G10-F	ATCGGTGTTCGGAATCGTAG
FuM-G10-R	CCGTTCCAGCATATGAACCT
FuM-G11-F	GGCCGAGGTACAGGAACTTT
FuM-G11-R	CACCTACGGCCTCACTCCT
FuM-G12-F	GGCATACAGGTCCTTCCTGA
FuM-G12-R	CTCTTCCAGCCCTCCTTCAT
FuM-G13-F	GGCATACAGGTCCTTCCTGA
FuM-G13-R	TCCCTGGAAAAGAGCTACGA
FuM-G14-F	CCGCACCCTCCTTGTAGTAA
FuM-G14-R	CTGGATGAGGGGAATCAAGA
FuM-G15-F	CACAAACCCACCCTTGACTT
FuM-G15-R	TGGCTTCGCCTAATGGTTAC
FuM-G16-F	CAACTCTCGGGAAACGGTAA
FuM-G16-R	GAAGACGTAGGGCGTTCTTG
FuM-G17-F	TCACAGGAGACACGAGGATG
FuM-G17-R	GCAGGCTATCTTCCTGCACA
FuM-G18-F	TTTCGGAAGCAACAGTGACA
FuM-G18-R	TCCAAGGCTGCTGCTTCTAC
FuM-G19-F	AGGCTCTCACCAAGAGGACA
FuM-G19-R	ATCAGGCACGCATTAAGACC
FuM-G20-F	GCGGACTCCAGCTAAACAAG
FuM-G20-R	TACAGCAGCCCTCCTTCACT
FuM-G21-F	TTTCTCTCTCCATGCACACG
FuM-G21-R	GCCTCGTCCTCAACTACTGC

TABLE 2

Evaluation of the subtraction efficiency

	Ct value (using unsubtracted cDNA as template)	Ct value (using subtracted cDNA as template)	The differential abundance of actin in unsubstracted cDNA relative to substracted cDNA
Forward subtraction	19.91±0.18	29.74±0.26	910 times
Reverse subtraction	19.56±0.28	29.39±0.27	910 times

was used as an endogenous control gene. We randomly selected 10 genes from forward library and 11 genes from the reversed library to perform qRT-PCR to evaluate the differential expression of these genes in samples cultured in nitrogen deplete and nitrogen replete media. The primers sequences for the 21 ESTs (FuM-G1-F and FuM-G1-R to FuM-G21-F and FuM-G21-R) and actin gene (actin-F2 and actin-R2) (listed in Table 1) were designed according to the EST and actin gene sequences. Relative gene expression levels were represented by the $2^{-\Delta\Delta Ct}$ method (Livak & Schmittgen, 2001).

RESULTS

Quality of total RNA and mRNA: Electrophoresis of the isolated total RNA on 1% agarose gels showed two bright bands corresponding to ribosomal 28s and 18s RNA, with an intensity ratio of 1.8-2.0:1, respectively. The purified mRNA appeared as a smear with normal dispersion (Photos not showed). These results indicated that total RNA and mRNA were intact.

PCR analysis of subtraction efficiency: The efficiency of the constructed SSH library was estimated by comparing the abundance of the housekeeping gene actin before and after subtraction. The actin gene was amplified with the templates of subtractive and unsubtractive products of the second round by qPCR. The results showed that for both forward and reversed subtraction, the differential abundance of unsubtracted and subtracted cDNA arrived at 910 times (Table 2). It indicated that a large number of constitutive genes were effectively removed in the SSH library, and the

few genes with differential expression were easily detected.

Screening transformants for insertion and sequencing: In forward subtraction, a total of 2 100 positive clones were identified with blue/white selection, while there were 2 650
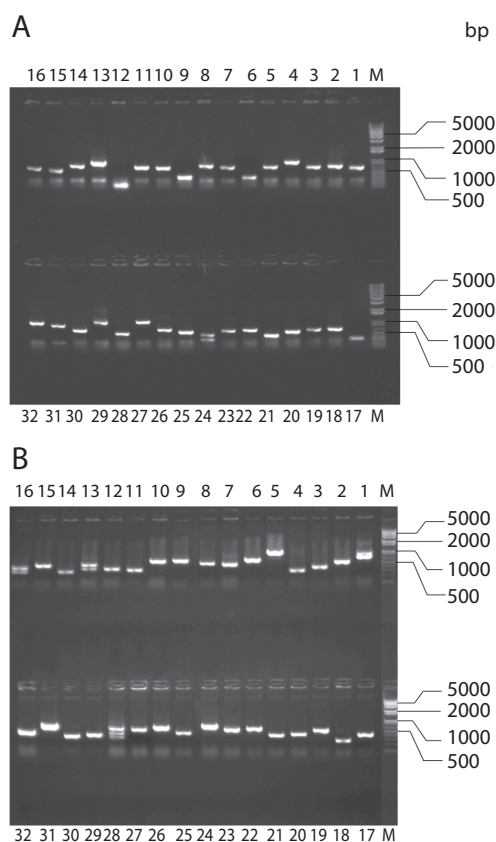

Fig. 1. PCR amplification of positive clones randomly selected from the forward and reversed libraries. M: 1kbp plus dna ladder (invitrogen), 1-32: PCR products of positive clones selected from forward (A) and reversed (B) libraries.

TABLE 3

Partial genes whose number of ESTs determined in our experiment through BlastX
was larger than 2 in the forward subtraction library

Gene annotation	Accession No. of best hit	Species
Similar to Adenosylhomocysteinase-like protein	NP_001067845.2	*Oryza sativa*
rRNA intron-encoded homing endonuclease	XP_003614387.1	*Medicago truncatula*
Mitochondrial protein, putative	XP_003588355.1	*Medicago truncatula*
ATP-dependent zinc metalloprotease FTSH 5, mitochondrial-like	XP_003569240.1	*Brachypodium distachyon*
predicted protein	XP_002328114.1	*Populus trichocarpa*
ZIP transporter	XP_002315981.1	*Populus trichocarpa*
with no lysine kinase 12	NP_001236036.1	*Glycine max*
triose phosphate isomerase cytosolic isoform	ACF06445.1	*Elaeis guineensis*
Multidrug resistance protein ABC transporter family	XP_003607667.1	*Medicago truncatula*
homeobox-leucine zipper protein HOX16-like	XP_003572981.1	*Brachypodium distachyon*
putative mitochondrial 2-oxoglutarate/malate carrier protein-like	XP_003531254.1	*Glycine max*
IAA9	AEV89263.1	*Musa acuminata*
hypothetical protein	XP_002464109.1	*Sorghum bicolor*
Pathogenesis-related protein PRB1-2	P35792.1	*Hordeum vulgare*
hypothetical protein	XP_002567556.1	*Penicillium chrysogenum*
phenylalanine ammonia-lyase	BAG70992.1	*Musa balbisiana*
pathogenesis-related protein	AEV89264.1	*Musa acuminata*
hypothetical protein	XP_002464109.1	*Sorghum bicolor*
Rb7	AAB53329.1	*Solanum lycopersicum*
S-adenosylmethionine synthase	O22338.1	*Musa acuminata*
hypothetical protein, partial	XP_003614388.1	*Medicago truncatula*
uncharacterized protein	XP_003572828.1	*Brachypodium distachyon*
putative glycosyltransferase	NP_001105849.1	*Zea mays*

clones for reversed subtraction. Among them, 1 000 clones were randomly selected from each library for sequencing. In order to estimate the rate of recombination and the length of the inserted fragment, we randomly selected 32 colonies each from the forward and reversed subtraction libraries for PCR. As shown by the electrophoresis results (Fig. 1A and 1B), the recombination rate of the library was above 95%, and the sizes of insert fragments were 300-600bp.

ESTs assembly: After preprocessing, ESTs clustering and ESTs assembly, 737 sequences (527 unigenes) from the forward library and 757 sequences (483 unigenes) from the reversed library were considered as informative ESTs. There were 75 contigs and 472 singletons in forward unigenes, and there were 105 contigs and 378 singletons in reversed

unigenes. The average length of all these unigenes were 479bp. The longest unigene had 2 064bp, and the shortest unigene had 100bp.

BlastX analysis: Our BlastX search against the NR database showed that 522 unigenes in the forward library and 479 unigenes in the reversed library had homologous sequences in GenBank. Table 3 (forward library) and Table 4 (reversed library) list the information of unigenes that had more than 2 ESTs in our experiment. Comparing these two tables, we could see there were more transporter proteins, such as ZIP transporter, multidrug-resistance protein ATP-binding cassette (ABC) transporter family, and putative mitochondrial 2-oxoglutarate/malate carrier protein in *E. crassipes* roots cultured under nitrogen deficient condition, suggesting that nitrogen transport may be related to these transporters.

TABLE 4

Partial genes whose number of ESTs determined in our experiment through BlastX
was larger than 2 in the reverse subtraction library

Gene annotation	Accession No. of best hit	Species
ATP-dependent zinc metalloprotease FTSH 5, mitochondrial-like	XP_003569240.1	*Brachypodium distachyon*
hypothetical protein, malate dehydrogenase	XP_002558886.1	*Penicillium chrysogenum*
hypothetical protein	NP_001067845.2	*Oryza sativa*
with no lysine kinase 12	NP_001236036.1	*Glycine max*
e3 ubiquitin-protein ligase UBR4-like	XP_002915692.1	*Ailuropoda melanoleuca*
hypothetical protein	XP_002567556.1	*Penicillium chrysogenum*
actin, alpha cardiac muscle 1-like isoform 2	XP_003442992.1	*Oreochromis niloticus*
translationally controlled tumor protein	ACF06596.1	*Elaeis guineensis*
hypothetical protein	XP_002559930.1	*Penicillium chrysogenum*
fasciclin-like arabinogalactan protein 8-like	XP_003580362.1	*Brachypodium distachyon*
putative glycosyltransferase	NP_001105849.1	*Zea mays*
NAC protein 1	AEQ94175.1	*Elaeis guineensis*
uncharacterized protein	XP_003572828.1	*Brachypodium distachyon*
rRNA intron-encoded homing endonuclease	XP_003614387.1	*Medicago truncatula*
tubulin alpha-1 chain-like, partial	XP_003635553.1	*Vitis vinifera*
Mitochondrial protein, putative	XP_003588355.1	*Medicago truncatula*
DC24	AAG44628.1; AF253979_1	*Homo sapiens*
uncharacterized protein	XP_003631610.1	*Vitis vinifera*
histone 2	refXP_002307619.1	*Populus trichocarpa*

In addition, adenosylhomocysteinase-like protein was also more abundant in nitrogen deficient *E. crassipes*. In those cultured in nitrogen replete medium, there were more proteins such as UBR4-like e3 ubiquitin-protein ligase and fasciclin-like arabinogalactan protein 8-like, as well as more cytoskeletal components, such as actin and tubulin.

COG analysis: After a homology search against the COG database, 195 and 148 ESTs in the forward and reversed libraries were identified to have best hits. These COG-informative ESTs were classified into several clusters according to their functions (Fig. 2A and 2B). In the forward library, ESTs involved in coenzyme transporter and metabolism (45 ESTs) were the most abundant, followed by ESTs participating in metabolism (37 ESTs). In the reversed library, cytoskeletal ESTs (22 ESTs) accounted for the largest number, followed by ESTs participating in information storage and processing (20 ESTs).

GO analysis: In GO analysis, all functions are clustered into three parts: cellular components, molecular functions, and biological processes. Overall, 239 and 183 unigenes in the forward and reversed libraries had GO information, respectively. Their categories are shown in Fig. 3. Unigenes related to binding and metabolic process were the most, followed by unigenes related to catalytic and cellular process. The most differentially expressed unigenes between forward and reversed libraries were those involved in binding, cellular process and electron carrier. The numbers of forward and reversed unigenes in these categories were 140 and 90, 109 and 66, 13 and 2 respectively. Their p-values were all below the significant level of 0.05 indicated remarkable relationship between the number of forward and reversed unigenes.

qRT-PCR analysis of differential unigenes: The results of qRT-PCR of ESTs are listed in Fig. 4. With regard to the forward
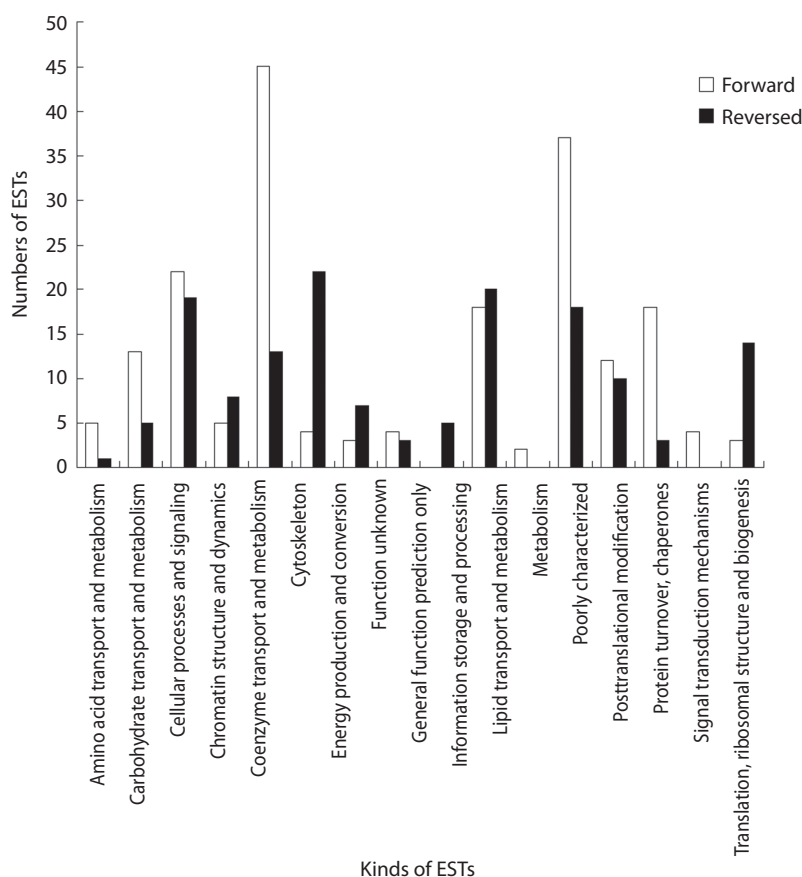
Fig. 2. Classification of all COG informative EST sequences from the forward and reversed subtraction libraries. E-value threshold was set to 10^{-10}.

library selections, the expression levels of all 10 unigenes in samples cultured in nitrogen deficient medium were higher than those from samples cultured in replete nitrogen. These 10 unigenes were selected from the forward library. For the reversed library selections, the expression levels of 10 out of 11 unigenes from samples cultured in nitrogen deficient medium were lower than that from *E. crassipes* roots cultured in replete; unigene 19 was the exception. These 11 unigenes were selected from reversed library. This result further confirmed the SSH construction.

DISCUSSION

Aquatic plants play important roles in water ecological system, and water environment can also influence the physiology and ecology of aquatic plants. Previous reports have demonstrated that aquatic plants could be used efficiently in phytoremediation of eutrophic water (Casabianca et al., 1995; El-Gendy et al., 2006; Lu et al., 2008; Chen et al., 2010; Wang et al., 2013), while water eutrophication was also closely related to algae blooms, as well as the decline and disappearance of
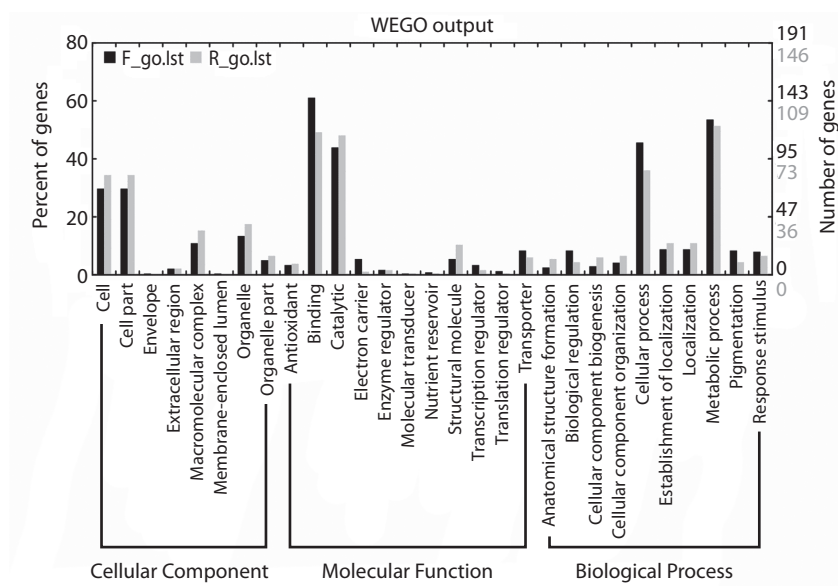
Fig. 3. Classification of all GO informative unigenes from the forward and reverse subtraction libraries on gene ontology. E-value threshold was set to 10^{-5}.
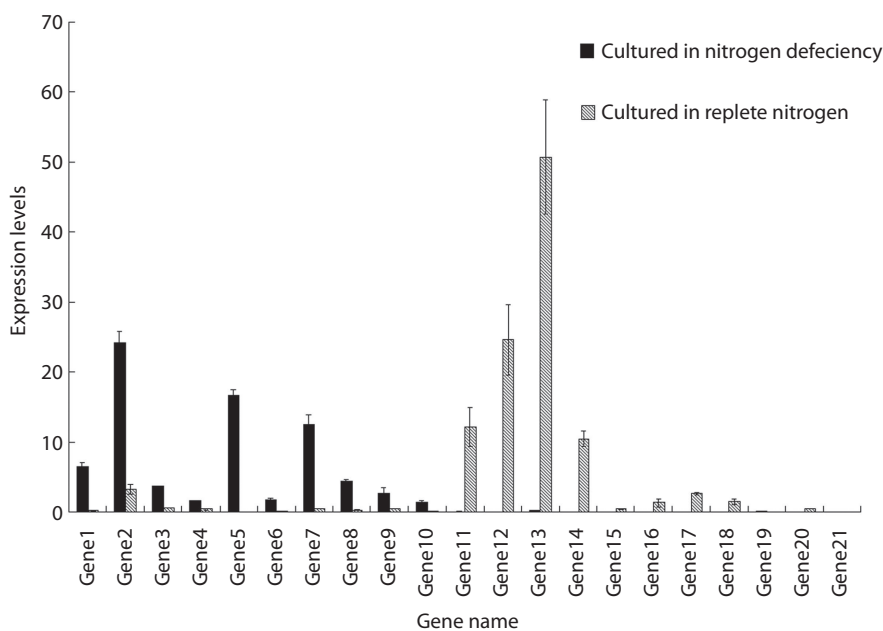

Fig. 4. qRT-PCR analysis of differential unigenes responsive to different nitrogen conditions. Samples were analyzed in triplicate. Data represent mean values, normalized with *E. crassipes* actin gene, and the error bars indicate the range of the replicate values.

submerged plants and uncontrollable increases in *E. crassipes* population (Dyhrman, 2008; Ripley et al., 2006; Wang, Zhang, Wang, Li, & Lu, 2010). The complexity of nutrient loading is matched by the complexity of nutritional strategies and capabilities of aquatic plants. Intensive study regarding the response at transcriptional level of aquatic plants to the status of different nutrients in water can help to elucidate the molecular mechanisms of the interaction between water environment and aquatic plants, and provide a theoretical basis for further develop aquatic plants in phytoremediation of eutrophic water. We can also use these differential expression genes as targets to regulate the physiology and ecology of aquatic plant from nutrient metabolism perspective in order to protect the balance of water ecology (Flores & Herrero, 2005; Dyhrman, 2008; Wurch, Haley, Orchard, Gobler, & Dyhrman, 2011).

Currently, research into the molecular mechanisms for aquatic plants to respond to nutrients in the environment has mainly focused on lower aquatic plants that could cause algae blooms. Wurch et al. (2011) used Long-SAGE (serial analysis of gene expression) to profile the transcriptome of the brown tide-forming algae *Aureococcus anophagefferens* and identified a suite of genes upregulated by nitrogen and phosphorus deficiency. Genes upregulated under nitrogen deficiency included an ammonium transporter, an acetamidase/formamidase, and two peptidases. Hildebrand (2005) also found an ammonium transporter expressed highly in nitrogen deficiency cell from the diatom *Cylindrotheca fusiformis*. Berg, Shrager, Glöckner, Arrigo, & Grossman (2008) found that in the algae *A. anophagefferens*, growth in ammonium solution resulted in an increase in the relative expression of an ammonium transporter, a novel ABC transporter, and a putative high-affinity phosphate transporter. Nitrogen limitation resulted in a 30- to 110-fold increase in the relative expression of nitrate, ammonium, urea, amino acid/polyamine, and formate/nitrite transporters.

In our experiment, there were also many transporters including ZIP transporter,

multidrug-resistance protein ABC transporter family, and putative mitochondrial 2-oxoglutarate/malate carrier protein in nitrogen-deficient *E. crassipes*. This was consistent with the reports described above. The 2-oxoglutarate/malate carrier protein can transport 2-oxoglutarate into the cytoplasm, where it may be involved in the process of nitrogen incorporation into carbon skeletons through the glutamine synthetase-glutamate synthase cycle, in which 2-oxoglutarate is the final nitrogen acceptor. Another function of 2-oxoglutarate is its role in nitrogen control. In cyanobacteria, the expression of many nitrogen-assimilation genes is subjected to regulation being activated by the nitrogen-control transcription factor NtcA, which is autoregulatory and whose activity appears to be influenced by 2-oxoglutarate and the signal transduction protein P(II) (Flores & Herrero, 2005; Herrero, Muro-Pastor, & Flores, 2001). In *E. crassipes*, whether 2-oxoglutarate is involved in nitrogen control still needs further study. ABC transporters utilize the energy of ATP hydrolysis to transport various substrates across cellular membranes. That nitrogen deficiency could increase the expression of ABC transporter gene suggests that this transporter might be involved in the transportation of nitrogen in *E. crassipes*. ZIP transporter can transport heavy metals such as Fe, Zn, Mn, Cu, Cd across the membrane. Why could nitrogen deficiency increase the expression of this gene in *E. crassipes*? Could it also transport ammonium into the cell or is there any connection between transportation of ammonium and heavy metals so that nitrogen deficiency can influence the expression of the ZIP transporter? Further study should solve this problem. Besides this, we also identified a novel protein adenosylhomocysteinase-like protein expressed at higher levels in nitrogen deficient *E. crassipes*. This protein is the key enzyme involved in the methylation reaction, indicating that nitrogen assimilation and utilization may be related to the process of methylation in *E. crassipes*. We did not identify increased ammonium transporters expression in nitrogen-deficient *E. crassipes*; perhaps ammonium

can be indirectly transported through other transporters (Bertl, Reid, Sentenac, & Slayman, 1997; Liu, Ludewig, Gassert, Frommer, & von Wirén, 2003; Loqué, Ludewig, Yuan, & von Wirén, 2005; Jahna et al., 2004). Another possible reason for this was that the differential expression of the ammonium transporters in *E. crassipes* was very low and was undetectable with the methods used here.

However, we found that UBR4-like e3 ubiquitin-protein ligase, fasciclin-like arabinogalactan protein 8-like, and some cytoskeletal protein such as actin and tubulin were upregulated in *E. crassipes* cultured in ammonium solution. E3 ubiquitin-protein ligase has many functions: it is a component of the N-end rule pathway, it forms meshwork structures involved in membrane morphogenesis and cytoskeletal organization together with clathrin, and regulates integrin-mediated signaling (Nakatani et al., 2005). Fasciclin-like arabinogalactan protein 8-like is a cell-surface adhesion protein and may be significant in the process of competence acquisition (Johnson, Johes, Bacic, & Schultz, 2003). Perhaps ammonium can efficiently improve certain metabolism processes, and enhance specific signal transduction pathways in *E. crassipes*. But which metabolic processes and which signal pathway could be influenced by ammonium in *E. crassipes* needs to be studied. It is also possible that higher levels of ammonium nitrogen allow *E. crassipes* to resist increases in osmotic pressure, partially due to alterations in cytoskeletal construction.

In conclusion, the function analysis of ESTs related to nitrogen deficiency and replete nitrogen in *E. crassipes* suggested that *E. crassipes* could respond to different nitrogen status by efficiently regulating and controlling some transporter gene expressions, certain metabolism processes, specific signal transduction pathways and cytoskeletal construction.

ACKNOWLEDGMENT

RESUMEN

Identificación de fragmentos de genes relacionados con la deficiencia de nitrógeno en *Eichhornia crassipes* **(Pontederiaceae).** Se ha convertido en una maleza importante en hábitats de agua dulce en ríos, lagos y embalses, tanto en zonas tropicales como templadas de todo el mundo. Algunas investigaciones han indicado que se puede utilizar para la fitorremediación de agua, debido a su fuerte asimilación de nitrógeno y fósforo, y la acumulación de metales pesados, su crecimiento y propagación puede desempeñar un papel importante en la ecología ambiental. Con el fin de explorar el mecanismo molecular de respuesta a la deficiencia de nitrógeno en *E. crassipes*, se construyeron bibliotecas de cDNA mediante síntesis adelantada y retrasada para raíces de *E. crassipes* en condiciones de deficiencia de nitrógeno mediante el método de hibridación supresiva sustractiva (SSH). Para este estudio se utilizaron 2 100 clones de síntesis adelantada y 2 650 de síntesis retrasada. De la biblioteca se escogieron al azar mil clones, 737 (527 unigenes) de síntesis adelantada y 757 (483 unigenes) de síntesis retrasada que fueron informativos. El análisis BLASTX mostró que había más transportadores y proteínas adenosilhomocisteinasa en *E. crassipes* cultivadas en un medio deficiente de nitrógeno; mientras que las cultivadas en un medio repleto de nitrógeno tenían más proteínas como UBR4 e3 ubiquitina-proteína ligasa y la proteína arabinogalactano 8 tipo fasciclina, así como otras proteínas del citoesqueleto, incluyendo la actina y la tubulina. Clúster del Grupo Ortológico (COG) también demostró que en la biblioteca de síntesis adelantada, la mayoría de los marcadores de secuencia expresada (ESTs) estaban involucrados en el transporte de coenzimas y el metabolismo.

Palabras clave: *Eichhornia crassipes*, SSH, deficiencia de nitrógeno, análisis BlastX, análisis COG, análisis GO.

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